observed at low temperature in a xenon matrix by Raman and infrared spectroscopy. Evidence for several lithiated first-row elements such as OLi_4, a 10-O-4 species, has also been discussed (*26*).

Conclusion

By the proper design of ligands, organo-nonmetallic species with unusual valence states or coordination numbers (or both) can be prepared and isolated. This article has illustrated this point by concentrating on a single class of compounds, the 10-*X*-*L* species with TBP geometry. Similar approaches are applicable to other classes of organo-nonmetallic species. The ability to fine tune the reactivity of such species offers considerable promise for the development of new reagents and catalysts of potential utility in synthetic chemistry. This promise has already been realized in applications of sulfurane reagents as dehydrating agents (*5*) and of organohalogen reagents as oxidizing agents (*15, 27*). Application of recent findings in hypervalent organosilicon chemistry (*18, 28*) in carbon-carbon bond-forming reactions currently appears promising.

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Ribonucleotide Reductase—A Radical Enzyme

Peter Reichard and Anders Ehrenberg

It was with some trepidation that we suggested in 1972 that one of the two subunits (protein B2) of the enzyme ribonucleotide reductase might contain a stable free organic radical as part of its structure (*1*). The protein had spent about 2 weeks in aqueous media during our efforts to prepare a homogeneous enzyme. We knew that protein B2 contained stoichiometric amounts of iron (*2*) and decided to apply electron paramagnetic resonance (EPR) spectroscopy to characterize the state of the iron. Much to our surprise, we found a signal consisting of an asymmetric doublet centered at $g = 2.0047$ (curve A, Fig. 1) that had all the hallmarks of an organic free radical and thus apparently did not derive from the iron of protein B2.

The occurrence of free radicals in biological systems, in particular in connection with redox processes, was of course firmly established ever since the pioneering work of Michaelis (*3*). Characteristic features of such radicals are, however, high chemical reactivity as well as instability in aqueous solution and short life time. The existence of a stable organic radical in an enzyme, as an intrinsic part of its protein structure, had never been observed before.

Nature of the Organic Radical

Ribonucleotide reductases from several sources (*4–6*) show EPR spectra strikingly similar to that of protein B2 of *Escherichia coli* (Fig. 1, curves B to D). The characteristic doublet structure suggested hyperfine interaction with a proton and the nature of the free radical was therefore investigated by EPR experiments involving deuterated protein B2 (*7, 8*). With a genetically manipulated strain of *E. coli* (*9*) containing about 3 percent of its total protein as ribonucleotide reductase, the characteristic EPR signal of protein B2 could be measured directly on thick cell suspensions. When the bacteria were grown on a synthetic medium in D_2O a complete collapse of the doublet structure occurred. This phenomenon could be traced to the incorporation of deuterated tyrosine into B2. Bacteria grown on synthetic media in H_2O to which specifically deuterated tyrosines were added gave spectra with a similar collapse of the doublet structure or loss of the superhyperfine structure, depending on the site of the substitutions (Fig. 2). From such experiments we could assign the radical to a tyrosine residue of the protein with its spin density delocalized over the aromatic ring. Similar isotope substitution experiments with ribonucleotide reductases from other sources identified tyrosyl radicals also in these enzymes (*4, 5*).

In Fig. 1 it is seen that spectra A and B have great similarities. The other two spectra, C and D, are also similar to each other, but clearly different from the first two. These spectral differences may be explained by a twist of approximately 10°

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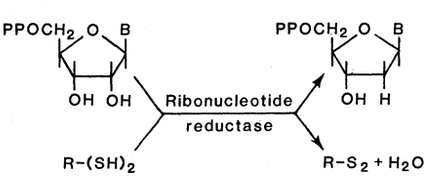
in the bond between the aromatic ring and the β-methylene group of the tyrosine residue (*4, 5*). The reductases from different sources probably vary in amino acid composition, but each enzyme must have a specific tyrosine residue involved in radical formation, and the major geometrical properties around the radical are conserved. The spin density distribution over the aromatic ring of protein B2 was estimated from the hyperfine couplings derived from the EPR spectra. The results suggest that the radical is an oxidation product of tyrosine with the proton still bound (*8*). A comparison with theoretical calculations of the spin density in this type of radical (*10*) further suggests the possibility of some stronger electrophilic interaction with the phenolic oxygen.

The tyrosyl radical depends on the presence of iron. Iron may be removed by various means that also lead to disappearance of the radical. In contrast, radical removal, for example, by treatment with hydroxyurea, leaves the iron center intact (*11*). The interaction between the radical and the iron center is discussed below, but first we briefly recapitulate the structure and general function of the *E. coli* ribonucleotide reductase.

The Enzyme Reaction

The ribonucleotide reductases discussed in this article catalyze the substitution of the hydroxyl at position 2′ of a ribonucleoside diphosphate by hydrogen (*12, 13*). The enzyme from *E. coli* has been studied most extensively and is the prototype for this class of reductases. Unless stated otherwise our discussion deals with this enzyme. Either of two known proteins [thioredoxin or glutaredoxin = $R(SH)_2$], containing redox active sulfhydryls, can function as immediate hydrogen donor in the following reaction:



Isotope studies of the stereochemistry demonstrated that only one new hydrogen atom is introduced during the reaction and that it enters at position 2′, with retention of the configuration at this carbon atom (*14*).

The four common ribonucleoside diphosphates are all reduced by the same enzyme. A balanced supply of the four deoxyribonucleoside triphosphate required for DNA synthesis is provided by an exquisite allosteric control of the enzyme (*12*), a control that regulates both its overall activity and substrate specificity.

Model of the *E. coli* Reductase

The active enzyme is a 1:1 complex of two nonidentical subunits, called proteins B1 and B2, each consisting of two identical or nearly identical polypeptide chains (Fig. 3). The complex dissociates easily and the two subunits are obtained as separate proteins during their purification (*12*).

Equilibrium dialysis experiments established that protein B1 (molecular weight, 160,000) binds both substrates (two sites) and effectors (four sites) with high affinities. The binding of individual substrates is strongly influenced by effectors. Binding data together with kinetic results obtained with various combinations of substrates and effectors led to the allosteric model of B1 shown in Fig. 3: two classes of effector sites exist, each with two subsites. Binding to one class (activity site) influences the overall activity of the reductase, with adenosine triphosphate (ATP) giving an active enzyme and with deoxyadenosine triphosphate (dATP) giving an inactive enzyme. Binding to the second class (specificity site) directs the substrate specificity toward reduction of either of the four common ribonucleoside diphosphates. Specificity sites can bind deoxythymidine triphosphate and deoxyguanosine triphosphate as well as ATP and dATP. The physiological implications of the allosteric regulation have been reviewed (*12*).

The main subject of this article is protein B2 (molecular weight, 78,000), the second subunit of the *E. coli* reductase (Fig. 3). In addition to the tyrosyl radical, one molecule of the protein contains close to two iron ions. The model shows one iron ion in each polypeptide chain. However, since separation of the two polypeptides results in loss of the metal ions, we have no direct evidence on this point.

The stoichiometry for the tyrosyl radical has proved troublesome. Early preparations of "pure" protein B2 contained varying substoichiometric amounts of the radical (*1*). These preparations contained close to two iron ions. For each preparation the enzymatic activity of B2 was proportional to the radical content. We subsequently found that both enzyme activity and radical content could be increased by removal and reintroduction of iron (*11*). Reactivated protein B2 then contained close to one radical per molecule. Taken together, these data suggest that preparations of pure B2 before reactivation consisted of two populations of molecules: one, lacking the tyrosyl radical, without enzyme activity; and the other with enzyme activity, containing the radical. In the model of B2 in Fig. 3 we assume the presence of one radical per molecule.

The catalytic site of the reductase is made up from parts of both protein B1 and B2. Protein B1 contributes two substrate binding sites and redox active SH groups; protein B2 contributes its tyrosyl radical and possibly the iron center. The isolated B1 subunit can bind substrates, but cannot reduce them. Addition of the radical containing B2 subunit resulted in the reduction of up to 3 moles of substrate per mole of B1 at the expense of redox active sulfhydryls of B1 (*15*). Addition of reduced thioredoxin or glutaredoxin to the B1:B2 complex leads to the regeneration of active SH groups on B1 and further reduction of ribonucleotides. Catalytic activity of the enzyme thus depends on the continued reduction of the redox active S-S groups of B1 by an external hydrogen donor.

So far we have not found changes in the EPR signal or the optical spectrum of B2 connected with the catalytic process, and the evidence for the presence of the tyrosyl radical in the catalytic site is indirect. It rests on the irreversible inactivation of the enzyme by the substrate

Summary. Ribonucleotide reductases catalyze the enzymatic formation of deoxyribonucleotides, an obligatory step in DNA synthesis. The native form of the enzyme from *Escherichia coli* or from mammalian sources contains as part of its polypeptide structure a free tyrosyl radical, stabilized by an iron center. The radical participates in all probability in the catalytic process during the substitution of the hydroxyl group at C-2 of ribose by a hydrogen atom. A second, inactive form of the *E. coli* reductase lacks the tyrosyl radical. Extracts from *E. coli* contain activities that interconvert the two forms. The tyrosyl radical is introduced in the presence of oxygen, while anaerobiosis favors its removal, suggesting a regulatory role in DNA synthesis for oxygen.

analog 2′-azidoCDP (CDP, cytidine diphosphate). This nucleotide scavenged the radical of the B1:B2 complex but had no effect on the radical of the B2 subunit in the absence of B1 (*16*). Under optimal allosteric conditions azidoCDP bound to the catalytic site reacted stoichiometrically with the tyrosyl radical. The interaction of this substrate analog with the radical is discussed later.

Much less is known about the structure of the other radical containing reductases. The mammalian enzyme also consists of two nonidentical subunits (M1 and M2) whose functions in most respects apparently parallel those of B1 and B2, respectively. Only pure M1 has been obtained in sufficient amounts for a more detailed characterization (*17*).

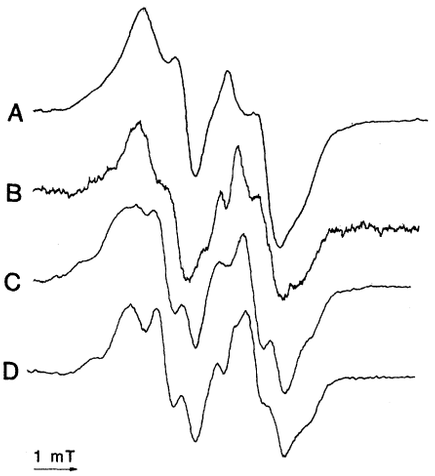


Fig. 1. EPR spectra of four different ribonucleotide reductases in frozen samples recorded at X-band frequency (9.5 GHz). (A) Isolated B2 subunit from *E. coli*. (B) Packed L cells, infected with pseudorabies virus and containing the virus-specific reductase (*6*). (C) Isolated bacteriophage T4 specific ribonucleotide reductase. (D) Packed hydroxyurea-resistant 3T6-HU11 cells, overproducing active M2, the mammalian counterpart of B2 from *E. coli* (*5*).

The Iron Center

Native protein B2 binds two iron ions strongly during the whole purification procedure. Efficient removal of the metal requires treatment with strong chelators under slightly denaturing conditions (*11*). No sulfide is released and reconstruction of the metal-free protein can be achieved simply by addition of ferrous iron under aerobic conditions. The nonheme iron of B2 is thus bonded directly to the protein structure and not via sulfur as in ferredoxin and similar proteins (*18*). In this and many other respects, the iron center of protein B2 resembles that of hemerythrin, a small oxygen-carrying protein of some invertebrates (*19*).

The iron ions showed no absorption by EPR spectroscopy, and other spectroscopic methods had to be used to elucidate the nature of the iron center. Since iron could be removed and reintroduced to form active protein, it was easy to prepare ^{57}Fe-labeled B2 for Mössbauer spectroscopy (*11*). With a weak applied transverse magnetic field two distinct quadrupole-split pairs of equal intensity were observed. This was clear evidence that the two iron atoms are in nonidentical sites. The effect of a strong applied magnetic field at a temperature of 4.2 K showed, in addition, that both sites are diamagnetic. The magnitude of the quadrupole splittings and their independence of temperature suggested two alternative structures as the only possibilities: two low spin Fe(II) complexes in independent sites, or a pair of inequivalent high spin Fe(III) ions in an antiferromagnetically coupled complex.

A distinction in favor of the second alternative could be made from results obtained by measurements of the magnetic susceptibility at temperatures below 200 K and from resonance Raman spectroscopy studies (*20*). Susceptibility measurements confirmed the diamagnetic ground state of the iron ions at low temperatures but also demonstrated that at higher temperatures paramagnetic excited states were populated. The data fitted the model of an antiferromagnetically coupled pair of high spin Fe(III) ions with an exchange coupling of $-J = 108$ cm^{-1}. Raman studies (*21*) demonstrated a spectral peak at 496 cm^{-1} after excitation of B2 at 406.7 or 413.1 nm. In $H_2{}^{18}O$, this peak shifted to 481 cm^{-1}. These data conclusively demonstrate the presence of a μ-oxo bridge linking the two Fe(III) ions.

The electronic spectrum of native B2 contains components from both the iron center and the radical. Treatment of the protein with hydroxyurea destroys the radical and the remaining spectrum above 300 nm can then be ascribed exclusively to the iron center (*20*). Figure 4 shows four peaks or shoulders at 325, 370, 500, and 600 nm. The radical spectrum shown in the inset of Fig. 4 was obtained by difference between the spectrum of native B2 and that of the radical-free protein containing the intact iron center. The inset also shows for comparison a closely similar spectrum reported for an oxidized phenoxy radical (*22*), confirming the nature of the tyrosyl radical as an oxidized species.

There is a striking similarity between the physicochemical data described above for the iron center of B2 and corresponding results reported for oxidized forms of hemerythrin (*19*). This analogy extends to optical, Mössbauer, and Raman spectra as well as paramagnetic susceptibility and magnitude of the exchange coupling. Both protein B2 and hemerythrin contain a binuclear iron center with the two nonidentical, high spin Fe(III) ions antiferromagnetically coupled by a μ-oxo bridge. Oxyhemerythrin and methemerythrin have also been investigated by x-ray crystallography (*23*) and x-ray absorption spectroscopy (*24*). The results suggest that the two iron ions have approximate octahedral symmetry, are coordinated to protein imidazolates, and are bridged by two protein carboxylates and a μ-oxo group. Investigations on protein B2 have not yet reached this refinement, but the extensive analogy between the properties of the iron centers of the two proteins may be based on some structural similarity in spite of apparent functional differences. The iron center of hemerythrin functions as an oxygen carrier, similar to heme in hemoglobin, and does not interact with a tyrosyl radical. There is also a second important structural difference: one subunit of protein B2 consists of two identical polypeptide chains (molecular weight, 39,000) and one binuclear iron center, while in hemerythrin one subunit consists of one polypeptide chain (molecular weight, 13,400) and one binuclear iron center.

Relation Between Iron Center and Radical

Several observations suggest a close connection between the iron center and the tyrosyl radical. The iron from native B2 can be efficiently removed by dialysis in strong imidazole buffer against 8-hydroxyquinoline (Fig. 5). The resulting preparation of the metal-free apoB2 then also lacks the radical and is enzymatically inactive (*11*). Reactivated protein B2 may be obtained from apoprotein and ferrous iron in a reaction requiring the participation of oxygen and leading to a simultaneous oxidation of the two irons and the tyrosine residue (*20*). From these results we conclude that the specific iron center in B2 is a requirement for the formation and stability of the radical.

On the other hand, it is possible to destroy the radical while leaving the iron center intact (*11, 21*). This can be done by treatment of native B2 with hydroxyurea or related compounds. The radical-free form of B2, which we have named B2/HU (Fig. 5), then gives Mössbauer and resonance Raman spectra identical to those of native B2.

The experiments with hydroxyurea analogs (*25*) also allowed some conclusions concerning the topology of the radical site of protein B2. The reactivity of these analogs was not only closely linked to their ability to undergo one-electron oxidation, but also to effects due to steric hindrance. These led to the conclusion that the tyrosyl radical is located in a pocket of B2 with a width of about 4 Å and a depth of more than 6 Å.

Evidence for interactions between the iron center and the radical have been difficult to obtain. Thus results obtained at temperatures below 77 K by both Mössbauer and EPR spectroscopy gave no indication that any spin density is delocalized to the iron site. Recent results suggest, however, that at higher temperature some magnetic interaction occurs between the radical and the iron center which, with increasing temperature, acquires paramagnetism (*20*). A careful analysis of the temperature dependence of the line shape and microwave saturation of the EPR signal of the radical tentatively suggests that a major contribution to the broadening of the signal is due to magnetic interaction between the radical spin and the thermally excited paramagnetic state of the iron center (*26*). From these data the distance between the iron center and the tyrosyl radical is estimated to be 6 to 9 Å.

For the mammalian protein M2 it was observed that much higher microwave power than in the case of B2 was needed to obtain saturation (*5*). This means that in M2 the interaction between the iron center and the radical is stronger than in B2, which may be due to different steric arrangements and speaks for different properties of the iron centers.

One remaining irritating uncertainty concerns the stoichiometry ratio between radical and iron. The bulk of our data suggests that the limiting ratio of the stoichiometry is one radical per Fe pair and B2 molecule. However, in some cases when B2 was prepared from a genetically manipulated overproducing strain of *E. coli*, the radical concentration actually slightly exceeded the B2 protein concentration (*20*). Even though the quantitation of radicals is fraught with considerable uncertainties, we cannot completely exclude the possibility that our best preparations of B2 consist of a mixture of molecules containing no or two tyrosyl radicals, or still more complex mixtures. However, most of our data favor the presence of one tyrosyl radical per B2. This important question may be settled in the near future with the aid of the radical-introducing enzyme system described below.

Radical Involvement in Ribonucleotide Reduction

Chemically, the formation of a deoxyribonucleotide from a ribonucleotide occurs through the transformation of a secondary alcohol to a methylene group. The reducing power is provided by the redox active dithiols located on the B1 subunit (Fig. 3). Hydroxyl (OH) groups are poor leaving groups, and early in our studies we looked for the possible existence of an activated intermediate, such as a 2′-phosphorylated or -pyrophosphorylated compound. This hypothesis was, however, effectively dispelled by negative experimental evidence. The subsequent discovery of the tyrosyl radical as part of the B2 subunit and the demonstration of its presence in the catalytic site opened up the possibility of a different kind of substrate activation. The presence of the radical makes it almost axiomatic that ribotide reduction occurs through a radical mechanism.

Unfortunately, all efforts to find changes in the EPR or optical spectra of the enzyme during the normal catalytic cycle have to this date been unsuccessful. Negative experiments of this kind are, of course, inconclusive since the rates of putative steps involving radical

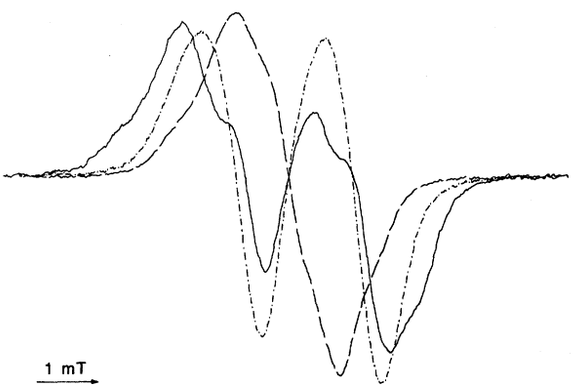
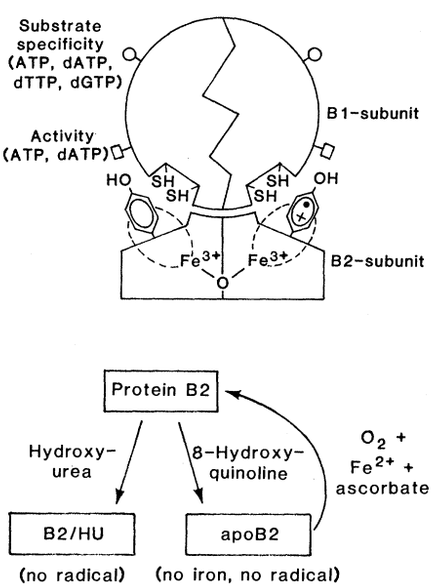


Fig. 2. EPR spectra (at 77 K) of centrifuged cells of *E. coli* KK-546 (*9*) grown in the presence of normal tyrosine (——), [3,5-D_2]tyrosine (–·–·–), or [β,β-D_2]tyrosine (– – –). The spectra are directly reproduced from experimental readings with approximately equal amplitudes.



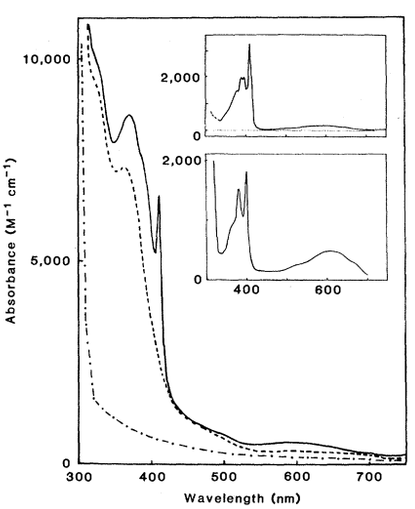
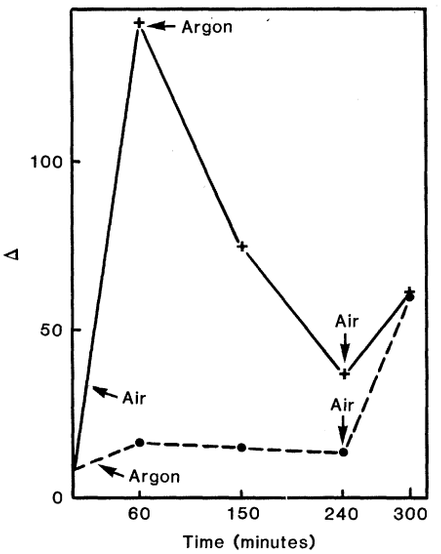


Fig. 3 (top left). Model of *E. coli* ribonucleotide reductase. The B1 subunit contains two classes of allosteric sites, regulating overall activity or substrate specificity. The B2 subunit contains the antiferromagnetically coupled iron center and the free tyrosyl radical. The catalytic site is constructed from parts of the B1 subunit contributing redox active sulfhydryls and parts of the B2 subunit contributing the tyrosyl radical. Fig. 4 (right). Light absorption spectra of the B2 subunit of ribonucleotide reductase from *E. coli* (native protein, ——), of the same sample incubated with hydroxyurea for 15 minutes at room temperature (radical-free protein, – – –), and of iron-free apoprotein (–·–·–). In the inset the top frame of the spectrum of the protein radical (difference spectrum is equal to the native minus the radical-free protein) is compared with the spectrum of 2,4,6-tritertiary butyl phenoxy radical in hexane (*22*) in the bottom frame. Spectra are redrawn from (*20*). [Courtesy of the *Journal of Biological Chemistry*] Fig. 5 (bottom left). Relations between different forms of protein B2. The native subunit loses both the iron center and radical on dialysis against 8-hydroxyquinoline and forms apoB2. Treatment of apoB2 with reduced Fe^{2+} in the presence of oxygen regenerates native B2. Treatment of native B2 with hydroxyurea destroys the free radical and results in the formation of radical-free B2/HU which still contains the iron center intact.

Fig. 6. Dependence of radical formation on oxygen (*31*). Radical-free B2/HU was incubated with an extract of *E. coli* in the presence of Mg^{2+} ions and dithiothreitol with air or argon bubbling as indicated by the arrows. The ordinate shows the amplitude of the EPR signal at different time intervals. [Courtesy of the *Proceedings of the National Academy of Sciences of the United States of America*]

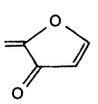


transfer might not favor accumulation of new radical species. We must therefore rely on indirect evidence both for our claim that a radical mechanism is involved at all and for any model postulating intermediate steps.

Any hypothesis must account for two established facts concerning the enzyme reaction: (i) as already mentioned, the OH-group at position 2′ is replaced stereospecifically by hydrogen. No other hydrogen atoms are introduced (or removed) during the reaction (*14*), which excludes mechanisms involving dehydration, or ketone or epoxide formation; and (ii) the tyrosyl radical of B2 is not lost during the reaction. If the radical is transferred to a different species during an intermediate step, it must again return to the original tyrosine residue during a later step.

Present ideas concerning the nature of the radical mechanism are fed by three lines of evidence:

1) Interaction of ribonucleotide reductase with nucleotide analogs containing Cl or F substitutions (*16, 27*) at C-2 in place of the OH group resulted not only in the release of the halogen but also in the cleavage of the bonds at C-1 between ribose and the base and at C-5 between ribose and pyrophosphate. In addition, the B1 subunit of the enzyme was inactivated. The reactions were postulated to occur via enzyme bound, substrate-radical intermediates that collapsed to form a 3′ ketone, responsible for the inactivation of the enzyme (*27*).

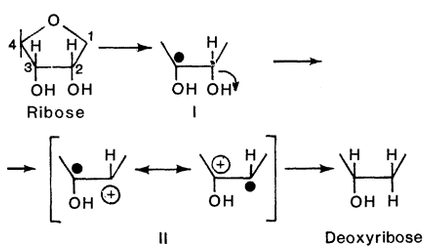


This postulate rests on a chemical analogy for facilitated elimination of a hydroxyl (or halogen) via a radical on an adjacent hydroxymethyl (*28*).

2) During the reduction of [3′-^{3}H]-uridine diphosphate by ribonucleotide reductase a more than threefold discrimination against the tritium-labeled substrate took place (*29*). This isotope effect again focuses attention on the 3′ carbon of the ribosyl moiety, suggesting cleavage of hydrogen bonded to this carbon as part of the overall reaction.

3) As mentioned earlier, the interaction of ribonucleotide reductase with substrate analogs containing an azido group at C-2 of the ribose in place of the OH group results in the stoichiometric loss of the tyrosyl radical. In this reaction a short-lived nucleotide radical was formed as an intermediate (*30*). The transient radical might be a counterpart to a substrate radical occurring in the normal reaction pathway.

All these findings are incorporated into the following hypothetical reaction scheme (*27, 29, 30*):



A pivotal idea here is the transient transfer of radical properties from the enzyme to the substrate. This occurs by abstraction of the hydrogen at C-3 of the ribosyl moiety by the tyrosyl radical of B2, resulting in the formation of substrate intermediate I. The presence of the radical at C-3 then facilitates the ejection of OH^- from C-2, with formation of cation radical intermediate II. Deoxyribose arises from II by the concomitant reduction of C-2 by thiols from subunit B1 and recapture of the hydrogen atom from the specific tyrosine of B2. The latter reaction both regenerates the tyrosyl radical and reintroduces the same hydrogen, originally present at C-3.

The reversible interaction between the tyrosyl radical and carbon 3 of the sugar moiety, crucial to the proposed mechanism, is an attractive hypothesis, but direct evidence for it is lacking. While the detailed scheme needs further corroboration the basic idea of a concerted action of redox active dithiols and the tyrosyl radical during a radical mechanism appears plausible.

A Radical-Introducing Enzyme System

Our understanding of the complexities of ribonucleotide reduction took a new and surprising turn when we found evidence for an enzyme system that may regulate the radical content of protein B2 (*31*). As mentioned earlier, "pure" preparations of B2 often contained mixtures of molecules, with or without the tyrosyl radical. Inactive, radical-free forms of B2 can be obtained from native B2 as shown in Fig. 5. Treatment with hydroxyurea generates the radical free form B2/HU, whereas 8-hydroxyquinoline gives rise to apoB2 that lacks both radical and iron; the latter reaction is reversible.

The formation of B2/HU from B2 was, in contrast, originally considered to be irreversible. Eventually, however, several phenomena became apparent that pointed to the possibility of a biological reversibility. Thus, hydroxyurea-treated bacterial (*32*) and mammalian (*33*) cells recovered their DNA synthesis so rapidly after removal of the drug that there was little time for the synthesis of a new enzyme. More recently, experiments with hydroxyurea-resistant mouse fibroblasts suggested that such cells might have become resistant by acquiring the capacity to overproduce an enzyme activity that could regenerate the radical of ribonucleotide reductase (*34*). We therefore looked for such an activity and found it in extracts from *E. coli*.

In these experiments, B2/HU prepared by treatment of B2 with hydroxyurea served as substrate in the putative enzyme reaction. The B2/HU was incubated with bacterial extracts and introduction of the radical function was monitored either by the appearance of the specific EPR signal or by the ability to form the catalytically active subunit of the enzyme. Both assays gave positive results. The reaction required addition of Mg^{2+} ions and a reducing agent, such as dithiothreitol, and depended on the presence of oxygen. The oxygen requirement was of particular interest and is exemplified in Fig. 6. After a rapid appearance of the B2 specific EPR signal under aerobic conditions, a switch to an argon atmosphere resulted in a decrease of the signal amplitude. On readmission of air, the signal reappeared. This behavior sug-

gested the presence of two opposing activities: one introducing the radical in the presence of oxygen, the second removing it. The second activity became apparent under anaerobic conditions.

The mechanism of this unusual radical-generating reaction remains a matter for speculation. Working on a purified enzyme system, we have so far found evidence for the involvement of several fractions, probably proteins, suggesting a complex kind of reaction. Chemically, the overall process with respect to protein B2 involves a one-electron oxidation of the aromatic tyrosine ring. It seems probable that oxygen is the final electron acceptor. Not unlikely, oxygen radicals function as intermediates with the iron of B2/HU, possibly participating in their generation. The requirements of the enzymatic process are very similar to the conditions used for the chemical radical introduction into apoB2. However, it seems clear that free apoB2 is not an intermediate in the enzymatic reaction since ^{59}Fe is not lost from labeled B2/HU during the process (*31*).

Results from mammalian hydroxyurea-resistant cells were a major impetus for our search for a radical-introducing enzyme system in *E. coli*. However, a similar system in mammalian cells has not yet been found. Significant amounts of the pure M2 subunit of the mammalian reductase, the counterpart of the B2 subunit of *E. coli*, are not yet available. Crude preparations of the mammalian reductase from hydroxyurea-resistant cells (*34*) contain the tyrosyl radical (Fig. 1) while purified preparations of the thymus enzyme lack the typcial EPR signal, suggesting that the radical was lost during the purification procedure (*35*). Aerobic incubation of such an enzyme preparation in the presence of dithiotreitol and iron rapidly restored the radical. On further anaerobic incubation, the radical was again lost, with a half-life of about 10 minutes. These results may indicate that the radical of the mammalian M2 subunit, in contrast to the B2 subunit of *E. coli*, is unstable and must be continuously regenerated. The transformations with the mammalian reductase appear to occur spontaneously, but may also be the result of the action of proteins still contaminating the highly purified enzyme. In any event, both the mammalian and bacterial enzymes can undergo oxygen-depending cycles between radical-containing (active) and radical-free (inactive) states.

What physiological significance in the intact cell has this newly discovered in vitro reaction? Modification by phosphorylation of tyrosine residues of proteins has recently attracted much attention as a regulatory mechanism for growth control. The "radicalization" of a specific tyrosine residue of ribonucleotide reductase and the removal of this radical also might serve a regulatory function. The balance between the two opposing reactions is influenced by oxygen, at least in vitro, suggesting the possibility that oxygen has a regulatory effect on DNA synthesis via its influence on the activity of the reductase.

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