The Function of Superoxide Dismutase during the Enzymatic Formation of the Free Radical of Ribonucleotide Reductase*

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An enzyme system from Escherichia coli activates an inactivation of ribonucleotide reductase by transforming a tyrosine residue of the enzyme into a cationic free radical. The process requires NAD(P)H, a flavin, dithiothreitol, and oxygen and at least three proteins. After purification to near homogeneity two of the proteins were identified as superoxide dismutase and NAD(P)H:flavin oxidoreductase (Fontecave, M., Ellason, R., and Reichard, P. (1987) J. Biol. Chem. 262, 12325-12331). The nature of the third protein, provisionally named Fraction b, is unknown. The flavin reductase is believed to reduce the ferric iron center of the ribonucleotide reductase as a prerequisite for radical generation. Here we demonstrate that the flavin reductase under aerobic conditions generates superoxide anions which inactivate ribonucleotide reductase. Superoxide dismutase protects the enzyme or a sensitive intermediate formed during the generation of the tyrosyl radical from the harmful effects of superoxide. Hydrogen peroxide, formed by superoxide dismutase, is also harmful. In this case, catalase present in Fraction b might protect the system. Fraction b has, however, an additional unknown function in the overall process of radical generation.

Ribonucleotide reductase from Escherichia coli consists of two nonidentical subunits, named proteins B1 and B2 (1). The enzymatically active form of protein B2 contains an iron center, consisting of two antiferromagnetically coupled Fe3+ ions, linked by a μ-oxo bridge, and a stable organic oxide anions which inactivate ribonucleotide reductase. Superoxide dismutase protects the enzyme or a sensitive intermediate formed during the generation of the tyrosyl radical from the harmful effects of superoxide. Hydrogen peroxide, formed by superoxide dismutase, is also harmful. In this case, catalase present in Fraction b might protect the system. Fraction b has, however, an additional unknown function in the overall process of radical generation.

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Incubation of B2/HU with an extract from E. coli under aerobic conditions regenerated the tyrosyl radical and enzymatically active B2 (4). During purification of the activity of the E. coli extract we separated three protein fractions which in concert, and in the presence of NADPH, FMN, dithiothreitol, and oxygen, transform B2/HU to active B2 (5). Two of the three proteins were purified to near homogeneity and identified as superoxide dismutase (5) and an NAD(P)H:flavin oxidoreductase (6). The third protein, Fraction b, has as yet not been purified, and its function remains unknown. The purification and characterization of the flavin reductase is given in the preceding paper (6). There we also propose the function of this enzyme to be to reduce the iron center of B2/HU. The tyrosyl radical is subsequently generated during reoxidation of the iron center by oxygen.

How does superoxide dismutase fit into such a reaction scheme? The enzyme is generally assumed to protect cells from harmful effects of superoxide radicals (7). This was previously considered a possibility for its function also during B2/HU activation, but a more specific and direct effect could not be excluded. Our present experiments address this question. We now demonstrate that large amounts of the superoxide radical are indeed produced by the flavin reductase and that they are harmful to protein B2. It appears likely that superoxide dismutase protects B2 or a sensitive intermediate formed during the activation of B2/HU. Superoxide dismutase generates hydrogen peroxide, also toxic for protein B2. Protection from it might be provided by the presence of catalase in Fraction b. This does, however, not fully explain the requirement for this fraction during activation of B2/HU.

EXPERIMENTAL PROCEDURES

Materials—The following enzymes were supplied by Sigma: catalase from bovine liver (16,000 units/mg), egg white lysozyme (40,000 units/mg), glucose oxidase from Aspergillus niger (250 units/mg), and glucose 6-phosphate from baker's yeast (340 units/mg). Superoxide dismutase from bovine erythrocytes (5,000 units/mg) was from Boehringer Mannheim. Horseradish peroxidase and lactoperoxidase were a gift from Dr. K. G. Paul (Department of Medical Chemistry, Umeå University, Umeå, Sweden). Thioredoxin from E. coli was a gift from Dr. Arne Holmgren (Department of Medical Chemistry, Karolinska Institute, Stockholm, Sweden). T4 lysozyme was a gift from L. Bértach (Department of Biochemistry, Stanford University). Proteins B1 and B2 and protein B2/HU were available in this laboratory.

Ferricytochrome c was from Sigma. Flavin reductase and Fraction b were prepared as described in the accompanying paper (6). All other chemicals were of the purest commercial grade available.

E. coli strain GG468, completely lacking superoxide dismutase activity (8), was a gift from Dr. D. Tounti (Institut Jacques Monod, Université Paris 7, 75005 Paris, France). The strain was grown anaerobically on LB medium. Extracts were prepared as described for E. coli C600 (7).

Assays—The rates of B2/HU activation and of NADPH:flavin oxidoreductase activity were measured as described previously (5, 6). One unit of B2/HU reactivation activity is defined as 1 nmol of dCDP formed under the conditions of the assay (5). One unit of flavin reductase activity is defined as 1 nmol of NADPH oxidized per min under the conditions of the assay (6). Specific activities are defined as units per mg of protein. Superoxide production was quantitated from the reduction of cytochrome c (9). Reaction mixtures contained in a final volume of 0.2 ml: 10 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 10 μM FMN, 1.5 mM NADPH, and 50 μM ferricytochrome c. This mixture was present both in a sample and a reference cuvette incu-
Superoxide Dismutase and Ribonucleotide Reductase

Fig. 1. Scheme for the preparation from an extract from E. coli C600 of the three fractions required for activation of B2/HU superoxide dismutase.

**RESULTS**

The requirement for superoxide dismutase during activation of B2/HU appeared during the first purification step by DEAE chromatography of an E. coli extract (5). A requirement for two other protein fractions, provisionally named b and c, was found in the subsequent step, gel exclusion chromatography on AcA-54. In the accompanying paper (6) Fraction c is identified as NAD(P)H:flavin oxidoreductase (briefly called flavin reductase), while the function of Fraction b remains unknown. Activation of B2/HU thus requires the concerted action of superoxide dismutase, the flavin reductase, and Fraction b. These interrelations are summarized in the scheme of Fig. 1.

**Genetic Evidence for the Involvement of Superoxide Dismutase in B2/HU Activation**—In the preceding paper it was demonstrated that the requirement for superoxide dismutase, originally found in experiments involving crude preparations of Fraction c (5), also was apparent when a close to homogeneity preparation of the flavin reductase was used to activate B2/HU. The recent construction of E. coli mutants completely lacking superoxide dismutase activity (8), now made possible a genetic approach to test for the requirement for superoxide dismutase during B2/HU activation. A crude extract from such a mutant strain displayed a weak but significant activity, but addition of superoxide dismutase led to a 3-fold increase (Table I). Also in the presence of NADPH and FMN, superoxide dismutase stimulated the activation of B2/HU approximately 3-fold. When an extract from wild type E. coli was assayed, addition of superoxide dismutase had no effect.

**Flavin Reductase Produces O2- and OH- Radicals**—Superoxide dismutase is generally considered to provide a defense mechanism against the potentially damaging effects of the superoxide radical O2- (7). Also here its effect might be related to such a function. Two kinds of experiments were performed to demonstrate that superoxide radicals indeed are formed by the action of the flavin reductase: spin trapping experiments with DMPO (12, 13) and quantitative determinations of O2- by the cytochrome c reduction assay.

In the first case the enzyme was incubated with NADPH, FMN, diethylenetriaminepentaacetic acid, and the spin trap DMPO at pH 7.5. As shown in Fig. 2 (scan a) these conditions generated an intense EPR signal, characteristic of the para-

**TABLE I**

| Bacterial strain | Specific activity |
|------------------|------------------|
|                  | No SOD | +SOD |
| GC4468 (= superoxide dismutase) |        |      |
| Experiment A     | 0.25   | 0.70 |
| Experiment B     | 0.30   | 0.85 |
| C600 (wild type) |        |      |
| Experiment A     | 1.4    | 1.3  |
| Experiment B     | 1.2    | 1.8  |

**Flavin Reductase Spectra**

The EPR spectra at 20 °C resulting from the activity of flavin reductase in the presence of the spin trap DMPO. The following conditions apply to all four scans. Flavin reductase (0.4 μg) was incubated for 10 min at room temperature in a final volume of 0.4 ml with 50 mM Hepes, pH 7.5, 0.1 mM DMPO, 1 mM diethylenetriaminepentaacetic acid, 0.5 mM NADPH, and 20 μM FMN. Further additions were: scan a, none; scan b, superoxide dismutase (20 μg/ml); scan c, superoxide dismutase (20 μg/ml) + ethanol (5%); scan d, catalase (50 μg/ml), the same spectrum being observed when catalase was replaced by Fraction b (20 μg/ml).
magnetic superoxide spin trapped adduct, DMPO-OH, indicating rapid formation of superoxide radicals by the flavin reductase. No other paramagnetic species were found. The three components of the system, enzyme, NADPH, and FMN, were found to be absolutely required for radical production, and no EPR signal was detected when either of them was omitted from the reaction mixture. Moreover, the intensity of the DMPO-OH signal was approximately proportional to the concentrations of NADPH and FMN (data not shown). When 20 μg/ml superoxide dismutase was added to the complete reaction mixture the DMPO-OH signal disappeared completely. Instead, a new intense EPR signal was detected (Fig. 2, scan b). This EPR signal is characteristic of the adduct of the hydroxyl radical, DMPO-OH, indicating significant generation of hydroxyl radicals in the reaction mixture. In order to verify that the DMPO-OH signal did not arise from degradation of DMPO-OH, ethanol was added to the reaction mixture. Under those conditions, production of DMPO-OH was completely inhibited, and a new EPR signal was detected due to the spin trapping of α-hydroxyethyl radicals by DMPO (Fig. 2, scan c). Addition of catalase to the reaction mixture containing superoxide dismutase, but not ethanol, led to the complete disappearance of any EPR signal characteristic of spin-trapped adducts (Fig. 2, scan d). Interestingly, Fraction b also efficiently inhibited the production of hydroxyl radicals by the flavin reductase system in the presence of superoxide dismutase since its addition in place of catalase also made the DMPO-OH radical signal disappear completely.

To quantify the production of superoxide radicals we used the cytochrome c reduction assay which is based on the ability of superoxide to reduce ferricytochrome. In Fig. 3 the appearance of an absorption at 550 nm in curve A indicates reduction of ferricytochrome c in the presence of flavin reductase (1 μg/ml), NADPH, and FMN. Addition of an excess of superoxide dismutase did not completely inhibit the reaction (Fig. 3, curve B), indicating that part of the reduction of cytochrome c was independent of superoxide. The superoxide-dependent reaction obtained from the difference between curves A and B, is shown as curve C in Fig. 3. From the slope of this curve we can calculate a rate of superoxide production of 3.2 nmol of O$_2^-$ (μg flavin reductase)$^{-1}$·min$^{-1}$ under the above conditions. Since the enzyme used in this experiment was material after the phenyl-Sepharose step with an estimated purity of 25%, the specific activity of pure flavin reductase can be calculated to be 13 nmol of O$_2^-$ (μg of protein)$^{-1}$·min$^{-1}$. No reduction of cytochrome c was observed when any component of the system was omitted.

**Protein B2 Is Inactivated by Oxygen Radicals Produced by the Flavin Reductase System**—Protein B2, and in particular its tyrosyl free radical, is a possible target for the harmful effects of oxygen radicals produced by flavin reductase during activation of B2/HU. When the protein was incubated for 2 h at 30 °C with flavin reductase, an NADPH-generating system, FMN, and dithiothreitol under conditions used for the activation of B2/HU but in the absence of superoxide dismutase and Fraction b, it lost one-third of its activity (Table II). Addition of superoxide dismutase alone did not provide any protection. However, complete protection was obtained by the combination of superoxide dismutase and catalase. Fraction b efficiently replaced catalase as a protective agent. Table II also demonstrates the harmful effects of ferrous iron in this context. In the presence of 2 mM Fe$^{2+}$ inactivation of B2 was accelerated greatly. Again, the combination of superoxide dismutase and catalase provided protection.

**The Tyrosyl Radical Is Destroyed by Flavin Reductase-dependent Oxygen Radicals**—We next investigated whether the inactivation of protein B2 by oxygen radicals generated by the flavin reductase was paralleled by a destruction of the tyrosyl radical of the protein. To this end protein B2 was incubated with NADPH, FMN, and flavin reductase at 30 °C, and the amount of free radical present in the protein was determined from the amplitude of its typical EPR signal after 15 min. Under the conditions shown in Table III the signal decreased with time, and after 15 min only 45% of the free radical remained. When present, neither superoxide dismutase nor catalase alone had an effect, while the combined two enzymes fully protected the tyrosyl radical. The destruction of the radical depended on the presence of the flavin reductase. Increasing concentrations of either FMN or the reductase accelerated the disappearance of the EPR signal (data not shown).

**Protein B2 Is Inactivated by Hydrogen Peroxide**—Since superoxide dismutase activity results in the production of hydrogen peroxide it seemed possible that also addition of H$_2$O$_2$ would destroy the tyrosyl radical of B2. This was found to be the case. Incubation of the protein with 5 mM H$_2$O$_2$ at

![Fig. 3. Quantitation of the amount of superoxide radicals formed by the flavin reductase. Conditions for measuring the reduction of ferricytochrome c are given under "Experimental Procedures." Curve A gives total ferricytochrome c reduction, curve B the reaction in the presence of an excess of superoxide dismutase. Curve C (difference between A and B) then gives the part of ferricytochrome c reduction that depended on superoxide radicals.](image-url)
TABLE III

Loss of the tyrosyl radical during incubation of protein B2 with flavin reductase

| Additions | Signal amplitude |
|-----------|------------------|
| None      | 100%             |
| Flavin reductase | 47               |
| + Superoxide dismutase | 31               |
| + Catalase | 35               |
| + Superoxide dismutase + catalase | 90               |

Protein B2 (120 μg, final concentration 10 μM) was incubated in a final volume of 0.16 ml at 30°C for 15 min in an EPR tube with 50 mM Tris-HCl, pH 7.5, 1.25 mM NADPH, and 50 μM FMN. Inactivation was started by addition of 2 μg of flavin reductase. Where indicated 30 μg/ml superoxide dismutase or catalase was added. After 15 min the sample was frozen and its EPR spectrum was recorded.

30°C for 2 h led to a 50% inactivation (Table IV). A very rapid inactivation of B2 was also observed when H2O2 was enzymatically produced by the system glucose + glucose oxidase. Upon incubation with Fenton’s reagent, hydrogen peroxide in the presence of ferrous ions, known to produce hydroxyl radicals, only 10% of the activity of B2 was recovered. Also inactivation by hydrogen peroxide was paralleled by destruction of the tyrosyl radical since the protein lost its typical EPR signal during incubation. As expected, catalase alone completely protected B2 from the harmful effects of hydrogen peroxide. Again, Fraction b gave the same protective effect.

Catalase, Peroxidases, or OH Scavengers Do Not Substitute for Fraction b during Activation of B2/HU—Since Fraction b protected protein B2 as effectively as catalase against oxygen radicals or H2O2, it seemed possible that the complementing activity of this fraction during the enzymatic generation of the tyrosyl radical of B2 might be related to such a protective function. By direct analysis we found that Fraction b contained catalase activity and could calculate that as much as 1–2% of the total protein mass was accounted for by this enzyme. We, therefore, investigated whether enzymes such as catalase or peroxidases that scavenge H2O2 or OH scavengers could substitute for Fraction b during B2/HU activation. The results in Table V clearly show that this was not the case. Thus, the presence of catalase in Fraction b may well contribute to its activity in the overall reaction but cannot be the complete explanation for its complementing activity.

TABLE IV

Inactivation of protein B2 by hydrogen peroxide

| Additions          | Activity | Signal amplitude |
|--------------------|----------|------------------|
| None               | 100%     |                  |
| Hydrogen peroxide  | 45       | 31               |
| + Catalase         | 92       |                  |
| + Fe²⁺             | 8        |                  |
| Glucose + oxidase  | 9        |                  |
| + Catalase         | 96       |                  |
| + Fraction b       | 96       |                  |

Protein B2 (3.6 μg) was incubated for 2 h at 30 min in a final volume of 12 μl of 70 mM Hepes, pH 7.5. Inactivation was started by addition of 5 mM H2O2 or by 0.1 mM glucose + 0.1 mg/ml glucose oxidase. Where indicated, 30 μg/ml superoxide dismutase or catalase, 5 μg of fraction b, or 2 mM ferrous ammonium sulfate was added. The remaining B2 activity was determined by CDP reduction. 100% activity corresponds to 25 units. For EPR experiments, 120 μg of B2 was incubated at 30°C for 15 min in 0.16 ml of 50 mM Tris-HCl, pH 7.5, with or without 30 mM H2O2. After 15 min the samples were frozen and their EPR spectrum was recorded.

TABLE V

Catalase, peroxidases, and hydroxyl radical scavengers do not substitute for Fraction b

| Additions          | Units    |
|--------------------|----------|
| None               | 4.0      |
| Mannitol (0.17 M)  | 4.0      |
| Thiourea (0.13 M)  | 5.1      |
| Catalase (3 g)     | 7.9      |
| + Mannitol (0.17 M)| 5.7      |
| + Thiourea (0.13 M)| 9.2      |
| Fraction b (5 μg)  | 34.5     |

Experiments 1: None catalyst, 1 μg fraction b, 5 μM Fe²⁺, 0.01 M glucose, 0.02 M glutathione, 50 mM Tris-HCl, pH 7.5, 50 μM FMN, 15 Units.

Experiment 2: None catalyst, 1 μg fraction b, 5 μM Fe²⁺, 0.01 M glucose, 0.02 M glutathione, 50 mM Tris-HCl, pH 7.5, 50 μM FMN, 15 Units.

Discussion

The active form of E. coli ribonucleotide reductase contains an organic free radical, identified as an oxidation product of tyrosine 122 of protein B2, one of the two nonidentical subunits of the enzyme (3). Treatment with hydroxyurea generates B2/HU, an inactive form of the subunit, in which the tyrosine radical has reverted to a normal tyrosyl residue (4). The radical is regenerated from B2/HU by an enzyme system present in E. coli consisting of superoxide dismutase (5), a flavin reductase (6), and a third Fraction b of unidentified function (cf. Fig. 1). In the accompanying paper (6) we discuss the function of the reductase; here we describe experiments aimed at an understanding of the requirement for superoxide dismutase.

This requirement first became apparent after fractionation of the E. coli extract (5). We now demonstrate that an extract from an E. coli mutant lacking superoxide dismutase (8) has a greatly diminished capacity to regenerate the tyrosyl radical and that addition of superoxide dismutase to the extract increases its activity approximately 3-fold. This result supports the importance of superoxide dismutase for the activation of ribonucleotide reductase under physiological conditions.

Previously, two main alternatives were presented for the role of superoxide dismutase: (i) superoxide dismutase destroys harmful superoxide radicals (7), formed during B2/HU activation; and (ii) superoxide dismutase fulfills a more specific but unknown function. The first alternative provided a simple explanation, but at that time no evidence was available that superoxide radicals were formed during the reaction. The subsequent identification of a flavin reductase as a component of the system by itself supported this alternative, since reduced flavins are known to produce superoxide during transfer of electrons to dioxygen (14, 15). We now demonstrate that large amounts of superoxide radicals are formed during aerobic incubation of the flavin reductase with NADPH and FMN (Figs. 2 and 3). In spin trapping experiments with DMPO the only detectable paramagnetic species was DMPO-OOH, produced upon trapping of superoxide. It is likely that the radical is formed by aerobic oxidation of reduced FMN (Fig. 4) since no reduction of oxygen into superoxide by the flavin reductase could be observed in the absence of FMN. As expected, superoxide radicals were efficiently scavenged by superoxide dismutase and no DMPO-OOH was detected when the spin trapping experiments were performed in the presence of the enzyme. In that case a different EPR signal, characteristic of
To B2. We could demonstrate that protein B2 itself indeed is present of ethanol an EPR signal characteristic of the spin action of the flavin reductase and superoxide dismutase (Fig. trap adduct of α-hydroxyethyl radicals was found (Fig. 2).

**FIG. 4.** Scheme for the generation of DMPO adducts resulting from the formation of superoxide and hydroxy radicals in the flavin reductase system. SOD, superoxide dismutase.

DMPO-OH, was detected indicating production of hydroxyl radicals. Its identity was confirmed by the finding that in the presence of ethanol an EPR signal characteristic of the spin trap adduct of α-hydroxyethyl radicals was found (Fig. 2). Since catalase completely inhibited the formation of this signal, the hydroxyl radicals probably arose from a one-electron reduction of hydrogen peroxide, in line with the expectation that H₂O₂ should be formed from the combined action of the flavin reductase and superoxide dismutase (Fig. 4). The source of electrons required for the reduction of H₂O₂ is not identified in Fig. 4. Usually transition metals catalyze this reaction, but this seems unlikely in our case since we employed diethylenetriaminepentaacetic acid, a strong iron chelator, during the reaction. Instead, reduced flavins present in the system could provide the required electrons. Clearly large amounts of oxygen radicals are produced by the flavin reductase supporting the idea of superoxide dismutase functioning as a protective agent.

What then can the target be for these radicals? One possibility might be the tyrosyl-free radical itself of protein B2 or a sensitive intermediate formed from B2/HU on the pathway to B2. We could demonstrate that protein B2 itself indeed is sensitive to superoxide radicals produced by flavin reductase as well as to hydrogen peroxide and hydroxyl radicals. This sensitivity correlated with the destruction of the tyrosyl radical, implying that the radical may be the primary sensitive target. However, we cannot exclude that this is a secondary effect caused by changes in the protein structure due to inactivation of some other sensitive groups by oxygen radicals. Superoxide dismutase alone was unable to provide protection of B2 from radicals produced by flavin reductase. However, this was not surprising since, as shown by the spin trapping experiments, superoxide dismutase activity gives rise to other deleterious species. For this reason, only the combination of superoxide dismutase and catalase (or Fraction b containing catalase) provided full protection of B2.

The fact that protein B2 is sensitive to oxygen radicals produced by the flavin reductase need not imply that it is the only or even main target. Earlier evidence suggested that B2-related intermediate(s) formed during the activation of B2/HU are more sensitive than B2 itself to inactivation by superoxide radicals (5). The experiments with protein B2 carried out now then might be looked upon as a model for the toxic effects of oxygen radicals.

Our present understanding of the involvement of superoxide dismutate in tyrosyl radical generation is shown in Fig. 5. The flavin reductase occupies a central position in the reaction. In addition to its positive role to generate reduced flavins active during radical introduction, a negative role derives from the production of harmful superoxide radicals during auto-oxidation of reduced flavins. Protein B2 and/or an intermediate between B2 and B2/HU must be protected. Superoxide dismutase destroys superoxide radicals but produces H₂O₂ (and OH⁻), and a second protective mechanism is required. This might be provided by catalase, present in Fraction b. However, this cannot be the only function of Fraction b since catalase is not able to substitute for Fraction b. Catalase activity thus appears to be only one function required from Fraction b, the other(s) remaining unknown. Their elucidation awaits the results of further protein purification. With respect to superoxide dismutate, the evidence now strongly supports a protective function from superoxide radicals.

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