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Review

Protein tyrosyl free radicals as active species in metalloenzyme catalysis†

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Summary — A number of metalloenzymes utilize a tyrosyl free radical as a catalytically competent cofactor. The chemistry and physicochemistry of tyrosyl radicals are summarized. Tyrosyl radical enzymes, galactose oxidase, ribonucleotide reductase, photosystem II and prostaglandine H synthase are described, with focus on the generation and reactivity of the free radical. This may serve as a basis for discussing an intriguing question: why are tyrosyl radicals found in metalloenzymes?

radical enzyme / tyrosyl radical / galactose oxidase / ribonucleotide reductase / photosystem II / prostaglandin H synthase

Résumé — Radicaux tyrosinyle comme espèces actives de la catalyse par métalloenzymes. *Un certain nombre de métalloenzymes impliquent un radical libre tyrosinyle au cours de leur cycle catalytique. La chimie et la physico-chimie des radicaux tyrosinyles sont résumées. Les enzymes à radical tyrosinyle (galactose oxydase, ribonucléotide réductase, photosystème II, prostaglandine H synthase) sont décrites avec une attention particulière à la formation et à la réactivité du radical. Ces données sont centrales pour débattre de la question: pourquoi des radicaux tyrosinyles dans les métalloenzymes?*

enzymes radicalaires / radicaux tyrosyle / galactose oxydase / ribonucléotide réductase / photosystème II / prostaglandine H synthase

Various strategies are employed by metalloenzymes to store oxidizing equivalents required for multielectron transfer [1]. In some cases, the number of metal ions matches the number of electrons to be transferred. For example, methane monooxygenase contains a non-heme binuclear iron center to catalyze the two-electron oxidation of methane to methanol by oxygen and tyrosinase, with two copper ions in the active site, catalyzes the *ortho*-hydroxylation of phenolic substrates. A second class of metalloenzymes involves an organic redox cofactor to provide or abstract electrons and complete the metal-driven electron transfer in the whole catalytic process. For example, cytochrome P-450 or heme peroxidases utilize the combination of an iron center and a porphyrin ligand, which can transiently exist in a rather stable cation radical form. Some metalloenzymes require quinone cofactors, such as topaquinone in copper amine oxidases which catalyze the oxidative deamination of primary or secondary amines.

A third class of metalloenzymes use their own polypeptide chain as a cofactor and generate organic free radicals on specific amino-acid residues. There is growing evidence that organic radicals play a role in the catalytic process [2–4]. The recent emergence of free radical enzymology is reflected in the rapidly increasing literature in the field, the publication of a book [5] and an international symposium [6]. The amino acids usually involved in such a redox process include tyrosine (ribonucleotide reductase, photosystem II, prostaglandin H synthase), modified tyrosine (galactose oxidase), tryptophan (cytochrome *c* peroxidase) and glycine (ribonucleotide reductase, pyruvate formate lyase). This review focuses on the chemistry of tyrosyl radical enzymes. Some comments have been added concerning non-catalytic tyrosyl radicals.

The tyrosyl radical

Simple phenoxyl radicals

The redox potentials for one-electron oxidation at pH 7 are 0.93 V/NHE for tyrosine, 0.77 V/NHE for *p*-cresol and 0.65 V/NHE for *p*-methoxy phenol [7–8] and

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Abbreviations: GOase: galactose oxidase; RNR: ribonucleotide reductase; PGH synthase: prostaglandin H synthase.

increase with decreased pH (the redox potential for tyrosine is 1.22 V/NHE at pH 2). It is likely that tyrosyl radicals in enzymes arise from one-electron oxidation of the phenolate form, thus implying a concomitant deprotonation. Several chemical one-electron oxidizing reagents (eg, ferricyanide) generate the phenoxyl radical from phenol in the presence of bases. A homolytic cleavage of the O–H bond is however possible. Bordwell [9] has estimated the homolytic bond dissociation energies for 35 phenol derivatives. The radicals are stabilized by *ortho* or *para* donor groups. Phenoxyl radicals are easily generated by photolysis or by pulse radiolysis [10]. It is well known that simple phenoxyl radicals are not stable and undergo dimerization reactions due to the intercoupling of the different canonical forms (fig 1).

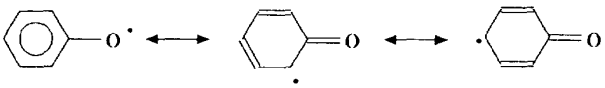

Fig 1. The phenoxyl radical.

The dimerization of the tyrosyl radical (which may be involved in protein cross-linking) gives dityrosyl units, with *o,o'*-biphenyl compounds as major products (fig 2).

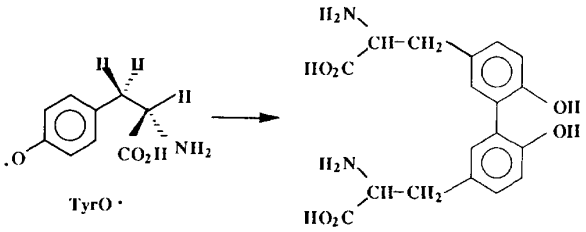

Fig 2. The tyrosyl radical and its major dimeric product.

Substitution at *ortho* and *para* positions stabilizes the phenoxyl radicals unless the substituents contain α hydrogen atoms [10] (fig 3). These radicals may undergo disproportionation reactions to phenol and cyclohexadienone. They react with oxygen to produce biscyclohexadienone peroxides [10].

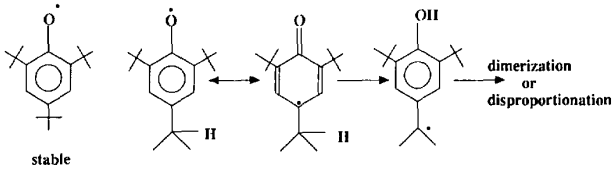

Fig 3. Substituted phenoxyl radicals.

Oxygen does not react with tyrosyl radical with a rate constant greater than 10^3 M^{-1} s^{-1} and this reaction is usually negligible [11]. Tyrosyl radical is reduced into the corresponding phenolate by ascorbate and other reducing agents [11]. Very recently, rate constants have been measured in non-aqueous media for hydrogen atom abstraction by the phenoxyl radical from some biologically important phenols and related compounds. These radicals exhibited a surprisingly high reactivity [12].

$$ArO^{\cdot} + RH \rightarrow ArOH + R^{\cdot} \text{ and } R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

Phenoxyl radicals exhibit a strong absorption band in the 390–430 nm region; this band is shifted to lower energies and its intensity increases as the interaction of the substitutent's $p\pi$ electrons with the phenoxyl π system increases.

Wheeler [13] has compared the calculated structures, electronic spin densities, vibrational frequencies and vibrational modes of the phenoxyl (PhO$^{\cdot}$) and tyrosine phenoxyl (TyrO$^{\cdot}$) radicals. Both radicals display substantial C=O double bond character. Calculated spin densities for the two radicals differ from each other by less than 0.03 and imply that the unpaired electron of TyrO$^{\cdot}$ resides entirely on its phenoxyl side chain. Calculated vibrational frequencies agree with the experimentally determined frequencies [14]. The two radicals have comparable vibrational frequencies and modes involving motions within the ring plane. The main differences can be attributed to interactions between the TyrO$^{\cdot}$ aliphatic chain and its phenoxyl side chain. This offers a potential to detect conformational differences between various TyrO$^{\cdot}$ in different proteins. The ESR spectrum of the tyrosyl radical in aqueous solution at pH 9.5 has been studied over a range of temperature [15]. The magnetic inequivalence of the diastereotopic methylene protons has been observed and, thus, ESR spectroscopy is a powerful tool for characterizing the conformation of the radical. The hyperfine coupling a_H of the unpaired electron to the diastereotopic protons is a function of the dihedral angle θ between the projection of the C–H bond and the ring carbon p_z orbital [16]; the most favored conformation for tyrosyl radical is that with $\theta = 60°$ (fig 4).

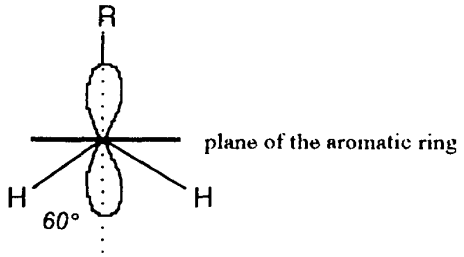

Fig 4. The favored conformation of the tyrosyl radical.

Tyrosyl radicals in enzymes

The identification of tyrosyl radicals in different proteins [17] has been carried out by a number of well-designed experiments combining isotopic substitution or site-directed mutagenesis with ESR, ENDOR, or vibrational spectroscopic measurements [18–24]. Resonance Raman spectroscopy and difference Fourier transform infrared vibrational spectroscopies have allowed comparisons of TyrO$^{\cdot}$ vibrational spectra in proteins

with those of simpler model structures [25–28]. Spin density distributions have been measured for TyrO$^{\cdot}$ in a number of proteins [29–32]. More recently, high field EPR has proved useful in evaluating the local electronic and magnetic environment of radicals, providing valuable information regarding the mechanism by which proteins are able to maintain and stabilize highly reactive radicals [33]. Some of these tyrosyl radical enzymes are described below.

Non-catalytic tyrosyl radicals in proteins

Peroxide-dependent oxidation of heme proteins has been shown in many cases to generate radicals on the polypeptide chain. Such processes participate in oxidative damage to proteins, membrane lipids and tissues. A famous case is that of myoglobin, a heme protein that is ubiquitous in aerobic muscle tissues. Since the 1950s, metmyoglobin has been known to react with hydrogen peroxide, resulting in the one-electron oxidation of ferric heme to form ferryl heme with the concomitant formation of a protein-centered radical. Many investigations have concluded that the free radical is centered on a tyrosine residue. However, site-directed mutagenesis studies, in which all of the tyrosine residues have been removed, and utilization of the ESR spin-trapping technique, unambiguously revealed that the globin radical is actually centered on a tryptophan residue [34].

Another recent observation of tyrosyl radical formation is during the oxidative deposition of iron in apoferritin. Ferritin is the principal reservoir for metabolic iron within the cell. Incorporation of iron consists of iron(II) oxidation by O_2 taking place on the ferroxidase sites of the protein. During this process radicals are formed but their functional roles are unknown [35]. However, there is now increasing evidence that these radicals are tyrosyl radicals arising from residue Tyr-34 located in the vicinity of the ferroxidase site and that they are not critically important in the protein-catalyzed mechanism of iron oxidation. The observed radical seems more likely to be the result of a side reaction, perhaps between hydroxyl radicals and the protein.

The tyrosyl radical enzymes

Galactose oxidase

Galactose oxidase (GOase) is an extracellular type II copper protein (68 kDa) of fungal origin [36]. GOase catalyzes the oxidation of several primary alcohols to aldehydes with the concomitant reduction of molecular oxygen to hydrogen peroxide, involving a two-electron transfer reaction. The crystal structure (1.7 Å resolution) of GOase [37] reveals unique mononuclear copper site with two histidine imidazoles, two tyrosines (one axial) and an exogenous water or acetate, in a distorted square-pyramidal coordination. The equatorial tyrosine ligand is covalently linked to a cysteine residue by a C–S bond at the *ortho* position of the hydroxyl group (fig 5).

In GOase, the number of metal ions involved in the reaction does not match the number of electrons

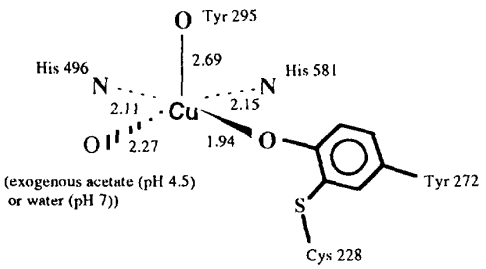

Fig 5. The active site of GOase.

transferred. This paradox has been solved with the identification of a tyrosyl free radical incorporated into the redox unit during the catalytic cycle (the pioneering work of Whittaker in the field of "the understanding of GOase" must be emphasized [36]). The enzyme exists in three well-defined and stable oxidation levels: the active oxidized form is EPR-silent indicating that the cupric ion is antiferromagnetically coupled to a free radical; the intermediate form shows a cupric EPR signal (type II copper protein); and the reduced form contains a cuprous center:

$$Cu^{2+}\text{-}Tyr^{\cdot} \overset{e^-}{\rightleftharpoons} Cu^{2+}\text{-}Tyr \overset{e^-}{\rightleftharpoons} Cu^{+}\text{-}Tyr$$

The enzyme could easily be interconverted between the active and the inactive forms in a redox titration using ferri/ferrocyanide solution [36]. Whittaker has shown that the tyrosyl free radical is located on the equatorial cysteine-substituted residue and is coordinated to the metal ion. In fact, the active enzyme has a diamagnetic (S = O) ground state. SQUID measurements gave evidence for the thermal population of a paramagnetic excited state at higher temperature with $-2J \geqslant 200$ cm^{-1}. The electronic spectrum of the active form displays a band at 445 nm which has been assigned by resonance Raman spectroscopy to the tyrosyl radical-to-metal charge-transfer band; the tyrosyl radical is ligated to the copper(II) center [38]. The redox potential for activation is 0.41 V/NHE. The thioether substitution could stabilize the radical by several hundred millivolts.

The specificity of the enzyme for primary alcohols is low, ranging from small molecules (eg, propanediol or dihydroxyacetone) to polysaccharides [39]. Nevertheless, GOase is stereospecific removing the pro-S methylene hydrogen of the C-6 alcohol in galactose. Specificity for oxidants is also broad; in the absence of O_2, several one-electron redox agents can act as electron acceptors.

The radical active form of GOase is ESR silent but the ESR signal of the tyrosyl radical can be generated from the apoenzyme [40]. When the metal-free GOase was exposed to mild oxidizing conditions (eg, ferricyanide) a sharp signal was detected in the ESR spectrum (fitting simulated with experimental ESR spectrum gave $g_1 = 2.0017$ and $g_2 = 2.0073$). The radical of the metal-free protein is stable; it is unreactive towards galactose; it may be reduced by hydroxyurea. Labeling experiments demonstrated that the radical was formed on the thioether-substituted tyrosine.

The mechanistic scheme for the catalytic cycle of GOase is shown in figure 6 (adapted with minor changes from reference [36]). Two electrons are transferred to the substrate, one from the free radical and one from the cupric ion. The oxygen then restores the active form by oxidation of the cuprous center and the modified tyrosine. In the proposed mechanism, the aldehyde release precedes dioxygen reduction. Under anaerobic conditions, galactose is oxidized into aldehyde and the copper(I) state is stable. The biological function of GOase is probably to serve as a generator of hydrogen peroxide (this accounts for the unusually low specificity of GOase for organic substrates) supporting lignin peroxidase reactions rather than as a source of aldehydes.

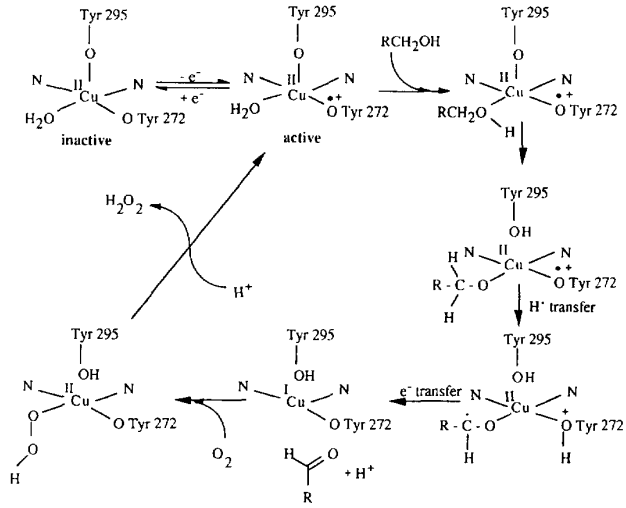

Fig 6. The possible catalytic cycle of GOase.

Ribonucleotide reductase

Ribonucleotide reductases (RNR) play a central role in DNA biosynthesis by catalyzing the conversion of ribonucleotides to deoxyribonucleotides [41]. The enzyme from *Escherichia coli* serves as a prototype for most eukaryotic reductases (mammals, viruses) and its structure has been determined by X-ray crystallography [42]. The reductase is composed of two homodimeric subunits. R1, the large subunit, provides redox active cysteines, which provide the reducing equivalents and binds the substrate. R2, the small subunit, contains a binuclear non-heme iron center, in which the two ferric ions are antiferromagnetically coupled through a μ-oxo bridge and a bridging carboxylate. Moreover, R2 contains a stable tyrosyl free radical located at residue 122, 5 Å away from the closest iron atom (fig 7).

ENDOR studies of the tyrosyl radical in RNR from *E coli* revealed an unusual value for the coupling of a methylene proton yielding an unusual conformation with $\theta = 33°$ and the second methylene proton situated in the plane of the aromatic ring ($\theta = 90°$) (fig 8).

This tyrosyl radical, which is essential for the catalytic activity, was the first free radical associated with a metalloenzyme to be recognized and characterized.

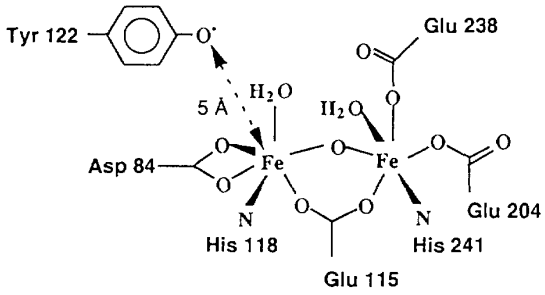

Fig 7. The active site in the R2 subunit of RNR.

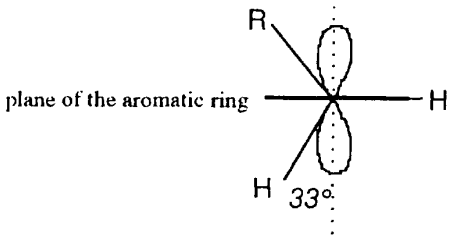

Fig 8. The conformation of the tyrosyl radical in RNR.

This radical is the most extensively studied protein radical so far, both in terms of its electronic properties and its reactivity. There are two reasons why this radical deserves so much attention. First, this oxidizing radical is absolutely required for the enzymatic reduction of ribonucleotides. This apparent paradox has been questioned and the fascinating chemistry of the reaction is now quite well understood, in particular from the exquisite studies from J Stubbe [43]. Second, this radical is essential for DNA synthesis and cell proliferation, and radical scavengers are potential anticancer and antiviral drugs.

The tyrosyl radical from protein R2 is generated during reaction of the apoprotein with ferrous iron in the presence of oxygen. The apoprotein binds two ferrous ions very efficiently and O_2 serves to oxidize the two ions into ferric ions and, concomitantly, the Tyr-122 residue into the tyrosyl radical. Since O_2 is a four-electron oxidant, an extraneous electron is required to split the O–O bond and is assumed to be supplied by a third ferrous ion during reconstitution (fig 9).

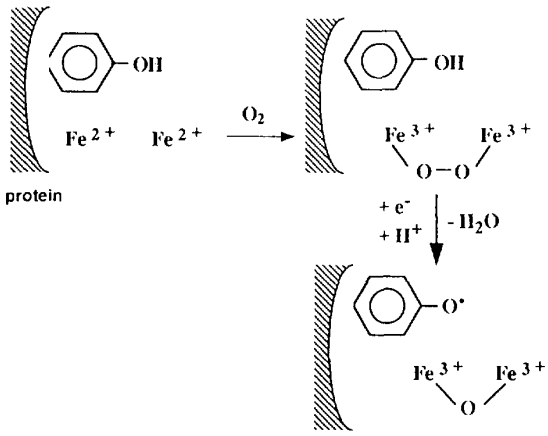

Fig 9. The generation of the tyrosyl radical in RNR.

The function of the tyrosyl radical during catalysis is to generate a radical on the ribose moiety of the substrate. This is achieved by abstraction of the hydrogen at the 3′ position. Only in the radical form, is the substrate activated for reduction. Formation of the deoxyribonucleotide then requires a three-electron reduction of the intermediate radical: two electrons are provided by two redox active cysteines and one electron by the tyrosine residue, which is recovered at the end of each catalytic cycle as a tyrosyl radical (fig 10). The radical X˙ in figure 10 has long been assumed to be the tyrosyl radical itself. However, three dimensional structures of both subunits R1 and R2 have revealed that the tyrosyl radical in R2 is located 30–35 Å from the substrate site in R1 [44]. Thus, the tyrosyl radical cannot abstract the hydrogen atom directly. In fact, through a long-range electron transfer pathway that remains to be identified but might involve tryptophan and tyrosine residues and a hydrogen-bonded array of invariant residues, the radical in R2 generates a radical at a third cysteine of the active site. X˙ is thus proposed to be a thiyl radical in R1 [45].

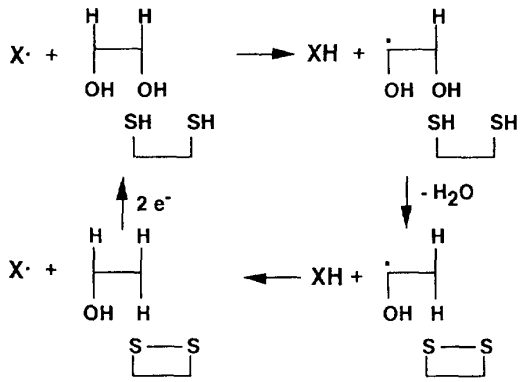

Fig 10. The catalytic cycle for ribonucleotide reduction by RNR.

The tyrosyl radical can be converted to tyrosine by reaction with radical scavengers, antioxidants or reducing agents. As a consequence, certain compounds like hydroxamates or phenols have antiproliferative properties. Hydroxyurea is used in clinics as an anticancer agent; the product of the reaction is a nitroxide radical which has been detected and characterized by ESR spectroscopy [46] (fig 11). It is interesting to note that hydroxyurea was recently found to greatly increase the anti-HIV properties of nucleosides such as AZT (azidothymidine) or DDI (dideoxyinosine).

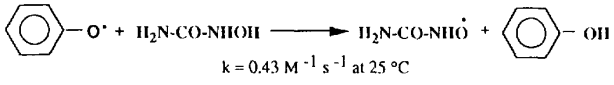

Fig 11. Reaction of RNR with hydroxyurea.

The radical is deeply buried inside the polypeptide chain. As a consequence, whether the mechanism involves direct abstraction of the hydrogen atom of hydroxyurea, within the protein or a long-range electron transfer (reduction to tyrosinate) followed by a proton transfer remains to be established.

Very recently, it has been shown that the tyrosyl radical of R2 can also react by coupling to radicals. The first example is the reaction with nitric oxide, NO. NO is a physiological inhibitor of ribonucleotide reductase and may be an essential mediator of the cytotoxicity of the activated macrophages. NO donors are antiproliferative agents. The inhibition involves a reversible coupling of NO to the tyrosyl radical, resulting in the formation of nitrosotyrosine [47] (fig 12).

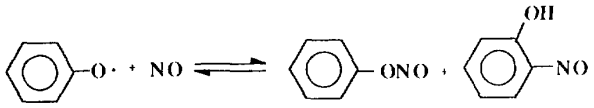

Fig 12. Coupling of NO with the tyrosyl radical of RNR.

Model reactions have confirmed such a reactivity of the phenoxyl radicals and shown that the coupling reaction to NO is very efficient (rate constant of the order of 10^9 M^{-1} s^{-1}) [48]. Tyrosyl radicals also couple to superoxide radical anions rather than being one-electron reduced. This may explain why, under oxidative stress conditions, the tyrosyl radical of ribonucleotide reductase is destroyed and the enzyme inactivated. This is an important observation since there are exceedingly few identified biological molecules which are inactivated by superoxide. Since ribonucleotide reductase needs to be protected, we can understand why aerobic living organisms have evolved superoxide dismutases to survive an oxygen atmosphere.

Photosystem II

The photosystem in higher plants, algae and cyanobacteria is a fascinating example in which tyrosine serves to catalyze electron transfer at high redox potential [49].

$$TyrOH \rightleftharpoons TyrO^{\cdot} + e^- + H^+$$

Such a property is similar to that of metal centers in terms of the ability to allow reversible one-electron transfer. The photosystem process operates by promoting electrons from the level of water to energies sufficient to reduce CO_2 to organic compounds. Photosystem II is a complex system composed of a minimum of seven polypeptides and multiple cofactors including chlorophyll, quinones, a tetranuclear manganese cluster and two tyrosyl radicals. Photon absorption by the photochemically active chlorophyll P680 generates its singlet excited state, which reduces the plastoquinone acceptor Q_A through a long-range electron-transfer pathway involving several cofactors. The oxidation of the excited state generates the strongly oxidizing $(P680)^{+\cdot}$ (+1.2 V) which initiates the one-electron abstraction from Y_Z, a specific tyrosine residue. Reduction of this tyrosyl radical to tyrosine involves an electron from the manganese cluster. This cluster serves as a charge accumulator that stores up to four oxidizing

equivalents to be used for O_2 evolution from H_2O. Nature's use of Y_Z as a charge-transfer interface between $(P680)^{+\cdot}$ and $(Mn)_4$ serves two important purposes: (i) by reducing $(P680)^{+\cdot}$ rapidly, it eliminates the wasteful $(P680)^{+\cdot}$ Q_A^- recombination reaction and allows the relatively slow water-oxidation chemistry; and (ii) its high redox potential preserves the oxidizing power necessary to drive the water-oxidation chemistry.

Prostaglandin H synthase

Prostaglandin synthase (PGH synthase) catalyzes the oxidation of arachidonic acid to prostaglandin endoperoxides, the initial step in prostaglandin, thromboxan and prostacyclin biosynthesis [50]. The protein is a glycoprotein composed of two 70 kDa subunits. Each subunit contains iron(III)-protoporphyrin IX as a prosthetic group. PGH synthase exhibits two catalytic activities: a cyclooxygenase that oxygenates arachidonic acid to a hydroperoxide and a peroxidase that reduces the hydroperoxide to an alcohol in the presence of a reducing substrate (fig 13).

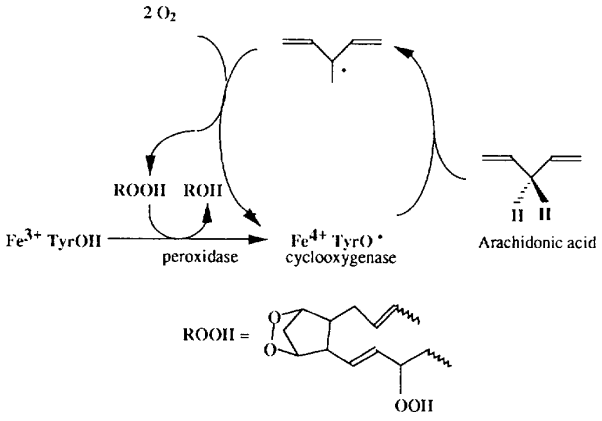

Fig 13. Peroxide-dependent activation of cyclooxygenase activity of PGH synthase (Fe^{3+} represents the heme moiety and TyrOH the acive tyrosine of the enzyme).

The crystal structure of PGH synthase indicates that the heme lies in a hydrophobic pocket. A tyrosine (Tyr 385) is positioned between the heme and the putative fatty acid binding site [50] (fig 14).

The mechanism by which PGH synthase oxidizes arachidonic acid is of considerable interest. Removal of the 13-pro-S hydrogen atom proceeds via a sizable

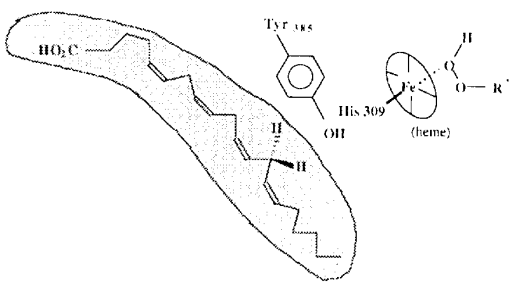

Fig 14. The active site of PGH synthase.

isotope effect, indicating that it is the rate-limiting step. The identity of the oxidizing agent responsible for this step is still uncertain. A possibility is that an iron-oxo complex oxidizes an amino acid residue on the protein to a radical that is the H-abstracting agent. This radical has been suggested to be a tyrosyl radical [51].

Tyrosyl radicals of PGH synthase are produced transiently after addition of arachidonic acid or a hydroperoxide such as PGG2 to the resting enzyme. A doublet ESR signal appears immediately but decays rapidly (1 min). Concomitant with decay of the doublet, a broad singlet ESR signal appears that persists and is detectable even at room temperature [52]. A third radical signal, a narrow singlet, occurs when PGH synthase is inactivated by indomethacin or incubated with a large excess of substrate. The broad singlet signal may well be a composite of the broad doublet and narrow singlet signals. These signals are caused by a tyrosyl radical but with different dihedral angles between the phenyl ring and the benzylic carbon and thus different hyperfine couplings. The splitting in the doublet signal is lost in PGH synthase-containing deuterated tyrosines.

Although it is clear that one or more tyrosyl radicals are produced as a result of the action of PGH synthase, their role as catalytically competent intermediates in the cyclooxygenase reaction is still debated. Tyrosyl radical formation could as well be a side reaction that occurs concomitantly with catalysis, reflecting oxidative damage and late inactivation of the protein.

In fact, only the wide doublet tyrosyl radical species is kinetically competent for participation in cyclooxygenase catalysis, whereas both the wide and narrow singlet-free radical species appear when much of activity has been lost or under conditions that enhance self-inactivation [53]. These signals may thus be indicators of self-destructive events in the enzyme.

Mutation of Tyr 385 to phenylalanine resulted in complete loss of cyclooxygenase activity, making this residue a likely candidate for involvement in radical formation [54–55]. Very recently, using single turnover experiments and EPR spectroscopy, it has been demonstrated that tyrosyl radicals react with arachidonic acid to form a carbon-centered radical, under anaerobic conditions [56]. The doublet EPR signal disappears during the reaction but is regenerated upon subsequent addition of oxygen to samples containing the fatty acyl radical.

In another context, it has recently been shown that free tyrosyl radicals generated by myeloperoxidase are catalysts for the initiation of lipid peroxidation in low-density lipoprotein [57]. The chemistry is relevant to that of PGH synthase. Actually, the tyrosyl radical is proposed to abstract hydrogen from bis-allylic methylene groups of polyunsaturated fatty acids. Reaction with O_2 yields peroxyl radicals and peroxides. This myeloperoxidase/tyrosine/H_2O_2 system provides a good model for the PGH synthase reaction.

Tyrosyl radicals in synthetic metal complexes (biomimetic models of radical enzymes)

If structural models for the first coordination sphere or the metal center in some of the radical enzymes

described herein have been described, a limited number of stable free-radical-containing models have been prepared and characterized. Lippard [58–59] synthesized and isolated as a stable solid a (μ-oxo)-bis-(μ-carboxylato)-diiron(III) complex with a tethered phenoxyl radical as a model for the active site of the R2 protein of ribonucleotide reductase. Magnetic susceptibility studies of this model revealed an overall magnetic behavior quite similar to that of the protein. Two tris-(phenolato)iron(III) complexes which could be oxidized to the $[Fe(L)]^{\cdot+}$ radical cation were described by Wieghardt [60]. In these species, the oxidizing equivalent is localized on one of the phenolate donors to give a phenoxyl radical which is coordinated to the Fe(III) atom through its oxygen atom. Whittaker [61–62] described the ESR and ENDOR spectra of a series of simple phenoxyl radicals including the *ortho*-(methylthio)phenoxyl radical which may be regarded as a model of the radical site in apogalactose oxidase.

One of us has described a stable phenoxyl-free radical-coupled copper(II) complex, modeling the active site in galactose oxidase [63].

Why are tyrosyl radicals found in metalloenzymes?

There is now ample evidence that tyrosyl radicals may serve important functions in catalysis. In most cases, a metal center is associated with the radical and is involved in the conversion of the tyrosine to the tyrosyl radical. However, it is striking that a large flexibility is allowed as far as the nature of the metal center is concerned. As we have seen above, heme iron, dinuclear non-heme iron or mononuclear copper may, for example, be utilized. This diversity may just reflect how it is easy to oxidize a phenol moiety by high-valent metal centers. Such a chemistry can easily be mimicked [64].

It is thus tempting to suggest that tyrosyl radicals appeared in redox active metal-containing proteins as a consequence of the increased oxidizing atmosphere, once molecular oxygen appeared at the surface of the earth. The various radicals had varied stabilities and reactivities. In most cases, the resulting radical proteins were useless and destroyed. A few tyrosyl radicals served for new functions.

What could be the advantages of maintaining a tyrosyl radical within an enzyme? A key property of a tyrosyl radical is its high and easy-to-modulate redox potential. As a consequence, this is an elegant way to preserve oxidizing equivalents, provided by metal centers. This high redox potential can be used for a variety of reactions which are summarized in the next paragraph. One important reaction used in biological catalysis (galactose oxidase, ribonucleotide reductase and prostaglandin synthase) is hydrogen atom abstraction from a C–H bond. Hydrogen atom abstraction by radicals such as alkoxy, phenoxy or hydroxyl radicals is energetically favored over a hydrogen atom transfer to an inorganic metal-based oxidant. The thermodynamic affinity of a tyrosyl radical for a hydrogen atom is high [12].

Another advantage of a tyrosyl radical resides in the possibility it offers to the metalloenzyme to delocalize, oxidizing equivalents through well-defined long-range electron-transfer pathways; these can then be stored at variable distances from the primary metal site. The polypeptide chain thus provides a mechanism for regulating the oxidizing reactivity of the system. For example, the specificity of the reaction can be modulated by utilizing tyrosyl radicals sites with different stereoelectronic properties.

Reactivity of the tyrosyl radical: a summary

Tyrosyl radicals in proteins are involved in electron transfers, hydrogen atom abstractions or coupling processes.

Electron transfer

With the exception of the radical of GOase, the redox potentials of the tyrosyl radical/tyrosine pair in proteins are not known but are assumed to be high (0.9–1 V), based on model studies. This explains why the radical may behave as a one-electron sink and catalyze high potential one-electron transfers. For example, electron transfer may arise between tyrosine and tryptophane residues [65]:

$$TrpH + TyrO^{\cdot} \rightleftharpoons Trp^{\cdot} + TyrOH$$

In the photosystem, the transfer may be written as:

$$Mn^{3+} + TyrO^{\cdot} \rightarrow Mn^{4+} + TyrOH$$

$$P680^{+\cdot} + TyrOH \rightarrow P680 + TyrO^{\cdot}$$

Hydrogen atom abstraction

$$TyrO^{\cdot} + RH \rightarrow TyrOH + R^{\cdot}$$

Hydrogen atom abstraction by phenoxyl radicals from some biologically important phenols is about 100–300 faster than hydrogen atom abstraction by peroxyl radicals (rate constants about 10^8–10^9 M^{-1} s^{-1} [12]). One example for H abstraction by a tyrosyl radical is depicted in figure 2, in the case of GOase. Another example is found in the catalytic cycle of PGH synthase. The reduction potential of the pentadienyl radical (+600 mV) makes this reaction thermodynamically favorable.

Coupling

While tyrosyl radicals are relatively unreactive with oxygen, they can couple to nitric oxide and superoxide radicals as shown in both the tyrosyl radical of ribonucleotide reductase and model systems. They can also react with a second tyrosyl radical to generate a bityrosine. Tyrosine cross-linking has been observed upon both UV- or ionizing irradiation and H_2O_2-induced oxidation of proteins.

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