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Radicals in Biological Catalysis

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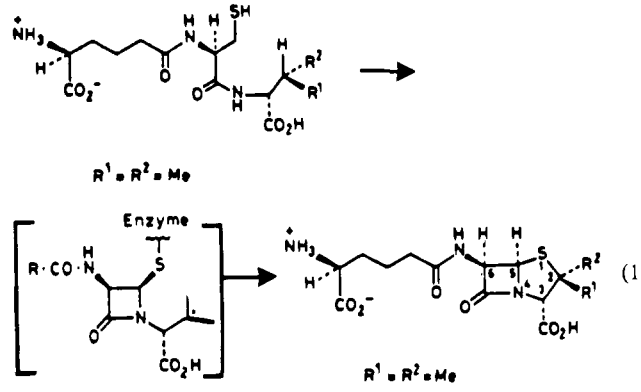
Radicals in Biological Catalysis. The "concept that free radicals are involved in biology has had a turbulent history" (Pryor, 1976). A classic paper of Michaelis in 1939 proposed that "all oxidation reactions of organic molecules proceed in successive univalent steps". A more recent, provocative review article by Hamilton (1971) postulated that the majority of redox reactions catalyzed by metallo- and nonmetalloenzymes proceed via ionic mechanisms. The truth most probably lies somewhere between these two extreme views. The thesis of this paper is that nature has designed a variety of ingenious cofactors to affect chemically difficult transformations through intermediate radical species. Furthermore, as has become apparent in the "synthetic organic" chemistry literature (Giese, 1986), radicals can be generated that exhibit regioselectivity, stereoselectivity, and chemoselectivity if the appropriate steric and stereoelectronic environment is chosen.

Obtaining evidence in support of kinetically and chemically competent radical intermediates along a reaction coordinate is a difficult task. Three general methods have been applied to solving this problem in biological systems. The most informative of these methods, direct observation of a radical intermediate, is applicable only if the proposed intermediates are observable by a spectroscopic method and if their concentrations are sufficient to allow detection (Beinert, 1972; Johnson, 1986). If these criteria are met, then use of, for example, stopped-flow visible spectroscopy or rapid-quench EPR methods should allow assessment of the kinetic competence of the proposed intermediate. Application of these rapid kinetic methods to adenosylcobalamin (AdoCbl)[1] and binuclear iron center–tyrosyl radical dependent proteins will be discussed subsequently.

A second, indirect method involves use of modified substrates whose products are indicative of a radical rearrangement process. Elegant studies of Baldwin and co-workers using such substrates have provided compelling evidence for radical involvement in an intriguing reaction catalyzed by isopenicillin N synthetase (eq 1) (Baldwin, 1984; Baldwin et al., 1984).



(1)

This protein in the presence of Fe^{2+} and O_2 catalyzes the conversion of the tripeptide δ-L-aminoadipoyl-L-cysteinyl-D-valine (ACV) to isopenicillin N. Incubation of the enzyme with the alternative substrate δ-L-α-aminoadipoyl-L-cysteinyl-D-allylglycine results in the production of the seven products shown in eq 2. A mechanism involving a common allyl radical intermediate can most easily accommodate the results in pathway b (Baldwin et al., 1984).

A subset of this second method, involving "radical clock substrates", can be used not only to provide support for the existence of but also to provide information about the lifetime of a proposed radical intermediate in a reaction. This method is based on the extensive kinetic studies of Ingold and co-workers, who have measured the absolute rates of radical rearrangements (Griller & Ingold, 1980). Ortiz de Montellano and co-workers have recently utilized this method to provide further support for radical involvement in cytochrome P-450 mediated reactions, as well as to establish a lower limit of 10^9 s^{-1} for the rate of recombination of the radical pair in the reaction sequence shown in eq 3 (Ortiz de Montellano &

[1] Abbreviations: AdoCbl, adenosylcobalamin; ACV, δ-L-aminoadipoyl-L-cysteinyl-D-valine; RDPR, ribonucleoside diphosphate reductase; RTPR, ribonucleoside triphosphate reductase.

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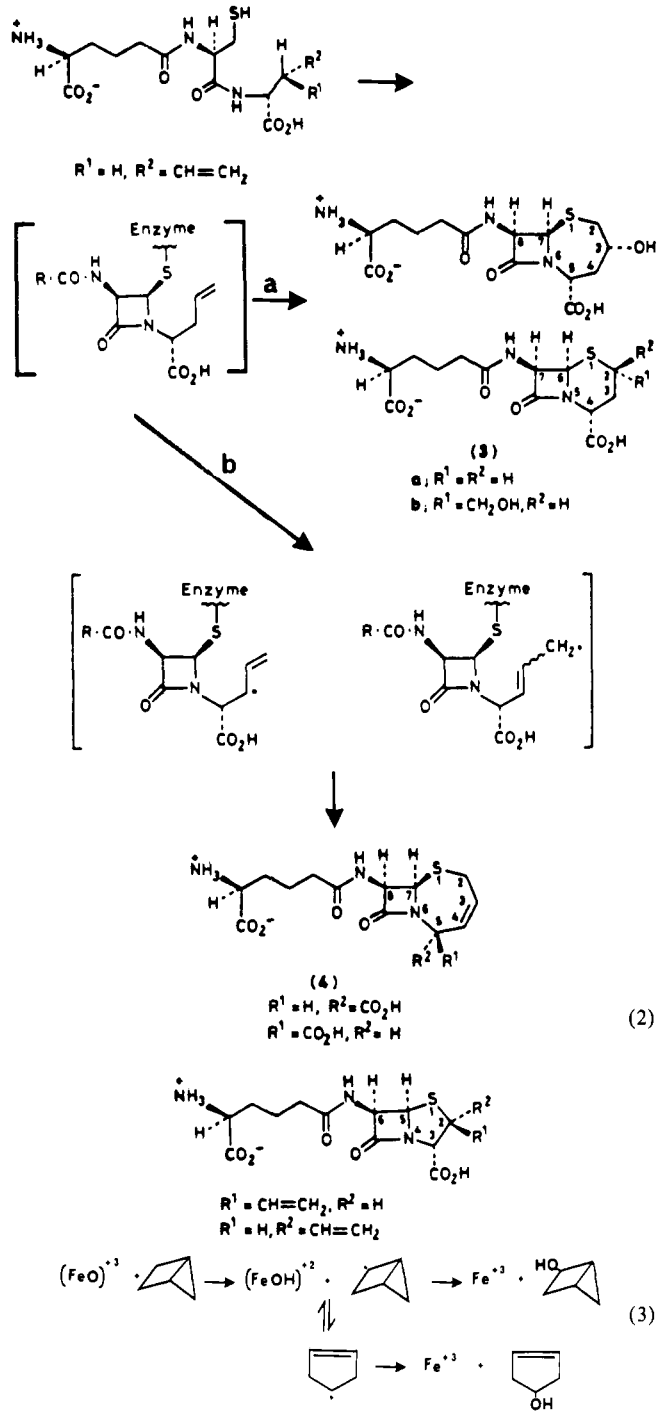

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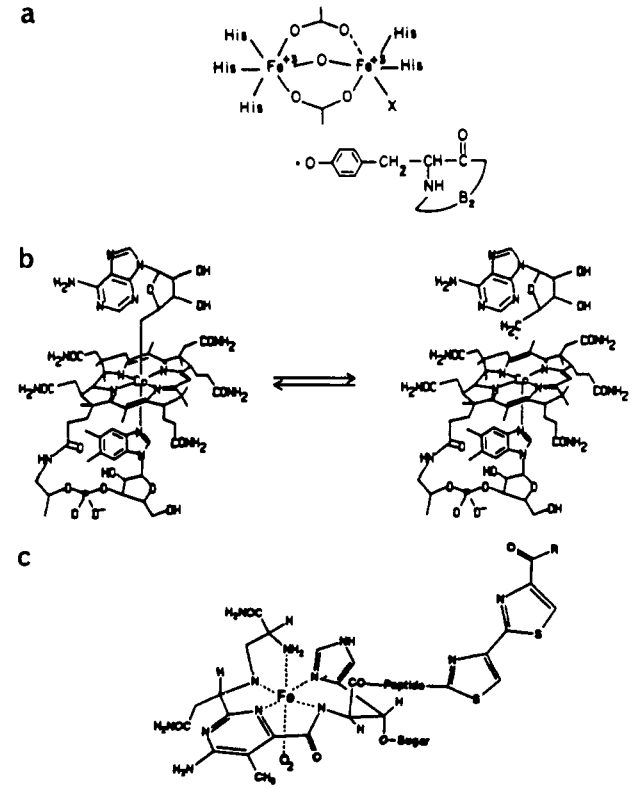


FIGURE 1: Proposed structures of cofactors: (a) *E. coli* RDPR; (b) *L. leichmannii* RTPR; (c) bleomycin.

Stearns, 1984). These conclusions were reached by investigating the reaction of nortricyclane, bicyclo[2.1.0]pentane, and methylcyclopropane with microsomal cytochrome P-450. Rearranged product was observed only with bicyclo[2.1.0]-pentane. Ingold and co-workers have measured a rate constant of 10^8 s^{-1} for the rearrangement of the cyclopropylmethyl radical to the 3-butenyl radical (Griller & Ingold, 1980). While the rate of rearrangement of the bicyclo[2.1.0]pentyl radical has not been measured, it is thought to be on the order of 10^9 s^{-1}. Hence, observed rearrangement of bicyclo-[2.1.0]pentane and not methylcyclopropane supports the conclusion that a radical intermediate exists having a half-life of approximately 10^{-10} s in the cytochrome P-450 catalyzed reaction.

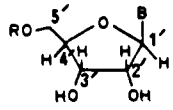
The general applicability of this method is unfortunately limited to enzyme systems that possess sufficient substrate flexibility to allow reporter groups to be built into an alternate substrate and to allow the chemistry that initiates radical production to still proceed on the alternate substrate. An additional limitation is that, in certain cases, identical products may arise by rearrangements involving cationic/anionic intermediates as well (Johns et al., 1987).

A third general/indirect method utilized to detect radical intermediates involves "spin traps" (Janzen, 1980). The basis for this method involves transformation of a transient radical R• into a persistent epr-observable radical RX•, usually a nitroxide radical, by allowing it to react with a spin trap X such as a nitrone or a nitroso compound. In addition, if RX•, or its reduction product, RXH, is stable, it can be chemically characterized and thus allow unambiguous identification of its precursor, R•. Unfortunately, this technique requires an enzyme with an accommodating active site allowing access to "bulky" spin traps, or it requires that the radical intermediate dissociate from the active site (an unlikely prospect) and be trapped by X prior to alternative chemistry with other components of the reaction mixture. These constraints and difficulties in quantitation of the amount of trapped species have thus far limited successful use of this technique to trapping radical intermediates in cytochrome P-450 (Augusto et al., 1982), prostaglandin H synthetase (Samokysyn & Marnett, 1987), dopamine hydroxylase (Fitzpatrick & Villafranca, 1986), and lipoxygenase-catalyzed reactions (de Groot et al., 1975). An additional, insurmountable limitation is that it is not possible to prove that the trapped product is on the normal catalytic pathway.

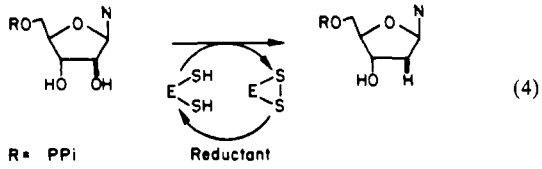
Constraints on present experimental methods have thus far "limited" compelling evidence for radical involvement in catalytic–biological systems.

Recent efforts from our laboratory have focused on catalytic systems involved in transformation of nucleosides(tides) via radical intermediates and the role of the novel cofactors in these transformations. A number of biological catalysts are being investigated that appear to effect radical transformations

at the 1′-, 2′-, 3′-, or 4′-position of the sugar moiety of the nucleoside(tide). In addition, the recent discovery of esperamycins (Golik et al., 1987a,b) and calichemicins (Lee et al., 1987a,b) and their similarity to the structure of neocarzinostatin, which Goldberg and co-workers (Kappen & Goldberg, 1985; Meyers, 1987) have shown mediates chemistry at the 5′-position of a nucleotide in DNA, also suggest that a radical intermediate can be specifically generated at C-5′.



Chemistry at C-3′. Our most extensive efforts have focused on the ribonucleotide reductases, the biological catalysts essential for the synthesis of the ultimate or penultimate monomeric precursors required for DNA biosynthesis (eq 4).



(4)

The *Escherichia coli* ribonucleoside diphosphate reductase (RDPR)[1] was discovered and has been extensively characterized by Reichard, Sjoberg, Thelander, Ehrenberg, and their collaborators (Reichard & Ehrenberg, 1983; Thelander & Reichard, 1979, and references cited therein). At present, this enzyme provides the only example of a well-characterized, stable protein free radical, although pyruvate formate-lyase (Knappe et al., 1984), cytochrome *c* peroxidase (Edwards et al., 1987), and dioldehydrase from *Clostridium glycolicum* (Hartmanis & Stadtman, 1987) also possess protein-derived radicals whose structures remain to be established. The radical in RDPR has been unequivocally shown to reside on a specific Tyr residue (122) of one subunit, designated B_2, to possess an EPR signal at $g = 2.0047$ and a visible absorption spectrum at 410 nm, and to be required for catalytic activity. The radical center is "stabilized" by an unusual binuclear iron center in which two high-spin irons have been shown to be inequivalent and antiferromagnetically coupled through a μ-oxo bridge (Figure 1a) (Reichard & Ehrenberg, 1983; Larsson & Sjoberg, 1986). On the basis of model chemistry (Ashley & Stubbe, 1987) we have proposed that the tyrosyl radical is intimately involved in the catalytic conversion of nucleotides to deoxynucleotides, through mediation of hydrogen atom abstraction from the 3′-position of the nucleotide, generating a 3′-nucleotide radical, as indicated in Figure 2a. This step is followed by protonation of the leaving group, perhaps catalyzed by one of the redox-active thiols, which subsequently results in loss of H_2O to generate a proposed cation radical intermediate. This intermediate is then reduced by either one or two electrons and H^+ or H^- transfers to produce a 3′-deoxynucleotide radical. The hydrogen originally abstracted from the 3′-position in the nucleotide is returned to the 3′-position in the product, and the tyrosyl radical is regenerated. While this overall scheme is consistent with extensive isotopic labeling experiments conducted in our laboratory that establish RDPR's ability to catalyze 3′ carbon–hydrogen bond cleavage, no direct evidence in support of radical intermediates during the normal reduction process presently exists. The mechanism in Figure 2a predicts that the tyrosyl radical should be reduced and reoxidized during each turnover. Extensive stopped-flow kinetic experiments undertaken to monitor the change in the absorbance at 410 nm of the tyrosyl radical have thus far been unsuccessful.[2] For

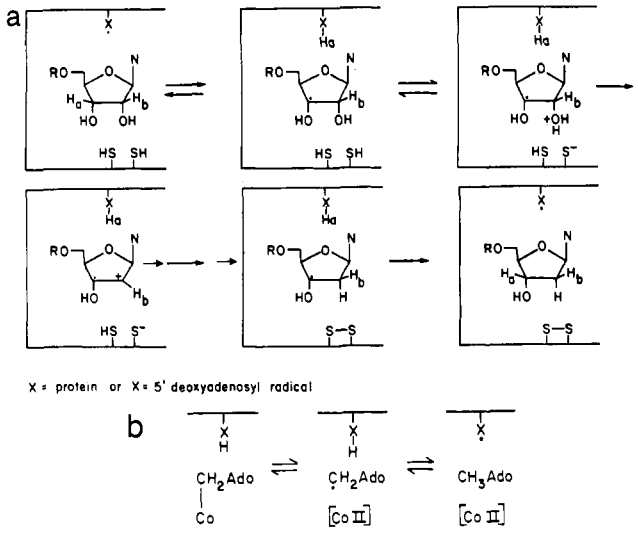


FIGURE 2: (a) Proposed mechanism of conversion of NDPs (NTPs) to dNDPs (dNTPs) catalyzed by RDPR (RTPR). X is a protein or 5′-deoxyadenosyl radical. (b) Proposed mechanism of protein radical production mediated by AdoCbl.

the mechanism in Figure 2a to be valid, therefore, one must propose that steps subsequent to 3′ C–H bond cleavage and prior to tyrosyl radical regeneration must be rapid (100× the rate of C–H bond cleavage). The concentration of reduced tyrosyl radical at any given time would then be sufficiently small that changes in tyrosyl radical concentration would be undetectable. Based on extensive model reactions for various steps proposed in this sequence and their measured rate constants (Ashley & Stubbe, 1987), it is not unreasonable to propose that tyrosyl radical changes may be kinetically invisible.

Evidence that indirectly supports the involvement of nucleotide radical intermediates in RDPR-catalyzed reactions is based on studies of the interaction of 2′-azido-2′-deoxynucleotides with RDPR, originally reported by Thelander et al. (1976). In this paper and the subsequent paper (Sjoberg et al., 1983), they reported that incubation of 2′-azido-2′-deoxycytidine 5′-diphosphate (N_3CDP) with RDPR resulted in enzyme inactivation, destruction of the tyrosyl radical (Thelander et al., 1976), and the formation of a new radical species (Sjoberg et al., 1983). We were especially intrigued by the latter two observations because our numerous stopped-flow kinetic experiments had failed to reveal any loss in the protein's tyrosyl radical under a wide variety of conditions in which RDPR was catalytically active. If a correlation could be established between cleavage of the 3′ C–H bond, destruction of the tyrosyl radical, and formation and identification of the structure of the new radical, then the enzyme's ability to mediate production of a substrate-derived radical would be established. Efforts in our laboratory have been focused on unraveling the products produced during inactivation of RDPR by N_3UDP and the mechanism of their production. These studies have established that 1 equiv of N_3UDP is sufficient to inactivate RDPR and that inactivation is accompanied by production of 1 equiv each of uracil, PP_i, N_2, and a sugar moiety that stoichiometrically and specifically modifies one of the two subunits, B_1. In addition, the 3′ carbon–hydrogen bond is cleaved, the tyrosyl radical on B_2 is destroyed, and a new nitrogen center radical derived from N_3UDP is produced. Interestingly, there is an isotope effect on the loss of tyrosyl radical when RDPR is incubated with [3′-^{2}H]N_3UDP, although the kinetics of this process are

[2] M. Ator, D. Ballou, and J. Stubbe, unpublished results.

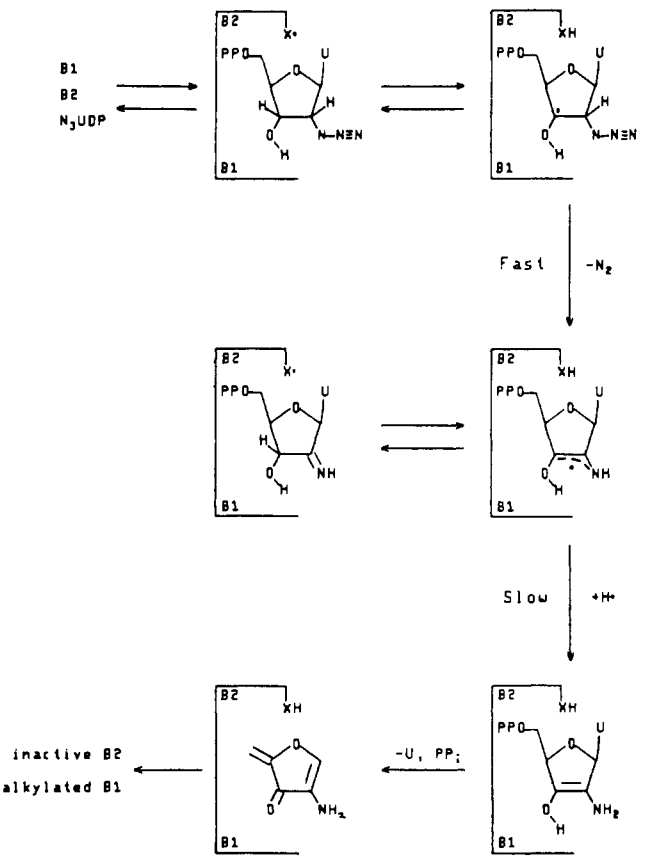


FIGURE 3: Proposed mechanism of inactivation of RDPR by N_3UDP.

complex and not as yet completely understood (Salowe et al., 1987).[3] A scheme accommodating these observations and the kinetic information thus far available is shown in Figure 3. The proposal is put forth that an alkyl azide moiety adjacent to a β-alkyl radical can act as an intramolecular radical trap resulting in the production of a nitrogen-centered radical. No chemical precedent for this proposal is to our knowledge available in the literature, and chemical model studies are presently being undertaken in our laboratory to test this hypothesis. If the model studies indicate the feasibility of this proposal, then alkyl azides β to a proposed radical center might serve as a general probe for radical intermediates. While the details of the reaction sequence remain to be unraveled, all of the evidence available to date is consistent with proposed cation radical intermediates in the RDPR-catalyzed reduction reaction (Figure 2a).

In nature at least one alternative method has evolved to effect the same transformation, conversion of NTP to dNTPs, using the best-characterized organometallic cofactor adenosylcobalamin (AdoCbl) (Figure 1b). As described in a recent review article by Halpern (1985), adenosylcobalamin appears to serve as a "reversible radical carrier". RTPR from *Lactobacillus leichmannii* is no exception, as early stopped-flow kinetic studies by Tamao and Blakely (1973) and rapid-quench EPR studies by Orme-Johnson et al. (1974) have established that homolytic cleavage of the carbon–cobalt bond of AdoCbl occurs in a kinetically competent fashion to produce cobalamin(II) and presumably the 5′-deoxyadenosyl radical. Our hypothesis in investigating the mechanism of ribonucleotide reductase catalyzed reactions is that even though the cofactors for the different classes of reductases are unique, in both prototypical cases (i.e., reductases from *E. coli* and *L. leichmannii*) there is a metal center "stabilizing" an organic radical that is capable of mediating radical transformations. While our initial hypothesis in the case of the *L. leichmannii* protein was that the 5′-deoxyadenosyl radical played a role analogous to that proposed for the tyrosyl radical in *E. coli* RDPR, recent studies from our laboratory have necessitated a modification of this proposal (Ashley et al., 1986). Results from studies with the normal substrate and the substrate analogue/mechanism-based inhibitor 2′-chloro-2′-deoxynucleotide are consistent with the proposal that the 5′-deoxyadenosyl radical acts as a radical chain initiator, producing a protein radical that, in analogy with the proposed role of the tyrosyl radical in *E. coli* reductase, mediates an analogous series of transformations (Ashley & Stubbe, 1987). Recent active site mapping studies (Lin et al., 1987) unexpectedly revealed the propinquity of three thiols: a third thiol in addition to the two expected thiols that are oxidized concomitant with substrate reduction. We would like to propose that X• in Figure 2a might therefore be a thiyl radical in the AdoCbl-dependent RTPR-catalyzed reaction. The thiyl radical could easily be generated by hydrogen atom abstraction by the 5′-deoxyadenosyl radical producing 5′-deoxyadenosine (Figure 2b). That the thiyl radical could mediate hydrogen atom abstraction from the 3′ carbon has excellent precedent in recent studies by von Sonntag and co-workers on thiol inhibition of ionizing radiation damage to DNA (Aklhaq & von Sonntag, 1986; Aklhag et al., 1987). The only unappealing step in the proposed sequence in Figure 2b is the re-formation of the 5′-deoxyadenosyl radical by hydrogen atom abstraction from the protein thiyl radical, a thermodynamically unfavorable process. However, this reaction, when coupled to the thermodynamically favorable regeneration of AdoCbl, should drive the reaction to the right (eq 5).

$$\begin{aligned} &E\text{–}S^{\bullet} + CH_3Ad + Co(II) \rightleftarrows \\ &\qquad {}^{\bullet}CH_2Ad + Co(II) \quad (+18\ \text{kcal}) \\ &\qquad Co(II) + {}^{\bullet}CH_2Ad \rightarrow [Co]\text{–}CH_2Ad \quad (-30\ \text{kcal}) \\ &\text{sum:}\quad ES^{\bullet} + CH_3Ad + Co(II) \rightarrow \\ &\qquad ESH + [Co]\text{–}CH_2Ad \quad (-12\ \text{kcal}) \end{aligned} \tag{5}$$

Further support for a radical mechanism similar to that postulated in Figure 2a is based on the extensive literature on the enzymes involved in AdoCbl-dependent rearrangement reactions (eq 6). Rapid-quench EPR studies and repetition

$$RCHXCH_aHOH \longrightarrow \left[RCHH_aCHOHX\right] \longrightarrow RCHH_aCHO \tag{6}$$

of these experiments with appropriate isotopically labeled substrates have established for ethanolamine ammonia lyase (X = NH_2; R = H) that a substrate-derived radical intermediate is produced and that it is converted to products in a kinetically competent fashion. Similar studies with dioldehydrase (X = OH; R = CH_3) have established the existence of substrate-derived radical intermediates; however, the question of kinetic competence has not yet been addressed. While a great deal has been learned about these rearrangement reactions, and compelling evidence exists to support the intermediacy of radicals, the extremely large selection effect on transfer of ^3H from [5′-^{3}H]AdoCbl to products in the case of both ethanolamine ammonia lyase and dioldehydrase has not been adequately addressed by the accepted mechanism (Figure 4) in which X is a 5′-deoxyadenosyl radical. In 1982, Cleland proposed that an alternative pathway (Figure 4), in which X is a protein radical generated by hydrogen atom abstraction by the 5′-deoxyadenosyl radical, occurring 9 out of 10 times could explain these large isotope effects. A reasonable alternative might now be suggested in analogy with the RTPR

[3] S. Salowe and J. Stubbe, unpublished results.

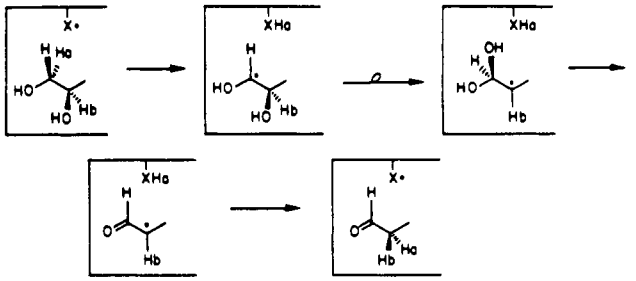
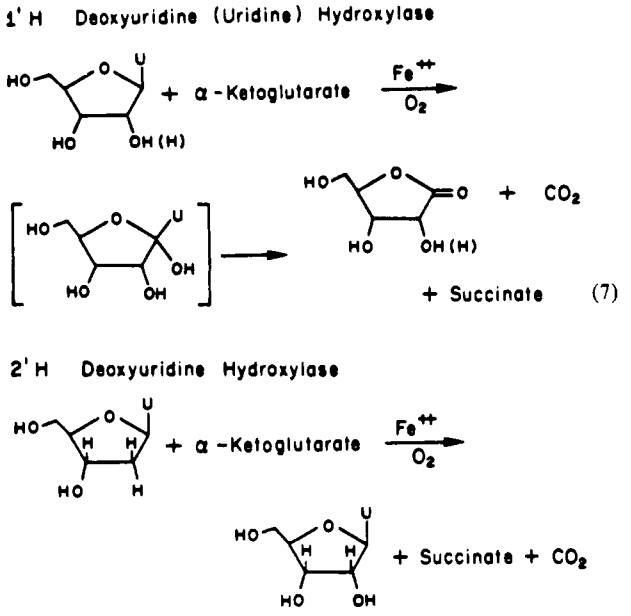


FIGURE 4: Proposed mechanism for B_{12}-dependent rearrangement reactions. Classic mechanism: X is a 5′-deoxyadenosyl radical. Cleland mechanism: X is a protein radical generated by hydrogen atom abstraction by a 5′-deoxyadenosyl radical.

system. The 5′-deoxyadenosyl radical could generate a thiyl radical that could orchestrate the rearrangement (Figure 4). The potential role for a protein radical is supported by recent ESEEM studies and isotopic labeling studies by Babior and colleagues on ethanolamine ammonia lyase (Tan et al., 1986; O'Brien et al., 1985). Additional support is provided by the recent intriguing report (Hartmanis & Stadtman, 1987) that in *Clostridium* there is a non-AdoCbl-dependent dioldehydrase that appears to possess a protein radical that is hydroxyurea sensitive, a hallmark of the binuclear iron center tyrosyl radical reductase (Reichard & Ehrenberg, 1983). Speculation that the protein radical may be a thiyl radical is based on the numerous reports that all AdoCbl-dependent enzymes are sensitive to thiol reagents. Whether protein radicals, and specifically thiyl or tyrosyl radicals, are involved in AdoCbl-requiring enzymatic reactions requires further experimentation.

Chemistry at C-4′. Bleomycin, an antitumor antibiotic, binds to double-stranded DNA and in the presence of Fe^{2+} and O_2 *catalytically* degrades DNA (Stubbe & Kozarich, 1987). The proposed structure of the ternary Fe^{2+}–O_2–BLM complex, shown in Figure 1c, is presumed to be "the non-heme iron" equivalent of the well-characterized heme-requiring enzyme cytochrome P-450. Recent investigations have allowed proposal of the mechanism shown in Figure 5 to account for the two types of monomeric products that have been isolated and identified subsequent to reaction of activated BLM with DNA: base and base–propenal. The first step in production of both monomeric products is hydrogen atom abstraction mediated by "activated bleomycin" [potentially, $(FeO)^{3+}$] to produce a 4′ nucleotide radical. This radical, as indicated in pathway b, can undergo radical combination and ultimately production of base, in analogy with mechanisms proposed for the extensively investigated cytochrome P-450 system (Guengerich & McDonald, 1985). Alternatively, in the presence of high O_2 concentrations the same 4′ radical intermediate can react with O_2 to form a peroxy radical, pathway a, which subsequent to a number of transformations produces base–propenal. The ability to manipulate the distribution of products by varying O_2 tension provides compelling evidence that the C-4′ radical is a common intermediate. It is interesting to note that, for many of the heme-requiring enzymes, there appears to be a "non-heme" iron-requiring protein capable of effecting similar transformations. At present the ligand arrangement of these non-heme iron systems and the mechanism by which the proposed active oxidant $(FeO)^{3+}$ species is stabilized in these systems remain to be unraveled.

Chemistry at C-1′ and C-2′. Chemistry at C-1′ and C-2′ of nucleosides might also be accomplished through radical intermediates by a novel, and still mechanistically unexplored, class of proteins: the α-ketoglutarate dioxygenases. The reactions catalyzed by two proteins in this class that we are presently investigating are shown in eq 7. The detailed



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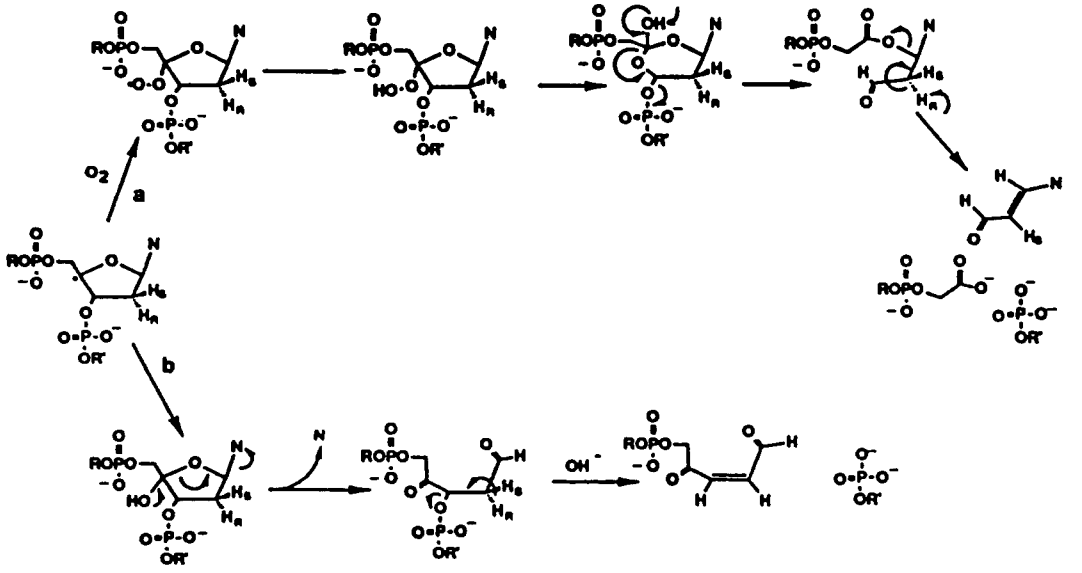

FIGURE 5: Proposed mechanism by which activated BLM degrades DNA.

Table I

protein	cofactor	reaction	lead ref
methane monooxygenase	binuclear iron center	$CH_4 \rightarrow CH_3OH$	*a*
pyruvate–ferredoxin oxidoreductase	thiamin pyrophosphate "FeS" center	$CH_3COCO_2H \rightarrow CH_3COSCoA$	*b, c*
lysine 2,3-aminomutase	*S*-adenosyl-methionine pyridoxal phosphate, Fe^{2+}	lysine $\rightleftarrows$ β-lysine	*d*
pyruvate formate-lyase	protein radical; structure unknown	$CH_3COCO_2H \rightleftarrows CH_3COSCoA + HCO_2^-$	*e*
cytochrome *c* peroxidase	heme and protein radical	cyt *c* $Fe^{2+} \rightarrow$ cyt *c* Fe^{3+}	*f*
lignin peroxidase "ligninase"	heme		*g*
prostaglandin H synthetase	heme	arachidonic acid → 12-hydroperoxy-5,8,11,14-eicosatetraenoic acid	*h*
cytochrome P-450	heme	RH → ROH	*i, j*
lactoyl-CoA dehydrase	cofactors unknown	lactoyl-CoA → acrylyl-CoA	*k*
dopamine β-hydroxylase	copper center	dopamine → norepinephrine	*l, m*
lipoxygenase	Fe^{3+}; structure unknown	linoleic acid → L-13-hydroperoxide and arachidonic acid → PGG_2 (9,10-*endo*-peroxy-5-hydroperoxyprostaglandin)	*n, o*
isopenicillin N synthetase	Fe^{2+}; structure unknown	δ-L-aminoadipoyl-L-cysteinyl-D-valine → isopenicillin N	*p, q*
RDPR and RTPR	(a) "binuclear iron center", (b) "Mn requiring center", (c) AdoCbl	NDP (NTP) → dNDP (dNTP)	*r, s, t*
dioldehydrase, ethanolamine ammonia lyase	AdoCbl	$RCHXCH_2OH \rightarrow RCH_2CHO$ (X = OH, NH_3; R = CH_3, H)	*u, v, w*
biotin biosynthesis; lipoic acid biosynthesis	cofactors unknown		*x*
degradation of alkylphosphonates	cofactors unknown	$CH_3PO_3 \rightarrow CH_4$	*y*
mitochondrial amine oxidase	FAD	$RCH_2NH_2 \rightarrow RCH{=}O$	*z, aa*
methanol oxidase	FAD or methoxatin	$CH_3OH \rightarrow HCHO$	*bb, cc*
E. coli DNA photolyase	flavin, pterin	reversal of thymine dimer formation	*dd, ee*
photosynthetic O_2-evolving system	tyrosyl radical		*ff*

[a] Green & Dalton, 1986. [b] Kerscher & Oesterhelt, 1981. [c] Docampo et al., 1987. [d] Frey, 1987; Moss & Frey, 1987. [e] Knappe et al., 1984. [f] Edwards et al., 1987. [g] Hammel et al., 1986. [h] Samokysyn & Marnett, 1987. [i] Guengerich & McDonald, 1984. [j] Groves et al., 1978. [k] Kuchta & Abeles, 1985. [l] Miller & Klinman, 1985. [m] Fitzpatrick & Villafranca, 1986. [n] Vliegenthart & Veldink, 1982. [o] de Groot et al., 1975. [p] Baldwin, 1984. [q] Baldwin et al., 1984. [r] Reichard & Ehrenberg, 1983. [s] Thelander & Reichard, 1979. [t] Ashley & Stubbe, 1987. [u] Cleland, 1982. [v] Tan et al., 1986. [w] O'Brien et al., 1985. [x] Parry, 1983. [y] Cordeiro et al., 1986. [z] Vazquez & Silverman, 1985. [aa] Silverman & Zieske, 1986. [bb] Sherry & Abeles, 1985. [cc] Duine et al., 1986. [dd] Heelis et al., 1987. [ee] Heelis & Sancar, 1987. [ff] Barry & Babcock, 1987.

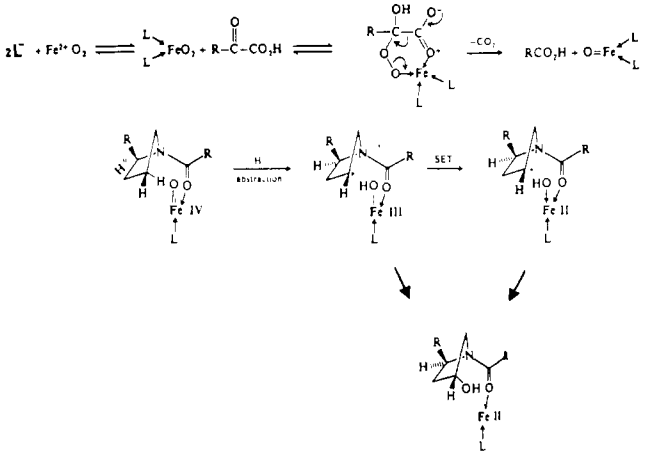

FIGURE 6: Proposed mechanism for α-ketoglutarate dioxygenases (Hanauske-Abel & Gunzler, 1982).

mechanisms of these dioxygenases and the ligands to the Fe^{2+} centers remain to be elucidated. A reasonable mechanistic proposal that is consistent with all available information has been put forth by Hanauske-Abel et al. (1982) (Figure 6) for prolyl hydroxylase, and its general applicability is presently under investigation in a number of laboratories (Englard et al., 1985; Stubbe, 1985).

While this paper has focused on efforts in our laboratory to elucidate involvement of unusual cofactors in the generation of radical intermediates in reactions on nucleosides(tides), a substantial number of other systems (Table I) with unusual cofactors exist in which studies by numerous investigators also implicate the involvement of radical intermediates. Perusal of Table I and examination of the more detailed examples cited above allow some generalizations to be made. (1) The majority of examples cited involve "metal cofactors": iron, cobalt, copper, and perhaps manganese, whose function involves reversible formation of an organic radical source that mediates hydrogen atom abstraction or whose chemistry with O_2 generates metal oxo species capable of hydrogen atom abstraction. Alternatively, the enzymes cited involve organic cofactors [flavin, thiamin pyrophosphate, methoxatin, pyridoxal phosphate (PLP)] that are capable of undergoing one-electron oxidations/reductions to produce resonance-stabilized radical intermediates. (2) In the majority of examples cited, the protein in conjunction with its cofactor(s) is required to cleave an unactivated carbon–hydrogen bond. (3) In approximately half of the cited examples evidence exists that thiols in some form appear to play an important role in the observed chemistry: as part of protein, as ligand to metal center, or as part of the substrate or cofactor. In the majority of the remainder of these systems, the potential role of thiols has not yet been addressed.

While radical intermediates have been postulated to exist in the reactions discussed above and in Table I, in no case has the detailed reaction mechanism been satisfactorily elucidated. This paper serves to stimulate enthusiasm for investigating the many novel mechanisms potentially involving free-radical intermediates that remain to be unraveled and whose solutions require development of new methodologies. The molecular biological revolution has made available large amounts of many of these enzymes, which should facilitate elucidation of the

structures of the unusual cofactors. Use of mutagenesis methods in conjunction with physical and synthetic chemical methods promises in the next few years to provide evidence for unique mechanistic sequences not previously described in the literature.

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Accelerated Publications

Physical Properties of DNA in Vivo As Probed by the Length Dependence of the *lac* Operator Looping Process†

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ABSTRACT: Plasmid constructs containing a wild-type (O^+) *lac* operator upstream of an operator-constitutive (O^c) *lac* control element exhibit a length-dependent, oscillatory pattern of repression of expression of the regulated gene as interoperator spacing is varied from 115 to 177 base pairs (bp). Both the length dependence and the periodicity of repression are consistent with a thermodynamic model involving a stable looped complex in which bidentate *lac* repressor interacts simultaneously with both O^+ and O^c operators. The oscillatory pattern of repression with distance occurs with a period approximating the helical repeat of DNA and presumably reflects the necessity for proper alignment of interacting operators along the helical face of the DNA. In the length regime examined, the presence of the upstream operator enhances repression between 6-fold and 50-fold depending upon phasing. This reflects a torsional rigidity of DNA in vivo that is consistent with in vitro measurements. The oscillatory pattern of repression is best fit with a period of either 9.0 or 11.7 bp/cycle but not 10.5 bp/cycle. This periodicity is interpreted as reflecting the average helical repeat of the 40-bp interoperator region of plasmid DNA in vivo, suggesting that the local helical repeat of DNA in vivo may differ significantly from 10.5 bp/turn. The *apparent* persistence length needed to fit the data (a_{app}) is only one-fifth the standard in vitro value. This low value of a_{app} may be due in part to DNA bending induced by catabolite activator protein (CAP) bound to its site between the interacting operators. Quantitative analysis of the dependence of loop formation on the interoperator spacing can thus be used to characterize the physical properties of the intervening DNA sequence in vivo.

The phenomenon of action-at-a-distance, i.e., the ability of a region of DNA to affect processes that occur hundreds to thousands of base pairs away, is of general importance in eucaryotic systems. Recent studies in several procaryotic transcriptional regulation systems have indicated that a similar phenomenon occurs in the *gal* (Irani et al., 1983; Majumdar & Adhya, 1984), *ara* (Dunn et al., 1984; Martin et al., 1986), *deo* (Dandanell & Hammer, 1985), *lac* (Besse et al., 1986; Mossing & Record, 1986; Krämer et al., 1987, 1988; Borowiec et al., 1987; Hsieh et al., 1987; Whitson et al., 1987b), and *glnA* (Reitzer & Magasanik, 1986) operons of *Escherichia coli*. Various mechanisms have been proposed to explain the ability of distant regions of DNA to influence each other, but recent evidence, primarily obtained for procaryotic systems, strongly implicates formation of a stable DNA loop [for a review, see Ptashne (1986)]. Evidence for loop formation in vitro includes the appearance of an altered pattern of nuclease sensitivity in DNA involved in highly bent loops (Hochschild & Ptashne, 1986; Krämer et al., 1987; Borowiec et al., 1987), direct observation of loops in electron micrographs (Krämer et al., 1987; Thêveny et al., 1987), and the observation of an oscillatory pattern of increased and decreased repression as operators are moved into and out of phase with one another (Dunn et al., 1984; Hochschild & Ptashne, 1986; Krämer et al., 1987, 1988).

The work presented here is a quantitative analysis of the effect of variation of the interoperator spacing on *lac* repression in vivo. The oscillatory dependence of repression on interoperator spacing that we observe strongly supports the quantitative model of DNA loop formation between *lac* operators in vivo proposed by Mossing and Record (1986) and provides a means of evaluating the helical periodicity and apparent lateral and torsional stiffness of DNA in vivo. These and other physical properties of DNA have been thoroughly examined in vitro [e.g., Wells and Harvey (1988)], but little information is available regarding their magnitudes or variability in vivo. This study demonstrates the use of the DNA looping assay to deduce the physical properties of DNA in vivo.

Materials and Methods

Strains and Media. All experiments were performed in *E. coli* strain HB101 (F^-, *hsd*S20 *rec*A *rps*L *lac*Y *leu pro gal*K2) containing the plasmid pi^Q (pACYC184 with an R1 insert

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