The Tyrosyl Free Radical in Ribonucleotide Reductase

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The enzyme, ribonucleotide reductase, catalyses the formation of deoxyribonucleotides from ribonucleotides, a reaction essential for DNA synthesis in all living cells. The Escherichia coli ribonucleotide reductase, which is the prototype of all known eukaryotic and virus-coded enzymes, consists of two nonidentical subunits, proteins B1 and B2. The B2 subunit contains an antiferromagnetically coupled pair of ferric ions and a stable tyrosyl free radical. EPR studies show that the tyrosyl radical, formed by loss of an electron, has its unpaired spin density delocalized in the aromatic ring of tyrosine. Effects of iron-radical interaction indicate a relatively close proximity between the iron center and the radical. The EPR signal of the radical can be studied directly in frozen packed cells of E. coli or mammalian origin, if the cells are made to overproduce ribonucleotide reductase.

The hypothetic role of the tyrosyl free radical in the enzymatic reaction is not yet elucidated, except in the reaction with the inhibiting substrate analogue 2'-azido-CDP. In this case, the normal tyrosyl radical is destroyed with concomitant appearance of a 2'-azido-CDP-localized radical intermediate. Attempts at spin trapping of radical reaction intermediates have turned out negative.

In E. coli the activity of ribonucleotide reductase may be regulated by enzymatic activities that interconvert a nonradical containing form and the fully active protein B2. In synchronized mammalian cells, however, the cell cycle variation of ribonucleotide reductase, studied by EPR, was shown to be due to de novo protein synthesis.

Inhibitors of ribonucleotide reductase are of medical interest because of their ability to control DNA synthesis. One example is hydroxyurea, used in cancer therapy, which selectively destroys the tyrosyl free radical.

Introduction

Ribonucleotide reductase is the only known enzyme that carries a stable free radical in its resting state. The radical can be observed in intact cells as well as in the isolated, homogeneous enzyme. Ribonucleotide reductase is an essential enzyme of all living cells. It catalyses the first step on the biochemical pathway leading to DNA synthesis by reducing ribonucleotides to their corresponding deoxyribonucleotides (Fig. 1). General reviews on the enzyme have been published recently (1–4). This review is more specifically devoted to the properties and functions of the free radical of the enzyme.

The most extensively studied ribonucleotide reductase is the one isolated from the bacterium Escherichia coli. It is the prototype of all known eukaryotic and virus-coded ribonucleotide reductases (Fig. 2). It consists of two nonidentical subunits, proteins B1 and B2, each of which are enzymatically inactive alone. The active enzyme is formed by a 1:1 complex of proteins B1 and B2, in which B1 contributes redoxactive sulphydryls and B2 contributes the unique tyrosyl radical stabilized by an adjacent binuclear iron center. Protein B1 contains two nearly identical polypeptide chains of 87 kDalton each. Protein B2 contains two identical polypeptide chains of 43 kDalton each. The amino acid sequences of the B1 and B2 polypeptide chains were recently deduced from the nucleotide sequence of the corresponding E. coli genes (5). Binding of the substrate molecules, ribonucleoside diphosphates, occurs at the B1 subunit. This subunit also contains two different classes of binding sites for the allosteric effector molecules, nucleoside triphosphates. One class of binding sites regulates the specificity of the enzyme to reduce any of the four possible ribonucleotide substrates. The other class of binding sites regulates the overall activity of the enzyme, which can be efficiently turned off in a feed back regulated manner by the nucleotide dATP. The allosteric regulation has been studied in considerable detail and variations on the general theme occur with enzymes from different origins (1,4,6).

In some bacteria and algae, other prototypes of ribonucleotide reductase are found (4). One type, first isolated from the bacterium Lactobacillus leichmannii,
and extensively studied, reduces ribonucleoside triphosphates. It consists of a single polypeptide chain and a vitamin B₁₂ cofactor. Other types of recently isolated and characterized enzymes are oligomeric and utilize B₁₂ or divalent metal ions like Mn²⁺ as cofactors. It seems as if microorganisms display a variety of evolutionary solutions to the chemically complex reaction of reducing a secondary alcohol. Of these only the *E. coli* variant has been selected to serve in the eukaryotic kingdom. The simplest common denominator of all types of ribonucleotide reductases seems to be redoactive sulfhydryls and a metal-containing cofactor.

The electrons needed for the reduction of ribonucleotides are transported through a redox chain utilizing NADPH as the ultimate reductant. Two hydrogen donor systems are known (7). One is the thioredoxin system composed of thioredoxin and thioredoxin reductase. The other is the glutaredoxin system composed of glutaredoxin, glutathione and glutathione reductase. The mutual importance of these two systems for ribonucleotide reduction is presently not clear.

The remarkable similarity in *E. coli* and eukaryotic ribonucleotide reductases is prominently manifested in the active site of the metal-containing subunit, which in all cases studied so far harbors a stabilized unpaired electron in a tyrosine side chain of its polypeptide backbone. Not only is the localization of the radical to a tyrosine residue specific, also the geometry of the tyrosyl radical is very similar in ribonucleotide reductases from *E. coli*, bacteriophage T₄, mouse cells and pseudorabies virus (8–11). For the first three of these enzymes it has also been shown that the tyrosyl radical coexists with an adjacent binuclear iron center (12–14).

Another remarkable similarity between species is evident in the primary structures of ribonucleotide reductases as deduced from their DNA sequences in *E. coli* (5), herpes simplex virus (15,16), Epstein Barr virus (17), and the surface clam *Spisula solidissima* (18) (Fig. 3). Although the N-terminal and C-terminal parts of the B₂-equivalent subunits show a low degree of homology, three short internal regions are strikingly conserved (19). Such distant evolutionary relationships have the advantage that most invariant residues are likely to be functionally important. In Figure 3 we want to emphasize residue Tyr 122 as a candidate for radical stabilization. This is the only conserved tyrosine residue besides Tyr 356, which can be excluded because B₂ lacking this C-terminal part still carries the tyrosyl radical (20). Close to Tyr 122 one finds the possible iron ligands His 118 and Glu 115. In one of the other conserved areas further candidates for iron ligandation are found in His 241 and Asp 237 or Glu 238. The definite proof of these residues being directly involved in the active site of protein B₂ must await the determination of its tertiary structure. Recently the crystallographic parameters were obtained for high resolution crystals of protein B₂ from *E. coli* (21).

**The Tyrosyl Radical**

**Spectroscopic Properties and Structure**

More than 10 years ago it was observed that purified preparations of ribonucleotide reductase from *E. coli* gave a characteristic EPR signal, which could not be explained by the known presence of iron in the sample. The EPR spectrum (Fig. 4a), most easily observed at low temperatures, was tentatively assigned to a free radical (22). The magnitude of the EPR signal was found to be correlated with the activity of the enzyme. Also associated with the EPR signal was a sharp peak at 410 nm in the optical absorption spectrum of the enzyme.

Due to the small amounts of ribonucleotide reductase in wild type *E. coli* cells, more detailed structural studies had to await the development of a bacterial strain which could overproduce the enzyme. This strain, which is lysogenic for a recombinant bacteriophage, carries the structural genes for the bacterial ribonucleotide reductase attached to the λ genome (23). Heat induction of the lysogenic strain gives about 50 times more of ribonucleotide reductase than what is produced in wild type cells. This makes it possible to study the ribonucleotide reductase-specific EPR signal directly in *E. coli* cell suspensions without previous enzyme purification, since the specific EPR signal greatly dominates over all other cellular contributions in the free radical region of the spectrum.
Taking advantage of the experimental possibilities opened by this overproducing bacterial strain, isotope substitutions were performed by adding labeled compounds to the bacterial growth medium (8, 24). When deuterium substitues for hydrogen in a free radical, the hyperfine pattern caused by protons changes in a characteristic way due to the different magnetic properties of H and D nuclei. The D nucleus with spin quantum number \( I = 1 \) has a magnetic moment which is only 15\% of that of the H nucleus with \( I = 1/2 \). An EPR doublet hyperfine splitting arising from H therefore, after D exchange, becomes a narrower triplet splitting, often coalescing into a single unresolved line. An effect of this was directly observed in cells grown in a medium based on 98\% \( \text{D}_2\text{O} \). The major hyperfine doublet splitting for cell suspensions grown in a \( \text{H}_2\text{O} \) medium (Fig. 4a) collapsed into an unresolved singlet line in a \( \text{D}_2\text{O} \) medium.

Further isotope substitutions localized the radical to a tyrosine residue. Figure 5 shows the effects in the EPR spectra of specifically deuterated tyrosines included in the growth media and incorporated into the enzyme: Deuterium at positions 2, 6, and 3\( \alpha \) gives only a slight decrease in the linewidth of an otherwise unchanged EPR spectrum (Fig. 5a; for tyrosine numbering see Fig. 6). Deuterium at positions 3 and 5 causes the 7 G triplet structure to disappear (Fig. 5b). Deuterium at positions \( \beta \) causes the major 19 G doublet splitting to disappear (Fig. 5c). Therefore it was concluded that the unpaired spin density is localized in the aromatic ring of tyrosine (8). By using equations relating hyperfine coupling constants and spin densities in a delocalized aromatic radical (25), spin densities were estimated as shown in Figure 6 (9). The spin density at C\(_1\) gives rise to the 19 G hyperfine coupling to one of the methylene protons. The second methylene proton in this case does not give any observable hyperfine coupling, for reasons that will be discussed later. However, the inequality of the two methylene protons indicates that, at least at low temperature, the aromatic ring is in a locked conformation relative to the methylene group.

The spin density distribution of the enzyme radical is similar to that of oxidized tyrosine (26–28). Tyrosine is an easily oxidizable amino acid and the oxidized tyrosyl radicals have been observed in several studies using 1-electron oxidation (26, 29, 30). Recently, Sealy et al. made an EPR study of the temperature dependence of the EPR parameters of chemically oxidized tyrosine at alkaline pH. Also in this case the results showed a basic inequivalence of the two \( \beta \) methylene protons even above room temperature (31).

The presence of a stable oxidized tyrosyl radical seems to be a common feature in all iron-containing ribonucleotide reductases studied so far. Figure 4 shows the EPR spectra of other reductases of widely different origins: bacteriophage T4-induced enzyme in \( E. \ coli \) cells (9), mammalian enzyme (10, 32) and pseudorabies-induced enzyme in mammalian cells (11). The T4-induced enzyme and the mammalian enzyme have been
duced enzyme and the mammalian enzyme have been shown to contain two nonidentical subunits, formally corresponding to the B1 and B2 proteins in the E. coli enzyme. In the T4-induced enzyme the two subunits are bound together more tightly than the subunits of E. coli (33). In the mammalian case, the subunits are called M1 and M2. Protein M1 is a dimer of molecular weight 170 kDalton (34, 35). Protein M2 has recently been purified to homogeneity and is a dimer of molecular weight 88 kDalton (34). For the T4 and mammalian enzymes, isotope substitution experiments similar to those already described for E. coli were used to assign the radical to a tyrosine residue (9, 10). The dominating doublet hyperfine splitting remains similar in all four cases, indicating a similar immobilized conformation at low temperature for the four enzymes.

The minor differences between the hyperfine patterns exhibited at low temperatures by the four different enzymes in Figure 4 are most likely due to small geometrical differences in the conformations of the immobilized tyrosine residues harboring the free radicals. The magnitude of the hyperfine coupling $a_H$ to each of the $\beta$ methylene protons, caused by the unpaired spin density at C1 (Fig. 6), is critically dependent on the angle $\theta$ between the orbital axis of the radical (perpendicular to the plane of the aromatic ring) and the $\cdot$$\cdot$H bond projected perpendicular to the C1-C9 bond. The following relationship holds: $a_H = \rho(B_o + B_2 \cos^2 \theta)$, where $\rho$ is the spin density at C1, $B_o \approx 0$ G, and $B_2 \approx 50$ G (9, 25, 36).

Applying this relation to the hyperfine couplings evaluated from the spectra of the T4-induced and native E. coli radicals, we obtained the following angles $\theta$ for the two methylene protons (9): T4-induced, 10° and 130° from two measurable couplings; E. coli, approximately 0° and 120°, since only one coupling can be measured.

Another possibility for the variability of the different radical EPR spectra could be different distributions on different conformational states already present in each sample (31). Although this possibility cannot be excluded as at least a partial explanation for the variability, we consider it less likely since each spectrum is

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**Figure 4.** EPR spectra of tyrosine free radicals of ribonucleotide reductase of various origins: (a) E. coli cells overproducing ribonucleotide reductase at 32°C. The wild type cell background was negligible. (b) Purified ribonucleotide reductase from bacteriophage T4-infected E. coli at 77°C. (c) Hydroxyurea-resistant mouse 3T6 cells at 32°C. A suitable background of wild type cells has been subtracted. (d) Pseudorabies virus-infected mouse L cells at 32°C. A background spectrum of mock-infected mouse L-cells has been subtracted.

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**Figure 5.** EPR spectra at 77°C of cell suspensions of E. coli KK546 which overproduce ribonucleotide reductase. The bacteria were grown in the presence of (a) [2,6,α-D$_3$]-tyrosine, (b) [3,5-D$_2$]-tyrosine, and (c) [β,β-D$_2$]-tyrosine (8).

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**Figure 6.** Estimated spin density distribution in the tyrosyl free radical of E. coli and bacteriophage T4-induced ribonucleotide reductase (8, 8, 9).
completely reproducible regardless of state of purification of the enzyme and velocity of freezing.

The optical spectra of the E. coli protein B2 and the mammalian protein M2 radicals are shown in Figure 7. The radical spectra are obtained as the differences between those of native and nonradical-containing B2 or M2 (37,12,14). Nonradical-containing protein was obtained through treatment with hydroxyurea which specifically destroys the ribonucleotide reductase radicals (see below). Both spectra are similar to the light absorption spectrum of a chemically induced 2,4,6-triteriary butyl phenoxy radical, shown in Figure 7c (12,38,39). This is also further proof that the enzyme radicals are formed through loss of an electron. However, whether the enzyme radicals also formally are phenoxy radicals or have hydrogen or a ligand bound to O₄ is in our opinion an open question.

Stability

It is unusual to find a stable free radical as an integral part of an enzyme. The stability of the protein B2 radical is closely connected to the presence of the iron center of the enzyme. The iron center of protein B2 has been characterized by Mössbauer spectroscopy (37), light absorption (12,37), magnetic susceptibility (18), and resonance Raman techniques (18,40). It is composed of a pair of high spin (S = 5/2) ferric ions, antiferromagnetically coupled through a μ-oxo bridge. When the iron is chelated out of the protein, the tyrosyl radical is also lost (37). The radical and enzyme activity may then be restored, if ferrous iron is presented to the protein in the presence of oxygen (12,37). In the mammalian enzyme, recent light absorption studies on purified protein M2 showed a similar iron center (14).

Hydroxyurea and hydroxylamine are radical scavengers which specifically destroy the tyrosyl radical in the ribonucleotide reductases (22,37). The radical scavengers probably act through 1-electron donation (41). In protein B2 the tyrosyl radical may be restored and the enzyme reactivated by iron chelation and subsequent addition of ferrous iron in the presence of oxygen. However, it was recently demonstrated that activation of nonradical-containing B2 is a pH-dependent reaction, which occurs spontaneously in the presence of dithiothreitol, oxygen, and Mg²⁺ above pH 8.0 (P. Reichard, personal communication).

In protein M2 the radical is less stable than in B2 and is easily lost, e.g., in enzyme purification procedures. The M2 radical is also easily restored in the presence of a reducing agent (dithiothreitol), oxygen, and iron (42), without previous removal of iron through chelating agents.

Radical Content and Localization

Early on, Ehrenberg and Reichard observed a linear correlation between specific activity and radical content of protein B2 (22). Our best preparations show a stoichiometry of 1 tyrosyl radical per dimeric iron center of B2 (12). Because each B2 protein consists of two identical polypeptide chains, each B2 polypeptide dimer most likely harbors 1 tyrosyl radical. The possibility of a mixed population of protein B2 with radical contents of 0, 1, and 2 is not likely, because the same average radical content of approximately 1 has repeatedly been obtained in native preparations and in chelated and reconstituted preparations.

Recently we encountered another form of protein B2 in the overproducing cells (20,43). This form arises from

![Figure 7](image_url)
a limited proteolysis of the normal B2 (ββ) dimers to give a B2 (β'β') dimer, which has lost 29 C-terminal amino acid residues. B2 (β'β') contains a normal iron center and a normal tyrosyl radical but has no enzymatic activity due to lack of binding to the B1 subunit. A hybrid dimer B2 (ββ') can be obtained in 50% yield from a stoichiometric mixture of normal B2 (ββ) dimers and defective B2 (β'β') dimers, which have been carried through a reconstitution cycle of iron chelation and reactivation. All three possible B2 dimers (ββ, ββ', and β'β') can be separated by ion exchange chromatography. It was possible to study the localization of the tyrosyl radical within the ββ' dimer by starting the reconstitution experiment described above from deuterated B2 (ββ) and protonated B2 (β'β'). Consequently, every full-length β-polypeptide was deuterated and every digested β'-polypeptide was protonated so that the mixed dimers ββ' must always incorporate one protonated and one deuterated polypeptide chain. All three types of dimers contained approximately 1 radical per dimer and in the B2 (ββ') dimers we observed a mixed EPR signal consisting of 58% protonated doublet and 42% deuterated singlet contribution. This implies a random generation of the tyrosyl radical at either polypeptide chain of the dimer during the reconstitution reaction, a situation which closely resembles a "half-site" reactivity feature as is frequently found in allosterically regulated enzymes (44). It probably also implies that protein B2 can only serve to reduce one substrate molecule at a time, albeit the B1 subunit contains two fully active substrate binding sites. One may in fact consider protein B2 as a high molecular weight cofactor for protein B1, a further support for the formal similarity between the E. coli and L. leichmannii enzymes (4).

Iron-Radical Interaction

A striking difference between the E. coli protein B2 and mammalian protein M2 radicals is apparent in their different EPR microwave saturation behavior. At 77°K, the EPR signal of the M2 radical is extremely difficult to saturate with available microwave powers (~100 mW), whereas the B2 radical shows microwave saturation effects above 0.3 mW.

Towards higher temperatures, the EPR spectra of the ribonucleotide reductases are considerably broadened and lose their low temperature hyperfine structure (compare Fig. 8a with Fig. 4a). For the E. coli and mammalian enzymes, a detailed study of the temperature dependence of EPR signal lineshapes and microwave saturation was recently conducted (45,46). For the free radicals of both B2 and M2, the unusual line broadening of the EPR signal and the difficulty to achieve saturation at higher temperatures may be explained by magnetic interaction between the free radical and the antiferromagnetic iron center. The latter exhibits an increasing effective magnetic moment at increasing temperatures (12). In protein B2, the theory of magnetic dipolar interaction was found to give a satisfactory ex-

![Figure 8. EPR spectra at room temperature of (a) the tyrosyl radical of protein B2 at a protein concentration of 112 μM, and (b) the azido-CDP substrate radical after 5 min reaction in an incubation mixture containing 20 μM of the E. coli ribonucleotide reductase (see text). The spectra were recorded in a flat cell using a TE-mode cavity. The microwave power was 20 mW and the modulation amplitude was 0.2 mT (59).](image_url)

planation for the results, which imply a rather close proximity between iron center and radical. In protein M2, the results are better explained by magnetic exchange interaction. Relatively minor steric differences in the iron center/radical geometry could explain why one or the other magnetic interaction mechanism dominates in a given case.

Reaction Mechanism

Hypothetical Role of the Tyrosyl Radical

The enzymatic reaction which is catalyzed by ribonucleotide reductase is formally a reduction of the secondary alcohol at 2'-C in the ribose ring to a hydroxycarbon with retention of configuration at this carbon. The reduction of nucleoside diphosphate substrates occurs at the expense of redoxactive sulphydryls of protein B1 (47). Although the fully active B2 subunit with its tyrosyl radical is needed for catalysis, we have not so far observed any changes in the EPR signal or the optical spectrum of B2 during the catalytic process (48). There are, however, several indirect lines of evidence for the involvement of the tyrosyl radical in the catalytic process. These studies deal with K_{cat} suicidal substrate analogs, such as 2'-halogenated or 2'-azido-substituted nucleoside diphosphates (49-52).

A few years ago, Stubbe et al. demonstrated that specifically 3'-tritiated substrates were selected against with an isotope effect of approximately 3 (53,54). This supports their hypothesis that cleavage of the 3'-carbon-hydrogen bond is a primary step in ribonucleotide reduction. The 3'-hydrogen is most likely abstracted by
the tyrosyl radical of B2 to generate a 3'-nucleotide radical.

A less significant selection against 3'-tritium occurred
with 2'-halogenated substrate analogs which specifically
inhibits the B1 subunit. Stubbe et al. proposed a de-
tailed mechanism for this suicidal reaction (54). The
mechanism involves, besides the 3'-nucleotide radical,
a radical cation intermediate which rapidly may decom-
pose into a reactive 3'-ketone. Recently several of the
hypothesized intermediates were observed (55). It was
also shown that 2'-fluoro analogs can partition between
a normal reduction to produce 2'-deoxy NDP and in-
activation of B1. Reactions involving normal substrate
are thus very likely to follow the same intermediary
steps at least in the outset of the reaction and probably
also involve a radical cation intermediate.

The suicidal reaction with azido-substituted analogs
specifically destroys the tyrosyl radical of the B2 sub-
unit. This K_{cat} inactivation involves a new radical in-
termediate which can be studied by EPR spectroscopy
(50). Its characteristics are a large (25 G) triplet further
split by a smaller (6 G) doublet (Fig. 8b). We proposed
a sugar-localized neutral nitrogen-centered radical with
an additional hyperfine coupling to a hydrogen. With
specifically 15N-labeled azido-UDP it was indeed cor-
rorobated that the new EPR species is localized to one
of the azido-nitrogens (56), but demonstration of a hy-
drogen coupling has been negative for the ribose hy-
drogens at carbon 1' through 4' (56,57). Taken together,
the results with 2'-halogenated and 2'-azido-substituted
substrate analogs as well as the isotope effect at the 3'-
C of the ribose ring are fully compatible with a radical
cation mechanism as outlined in Figure 9.

In the mammalian ribonucleotide reductase prepared
from calf thymus the M2 radical is labile and needs
oxygen and a reducing agent to be continuously regen-
erated. The halflife of the radical under anaerobic con-
ditions was found to be of the order of 10 min (42). The
continuous presence of oxygen is also needed during the
catalytic action. However, there is no specific con-
sumption of oxygen in the catalytic cycle, since the ini-
tial reaction rate was approximately unchanged under
anaerobic conditions compared to aerobic conditions
(42).

Experiments were also performed with the E. coli
ribonucleotide reductase to determine a possible oxygen
requirement during catalysis (58). The EPR signal was
monitored at 77°C after aerobic or anaerobic incubation
of an enzyme reaction mixture at 25°C in the time in-
terval 0–30 min. Samples were also tested for enzymatic
activity during the same time interval. To ensure ab-
solute anaerobicity, in one experiment, a system with
addition of glucose oxidase, glucose and catalase was
used. It was not possible in any experiment to dem-
strate a requirement for oxygen in the E. coli system
even after 3000 turnovers (58).

Spin-Trapping Experiments

In order to try to reveal the nature of the hypothet-
ical substrate radical, spin trapping experiments were
performed at room temperature (59). Three different spin
traps were used, namely 5,5-dimethylpyrroline-N-oxide
(DMPO), phenyl-tert-butyl nitrotrone (PBN), and tert-
nitrobutane (MPN). Reaction conditions were equimolar
concentrations of proteins B1 and B2 in a mixture con-
taining the appropriate effector for assay conditions at
pH 7.6 (60) but with dithiothreitol as a reducing agent
instead of the thioredoxin system. Both normal sub-
strate, CDP, and the substrate analog azido-CDP were
used. No substrate radical could be trapped with any
of the spin traps. Likewise, it was not possible to trap
the azido-CDP radical. However, it could be observed
that the azido radical spectrum at room temperature
(Fig. 8b) is in principle the same as that obtained at
77°C, showing the same anisotropy of the major nitro-
gen hyperfine splitting (50). This is in contrast to the
loss of structure observed for the B2 radical at higher
temperatures (Figs. 8a and 4a). Thus the substrate an-
alog radical appears to be in a locked conformation at
the active site of the enzyme even at room temperature.
Furthermore the radical is not close enough to the bin-
nuclear iron center for its EPR spectrum to be broadened
at higher temperatures.

Since the EPR signal from protein B2 disappears
upon addition of the radical scavenger hydroxyurea,
spin-trapping experiments with MNP were also per-
formed during the hydroxyurea reaction. However, no evidence for any trapped radical was found.

Finally, reconstitution of protein B2 from apoprotein was performed with a reactivation mixture containing Mohr's salt (37) and with DMPO as a spin trap. The results were immediate reconstitution with no radical trapped.

To summarize the spin-trapping experiments, we conclude that the E. coli ribonucleotide reductase keeps its tyrosyl radical in a rather rigid and protected conformation. We also infer that the substrate radical (as observed with azido-CDP and postulated with normal substrate) must be inaccessible for the spin traps at the conditions used, probably due to the geometry of the active site and/or the short lifetime of the substrate radical.

Similarities with Reaction Mechanism of Ribonucleotide Reductase from L. leichmannii

In discussing the reaction mechanism of E. coli ribonucleotide reductase it is worthwhile to consider the remarkable similarities to the reaction mechanism of the L. leichmannii enzyme. Isotope labeling studies indicate that also this enzyme cleaves the 3'-C-H bond of the ribose moiety (61). A radical cation mechanism for the L. leichmannii enzyme is particularly intriguing, since the adenosylcobalamin cofactor in this case has been shown to undergo a homolytic scission of the cobalt-deoxyadenosine bond during catalysis (62). The function of adenosylcobalamin may be as a radical chain initiator. Its hypothetical role could be to facilitate the formation of a protein localized but exceedingly short-lived equivalent of the tyrosyl radical (4, 61).

In Vivo Regulation of the Tyrosyl Radical

The B2-equivalent subunit of any ribonucleotide reductase has to undergo post-translational modification in order to become a fully active subunit with iron and tyrosyl radical from the nascent ApoB2 form. As pointed out earlier, in vitro reconstitution of the E. coli ApoB2 form occurs spontaneously in the presence of ferrous iron and oxygen, constituents which are certainly available in anaerobically grown E. coli cells. It is presently not known which oxidizing agent might take the role of oxygen in anaerobically grown cells.

Protein B2 of E. coli

The tyrosyl radical of B2 is remarkably stable during storage conditions. For instance, a sample of 1 mM B2 in deuterated phosphate buffer retained 80% of its free-radical content after storage at 4°C for about one month (58). Slow but measurable half-lives can, however, be observed at ambient temperatures and, e.g., during catalysis (23, 37). This leads to an inactive form of B2 lacking the tyrosyl radical. Similar forms may also exist in an in vivo situation.

Extracts from E. coli were recently shown to contain activities that interconvert the nonradical-containing and the fully active forms of protein B2 (3, 63). The inactivating system has not yet been studied in any depth. The radical introducing system on the other hand has been studied and turns out to be complex. It involves several enzyme activities, a few of which are flavine dependent, and it requires the presence of a nicotinamide cofactor, reducing agent, oxygen and Mg²⁺ (64). This enzyme-catalyzed reactivation proceeds well at pH 7.0 in contrast to the chemical activation mentioned above which requires a pH above 8.0.

Another radical-containing enzyme requiring an activating system is pyruvate formate-lyase from E. coli which is induced during anaerobic cultivation of the bacteria. Knappe et al. recently showed that the activating reaction in this case resulted in the introduction of a free radical into the inactive form of the enzyme (65). The assignment of its doublet EPR spectrum has not been clarified but preliminary experiments indicate that it is not localized to a tyrosine residue (66). Pyruvate formate-lyase is the second example of an enzyme having a stable free radical as an integral part. Since EPR spectroscopy is not a routine technique to characterize enzymes, the future might reveal yet more catalytically essential free radicals of this kind.

Protein M2 and the Mammalian Cell Cycle

For the mammalian ribonucleotide reductase a study of the cell cycle regulation of the enzyme has been carried out in mouse mammary tumor cells (67). The cells were selected for overproduction of protein M2 by continuous culturing over a period of one year in stepwise increasing concentrations of the radical scavenger hydroxyurea. The M2 radical can be determined quantitatively "in vivo" by EPR measurements of such frozen, packed cells. When the cells are grown in a medium containing deuterated tyrosine instead of normal tyrosine, the characteristic collapse of the EPR hyperfine doublet spectrum into a singlet is observed. This fact allows discrimination between de novo synthesis of protein M2 versus possible reactivation of "old" inactivated nonradical-containing protein upon change of cell medium from containing normal to deuterated tyrosine. A 3 to 7-fold increase in M2 radical was observed when synchronized mouse cells passed from the G1 to the S phase of the cell cycle and this increase was due to de novo synthesis. In contrast it was observed that after hydroxyurea inactivation and removal of the drug, the cells were able to rapidly regenerate the tyrosyl radical from "old" nonradical-containing M2 protein.

Medical Aspects

The ability to control DNA synthesis is of considerable medical interest. A selective inhibition of DNA
synthesis is desired, e.g., in clinical applications such as restriction of tumor growth or virus replication. In this context ribonucleotide reductase occupies a crucial stand at the intersection between RNA and DNA precursor biosynthesis. Specific inhibitors of ribonucleotide reductase like hydroxyurea and azidocytidine have been used as agents in cancer therapy (4). The accessibility of the tyrosyl radical of E. coli ribonucleotide reductase has been thoroughly explored by use of a series of hydroxyurea analogs (41). These compounds can donate an electron to the tyrosyl radical. The reaction rate with B2 is essentially proportional to the reaction rate between the same analog and the radical compound Fremy's salt. Very bulky side chains on chemically reactive analogs however are completely inactive with the tyrosyl radical of B2. From such experiments the tyrosyl radical of B2 could be assigned to a pocket in the protein of approximately 4 × 6 Å.

In the mammalian enzyme the localization of the tyrosyl radical and the iron center must be more exposed, since chemically reactive and bulky compounds such as polyhydroxybenzohydroxamic acids exert potent inhibition at concentrations as low as 10 μM. The same compounds are virtually inactive with E. coli B2, most likely due to steric hinderance. Polyhydroxybenzohydroxamic acids are presently explored as antiproliferative drugs because of their low general toxicity to mammalian cells.

One of the most potent inhibitors known for the mammalian ribonucleotide reductase is the iron chelate of 1-formylisooquinoline thiosemicarbazone (IQ-1). It has been shown that the target of this drug is the tyrosyl radical of protein M2 (68). After removal of the drug, regeneration of the radical could be achieved during normal assay conditions. A closer analysis showed that the reducing agent dithiothreitol under aerobic conditions was responsible for the regeneration, i.e., the same conditions as after hydroxyurea inactivation were needed. It was also found that oxygen was needed for the tyrosyl radical destruction by IQ-1. It was therefore postulated that the iron chelate of IQ-1 destroys the M2 tyrosyl radical by a one-electron redox process with participation of iron and oxygen.

Further exploration of specific inhibitors for ribonucleotide reductases seems promising, especially since it has been demonstrated that several medium-sized virus genomes (pseudo-rabies, herpes simplex and Epstein-Barr) code for virus-specific ribonucleotide reductase of the common prototype but with some distinctly virus-specific characteristics (11,16,17).

Up to now, no enzymes induced by eukaryotic viruses have been produced in amounts sufficient to make meaningful quantitative comparative inhibition studies. In the E. coli case, which is the only one known, it is however clear that the bacteriophage T4-induced enzyme is considerably more sensitive to hydroxyurea than is the host enzyme (9,41). If this is a more general phenomenon, one may look for other radical scavengers which are more potent against viral enzymes than against their host enzymes.

Ribonucleotide reductase is one of approximately a handful of enzymes specific for active DNA synthesis in living organisms. Due to its unique tyrosyl radical and delicate allosteric regulation it comprises a very promising target for dedicated inhibition of DNA synthesis.

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