Protein Radicals in Enzyme Catalysis

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Contents

I. Introduction

The interest in the involvement of protein radicals, stable and transient, in catalysis has skyrocketed since our initial reviews on this topic in 1988 and 1989.[1,2] This explosion of interest in the past few years has thus elicited a number of important reviews on this topic.[3−7] This review will focus on the general principles that have evolved governing the formation of protein radicals and their roles in catalysis. New experimental information that has emerged since 1988 will then be summarized for each system that has been thus far characterized. We will limit the scope of this review to enzymatic systems that utilize amino acid or modified amino acid based radicals that are covalently linked to the protein. Other enzymatic systems in which the involvement of non-amino acid based organic radicals have been proposed such as the cytochrome P450 enzyme family,[8] flavin and pyrroloquinoline quinone (PQQ) dependent enzymes,[9,10] heme peroxidases,[11] the pyridoxal phosphate dependent 2,3-lysine aminomutase,[3,12] pyruvate:ferredoxin oxidoreductase,[13] and enzymes

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involved in 3,6-dideoxyhexose biosynthesis[14] have been extensively reviewed elsewhere. It is hoped that the general principles and recent experimental methods described herein will serve as a guide for examining new protein radical containing systems that will undoubtedly be discovered.

II. General Principles

A. One Electron Oxidized Amino Acids Thus Far Identified in Proteins

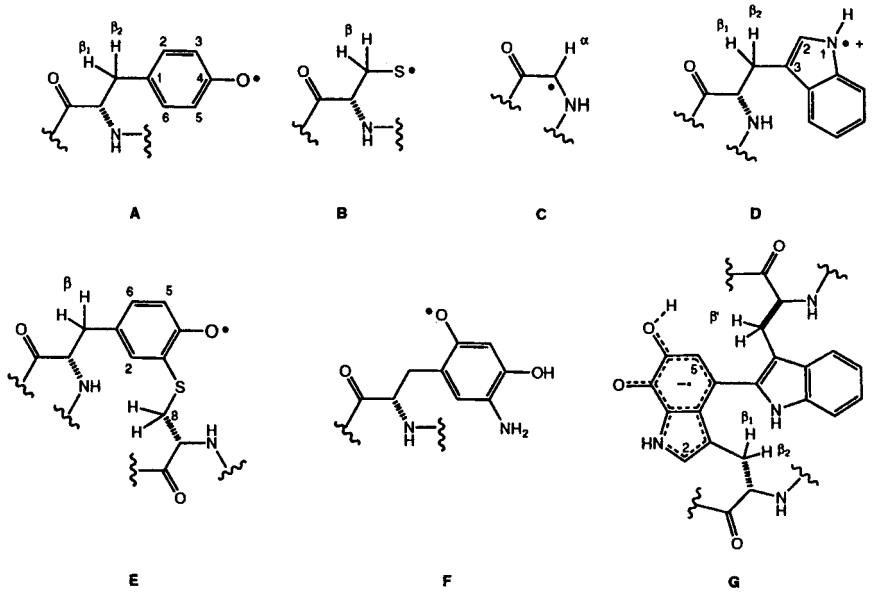
Since the seminal experiments of Sjöberg et al. identifying the organic radical essential for ribonucleotide reduction as being a one electron oxidized tyrosine residue,[15] stable and transient amino acid radicals have been localized to glycines,[16,17] cysteines,[18] tyrosines,[19,20] and tryptophans[21] as well as a variety of modified tyrosine[22,23] and tryptophan[24,25] residues within proteins. These species have been identified predominantly using electron paramagnetic resonance (EPR) spectroscopy of specifically isotopically labeled proteins. Table 1 summarizes the information available about their spectroscopic properties, the reduction potentials of these radicals, and the protein with which they are associated. Table 2 summarizes the available information about the corresponding free amino acid radicals or amino acid radicals within small peptides in most cases generated using pulse radiolysis experiments.

The protein environment, in conjunction with natural amino acids that have been modified (Tables 1 and 2, Figure 1), provide a method to dramatically alter the reduction potential and thus the accessible chemistries. As summarized in Table 1, tyrosines are the most prevalent of the oxidized amino acids thus far identified and will serve as a case in point. Specifically, in the photosynthetic (PS) reaction center II involved in the oxidation of H_2O to O_2, two tyrosyl radicals are present with reduction potentials that vary from 750 to 1000 mV. A cysteine-modified tyrosine residue with the thiol ortho to its hydroxyl group has been identified in galactose oxidase (GAO) (Table 1), shifting the reduction potential to 400 mV, 600 mV lower than tyrosyl radicals generated in model peptides. Model studies of modified amino acids within peptides have begun to address the effects of substitution and environment on reduction potential and this remains a fruitful area for further investigation. Additional electrochemical measurements on the proteins themselves (Table 1) are also essential, although the stability of the protein radical remains a problem in making such measurements.

B. Biosynthesis of (Modified) Amino Acid Radicals

All of the amino acid derived radicals are synthesized by a posttranslational process involving metallo cofactors located either adjacent to the amino acid being oxidized or on a second subunit or "activating enzyme" required for oxidation. The metallo cofactors come in many varieties (Fe, Cu, Co, and Mn) and are indicated in Figure 1. Aerobic ribonucleotide reductases (RNRs) contain diferric-tyrosyl radical cofactors (class I RNRs, Figure 1A). Their generation requires O_2, a reductant and the diferrous form of a metallo cofactor on one of its subunits (R2),[26,27] which is located 5.3 Å from tyrosine 122 (*Escherichia coli* numbering) that becomes oxidized to the tyrosyl radical ($Tyr^{\bullet}$).[28] Recent studies have shown that the biosynthesis of topa quinone (TPQ) (Figure 1H) in plasma amine oxidase places the tyrosine residue to be modified adjacent to the essential copper and requires O_2 and a reductant for self-assembly.[29−32] The radical form of the cofactor has been reported to be generated transiently during the self-catalytic biosynthesis of TPQ as well as in the reoxidation of the reduced form of the cofactor.[30] The generation

Table 1. Redox Potentials and Spectroscopic Data for the Protein Radicals in Various Enzyme Systems

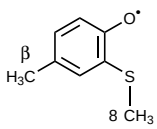


radical	protein source	$E^{o\prime}$ (mV)	ref(s)	UV−vis: λ_{max} (nm) [ϵ_{max} (M^{-1} cm^{-1})]	EPR principal g values	EPR principal hyperfine values[a]			ref(s)
A	class I	1000 ± 100	*c*	410	2.00912	A_x^{β} 21.8 G	$A_x^{H3,5}$ −9.5 G	$A_x^{H2,6}$ 1.8 G	7,110,112
	ribonucleotide			[1,800][d]	2.00457	A_y^{β} 19.1 G	$A_y^{H3,5}$ −3.0 G	$A_y^{H2,6}$ 2.7 G	
	reductase (RNR)[b]				2.00225	A_z^{β} 19.1 G	$A_z^{H3,5}$ −7.0 G	$A_z^{H2,6}$ 0.8 G	
A	photosystem II				2.00745	A_x^{β} 11.3 G	$A_x^{H3,5}$ −9.4 G	$A_x^{H2,6}$ 1.5 G	7,372,377
	$Y_D^{\bullet}$	720−760	*e*		2.00422	A_y^{β} 9.7 G	$A_y^{H3,5}$ −2.8 G	$A_y^{H2,6}$ 2.5 G	
					2.00211	A_z^{β} 9.7 G	$A_z^{H3,5}$ −7.0 G		
	$Y_Z^{\bullet}$	~1000	*f*		2.00750	A_x^{β} 12.6 G	$A_x^{H3,5}$ −9.6 G	$A_x^{H2,6}$ 1.8 G	7,368,369
					2.00422	A_y^{β} 10.4 G	$A_y^{H3,5}$ −3.0 G	$A_y^{H2,6}$ 2.7 G	
					2.00225	A_z^{β} 10.4 G	$A_z^{H3,5}$ −7.0 G	$A_z^{H2,6}$ 0.5 G	
A	prostaglandin H				2.008	$A_{\|}^{\beta}$ 24.6 G	$A_x^{H3,5}$ −9.2 G	$A_x^{H2,6}$ 1.8 G	7,20,331
	synthase[g]				2.003	$A_{\perp}^{\beta}$ 20.8 G	$A_z^{H3,5}$ −7.0 G	$A_y^{H2,6}$ 2.5 G	
					2.003				
B	class II RNR[h]				1.984	A_x^{β} 38.5 G			177
					2.001	A_y^{β} 35.3 G			
					2.230	A_z^{β} 30.9 G			
C	class III RNR[i]				g_{iso} 2.0033	a^{α} 14 G			17
C	pyruvate formate			365	g_{iso} 2.0037	a^{α} 15 G	a^{NH} 6 G	4.5 G	16
	lyase			[8,000]					
D[j]	cytochrome	655[k]	261		$g_{\|}$ 2.04	a^{β_1} 7.5 G[j]	a^{H1} 5.7 G		21
	peroxidase				$g_{\perp}$ 2.01	a^{β_2} 4.6 G	a^{H2} 5.3 G		
E	galactose	410	420	445, 810[l]	2.00741	A_x^{β} 9.5 G	A_x^{β} 15.5 G		428
	oxidase	400	429		2.00641	A_y^{β} 8.0 G	A_y^{β} 14.2 G		
					2.00211	A_z^{β} 8.5 G	A_z^{β} 14.2 G		
F	plasma amine oxidases			360, 438, 464	g_{iso} ~2.0				22,457,484
G	methylamine		517	428	g_{iso} ~2.0	a^{β_1} 2.1 G	$a^{\beta'}$ 0.5 G	$a^{H2/H5}$ 2.3 G	522
	dehydrogenase					a^{β_2} 1.2 G		$a^{H2/H5}$ 4.2 G	

[a] Anisotropic hyperfine values are denoted A, while isotropic values are given as *a*. Principal axes for *g* values and various hyperfine interactions are not necessarily collinear. [b] Protein from *E. coli*. [c] Silva, K. E., Elgren, T. E.; Que, J. L., Stankovich, M. T. *Biochemistry* **1995**, *34*, 14093−14103. [d] Extinction coefficient for $Y_{122}^{\bullet}$ corrected for absorption by the diferric cluster at the same wavelength.[26] [e] Vass, I., Styring, S. *Biochemistry* **1991**, *30*, 830−839. [f] Boussac, A., Etienne, A. L. *Biochim. Biophys. Acta* **1984**, *766*, 576−581. [g] Data for the "wide doublet (WD)" radical signal. [h] Protein from *L. leichmannii*. Data for a cysteinyl radical exchange coupled to an $S = {}^1/_2$ Co(II) center. [i] Protein from *E. coli* grown under anaerobic conditions. [j] Data for a tryptophan radical cation exchange coupled to the $S = 1$ Fe^{IV}═O center. [k] This value was obtained indirectly using rate constants for the reduction of the porphyrin radical in CcP by an endogenous electron donor in the mutant W191F. [l] All hyperfine values at g = 2.01. [m] Data for modified tyrosyl radical antiferromagnetically coupled to the Cu^{II} center.

of the modified tyrosyl radical in galactose oxidase (Figure 1G) is another example of an O_2-dependent process that is facilitated by an adjacent copper center.[33,34]

Table 2. Redox Potentials and Spectroscopic Data for Various Amino Acids and Model Compounds

radical	$E^{o\prime}$	ref	UV−vis: λ_{max} (nm) [ϵ_{max} (M^{-1} cm^{-1})]	ref	*g* values	EPR proton hyperfine constants		ref
						crystal		*c*
Tyr•	0.94 V[a]	430	407 [3200]	*b*	2.0067	$A_x^{3,5}$ 9.0 G	a_β14.0 G	
	0.93 V[b]	*a*			2.0042	$A_y^{3,5}$ 3.1 G		
					2.0023	$A_z^{3,5}$ 6.5 G		
						crystal		*e*
Cys•	1.33 V	215	300−330 [400−1200]	*d*	2.2441	A_x 34.3 G		
					2.0006	A_y 34.4 G		
					1.9837	A_z 34.7 G		
						crystal	solution	*g,h*
Gly•	1.22 V (pH 10.5)	*f*			2.0042	$a_{\alpha H}$ 23.6 G	$a_{\alpha H}$ 6.0 G	
					2.0035	a_{NH_3} 17.6 G	a_{NH_3} 6.0 G	
					2.0032	a_N 3.2 G	a_N 5.8 G	
Trp•	1.05 V[i]	430	320 [2800]	*k*		a_β 15.5 G		*m*
	1.01 V[j]	*a*	510 [1750]					
			325 [3670]	*l*				
			510 [2300]					
$TrpH^{+\bullet}$	1.15 V[j] (pH 3)	431	340 [3900]	*n*				
			580 [2600]					
	1.15 V[j] (pH 2)	*a*	335 [4750]	*l*				
			560 [3000]					
	~0.6 V	432	400	426	2.0072	A_x^8 7.5 G	$A_{x'}^\beta$ 10.0 G	427
			830		2.0062	A_y^8 2.5 G	$A_{y'}^\beta$ 9.0 G	428
					2.0019	A_x^8 7.5 G	$A_{z'}^\beta$ 9.0 G	

[a] Harriman, A. *J. Phys. Chem.* **1987**, *91*, 6102−6104. [b] Bansal, K., Fessenden, R. W. *Radiat. Res.* **1976**, *67*, 1−8. [c] Fasanella, E. L., Gordy, W. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *62*, 299−304. [d] Hoffman, M. Z., Hayon, E. *J. Phys. Chem.* **1973**, *77*, 990. [e] Kou, W. W. H., Box, H. C. *J. Chem. Phys.* **1976**, *64*, 3060−3062. [f] Zhao, R., Lind, J., Merényi, G., Eriksen, T. E. *J. Am. Chem. Soc.* **1994**, *116*, 12010−12015. [g] Armstrong, W. A., Humphreys, W. G. *Can. J. Chem.* **1967**, *45*, 2589−2597. [h] Collins, M. A., Whiffen, D. H. *Mol. Phys.* **1966**, *10*, 317−325. See also: Gramza, M.; Hilczer, W.; Goslar, J.; Hoffmann, S. K. *Acta Chem. Skand.* **1997**, *51*, 556−561. [i] Determined by pulse radiolysis. [j] Determined by cyclic voltammetry. [k] Redpath, J. L., Santus, R., Ovadia, J., Grossweiner, L. I. *Int. J. Radiat. Biol.* **1975**, *27*, 201−204. [l] Solar, S., Getoff, N., Surdhar, P. S., Armstrong, D. A., Singh, A. *J. Phys. Chem.* **1991**, *95*, 3639−3643. [m] Flossman, W., Westhof, E. *Radiat. Res.* **1978**, *73*, 75−85. [n] Santus, R., Grossweiner, L. I. *Photochem. Photobiol.* **1972**, *15*, 101.

In contrast, a number of amino acid radicals can be generated under anaerobic conditions, and in fact O_2 causes destruction of the metal center, the protein, or both. Recent studies of Licht et al. have indicated that the function of adenosylcobalamin (AdoCbl) (Figure 1B) in the *L. leichmannii* RNR (a member of class II RNRs) is to generate a thiyl radical exchange coupled to cob(II)alamin.[18] Under anaerobic conditions this system is relatively stable ($t_{1/2}$ = 0.5 h at 37 °C). However, at 37 °C under aerobic conditions the cob(II)alamin is slowly oxidized to hydroxycobalamin. Glycyl radicals have recently been shown to be essential for catalysis in both pyruvate formate lyase (PFL)[16] and RNR from *E. coli* grown under anaerobic conditions (class III RNR).[17] In both cases, *S*-adenosylmethionine (SAM) and an iron−sulfur cluster are required for the oxidation process.[17,35] This cluster is present on a polypeptide chain (an activating enzyme or a second subunit[36]) other than the one containing the glycine to be oxidized (Figure 1C). In the case of PFL, SAM is stoichiometrically converted into 5′-deoxyadenosine (5′-dA) and methionine, and it has been demonstrated that the *pro-S* hydrogen atom of glycine 734 is transferred to the 5′-position of 5′-dA consistent with a hydrogen atom abstraction process mediated by a 5′-dA radical (5′-dA•).[35] The mechanism by which the putative 5′-dA• is generated remains to be elucidated. A similar mechanism is also applicable in the case of the anaerobic RNR. In the case of both PFL and the anaerobic RNR, in contrast to the requirement for O_2 in the systems described above, O_2 initiates a chemical, nonenzymatic reaction, resulting in peptide bond cleavage at the glycine residue, and consequently enzyme inactivation.[37,38]

The common theme in nearly all cases thus far examined is that amino acid oxidation requires a metal cofactor. The ability to obtain large amounts of apoproteins via genetic engineering has greatly facilitated the investigation of the detailed mechanisms of amino acid oxidation using a wide variety of physical and biochemical methods. The time scale for many of these processes are within reach of conventional stopped-flow (SF) UV−vis spectroscopy and rapid freeze−quench (RFQ) technologies (see section II.D), and thus the mechanism of amino acid oxidation is a major focus of investigation in almost all of the systems in Figure 1.

C. Emerging General Chemical Properties of Enzymes Utilizing Protein Radicals for Catalysis

In many enzymes discussed in this review, the function of the cofactor is to generate a transient protein radical that interacts directly with the substrate. The transient nature of these species (Table 3) has made them difficult to detect and, therefore, to define their functions. The identity and kinetic competence of these transient radicals, as described in detail subsequently, has been delineated for a

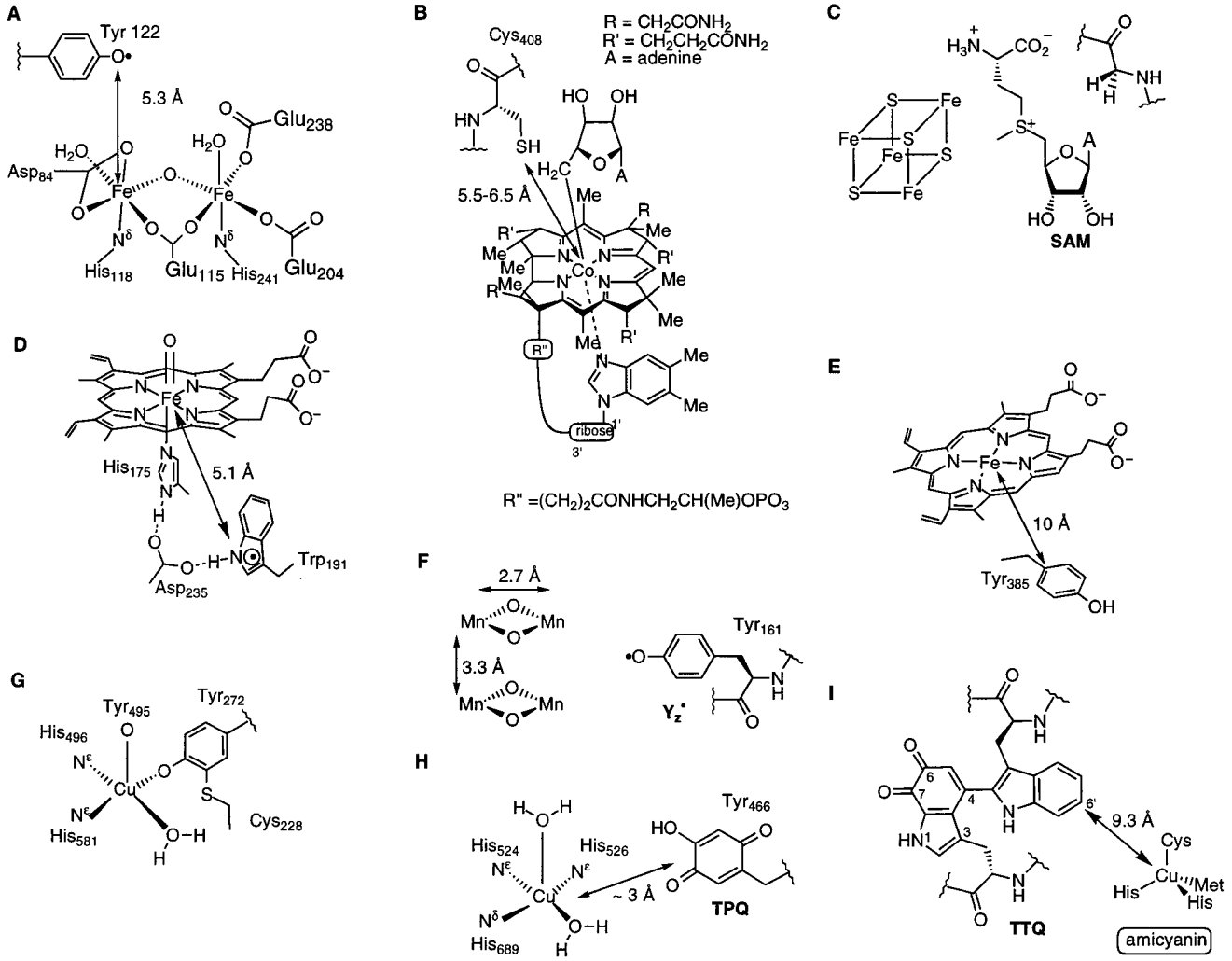


Figure 1. The structures of the metallocofactors and the (modified) amino acids that they oxidize. (A) The diferric cluster and stable tyrosyl radical of class I RNR from *E. coli*.[28] (B) Adenosylcobalamin generates a thiyl radical at Cys408 in class II RNR from *L. leichmannii*. EPR studies of the resulting exchange coupled system have established a distance of 5.5−6.5 Å between the cobalt and the radical.[18,177] (C) Schematic representation of the FeS cluster on the activating enzyme (or subunit) and the glycine to be oxidized in pyruvate formate lyase and class III RNR. (D) The exchange coupled tryptophan radical cation and ferryl heme center of cytochrome *c* peroxidase from bakers' yeast.[245,276] (E) Schematic representation of the heme and Tyr385 in mature ovine prostaglandin endoperoxide synthase-1.[316] The side chains of the protoporphyrin IX are omitted for clarity. (F) Schematic representation of the manganese cluster and $Y_Z^{\bullet}$ based on EXAFS[349−352] and ENDOR studies.[372] (G) The unusual cysteine-tyrosine cross-linked copper ligand in galactose oxidase from *Fusarium* spp.[79] (H) Topaquinone and the nearby copper site in copper amine oxidase from *E. coli*.[31] (I) Schematic representation of tryptophan tryptophylquinone from methylamine dehydrogenase and the copper site of the physiological electron acceptor amicyanin.[526]

number of proteins: the class II RNR from *L. leichmannii* generates a thiyl radical, cob(II)alamin;[18] the prostaglandin synthase (PGHS), a tyrosyl radical;[39] cytochrome *c* peroxidase (CcP), a tryptophan cation radical; and photosystem II (PSII)/O_2 evolving system, a tyrosyl radical. While thiyl radicals have been proposed to play a role in *E. coli* aerobic RNR[40] and PFL,[12,41,42] and by inference in the anaerobic RNR,[43] evidence of their formation in a kinetically competent fashion has not yet been demonstrated.

A second feature of these systems is the possibility that a transient radical catalyzes multiple turnovers, prior to regeneration of the stable form of the cofactor. Only in the case of PGHS has this ability been demonstrated.[44] In contrast, very recent studies on the class II RNR have established that AdoCbl produces a thiyl radical which regenerates the cofactor after each turnover.[45] A similar case is found in PSII where a tyrosine residue is oxidized to an intermediate tyrosyl radical which is subsequently reduced back to tyrosine before the next turnover.[46] Thus, whether a general feature will arise when all or most of these systems have been examined is the subject of ongoing investigation.

A third general characteristic of these proteins is their propensity to self-inactivate during turnover or in a primed position to initiate turnover when substrate is absent. The problem is extensively exacerbated when an alternate substrate is used to examine the mechanism or when a site-directed mutant of a protein is used for mechanistic analysis. Nature has developed methods to harness the reactivity of radical intermediates, but slight perturbations in the active site environment result in self-destruction. Specific examples of this phenomenon is provided by PGH synthase which self-inactivates very rapidly (10 s to 1 min),[47] and AdoCbl-dependent

Table 3. Transient Protein Radicals and the Cofactors from Which They Are Generated in Several Enzymatic Systems

enzyme	source	cofactor	protein radical involved in turnover	ref(s)
class I RNR	*E. coli*	Tyr$^{\bullet}$	Cys$^{\bullet}$	40,56
class II RNR	*L. leichmannii*	AdoCbl	Cys$^{\bullet}$	18
class III RNR	*E. coli*	Gly$^{\bullet}$	Cys$^{\bullet}$[a]	227
PFL	*E. coli*	Gly$^{\bullet}$	Cys$^{\bullet}$[a]	12,213
PGH synthase	mammals	heme/Fe	Tyr$^{\bullet}$	20,322
CCP	bakers' yeast	heme/Fe	TrpH$^{\bullet+}$	21
PSII	plants	P_{680}	Tyr$^{\bullet}$	19,365

[a] Cysteinyl radicals have been proposed for class III RNRs and PFL, but no direct evidence is available at present.

RNR which self-inactivates after about 1 h at 37 °C.[48,49]

D. Methods To Examine Radical Dependent Reactions

The ability to detect the presence of radical intermediates and determine their chemical and kinetic competence has been greatly facilitated by the ability to overexpress wild-type (wt) and mutant proteins. These methods have allowed acquisition of gram quantities of the protein required for physical biochemical analysis. Two methods have been frequently utilized to provide mechanistic insight about potential radical intermediates: stopped-flow (SF) UV−vis spectroscopy[50] and rapid freeze−quench (RFQ) electron paramagnetic resonance (EPR) spectroscopy.[51] As indicated in Table 1, many of the cofactors are colored and therefore their spectral changes in the visible region can be monitored continuously during the first turnover. Furthermore, many of the cofactors and all of the transient radicals (Tables 1 and 3) are paramagnetic and therefore the kinetics of their appearance and/or disappearance can be monitored in a discontinuous fashion by quenching the reaction in liquid isopentane at −140 °C followed by analysis via EPR spectroscopy. The time scale of almost all of these reactions is milliseconds; rate constants of $\leq 500\ s^{-1}$ can be measured, and thus both methods have been very informative.

In many instances use of active-site mutants or alternative substrates, as discussed below case by case, has greatly facilitated detection of intermediates. Propitious choice of mutants can block a particular step in the reaction and allow buildup of an intermediate and hence enhance its detection. However, what is also clear is that if a "reactive paramagnetic intermediate" builds up and its normal reductant is not present due to mutagenesis, that other amino acid residues in the vicinity of the active species can become oxidized. These reactions usually occur at a reduced rate relative to the normal pathway, and are mechanistically uninformative. Thus a kinetic analysis must accompany the spectroscopic detection.

Recent studies have also suggested that appropriately designed substrate analogues can provide important information about the involvement of radical intermediates in enzyme-catalyzed reactions. If the enzyme has relaxed substrate specificity, then functionality can be chemically built into the substrate to enhance the lifetime of a radical or to generate a predictable product from the radical. Many of the recently reported radicals detected during enzyme-mediated transformations have been quenched on the second time scale for analysis by EPR spectroscopy. In most of these cases the radical observed is many steps removed from the initially generated radical making it difficult to assign a structure.[42,52−54] The initial "transient" radical that is of most interest mechanistically, undergoes chemical transformations until it finds an "environment" in which it is more stable. It is usually this latter species that is actually detected. Thus, the RFQ method may facilitate detection and structural assignment of mechanistically more important radical intermediates. A key component of any analysis is the ability to simulate EPR spectra at different microwave frequencies using parameters consistent with a postulated molecular structure.

Recent studies on ribonucleoside 5′-triphosphate reductase (RTPR) from *L. leichmannii*, CcP, and GAO have revealed another important feature that some of these systems share in common. In the case of RTPR and CcP the assignment of the radical signals observed to specific protein residues was hampered by their appearance which eluded simulation, and by their physical properties. Although many years ago their unusual behavior was attributed to exchange coupling between the protein radical and a nearby metal center (Co(II) and Fe^{IV} in the cases of RTPR and CcP, respectively), only recently has it been possible to provide conclusive evidence for this supposition through satisfactory simulations of these signals. An extreme example of this phenomenon is GAO in which strong antiferromagnetic coupling between a Cu^{II} ion and a modified tyrosyl radical renders the active protein apparently EPR-silent.[23]

With these general features of protein radical requiring enzymes in mind, recent work on each of the systems from which these general principles were derived will be presented.

III. Background on Ribonucleotide Reductases

Ribonucleotide reductases catalyze the conversion of nucleotides to deoxynucleotides in the rate-determining step in DNA biosynthesis.[55−59] Despite this central role in metabolism, reductases possess a diverse array of primary and quaternary structures. The RNRs have been categorized into four classes based on the unusual cofactors essential for catalysis (Figure 2).[57] The class I reductases, discovered by Reichard in the early 1950s,[60,61] possess the stable tyrosyl radical adjacent to a diferric cluster (Figure 1A). Recently, it has been reported that several *Enterobacteracae* contain two class I RNRs,[62−64] that differ in their reductant sources,[65,66] and in their

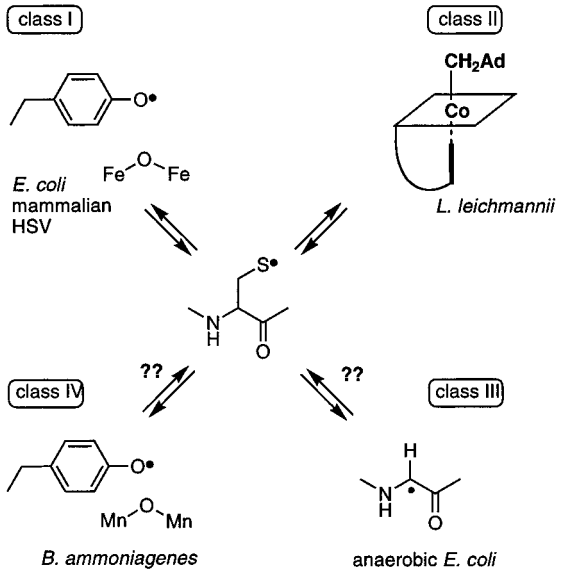
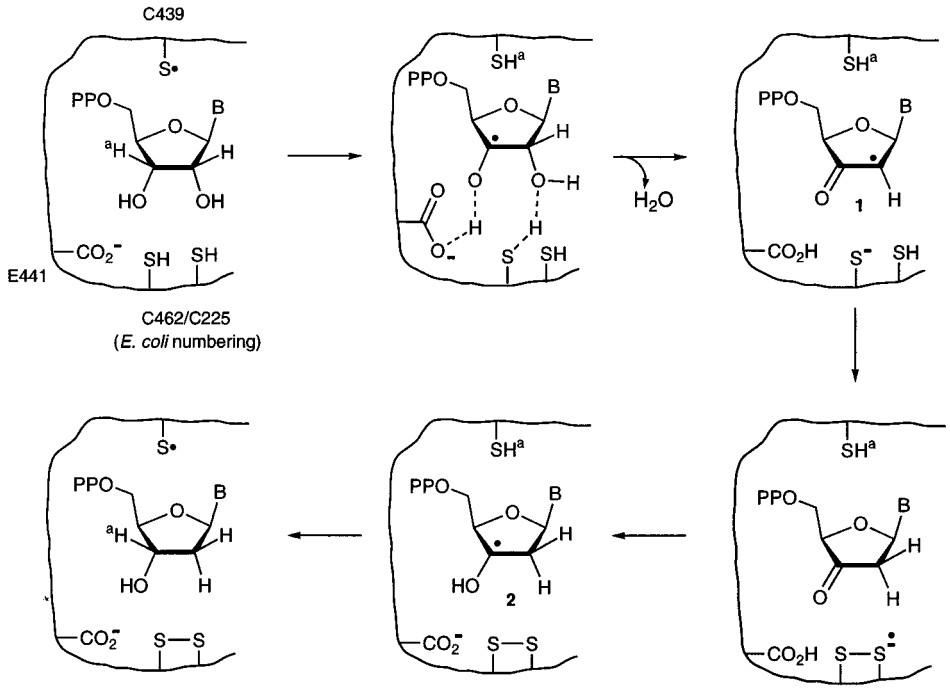


Figure 2. Classification of the various ribonucleotide reductases according to the cofactors used in catalysis. It has been shown for class I and II RNRs, and proposed for class III and IV RNRs, that the function of all of these cofactors is to generate a thiyl radical that initiates nucleotide reduction.

mechanism for expression,[67] while exhibiting similar allosteric regulation.[62,68] Consequently, this class has been subdivided into class Ia (*nrdAB* genes) and class Ib (*nrdEF* genes). The class II reductases, discovered by Blakley[69] and Beck,[70] utilize AdoCbl. Class III enzymes (*nrdDG* genes), first reported by Barlow,[71] and studied in detail in the laboratories of Fontecave, Eliasson, and Reichard, is found in *E. coli* grown under anaerobic conditions.[72] This class of enzymes employs a glycyl radical and an FeS cluster. Finally class IV reductases, discovered by Auling and Follmann in *coryneform* bacteria,[73,74] possess a putative dimanganese cluster and a protein radical that recent studies suggest is a tyrosyl radical.[75] An interesting development from an evolutionary point are the recent independent discoveries by Tauer and Benner[76] and by Fontecave and co-workers[77] of two related ribonucleotide reductases from archaebacteria that appear to be a conglomerate of class I, II, and III enzymes. Both proteins (from *Thermoplasma acidophila* and *Pyrococcus furiosus*) are related by common ancestry to the class I enzymes conserving the catalytic domain for nucleotide reduction, but use AdoCbl instead of a tyrosyl radical/diferric cofactor to initiate catalysis.[76,77] Furthermore, the enzymes have sequence homology at their N-terminal domain with the class III proteins.

The function of the different cofactors has been shown in the case of class I, II, and III RNRs to initiate a radical dependent nucleotide reduction process which is mechanistically quite similar for all the enzymes thus far examined.[40] This review will focus specifically on the formation and function of the protein radicals in each class, and not on the nucleotide reduction process itself, which has been extensively examined in other reviews.[56,58,78] However, for a framework to evaluate the function of radicals in these reactions, the working model for the nucleotide reduction process is presented in Scheme 1. Catalysis is initiated by hydrogen atom abstraction from the 3′ position of the substrate by the thiyl radical generated by one of the metallo cofactors (Figure 1).[79,80] Theoretical studies have suggested that the subsequent elimination of the 2′-hydroxyl group as water occurs simultaneously with deprotonation of the 3′-hydroxyl.[81] Alternatively, mechanisms involving either a radical cation or radical anion (i.e., general acid or general base catalysis, respectively) can be invoked in this step on the basis of model systems (for review see refs 56 and 82). Reduction of the intermediate α-keto radical **1** via oxidation of two cysteines to the corresponding disulfide then

Scheme 1



generates a 3′-deoxynucleotide radical **2**, which reabstracts the originally removed hydrogen atom from the third cysteine providing the product and regenerating the thiyl radical. This working model has been supported by the results of extensive investigation over the past two decades, including biochemical studies,[80,83−86] use of mechanism-based inhibitors,[52,87−98] site-directed mutagenesis,[99−104] model studies in solution,[105] and crystallographic studies.[28,106] However, until recently, no conclusive evidence had been obtained for the involvement of thiyl radicals and the intermediacy of substrate-based radicals. As discussed in the following two sections, such evidence is now available for class I and II RNRs.

IV. Class I Ribonucleotide Reductases

The best characterized reductases are those in class I, particularly the enzyme from *E. coli*.[40,55,56] They are composed of two homodimeric subunits: R1 and R2. R1 is the business end of the protein where nucleoside diphosphate reduction occurs (Scheme 1), and R2 is the subunit that contains the tyrosyl radical (located on residue 122 in *E. coli*) and metallo cluster (Figure 1A). Several sets of seminal experiments using EPR spectroscopy and isotopic labeling of R2 revealed that the doublet EPR signal (Figure 3, Table 1)[107] observed with wild-type enzyme is associated with a tyrosyl radical.[15,108,109] Electron nuclear double resonance (ENDOR) experiments[110,111] in conjunction with high-frequency (140 GHz) EPR studies[112] have resulted in a complete description of this species. The electron distribution over the atoms of the tyrosine radical is shown in Table 4. The large hyperfine interaction (∼20 G) that dominates the observed doublet signal at 9 GHz (Figure 3) originates from one of the two β-methylene hydrogens which is oriented with a dihedral angle of 33° with respect to the p_z orbital on C1 of the aromatic ring.[110] Smaller hyperfine interactions are associated with the protons in the 3 and 5 positions of the aromatic ring (Table 1).

After the initial discovery of a tyrosyl radical in ribonucleoside 5′-diphosphate reductase (RDPR) from *E. coli*, tyrosyl radicals have been detected in several other enzymatic systems, discussed in sections IX and X. Detailed examination of the characteristics of these radicals by EPR and ENDOR spectroscopies have allowed distinctions to be made between these systems. Comparison of the *g* values of the tyrosyl radical of RDPR with those of tyrosyl radicals in model peptides and the $Y_D^{\bullet}$ (the stable tyrosyl radical not involved in electron transfer) in PSII (see Tables 1 and 2) reveals that the values of g_x vary, while those for g_y and g_z are almost identical. The lower values of g_x in $Y_D^{\bullet}$ and in a single crystal of a tyrosyl radical compared to that of $Y_{122}^{\bullet}$ in RDPR have been interpreted to reflect stabilization of the nonbonding orbitals of the oxygen in the former radicals through electrostatic and hydrogen-bonding interactions.[112,113] This hypothesis is supported by theoretical studies and by ENDOR investigations of the tyrosyl radicals of RDPR and PSII which indicate that the former is present as a neutral non-hydrogen-bonded species, whereas the latter, while still a neutral radical, forms a hydrogen bond to proton donors in the protein.[110] Interestingly, these tyrosyl radicals display remarkably similar distributions of the unpaired spin density (Table 4) given that they are located in different environments, and are proposed to participate in distinct types of chemistry (see sections IV.B, IX.B, and X.B).

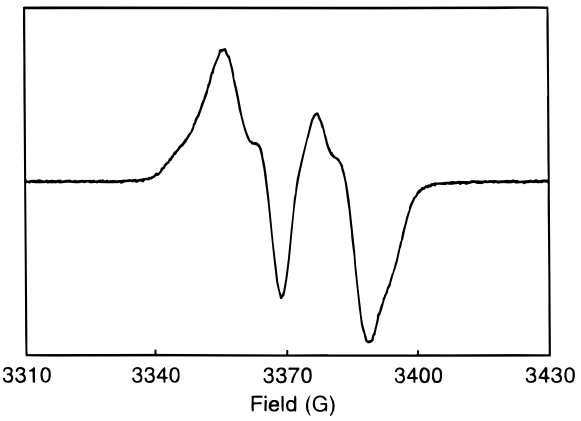
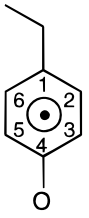


Figure 3. EPR spectrum of the tyrosyl radical in ribonucleotide reductase from *E. coli*. Conditions: microwave frequency, 9.428 GHz; temperature, 20 K; power, 10 μW, modulation amplitude, 4 G; modulation frequency, 100 kHz; time constant, 0.126 s.

Table 4. Spin Density Distribution in Various Tyrosyl Radicals



radical	source	C_1	C_2/C_6	C_3/C_5	C_4	O	H bonding	θ	ref(s)
$Y_{122}^{\bullet}$	RDPR	0.38	−0.08	0.25	−0.05	0.29	no	33°	111
$Y_D^{\bullet}$	PSII	0.37	−0.07	0.24	−0.01	0.26	yes		378
$Y_Z^{\bullet}$	PSII	0.37	−0.07	0.26	−0.01	0.26	no		368
$Y^{\bullet}$	crystal	0.32	−0.06	0.25		0.30	yes	45	*a*
$Y^{\bullet}$	solution	0.34	−0.07	0.24	−0.02	0.26		60	111, *b*

[a] Fasanella, E. L.; Gordy, W. *Proc. Natl. Acad. U.S.A.* **1969**, *62*, 299−304. [b] Sealy, R. C.; Harman, L.; West, P. R.; Mason, R. P. *J. Am. Chem. Soc.* **1985**, *12*, 3401−3411.

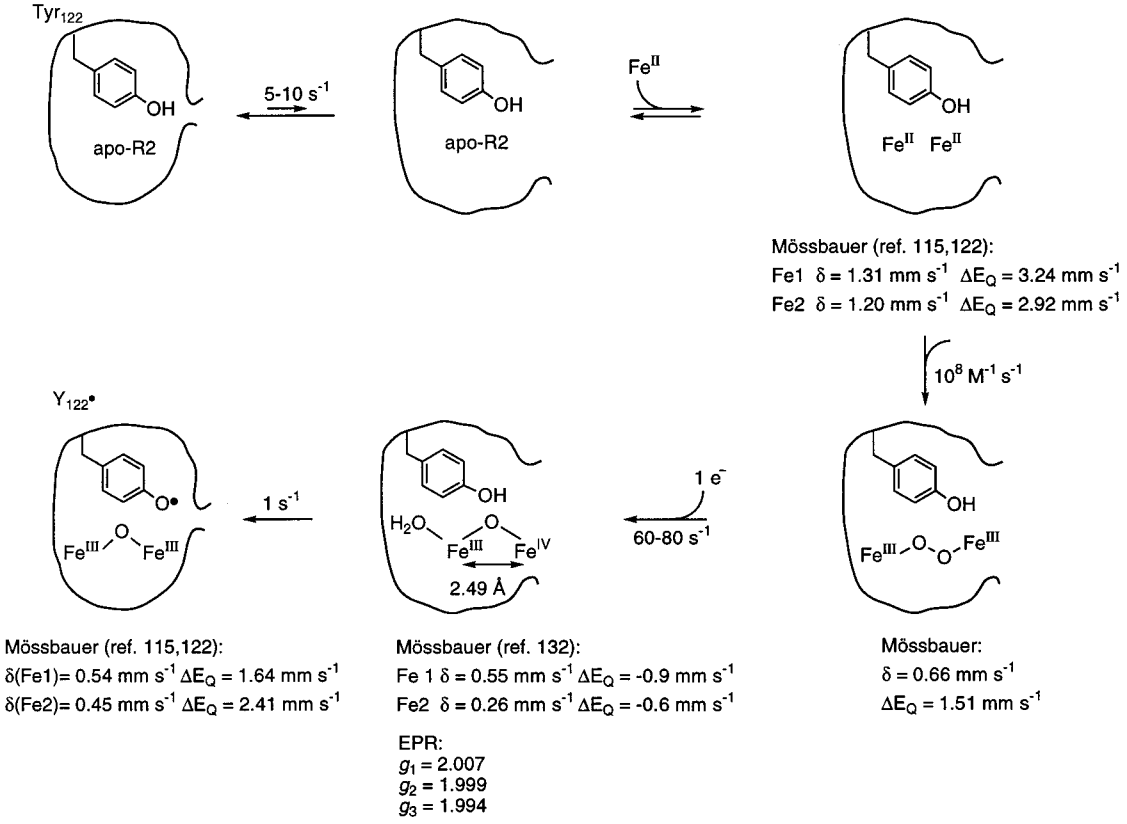
A. Formation of the Tyrosyl Radical

In 1973 Atkin et al. reported the important observation that incubation of apo-R2 with Fe^{II} and O_2 in the presence of ascorbate led to the formation of the diferric tyrosyl radical cofactor.[114] The structure of the metal cluster of met-R2 (R2 in which the tyrosyl radical is reduced) is shown in Figure 1A.[28] Spectroscopic methods including Mössbauer,[114,115] resonance Raman (RR),[116] and magnetic susceptibility measurements[117] revealed that the final diiron cluster is composed of two high-spin irons antiferromagnetically coupled ($J = -108$ cm^{-1})[114,118] through a μ-oxo and a carboxylate bridge. In the X-ray crystal structure of R2 the oxygen of the tyrosine (Tyr122 in *E. coli*) is 5.3 Å from the closest iron, and buried inside the protein 10 Å from the nearest surface.[28,119]

The mechanism of assembly of the cofactor from apo-R2, Fe^{II}, O_2, and reductant, and more recently from the diferrous form of R2,[120] has been studied using SF−UV−vis spectroscopy and RFQ-EPR,[27,121,122] Mössbauer,[120,122,123] ENDOR[124] and extended X-ray absorption fine structure (EXAFS) experiments.[125] Collectively, they have suggested the model for the assembly process outlined in Scheme 2. Apo-R2 is proposed to undergo a slow conformational change to a form that can bind two ferrous ions at a site close to Tyr122.[120] The diferrous cluster then very rapidly reacts with O_2 to produce a putative short-lived peroxo intermediate which is reduced to a paramagnetic intermediate designated X. The structure of the putative peroxo species is unknown but can be drawn as an μ-1,2-peroxo compound based on model systems[126−129] and the observation of a potentially similar intermediate in studies of methane monooxygenase from *M. capsulatus* (Bath).[130,131] Mössbauer studies suggest that the source of the reducing equivalent required to reductively activate the putative peroxo intermediate can be supplied by Fe^{II} from multiple locations or by ascorbate in vitro.[122] The source of the reducing equivalent in vivo is unknown. Intermediate X, the structure of which is currently the topic of extensive investigation,[124,125,132] is then converted into the diferric cluster by oxidation of Tyr122 to the radical form. This assembly of the tyrosyl radical−diferric cluster cofactor represents an example of an emerging general theme: the self-catalytic biosynthesis of a cofactor which requires a metal and O_2 (for another, putative, example see section XII.A).

The structure of X was originally formulated, on the basis of its EPR and Mössbauer spectra, as a diferric radical species in which two high-spin irons were coupled to a spin $^1/_2$ radical.[123,133] This formulation assumed that both irons were isotropic. Recent further characterization of this intermediate using isotopic labeling with ^{57}Fe and $^{17}O_2$, $H_2{}^{17}O$, and RFQ-ENDOR spectroscopy,[124,132] has indicated that one of the irons is anisotropic, requiring a reinterpretation of our earlier studies. At present the best formulation for X is as an Fe^{4+}/Fe^{3+} species in which both irons are high spin, containing one oxygen derived from O_2 in the form of a μ-oxo bridge, and the second oxygen from O_2 as a water or hydroxide ligand to the Fe^{3+}.[134,135] This water (hydroxide) ligand originally derived from O_2 can exchange with solvent. Interestingly, a short Fe−Fe distance of 2.5 Å has recently been determined for X by RFQ-EXAFS studies.[125]

Scheme 2



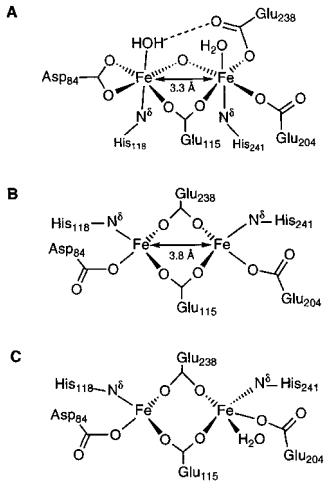


Figure 4. (A) The structure of the diferric cluster in R2 from *E. coli* determined by X-ray crystallography,[28] (B) the diferrous form of the cofactor determined by X-ray crystallographic studies,[138] and (C) proposed structure of the diferrous form of R2 based on CD and MCD studies.[137]

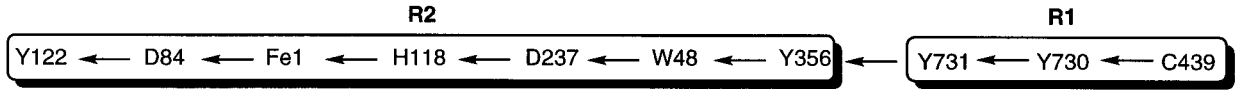
Conflicting structural information on the diferrous form of R2 has recently been reported from studies using X-ray crystallography,[136] Mössbauer,[115] circular dichroism (CD),[137] and magnetic circular dichroism (MCD) spectroscopies.[137] Comparison of the X-ray structures of the diferrous and diferric forms of R2 indicate that they have significantly different iron coordination.[138] The two irons in reduced R2 are highly symmetrical with an unusually low coordination number of four, and the water and oxo ligands observed in the diferric cluster are absent (Figure 4A,B). Furthermore, Glu115 and Glu238 carboxylates bridge between the irons in a bidentate mode in diferrous-R2, whereas only Glu115 bridges the two irons in diferric-R2 with Glu238 coordinated to a single iron in a monodentate mode. This "carboxylate shift"[139] was first identified in model systems and has recently been observed in the reduced and oxidized forms of methane monooxygenase.[140] In contrast Mössbauer,[115] CD, and MCD studies of the reduced form of R2 have been interpreted in terms of one five- and one four-coordinate Fe^{II} (Figure 4C).[137] The different methods by which the diferrous-R2 was generated in these studies may provide an explanation for these discrepancies. In the X-ray structure work, the diferrous form was obtained by exposure of a crystal of met-R2 (diferric cluster) to intense synchrotron X irradiation at 100 K and subsequent warmup to room temperature.[136] Conversely, the diferrous form of R2 for the CD and MCD studies was produced via either reduction of the diferric form or by addition of Fe^{II} to apo-R2. Thus, further studies must be carried out to reconcile these differences. What is most amazing about the system is the structural reorganization of the irons during cofactor assembly. They are 3.8 Å apart in diferrous R2, collapse to 2.5 Å in intermediate X, and reorganize to 3.3 Å in diferric R2. The chemical basis for this process and for the reactivity of intermediate X is a major focus of attention.

B. Function of the Tyrosyl Radical

Studies of Ehrenberg and Reichard in 1972 revealed that one electron reduction of the tyrosyl radical of the dinuclear iron cofactor to tyrosine

nrdA (class Ia)				
HSV 1	IAGSNLCTEI	VHP.....	.....ASK	RSSGVCNLGS
HSV 2	IAGSNLCTEI	VHP.....	.....SSK	RSSGVCNLGS
Equine HSV 1	IMGSNLCTEI	IQH.....	.....ADE	TQNGVCNLAS
Equine HSV 2	ISYANLCAEV	VQQ.....	.....PHE	FT.STCNLAN
Gallid HSV	IAGSNLCTEI	IQK.....	.....TNE	STNGVCTLAS
Epstein-barr virus	MNAANLCAEV	LQP.....	.....SRK	SV.ATCNLAN
Varicella virus	ITGSNLCTEI	VQK.....	.....ADA	HQHGVCNLAS
Vaccinia-Zoster virus	IKCSNLCTEI	IQ.....	....YADA	NEVAVCNLAS
Variola virus	IKCSNLCTEI	IQ.....	....YADA	NEVAVCNLAS
H. Influenzae	VRQSNLCLEI	ALPTKP....	LQHINDEN	GEIALCTLSA
Mouse	IKCSNLCTEI	VE.....	....YTSK	DEVAVCNLAS
Human	IKCSNLCTEI	VE.....	....YTSK	DEVAVCNLAS
S. pombe	IRSSNLCTEI	VE.....	....YSSP	DEVAVCNLAS
S. cerevisiae	IKSSNLCCEI	VE.....	....YSSP	DETAVCNLAS
P. falciparum	IKCSNLCCEI	IE.....	....YTSP	DEVAVCNLAS
S. typhimurium	VRQSNLCLEI	ALPTKP....	LNDVNDEN	GEIALCTLSA
African swine fever	ITNSNLCIEV	TIPCW.....	....EGDK	AEQGVCNLAA
Bacteriophage T4	IRQSNLCCEI	AIPT.....	.NDVNSPD	AEIGLCTLSA
M. tuberculosis	ITHSNLCSEI	LQVSTPSLFN	EDLSYAKV	GKDISCNLGS
E. coli	VRQSNLCLEI	ALPTKP....	LNDVNDEN	GEIALCTLSA
Synechocystis sp.	PGTNLCCES	FSNVTP	G	QDAHCCNLVS
E. coli numbering				
consensus	NLC E			C L
nrdE (class Ib)				
M. genitalium	IVMSNLCSEI	VQPSLPSEFY	SDLTFKKV	GSDISCNLGS
S. typhimurium	INMSNLCSEI	LQVNSASRYD	DNLDYTHI	GHDISCNLGS
B. subtilis	VKFSNLCSEV	LQASQVSSYT	DYDEEDEI	GLDISCNLGS

Figure 5. Sequence alignment of the R1 subunit of class I RNRs from various organisms showing two of the three fully conserved cysteine residues and the conserved glutamate in the active site. *nrdA* are class Ia genes; *nrdE*, class Ib genes.



	R2	R1
P. falciparum	EACFWTAE---LAFFAASDGI---IHSETYSLLI---LISRDEGLHT---VADYQKSGVM---TGAYYLRTQA	
HSV 1	ILNRWLET---FAFLSAADDL---AHSRVYNIIQ---LISRDEAVHT---STSYAGAVVN---TGMYYCKVRK	
Equine HSV 1	VANRWLDT---FAFLSAADDL---THSRVYSAIQ---LISRDEAIHT---STAYSGTLIN---TGMYYCKIKK	
Equine HSV 2	WQNRWFPS---FSFLAMAEKL---IHGKVYANIL---YISRDEWVHT---SSDYTTAVDN---TIMYYCRIQK	
Varicella	ISNRWLES---FTFLSAADDL---VHARVYSQIQ---LISRDEAIHT---STSYAGTVIN---TGMYYCKIRK	
Gallid HSV	IMNRWTET---FTFLSAADDL---VHGETYANIL---YISRDELLHT---SSDYTMLVVD---TIMYYCRIEK	
Vaccinia-zoster	EASFWTVE---LAFFAASDGI---IHSEMYSLLI---LISRDEGLHC---VGEYQKMGVM---TGMYYLRTKP	
Mouse	EASFWTAE---LAFFAASDGI---IHSEMYSLL.---LISRDEGLHC---VGEYQRMGVM---TGMYYLRTRP	
Human	EASFWTAE---LAFFAASDGI---IHSEMYSLL.---LISRDEGLHC---VGEYQRMGVM---TGMYYLRTRP	
S. pombe	EASFWTAE---LAYFAASDGI---IHSETYSLLL---LICRDEGLHT---VSDYQIAGVM---TGMYYLRTMA	
S. cerevisiae	EASFWTAE---LAFFAASDGI---IHSETYSLLI---LICRDEGLHT---VSDYQKAGVM---TGMYYLRTQA	
Bacteriophage T4	ISFFWRPE---LKYQSLLDSI---IHSRSYTHIM---FIARDEQLHL---LSSYLVAQID---N.FYYHNTRD	
S. typhimurium[a]	TSNFWLPE---FTGLTLLDTI---VHARSYSSIF---LIIRDEAVHG---GSSYVMGKTV---S.LYYIRLRQ	
M. genitalium[a]	VQNFWLPE---FTGLTLLDTI---VHARSYGTIF---LILRDKVIHN---GSSYVMGVSE---S.IYYVRVRQ	
B. subtilis[a]	VKQFWLPE---LAGLTLLDTE---VHAKSYSNIF---LILRDEAIHG---GNGYKKATVE---T.IYYARTKD	
E. coli	LSFFWRPE---LKYQTLLDSI---IHSRSYTHII---FIARDEALHL---VSSYLVGQID---T.LYYQNTRD	
	W D H Y D Y YY	
E. coli numbering	48 84 118 122 237 356 730/731	

Figure 6. Sequence alignment showing the residues proposed by Uhlin and Eklund[106] to form the coupled electron/proton-transfer pathway from the tyrosyl radical on R2 to cysteine 439 on R1. Superscript a's indicate *nrdE* (class Ib) genes.

resulted in complete loss in the ability of RDPR to mediate nucleotide reduction.[107] While it was initially thought that the active site for substrate reduction was at the interface of the two subunits, biochemical studies,[56] and more recently, crystallographic studies of R1 by Uhlin and Eklund revealed that the active site is located exclusively in R1 and is buried.[106,141,142] Docking of the two crystal structures of R1 and R2 from *E. coli* suggests that the tyrosyl radical on R2 is 35 Å from the active site in R1.[106] Thus, the tyrosyl radical *cannot* be directly involved in the reduction process. Extensive site-directed mutagenesis studies on R1 from *E. coli* have suggested that the fully conserved Cys439 is essential for catalysis (Figure 5).[102] The hypothesis was therefore put forth that the function of the tyrosyl radical on R2 is to generate a thiyl radical on Cys439 of R1 which then initiates the nucleotide reduction process (Scheme 1).[40,100] With the structures of both R1 and R2 available, a possible electron transfer pathway via a number of fully conserved residues on both subunits has been proposed as outlined in Figure 6.[106,143] Site-directed mutagenesis studies in which some of these residues were mutated (D84A-R2,[144] H118A-R2,[144] Y356A-R2,[145] W103Y-R2 and D266A-R2 from mouse,[146] and the R1 mutants Y730F and Y731F[147,148]) indicate nucleotide reduction activity at the level of contaminating wild-type in these mutant proteins. (Since R1 and R2 mutant proteins from *E. coli* are overexpressed in *E. coli*, contaminating wild-type proteins prevent determination of specific activities that are <3% of wt.[102]) Efforts to detect transient reduction of the tyrosyl radical or transient formation of radical intermediates in the mutants in the presence of the normal substrate have failed thus far. Furthermore the distance between these two residues (35 Å), the observed rates of electron transfer in proteins,[149] and the observed rate constant for nucleotide reduction ($\sim$4−5 s^{-1}) require that intermediates are generated. Thus while the hypothesis of an electron-transfer pathway is appealing, there is no conclusive evidence supporting it at this time.

Indirect support for the model comes from recent studies of Cooperman and co-workers studying the mammalian equivalent of Y122F-R2 in *E. coli*.[150] They showed that despite the inability of this mutant protein, Y177F-R2, to generate a Tyr• at that position, it was capable of catalyzing nucleotide reduction at 1/10 the rate of the wt-RDPR! The diiron cluster in the mammalian RDPR is much more labile than the cluster in the *E. coli*. Thus, under aerobic conditions in the presence of reductant the cluster was proposed to assemble and disassemble through intermediate X. This intermediate is unable to oxidize Phe122, and in a much slower process is proposed to oxidize a second Tyr residue. In fact a transient species with UV−vis characteristics of a Tyr• has been detected during the investigation of the assembly of the diferric cluster in *E. coli* Y122F,[27] and recent mutagenesis studies of the Stubbe[151] and Sjöberg[152] laboratories have suggested that this transient might be associated with Tyr356. This tyrosine is proposed to be on the surface of R2 that interacts with R1, is conserved in all R2s sequenced to date, and is proposed by Cooperman to act as an electron conduit between Cys439 in R1 and the cofactor center in R2. The facility with which the cofactor in mammalian R2 assembles and disassembles could allow generation of Y_{356}• under multiple turnovers and hence allow detection of dNDP formation.

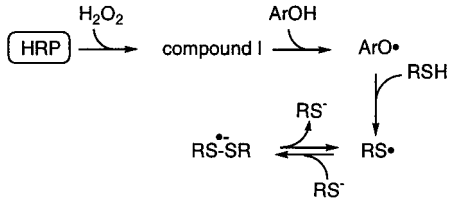
Conclusive evidence for a thiyl radical on Cys439 has thus far not been obtained for class I RNRs. However, as described in detail in section V.A, there is now compelling evidence for a thiyl radical for class II RNRs. The chemical similarities of substrate reduction, and the sequence similarity in the region of the putative cysteine radicals (Figure 7) between these two classes of enzymes, provide by analogy

	gene	source	sequence
class Ia	*nrdA*	*E. coli*	RQSNLCLEIA
class Ia	*nrdA*	Human	KCSNLCTEIV
class Ia	*nrdA*	HSV 1	AGSNLCTEIV
class Ia	*nrdA*	Bacteriophage T4	RQSNLCCEIA
class Ib	*nrdE*	*S. typhimurium*	NMSNLCSEIL
class Ib	*nrdE*	*B. subtilis*	KFSNLCSEVL
class II		*L. leichmannii*	EGTNPCGEIS
class II		*T. acidophila*	ESTNPCGEQP
class II		*P. furiosus*	RATNPCGEEP
class III	*nrdD*	*E. coli*	PSENLCDRFC
class III	*nrdD*	*H. influenzae*	PSENLCDRFC
class III	*nrdD*	Bacteriophage T4	PAENLCYRFC

Figure 7. Amino acid sequence alignment for some selected members of the different classes of RNRs. The alignment for the class III RNRs is speculative at present. The cysteine residues with a similar sequence environment as Cys439 (*E. coli*) or C408 (*L. leichmannii*), the residues proposed to initiate nucleotide reduction in the form of a thiyl radical,[102,104] are underlined.

strong evidence that a thiyl radical is generated on Cys439 via a coupled electron and proton-transfer mechanism to the tyrosyl radical. The reduction potentials in Table 5 indicate that while this electron transfer is thermodynamically uphill, given the perturbations of reduction potentials imposed by protein environments it is certainly within the realm of feasibility. In addition, this reaction would be thermodynamically favorable if Cys439 were present as a thiolate prior to electron transfer (Table 5). Mason and co-workers have inadvertently provided us with a model for this process. They reported that when compound I of horseradish peroxidase (HRP) was reduced by acetaminophen to generate phenoxyl radical in the presence of glutathione and/or cysteine, disulfide radical anions were detected.[153] These species are proposed to arise from rapid (10^8 M^{-1} s^{-1}) reaction of a thiyl radical with a thiolate (Scheme 3).

Scheme 3



C. Thiyl Radical Involvement in Catalysis

The acceptance of the involvement of thiyl radicals as radical chain initiators has received much resistance from the chemical community. In part this is

Table 5. Redox Potentials for One-Electron Processes That Are Relevant to the Enzymatic Systems Discussed in This Review

entry	oxidant	reductant	E_m vs NHE (V)[a]	ref(s)
1	$(RS)_2^{\bullet-}$	2 RSH	1.72	215
2	$^{-}O_2C^{\bullet}$	HCO_2^-	1.49	215,*b*
3	$RS^{\bullet}$	RSH	1.33 (1.42)[c]	215
4	$Gly^{\bullet}$	GlyH	1.22	*d*
5	$P_{680}^{\bullet+}$	P_{680}	1.17[e]	*f*
6	$TrpH^{\bullet+}$	TrpH	1.15	431
7	compound II (CcP)	CcP (ferric)	1.08 [g]	*h*
8	$Trp^{\bullet}$	TrpH	1.05	430,*i*
9	$ROO^{\bullet}$	ROOH	1.02−1.11	*j*
10	propenyl$^{\bullet}$	propene	0.96	*b*
11	compound (II) (HRP)	HRP (ferric)	0.96[k]	*l*
12	$TyrO^{\bullet}$	TyrOH	0.94	430,*i*
13	compound I (HRP)	compound II (HRP)	0.94[k]	*l*
14	$O_2^{\bullet-}$	H_2O_2	0.89	*m*
15	$RS^{\bullet}$	RS^-	0.77 (0.84)[c]	*n*
16	pentadienyl$^{\bullet}$	pentadiene	0.60	*b*
17	Cu^{II} DβH (type 2)	Cu^{I}-DβH	0.31−0.38	*o,p*
18	Cu^{II} laccase (type 2)	Cu^{I}-laccase	0.37	*q,r*
19	Cu^{II} plastocyanin (type 1)	Cu^{I}-plastocyanin	0.36	*s*
20	Cu^{II} amicyanin (type 1)	Cu^{I}-amicyanin	0.294	*t*
21	Fe^{III} cytochrome *c*	Fe^{II}-cytochrome *c*	0.25	*u*
22	Fe^{III} cytochrome *c*551i	Fe^{II}-cytochrome *c*551i	0.19	*t*
23	O_2	$O_2^{\bullet-}$	−0.16	*m*
24	Fe^{III} CcP	Fe^{II} CcP	−0.194	*v*
25	Fe^{III} protoporphyrin IX	Fe^{II}-protoporphyrin IX	−0.226	*u*
26	$P_{680}^{\bullet+}$	P_{680}*	−0.7	*f*
27	RSSR	$(RS)_2^{\bullet-}$	−1.60	215
28	CO_2	$^{\bullet}CO_2^-$	−1.85	216

[a] At pH 7 unless indicated otherwise. [b] Koppenol, W. H. *FEBS Lett.* **1990**, *264*, 165−167. [c] Calculated. [d] Zhao, R.; Lind, J.; Merényi, G.; Eriksen, T. E. *J. Am. Chem. Soc.* **1994**, *116*, 12010−12015. [e] From studies on the $P_{680}^{\bullet+}$/pheophytin redox couple at pH 10.5. [f] Klimov, V. V.; Allakhverdiev, S. I.; Demeter, S. Krasnovsky, A. A. *Dokl. Akad. Nauk. USSR* **1979**, *249*, 227−230. [g] At pH 5.26. [h] Purcell, W. L.; Erman, J. E. *J. Am. Chem. Soc.* **1976**, *98*, 7033−7037. [i] Harriman, A. *J. Phys. Chem.* **1987**, *91*, 6102−6104. [j] Jovanovic, S.; Jankovic, I.; Josimovic, L. *J. Am. Chem. Soc.* **1992**, *114*, 9018−9021. [k] At pH 6.5. [l] Hayashi, Y.; Yamazaki, I. *J. Biol. Chem.* **1979**, *254*, 9101−9106. [m] Sawyer, D. T. In *Oxygen Complexes and Oxygen Activation by Transition Metals*; Martell, A. E., Sawyer, D. T., Eds.; Plenum: New York, 1988. [n] Armstrong, D. A. In *Sulfur-Centered Reactive Intermediates in Chemistry and Biology*; Chatgilialoglu, C., Asmus, K.-D., Eds.; Plenum Press: New York, 1990; pp 121−134. [o] Walker, G. A.; Kon, H. Lovenberg, W. *Biochim. Biophys. Acta* **1977**, *482*, 309−322. [p] Ljones, T.; Flatmark, T.; Skotland, T.; Petersson, L.; Bäckström, D.; Ehrenberg, A. *FEBS Lett.* **1978**, *92*, 81−84. [q] Reinhammer, B. R. M.; Vänngård, T. I. *Eur. J. Biochem.* **1971**, *18*, 463−468. [r] Reinhammer, B. R. M. *Biochim. Biophys. Acta* **1972**, *275*, 245−259. [s] Wood, P. M. *Biochim. Biophys. Acta* **1974**, *357*, 370−379. [t] Gray, K. A.; Knaff, D. B.; Husain, M.; Davidson, V. L. *FEBS Lett.* **1986**, *207*, 239−242. [u] Loach, P. A. In *Handbook of Biochemistry*; pp J27−37. [v] Conroy, C. W.; Tyma, P.; Daum, P. H.; Erman, J. E. *Biochim. Biophys. Acta* **1978**, *537*, 62−69.

Table 6. Rate Constants for Hydrogen Atom Abstraction from Alcohols, Dienes, and Formate by Various Thiyl Radicals

radical[a]	H donor	k (M^{-1} s^{-1})	ref
GS•	2-propanol	1.2×10^4	159
GS•	linoleic acid	0.8×10^7	*b*
GS•	arachadonic acid	3.1×10^7	*b*
DTT•	2,3-dimethyltetrahydrofuran	5×10^3	164
Cys•	2-propanol	2.0×10^4	159
Cys•	linoleic acid	0.6×10^7	*b*
Cys•	arachadonic acid	1.6×10^7	*b*
RS•	HCO_2^-	$\geq 3.6 \times 10^4$	216

[a] Abbreviations GS•, glutathionyl radical; DTT•, dithiothreitol thiyl radical; Cys•, cysteinyl radical; RS•, thiyl radicals of penicillamine or β-mercaptoethanol. [b] Schöneich, C.; Asmus, K.-D.; Dillinger, U.; von Bruchhausen, F. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 113.

Table 7. Gas-Phase Bond Dissociation Energies

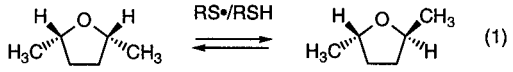
bond	dissociation energy (kcal mol^{-1})
H−OH	119
H−OR	104
H−COO^-	94.8,[216] 92[a]
H−CH(R)OH	93
H−NR_2	91
H−SR	91
H−$COH(R)_2$	91
H−OOR	88−92[b]
H−OPh	86
H−CH(R)Ph	85
H−CH_2O^-	84[161]
H−CH(R′)C=CR_2 (allylic)	81−86
H−$CH(C{=}CR_2)_2$ (bisallylic)	76−82
RS−SR	72
Co−C (AdoCbl)	30[c]

[a] Koppenol, W. H. *FEBS Lett.* **1990**, *264*, 165−167. [b] Jovanovic, S.; Jankovic, I.; Josimovic, L. *J. Am. Chem. Soc.* **1992**, *114*, 9018−9021. [c] Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *108*, 4820. This value was determined for adenosylcobalamin with the benzimidazole nitrogen as its axial ligand (i.e., "base on").

related to the known propensity of RSH compounds to quench carbon-centered radicals with diffusion controlled rate constants (10^8 M^{-1} s^{-1}).[154,155] Three factors regarding this quenching process, based on chemical evidence, have been under appreciated. The first is that the S−H homolytic bond dissociation energy (BDE) is between 88 and 91 kcal mol^{-1}.[156,157] Thus, these radicals are thermodynamically capable of abstraction of a hydrogen atom from bisallylically activated positions (82 kcal mol^{-1})[158] or the hydrogen of a deoxyribose sugar (BDE for other secondary alcohols or ethers: 91 kcal mol^{-1} for 2-propanol, 92 kcal mol^{-1} for tetrahydrofuran).[159] The second is that thiyl radicals can abstract hydrogen atoms with rate constants of $\sim 10^4$ M^{-1} s^{-1} (Table 6), and thus their ability to do so is frequently masked by their ability to more rapidly donate a hydrogen atom. Third, if the hydrogen atom abstraction process is coupled to a second rapid irreversible process, then the initial reaction is pulled to the right, allowing the reaction to go to completion.[160]

In the case of RNRs hydrogen atom abstraction by a thiyl radical from the 3′ position of a ribonucleotide may not be up hill thermodynamically depending on the protonation state of the 3′-hydroxyl of the nucleotide (Table 7).[161] Even in the protonated form, where the reaction could be unfavorable by as much as 3 to 6 kcal mol^{-1}, several pieces of literature precedent suggest that loss of H_2O from the 2′ position of the 3′-nucleotide radical will be very rapid and irreversible, driving the hydrogen atom abstraction reaction to the right. First, base-catalyzed elimination of OH^- from α,β-dihydroxyalkyl radicals generated by pulse radiolysis of ethylene glycol and other 1,2-diols has been shown to occur rapidly with rate constants of 10^6 M^{-1} s^{-1}.[162] Recently, these model studies have been extended to chemically generated 3′-nucleoside radicals in Giese's laboratory, giving rate constants on the order of 10^6-10^7 s^{-1} for the elimination step.[105] Alternatively, acid-catalyzed elimination of H_2O from these type of radicals has also been reported with rate constants of 10^6-10^9 M^{-1} s^{-1}.[162,163] In addition to these acid, or base-catalyzed eliminations, theoretical studies by Zipse suggest that a concerted combination of both types of catalysis may be energetically most favorable.[81]

Von Sonntag and co-workers have shown in a model study that a thiyl radical generated by pulse radiolysis mediated the epimerization of *cis*-2,5-dimethyltetrahydrofuran to a mixture of cis and trans isomers. This process presumably occurred via reversible hydrogen atom abstraction (eq 1).[164] In



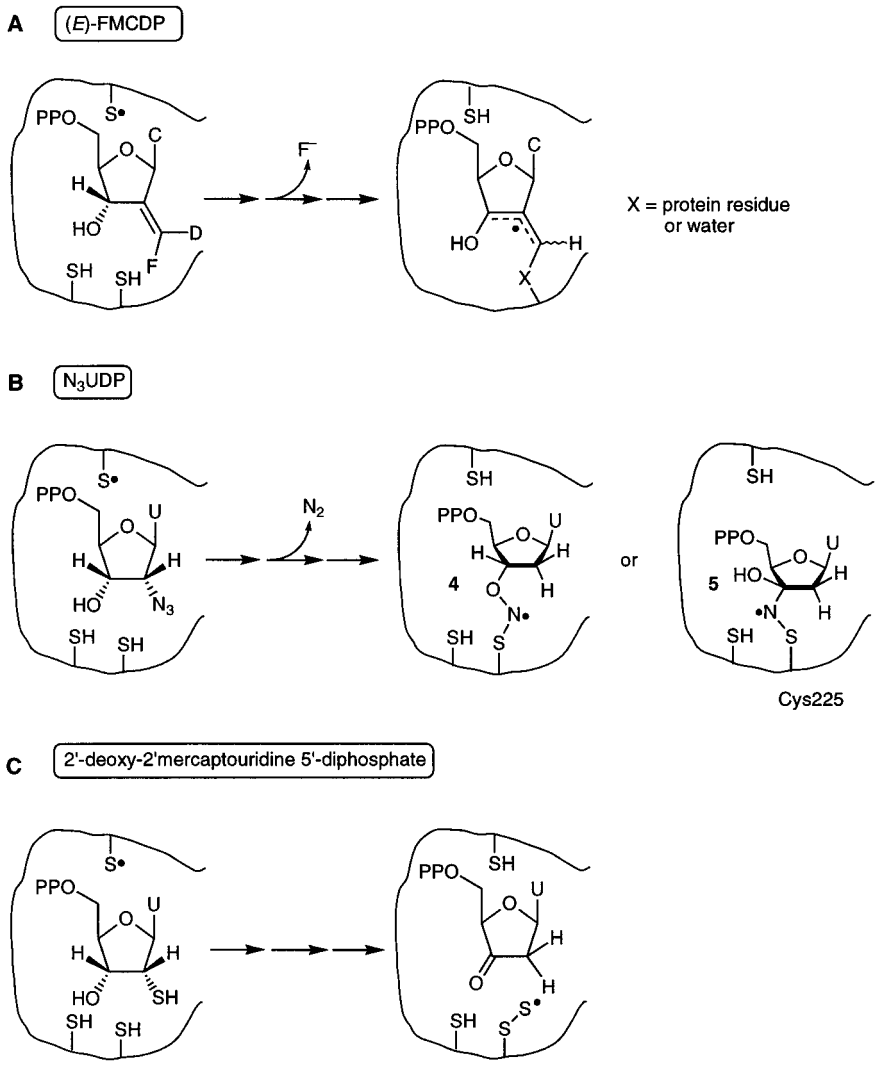
addition Schöneich and Asmus determined the rates of hydrogen atom abstraction from a variety of substrates including secondary alcohols by thiyl radicals (Table 6).[159] Thus these model systems and the extensive studies on class I and II RNRs provide compelling evidence for thiyl radical involvement in catalysis.

D. Evidence for Enzyme-Mediated Radical Chemistry Using Nucleotide Analogues

Direct evidence for 3′-hydrogen atom abstraction has recently been obtained using (*E*)-2′-(fluoromethylene)-2′-deoxycytidine 5′-diphosphate (*E*)-FMCDP, a mechanism-based inhibitor of the class I and II RNRs.[165] One and a half equivalents of this nucleotide analogue inactivate *E. coli* RDPR with the major mode of inactivation involving loss of the tyrosyl radical.[98] Concomitant with this loss is the generation of a new radical that is substrate derived on the basis of isotopic labeling experiments with deuterated inhibitor [6′-^{2}H]-(E)-FMCDP. High-frequency EPR studies and simulation of spectra, recorded at 9 and 140 GHz, have established that the signal is consistent with an allyl radical **3** in which the fluorine atom has been replaced with an active site residue (Scheme 4A). If this assignment is correct, this result provides the first support for abstraction of the hydrogen atom from the 3′ position.[166] Unambiguous proof of this hypothesis awaits synthesis of [2′-^{13}C]-(*E*)-FMCDP.

Two other mechanism-based inhibitors, 2′-azido-2′-deoxyuridine 5′-diphosphate (N_3UDP)[87] and 2′-deoxy-2′-mercaptouridine 5′-diphosphate,[54] have re-

Scheme 4



cently provided indirect support for the intermediacy of thiyl radicals in nucleotide reduction. As in the case of (*E*)-FMCDP, incubation of RDPR with N_3UDP leads to loss of the tyrosyl radical concomitant with the production of a new radical (Figure 8A).[52,91,92] While the large anisotropic hyperfine coupling observed in this signal (~25 G) had been unambiguously assigned to the interaction with a nitrogen nucleus in studies with [$^{15}N_3$]-N_3UDP,[92] the origin of a second, smaller hyperfine interaction (~5 G) had remained elusive despite extensive isotopic labeling experiments involving both inhibitor and R2.[91,167] Recent experiments with [3-{^{2}H}$_2$]cysteine-labeled R1 in conjunction with earlier studies using site-directed mutants have finally established that one of the β-protons of Cys225 is the source of this second hyperfine interaction (Figure 8B).[52] The EPR spectral parameters at 9 and 140 GHz and the products observed during the inactivation have allowed the tentative assignment of the new radical to structures **4** or **5** (Scheme 4B). Thus, these studies with N_3UDP indicate the feasibility of nucleotide reduction occurring via one electron chemistry involving thiyl radicals. Similar results have recently been reported for the inactivation of RDPR by 2′-deoxy-2′-mercaptouridine 5′-diphosphate. In this case, a transient new radical is formed which has been proposed to be associated with a perthiyl radical on the R1 subunit (Scheme 4C). This assignment is supported by isotopic labeling of all cysteines of R1, and by comparison of the EPR parameters with values previously reported for perthiyl radicals.[54]

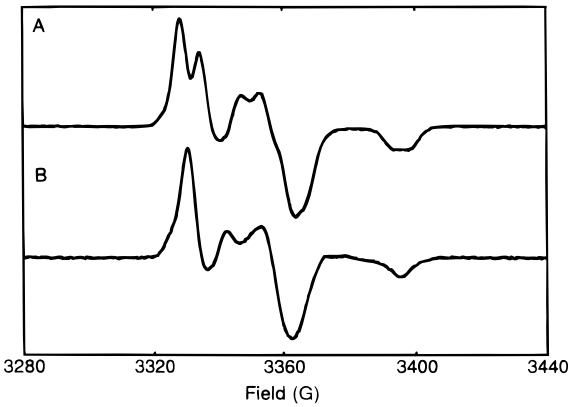


Figure 8. EPR spectra of the new radical produced after 3 min incubation of N_3UDP with R2 and (A) wt-R1, and (B) [3-{^{2}H}$_2$]cysteine labeled R1 from *E. coli*.[52] Conditions: temperature 101 K; microwave frequency 9.43 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 1.5 G; and time constant, 0.126 s.

The results with (*E*)-FMCDP, N_3UDP, and 2′-deoxy-2′-mercaptouridine 5′-diphosphate illustrate one important caveat of using mechanism-based inhibitors for the study of radical-based reactions mentioned in section II.D: the putative transient radical species that are observed in these hand-quench experiments are several steps removed from the chemistry that is informative for the understanding of the mechanism of turnover with normal substrate (Scheme 1). To detect the precursors to these radicals, rapid quench methods need to be attempted. However, it might well be that the precursor radicals are too reactive to trap.

E. Structure

Very recently, the long-awaited structure of the R1 subunit was presented by Uhlin and Eklund.[106,168] While the actual structure did not have a nucleotide bound, modeling studies revealed an active site shown in Figure 9. In this model Cys439 is poised to abstract the 3′-hydrogen atom from the β-face of the substrate. Cysteines 225 and 462, proposed to provide the reducing equivalents on the basis of biochemical studies[84,86] and site-directed mutagenesis,[100] are found as a disulfide in the structure. The only remaining group in the active site that could function as an acid/base catalyst is Glu441, located on the α-face of the substrate in the modeling studies. Recent site-directed mutagenesis studies suggest that this Glu is not essential for 3′-hydrogen atom abstraction.[148] The structural data require that Cys439 initiates the catalytic process. The experiments with (*E*)-FMCDP described above make a strong argument for its ability to catalyze 3′-hydrogen atom abstraction. Therefore, while the details of the nucleotide reduction process may never be completely understood, the data obtained in physical organic chemical studies using isotopically labeled nucleotides, with site-directed mutants,[100−102] and with nucleotide analogues that partition between turnover and inactivation,[56] have revealed that while this system is amazing, it is no longer confusing.

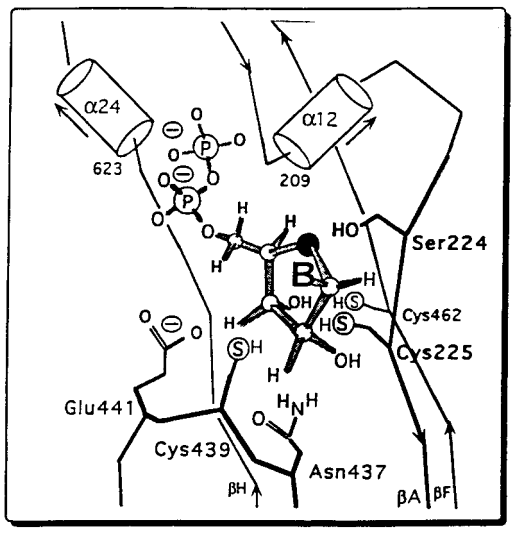


Figure 9. Active site of the R1 subunit of ribonucleotide reductase from *E. coli* showing Cys439, the putative thiyl radical in Scheme 1, and Cys225 and Cys462 that provide the reducing equivalents for nucleotide reduction.[141,142] (Reprinted from ref 106. Copyright 1994 Nature.)

V. Class II Ribonucleotide Reductases

As indicated in Figure 2, this class of reductases utilizes AdoCbl as a cofactor. In contrast to all other enzymatic reactions utilizing AdoCbl,[171] the nucleotide reduction process is not accompanied by a rearrangement reaction, and hence until recently this enzyme had been put in a category by itself. We will return to this point at the end of this section and address the question as to whether this is an appropriate classification.

The AdoCbl-dependent RNRs can be a diphosphate or triphosphate reductase (RTPR) and the one from *Lactobacillus leichmannii*, an RTPR, has been studied extensively by Blakley and co-workers in the early 1970s.[172] Recent developments in the field have now shown that their important work was ahead of its time. As outlined below, revisiting those studies within the framework of recently obtained information about the structure and function of class I and II RNRs has allowed us to understand the mechanism of the reduction process in some detail.

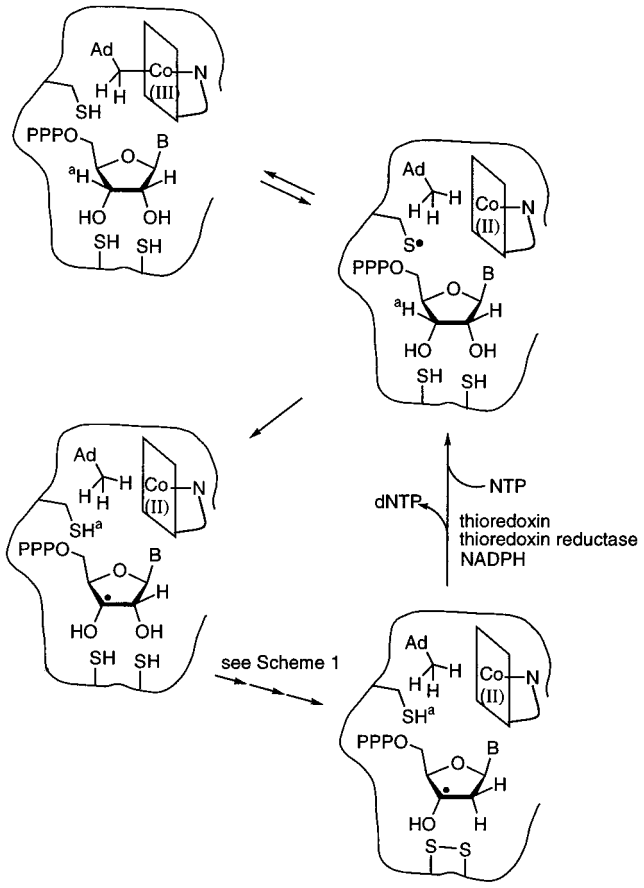
The AdoCbl-dependent reductase catalyzes three reactions: nucleotide (NTP) reduction, exchange of tritium from the 5′ position of [5′-^{3}H]-AdoCbl with solvent in the presence of allosteric effector and in the absence of substrate, and the slow conversion of AdoCbl to 5′-deoxyadenosine and cob(II)alamin.[48,49] As outlined subsequently, the exchange reaction has played a very important role in defining the catalytic capabilities of RTPR.

A. Exchange Reaction: Detection of the Elusive Thiyl Radical

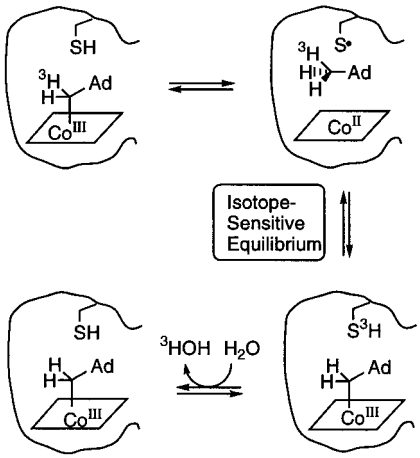
The exchange reaction is chemically a remarkable process. We have reexamined this reaction quantitatively, and as outlined below and in Scheme 5, recent compelling evidence suggests that it represents the first step in the nucleotide reduction process: the B_{12}-dependent formation of a thiyl radical. Studies of Abeles and Beck,[173] and Hogenkamp[174] and co-workers made the unusual observation that RTPR in the presence of reductant, allosteric effector and [5′-^{3}H]-AdoCbl catalyzes the washout of the extremely nonacidic 5′-hydrogens of AdoCbl to solvent. This reaction occurs in the absence of substrate, but does not require a reductant as previously reported.[174] The rate of ^3H_2O formation with dGTP as effector (0.3 s^{-1}) is 20% the rate of nucleotide reduction (1.5 s^{-1}). This means that the rate of hydrogen exchange can be estimated to be at least 9 s^{-1} given the expected selection effect for cleavage of a C−H bond over a C-^{3}H bond, and a statistical correction for the probability of abstracting a tritium over a hydrogen from three equivalent positions at the 5′ positions of dA in the reverse reaction (Scheme 6). Thus this exchange reaction is fast enough to be informative about the mechanism of catalysis.

The results from examining this exchange reaction using SF−UV/vis spectroscopy, rapid chemical quench experiments, and RFQ-EPR spectroscopy revealed that cob(II)alamin is formed with an apparent rate constant of 40 s^{-1}, as is 5′-dA. This apparent rate constant is obtained from fitting the data to a single

Scheme 5



Scheme 6



exponential. In fact, the reaction is more complex and a detailed kinetic analysis is in progress.[45] In the experiments using rapid acid quench techniques no other products were detected, establishing that 5′-dA or 5′-dA• is generated during this process. Thus the fate of the axial ligand has been defined for the first time. The SF data agreed well with those previously reported by Tamao and Blakley.[175] This process was also examined by EPR spectroscopic analysis and revealed a signal identical with that first reported by Orme-Johnson et al. in 1974 (Figure 10).[176] Isotopic labeling of RTPR with [3-{^{2}H}$_2$]-cysteine incorporated into all cysteine residues revealed a sharpening of the hyperfine features associated with cobalt, suggesting that the observed

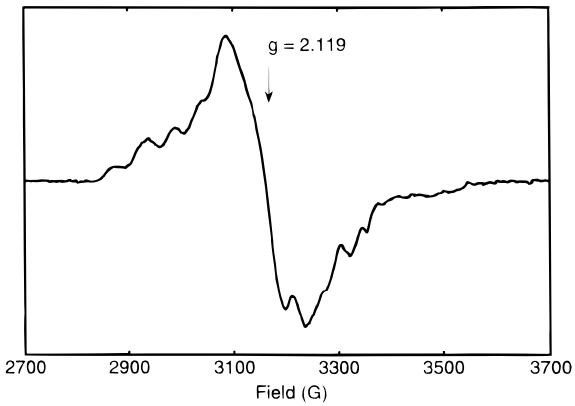


Figure 10. EPR spectrum of cob(II)alamin exchange coupled to the thiyl radical on Cys408 in class II RNR from *L. leichmannii*. Conditions: temperature, 100 K; microwave frequency, 9.41 GHz; microwave power, 10 mW; modulation frequency 100 kHz; modulation amplitude, 4 G; time constant, 1.3 s; and scan time 671 s.[18]

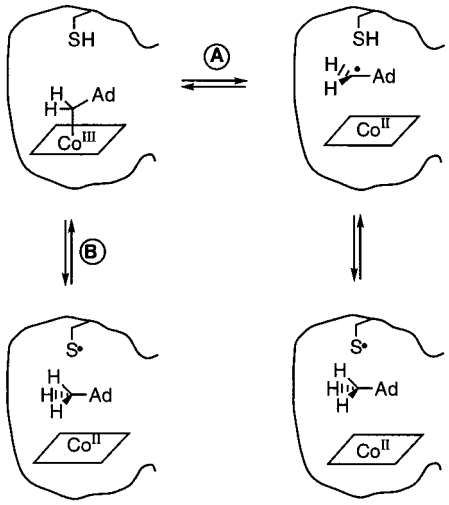


Figure 11. Two possible pathways for the generation of the exchange coupled cob(II)alamin and thiyl radical: (A) stepwise and (B) concerted.

signal results from a thiyl radical interacting with cob(II)alamin.[18] Recently, spectra of this species acquired at multiple frequencies (9 and 35 GHz) were simulated adequately for the first time by Gerfen et al.[177] revealing that the spectra are the result of exchange coupling between the two spin systems with $|J_{ex}| > 4$ cm^{-1} and a dipolar coupling with $D = 0.002$ cm^{-1}. These results establish that the function of AdoCbl is to generate a thiyl radical. Two pathways can be envisioned for this process: the first involves a stepwise mechanism and the second a concerted mechanism (Figure 11). Site-directed mutagenesis studies in which Cys408 (the putative radical) is replaced by serine, and the results of studies in ^2H_2O and using [5′-^{2}H] and/or [5′-^{1}H]-AdoCbl are best interpreted in terms of the concerted pathway.[45] When the mutant RTPR C408S was examined for cob(II)alamin formation under conditions identical with those of the exchange reaction discussed above, none was detected.[104] If the reaction were stepwise generating a distinct 5′-dA• (pathway A), then it might have been expected that cob(II)alamin would have been observed.

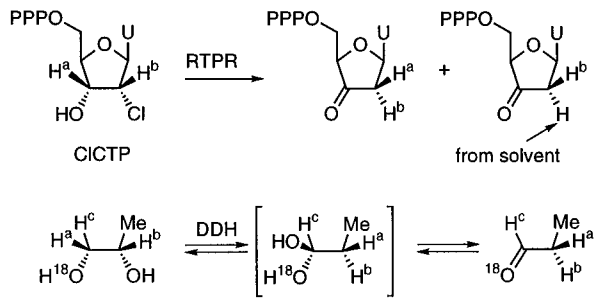
The concerted mechanism predicts a multiplicative isotope effect on thiyl radical formation when the reaction is carried out with [5′-^{2}H]-AdoCbl in ^2H_2O. The solvent isotope effect observed on cob(II)alamin formation by SF was 1.8, and the putative secondary isotope effect with [5′-^{2}H]-AdoCbl in H_2O revealed $k_H/k_D = 1.6$.[45] When the same experiment was carried out in ^2H_2O with [5′-^{2}H]-AdoCbl, the observed isotope effect was 3.1. Furthermore, given the fractionation factors of thiols and of a methyl group, ^{2}H would rather be bonded to carbon than to sulfur and one would predict that the amount of cob(II)alamin would increase by a factor of 2 at equilibrium. The observed cob(II)alamin content of the reaction mixture at equilibrium changed from ~0.15 equiv in H_2O to ~0.3 equiv in ^2H_2O.[45] Thus, these data allow us to favor the concerted model for thiyl radical formation in which the elusive 5′-dA• is never present.

B. Role of the Thiyl Radical in Catalysis

How do the results from the exchange reaction relate to the nucleotide reduction process? To address this question the rapid kinetics studies have been repeated in the presence of the substrate ATP in addition to the allosteric regulator dGTP. In this case cob(II)alamin is rapidly generated ($t_{1/2} < 3$ ms) and then converted to the steady-state level. A RFQ-EPR spectrum of a sample quenched at 25 ms revealed a species similar, but not identical with that observed in the exchange reaction. Repetition with [^{2}H]cysteine containing RTPR results in extensive sharpening of the features associated with the cobalt hyperfine interactions. These results indicate, as postulated in Scheme 5, that the cob(II)alamin exchange coupled to a thiyl radical is also on the pathway of the nucleotide reduction process. It is interesting to note that rapid chemical quench experiments indicate that product dATP is generated with a rate constant of 15−20 s^{-1} in the pre-steady state, and that the observed k_{cat} under steady-state conditions (1.5 s^{-1}) is limited by rereduction of the disulfide bond in the active site to the dithiol form, or by a conformational change.

While a variety of experiments support the mechanism postulated in Scheme 5, until very recently no evidence for substrate derived radicals had been available for class II RNRs. However, recent studies using several different mechanism-based inhibitors (the 5′-triphosphates of 2′-(fluoromethylene)-2′-deoxycytidine,[148] 2′-methylene-2′-deoxycytidine, 2′-spirocyclopropyl-2′-deoxycytidine,[178] and 2′-difluoro-2′-deoxycytidine (dF_2CTP)[148]) reveal that inactivation is accompanied by formation of cob(II)alamin. The chemistry requires that for every equivalent of cob(II)alamin formed, 1 equiv of a different radical species must be generated. The very rapid rate of cob(II)alamin formation in the case of dF_2CTP (50 s^{-1})[148] suggested that this second radical species might be detectable by RFQ-EPR experiments. While the results of such an experiment are complex due to interaction of two spin systems, early time points revealed a signal remarkably similar to that detected during substrate turnover and is attributed to a thiyl radical coupled to cob(II)alamin. With time (over 140 ms) this signal disappeared and a new signal appeared consistent with cob(II)alamin interacting with a carbon-centered radical via dipolar coupling. This signal is very similar to the kinetically competent signals observed with dioldehydrase (DDH)[179] and ethanolamine ammonia lyase (EAL),[180,181] two other AdoCbl-dependent enzymes (see below). Isotopic labeling experiments and comparison of the EPR spectra taken at multiple frequencies will be required to assign structures that accommodate the observed spectra.

Scheme 7

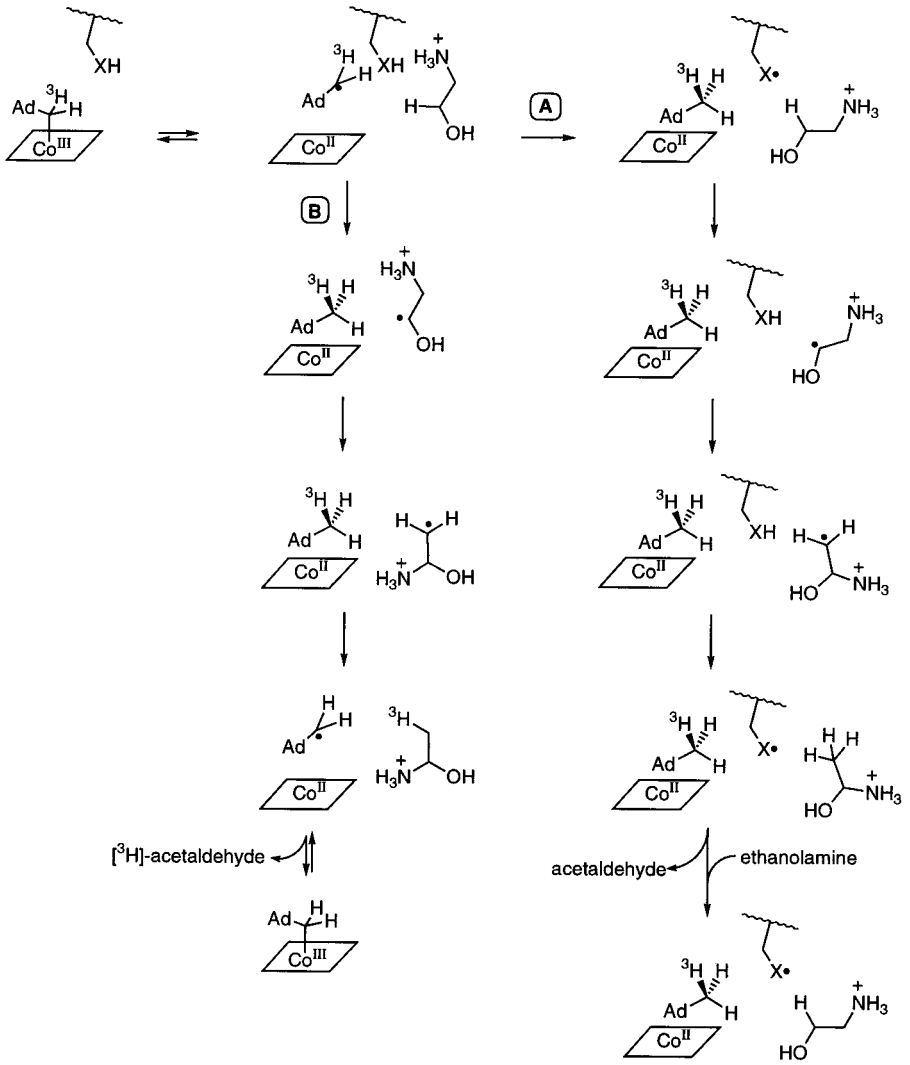


In the past two years much insight has been obtained about the chemical mechanism of nucleotide reduction and the function of AdoCbl in this process. From studies thus far it seems clear that although the mechanism of thiyl radical formation is different in the class I and class II proteins, the mechanism of nucleotide reduction is remarkably congruent.

Finally, let us return to the question posed in the beginning of this section, should AdoCbl RNR be considered in a separate mechanistic class relative to other AdoCbl requiring proteins? In 1984, it was demonstrated that RNR can catalyze a rearrangement reaction with a substrate analogue, the mechanism-based inhibitor 2′-chloro-2′-deoxyuridine 5′-triphosphate (ClUTP). The product of the reaction is a 3′-keto-2′-deoxynucleotide with the stereochemistry shown in Scheme 7.[93,97] This reaction is remarkably similar to that observed with dioldehydrase.[182] In the case of the latter enzyme, in addition to a 1,2 hydrogen shift, the hydroxyl group can also migrate, determined using appropriately ^{18}O-labeled (*R*)- and (*S*)-propanediol.[183] A similar migration of chloride from C2′ to C3′ of the nucleotide cannot be experimentally addressed in the RNR case due to stereochemical constraints on the nucleotide substrate.

The similarities between these two systems was deemed even more compelling when Stadtman's laboratory reported the discovery in *Clostridium glycolicum* of a DDH that does not use AdoCbl, but appears to require a "protein−radical" cofactor.[184,185] Furthermore, in the case of AdoCbl-dependent DDH and EAL extremely large isotope effects on the transfer of ^{3}H from the 5′ position of AdoCbl to the product aldehydes ($k_H/k_T = 125$ and 160, respectively) have been observed.[186,187] Cleland has put forth a proposal to accommodate the isotope effects observed in the EAL reaction. In this hypothesis the initially formed [^{3}H]-5′-dA• can propagate the reaction via two pathways (Scheme 8). In the major pathway A 5′-dA• abstracts a hydrogen atom from a protein residue resulting in the formation of a protein radical. This radical abstracts a hydrogen atom from substrate,

Scheme 8

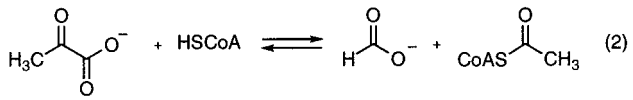


and after rearrangement of the substrate radical, the product radical reabstracts a hydrogen atom from the protein residue. The generated protein radical then initiates the next turnover rather than abstracting a hydrogen atom/tritium from [^{3}H]-dA. However, in the minor pathway (about one out of nine turnovers) [^{3}H]-dA$^\bullet$ abstracts a hydrogen atom directly from substrate (Scheme 8, pathway B). After rearrangement of the substrate radical, the product radical abstracts a hydrogen atom or tritium from [^{3}H]-dA resulting in the incorporation of ^{3}H into the product aminal, and eventually acetaldehyde (pathway B, Scheme 8).[188]

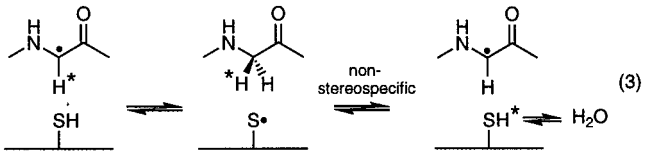
Despite all of this indirect evidence for a common mechanism, recent sequencing of the genes for all of these proteins failed to reveal any readily detectable regions of homology with respect to an AdoCbl binding domain or a thiyl radical that could function in a fashion similar to Cys408 in RTPR.[189−192] Finally, it is clear from studies carried out on other AdoCbl-requiring proteins that there are two classes of B_{12}-dependent rearrangement reactions.[193,194] While the details of these differences are beyond the scope of this review, those similar to methylmalonyl CoA mutase utilize a His as an axial ligand to the cobalt,[195] while RNR and presumably DDH and EAL, utilize their dimethylbenzimidazoles as axial ligands. Thus the jury is still out with respect to the involvement of protein radicals in DDH and EAL dependent rearrangement reactions.

VI. Pyruvate Formate Lyase

Pyruvate formate lyase plays a central role in anaerobic glucose fermentation, catalyzing the reversible conversion of pyruvate and coenzyme A (CoA) to acetylCoA (AcCoA) and formate (eq 2). This



enzyme requires a glycyl radical for catalysis, located at residue 734. In addition, PFL catalyzes an unusual exchange reaction, the wash-in of ^{2}H from ^2H_2O into the essential glycyl radical (eq 3). This reaction



at face value appears reminiscent of the exchange reaction catalyzed by the class II RNRs discussed in section V. However, the rate of this exchange reaction is 10^{-4} to 10^{-5} that of turnover in contrast to the B_{12}-dependent RNR. Several excellent reviews have appeared on this system[4,12,196] and emphasis herein will be placed on new aspects of the mechanism that have recently emerged.

A. Characterization of the Glycyl Radical

The glycyl radical of PFL has been characterized by EPR spectroscopy (Table 1) and appears as a doublet with a proton hyperfine interaction of 15 G. One property of the radical initially confounded its structural assignment: when activated PFL was incubated in D_2O, the doublet EPR spectrum collapsed to a singlet,[16] indicating that the proton associated with the observed hyperfine interaction is exchangeable. The half-life for the exchange reaction is slow ($\sim$0.14 min^{-1} at 0 °C), 1.8×10^{-5} the rate of turnover. Recent site-directed mutagenesis studies from the Kozarich laboratory have suggested a mechanism for this exchange reaction, implicating the involvement of Cys419, as the C419S mutant abolished this process.[41] As indicated in eq 3, the postulated mechanism of exchange requires nonstereospecific removal of a hydrogen atom from Gly734, unusual for an enzyme catalyzed reaction. Whether this slow exchange defines a catalytic function for Cys419 is open for discussion. However, these results support the proposal that Gly734 is juxtaposed to Cys419 in three-dimensional space during the exchange reaction, which has been interpreted to represent the initial step of a radical-dependent conversion of pyruvate to acetylCoA in the normal reaction as discussed in section VI.C.

B. Formation of the Glycyl Radical

PFL from *E. coli* is a homodimeric protein of 170 kDa. Over the past decades elegant studies from the Knappe laboratory have clearly defined the requirements for its activity. The enzyme can exist in an active form (E_a) which contains one glycyl radical per dimer and an inactive form (E_i) in which the glycyl radical is reduced.[197] The interconversion of these forms requires an activating enzyme (AE, 28 kDa based on its DNA sequence), SAM, flavodoxin, and a flavodoxin reductase (Figure 12).[197] The AE catalyzes

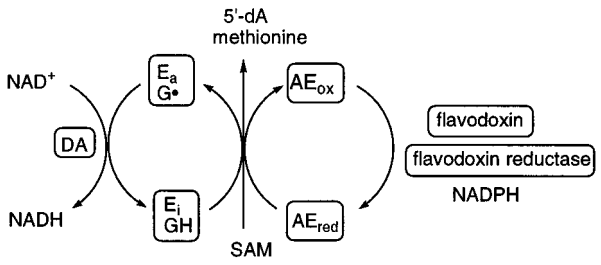


Figure 12. Requirements for activation/deactivation of PFL by generation or quenching of a glycyl radical (Gly•). E_a = active PFL with Gly•, E_i = inactive PFL in which Gly• is reduced to glycine, AE_{red} = activating enzyme in reduced state, AE_{ox} = activating enzyme in oxidized state, DA = deactivase.

PFL

E. coli	LTIRVSGYAVRFN
H. influenzae	LTIRVSGYAVRFN
C. pasteuranium	LTIRVSGYAVNFN

Class III RNR α-subunit

E. coli	VTRRVCGYLGSPD
H. influenzae	VTRRVCGYLGSPD
Bacteriophage T4	TIRRTCGYLGNPN

Figure 13. Alignment of pyruvate formate lyase and anaerobic ribonucleotide reductase from various organisms showing the amino acid sequence homology around the glycyl radical (underlined).

PFL-AE

E.coli	DGPGIRFITF FQGCLMRCLY CHNRDT
H. influenzae	DGPGIRFILF MQGCLMRCKY CHNRDT
C. pasteuranium	DGPGIRTVVF FQGCGLRCSY CHNPDT

Class III RNR β-subunit

E. coli	NGPGTRCTLF VSGCVHECPG CYNKST
H. influenzae	NGEGTRCTLF VSGCTHACKG CYNQKS
Bacteriophage T4	NGPGCRVVLF VTGCLHKCEG CYNRST

Figure 14. Amino acid sequence alignments for several pyruvate formate lyase activating enzymes (PFL-AE), and anaerobic ribonucleotide reductase activating enzymes (α-subunit). The cysteine residues that are proposed to be involved in an iron−sulfur cluster are underlined.

the conversion of Gly734 of PFL to a glycyl radical concomitant with the conversion of SAM to 5′-deoxyadenosine and methionine.[37,198] The AE purified by Conradt et al. possesses an essential iron−sulfur cluster based on metal analysis and its UV−vis spectrum (λ_{max} at 388 nm).[197] Conversely, studies on a recombinant AE reported no apparent chromophore.[199] However, this enzyme was found in inclusion bodies,[199] and the resolubilized material had a specific activity of only $^1/_2$ that previously reported. Recent studies on the soluble form of the recombinant enzyme are in agreement with the earlier studies by Conradt et al. (J. Kozarich, J. Broderick, personal communications). Finally as discussed in section VII, the RNR isolated from *E. coli* grown under anaerobic conditions also possesses a glycyl radical (Figure 13). Very recent studies suggest that it is generated by a second subunit of the enzyme that is homologous to the AE required for PFL (Figure 14).[17,36] The structure of the iron center and its role in glycyl radical formation is as outlined subsequently the subject of ongoing investigation.

As in the case of the other protein radicals discussed in this review, the generation of the glycyl radical requires a metal cofactor. Uniquely in this case, however, O_2 is not a key component in the oxidation process. In fact, O_2 initiates cleavage of the PFL polypeptide into two pieces at the site of G_{734}• resulting in its inactivation.[37] The mechanism of the glycyl oxidation to a glycyl radical is chemically unprecedented. Studies in the Kozarich laboratory have shown that the glycyl radical is generated stereospecifically. Synthesis of *pro-R* and *pro-S* [^{2}H]-labeled Gly-PFL indicate that the *pro-S* hydrogen of glycine 734 is specifically removed.[12,200] Interestingly studies by Knappe and co-workers indicate that the AE can also use as substrates small peptides with

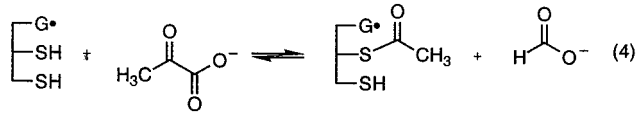
the sequence RVSGYAV corresponding to residues 731−737 of PFL. While the glycyl radical has not been detected with these small peptides, SAM is cleaved to 5′-dA and methionine.[35] Replacement of Gly with D or L-Ala in this peptide results in cleavage to 5′-dA and methionine only in the former case. Thus, this model study is consistent with the finding that the *pro-S* hydrogen atom is abstracted from the glycine of natural substrate PFL.[35]

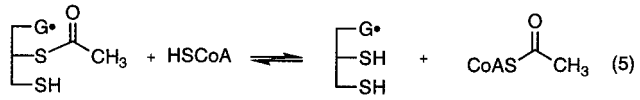
Recent studies from the Knappe and Kozarich laboratories have provided much insight into the requirements of this process. They have shown that [^{2}H] from [^{2}H]-Gly-PFL is found in the methyl group of 5′-dA, indicating that the iron−sulfur cluster can convert SAM into a hydrogen atom abstractor.[35,200] One possible scenario is the reductive cleavage of SAM to 5′-dA$^\bullet$ and methionine by one-electron transfer from the FeS cluster. However, the redox potential of SAM is believed to be on the order of −1 V, on the basis of electrochemical studies of sulfonium compounds,[201] while studies with other proteins with similar FeS clusters have redox potentials between −110 and +300 mV.[202] Therefore, thermodynamically such electron transfer appears not feasible, unless the protein significantly alters the redox potentials of the cluster or SAM. Examples of the extremes in enzyme-induced modulation of redox potentials are provided by flavin-dependent proteins in which the potentials have been found to vary between +150 and −450 mV.[203] Thus, in the case of PFL it remains to be established if the redox potential could be modulated by 700−1100 mV by the protein's environment. At present, the function of the iron−sulfur cluster is still unresolved and a major focus of investigation. Recent studies of Johnson and co-workers on a ferredoxin:thioredoxin reductase suggest that FeS clusters can stabilize a one electron reduced species, in this particular case a cysteinyl radical.[204] Therefore it is possible that the FeS cluster in the AE enzyme of PFL is involved in the formation of the glycyl radical.

An additional or alternative postulate for the function of this cluster can be made when considering several other FeS requiring proteins with well-established functions and relates to protection of the glycyl radical by the cluster from O_2 mediated destruction. First, a "deactivase", a regulatory component of the PFL system, very efficiently reduces the glycyl radical of PFL to glycine preventing its destruction by O_2.[205,206] Thus the deactivase functions as an oxygen sensor and a reductant. The mechanistic details of these reactions, and whether the iron−sulfur cluster of PFL-AE is involved, is at present not understood. Second a number of additional FeS requiring proteins, *B. subtilis* phosphoribosylpyrophosphate amidotransferase[207] cytosolic aconitase,[208,209] and the transcription factor SoxR[210] also appear to act as O_2-sensing devices. Thus, it is possible that one role of the cluster in PFL is to mediate the reduction of the glycyl radical by the deactivase and prevent its rapid inactivation by O_2-mediated chemistry resulting in irreversible peptide backbone cleavage.

C. Catalytic Mechanism

In the past decade a variety of biochemical and molecular biological studies have been carried out providing information that must be accommodated in any working hypothesis for the mechanism of conversion of pyruvate to AcCoA and formate. Early studies by Knappe and co-workers indicated that the overall reversible reaction can be divided into two half-reactions (eqs 4 and 5).[211] The first half-reaction

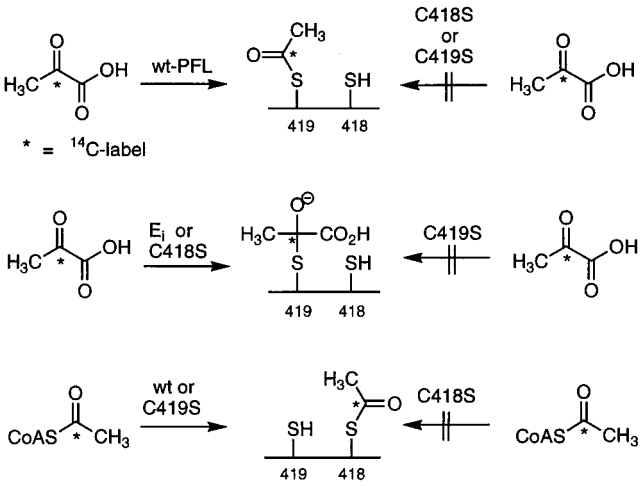




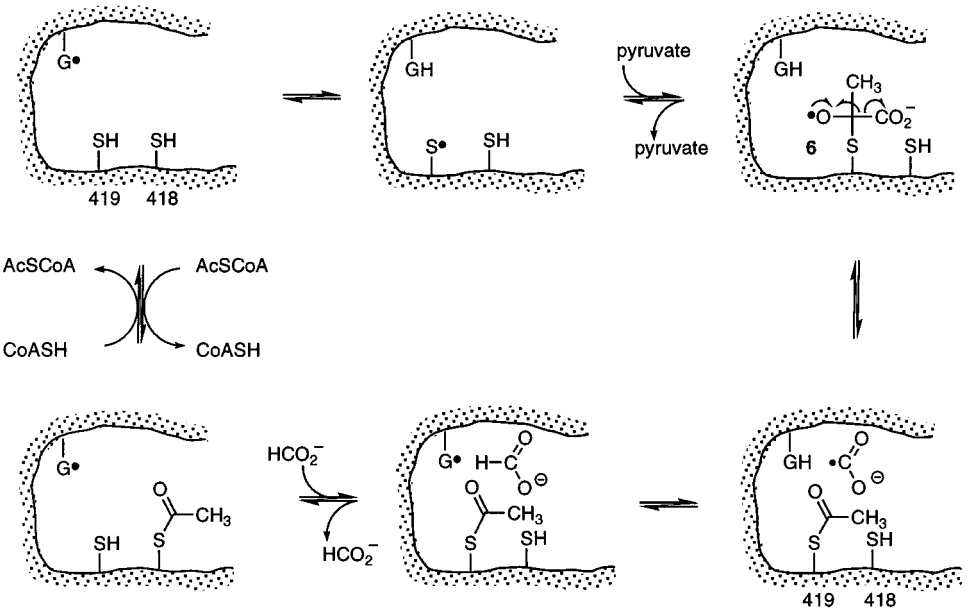
converts pyruvate to acetyl-PFL and formate, and the second half-reaction is a thiotransesterification involving the conversion of acetyl-PFL and HSCoA to AcCoA and free PFL. Both half-reactions require the presence of the glycyl radical.

A number of biochemical studies have implicated Cys418 and Cys419 as playing essential roles in this reaction (Scheme 9). Incubation of AcCoA or pyruvate in the absence of formate and/or HSCoA respectively, resulted, after proteolytic degradation of PFL, in recovery of a peptide in which Cys419 was predominantly acylated (∼0.85 equiv).[212] Recent site-directed mutagenesis studies substituting Cys418 or Cys419 with serine, indicated that both cysteines are required for the overall reaction and that the corresponding mutant proteins can each be converted into the glycyl radical form of the protein with similar efficiencies to the wt-PFL.[213] When [^{14}C]pyruvate was incubated with either C418S or C419S mutant, in contrast with the wild-type enzyme, no acylated protein was recovered (Scheme 9). However, starting with [^{14}C]-AcCoA and C419S PFL, 0.3 equiv of acylated Cys418 was recovered. With glycine inactivated PFL (E_i, no radical) incubation of the enzyme with [^{14}C]pyruvate resulted in 1 equiv of radiolabel "covalently bound" to the protein, presumably as the

Scheme 9



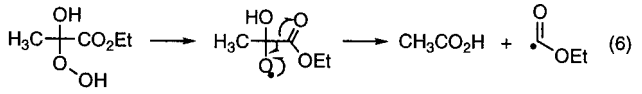
Scheme 10



thiohemiacetal. When the same experiment was repeated with C418S, 0.9 equiv was still bound consistent with the thiohemiacetal being located on Cys419. Also in agreement with this proposal is the observation that the C419S mutant contained no radiolabel (Scheme 9). These studies have stimulated two different working models for the mechanism of this reaction with some common themes (Schemes 10 and 11). In both models, the reaction starting with pyruvate requires acylation of Cys419, while in the reverse reaction starting with acetylCoA, acylation of Cys418 is involved. During the transesterification, the acyl group can be transferred between cysteines 418 and 419.

The model of the Kozarich laboratory proposes that the function of the glycyl radical is to generate a thiyl radical on Cys419 which then initiates the radical dependent C−C bond cleavage process (Scheme 10).[12,214] While the ability of a glycyl radical to generate a thiyl radical appears thermodynamically reasonable (Tables 6 and 7),[215,216] the equilibrium constant between a cysteinyl radical and an α-glycyl radical in glutathione at pH 7 has been reported to be about $>10^4$ in favor of the capto-datively stabilized carbon radical.[217,218] The role for Cys419 in the Kozarich model has been assigned on the basis of its requirement for the exchange reaction (washout of [^{2}H] from [^{2}H]-Gly734) discussed above. However, recall that the rate of this exchange reaction is very slow, 10^{-5} that of turnover, and thus calls into question its importance in the overall reaction. This exchange reaction could be an aberrant side reaction, albeit an interesting one. There are now many examples of radical-containing enzymes that "slowly" catalyze their own self-inactivation (PGHS, GAO, class II RNR). Furthermore, studies by both Knappe and Kozarich indicated that this exchange is not effected by acylation of the protein. From Knappe's published data it would seem that acylation occurs on Cys419.[212] If this is true, another mechanism of exchange other than through the thiyl radical at Cys419 must be considered.

The second step in the Kozarich model involves addition of a thiyl radical to a carbonyl. Thiyl radical addition to olefinic species is well precedented[219] (ΔH C=C π-bond = 54−59 kcal mol^{-1},[220] S−C σ-bond = 88−91 kcal mol^{-1}); reversible addition to a carbonyl would be thermodynamically less favorable (ΔH C=O π bond = 72−80 kcal mol^{-1}),[220] but certainly feasible. Thiolates readily add to carbonyls to form thiohemiacetals; however, we were unable to find precedent for a similar thiyl radical addition. For this reaction to be viable, it would need to be coupled to a favorable reaction such as the proposed β-scission of the radical **6** to drive the reaction to the right. Precedent for such a reaction is the reported cleavage of α-keto esters with Fenton's reagent described by Minisci (eq 6),[221−223] suggesting that for the β-scission reaction to be most effective, the carboxylate of pyruvate would have to be protonated. The fourth step in the

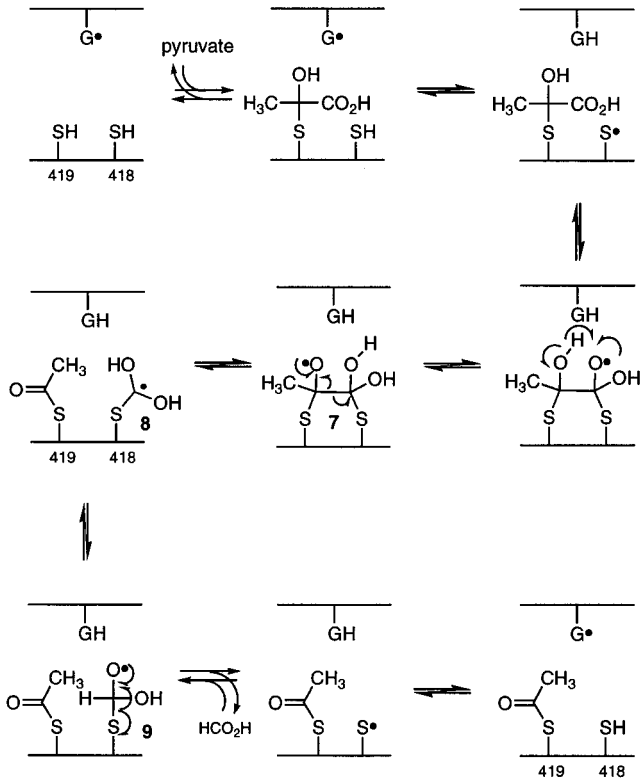


(6)

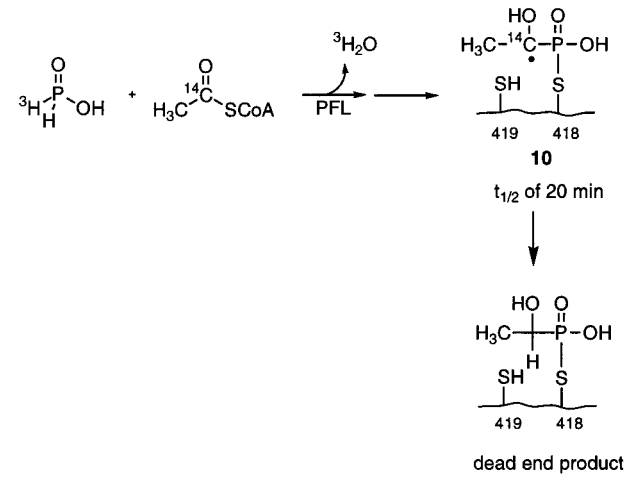
Kozarich model involves reduction of the resulting $^{\bullet}CO_2H$ by glycine to regenerate the glycine radical required for the second half-reaction. This mechanism to regenerate the Gly$^{\bullet}$ is unappealing from the perspective of symmetry. Specifically, if the Gly$^{\bullet}$ abstracts a hydrogen atom initially from Cys419 as proposed in the Kozarich model, one might expect it to be regenerated by the reverse of this process.

A second mechanism has been proposed by Knappe and co-workers and is shown in Scheme 11.[213] The first step is addition of the thiolate of Cys419 to the carbonyl of pyruvate to form a thiohemiacetal. $Gly_{734}^{\bullet}$ then generates a $Cys_{418}^{\bullet}$, that adds to the carboxylic acid of pyruvate bound covalently to Cys419. While the ability of a glycyl radical to generate a thiyl radical is thermodynamically reasonable (Tables 6 and 7),[215,216] the addition of the thiyl radical to the carboxylic acid is the least appealing step in this proposal, and for which we were unable to find

Scheme 11



Scheme 12



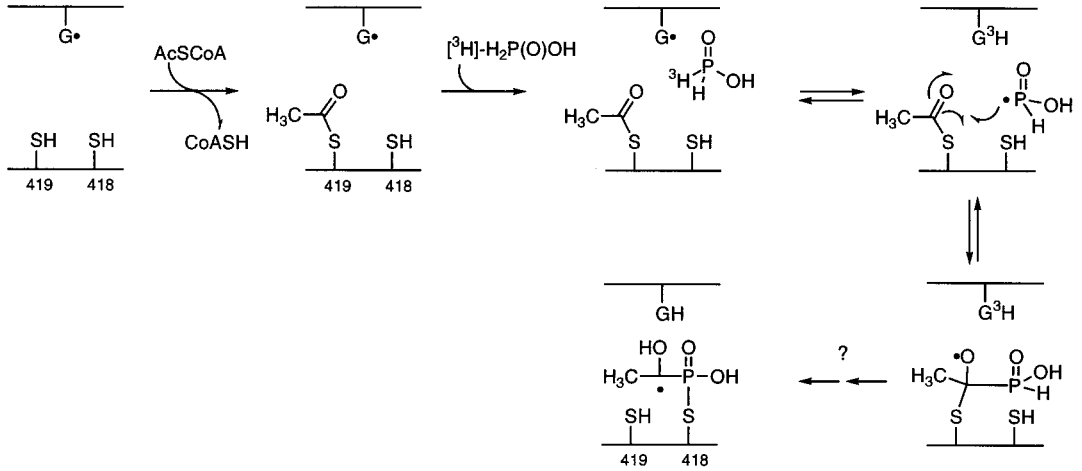
chemical precedent. The resulting radical is then proposed to rearrange to **7**, which then undergoes a β-scission reaction that leads to acylated Cys419 and a new radical **8**. Another rearrangement then produces **9** which disproportionates into formic acid and $Cys_{418}^{\bullet}$. In the final step, this thiyl radical reabstracts a hydrogen atom from Gly734 regenerating the glycyl radical. This last step is precedented by studies with glutathione and penicillamine radicals in the presence of ammonium formate.[224]

The Knappe mechanism has been strongly influenced by observations made with the formate analogue, hypophosphite, in the presence of AcCoA[16,198,212,214] or, in the reverse direction with the corresponding pyruvate analogue, acetyl phosphinate (Scheme 12).[213,225] Incubation of E_a with [^{3}H]-H_3PO_2 in the presence of [^{14}C]acetylCoA results in rapid inactivation, formation of volatile ^{3}H, presumably ^3H_2O, and ^{14}C-labeled protein. The label is stable at neutral and acidic pH, and peptide mapping with chymotrypsin identified a ^{14}C-labeled peptide. Enzymatic or chemical degradation of this intermediate using a phosphodiesterase or Br_2 resulted in the formation of 1-hydroxyethylphosphonate. Further studies revealed that this analogue is present in a thioester linkage to Cys418,[212] and that the initially formed product is EPR-active with $g_{iso} = 2.0037$ and $a(3H) = 14.6$ G $a(P) = 27.2$ G. Formation of this radical is accompanied by quenching of the glycyl radical.[16] The assignment of this PFL-generated radical to the structure **10** appears to be on firm ground based on isotopic labeling studies (Scheme 12). Two mechanisms by which it is generated have been proposed based on either the Knappe or Kozarich models for the normal reaction (Schemes 13[213] and 14[12]). However, many aspects of these mechanisms are unprecedented and the details need to be studied further. In addition, how the inactivation by hypophosphite relates to the normal reaction mechanism remains to be elucidated.

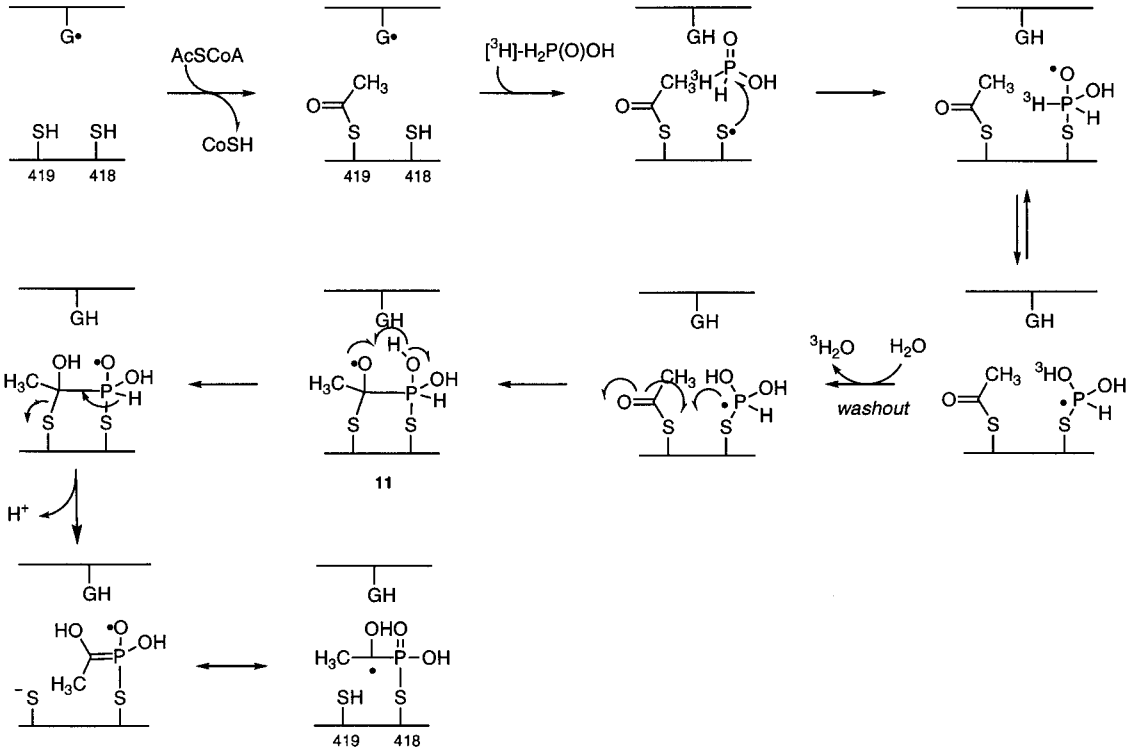
The mechanism of Knappe is much more complex than the Kozarich model and has been formulated on the basis of the belief that the hypophosphite/phosphinate system is an excellent mechanistic model from the point of view that the glycyl radical is reduced and reoxidized via its interaction with Cys418. If the Knappe model is valid, then the requirement for Cys419 in the washout of [^{2}H]-label from [^{2}H]-Gly-PFL described by Kozarich and co-workers must be a side reaction to the overall reaction. Furthermore the Kozarich mechanism, while appealing in many of its aspects, does not appear to readily accommodate the studies with [^{3}H]-H_3PO_2.[198] As described above, Knappe reported that incubation of [^{3}H]-H_3PO_2 with PFL resulted in ^3H_2O release and that no ^{3}H is found in PFL. In the Kozarich model, the glycyl radical would abstract a $^3H^{\bullet}$ from the formate analogue yielding [^{3}H]-Gly734-PFL (Scheme 13).[12] No exchange with the solvent would be observed and [^{3}H] would end up labeling the protein. The Knappe model on the other hand in which the $Gly^{\bullet}$ generates a $Cys_{418}^{\bullet}$ which then adds to H_3PO_2 and rearranges to **11**, could account for the observed results (Scheme 14). Once again it should be emphasized, however, that the observed results with hypophosphite could yet be another example of an observed radical that is many steps removed from the initially generated radical. Relating it to the normal mechanistic pathway can only be done with caution.

In summary, although a tremendous amount has been learned about the details of the PFL reaction mechanism several questions still remain. The availability of large amounts of homogeneous protein and mutant proteins combined with the use of physical methods, will in the not too distant future reveal the secrets of the intriguing mechanism by which the essential glycyl radical of PFL is generated and by which pyruvate is converted to acetylCoA and formate.

Scheme 13



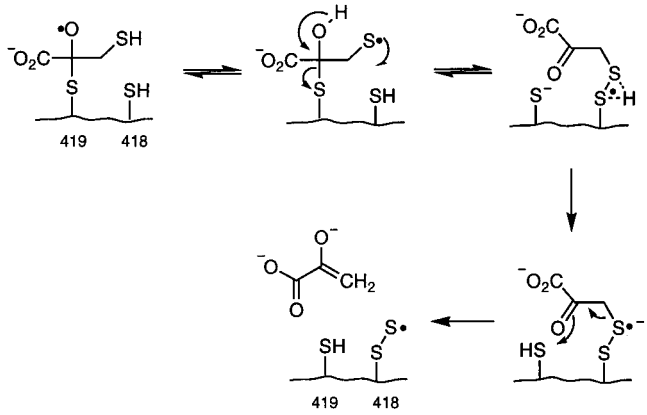
Scheme 14



D. Enzyme-Mediated Radical Chemistry with Pyruvate Analogues

Two recent interesting papers have appeared using pyruvate analogues that partition between turnover and enzyme inactivation, the latter resulting from loss of the required Gly• and formation of a new stabilized, substrate-derived radical. Incubation of E_a with mercaptopyruvate resulted in loss of the Gly• and formation of two sulfur-based radicals (Scheme 15).[42] Isotopic labeling experiments using [β-^{2}H]-Cys-PFL indicate that the new radicals are attached to the protein, presumably at Cys419 or 418. The observed g values for one of these species (2.057, 2.023, and a third feature at ~2.0) and the loss of the observed hyperfine interaction of 10 G, when the experiment was repeated using [β-^{2}H]-Cys-PFL, suggested that at least one of these species is a persulfide radical.[42] A second species with $g = 2.01$ was also

Scheme 15

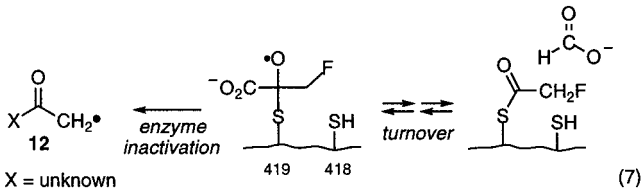


reported and remains to be structurally characterized. The use of [β-^{2}H]mercaptopyruvate had no effect on the observed EPR spectrum of either species.

A mechanism to accommodate the available results was proposed and is shown in Scheme 15.[42] The first few steps are identical with those proposed in Scheme 10 for the normal reaction mechanism. For mercaptopyruvate it is proposed, however, that the alkoxy radical generates a substrate-derived thiyl radical that reacts with Cys418 within the active site to form a disulfide radical anion. The last step in the proposed mechanism, heterolysis of the C−S bond to generate pyruvate and the observed persulfide radical, is to our knowledge chemically unprecedented. This step is required as [^{2}H]-labeling of mercaptopyruvate did not alter the observed EPR spectrum as might have been expected if the persulfide radical were attached to the substrate. Their mechanism predicts that pyruvate is generated during inactivation, a point which should be easy to test experimentally.

As has been seen previously in the case of RNRs,[52] the EPR-detectable radical species are many steps removed from the initial radical intermediate generated and have fortuitously ended up within the active site of PFL in a stabilized detectable form. These experiments, as in the case of RNRs, need to be examined using rapid freeze quench EPR methods in an effort to detect potential precursor radicals such as disulfide radical anions. The latter species might also be observable by SF UV/vis spectroscopy as disulfide radical anions have absorption features at ~450 nm with moderately large extinction coefficients (7000−9000 M^{-1} cm^{-1}).[226] Regardless of the details of this interesting inactivation, the results provide very strong evidence for the involvement of thiyl radicals in the PFL reaction sequence.

Recently Parast et al. reported that inactivation of PFL with fluoropyruvate resulted in the formation of the normal product,[53] fluoroacetylCoA and enzyme inactivation. The inactivation process, as in the case of mercaptopyruvate, is accompanied by loss of the glycyl radical and formation of a new species assigned to be a ketyl radical **12** based on experiments with [^{2}H]-labeled fluoropyruvate (eq 7).



(7)

This assignment requires loss of fluorine by an unknown mechanism. Both fluoride and formate can be monitored and can define the number of turnovers per inactivation event. Once again it is not easy to accommodate these results using a simple mechanism resulting from the partitioning of an intermediate that is common to the normal reduction process. As mentioned before, use of RFQ methods to look for the precursor radicals and the determination of the kinetic relationship of the initially generated radicals to the experimentally detected radicals will eventually provide a wealth of mechanistic insight into this intriguing system.

VII. Anaerobic Ribonucleotide Reductase

A. Background

The third class of RNR (*nrdD* and *nrdG* genes), in a beautiful set of biochemical studies, has recently been isolated and characterized by Reichard and Fontecave and their colleagues.[227] Despite extreme O_2 sensitivity,[228] amazing progress has been made in defining the components required for the conversion of NTP to dNTPs. Although some confusion concerning the subunit composition was initially apparent, the RNR has recently been shown to have $\alpha_2\beta_2$ structure, with a glycyl radical located on residue 681 of the α_2 subunit ($M_r \approx 160$ kDa) that is essential for catalysis.[229] UV−vis spectroscopic analysis, metal analysis, and EPR spectroscopic characterization suggest that the β_2 homodimeric subunit contains a single [4Fe-4S] cluster that is essential for the generation of the glycyl radical.[36,229]

B. Requirement for a Glycyl Radical

PFL (discussed in section VI) has provided an excellent framework for making rapid progress in elucidating the structure of the glycyl radical required for nucleotide reduction, and the protein(s) and small molecules required for its generation. The RNR as isolated contains a doublet EPR signal (g = 2.0033, a_H = 14 G)[17,229] reminiscent of the radical in PFL. In contrast to PFL, however, incubation of active RNR in D_2O does not collapse the doublet into a singlet. Given that recent work by Kozarich suggests that this exchange reaction in PFL is very slow and hence may not be on the normal pathway, perhaps it is not surprising that the α-hydrogen of the glycyl radical in RNR is not exchanged.

The genes for the anaerobic RNR have been identified from *Lactococcus lactis*,[230] *E. coli*,[230], *H. influenzae*,[231] and bacteriophage T4.[232,233] As shown in Figure 13, a comparison of the sequence homology in the environment of the glycyl residue that becomes oxidized in comparison with the same region of PFL reveals significant similarities.

Insight into the mechanism by which the glycyl radical of the anaerobic RNR is generated was provided by detection of an open reading frame within the same operon, downstream of the *nrdD* gene (α_2 subunit). This open reading frame encodes the β_2 subunit (designated *nrdG* genes) and has considerable sequence homology with the AE of PFL. The M_r of the β_2 subunits of the anaerobic RNR from *E. coli* and *L. lactis* are 36 and 46.6 kDa, respectively, in comparison with 28 kDa for the AE of PFL. The sequence similarities are most striking in the N-terminal region of these proteins (26% position identity) with a CX_3CX_2C motif being apparently conserved and providing a binding site for an FeS cluster (Figure 14). The β_2 subunit of the RNR from *E. coli* has been overexpressed and purified.[230] It exhibits a visible absorption spectrum that displays three absorption bands between 380 and 620 nm, and contains 2 Fe per mole of polypeptide in the most active enzyme preparations. Recent studies suggest that the irons are present in a [4Fe-4S] cluster that

links the two polypeptides of the β_2 subunit forming a small homodimeric protein (35 kDa) that binds tightly to the α_2 subunit (160 kDa).[36] Thus, as in the case of class I RNRs, the activating enzyme is a subunit of the anaerobic RNR with the active enzyme having an $\alpha_2\beta_2$ structure with nucleotide reduction activity located on the large subunit.

SAM,[234,235] flavodoxin,[236] flavodoxin reductase, and β_2 are required to generate the Gly$^\bullet$ on α_2 in the anaerobic RNR. However, as in the case of PFL the detailed mechanism remains to be established.[237] Examination of the protein databases for potential glycyl radical containing proteins reveals a surprising number of proteins with very similar sequence contexts.[12,199] Thus the role of this radical is more prevalent than initially imagined and determination of the functions of these proteins and whether in fact they use glycyl radicals in catalysis or as O_2 sensing devises awaits further experimentation that promises to be intriguing.

C. Mechanism of Nucleotide Reduction

Recent studies[43,238] have suggested that the mechanism of nucleotide reduction is very similar to that described for the RNRs from aerobically grown *E. coli* and *L. leichmannii*. Experiments carried out in D_2O have shown that the hydroxyl of the nucleotide in the substrate is replaced with retention of configuration by a proton in the product that is derived from solvent.[238] Careful analysis of the deoxycytidine (the three phosphates of the triphosphate were removed by alkaline phosphatase treatment) by NMR spectroscopy revealed that the 3′-H had been replaced with deuterium in 1−2% of all products.[238] These results are similar to those previously observed using [3′-^{3}H]-NDP or [3′-^{3}H]-NTP and release of label to solvent catalyzed by the *E. coli* and *L. leichmannii* RNRs, respectively.[80] Given this precedent, the results with the anaerobic RNR suggest that its 3′ carbon−hydrogen bond is cleaved during nucleotide reduction.

Further evidence for commonality of its mechanism with other reductases is provided by studies with 2′-chloro-2′-deoxycytidine 5′-triphosphate (ClCTP), 2′-fluoro-2′-deoxycytidine 5′-triphosphate (FCTP), and 2′-azido-2′-deoxycytidine 5′-triphosphate (N_3CTP) nucleotide analogues[43] previously shown to be mechanism-based inhibitors of the *E. coli* and *L. leichmannii* reductases (see sections IV.D and V.B and Scheme 7).[58]

The experiments are much more complex with the class III RNRs due to the initial inability to separate the activation process (generation of the glycyl radical) from the reduction process. Despite these difficulties the results are quite similar to those anticipated from similar studies with other reductases: ClCTP inactivates the enzyme in ~20 turnovers and generates cytosine and tripolyphosphate. The FCTP partitions between nucleotide reduction to produce dCTP and inactivation of the enzyme. Incubation of the enzyme with N_3CTP results in destruction of the glycyl radical and inactivation of the enzyme. These studies thus suggest that the mechanism of nucleotide reduction in the case of all three classes of reductases is very similar (see section III).

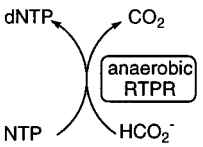


Figure 15. Formate delivers the reducing equivalents required for nucleotide reduction in the anaerobic RNR (class III) from *E. coli*.

One striking difference between this system and those examined previously involves the source of the reducing equivalents for the nucleotide reduction process. Previous studies with class I and II reductases (sections IV and V) have shown that the reducing equivalents are supplied by two cysteines that are oxidized to a disulfide during each turnover and that a small thiol dependent protein (thioredoxin or glutaredoxin) is required to reduce this disulfide so that multiple turnovers can occur. The anaerobic RNR was recently shown to require formate which is stoichiometrically oxidized to CO_2 during each conversion of NTP to dNTP (Figure 15).[239] Studies using [^{3}H]formate showed that all of the ^{3}H is transferred to solvent, consistent with the D_2O experiments described above, indicating that it is not the direct reductant. While the details of the reduction process are still unknown, a reasonable model based on the redox potentials involves reduction by formate of the disulfide generated concomitant with dNTP production. Accessibility of the active site disulfide to formate would be analogous to the unusual observation with both the class I and II reductases that dithiothreitol can replace the protein thioredoxin (or glutaredoxin) as a reductant.

An additional point can be raised regarding the mechanism of the anaerobic RNR. In the case of both *E. coli* and *L. leichmannii* RNRs, the cofactor's (Tyr$^\bullet$ and AdoCbl, respectively) function is to generate a thiyl radical. Sequence analysis of the environment of each cysteine in the anaerobic RNR reveals that of the 16 cysteines only one (Cys448 in bacteriophage T4, and Cys551 in *E. coli* and *H. influenzae*) is in a sequence environment with some homology to the putative thiyl radicals Cys439 or Cys408 of the *E. coli* and *L. leichmannii* RNRs, respectively (Figure 7). It should further be recalled that evidence is moderately strong that the function of the glycyl radical in PFL is to generate a thiyl radical which initiates the normal catalytic function. A search of the sequence environment of the cysteines of anaerobic RNR fails to reveal any primary sequence similarity to the putative active site of PFL. Finally, as has been pointed out by others,[239] it is probably not an accident that a product of the PFL reaction, formate, is the required reductant for the anaerobic RNR, and that both of these key enzymes in anaerobic metabolism possess an essential glycyl radical in similar sequence contexts. The metabolic link between these two systems and the mechanism that functions to make the glycyl radical a sensor of anaerobiosis within the cell remains to be established.

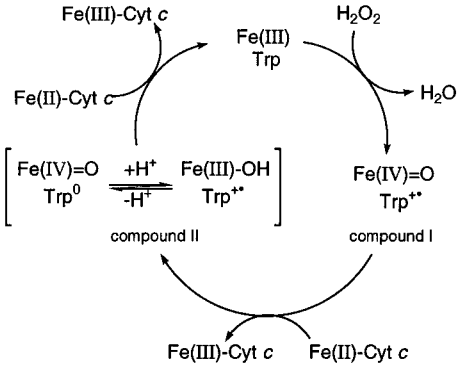
VIII. Cytochrome c Peroxidase

Cytochrome *c* peroxidase (CcP) is a heme protein isolated from yeast that catalyzes the oxidation of two molecules of ferrocytochrome *c* to ferricytochrome *c* (eq 8) concomitant with the reduction of hydrogen peroxide to water. An excellent review has recently

$$H_2O_2 + 2\ \text{Cyt-}c(Fe^{II}) + 2\ H^+ \xrightarrow{CcP} 2\ H_2O + 2\ \text{Cyt-}c(Fe^{III}) \quad (8)$$

been published on the structural similarities and differences between several peroxidases and their respective catalytic mechanisms.[240] As in other peroxidases the first step in this process involves a H_2O_2-mediated two-electron oxidation of the ferric form of the enzyme to an intermediate called compound I (Scheme 16). This intermediate is reduced back to the resting state of the enzyme by two sequential one-electron transfers from ferrocytochrome *c* through a second intermediate, compound II. One of the two oxidizing equivalents in compound I is stored as an oxyferryl-heme (Fe^{IV}=O).[241] In most peroxidases, such as horseradish peroxidase[242] and catalase,[243] the second equivalent is stored in the form of a porphyrin radical cation. However, as first suggested by Yonetani and co-workers in 1966,[244] in compound I of CcP the second oxidizing equivalent is present as an oxidized amino acid residue. The identity of this protein radical remained elusive for more than two decades although numerous proposals were put forth regarding its identity including tryptophanyl,[245] peroxy,[246] and nucleophilically stabilized methionyl radicals.[247] One of the problems hampering the assignment of a structure was the anomalously broad EPR envelope of the axially symmetric signal (Table 1), which seemed inconsistent with proposals that the observed EPR spectrum was associated with an aromatic π-type radical. The crystal structure of the resting Fe^{III} CcP and of compound I,[248] site-directed mutagenesis studies,[249−253] and ENDOR studies on compound I of the wild-type enzyme have now unambiguously assigned the radical to be associated with protonated Trp191.[254] In the following section we will briefly summarize the evidence for the assignment of the radical to Trp191, and what is understood about the chemistry involved in cytochrome *c* reduction of compounds I and II of CcP. Furthermore, the issues will be outlined that are still surrounded by controversy.

Scheme 16



A. Formation of the Ferryl Heme/Tryptophan Cation Radical

In 1980 Poulos and Kraut, on the basis of the crystal structure of CcP,[255] postulated a mechanism for the formation of the oxyferryl-heme intermediate (Figure 16 and Scheme 16).[256] The N^{ϵ} of the distal His52 was proposed to function as a general base catalyst to deprotonate the peroxide oxygen of the substrate, allowing it to bind to the iron center in the first step. Subsequently the protonated His52 can act as a general acid catalyst, returning this proton to the terminal oxygen during the heterolytic O−O bond scission of the peroxide to generate the oxyferryl-heme. The positive charge on the nearby Arg48 was proposed to provide electrostatic stabilization of the incipient negative charge during O−O bond cleavage. Mutagenesis studies in which His52 was replaced by Leu, gave protein with a specific activity for compound I formation that was decreased by 5 orders of magnitude in comparison with wt-CcP.[257] These studies supported the importance of this residue in catalysis, specifically its function as a general base catalyst. The role of Arg48 in compound I formation is masked due to the relative rates of heterolytic O−O bond cleavage and peroxide binding. The mutant R48L CcP has a specific activity of about 5.0% that of the wild-type,[258] not unreasonable given the role of His52 as a general acid catalyst in this step in conjunction with Arg48.

The oxyferryl/tryptophan cation radical intermediate in CcP is remarkably stable ($t_{1/2}$ of 6.6 ± 1.4 h),[259] considering the high reduction potential found for compound I in HRP (∼0.96 V) and for free tryptophan and tryptophan cation radicals (1.0−1.15 V) (Table 6). Direct measurements of this potential for CcP by cyclic voltammetry indicate that those values are overestimates for its compound I.[260] The oxidation of the heme and Trp191 occur in a cooperative fashion with $E^{o\prime}$ (first oxidation) ≤ 0.740 V ≤ $E^{o\prime}$ (second oxidation). A similarly depressed midpoint potential of 650 mV was also calculated for Trp191 by Kraut and co-workers.[261] The stabilization of

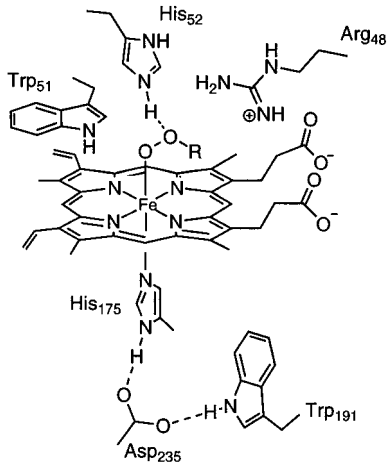


Figure 16. Schematic representation of the active site of CcP from yeast showing the hydroperoxide substrate (R = H or alkyl) coordinated to the ferric heme and the active site residues proposed to be involved in catalysis. (Adapted from ref [256].)

$Trp_{191}^{\bullet+}$ by ~0.35 V (7.8 kcal/mol) relative to free $Trp^{\bullet}$ or 0.5 V relative to a free $TrpH^{\bullet+}$ (Table 2) has been attributed to a strong negative electrostatic potential in the vicinity of this residue.[261] In addition, Peisach proposed that stabilization of the oxyferryl heme may be achieved by deprotonation of a proximal His ligand to the iron, adding electron density to the metal.[262,263] Tests of this proposal with model porphyrin complexes showed that the presence of an imidazolate axial ligand indeed stabilizes the ferric state,[264] lowered their affinities for CO,[265,266] increased their ν_{Fe-N} frequencies,[263] and red-shifted their Soret bands.[265] Support for such a proximal ligand effect in CcP was subsequently provided by the refined structure (1.7 Å resolution) of the enzyme which demonstrated the presence of Asp235 within hydrogen-bonding distance from the proximal His175. Resonance Raman studies on the ferrous forms of wt-CcP and the mutant D235N,[267,268] and NMR studies of these proteins in the presence of cyanide further corroborated this hydrogen-bonding interaction. The Asp-His-Fe triad was also found to control the reduction potential of the heme on the basis of the increase for the Fe^{III}/Fe^{II} heme couple in the mutants D235N, D235E and H175Q ($\Delta E_m = +103$, +70, and +75 mV, respectively).[269,270] However, despite these studies supporting the importance of the proximal residues His175 and Asp23, they are not essential for *catalysis*. For instance, compound I formation in CcP decreased only by a factor of 4−5 for D235N and about 3-fold for H175Q compared to wt-CcP,[258,270] and k_{cat} for the overall reaction between hydrogen peroxide and reduced cytochrome *c* has been reported to be 1463 s^{-1} for wild-type and 1585 s^{-1} for H175Q.[270,271] Thus, these results show that the residues in the proximal heme pocket do not play a significant role in the activation of hydrogen peroxide or the overall process. They are believed to be important for the stability of compound I, and are thought to play a significant role in the electron-transfer pathway (see below).[269,272]

As discussed above, the identity of the organic radical in CcP-compound I has been the topic of great interest and discussion for many years. Structural information suggested that Trp151, Met172, and Trp191 were candidates for the observed amino acid radical. Mutagenesis studies ruled out the first two candidates from further consideration.[249,250,273] Assignment of Trp191 as the site of the radical received strong support from mutagenesis studies with W191F CcP.[251,252] This mutant still formed an oxyferryl-heme during its interaction with H_2O_2, but it did not generate the radical signal characteristic of wild-type compound I. Instead, a transient porphyrin π-cation radical was observed that rapidly decayed ($t_{1/2} = 20$ ms), indicating the inability of the mutant to store both oxidizing equivalents.[274]

Recent ENDOR studies have now unambiguously established that the radical is located on a protonated tryptophan residue. This assignment appears contradictory to earlier reports that had ruled out an aromatic π-cation radical based on the *g* values and hyperfine tensors of the observed EPR spectrum.[247,275] However, in an extensive reexamination of their earlier conclusions using multi-frequency ENDOR and EPR spectroscopies, Hoffman and co-workers have provided an explanation for the unusual EPR signal.[21,276] This model requires that the organic radical ($S = {}^1/_2$) is weakly exchange coupled to the oxyferryl-heme ($S = 1$). A distribution of different protein conformations is proposed to give rise to a range in magnitude of this coupling from $J = -0.049$ cm^{-1} (antiferromagnetic coupling) to $J = +0.098$ cm^{-1} (ferromagnetic coupling), and such a distribution can qualitatively explain the broad EPR envelope.[276]

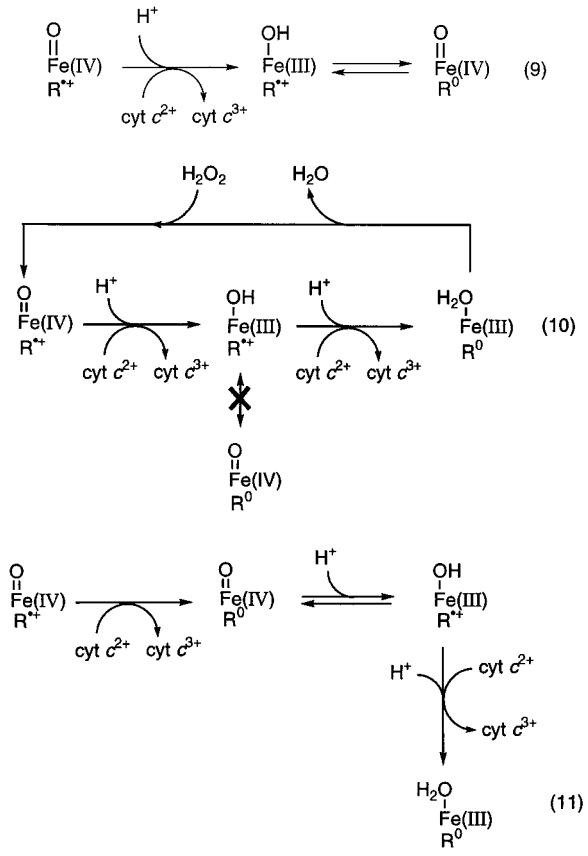
The importance of the hydrogen bonding network in the exchange coupling between the heme and the Trp191 radical site was illustrated by Goodin and co-workers in their studies of Asp235 mutants.[269,276] Crystallographic data show that replacement of this residue by glutamate introduces no significant changes at the Trp191 site. However, the EPR envelope of the organic radical signal in compound I was sharpened substantially, and the sense of the axial anisotropy was reversed (i.e., $g_{\|} > g_{\perp}$). ENDOR analysis of this signal indicated that it is still associated with Trp191, but that the nature of the exchange coupling has been significantly altered.[276]

Why does CcP form a tryptophan cation radical when most other peroxidases form porphyrin cation radicals? Comparison of sequence and structural data of peroxidases from plants, mammals, and fungi shows a high degree of homology around the heme active sites.[240] Until recently, CcP was the only protein in this superfamily that contained tryptophan residues (Trp51 and Trp191) in the proximal and distal heme pocket. In all other peroxidases examined structurally these residues are phenylalanines. These observations thus offered a reasonable explanation for the unique involvement of a tryptophan radical cation in compound I from CcP. The discovery of 33% amino acid sequence identity between CcP and pea cytosolic ascorbate peroxidase (APX), including a tryptophan residue (Trp179) in its proximal heme pocket, created the opportunity to test this hypothesis.[277,278] In 1995, the structure of this enzyme was reported by Patterson and Poulos.[279] Surprisingly, although Trp179 occupies an identical position to Trp191 in the CcP structure, it forms a porphyrin π cation radical in its reaction with hydrogen peroxide rather than a tryptophan cation radical.[280] The only obvious difference between the structures of the two enzymes is the presence of a cation binding site in APX at a distance of 8 Å from Trp179. It was proposed that a cation at this site raises the oxidation potential of Trp179 such that the intermediate porphyrin cation radical cannot oxidize the proximal Trp.[280] This proposal has recently been substantiated by site-directed mutagenesis studies on CcP.[281]

B. Proposed Function of the Tryptophan Radical in Electron Transfer

While our understanding of the structure of compound I and the chemistry involved in its formation has greatly increased in the past 10 years, the mechanisms of the reduction of compound I to

compound II, and of compound II to the resting state of the enzyme are still controversial. In contrast to HRP and other plant peroxidases in which the one-electron reduction of compound I leads to specific reduction of the porphyrin radical cation, the one-electron reduction of compound I in CcP under certain conditions results in a pH-dependent equilibrium mixture of oxyferryl/TrpH and ferric/$Trp^{\bullet+}$ forms of compound II (Scheme 16).[282,283] The question whether ferrocytochrome *c* transfers an electron to the oxyferryl-heme or to the $Trp^{\bullet+}$ of compound I is the subject of much debate. These controversies in part relate to the choice of experimental conditions, particularly the ionic strength, and the differences in methods used to make the required measurements. Several mechanistic options are being considered and alternative mechanisms appear possible in the test tube (see eqs 9−11 for possibilities).



As noted above, the pathway of reduction of compound I in vitro has as many postulated mechanisms as investigators, and appears to be strongly dependent on the experimental conditions. SF studies by Erman and co-workers using horse heart cytochrome *c* as reductant have been interpreted as support for the direct one-electron reduction of the oxyferryl-heme group to the ferric heme, which can then in turn reduce the tryptophan radical cation (eq 9).[284] Similar conclusions were reported by Hazzard and Tollin based on flash-photolysis studies with deazaflavins as reductant.[285] In addition these workers concluded that there is no need to invoke intramolecular electron transfer from the ferric heme to the radical under steady-state conditions (eq 10). In direct contradiction, Durham and Millet and co-workers have concluded from their SF experiments that the electron transfer (et) to the oxyferryl-heme is about 3 orders of magnitude slower than that to the radical site ($TrpH^{\bullet+}$) of compound I.[286] It was proposed that the reduction of the oxyferryl-heme involved a slow reversible intramolecular et from the ferryl site to Trp191, followed by a fast reduction of the $Trp^{\bullet+}$ by cytochrome *c* (eq 11). Flash-photolysis studies with ruthenated cytochrome *c* derivatives were conducted that supported the model in eq 11.[283] From their experimental results Millet and colleagues concluded that the tryptophan radical cation and not the oxyferryl species is the kinetically competent intermediate in the et reaction and that the role of Trp191 is that of an electron gate.[283]

A second question that is subject to much debate is whether CcP and cytochrome *c* form a single static complex to promote electron transfer, or whether multiple complexes, involving different electron pathways, can accommodate the observed results. In 1980 Poulos and Kraut proposed a model for the CcP/cytochrome *c* complex based on electrostatic complementarity between patches of negative charge on the surface of CcP and patches of positive charge on the surface of cytochrome *c*.[287] Subsequently, Pelletier and Kraut have solved several crystal structures of binary complexes of CcP with yeast and horse cytochrome *c*, giving results that differed from those predicted from electrostatic considerations.[288] The structural investigations suggested a distinct et pathway from Trp191 to the heme of cytochrome *c* based on the assumption that the crystal structure represents a unique binding mode.[288] These studies have recently been supported by site-specific cross-linking experiments in which cytochrome *c* and CcP were covalently linked at the putative interaction site.[289,290] This cross-linked complex showed a similar rate of et from the tethered ferrocytochrome *c* to compound I of CcP as that observed in the noncovalent complex. Furthermore, the covalently linked complex was inactive toward exogenous horse heart cytochrome *c*, suggesting blocking of an essential binding site.

Several groups have provided experimental support for the presence of two cytochrome *c* binding sites.[291−297] Hoffman and co-workers have proposed that CcP has two distinct cytochrome *c* binding sites, one with high affinity but relatively low reactivity particularly at low ionic strength, and one with low-affinity but high electron-transfer reactivities.[292,293,298] In these studies the et rates from photoinduced ^{3}Zn cytochrome *c* to ferric CcP were investigated for both wt-CcP and site-directed mutants. Substitution of Asp37 of CcP located at the binding site of the Pelletier−Kraut structure with a lysine residue lowered the binding 10-fold, but increased the et rate about 1000-fold. These and other results were interpreted as resulting from an increased binding of cytochrome *c* to the low-affinity, high reactivity site. Millet, Miller, and co-workers on the other hand have reported that release of oxidized cytochrome *c* is rate limiting at the low ionic strength used in the Hoffman studies.[299,300] They argue that binding of cytochrome

c to the low-affinity binding site increases the dissociation rate at the high-affinity binding site thereby leading to faster observed rates of turnover. Thus, in their view electron-transfer still occurs at the high-affinity Pelletier−Kraut binding site, even at low ionic strength.

Northrup and co-workers used Brownian dynamics simulations to provide insight into potential alternative sites of interaction.[301] These studies predicted the Pelletier−Kraut complex as the preferred mode of binding, and a second potential site near Asp148. To test this alternative site, Poulos and co-workers engineered a complex covalently cross-linked through a disulfide at position 149.[290] This complex showed a rate of et from the tethered yeast ferrocytochrome *c* to compound I that was reduced by about 2 orders of magnitude compared to the wild-type. Furthermore, this complex behaved like the wild-type when exogenous cytochrome *c* was used as substrate. Therefore, the region blocked in this complex is not functionally important. Further support for the Pelletier−Kraut model has been presented recently in site-directed mutagenesis studies by Miller et al. and Wang et al.[302,303] It should be pointed out, however, that it is becoming increasingly clear in this system and in other systems, that alternate pathways for electron transfer can occur when a block is placed in the normal pathway. The following is a case in point. The single mutant of CcP, W191F, is essentially inactive under steady-state conditions.[251] The double mutant W191F/H175Q, however, displays 20% of the activity of the wt-CcP.[270,304] It was proposed that this indicates the availability of an alternative electron-transfer pathway in the double mutant. This result also implies that while Trp191 is important for efficient electron transfer, it is not essential. This is consistent with an alternative view in which the interaction of CcP and cytochrome *c* is not unique, but a dynamic interconversion of many binding orientations with several possible electron-transfer pathways.[240,301]

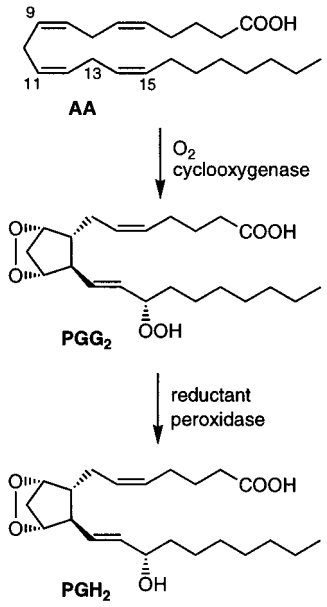
Clearly, at present there is no consensus regarding the in vitro mechanism(s) and pathways of et from cytochrome *c* to CcP. In addition, the pathway in vivo might not be related to the pathways being examined in vitro, although these latter studies are essential for considering all viable options. Studies with CcP mutants placed back inside yeast cells and subsequent examination of the phenotypic consequences of these mutations in analogy to similar studies on cytochrome *c*[305,306] will eventually play a key role in understanding the reduction pathways in Nature.

IX. Prostaglandin H Synthase

Prostaglandin H synthase (PGHS) is a bifunctional protein that catalyzes the first committed step in prostaglandin biosynthesis.[307−309] In the cyclooxygenase center it catalyzes the conversion of arachadonic acid (AA) and 2 O_2 to prostaglandin endoperoxide G_2 (PGG_2), which in the presence of a cosubstrate (reductant) can then be reduced to prostaglandin endoperoxide H_2 (PGH_2) in the peroxidase active site (Scheme 17).

The PGHS (PGHS-1, *cox*1 gene), which was the major focus of investigation for years, is now thought

Scheme 17



to be the housekeeping enzyme present in all tissues. A second, recently discovered PGH synthase, (PGHS-2, *cox2* gene) is induced in a few specific tissues by mitogens, growth factors, and cytokines.[310−313] The discovery of this inducible PGH synthase was unanticipated and very exciting as this protein is believed to be the target of nonsteroidal antiinflammatory drugs (NSAID) such as ibuprofen and aspirin. Despite the 60% sequence identity between the two synthases, specific inhibitors of PGHS-1 have only recently been identified and give hope that the selective inhibitors of PGHS-2 will soon be on hand.[309,314,315] Inhibition of PGHS-1 may be associated with the gastric and renal toxicity of the NSAI agents, and thus specific inhibitors of PGHS-2 could alleviate many unwanted side effects of antiinflammatory agents.

Exciting developments have also emerged in the past few years in our understanding of both the structure and the function of PGH synthases. Garavito and co-workers[316] have solved the first structure of this enzyme (3.5 Å resolution) and studies from the groups of Tsai and Kulmacz have established the chemical competence of the involvement of a tyrosyl radical located on Tyr358 of PGHS-1[39,317] and Tyr371 of PGHS-2[318,319] in the cyclooxygenase reaction.

A. Structure: A Tyrosine Residue Revealed

The sheep seminal vesicle PGH synthase is a homodimeric integral membrane protein of 70 kDa with one heme per subunit. Each subunit is divided into three domains: an EGF-like (epidermal growth factor) domain composed of residues 34−72, a membrane binding domain composed of residues 75−116 generating 3 amphiphatic helixes, and the catalytic domain containing residues 117−587 and both the cyclooxygenase and peroxidase active sites. This protein is a rare example of an integral membrane protein with no transmembrane segments. The structure supports the proposed model from biochemical studies that the peroxidase and cyclooxygenase

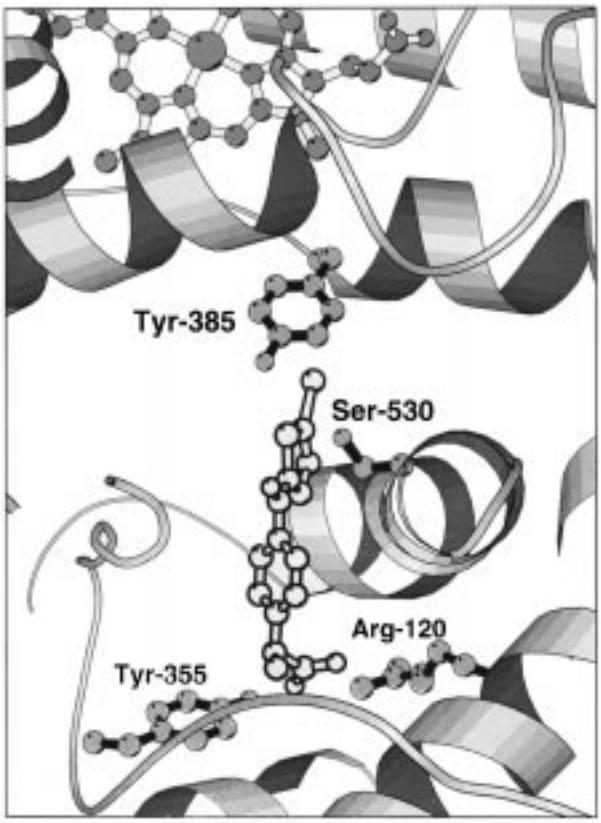


Figure 17. View of the cyclooxygenase active site of PGHS in the presence of iodosuprofen, an iodinated NSAID.[555] The tyrosine residue that is postulated to initiate catalysis as a tyrosyl radical is indicated. Also shown is Ser530, the residue that is acylated by aspirin blocking the access of arachadonic acid to Tyr385.[556]

active sites are distinct but share a common heme cofactor. Despite the low sequence homology between PGH synthase and other peroxidases,[320] the secondary and tertiary structure of PGH synthase is similar to the structure of myeloperoxidase[320] and yeast cytochrome *c* peroxidase.[245] However, in contrast to these other systems, the heme is very exposed to solvent.

The cyclooxygenase active site, on the other hand, is located on the opposite side of the heme from the peroxidase active site (Figure 17), with a channel of 25 Å leading to this site from the center of the membrane binding domain. This channel is proposed to allow access of AA to the essential heme.[321] Interestingly, Tyr358, proposed to play a key role in catalysis, lies in this channel and prevents direct access of AA to the heme (Figure 17). Thus the structure, as outlined subsequently, supports the proposal made by Ruf and co-workers in 1988,[322] that a tyrosyl radical plays a key role in the cyclooxygenase activity of PGH synthase.

Before discussing the mechanism of PGH synthase, a few comments about the properties of the protein are required to provide a setting for the contradictory results that have appeared concerning mechanistic issues. The enzyme is very unstable in the presence of hydroperoxide and both the peroxidase and cyclooxygenase activities can be lost independently of one another, depending on the conditions.[47,323,324] Loss in activity can occur in several to several hundred turnovers depending on the concentrations of the protein and the ratios of peroxide, AA and cosubstrate employed. The integral membrane characteristics of this protein and the inability to obtain large amounts of the protein through genetic engineering have all made a quantitative analysis of this system difficult. Despite this complexity, a general consensus for the mechanism of substrate oxidation is now emerging.

B. Proposed Role of the Tyrosyl Radical in Catalysis

In 1988 Ruf and co-workers proposed the catalytic cycle shown in Figure 18A for PGH synthase.[322] The product hydroperoxide PGG_2 is required to initiate the reaction, presumably by converting the ferric heme first to oxyferryl-heme and a porphyrin radical cation via heterolytic cleavage of the O−O bond.[322,325,326] This intermediate can then undergo intramolecular electron transfer from a nearby tyrosine residue to the porphyrin generating a tyrosyl radical (Tyr•) similar to compound I in CcP. The Tyr• of this intermediate is then proposed to abstract the *pro-S* hydrogen atom from C13 of AA (Scheme 18),[327] generating a pentadienyl radical which reacts with

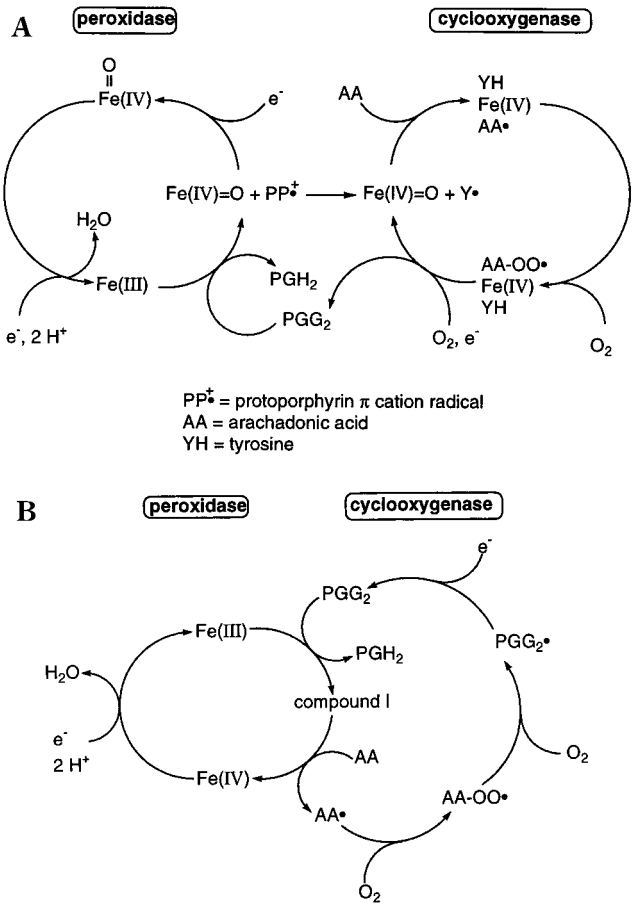
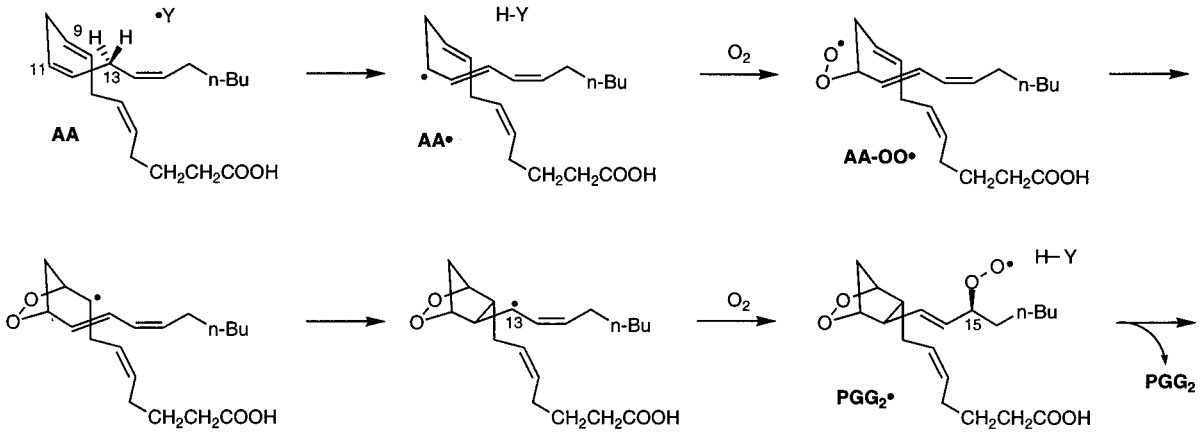


Figure 18. (A) Catalytic cycle of the proposed mechanism in which compound I, formed in the reaction of the peroxidase with a catalytic amount of peroxide, primes the cyclooxygenase by generation of a tyrosyl radical. (Adapted from refs 557 and 44.) (B) Catalytic cycle of the proposed mechanism in which the peroxidase and cyclooxygenase activities are tightly coupled. (Adapted from refs 328 and 44.)

Scheme 18



O_2 specifically at C11 to form a peroxy radical. This radical then attacks itself intramolecularly at C9 to generate a new radical which cyclizes and is trapped by a second molecule of O_2 on C15 to form a second peroxy radical. This species is reduced by Tyr358 to generate PGG_2 and regenerate the Tyr• (Scheme 18). During the entire process the oxyferryl-heme is sitting as an innocent bystander. The PGG_2 dissociates and AA can then bind to compound I, without regenerating the resting protein. The cyclooxygenase cycle can then continue independently of the peroxidase activity (Figure 18A).

An alternative second model invokes a one to one correspondence between the cyclooxygenase and peroxidase activities (Figure 18B).[328] In this case two cosubstrates are required at the end of the cyclooxygenase reaction to reduce the oxoferryl-heme and Tyr• back to the resting state. The hydroperoxide, PGG_2, is therefore not a priming reagent, but is essential for each catalytic step. In this model the cyclooxygenase and peroxidase activities are tightly coupled.

An alternative explanation for the observation of a tyrosyl radical signal has been offered by the groups of Lassmann, Marnett, Mason, and Eling.[329] They proposed that the Tyr•, originally observed by Ruf and postulated to play an essential role in both mechanisms shown in Figure 18, is not on the normal catalytic pathway, but is on a pathway that ultimately leads to enzyme inactivation.

Since the original report of Ruf et al. of an EPR-active species attributed to a Tyr• during the turnover of PGHS,[20] several groups have made a concerted effort to define the structure of this paramagnetic species and to address the question of its chemical and kinetic competence. The assignment of the EPR-active signal to a Tyr• has recently been unambiguously established by Tsai et al.[330] Transient expression of PGHS in COS cells grown in [U-^{2}H]tyrosine altered the observed EPR spectrum in a predictable fashion.

As outlined above, the experimental difficulties associated with this system have made the question of the chemical and kinetic competence of this Tyr• more difficult. Three different spectra of putative tyrosyl radicals have been reported for PGHS-1: a wide doublet (WD), a wide singlet (WS), and a narrow singlet (NS).[317,329,331] Several recent experiments suggest that the WD exhibits the required characteristics of an intermediate in the cyclooxygenase pathway as its appearance correlates with peroxide consumption,[332] and its disappearance appears to be correlated with PGG_2 formation.[317] The NS signal appears after completion of PGG_2 formation and is proposed to be associated with enzyme mediated self-inactivation.[317,329,331] Site-directed mutagenesis of Tyr358 in PGHS-1[333] to phenylalanine resulted in complete loss of cyclooxygenase activity, and complete loss of the appearance of the WD signal.[330,334] Peroxidase activity was still present at 57% that of the wild-type enzyme PGHS-1, supporting the postulate that the active sites are independent of one another, but share the heme cofactor.[333] The strongest support in favor of the importance of the WD signal was provided by an experiment run under anaerobic conditions, in the absence of AA, in which the WD could be generated. When the substrate (AA) was added to this mixture, the loss of the WD was accompanied by formation of a new radical species.[39] Repeating this experiment with [^{2}H]-labeled AA suggested that this new species is an AA-derived radical (AA•). On the other hand, in a similar experiment in which the NS signal was generated, *no* new radical signal was observed on addition of substrate.[39] The origin of the WS species is at this stage still confusing. It has been proposed that this signal is composed of at least two signals including the WD and an additional singlet.[331] Thus this signal also gives rises to AA•. In conclusion, the WD signal appears to be able to initiate chemistry on the substrate as postulated in the mechanisms depicted in Figures 18A, B.

Similar studies with PGHS-2 provided even more convincing EPR spectra.[318] Incubation of the enzyme with ethyl peroxide produced a wide singlet tyrosyl radical (29−31 G) unlike PGHS-1. However, subsequent reaction of this radical with AA generated a well-resolved seven line EPR signal which could be simulated as a planar pentadienyl radical. This seven-line signal collapsed to a five-line signal when AA-d_8 was used supporting the assignment to a pentadienyl rather than a allyl radical species. The reason for the different line shapes of the radicals observed in PGHS-1 and PGHS-2 is at present unclear.

The kinetics of the system are at present still confusing. The disappearance of the WD in PGHS-1 is not associated with stoichiometric production of an AA•. If the AA• was rapidly carried on to product this would not be a problem. However, the anaerobic conditions preclude this possibility, and under these conditions the AA• would be expected to be moderately stable. The time scale of these reactions is such that the RFQ EPR methods could provide a more reliable kinetic analysis of the reaction events, if no freezing artifacts are encountered. Second, it has also been reported that there is a substantial isotope effect on AA• formation.[327] Once again, the RFQ methods ought to allow detection of this predicted effect. While the PGH synthase needs to be further examined, the set of experiments carried out by Tsai et al. provides the most compelling evidence to date for the direct role of Tyr385 in initiating the radical-dependent cyclooxygenase reaction.

The question then arises as to whether a distinction can be made between the mechanisms in Figure 18A, B, that is, whether once the protein radical is generated, it can participate in multiple turnovers or must be regenerated each cycle. Recent evidence of Wei et al. supports the former hypothesis as briefly summarized.[44] The tightly coupled system (Figure 18B) requires that PGG_2 does not accumulate, but is reduced directly to PGH_2, a process requiring the consumption of two cosubstrates per turnover. Recent studies reveal that under certain sets of conditions PGG_2 can accumulate, and that cosubstrate is not stoichiometrically consumed in the predicted fashion according to Figure 18B.[44] Thus these studies, and additional ones, support the catalytic mechanism in Figure 18A. Thus far, this is the only protein radical requiring enzyme system in which the radical has been demonstrated to function catalytically.

Finally, another one of the general principles of protein radical requiring enzymes is illustrated with PGHS. In the study on PGHS, in which Tyr385 was replaced with a Phe, the perferryl species generated by addition of a peroxide, is reduced by amino acids in the vicinity of the heme.[333] Thus this system and a variety of additional systems discussed in this review (RNR, PFL, and CcP) clearly indicate that when site-directed mutants are prepared to block specific steps in a pathway, allowing accumulation of a reactive intermediate, that alternative chemistry, albeit slower, occurs generating amino acid radicals.

X. Photosynthetic Oxygen Evolution

The conversion of water to O_2 (eq 12), mediated by

$$2\,H_2O \longrightarrow O_2 + 4\,H^+ + 4\,e^- \quad (12)$$

photosystem II and the oxygen-evolving complex (OEC) occurs at a rate of 10^4 tons of O_2 per second and plays a major role in the environmental homeostasis. This process is accompanied by proton release and plastoquinone reduction, ultimately playing a key role in CO_2 fixation. Recent advances in membrane biochemistry and molecular biology coupled to time-resolved biophysical methods have begun to reveal some of the details of this amazing machine. Many comprehensive reviews have been written in the past few years.[7,335−343] The complexity of the experimental system makes it difficult for an "outsider" to critically evaluate the published data. In the present article new ideas proposed in the past few years focusing on the role of the tyrosyl radicals and a putative histidine radical in PSII will be presented.

A. Background

This machine is composed of a multicomponent protein complex located in the thylakoid membranes of chloroplasts and cyanobacteria. PSII couples light-induced charge separation with the reduction of plastoquinones (Q_A and Q_B) and oxidation of H_2O. There are a number of chlorophyll (Chl) binding proteins that absorb light and transfer the energy to PSII containing a specialized monomer or dimer of chlorophyll. The OEC, the site for the oxidation of H_2O to O_2, is believed to be composed of four manganese ions, calcium, and chloride in a structural organization that remains to be elucidated. The key polypeptides required for this process have been designated D_1 and D_2, which together with several additional essential proteins (33, 23, CP43 and CP47) form the PSII core complex (Figure 19). These proteins bind all of the cofactors required for this machine to function. While no structural information is available for PSII, at present there are sequence and secondary structure analogies between D_1 and D_2 and the corresponding L and M polypeptides of the bacterial reaction center core,[344] whose structure has been solved crystallographically to high resolution.[345,346] Modeling of PSII/OEC with respect to this system has provided many investigators with a tool to think about experimental design.

The sequence of chemical events in PSII/OEC has been delineated and proceeds as follows (Figure 20). Chlorophyll P_{680} absorbs light and transfers an electron to a pheophytin (pheo), which in turn reduces a bound plastoquinone (Q_A), a one-electron acceptor. Q_A then reduces Q_B, which can act as a two-

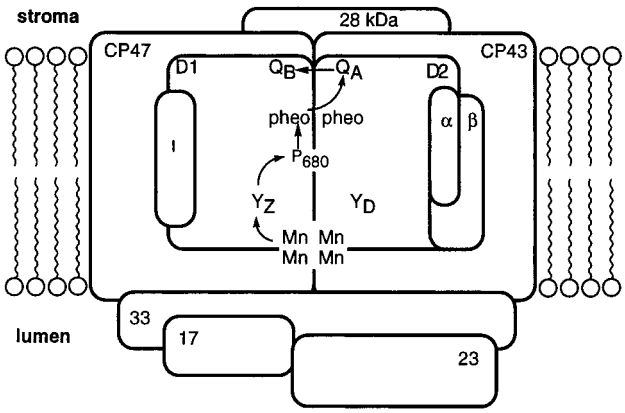


Figure 19. Schematic representation of the photosystem II core complex, and of the electron-transfer pathway from the Mn cluster to Q_B.

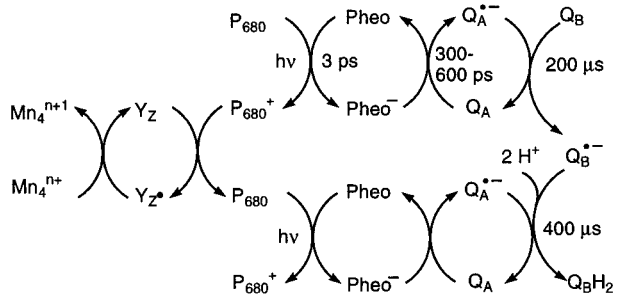


Figure 20. Light-induced electron transfer from chlorophyll P_{680} to quinone Q_B with pheophytin (Pheo) and quinone Q_A as electron carriers generates the Q_B semiquinone ($Q_B^{\bullet -}$). Subsequent one electron oxidation of the manganese cluster reforms P_{680} through the redox active tyrosine Y_Z. A second light induced electron transfer combined with uptake of two protons leads to the two-electron reduced Q_BH_2.

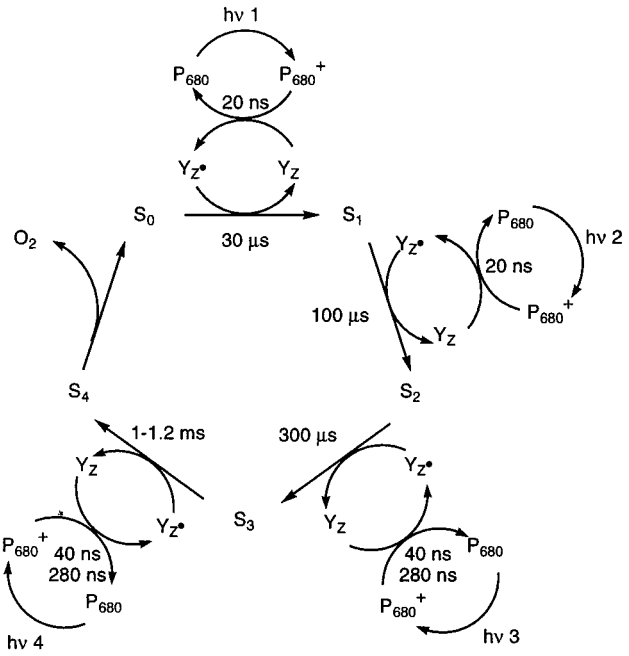


Figure 21. Redox cycle of the manganese cluster of the OEC, and the electron transfer from the manganese cluster via Y_Z to P_{680}. The half-lives for these processes are indicated and were taken from refs 335 and 558. The kinetics of the $S_2'S_3$ and $S_3'S_4$ are reported to be biphasic with half-lives of 40 ns (60% of total) and 280 ns (40%).[558]

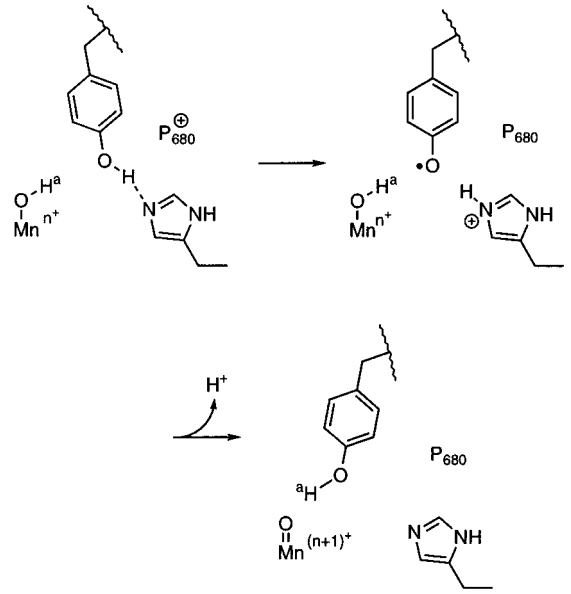
electron acceptor generating a plastoquinol which is a diffusable electron and proton carrier. The oxidized form of P_{680} ($P_{680}^{\bullet +}$) oxidizes the manganese cluster of the OEC via a redox active tyrosine (Y_Z). The oxidation/reduction processes occur in a sub-microsecond time domain, differing substantially from the previously discussed systems in this review. Four successive oxidations of P_{680} are required prior to release of O_2, and therefore the OEC cycles through five oxidation states (Figure 21), designated S_0 through S_4.[347] The oxidizing equivalents accumulate within the manganese cluster/substrate complex to the S_3 state. The subsequent oxidation of S_3 is thought to involve a transient S_4 state before the cluster rapidly decays back to S_0, releasing O_2.[347] The structure of the Mn cluster,[348−353] its changes in oxidation state during the cycle,[349,354,355] the timing of the proton release accompanying H_2O oxidation,[356−358] the role of the required Ca^{2+} and halide ions, and their relationship to the Mn cluster have all been the focus of intense investigation.[335−341,359−361]

B. Proposed Roles of Tyrosyl Radicals $Y_Z^\bullet$ and $Y_D^\bullet$

Studies of the bacterial reaction center, containing the symmetry-related peptides L and M have suggested that D_1 and D_2 might also be symmetry-related. In fact two tyrosine radicals have been detected,[19,362] and their positions identified by site-directed mutagenesis: $Y_Z^\bullet$ at position 161 of D_1,[363,364] and $Y_D^\bullet$ at position 160 in D_2.[365,366] $Y_Z^\bullet$ is directly involved in oxygen evolution and exists only transiently. In contrast, $Y_D^\bullet$ is present as a stable tyrosyl radical, and its direct involvement in the O_2 evolution process has not been observed, not even as a backup to $Y_Z^\bullet$ in proteins in which Y_Z has been replaced by phenylalanine. The biological rationale for evolving a system with "half-site" reactivity, as in the bacterial reaction center, RNRs, and PFL, is at present a mystery. The observation that a number of complex biological systems appear to have chosen this approach, suggests that it is not an in vitro experimental artifact, and that an understanding of this choice could be mechanistically important.

In the past few years the properties of $Y_Z^\bullet$ have been compared with those of $Y_D^\bullet$, and a variety of additional tyrosyl radicals (Table 1).[367−370] The results of these studies are the basis for two closely related models that have been proposed for the role of $Y_Z^\bullet$ in the oxygen evolving reaction by Babcock and co-workers,[46,371] and Britt and co-workers.[372,373] The key reaction in both models is illustrated in Scheme 19.[46,372,373] Light-induced charge separation leads to the formation of P_{680}^+ followed by coupled electron and proton transfer from Y_Z that is hydrogen bonded to an active-site base, possibly His190 of D1 based on site-directed mutagenesis studies.[374,375] This generates a non-hydrogen-bonded $Y_Z^\bullet$ that is proposed to account for the motional flexibility of this radical observed in various magnetic resonance stud-

Scheme 19



ies.[368,370,376] After release of the proton from the protonated active site base via an unknown mechanism, $Y_Z^{\bullet}$ is postulated to be reduced by the cluster. This process could take place via an uncoupled or weakly coupled electron transfer and protonation as proposed by Britt and co-workers.[46,372,373] Alternatively, Babcock has proposed that $Y_Z^{\bullet}$ abstracts a hydrogen atom from water or hydroxyl coordinated to the manganese cluster.[46] As discussed below, these mechanistic models are based on studies on the distance of $Y_Z^{\bullet}$ to the manganese cluster, and the belief that the experimentally observed motional lability of $Y_Z^{\bullet}$ is the result of directional deprotonation and reprotonation processes.

C. Spectroscopic Studies on $Y_Z^{\bullet}$ and $Y_D^{\bullet}$

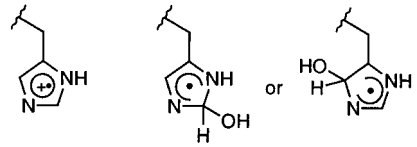
To examine the properties of $Y_Z^{\bullet}$ a manganese-depleted PSII core complex and a D_2 mutant protein in which Tyr160 was replaced by phenylalanine were required. These experimental manipulations allowed detection of the transient $Y_Z^{\bullet}$ (in the Mn-depleted complex the radical is longer lived), which would otherwise be masked by the spectrum of the stable $Y_D^{\bullet}$.[365] However, these modifications may also have introduced changes to the wild-type system that are difficult to evaluate. Using [^{2}H]-ESEEM (electron spin−echo envelope modulation), ENDOR and EPR spectroscopies $Y_Z^{\bullet}$ has been shown to exhibit considerable motional flexibility with no well-ordered hydrogen-bonding interactions,[368,370] in contrast with similar experiments on $Y_D^{\bullet}$ which appears to be rigidly fixed and hydrogen bonded within its binding domain.[113,367,376−379] As outlined below these differences in environment have been interpreted by Hoganson et al. to indicate that $Y_Z^{\bullet}$ is involved in chemistry other than electron transfer reactions which generally display minimal nuclear motion. Recent high-field EPR[369] and triple ENDOR studies,[370] however, suggest that $Y_Z^{\bullet}$ is in fact involved in hydrogen bonding, but with a distribution in distances between the hydrogen-bond donor and acceptor or heterogeneity in their angular orientation. The origin of this disordered environment was attributed in one case to manganese depletion,[369] which electron diffraction studies have indicated induces a large disruption of the PSII system.[380] Thus, while the analysis of the spectroscopic data is interesting, its relationship to the actual intact PSII/OEC remains unclear.

The potential perturbation of the system by Mn depletion is further highlighted by recent studies of Britt and co-workers using ESE-ENDOR (electron spin−echo-electron nuclear double resonance) spectroscopy that were interpreted to indicate that the Mn cluster is ~4.5 Å from $Y_Z^{\bullet}$.[372,381] Its removal might therefore be expected to alter the flexibility of $Y_Z^{\bullet}$. It should be noted, however, that the studies of Britt and co-workers also required manipulation of the PSII system to allow spectroscopic analysis. In this case the samples were Ca^{2+}-depleted, a manipulation that prohibits O_2 evolution and allows the OEC to pause in the S_3 state.[336,382] These experiments of Britt et al. indicating a short distance between the $Y_Z^{\bullet}$ and manganese cluster have been cited by Babcock and co-workers as additional evidence that $Y_Z^{\bullet}$ can function as a hydrogen atom abstractor from a water or hydroxide ligand bound to the Mn cluster.

As is apparent from the studies just presented, the PSII/OEC is sufficiently complex that to succeed in spectroscopic analysis a reductionist approach must be taken to minimize interference from its multiple additional components. In addition, blocks in a pathway, imposed on the system by Ca^{2+} or halide depletion, alter the system in ways not yet understood. In this system in which the reactions are exceedingly rapid, side reactions, that is quenching of the intermediate(s) is or can be prevalent.

The Ca^{2+}-depleted PSII provides an example of problems that may be encountered. As described above, no O_2 is evolved by PSII that has been Ca^{2+}-depleted. Using several different methods to achieve the S_3 state a "split" $g = 2$ EPR signal is observed,[383] which has attracted much attention. The signal is markedly different from the EPR spectra observed in the S_2 state, and has been suggested to arise from the Mn cluster interacting with an amino acid radical derived from D_1.[384] This signal has been investigated by UV−vis and by ESE-ENDOR spectroscopies.

Studies of Boussac et al. indicate that the amino acid radical gives rise to a visible spectrum with a λ_{max} at about 315 nm, which they suggested to be associated with an oxidized histidine radical.[383,385] The spectrum, however, is inconsistent with that of **13** and can be accommodated only by a hydroxylated, one-electron oxidized histidine radical (**14**).[386] How such a species would be generated was not addressed.



13 **14**

EPR,[387] and more recently ESE-ENDOR studies[372] of this same signal, have been interpreted to indicate that it does not come from a histidyl radical, but rather from $Y_Z^{\bullet}$. Very recent ESEEM studies using PS II core complexes isolated from *Synechocystis* cells grown on d_7-deuterated tyrosine has provided strong support for the assignment of the "split" signal to a tyrosyl radical species.[373] The increase in the width of the signal proposed to be $Y_Z^{\bullet}$ relative to that observed in the Mn-depleted system in the ESE-ENDOR experiments suggested a magnetic interaction between the Mn cluster and $Y_Z^{\bullet}$. With the assumption of only dipolar interactions between the manganese cluster and the Tyr$^{\bullet}$ the authors suggested that they are at a distance of 4.5 Å from one another.[372] It should be noted that this distance using other techniques and different preparations has been proposed to be 16 Å[388,389] and >30 Å.[390] As in the case of cytochrome *c* peroxidase where compound II exists as either a ferryl heme intermediate adjacent to Trp191 or a ferric heme intermediate and Trp$^{\bullet+}$ (section VIII.B), a similar equilibrium could be present in this locked S_3 state (eq 13), or the observed

$$Mn^{(n+1)+} + L \rightleftharpoons Mn^{n+} + L^{\bullet} \quad (13)$$

species might not be on the normal reaction pathway at all. Therefore, while this signal has received much attention, its relevance to the normal catalytic cycle remains to be established.

D. Proposed Mechanisms for Water Oxidation and O_2 Evolution

A number of molecular mechanisms have been proposed for the oxidation of water at the OEC. The models proposed by Brudvig and Crabtree,[391] Vincent and Christou,[392] Pecoraro,[393] and Proserpio et al.[394] have been reviewed previously,[343,395,396] and will not be discussed here. Instead we will focus on two more recent proposals from Babcock's laboratory,[46,371,397] and a model put forth by Yachandra, Sauer, and Klein (abbreviated YSK).[341]

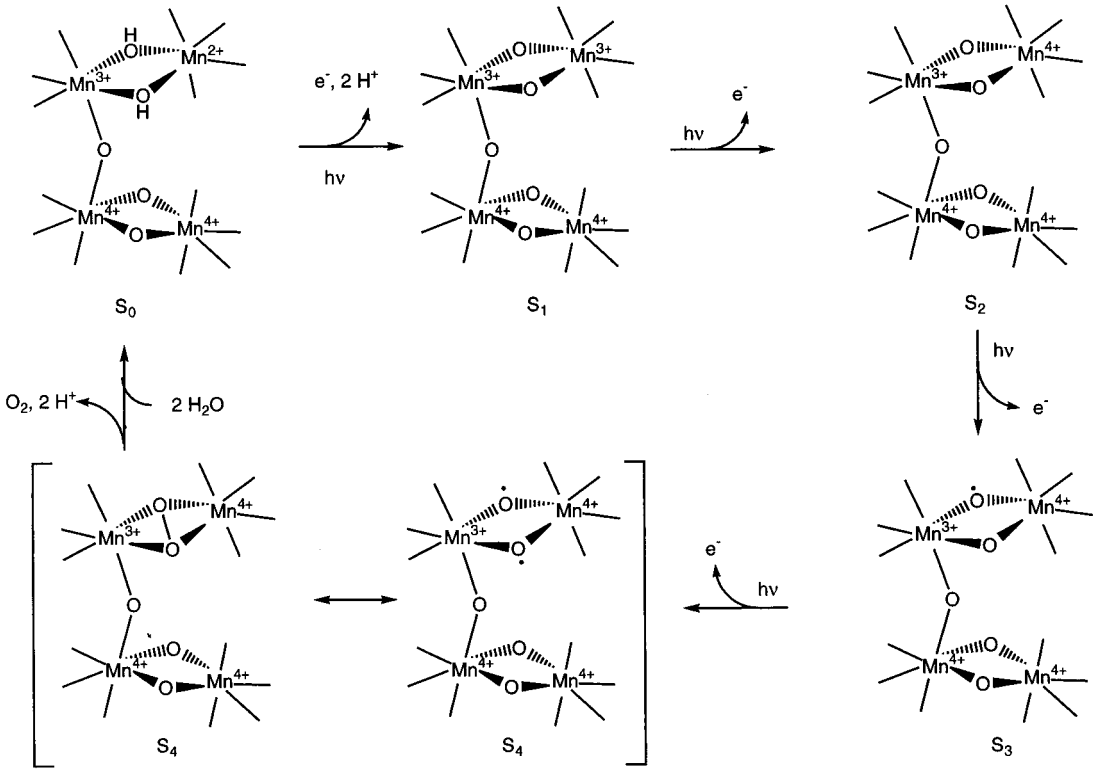
The starting point for both mechanisms is the "dimer of dimers" model first proposed by Sauer and Klein and co-workers for the manganese cluster[349,398] (Schemes 20 and 21). Several other structures had been suggested previously for the manganese cluster based in part on model chemistry (reviewed in refs 339, 341, 343, and 393), but it is now believed on the basis of extensive EXAFS studies that a short Mn−Mn distance of 2.7 Å is consistent with a di-μ-oxo-bridged dimer structure (Figure 1F) and that two such dimers might be linked by carboxylate or oxo bridges with a Mn−Mn distance of 3.3 Å.[348,350,399,400] This latter interaction at >3 Å has also been suggested to arise from a Ca−Mn distance.[349,353]

The YSK mechanism is shown in Scheme 20.[341] This model is based to a large extent on Mn−Mn distances deduced from EXAFS studies and on changes in the oxidation state of the cluster during the various S-state transitions using XANES. These studies have led to the hypothesis that the two Mn atoms in one of the bis-μ-oxo dimers are redox-inert during the oxygen-evolving process. One of the Mn atoms in the second dimer undergoes two one electron oxidations during the $S_0 \rightarrow S_1$, and $S_1 \rightarrow S_2$ transitions (Scheme 20). The bridging oxygen atoms are then oxidized to oxyl radicals during the $S_2 \rightarrow S_3$, and $S_3 \rightarrow S_4$ transitions. These radicals are proposed to combine producing O_2 and allowing the reduction of the cluster to the S_0 state.

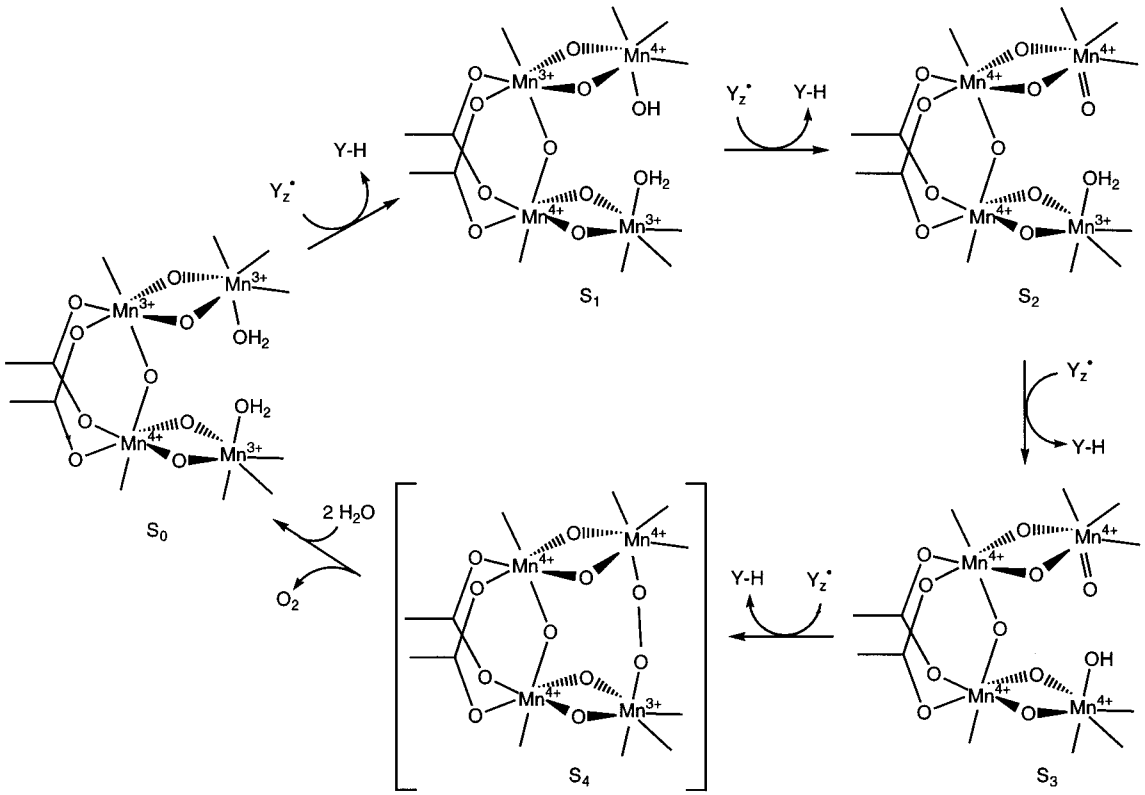
As outlined previously in section X.B, Babcock has put forth an alternative model which postulates a direct role of $Y_Z^{\bullet}$ in oxygen evolution (Scheme 21).[46,371,397] In this mechanism one manganese ion in the dimer of dimers is oxidized in each transition from S_0 through S_3 via abstraction of a hydrogen atom from a coordinated water or hydroxide ligand by $Y_Z^{\bullet}$ (Scheme 21). Thus, these S-state transitions are charge neutral unlike the mechanism in Scheme 20 in which each step involves changes in the overall charge of the cluster. In the $S_3 \rightarrow S_4 \rightarrow S_0$ transition two Mn=O species held in close proximity are transiently formed and combine to produce O_2 and reduce the Mn cluster back to the S_0 state.

The changes in the manganese oxidation states through the OEC cycle are critical in the models described above. A consensus is building on the basis of EXAFS and X-ray absorption near edge spectroscopy (XANES) studies,[349−352,355] that the early S states are predominated by average Mn^{III} oxidation states, while the later S states are predominated by average Mn^{IV} oxidation states. Furthermore, it is generally believed that the $S_0 \rightarrow S_1$, and $S_1 \rightarrow S_2$ transitions are accompanied by a one-electron oxidation of the manganese cluster. Various groups disagree, how-

Scheme 20



Scheme 21



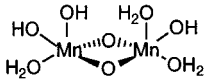
ever, about the nature of the change in oxidation state of the cluster associated with the $S_2 \rightarrow S_3$ transition. The YSK model is based in part on their belief that the manganese ions are not oxidized during this transition based on X-ray absorption near-edge spectroscopy (XANES) studies, and on the increase in the Mn−Mn distance from 2.7 to 2.9 Å. Instead they propose that the oxidizing equivalent is stored on one of the bridging oxygens generating an oxyl radical. The Babcock model on the other hand invokes a one electron oxidation of a manganese ion in this step based on the shift in Mn K edge in the XANES spectrum during this transition reported by Ono et al.[355]

In Scheme 20, oxygen is formed from two bridging oxyls, while the mechanism in Scheme 21 proposes oxygen formation from two terminal Mn-oxo species. Both groups site model studies on binuclear complexes from metals other than Mn as support for these possibilities. Tolman and co-workers have reported the reversible interconversion of a μ-η^2:η^2-peroxo dinuclear Cu^{II} complex with a di-μ-oxo core, and determined a Cu−Cu bond shortening from 3.5 to 2.8 Å during this process.[401,402] They also note the structural similarities between bis(μ-oxo)dicopper and bis(μ-oxo)dimanganese complexes.[403] However, at present no evidence is available for oxygen formation from two bridging oxygens in manganese model systems. Support for oxygen formation from terminal metal-oxo's can be found in studies on dinuclear Ru complexes,[404−406] during which it was found that a $O{=}Ru^{V}(\mu\text{-}O)Ru^{V}{=}O$ core can evolve oxygen. At present one structurally characterized dinuclear Mn complex has been reported that can function as a water oxidation catalyst.[407] However, mechanistic studies on this system have not been reported to date.

The two models described above also differ in the timing of proton release. In the YSK model two protons are released from the OEC in both the $S_0 \rightarrow S_1$ and $S_4 \rightarrow S_0$ transitions. In contrast every S-state transition involves proton removal from the manganese cluster in the Babcock model. This latter view is supported by studies from Junge's laboratory on PSII core complexes that have demonstrated stoichiometric proton release accompanying each S-state transition.[356,358] This pattern is not observed, however, in proton release studies on intact PSII membranes and thylakoids which show more complex behavior that is dependent on solution pH and sample preparation.[357]

Finally, the Babcock model requires that hydrogen atom abstraction from water or hydroxide liganded to one or more Mn atoms by a tyrosyl radical is thermodynamically and kinetically feasible. As described in sections IV and IX, two other well-characterized enzyme systems involve tyrosyl radicals. In the case of class I RNR (section IV), structural evidence suggests that its involvement in catalysis must be via electron transfer. In the case of PGH synthase (section IX), it has been proposed by Ruf et al. that it is directly involved in hydrogen atom abstraction from arachadonic acid, generating a substrate radical.[20,322] Thus at this stage there is no paradigm with respect to the role of tyrosyl radicals in biological systems. In the latter case, the bond dissociation energy (BDE) for the bisallylic carbon−hydrogen bond being cleaved is 76−81 kcal mol^{-1} and the BDE of tyrosine has been reported as 86.5 kcal mol^{-1}.[408] Recent determination of the pK_a's and redox potentials of a number of $Mn^{III}(\mu\text{-}O,\mu\text{-}OH)Mn^{IV}$ and

$Mn^{III}(\mu\text{-}O,\mu\text{-}OH)Mn^{III}$ model systems[343,409−411] has allowed estimation of the O−H bond enthalpies by use of a thermochemical cycle.[412] The BDE's so determined range from 77 to 84 kcal mol^{-1}, while a stronger bond strength (78−93 kcal mol^{-1}) was found in corresponding $Mn^{IV}(\mu\text{-}O,\mu\text{-}OH)Mn^{IV}$ complexes.[343] These values all apply to the O−H bond strength for μ-OH bridges. The O−H BDE for a water or hydroxide terminally ligated to bis μ-oxo bridged Mn_2 clusters in various oxidation states as proposed in the Babcock model has at present only been determined for one class of dinuclear manganese complexes.[413] Caudle and Pecoraro found the O−H BDE of water coordinated to alkoxide bridged Mn_2L_2 complexes (L = 2-hydroxy-1,3-bis(3,5-X_2-salicylideneamino)propane, X = H, Cl, *t*-Bu) to be between 82 and 94 kcal mol^{-1} in two cluster oxidation states (III,III, and III,IV). These values are close to the reported O−H BDE for $Y^{\bullet}$ (86 kcal mol^{-1}),[408,414] which is necessary to ensure optimum energy transfer during each S-state advancement. The O−H BDE of water or hydroxide terminally ligated to manganese complexes has also been examined by quantum chemical calculations using the model structure **15**.



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These studies have shown a bond strength of 84.3 kcal mol^{-1} for the first abstraction of a hydrogen atom from water, and 85.8 kcal mol^{-1} for the second abstraction from the resulting hydroxide.[415] However, as pointed out by the authors these theoretical studies were by necessity performed on structures that may not be good model systems for the OEC. In particular, substantial changes in the O−H BDE's were found upon introduction of formate instead of hydroxyl in order to better mimic carboxylate ligands believed to coordinate to the Mn in the OEC. In this model the second hydrogen atom abstraction was increased by 11 kcal mol^{-1} to 96.6 kcal mol^{-1}. It should be pointed out that these same calculations do not support the presence of significant unpaired spin density on the two bridging oxygen atoms in a $Mn^{III}(\mu\text{-}O)_2Mn^{IV}$ structure as proposed in the YSK model.

In summary, many important experimental observations have been reported recently on PSII, which have led to several thought-provoking proposals for the mechanism of oxygen evolution. Continued development of synthetic models and the ability to understand the mechanisms by which these function,[343,416,417] in addition to a structure of the Mn cluster itself, are essential components to ultimately understanding this complex machine.

XI. Galactose Oxidase

Galactose oxidase is an extracellular copper-containing enzyme secreted by a *Fusarium* spp. (The organism from which GAO is secreted has been referred to in the literature as *D. dendroides*; however, recent studies have shown it to be a *Fusarium* spp.[418]) It catalyzes the two-electron oxidation of a variety of primary alcohols to their corresponding aldehydes concomitant with reduction of O_2 to H_2O_2 (eq 14).[419]

$$RCH_2OH + O_2 \longrightarrow RCHO + H_2O_2 \quad (14)$$

It is a 68.5 kDa monomeric protein, containing a single copper binding site, and has attracted a great deal of interest since it appears to catalyze a two-electron oxidation using a single copper. As discussed subsequently, the recent spectroscopic studies of Whittaker and co-workers[419] in conjunction with crystallographic determination of its structure[79] have indicated that GAO utilizes an unique and unusual protein radical cofactor during substrate oxidation.

A. Different Redox States of Galactose Oxidase

Initial purifications of GAO by different laboratories resulted in protein with widely differing specific activities (Figure 22). Hamilton and co-workers noted that the activity of GAO could be increased 20- or 30-fold in the presence of oxidants such as ferricyanide, hexachloroiridate, or the EDTA complex of Mn(III).[420] Conversely, activity was completely inhibited in the presence of the reductant, ferrocyanide. These studies led Hamilton to propose that GAO as isolated was heterogeneous, containing two interconvertible forms in differing oxidation states. Furthermore, their studies established that the interconversion of these two forms occurred by a one-electron process with an oxidation potential of 0.41 V. Since the reduced form of the protein was shown to contain Cu^{II}, Hamilton proposed that the one-electron oxidized form contained a Cu(III).[420]

In 1988, Whittaker succeeded in the isolation of homogeneous preparations of these two redox forms of GAO, which has allowed their detailed characterization using a number of spectroscopic techniques (Figure 22).[33] The presence of a cupric metal site in reductively inactivated protein was confirmed by XANES,[421] and by EPR spectroscopy which displayed a signal characteristic of type 2 copper. No spectral changes were observed upon incubation of this form of the protein with galactose and/or O_2, not surprising since this form of the protein is inactive.

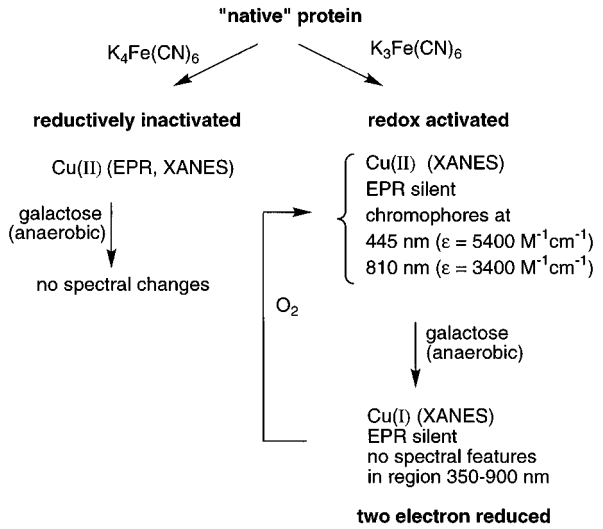


Figure 22. Galactose oxidase can exist in three different redox forms.

XANES studies, however, established that redox-activated GAO also contains Cu^{II}, not Cu^{III} as originally proposed.[420] Despite the presence of Cu^{II}, the activated GAO did not contain the expected EPR signal for a cupric metal site. In addition it displayed a distinctive visible spectrum with λ_{max} at 445 (ϵ = 5400 M^{-1} cm^{-1}) and 810 nm (3400 M^{-1} cm^{-1}). To explain this apparent contradiction, the presence of a one electron oxidized, protein-derived, organic radical antiferromagnetically coupled to the Cu^{II} was proposed.[33]

In subsequent studies, evidence for the postulated protein-based radical was obtained by one-electron oxidation of metal-free apo-GAO.[23] EPR analysis of this oxidized apoenzyme revealed a spectrum characteristic of an aromatic free radical, produced in yields up to 40% of the total enzyme concentration. Isotopic labeling studies using [β,β-^{2}H]tyrosine unambiguously established that this radical was derived from a tyrosine residue. The relevance of this species to the active metallo cofactor remained to be established, however, since no reaction was observed when this radical in the copper-free enzyme was incubated with galactose.

Further evidence that the active form of GAO is a coupled Cu^{II}−amino acid radical, comes from anaerobic reconstitution experiments of the aporadical-containing GAO with cuprous ions. Addition of oxygen resulted in active enzyme with about 80% of the activity of the starting enzyme, and containing 1 equiv of cupric ion while lacking an EPR signal.[23] These results provide strong support for the proposed antiferromagnetically coupled free radical copper cofactor.

A third, oxygen-sensitive, redox form of the protein was produced by anaerobic incubation of the redox-activated holoenzyme with substrate. This protein contained a cuprous site by XANES [241] and did not exhibit an EPR signal, suggesting that it corresponded to a two-electron reduced form of the protein. This cuprous enzyme was then transformed by admission of oxygen into a form of the protein with identical spectroscopic characteristics as the redox-activated GAO (Figure 22). The low molecular weight products of this reaction, however, were not isolated and characterized. These results indicate the chemical competence of the EPR-silent form of GAO and provide the first evidence of participation of the copper in the oxidation process. Furthermore they suggest that catalysis occurs in two distinct half-reactions.

B. Structure: A Modified Tyrosine Residue Revealed

Recently, the crystal structure of galactose oxidase was reported to 1.7 Å resolution.[34,79] The ligands coordinating the copper ion in this structure confirmed earlier predictions based on extensive spectroscopic investigations by Kosman et al.[422] At pH 4.5, the copper is coordinated by five ligands in a square-pyramidal geometry (Figure 23). Tyr272, His496, His581, and acetate ion from the crystallization buffer form an almost perfect square with distances to the copper of 1.94, 2.11, 2.15, and 2.27 Å respectively, with the fifth ligand, Tyr495, occupying the axial position at a longer distance (2.7 Å). After the crystals were soaked in Pipes buffer at pH 7.0, the acetate ion is displaced by a water molecule that is located 2.8 Å from the copper. The coordination geometry in this structure is better described as a distorted tetrahedron.

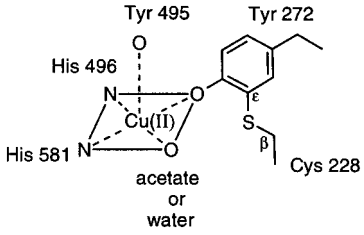


Figure 23. Schematic representation of the coordination sphere around the copper of GAO.[79]

The most exciting revelation about the cofactor structure was the detection of a very unusual, and unprecedented, thioether bond between the sulfur atom of Cys228 and the C_ϵ of Tyr272. The sulfur and C_β of Cys228 lie in the plane of the aromatic ring of Tyr272, suggesting partial double-bond character for the S−Cϵ bond. In addition, the indole ring of Trp290 is π-stacked above this extended planar structure with a distance of 3.4 Å between the planes. Whether this crystal structure is a good representation of the active form of GAO is subject to some debate. Knowles, McPherson, and Phillips and their coworkers reported that the heavy-atom derivative structures obtained for GAO prepared with hexachloroiridate, a strong one-electron oxidant, and that obtained in the presence of 100 mM ferricyanide, a strong one-electron reductant, are indistinguishable from that of the native enzyme.[79,423] Since native enzyme exists as a mixture of inactive and active forms, they concluded that the active-site structure of these two redox forms must be very similar. However, Whittaker contends that the hexachloroiridate would have been reduced by the glycerol present in the crystallization buffer, and that the structure represents inactive, one-electron reduced GAO.[419] Spectroscopic data from CD and EXAFS studies in his laboratory suggest that the coordination sphere around copper changes upon redox activation which would argue for a significant difference in structure of the two forms.[424]

Recently a glyoxal oxidase was discovered, which catalyzes the oxidation of aldehydes to acids (eq 15).[425] This enzyme, in analogy with GAO, is also

$$RCHO + O_2 + H_2O \longrightarrow RCO_2H + H_2O_2 \quad (15)$$

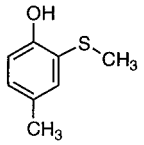
secreted by a wood-metabolizing fungus, *Phanerochaete chrysosporium*, and has properties very similar to those of galactose oxidase despite having no obvious sequence homology.

Glyoxal oxidase is isolated in an inactive form exhibiting a Cu^{II} EPR signal. The enzyme is activated by one-electron oxidation, resulting in a protein that is EPR silent. The redox potential for this

activation is 0.64 V, about 200 mV higher than found for GAO. Resonance Raman spectra of redox-activated glyoxal oxidase show remarkable similarities with those of activated GAO. Vibrational modes characteristic for a phenolate copper ligand-to-metal charge transfer (LMCT) transition have been observed in addition to features that are diagnostic of the presence of the modified cysteinyl-tyrosine. These results therefore strongly imply that, in analogy with GAO, glyoxal oxidase contains a modified tyrosyl radical antiferromagnetically coupled to a Cu^{II} ion, and suggest that this cross-linked Cys-Tyr radical could represent a cofactor common to a family of copper-containing oxidases.

C. Is the Oxidized Amino Acid Associated with Apo Oxidized GAO the Novel Ortho-Thiol-Substituted Tyrosine?

Subsequent to the discovery of the thiol-substituted Tyr272 in the crystal structure, several studies have provided strong support that this is the location of the oxidized amino acid radical found in apo-GAO. A model system in which (methylthio)cresol (**16**) has



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been oxidized by UV irradiation in a frozen matrix yielded a phenoxyl free radical with an optical absorption and EPR spectrum showing a striking resemblance to that obtained from radical containing apo-GAO.[426] In addition, ENDOR and high-field EPR (140 GHz) spectroscopy of the model system revealed that the observed hyperfine tensors[427] and g values[428] are strikingly similar to those of the radical in apo-GAO. The high-field EPR studies in combination with molecular orbital calculations support the hypothesis that the EPR-active species is in fact a tyrosine with significant unpaired spin density on the sulfur substituent.[428] Combined, these results strongly suggest that the tyrosyl radical observed after one-electron oxidation of apo-galactose oxidase is located on Tyr272.

Knowles and co-workers proposed that the stability of this unusual radical can be attributed to a π-stacking interaction with Trp290.[79] However, no evidence for spin density on Trp290 was found in either the ENDOR or the EPR studies.[427,428] The recent observation of Knowles and co-workers that the radical can still be produced in the W290H-GAO, albeit at a much slower rate (P. Knowles, personal communication) also suggest that Trp290 is not essential for the stability of the radical.

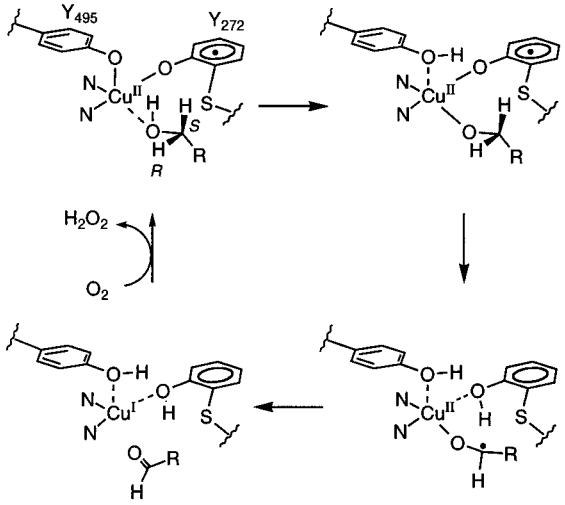
One of the most intriguing consequences of this unusual structure is that the oxidation potential of this altered Tyr272 is $\sim$+ 0.4 V,[420,429] some 0.5−0.6 V lower than a normal tyrosyl residue (Table 2).[430,431] This reduced value is in part the result of the thiol substituent *ortho* to the phenolic hydroxyl, since model studies with phenol and methylthiocresol indicate that the oxidation potential for the latter is lower than the former by about 120 mV.[432] The protein environment thus must also play an essential role in modulating the redox potential.

D. Catalytic Mechanism

Studies by Perlin and co-workers using stereospecifically deuterium-labeled methyl β-D-galactopyranosides established that GAO stereospecifically removes the *pro-S* hydrogen from C6 with a primary kinetic isotope effect of 7.7 on this reaction.[433] On the basis of these data and the geometry of the active site in the crystal structure, Whittaker has proposed a model for substrate oxidation shown in Scheme 22.[419] In this model, the substrate alcohol is coordinated to the equatorial copper site that is occupied by an acetate ion in the crystal structure (Figure 23). This mode of substrate binding had also been suggested independently by Knowles on the basis of docking experiments of galactose into the active site.[79] Tyr495 could then deprotonate the alcohol, rendering the hydrogen atom to be abstracted more acidic, followed by hydrogen atom abstraction from C-6 of galactose by the modified $Y_{272}^{\bullet}$. The substrate radical is then oxidized to the product aldehyde by one-electron reduction of Cu^{II} to Cu^{I}. The cuprous ion and the reduced Tyr272 are then reoxidized by molecular oxygen to regenerate Cu^{II} and the modified $Tyr^{\bullet}$.

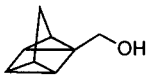
The role of the Tyr495 as a general base catalyst is supported by recent site-directed mutagenesis studies in which Tyr495 is replaced with a Phe. The k_{cat}/K_m for the Y495F GAO is reduced 1100-fold in comparison with wt-GAO.[434] Spectroscopic studies on this mutant show that the tyrosyl radical can still be generated under redox-activating conditions, and that the copper content is comparable with wild-type enzyme. Thus the reduced rate is most likely associated with an altered catalytic and not an altered structural perturbation of the mutant. Additional support for the role of Tyr495 as a general base catalyst comes from azide titrations of the cupric form of the enzyme by Whittaker, which was accompanied by stoichiometric uptake of a proton by a residue with

Scheme 22



a pK_a > 9.[435] This proton uptake was abolished with Y495F suggesting Tyr495 dissociates as a copper ligand during the titration generating a protonated phenol.[434] Crystallographic studies indicate that azide occupies the same equatorial copper site that is the proposed substrate binding site. Therefore, the dissociation of the axial Tyr495 upon azide binding suggests a similar event upon substrate binding.

Although the involvement of a substrate radical is likely, no such species has been detected. Intermediates in either of the two half-reactions might be observable by RFQ EPR experiments. Alternatively, design of mechanism-based inhibitors that can stabilize substrate based radicals could prove valuable, given the success of this approach with RNR and PFL and the known broad substrate specificity of GAO. A stoichiometric mechanism based inactivator, the radical probe **17**, has been reported, but the inactiva-



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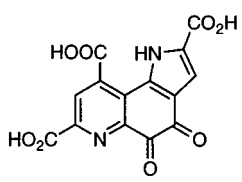
tion process was not investigated by EPR spectroscopy.[436] On the basis of the formation of the one electron reduced form of GAO during inactivation and the detection of a large primary deuterium isotope effect on this process radical chemistry was invoked. Similar support for radical intermediates was presented recently in studies with β-haloethanol inhibitors.[437]

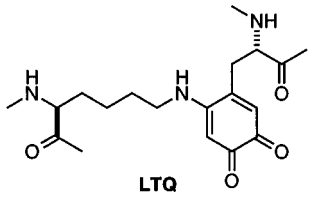
Finally, at present the chemistry by which the unusual cofactor is formed has not been investigated. By analogy with the assembly of the cofactors of class I RNR (section IV.A) and copper-dependent amine oxidases (section XII.A) it seems likely that copper and oxygen are involved in this process.

XII. Quinoproteins

Pyrroloquinoline quinone (PQQ) is a cofactor independently discovered in two laboratories in 1979.[438−440] Since that time studies have revealed that PQQ is only one of a class of *o*-quinone cofactors generated from tyrosine and tryptophan in oxidases and dehydrogenases, via metal, O_2 based posttranslational modification processes. The structures of two of these novel protein-based *o*-quinones, 2,4,5-trihydroxyphenylalanine quinone (TPQ, Figure 1H) in copper amine oxidases (AO), and tryptophan tryptophylquinone (TTQ Figure 1I) in methylamine dehydrogenase (MADH), have recently been unambiguously established by crystallographic analysis. Very recently, lysine tyrosylquinone (LTQ), yet another *o*-quinone, has been identified as the cofactor of mammalian lysyl oxidase.[441] Studies on the mechanism of the oxidation of the reduced form of the cofactors, aminoquinols, have provided strong support for the intermediacy of semiquinone radical forms of these cofactors during their reoxidation to the oxidized forms. In the next two sections, the current understanding of the mechanistic details for these processes are summarized.



PQQ



LTQ

A. Copper-Dependent Amine Oxidases

Copper-containing amine oxidases catalyze the two-electron oxidation of primary amines to their corresponding aldehydes and the aminoquinol form of the cofactor.[442−446] The reduced cofactor is subsequently reoxidized by O_2 resulting in the production of NH_3 and H_2O_2. These proteins are homodimeric containing one copper atom and one organic cofactor per monomer in the most active enzyme preparations.[447] In 1990 Klinman and co-workers, through a seminal set of biochemical experiments, unambiguously identified the cofactor in bovine plasma amine oxidase (BPAO)[448] as TPQ (Figure 1H), an enzyme-bound modified tyrosine residue. The cofactor is found within the peptide, TXXNZ(D/E), where Z is TPQ. Recent sequence comparisons with other amine oxidases has revealed this to be a general sequence context for this cofactor. However, searches of protein and gene databases have indicated that this sequence is also present in nonquinoproteins, demonstrating that the primary consensus sequence by itself is insufficient for posttranslational formation of TPQ. At present, the presence of TPQ has been demonstrated in amine oxidases from plants (pea seedlings, PSAO),[449] in an intracellular diamine oxidase from pig kidney (PKAO),[449] in bacterial enzymes (*E. coli*, ECAO,[450] and *Arthrobacter* P1, APAO[451]), and in yeast (*H. polymorpha*),[452] indicating that it is ubiquitous. Mammalian lysyl oxidase also contains copper and displays characteristics very similar to the other members of the enzyme family. However, it lacks the consensus sequence for the cofactor found in all other amine oxidases. Recent studies have revealed it to be a different variation on the TPQ theme in which a lysine residue is cross-linked with a modified tyrosine residue (LTQ).[441]

1. Structure

Very recently the first two structures of a TPQ requiring enzyme have been solved to atomic resolution. ECAO consists of a symmetrical dimer containing four domains per subunit.[31] The largest domain (residues 286−725 at the C-terminus) consists of an extensive β-sandwich that contains the active site and the dimer interface. Two pairs of adjacent β-strands on this domain form two β-hairpins of about 20 amino acids stretching from one monomer and embracing the other monomer. Each of these "arms" reaches from the active site of one subunit to that of the other over a distance of 35 Å. The more recent structure of PSAO has a very similar fold and is shown in Figure 24. The conserved His357 of one subunit makes an hydrogen bond with another conserved residue, Asp388, of the second subunit which is located within the TPQ consensus sequence

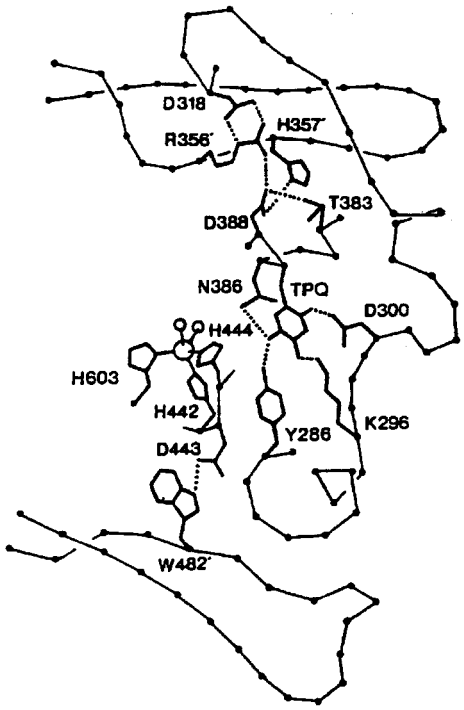
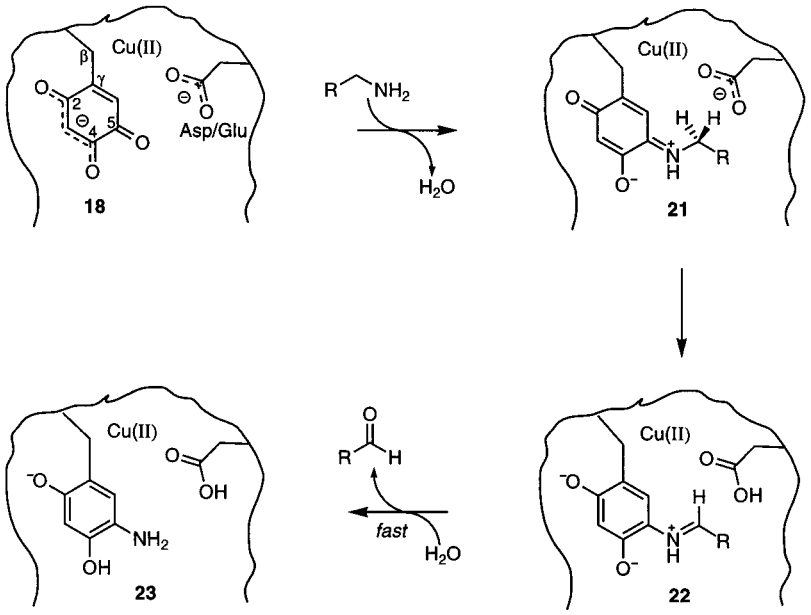


Figure 24. Pea seedling amine oxidase (adapted from ref 459) detailing the relationship between the active site and the two β-ribbon arms of the other subunit (residues identified by primes). (Reprinted from ref 459. Copyright 1996 Structure.)

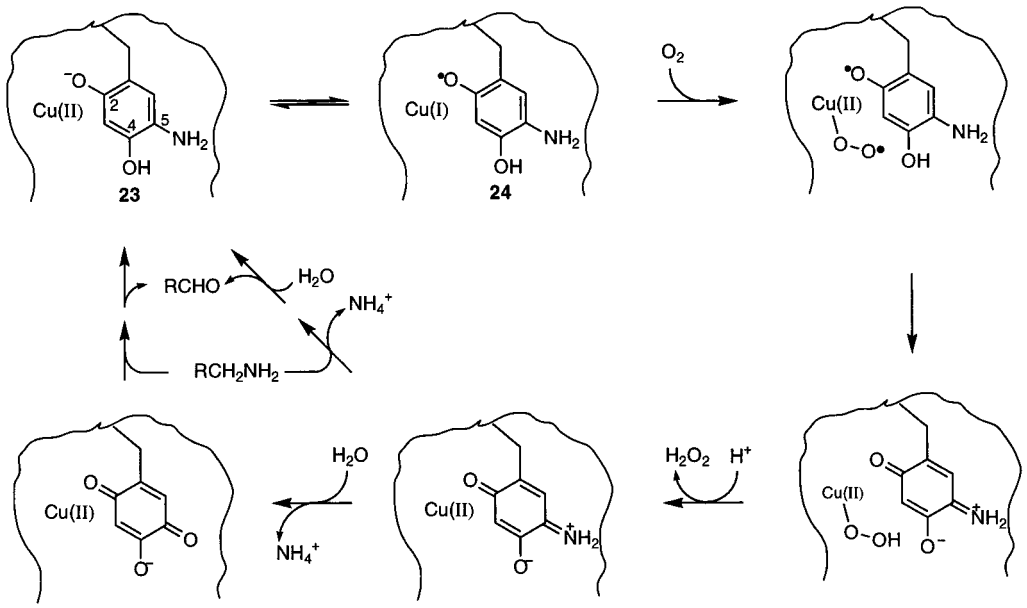
$T_{383}XXNZD_{388}$.[449] The conserved residues Thr383 and Asp388 in this motif are situated on opposite sides of a β-turn and are involved in another hydrogen bond (Figure 24). This intimate relationship between the two active sites could provide a mechanism for subunit cooperativity (a point of disagreement within the amine oxidase community).[447,453,454]

The copper is ligated by His442, His444, and His603 and two water molecules in a distorted square-pyramidal geometry in agreement with previous predictions from extensive spectroscopic investigations.[170,455−458] TPQ is located close to the copper center, but is not a ligand in crystals that exhibit catalytic activity. Unfortunately, the ring orientation of TPQ in the active form of ECAO could not be determined unequivocally due to disorder in the crystal. However, the position of TPQ has been unambiguously defined in the crystallographic study on PSAO.[459] In this structure the copper atom is located approximately 6 Å from the O2 oxygen atom of TPQ. On the basis of the observed interatomic distances, hydrogen-bonding interactions occur between the hydroxyl oxygen O4 of TPQ and the side chain amino group of Lys296, and between the quinone oxygen O5 to the hydroxyl moiety of Tyr286 and the amide group of Asn386. These hydrogen bonds are consistent with EPR and ENDOR studies of the Cu(I) semiquinone state of the cofactor.[460] The hydrogen bonds to O5 of TPQ have been proposed to enhance the electrophilicity at this position consistent with chemical studies described below which strongly suggest that catalysis is initiated at O5 of the quinone. The quinone oxygen O2 appears to be involved in a hydrogen-bonding interaction with the carboxyl group of Asp300. This carboxylate is proposed to be the general base responsible for deprotonation of the substrate (Scheme 23). This role is consistent with the pH dependence of kinetic studies on steady-state turnover that implicated an active site base with a pK_a of ~5[461] and the presence of a hydrogen bond in the crystal at pH 4.8.[459] In order for the deprotonation to occur, however, the TPQ ring must rotate 180° around the $C_\beta-C_\gamma$ bond since the substrate initially forms a Schiff base at O5. Such flexibility of the TPQ ring is supported by the above-average temperature factors of the TPQ side-chain atoms in the structure of PSAO, and the even higher degree of disorder in ECAO. Furthermore, the crystal structure of the complex between ECAO and the inhibitor 2-hydrazinopyridine covalently bound to C5 of TPQ has recently been reported.[462] In this

Scheme 23



Scheme 24



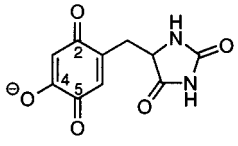
inhibitor the methylene group normally carrying the proton to be abstracted is replaced by a nitrogen, which is hydrogen bonded to the carboxylate of Asp383 (Asp300 in PSAO) in the structure. As will be discussed below, rotation of the ring is also proposed to occur during the biosynthesis of TPQ.

2. Catalytic Mechanism

As outlined in Schemes 23 and 24, amine oxidase reactions can be divided into two half-reactions. The first half-reaction involves oxidation of the amine to the aldehyde, concomitant with the reduction of TPQ to an aminoquinol (Scheme 23). The amino group is derived from substrate and provides important insight about the catalytic mechanism. Studies on PKAO,[463] PPAO,[464,465] BPAO,[447] and lentil seedling amine oxidase (LSAO)[466] have established that incubation of enzyme and substrate under anaerobic conditions leads to production of the aldehyde, but that oxygen is required for the release of ammonia (Scheme 24). In addition, kinetic studies have previously implied a ping−pong mechanism in agreement with the postulated mechanism in Schemes 23 and 24.[442,467]

Stereochemical analysis of amine oxidation catalyzed by a variety of oxidases have revealed no stereochemical imperative despite the similarities in cofactor and metal requirements of these proteins. Furthermore, the stereochemistry of substrate oxidation is substrate dependent. Stereospecific abstraction of the *pro-S* hydrogen from C1 is generally observed for benzylamines, but with phenethylamines proton abstraction has been reported to be *pro-S*, *pro-R*, and/or nonstereospecific.[468,469] In addition, the relative rates of the first and second half-reactions also depend on the source of the enzyme with the first half-reaction being much faster than the second for plant amine oxidases and the converse with PPAO.

a. Oxidation of Amine to Aldehyde. With the identification of the cofactor as TPQ, a mechanism for the oxidation of an amine to the corresponding aldehyde has been proposed (Scheme 23).[470,471] Rapid-scanning SF experiments showed bleaching of the characteristic broad absorbance at 480 nm, associated with the conjugate anion (**18**) of the cofactor,[472] concomitant with the transient appearance of a new chromophore at 340 nm.[473] This new chromophore has been assigned to the formation of a Schiff base complex between the amine substrate and carbonyl at C5 of the cofactor. Formation of this imine, rather than the one at C2, is supported by model studies with hydantoin **19** and by reductive trapping experiments of the substrate−enzyme Schiff base using [^{3}H]-$NaBH_3CN$ in BPAO.[474] Moreover, the model studies with **19** indicate that deprotonation of the 4-hydroxyl group directs the formation of this Schiff base to C5 of the cofactor.[472,475]



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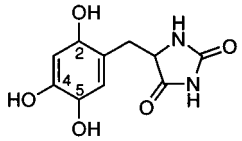
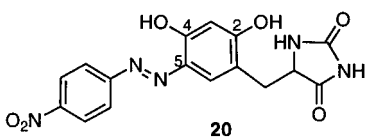


19-H_2

Additional support for imine formation at C5 was obtained from studies using resonance Raman (RR) spectroscopy in which AOs from a variety of sources had been inactivated with *p*-nitrophenylhydrazine to generate the phenylhydrazone adducts.[451] These RR studies produced essentially identical spectra as those of the azo tautomeric form of the hydrazone (**20**) produced in the reaction of topaquinone hydantoin with *p*-nitrophenylhydrazine. The position of



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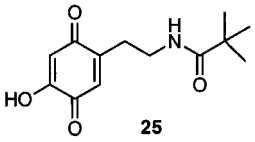
this adduct has been unambiguously assigned to C5 by 2D NMR spectroscopy.[472] More recent RR inves-

tigations of TPQ specifically ^{18}O-labeled at the C2, C4, and C5 positions showed that the C5 carbonyl has significantly more double-bond character explaining the exclusive attack of substrates and analogues at this position.[476−478]

Once the Schiff base is formed, the second step in the reaction involves abstraction of the hydrogen from C1 of the Schiff base complex (**21**) by a protein residue with a pK_a ~5, possibly Asp383 (*E. coli* numbering, see above), producing a new Schiff base **22,** the direct precursor to the product aldehyde. Studies with **19** indicate that the second imine is much shorter lived than the first one generated from the substrate. This had also been postulated previously for the enzyme system on the basis of kinetic isotope effect studies.[461] Proton abstraction at C1 is the rate determining step in the first half-reaction allowing build up of **21**. A short lifetime of **22** also explains the failure to reductively trap this second imine with [^{3}H]-$NaBH_3CN$.[475] However, very recent studies with a mutant form of yeast methylamine oxidase have provided the first observation of an intermediate that has all the characteristics of a product Schiff base complex.[446 479] Hydrolysis of the product Schiff base then produces the aldehyde and the aminoquinol **23**. While the copper center is not involved in any of the chemical steps in the first half reaction, it is required for this half-reaction in all but lentil seedling amine oxidase studied to date.[466,480] Furthermore, model studies on the hydantoin **19** or the pivalamide **25** indicate that both are able to catalytically oxidize primary amines to aldehydes in the absence of copper.[475,481,482] Thus, a structural role for the metal in the first half reaction most reasonably accounts for these results.



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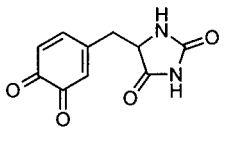
b. Reoxidation of the Reduced Amino-TPQH$_2$. While studies using mammalian serum amine oxidases have proven most informative for the first half-reaction, key observations for the reoxidation of the enzyme (second half-reaction, Scheme 24) have been provided by studies of the plant amine oxidases. In 1984 Finazzi-Agrò and co-workers reported the detection of an organic radical during anaerobic incubation of LSAO with its natural substrate putrescine in the presence of cyanide.[483] On the basis of the observation that incubation with other amine substrates produced an identical radical, they suggested that this species was enzyme based, possibly a semiquinoid form of the cofactor. Formation of this radical was accompanied by a new UV-vis absorbing species with λ_{max} at 464 and 432 nm, and a shoulder at 360 nm.[484] Both the radical signal and the new chromophore disappeared rapidly upon admission of oxygen. These observations remained restricted to plant amine oxidases until Dooley and co-workers detected the same radical signal in the absence of cyanide with a variety of amine oxidases (BPAO, PPAO, PSAO, PKAO, and APAO).[22,457] Addition of cyanide greatly increased the intensity of the radical signals with concomitant decrease of the signal associated with Cu^{II}.[22,457,485] Furthermore, in these additional systems, a direct correlation of the intensity of the visible absorbance features with the intensity of the radical signal was established. To explain these results it was proposed that the reduced form of the cofactor generated in the first half-reaction, Cu^{II} aminoquinol (**23**), exists in an equilibrium with a Cu(I) aminosemiquinone form (**24**, Scheme 24), which is stabilized by the addition of cyanide.[457] The elusiveness of this latter form was shown to be due to a strong temperature dependence of the equilibrium producing almost exclusively the Cu^{II} aminoquinol form at the low temperatures at which EPR experiments had been carried out previously.[22] The assignment of the new radical to **24** is strongly supported by the observed alteration of the EPR spectrum with ^{15}N-labeled substrate,[457] and more recently by studies using ESEEM and ENDOR spectroscopies.[460,486]

The results described above formed the foundation for a mechanistic model for the second half-reaction (Scheme 24). The Cu(I) aminoquinol radical intermediate reacts with O_2 to generate the TPQ-imine, Cu^{II}, and H_2O_2. The iminoquinone produced then undergoes hydrolysis regenerating the TPQ form of the cofactor. Alternatively, the quinone imine could directly enter the first half-reaction via a transimination with substrate (Scheme 24). The kinetic competence of the Cu(I) aminosemiquinone species as an intermediate in the overall reaction was demonstrated by measuring the rate of electron transfer from Cu^{II} to the aminoquinol using temperature-jump relaxation techniques monitoring the characteristic absorbance at 464 nm.[487,488] However, the details of the reduction of oxygen to H_2O_2 via this intermediate are not well understood. Thus the data at present support a chemical role for the copper and a protein radical in the second half-reaction. It should be noted, however, that there are still some investigators who dispute this contention.[489,490]

As described for GAO (section XI), Nature has altered the redox potential of one of its tyrosines by C6 substitution with a thiol of a cysteine. In the case of TPQ, the redox potential has been altered by C2 and C5 substitution with a hydroxyl group. Unfortunately, the redox potentials have not yet been measured for any amine oxidases. However, the model **19** indicates that the hydroxyl group at C2 alters the oxidation potential by 0.3 V relative to the non-hydroxylated dopa quinone hydantoin **26** (Table 8).[472] Thus, TPQ provides another example of post-translational modification to grossly tune the redox capabilities of natural amino acids.



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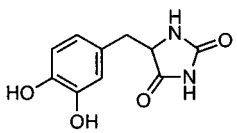


26-H$_2$

Table 8. Redox Potentials for Two-Electron Processes That Are Relevant to the Enzymatic Systems Discussed in This Review

oxidant	reductant	$E^{\circ\prime}$ vs NHE (V)[a]	ref
H_2O_2	2 H_2O	1.394	*b*
O_2	H_2O_2	0.31	*b*
TTQ_{ox} (*P. denitrificans*)	TTQ-H_2 (*P. denitrificans*)	0.126	517
26	**26-H_2**	~0.36[c]	472
29	**29-H_2**	0.107	*d*
Topa quinone	Topa	0.079	*e*
19	**19-H_2**	0.075	472
PQQ	PQQ-H_2	0.066	*f*
CysSSCys	2 CysSH	−0.34	*g*
CO_2	HCO_2^-	−0.41	*g*

[a] At pH 7 unless indicated otherwise. [b] At pH 6.78. [b] Sawyer, D. T. in *Oxygen Complexes and Oxygen Activation by Transition Metals*; Martell, A. E., Sawyer, D. T. Eds.; Plenum: New York, 1988. [c] Deduced from E_m vs pH diagram for **26**. [d] Itoh, S.; Ogino, M.; Haranou, S.; Terasaka, T.; Ando, T.; Komastu, M.; Ohshiro, Y.; Fukuzumi, S.; Kano, K.; Takagi, K. Ikeda, T. *J. Am. Chem. Soc.* **1995**, *117*, 1485−1493. [e] Kano, K.; Mori, T.; Uno, B.; Goto, M.; Ikeda, T. *Biochim. Biophys. Acta* **1993**, *1157*, 324. [f] Kano, K.; Mori, T.; Uno, B.; Kubota, T.; Ikeda, T.; Senda, M. *Bioelectrochem. Bioeng.* **1990**, *24*, 193. [g] Loach, P. A. *Handbook of Biochemistry*.

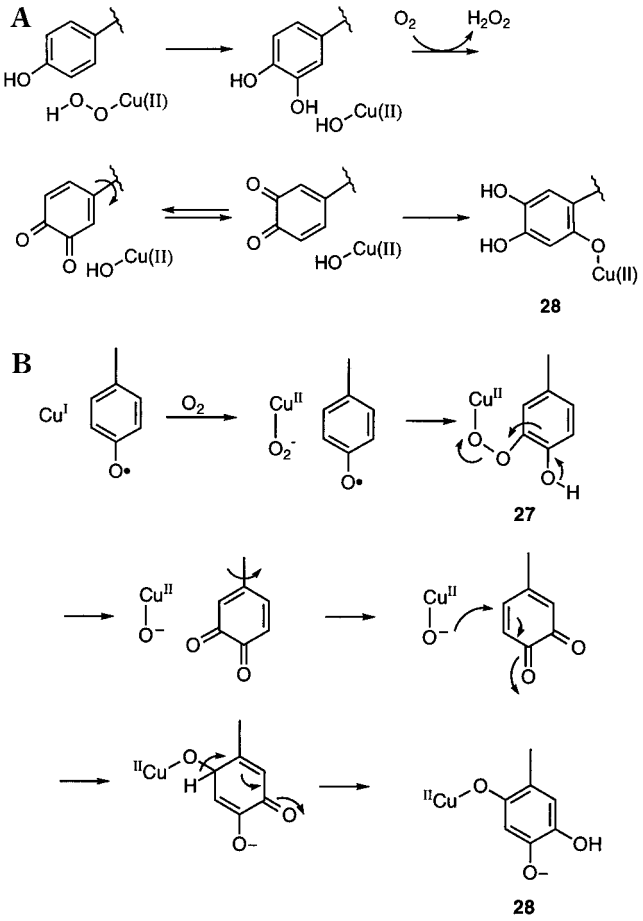
3. TPQ Formation: Metal and O_2 Based Posttranslational Modification

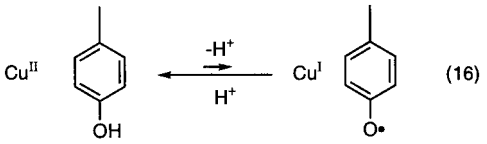
DNA sequence alignments of all known amine oxidase genes reveal that a tyrosine is the precursor to TPQ.[452,491−496] Several recent studies as summarized subsequently, suggest that conversion of Tyr to TPQ can occur by a metal and O_2 dependent self-assembly process. Klinman and co-workers were able to express active amine oxidase from *Hansenula polymorpha* in *S. cerevisiae*,[497] an organism with no known endogenous amine oxidases. Thus, its successful expression implicates a self-assembly process. The role of the copper in this process was suggested by mutagenesis studies in which His456, one of the copper ligands, was mutated to Asp. The resulting protein in addition to not binding copper (4% compared to the wild-type) was unable to convert the Tyr to TPQ.[29]

The most compelling evidence thus far for a self-assembly process requiring Cu and O_2, however, has been provided by recent studies from the laboratories of Tanizawa and Dooley on phenylethylamine oxidase from *Arthrobacter globiformis*.[32] They were successful at expressing this protein in a copper-depleted media allowing isolation of an inactive amine oxidase with its Tyr382 (the TPQ precursor) unmodified and containing no copper.[493] Upon incubation of this protein with Cu^{II} and O_2 one TPQ/subunit was generated, and the specific activity of the protein was similar to that of the wild-type.[32] This process was shown to be independent of the amount of copper added as long as at least 1 equiv of copper was present per subunit. These results suggest that tyrosine oxidation to TPQ requires only active site bound copper.

A mechanistic model for this self-assembly process has been proposed by Cai and Klinman (Scheme 25A),[497] and expanded upon by others (Scheme 25B).[30,32,498] The tyrosine is proposed to be hydroxylated to dopa in a copper and O_2 mediated process that involves a copper peroxo intermediate. The source of the extra electron necessary to reduce O_2/Cu^I to this Cu^{II}-OOH is at present unknown. Several researchers have independently suggested that the tyrosine to be oxidized may provide this electron.[30,32,498] In these proposed models the Cu^{II}/Tyr system would be in equilibrium with Cu^I/Tyr$^\bullet$ (eq 16).

Scheme 25





For this to occur the redox potential of the copper center would need to be modulated by about 600 mV on the basis of the redox potential of Y$^\bullet$ and known copper proteins (Table 5). This oxidation of tyrosine differs markedly from the second half reaction of the amine oxidation process, the reoxidation of reduced cofactor, as the redox potential of an aminoquinol is 111 mV, some 900 mV lower than tyrosine. If such an equilibrium (eq 16) exists, Cu^I may react with oxygen, forming, a copper superoxide complex which could combine with the Tyr$^\bullet$ to form intermediate **27** (Scheme 25B).[32] Heterolytic cleavage of the O−O bond would then generate dopa and a copper oxide or hydroxide. Rotation and subsequent hydration of dopa by Cu^{II}−OH or H_2O would yield **28** which can be oxidized in an O_2-dependent process to generate

TPQ. The hydrogen peroxide formation that is predicted during this process has yet to be confirmed experimentally.

Recently RR studies were carried out to determine the source of the oxygens in TPQ.[477] The authors were able to detect ^{18}O derived from $H_2{}^{18}O$ in both the C5 and C2 of TPQ. Unfortunately, these are mechanistically uninformative results given that the carbonyl group at C5 could exchange by hydration/dehydration and that a copper bound hydroxide derived from O_2, initially proposed by Klinman as the source of the C2 oxygen, can also exchange with solvent.

Given the availability of recombinant precursor protein, the details of this assembly process in vitro will soon be elucidated, and the experimental observations should allow distinction between the various proposed mechanisms. How the in vitro reconstitution relates to the biosynthetic pathway of cofactor assembly in vivo is the subject of ongoing investigation.

B. Methylamine Dehydrogenase

MADHs, found in gram-negative methylotropic bacteria, are a second class of enzymes containing a covalently bound quinone cofactor. They catalyze the deaminative oxidation of methylamine to formaldehyde and ammonia (eq 17).

$$CH_3NH_2 + H_2O \xrightarrow{2e^-,\ 2H^+} HCHO + NH_3 \qquad (17)$$

In contrast to the copper amine oxidases discussed in the previous section, the reoxidation of the cofactor does not require O_2 and is catalyzed by either the copper protein amicyanin (Figure 25),[499−502] or by a heme requiring cytochrome *c* type protein.[503,504] A combination of biochemical, molecular biological, and crystallographic studies have established the structure of the orthoquinone cofactor as tryptophan tryptophylquinone (TTQ, Figure 1I) and have facilitated our understanding of the mechanism of the reoxidation of the reduced form of the cofactor by one electron transfer mechanisms.

1. Structure

MADHs possess an $\alpha_2\beta_2$ subunit structure, with M_r = 39−50 kDa for the α-proteins and M_r =13−18 kDa for the β-proteins which contain the covalently bound TTQ. Early studies of Tobari and co-workers on the enzyme from *Methylobacterium extorquens* AM1 revealed that this cofactor was linked to the protein at the unidentified residues 55 and 106,[505] and spectroscopic studies of the cofactor adducts generated upon incubation of the enzyme with phenylhydrazine suggested that the cofactor might be a modified PQQ.[506] Several additional studies, solution of a high-resolution crystal structure of MADH from *Thiobacillus versutus*,[507] and sequencing of the MADH genes from *M. extorquens*,[508] set the stage for the identification of the structure of the cofactor by McIntire and Lidstrom and co-workers.[24] The structure of the cofactor could not be deduced from the crystallographic analysis in part because the sequence of the gene for the β-subunit was not established.[507] However, the electron density map established that it was not a covalently bound version of PQQ. Subsequent cloning of the gene for the β-protein of MADH from *M. extorquens* by Lidstrom and co-workers identified the importance of two tryptophan residues.[508] Isolation of a peptide containing the cofactor derivatized as a semicarbazide-derived cofactor, and its characterization by NMR and mass spectrometric techniques identified the cofactor as two C4−C2 cross-linked tryptophans with the orthoquinone moiety residing at C6 and C7 of residue 55 (Figure 1I).[24] A reevaluation of the crystallographic data confirmed this proposed structure.[25]

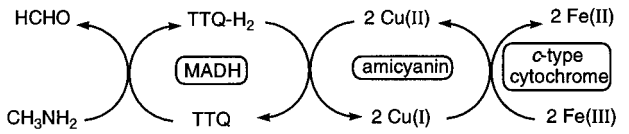


Figure 25. Electron flow during the oxidative deamination of methylamine catalyzed by methylamine dehydrogenase.

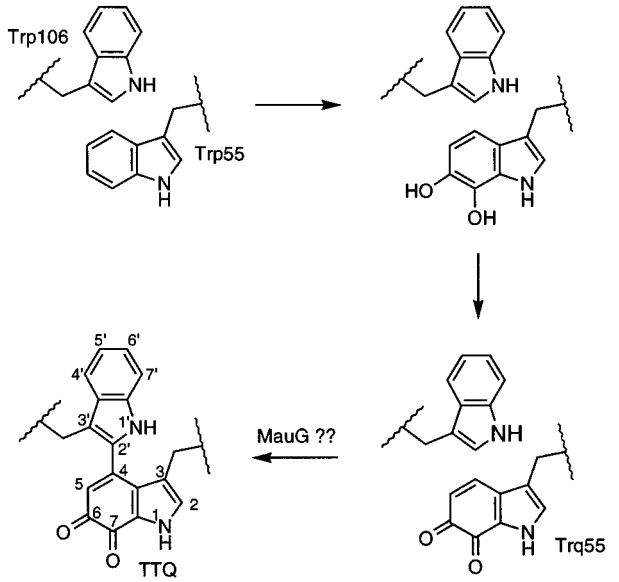


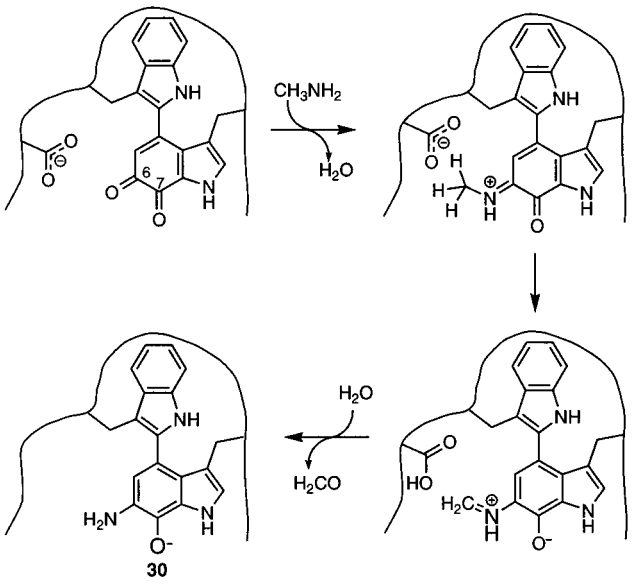
Figure 26. Proposed steps in the biosynthesis of TTQ.[509,510] (Trq = tryptophan quinone.)

Genetic analysis of the DNA sequences contiguous to the genes for the two subunits of methylamine dehydrogenase (*mauA* and *mauB*) revealed 8−11 open reading frames depending on the organism and suggested that the biosynthesis of TTQ requires at least two gene products: MauG, a protein with sequence similarity to a cytochrome *c* peroxidase from *Pseudomonas* sp., and MauL with no known homology to any proteins in the protein databases.[509,510] MauG has been proposed to be involved in the cross-linking of tryptophylquinone (Trq) 55 and Trp106 (*M. extorquens* numbering),[509,510] after hydroxylation and autoxidation of Trp55 to its orthoquinone (Figure 26).[511] Support for the role of MauG comes from studies that indicate peroxidases can catalyze cross-linking of indoles at these positions.[512] Furthermore, although MADH activity was absent from crude extracts of *mauG* mutants, redox-cycle staining still allowed detection of a quinone functionality in the β-protein, possibly the putative non-crossed-linked orthoquinone precursor to TTQ.[509]

2. Catalytic Mechanism

Substantial evidence exists to suggest that the mechanism of the oxidation of amine to aldehyde in methylamine dehydrogenase (Scheme 26) is similar to the first half-reaction catalyzed by the copper amine oxidases (section XII.A).[24,513] The amine is proposed to form a Schiff base at C6 of TTQ, detected as a transient intermediate at 425 nm when the reaction is run in the absence of amicyanin.[514] As in the case of BPAO,[515] a large kinetic isotope effect ($k_H/k_D = 17.2$)[516] was detected on removal of a proton by a general base catalyst thought to be Asp70 (*T. versutus*), again in analogy with AOs. Proton removal would allow formation of a new imine, thus far not detected. Hydrolysis of the imine gives the reduced amino form of the cofactor and formaldehyde (Scheme 26).

Scheme 26



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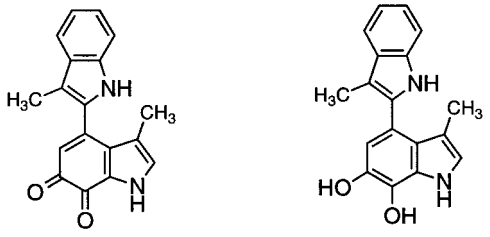
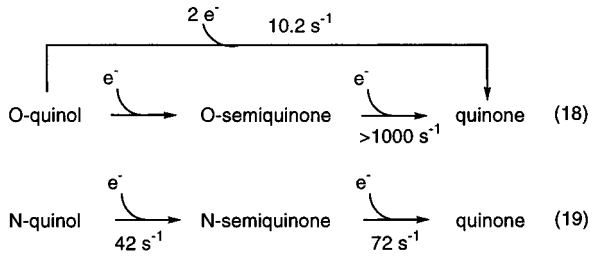
Recent model studies with the TTQ derivative **29** provide additional support for this proposal with a λ_{max} for the oxidized (434 nm) and reduced forms (300 nm) that is very similar to those reported for the cofactor of MADH (440 and 326 nm, respectively for the protein from *P. denitrificans*). This model system has an $E^{o\prime} = 107$ mV for a $2e^-$ reduction compared to $E^{o\prime} = 126$ mV for the cofactor of *P. denitrificans*,[517] and **29** can rapidly oxidize benzylamine to benzaldehyde.[518]



29 **29-H_2**

As indicated in eq 17 the mechanism of the second half-reaction, reoxidation of **30** to TTQ, must differ from that of AO. The requirement of the MADH enzyme from *P. denitrificans* for amicyanin and cytochrome c_{551i},[519] and the characterization of amicyanin as a type I copper protein with a single copper requires that the reoxidation involves two consecutive one electron transfer steps (Figure 25). Indirect evidence for the ability of the cofactor to undergo one electron as well as two electron transfers has been provided in a study of MADH treated with 0.5 equiv of methylamine. A semiquinone radical was observed resulting from comproportionation of the oxidized and reduced forms of the cofactor.[514,520] The presence of a nitrogen derived from substrate in the semiquinone radical generated by incubation of the enzyme with [^{15}N]-CH_3NH_2 was demonstrated by ESEEM spectroscopy.[521,522] These experiments, however, also generate 0.5 equiv of *O*-semiquinone radical in addition to the *N*-semiquinone radical.

In very recent work, Davidson and co-workers have provided evidence that a *N*-semiquinone radical, and not an *O*-semiquinone as argued by Duine and coworkers,[523,524] is a true intermediate in the reoxidation of the cofactor.[525] Using SF−UV−vis spectroscopy they examined the two-electron oxidation of MADH by amicyanin. In these experiments MADH was present in either the *O*-quinol state (eq 18), generated by dithionite reduction of TTQ, or in its N-quinol state (eq 19), generated by methylamine reduction of TTQ.



Only in the latter case did an intermediate with spectral properties similar to a semiquinone radical (λ_{max} ~425 nm) accumulate with $k_{obs} = 42$ s^{-1}. This intermediate was then further oxidized to TTQ at a rate of 72 s^{-1}. In a separate experiment the authors reacted amicyanin with MADH in which the cofactor was present in its *O*-semiquinone radical state. The observed rate of electron transfer from this *O*-semiquinone radical to amicyanin was too fast to measure (>1000 s^{-1} at 25 °C). On the other hand, when amicyanin was reacted with MADH in which the cofactor was present as a mixture of *N*-semiquinone radical and *O*-semiquinone radical by comproportionation as discussed above, a fast and a slow phase of oxidation to quinone were observed. Only the kinetics of the slow phase could be deconvoluted, providing a rate constant of 70 s^{-1}. Assuming that this slow reaction corresponds to the oxidation of the *N*-semiquinone radical and the fast phase to that of the *O*-semiquinone radical, the authors concluded that the intermediate observed in the two electron oxidation of methylamine reduced MADH corresponds to an *N*-semiquinone radical.

Our understanding of the mechanism of electron transfer between MADH and amicyanin and amicyanin and cytochrome c_{551i} has been aided by the

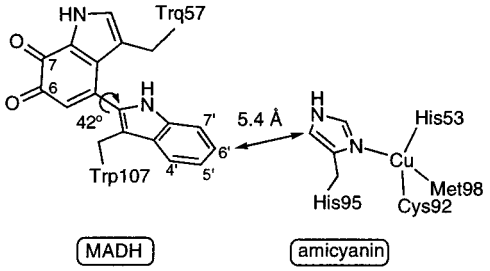


Figure 27. Schematic representation of the orientation of TTQ of methylamine dehydrogenase and the copper site of its physiological substrate amicyanin from *P. denitrificans*. Adapted from ref 526. The corresponding residues comprising TTQ in MADH from *Methylobacterium extorquens* AM1 are Trq55 and Trp106.

recent structures at high resolution of the binary and ternary complexes of the proteins from *P. denitrificans*.[526,527] However, despite this spectacular set of structures, the mechanism and pathway(s) involved in electron transfer remain to be established. The copper site of amicyanin in the crystal structure of the binary complex $(MADH)_2(amicyanin)_2$ is located close to the interface of the two proteins, which is composed of largely hydrophobic residues. One of the copper ligands, His95, is part of this hydrophobic patch with its N^{δ} ligated to the metal and its N^{ϵ} exposed to the interface (Figure 27).[526] At the MADH side, the *o*-quinol of Trq57 (Trq55 in *M. extorquens*) is pointed away from the copper, and the distance from its C6, the putative site of transamination in the reductive half-reaction, to the copper is 15.8 Å. Interestingly, the indole moiety of Trp107 (Trp106 in *M. extorquens*) is oriented toward the copper site at a distance of 9.4 Å. The closest distance between the two groups is 5.4 Å between the C^{δ} of His95− and the C6′ of Trp107. Another interesting observation is the nonplanarity of the two indole rings of TTQ which make a dihedral angle of about 42°.[25]

In the ternary complex $(MADH)_2(amicyanin)_2$-(cytochrome $c_{551i})_2$ the cytochrome and MADH are both in contact with amicyanin but not with each other.[527] The three centers of the electron-transfer pathway are situated roughly in one line, with a distance of about 40 Å between TTQ and the heme iron, while the copper and the iron are 24.8 Å apart. It has been proposed that the electron-transfer pathway from reduced TTQ and amicyanin involves the indole ring of Trp107 and the imidazole ring of His95 (Figure 27). Calculations have indicated that this pathway is about three times less efficient than an alternative pathway following the main chain of the β subunit of MADH.[527] These same calculations predicted the electron transfer from MADH to amicyanin to be about 10^6 times faster than from amicyanin to the cytochrome. Experimentally, this prediction is not observed, however, with approximately equal rates for both reactions. Thus, although the proposed pathway involving Trp107 is attractive, at present the details of this process are not well-understood.

XIII. Other Systems in Which Protein-Based Radicals Have Been Proposed or Detected

A. Bovine Liver Catalase

Catalases promote the disproportionation of hydrogen peroxide to water and O_2. As in the case of peroxidases (section VIII) this reaction takes place at an iron-protoporphyrin IX cofactor. In native catalase the iron is in a high spin ferric form which reacts with hydrogen peroxide to form compound I. In the enzyme from *M. luteus*, EPR studies have demonstrated that compound I consists of an oxyferryl ferromagnetically exchange coupled to a porphyrin π radical cation.[243] In contrast, a study by Ivancich et al. reported the formation of a different EPR signal in the reaction of bovine liver catalase with peroxyacetic acid.[528] Comparison of the principal values of the g tensors and the hyperfine tensors determined by ENDOR spectroscopy with those of known tyrosyl radicals led the authors to assign the EPR signal to a tyrosyl radical.[529] The X-ray structure of this enzyme shows an axial tyrosinate ligand to the heme iron.[530] Thus, as in the case of CcP and PGHS, bovine liver catalase may store the two oxidation equivalents from peroxide in the form of an oxyferryl-heme and a protein radical. Recent rapid kinetics studies suggest that initially a oxyferryl-heme and a porphyrin π-cation radical is formed, which then undergoes intramolecular electron transfer to generate the tyrosyl radical species.[529] Further studies on this system are required to examine this interesting observation and to investigate the differences between the enzymes from *M. luteus* and bovine liver.

B. DNA Photolyase

Ultraviolet light causes DNA damage by the formation of *cis,syn*-cyclobutane dimers of pyrimidine bases (Pyr<>Pyr, Figure 28). Cells can repair this damage by photoreactivation mediated by the enzyme DNA photolyase.[531] During this process, the enzyme binds to the damaged DNA site in a light independent step. Catalysis is initiated by absorption of a blue-light (350−450 nm) photon by a light-harvesting cofactor, 5,10-methenyltetrahydrofolate (MTHF)[532] or 7,8-didemethyl-8-hydroxy-5-deazariboflavin[533] depending on the enzyme source. The excitation energy is then transferred from this "antenna" to the two electron reduced deprotonated cofactor, flavin adenine dinucleotide ($FADH^-$), generating the excited-state $FADH^{*-}$, which in turn transfers an electron to the pyrimidine dimer producing the neutral semiquinone radical $FADH^{\bullet}$ (Figure 28). The cyclobutane is split, and back electron transfer from the resulting pyrimidine radical anion to $FADH^{\bullet}$ yields the repaired DNA and regenerates the resting form of the enzyme.

Sancar and co-workers have shown that in addition to this normal turnover reaction, photolyase can catalyze two other electron-transfer reactions in vitro that involve tryptophan radicals. The first was observed during reactivation of inactive enzyme that is produced during purification. In this protein the $FADH^-$ cofactor has been oxidized to the catalytically

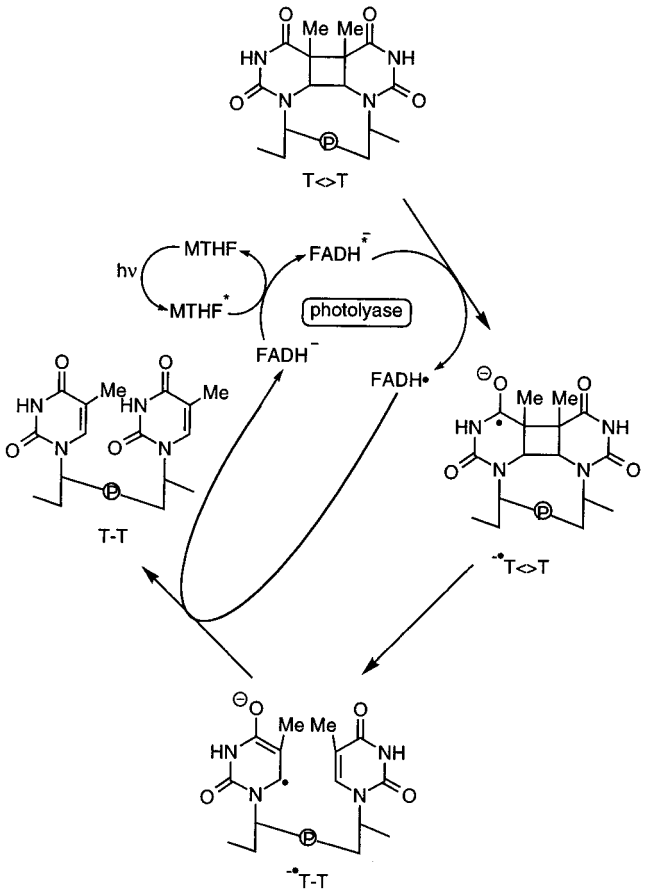
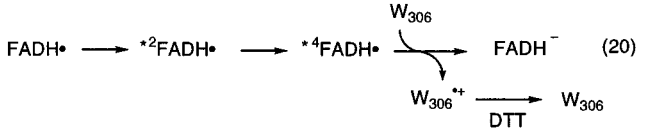


Figure 28. Proposed mechanism of the light-induced repair of thymidine dimers in DNA by photolyase.[531] The light-harvesting cofactor MTHF is excited by light with $\lambda = 350-450$ nm. The excitation energy is transferred to the deprotonated reduced form of flavin adenine dinucleotide, generating its excited singlet state $FADH^{-*}$. This then transfers an electron to T<>T generating the neutral semiquinone radical FADH•, and a T<>T radical anion ($^{-}$•T<>T). The radical anion then splits to form $^{-}$•T−T which transfers an electron to the FADH• radical to regenerate $FADH^-$ and the product T−T.

inactive semiquinone radical FADH•. This inactive form of the protein can be activated by photoreduction of FADH• to $FADH^-$ in a MTHF-independent process. Irradiation at 360−600 nm yields the excited doublet state of FADH• which undergoes intersystem crossing to the excited quartet state (eq 20).[532]



(20)

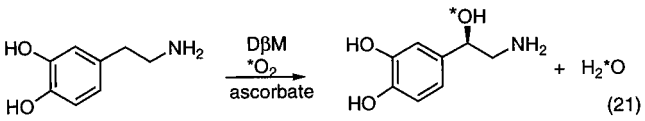
This quartet state is reduced by a nearby amino acid residue generating a protein radical and $FADH^-$. In the presence of exogenous electron donors such as DTT, the protein radical is reduced, producing the active form of photolyase. In vitro this photoreduced photolyase can catalyze several turnovers under aerobic conditions before reoxidation of $FADH^-$ to FADH• by molecular oxygen.[534]

Laser flash photolysis studies of this process revealed a difference spectrum with a maximum at 510 nm that was initially ascribed to a neutral tryptophan radical.[535] Site-directed mutagenesis studies in which 9 of the 15 tryptophan residues of photolyase from *E. coli* were replaced by phenylalanine identified one mutant protein, W306F, that was incapable of photoreduction of FADH•, while it was still able to bind substrate and repair Pyr<>Pyr dimers in the presence of chemical reductants.[536] Subsequently, the redox active residue was unambiguously identified as Trp by time-resolved EPR studies on photolyase containing isotopically labeled tryptophan residues.[537] These studies also indicated that FADH• is reduced by electron-transfer rather than hydrogen atom transfer from tryptophan, generating a cationic tryptophan radical ($Trp^{\bullet+}$) and $FADH^-$ (eq 20). In the recently reported crystal structure of DNA photolyase from *E. coli*, Trp306 is 13 Å removed from the flavin.[538] Several other residues that can undergo redox chemistry (Trp359 and Trp382) are located between Trp306 and the flavin, but site-directed mutagenesis studies suggest that they are not involved in the electron transfer. Thus, the exact pathway of the et in this photoreduction of FADH• remains to be elucidated. Whether this in vitro reduction of the semiquinone form of FAD is also important during in vivo repair of pyrimidine dimers is at present unclear as the physiological form of FAD in photolyases is $FADH^-$.[534,537]

A second pathway to repair Pyr<>Pyr dimers involving a tryptophan radical apparently circumvents both the MTHF and $FADH^-$ cofactors of photolyase. Irradiation at 280 nm leads to repair with a quantum yield of 0.56 under conditions where repair by $FADH^-$ is absent.[539] The absolute action spectrum, Trp fluorescence and fluorescence quenching by substrate suggested that a single Trp residue functioned as the photosensitizer during this repair. Mutagenesis studies suggested that the excited singlet state of Trp277 is the electron donor in the repair process. In support of this postulate, Trp277 is close to the putative substrate binding site in the crystal structure. However, taking into account the low <300 nm flux in sunlight, the contribution of this Trp277-mediated photoreversal of Pyr<>Pyr dimers by photolyase is <0.01% compared to repair involving the MTHF and $FADH^-$ cofactors.[539]

C. Dopamine β Monooxygenase

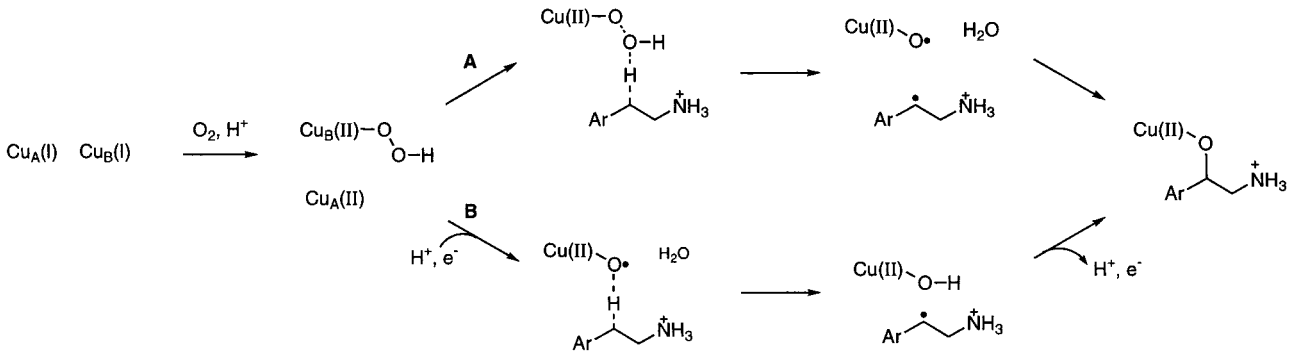
Dopamine β monooxygenase (DβM) catalyzes the conversion of dopamine and oxygen to norephinephrine and water (eq 21). In vitro the two reducing



(21)

equivalents required for this process can be provided by ascorbate,[540] which is also thought to be the in vivo reductant. The enzyme exists as active dimeric and tetrameric species, and contains two essential copper atoms per subunit.[541] These two coppers are believed to be at least 4 Å apart as no spin coupling could be detected by EPR spectroscopy in the ascorbate-

Scheme 27

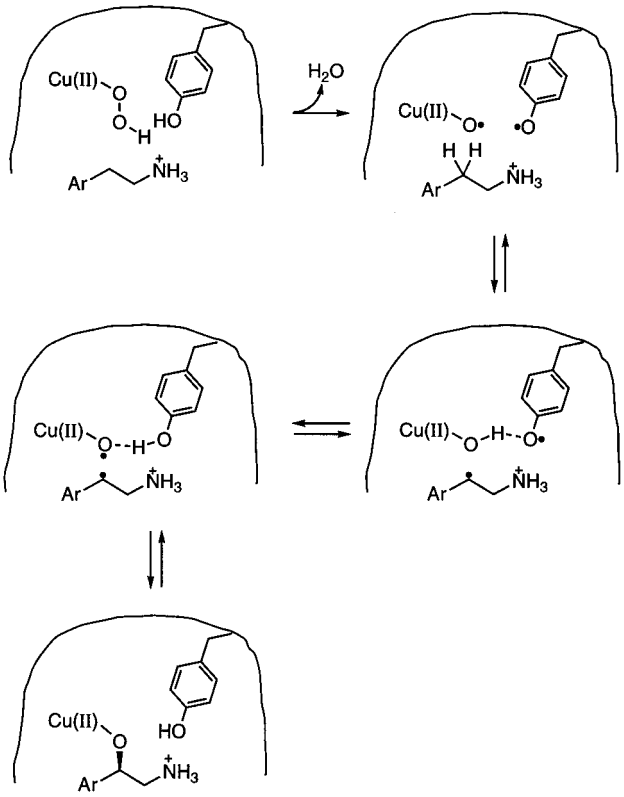


reduced Cu(I) state of the resting enzyme,[542,543] nor in the catalytically generated enzyme−product complex.[543,544] Furthermore, EXAFS experiments failed to detect any backscattering between the two copper centers.[545] These results suggest therefore, that a binuclear center, as observed in other copper proteins such as tyrosinase and hemocyanin,[546] is not involved in catalysis in the case of DβM. It has been proposed that the two copper centers perform separate functions with one site (Cu_A) involved in ascorbate binding and electron transfer from ascorbate to the second site (Cu_B). This second copper center is generally believed to be the site of substrate binding and hydroxylation.[541] Studies of pH-dependent isotope effects[547] and structure−function studies with substrates and mechanism-based inhibitors[548,549] have suggested the intermediacy of a benzylic radical formed by abstraction of the *pro-R* hydrogen atom in the initial step of catalysis. The reactive species responsible for this process has been proposed to be a copper hydroperoxide generated via the reaction of the two-electron reduced dicuprous form of the enzyme with molecular oxygen (Scheme 27, pathway A). This hypothesis is supported by kinetic studies that exclude the possibility of binding of external reductant between substrate binding and oxygen activation, and therefore rule out a reductive cleavage of the CuO−OH to generate a reactive copper-oxo species (Scheme 27, pathway B).[550] Furthermore, it has been shown that the Cu(I) form of DβM is catalytically fully active in the absence of external reductant.[543]

An alternative mechanism that is consistent with the experimental data was proposed recently by Klinman and co-workers and is shown in Scheme 28.[551] This model postulates that the copper hydroperoxide abstracts a hydrogen atom from a nearby tyrosine residue rather than from the substrate leading to the formation of a copper-oxo species, a tyrosyl radical, and water. Subsequent hydrogen transfer from the benzylic position of the substrate to the Cu^{II}-O$^{\bullet}$ generates an intermediate that can equilibrate between a Cu^{II}-OH/Tyr$^{\bullet}$ form and a Cu^{II}-O$^{\bullet}$/Tyr form (Scheme 28). Oxygen rebound from the Cu^{II}-O$^{\bullet}$ species with the benzylic substrate-derived radical then produces the product alcohol.

This postulate is supported by recent studies on the correlation of reaction rates with ^{18}O kinetic isotope effects observed with several substituted phenethylamines that indicated cleavage of the O−O

Scheme 28



bond occurs prior to hydrogen atom abstraction.[551] As outlined above, kinetic investigations have ruled out reductive cleavage of the O−O bond by external reductants. However, these experiments do not rule out activation of the peroxide to a copper−oxo species by a redox active residue on the protein. The presence of two tyrosine residues at or near the active site had been previously established in studies with the mechanism-based inhibitors *p*-cresol, phenylhydrazine, and 6-hydroxybenzofuran which covalently labeled Tyr216 and Tyr357.[552,553] The second step in the proposed mechanism, abstraction of the benzylic substrate hydrogen by the tyrosyl radical, is feasible on thermodynamic grounds as the bond dissociation energies for a benzylic C−H bond and a phenolic O−H bond are ~85 kcal mol^{-1} and 86 kcal mol^{-1}, respectively (Table 7).

While the proposed involvement of a tyrosyl radical in the hydroxylation of dopamine can account for these experimental data, no direct evidence for this model has been obtained to date.

XIV. Summary and Outlook

Since the discovery and identification of the essential tyrosyl radical in ribonucleotide reductase, modified tyrosyl radicals, tryptophan radicals, glycyl radicals and thiyl radicals have been characterized and shown to play essential roles in enzymes involved in primary metabolic pathways. All have in common that their biosynthesis occurs via metal-mediated transformations involving Co, Cu, Fe, and Mn. In some cases additional protein factors and small molecules are required, but in other cases the systems self-assemble. RNRs and PFL, both of which are primary control points in metabolism, have evolved sophisticated pathways not only for the synthesis of the cofactor, but also for its reduction as a regulatory switch when activity is not required. The reactions catalyzed by radical-dependent proteins are very diverse and in many cases there exist comparable transformations that involve heterolytic chemistry. Examining the variety of reactions—oxidations, reductions, electron transfer, carbon—carbon bond cleaving, O_2 evolution, and lyases—no general paradigm seems to emerge.

What has changed since the discovery of many of these systems is the facility with which we can now study their mechanisms. In many of the systems discussed, molecular biology has provided us with gram quantities of proteins, and with the ability to isolate unmodified forms of these in order to study their posttranslational modifications. Furthermore, it has made possible the production of isotopically labeled amino acid residues within these proteins and the build up of reactive intermediates by blocking a pathway using site-directed mutants. Many of these species will be very difficult to model chemically due to our inability to mimic active site cavities. The time scale of many of these reactions, the O_2 evolving system being an exception, is well within the range of conventional instrumentation where rate constants of up to 500 s^{-1} can be determined. More recently instrumentation and methodology has become available that has facilitated the analysis of intermediates formed during these processes. High-frequency EPR spectrometers (140 and 250 GHz) are now accessible in addition to the more conventional 3, 9, and 35 GHz instruments. The complexity of many of the observed signals due to the powder patterns resulting from the freeze—quenching required to trap intermediates, and in some cases because of the presence of exchange coupled systems, requires analysis at these multiple frequencies. In addition the ENDOR spectroscopy now available at 35 GHz is being extended to 140 GHz. This in conjunction with the ability to isotopically label and trap transient intermediates is allowing insight into the unprecedented structures of reactive intermediates. The input of all these techniques is essential for accurate spectral simulations, which given the advances in computer technologies, should now accompany spectral presentations. These advances along with the revolution in structural biology that has allowed us to examine the structures of proteins before and after a reaction at high resolution (<1.8 Å) are now providing us with the key framework to evaluate catalytic transformations.

With the rapid pace at which more and more genes are cloned, proteins are expressed, and new metal centers are revealed, the field of understanding protein radicals and their roles in catalysis will remain a vigorous one for some time to come.

XV. Abbreviations

AA	arachadonic acid
AcCoA	acetyl coenzyme A
AdoCbl	5′-deoxy-5′-adenosylcobalamin
AE	activating enzyme
AO	amine oxidase
APAO	arthobacter plasma amine oxidase
APX	ascorbate peroxidase
BDE	bond dissociation energy
BPAO	bovine plasma amine oxidase
B. subtilis	*Bacilus subtilis*
Cys$^{\bullet}$	cysteinyl radical
CcP	cytochrome *c* peroxidase
CD	circular dichroism
Chl	chlorophyll
ClUTP	2′-chloro-2′-deoxyuridine 5′-triphosphate
CoA	coenzyme A
5′-dA	5′-deoxyadenosine
5′-dA$^{\bullet}$	5′-deoxyadenosyl radical
DβH	dopamine β-hydroxylase
DDH	dioldehydrase
dF_2CTP	2′-difluoro-2′-deoxycytidine 5′-triphosphate
Dopa	3,4-dihydroxy phenylalanine
EAL	ethanolamine ammonia lyase
ECAO	*E. coli* amine oxidase
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
(*E*)-FMCDP	(*E*)-2′-(fluoromethylene)-2′-deoxycytidine 5′-diphosphate
(*E*)-FMCTP	(*E*)-2′-fluoromethylene-2′-deoxycytidine 5′-triphosphate
EGF	epidermal growth factor
E_m	midpoint potential
ENDOR	electron nuclear double resonance
EPR	electron paramagnetic resonance
ESEEM	electron spin—echo envelope modulation
ESE-ENDOR	electron spin—echo-electron nuclear double resonance
et	electron transfer
EXAFS	extended X-ray absorption fine structure
FAD	flavin adenine dinucleotide
FCTP	2′-fluoro-2′-deoxycytidine 5′-triphosphate
Gly$^{\bullet}$	glycine radical
GAO	galactose oxidase
GS$^{\bullet}$	glutathionyl radical
H. influenzae	*Haemophilus influenzae*
HoSF	horse spleen ferritin
HRP	horseradish peroxidase
HSV	herpes simplex virus
HuHF	human H-chain ferritin
L. lactis	*Lactococcus lactis*
L. leichmannii	*Lactobacillus leichmannii*
LSAO	lentil seedling amine oxidase
LMCT	ligand-to-metal charge transfer
LTQ	lysine tyrosylquinone
MADH	methylamine dehydrogenase
mau	methylamine utilizing genes
MCD	magnetic circular dichroism
met-R2	R2 of which the tyrosyl radical has been reduced
M. extorquens	*Methylobacterium extorquens*
M. genitalium	*Mycoplasma genitalium*

MMO	methane monooxygenase
M. tuberculosis	*Mycobacterium tuberculosis*
MTHI	5,10-methenyltetrahydrofolate
NHE	normal hydrogen electrode
nrd	nucleotide reduction genes
NS	narrow singlet
NSAID	nonsteroidal antiinflammatory drug
N_3UDP	2′-azido-2′-deoxyuridine 5′-diphosphate
N_3CTP	2′-azido-2′-deoxycytidine 5′-triphosphate
OEC	oxygen evolving complex
ORF	open reading frame
P. denitrificans	*Paracoccus denitrificans*
P. falciparum	*Plasmodium falciparum* (malaria)
PFL	pyruvate formate lyase
PGG_2	prostaglandin endoperoxide G_2
PGH_2	prostaglandin endoperoxide H_2
PGHS	prostaglandin H synthase
pheo	pheophytin
PIPES	piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)
PPAO	porcine plasma amine oxidase
PPIX	protoporphyrin IX
PKAO	pig kidney amine oxidase
PQQ	pyrroloquinoline quinone
PSAO	pea seedling amine oxidase
PSII	photosystem II
Q_A	plastoquinone A
Q_B	plastoquinone B
RFQ	rapid freeze quench
RDPR	ribonucleoside 5′-diphosphate reductase
RNR	ribonucleotide reductase
RR	resonance Raman
RTPR	ribonucleoside 5′-triphosphate reductase
SAM	*S*-adenosyl methionine
SF	stopped flow
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
S. typhimurium	*Salmonella typhimurium*
T. versutus	*Thiobacillus versutus*
topa	1,3,4-trihydroxy-phenylalanine
TPQ	topa quinone
Trq	tryptophan quinone
TTQ	tryptophan tryptophylquinone
UV	ultraviolet
WD	wide doublet
WS	wide singlet
XANES	X-ray absorption near edge spectroscopy
Tyr$^\bullet$	tyrosyl radical
$Y_D^\bullet$	stable tyrosyl radical on subunit D_2 in photosystem II
$Y_Z^\bullet$	transient tyrosyl radical on subunit D_1 in photosystem II

Note Added in Proof

Since submittal of this review a number of reports have appeared that are relevant to the mechanistic scenarios depicted here. The reader is referred to the following references: amino acid radicals,[560,561] RNR,[562−567] PFL,[568−571] GAO,[572,573] PSII,[574−584] and AO.[585]

XVI. Acknowledgments

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