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# Enzymic modification of a tyrosine residue to a stable free radical in ribonucleotide reductase

(enzyme regulation/DNA synthesis/hydroxyurea)

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**ABSTRACT** **Protein B2, a subunit of ribonucleotide reductase from *Escherichia coli*, contains in its active form a tyrosyl free radical as part of the polypeptide chain and a dimeric iron center that stabilizes the radical. The enzyme depends on this radical for its catalytic activity. Treatment with hydroxyurea scavenges the radical without disturbing the iron center and, thereby, results in an inactive form of the subunit, B2/HU. A second inactive form, apoB2, lacking both the radical and the iron center, is obtained by treatment of B2 with 8-hydroxyquinoline. Here we describe an enzyme activity in extracts from *E. coli* that transforms the catalytically inactive B2/HU form into the active B2 subunit by regeneration of the tyrosyl radical. This reaction requires the presence of oxygen, dithiothreitol, and $Mg^{2+}$ and does not proceed through apoB2. Under anaerobic conditions, we obtained evidence for a second activity in the bacterial extract that destroys the free radical and transforms B2 into B2/HU. We suggest that this novel type of protein modification is functionally related to the synthesis of deoxyribonucleotides and DNA.**

Ribonucleoside diphosphate reductase from *Escherichia coli* consists of two nonidentical subunits, named protein B1 and B2, in a 1:1 stoichiometry (1). Each subunit contains two identical or nearly identical polypeptide chains. The two catalytic sites of the enzyme are made up from part of each subunit: B1 contributes substrate binding sites in addition to redox-active sulfhydryl groups that furnish the reducing hydrogens; B2 contributes one or possibly two tyrosyl radicals, which are part of the polypeptide chain of the protein. The radical is believed to participate in the reduction of the hydroxyl group at the 2′ carbon of the ribose moiety (2–4). Protein B2 also contains two $\mu$-oxobridged, antiferromagnetically coupled iron(III) ions that stabilize the tyrosyl radical (5).

Protein B2 can occur in different forms. The catalytically active B2 subunit contains in its polypeptide structure the tyrosyl radical that is absent in the inactive form of the subunit. Protein B2 is usually obtained as a mixture of the active and inactive forms. The balance between the two depends on the isolation procedure and, in particular, on the time taken for purification. The specific activity of the highly purified subunit is directly proportional to the amount of radical-containing protein (6).

The balance between the different forms of B2 can be manipulated in the test tube. Incubation of active B2 with hydroxyurea, hydroxylamine, and certain other chemicals results in the loss of the radical without disturbing the iron center (5, 7). We will call this form of the subunit B2/HU. Dialysis against 8-hydroxyquinoline or other chelating agents further degrades the enzyme by removing the iron atoms and results in the formation of apoB2 (7). The iron center may be restored by simply adding iron(II) and ascorbic acid in the presence of oxygen. Such treatment also reintroduces the free radical, thus transforming apoB2 to active B2.

We report here that extracts of *E. coli* contain an enzyme activity capable of modifying the critical tyrosine residue of B2/HU by transforming it to a radical and, thereby, reactivating the subunit. This reaction requires $Mg^{2+}$, dithiothreitol, and oxygen. We also have obtained evidence for a second enzyme activity that, in the absence of oxygen, removes the radical function from active B2. The interrelationships between the different forms of protein B2 are shown schematically in Fig. 1.

## MATERIALS AND METHODS

**Protein Preparations.** *E. coli* C600 wild type and C600 carrying plasmid pPS2 were grown on LB medium (8). For the preparation of bacterial extracts, cells were harvested at an $OD_{640}$ of 1.5, cooled on ice, and immediately centrifuged at 4,000 × *g* for 10 min at 2°C. The pellet was suspended to give a calculated $OD_{640}$ of ≈400 and quick-frozen.

Bacterial extracts were prepared from pelleted bacteria by treatment with lysozyme (9). For some experiments, the protein of the extract was precipitated to 70% saturation with solid ammonium sulfate, dissolved in buffer A (25 mM Hepes, pH 8.0/20% glycerol), and dialyzed until free of sulfate.

Protein B2 was prepared from *E. coli* C600 carrying plasmid pBS1 (unpublished data). The preparation used was about 50% pure, as judged from its specific activity. One milligram of protein contained 14.9 nmol of iron and 7.2 nmol of radical (see below). ApoB2 was made from B2 as described earlier (7). B2/HU was prepared by treating a solution of B2 (8 mg/ml) in buffer A for 2 hr with 0.1 M hydroxyurea. The protein was then precipitated by addition of an equal volume of saturated ammonium sulfate and centrifuged; the precipitate was subsequently dissolved in buffer A and dialyzed for 6 hr against three changes of the same buffer until free of sulfate. All of these operations were made at 2°C. All B2 preparations were stored at −70°C.

**Standard Conditions for Reactivation of B2/HU.** Bacterial protein in buffer A was incubated with gentle rocking in a 30°C water bath with B2/HU (50–60 $\mu$g unless stated otherwise) together with 10 mM $MgCl_2$ and 10 mM dithiothreitol in a final volume of 0.22 ml in siliconized 3-ml test tubes. The reaction was stopped at a given time by transfer of the tubes to an ice bath. The extent of B2 reactivation was determined from the reappearance of the tyrosyl radical or from its catalytic activity. In the former case, the incubation mixture was rapidly transferred to standard quartz tubes of 3-mm inner diameter, frozen in liquid nitrogen, and scanned for the typical EPR spectrum at $g = 2$ (6). First-derivative EPR spectra were recorded at 77 K on a Bruker ER 100D EPR spectrometer with 100-kHz field-modulation frequency. The microwave power was set at 4.8 mW, and a modulation amplitude of 0.8 mT was used. Under these

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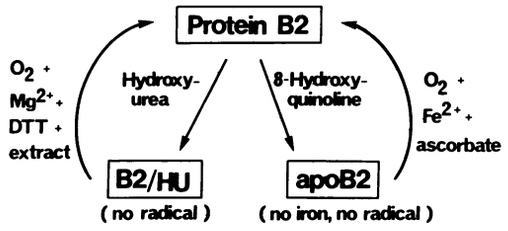


FIG. 1. Interrelationships between different forms of protein B2. DTT, dithiothreitol.

conditions, maximal signal amplitude was obtained while the fine structure of the signal was lost (see Fig. 2). The free radical concentration could be calculated from the signal amplitude as described (10, 11). For practical reasons, we define Δ as equivalent to half the signal amplitude, as shown in Fig. 2 *Inset*. A Δ of 54 was obtained for a concentration of tyrosyl radical of 1 μM. The ability of B2 to function as an active subunit of ribonucleotide reductase was measured in the standard assay with an excess of the B1 subunit and expressed as specific B2 activity (12). The protein concentration of the B2 subunit was determined from its absorption at 280 nm (13).

Gel electrophoretic separations under nondenaturing conditions were made on cylindrical gels as described (14). The gels were sliced with a Gilson gel-slicer apparatus, and each slice was analyzed for $^{59}Fe$ by liquid scintillation counting.

## RESULTS

**The Free Radical Is Rapidly Regenerated *in Vivo*.** The B2-specific tyrosyl radical of *E. coli* can be measured directly by EPR spectroscopy of packed cells genetically manipulated to overproduce this protein (15, 16). In preliminary experiments, when such an overproducing strain containing the multicopy plasmid pPS2 (8) was treated with hydroxyurea, the EPR signal characteristic for the tyrosyl radical of B2 disappeared completely. The cells were washed with a salt solution containing chloramphenicol (170 μg/ml) to inhibit the synthesis of new B2 and then resuspended in the same salt solution at 37°C. This resulted in a rapid reappearance of the EPR signal (data not shown).

Similarly, inactive extracts from such cells, after precipitation with ammonium sulfate and passage through a column of Sephadex G-25 equilibrated with a buffer containing dithiothreitol, rapidly regenerated the EPR signal and enzyme activity (data not shown).

***In Vitro* Regeneration of the Radical in Protein B2/HU.** The experiments described above may be explained by assuming that the bacteria contained an enzyme activity that rapidly restored the tyrosyl radical in the B2 subunit, which had been stripped of its radical function by hydroxyurea treatment. Therefore, we looked for such an enzyme activity in crude extracts from the overproducing strain of *E. coli* and wild-type *E. coli*. To this end, a preparation of highly purified protein B2 was treated with 0.1 M hydroxyurea and, after ammonium sulfate precipitation, dialyzed extensively against a glycerol-containing buffer. The preparation was now devoid of both enzyme activity and EPR signal, and protein B2 had been transformed to B2/HU (Fig. 1). Addition of the extract from either strain of *E. coli* to solutions containing B2/HU resulted in the rapid appearance of the characteristic EPR signal and regeneration of catalytic activity.

Fig. 2 compares EPR spectra of protein B2 before and after HU treatment with a spectrum obtained from B2/HU after incubation with 1.2 mg of protein from an extract of wild-type *E. coli*. The characteristic doublet of the tyrosyl radical of B2 disappeared entirely during hydroxyurea treatment and was completely regenerated after incubation of B2/HU. Fig. 2 *Inset* shows the regenerated spectrum, measured under conditions that maximized its amplitude even though much of its fine structure was lost. The arbitrary Δ value indicated in the curve was directly proportional to the concentration of the tyrosyl radical and was used for its quantification in the experiments described below.

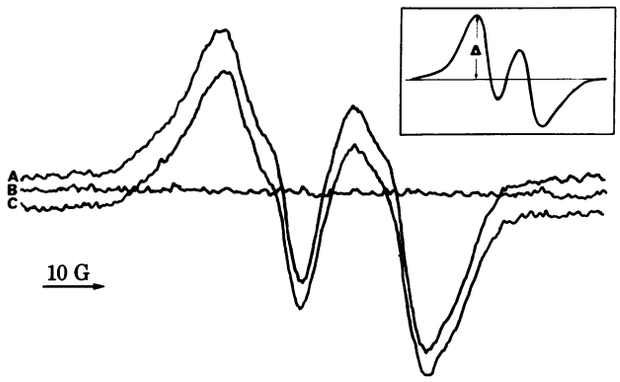


FIG. 2. EPR spectra at 77 K of protein B2 (spectrum A), hydroxyurea-inactivated B2 (spectrum B), and enzymatically reactivated B2 (spectrum C). The microwave power was 1.2 mW, and the modulation amplitude was 0.25 mT. (*Inset*) Spectrum of reactivated B2 at increased field-modulation intensity and microwave power.

The time course for the regeneration of the EPR signal was complicated and depended on the amount of bacterial extract added (Fig. 3). At low concentrations, a short lag period preceded a roughly linear increase in signal amplitude that reached a plateau, the value of which approximated the level of the original B2 signal (Fig. 3*A*). This maximum was reached earlier at higher protein concentrations. With the highest concentrations, the signal amplitude eventually decreased, suggesting that the extract also contained an activity that removed the radical—a point to which we shall return.

The time course of the reaction also depended on the concentration of B2/HU. At a fixed concentration of extract, both the final plateaus and the rates of the reaction increased with increasing amounts of B2/HU (Fig. 3*B*).

**Requirements of the Reaction.** After ammonium sulfate precipitation of the bacterial extract, the enzyme reaction was de-

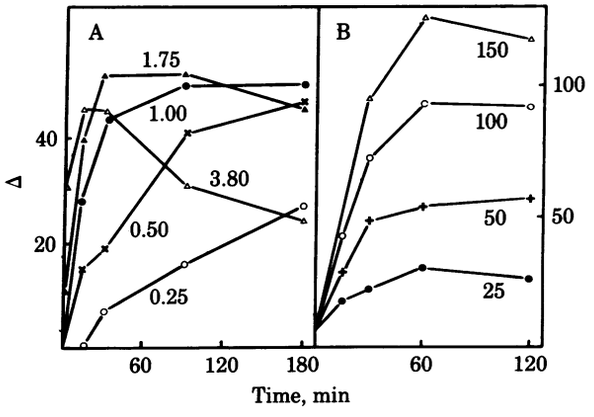


FIG. 3. (*A*) Dependence of radical appearance on enzyme concentration. B2/HU (32 μg) was incubated under standard conditions for various times with the amounts of bacterial extract (mg of protein) indicated. (*B*) Dependence on concentration of B2/HU. Different amounts of B2/HU (shown in μg) were incubated with 1.1 mg of bacterial protein under standard conditions. Δ, Half the signal amplitude.

Table 1. Requirements for radical regeneration

| | Activity,* % |
|---|---|
| Complete | 100 |
| No incubation | 11 |
| Incubation at 0°C | 11 |
| Enzyme preincubated 60 min at 40°C | 33 |
| Enzyme preincubated 20 min at 60°C | 14 |
| No $Mg^{2+}$ | 14 |
| No dithiothreitol | 29 |
| No oxygen | 30 |

* The data are compiled from several experiments with 100% Δ value ranging from 70 to 104.

pendent on the presence of $Mg^{2+}$ and dithiothreitol (Table 1). The reaction was also dependent on the presence of oxygen. Incubation in a Thunberg tube filled with argon instead of air decreased the Δ value to about one-third. Heating the extract for 60 min at 40°C decreased its activity to less than one-third and, for 20 min at 60°C, completely destroyed its activity. When incubation was carried out in an ice-bath, no reaction occurred.

The discovery of an oxygen requirement resulted from the unexpected finding that no radical was generated when the reaction was carried out in the narrow capillary tubes used for EPR spectroscopy instead of small centrifuge tubes. However, when air was slowly bubbled through the solution in the EPR tubes, the reaction proceeded smoothly (Fig. 4*A*). When air, after 60 min, was replaced by argon, the EPR signal disappeared but increased again on readmission of air. The bacterial extract under anaerobic conditions rapidly removed the radical from native protein B2 not treated with hydroxyurea (Fig. 4*B*). Taken together, these data suggest the presence in the bacterial extract of two activities with opposing effects: one activity, maximally active under anaerobic conditions, removed the radical function of B2; the second activity introduced the radical into the inactive protein in the presence of oxygen.

**Radical Formation Results in an Active Enzyme.** Treatment with hydroxyurea functionally destroys both radical and subunit activity. It remained to be shown that the presumed enzymatic reintroduction of the tyrosyl radical restores the catalytic activity of B2 as an active subunit of ribonucleotide reductase. For this purpose B2/HU was incubated with two concentrations of bacterial protein, and radical formation and the ability to reconstitute active ribonucleotide reductase with an excess of the complementary B1 subunit were determined at different time intervals. Fig. 5 shows a striking parallelism for the recovery of the two activities in the experiments involving B2/HU. At the end of the incubation, both radical content and enzyme activity approach the corresponding values observed for native B2, incubated in the same way. For each time point and each curve, the data give a constant ratio between enzyme activity and radical content. Finally, one notices a slow reactivation of B2/HU also in the absence of any added bacterial protein. However, it is not clear whether this represented a spontaneous reactivation or was due to contaminating proteins.

**ApoB2 Is Not Involved in Radical Generation.** Earlier work had demonstrated that the tyrosyl radical could be reintroduced nonenzymatically into B2/HU by first generating iron-free apoB2, followed by addition of iron(II) in the presence of ascorbate, a reducing agent, and oxygen. Two types of experiments were carried out to test whether the enzymic radical generation depended on the removal and reintroduction of iron, both involving the use of radioactive [$^{59}$Fe]B2: In the first instance, [$^{59}$Fe]B2 was prepared from highly purified apoB2 by chemical regeneration with $^{59}FeCl_3$ and ascorbate; in the second case, the B2-overproducing strain of *E. coli* was grown on LB medium, labeled with $^{59}$Fe, and a bacterial extract containing [$^{59}$Fe]B2 was prepared. Because protein B2 constitutes about 1% of the total soluble protein of these bacteria, a major part of the protein-bound $^{59}$Fe was [$^{59}$Fe]B2.

In both cases [$^{59}$Fe]B2/HU was prepared by treatment with hydroxyurea, ammonium sulfate precipitation, and dialysis. The tyrosyl radical was then regenerated enzymatically, the extent of which was ascertained from the amplitude of the EPR signal. The protein distribution of $^{59}$Fe was investigated by gel electrophoresis. In each set of experiments, three protein samples corresponding to the original [$^{59}$Fe]B2, [$^{59}$Fe]B2/HU, and reconstituted [$^{59}$Fe]B2 were run on nondenaturing cylindrical gels. The distribution of radioactivity on each gel is depicted in Fig. 6 *A–F*.

Fig. 6 *A–C* describes the results of the experiment with [$^{59}$Fe]B2 obtained from apoB2. All three samples showed a major peak of radioactivity at the position of protein B2. The starting material (Fig. 6*A*) contained only 55% of the total radioactivity in this peak, the remainder being present in a broad, rapidly moving peak close to the dye marker, which probably

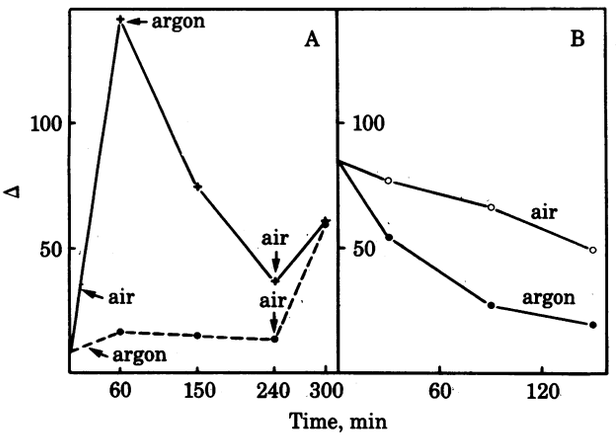


FIG. 4. Dependence of radical generation on oxygen. (*A*) B2/HU (108 μg) was incubated under standard conditions with 1.85 mg of bacterial protein in EPR tubes. Air or argon, as indicated in the figure, was bubbled slowly through the solutions. (*B*) Protein B2 (49 μg) was incubated with 2.5 mg of bacterial protein as described above. Δ, Half the signal amplitude.

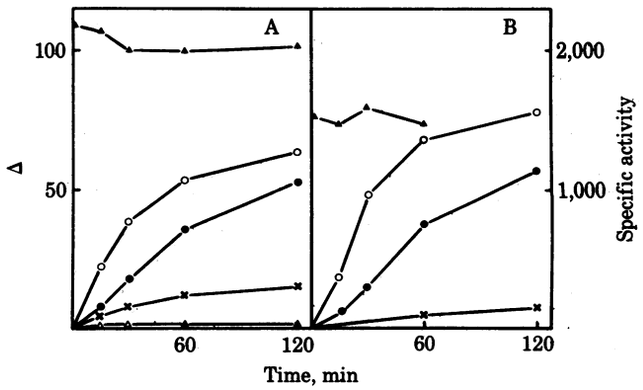


FIG. 5. Radical appearance in relation to enzyme activity. Incubations were carried out under standard conditions. At the indicated times, 1- and 2-μl portions were removed and analyzed for B2 activity (*B*), while the remainder was frozen and analyzed by EPR (*A*). ▲, Incubation of 76 μg of B2 with 0.63 mg of bacterial protein; ●, 55 μg of B2/HU with 0.63 mg of bacterial protein; ○, 55 μg of B2/HU with 1.26 mg of bacterial protein; x, 110 μg of B2/HU; and △, 0.63 mg of bacterial protein. Δ, Half the signal amplitude.

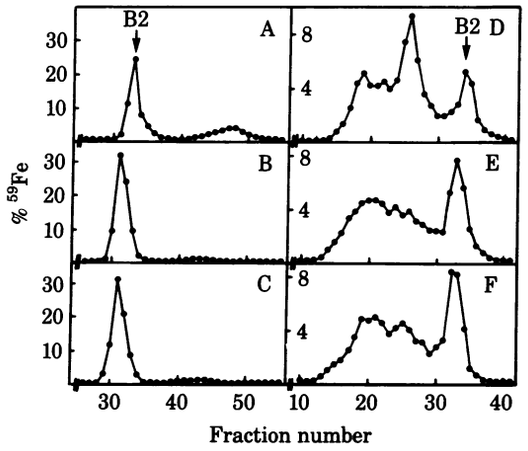


FIG. 6. Electrophoresis on nondenaturing gels of $^{59}$Fe-labeled protein B2, B2/HU, and reactivated B2. All panels show the distribution of $^{59}$Fe in the sliced gels, numbered from the top, with the arrow showing the position of protein B2. (*A–C*) An experiment with highly purified [$^{59}$Fe]B2. (*D–F*) An experiment in which [$^{59}$Fe]B2 was present in a crude extract from a B2-overproducing strain of *E. coli* grown on $^{59}$Fe-containing medium. (*A* and *D*) Results for the two original [$^{59}$Fe]B2 preparations. (*B* and *E*) Results after treatment with hydroxyurea. (*C* and *F*) Results after final reactivation.

is the unbound $^{59}$Fe left after the reactivation of apoB2. In the two other samples, namely B2/HU (Fig. 6*B*) and reconstituted B2 (Fig. 6*C*), 80% of the radioactivity migrated as B2. From the known specific activity of $^{59}$Fe, the recovery of radioactivity on the gel, and the EPR spectra of the materials subjected to electrophoresis, we could calculate that 1 mg of the initial [$^{59}$Fe]B2 preparation contained 11.6 nmol of iron and 3.3 nmol of radical, that 1 mg of [$^{59}$Fe]B2/HU contained 11.5 nmol of iron and less than 0.2 nmol of radical, and that, after reactivation, 1 mg contained 11.5 nmol of iron and 5.1 nmol of radical. These results demonstrate that no $^{59}$Fe was lost during the overall process and that enzymatic reactivation had increased the radical content of the initial [$^{59}$Fe]B2 preparation.

The same conclusion derives from the second experiment, depicted in Fig. 6 *D–F*. Fig. 6*D* shows the isotope pattern from the gel containing the crude extract from the $^{59}$Fe-labeled bacteria. Radioactivity was distributed through a large part of the gel, but a major peak was found at the position of B2. Also, after hydroxyurea treatment (6E) and after subsequent reactivation (6F), the isotope patterns showed clear peaks at the position of B2. When the EPR signals of the materials were related to the total radioactivities, Δ values of 7.1 and 10.6 per 1,000 cpm were obtained for the B2 peaks in the original extract and the reactivated material, respectively.

## DISCUSSION

Our results point to an enzymatic transformation of a tyrosine residue of the B2 subunit to a tyrosyl radical, essential for the catalytic activity of ribonucleotide reductase. In addition, some experiments are suggestive of the presence of a "deactivating" enzyme that destroys the tyrosyl radical. It seems possible that this unusual enzyme and its antagonist play an important role in the regulation of ribonucleotide reductase, either by influencing its overall activity or by directly participating in the reaction.

We presume that radical introduction occurs at one specific residue of the polypeptide chain, but direct evidence on this point is difficult to obtain. Strong support for a specific tyrosine residue being involved comes from the characteristic hyperfine structure of the EPR spectrum, which reflects a specific radical geometry within the tyrosine residue (4) and which, furthermore, is exactly reproduced in the reconstitution process.

The need for oxygen during reactivation of B2/HU can be explained by direct involvement in the reaction as the tyrosine in effect is oxidized. The additional requirement for dithiothreitol could indicate the participation of a reductive step in the overall process but might also have a more trivial explanation, such as enzyme protection.

Our last set of experiments (Fig. 6) investigated the behavior of the iron center during reactivation, and the results suggest that radical formation does not lead to removal of iron. This implies that although the iron may be involved in the overall process, the enzymatic reaction does not proceed through the formation of apoB2 and that its mechanism is, in principle, different from the chemical regeneration of active B2 described earlier (7).

What is the functional significance of the novel enzyme(s)? Recent evidence indicates the formation of a transient ribose radical intermediate during ribotide reduction; thus, the highly reactive tyrosyl radical can be postulated to accept an unpaired electron from the ribose moiety (2–4). The enzyme described here might then be involved in the regeneration of the radical and be required for an optimally functioning reductase system.

A second hypothesis takes account of our finding of two opposing activities, one requiring oxygen and leading to the formation of an active radical containing reductase and the other inactivating the enzyme and occurring optimally in the absence of oxygen. One can visualize that the balance of opposing activities regulates reductase function in step with changing requirements for DNA synthesis depending on the availability of oxygen.

However, a better understanding of the mechanism of radical formation and the physiological function of this process must await purification and characterization of the enzymes involved.

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