The irreversible inactivation of ribonucleotide reductase from *Escherichia coli* by superoxide radicals

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Abstract The expression of superoxide dismutase in all aerobic living organisms supports the concept that superoxide radicals are toxic species. However, because of the limited chemical reactivity of superoxide, the mechanisms of this toxicity are still uncertain. Protein R2, the small component of ribonucleotide reductase, a key enzyme for DNA synthesis, is shown here to be irreversibly inactivated during incubation with an enzymatic generator of superoxide radicals, at neutral pH. During inactivation the essential tyrosyl radical of protein R2 is irreversibly destroyed. Full protection is afforded by superoxide dismutase. It is proposed that coupling between superoxide radicals and the radical protein R2 generates oxidized forms of tyrosine, tyrosine peroxide and 3,4-dihydroxyphenylalanine.

Key words: Superoxide radical; Flavin reductase; Ribonucleotide reductase; Tyrosyl radical; *Escherichia coli*

1. Introduction

The superoxide radical $O_2^{\bullet-}$ is believed to be a toxic oxygen-derived species since all aerobic cells have evolved a specific enzyme, superoxide dismutase (SOD), for protecting themselves from $O_2^{\bullet-}$ [1,2]. Because of the limited chemical reactivity of $O_2^{\bullet-}$ its importance in oxygen toxicity has been questioned. The currently persisting dominant view of $O_2^{\bullet-}$ toxicity is that it damages cells only indirectly, by giving rise to the hydroxyl radical OH• [3]. However, the seminal work of Fridovich has demonstrated that $O_2^{\bullet-}$ itself can exert direct deleterious effects in biological systems [1]. A number of enzymatic targets of $O_2^{\bullet-}$ have been identified, such as α,β-dihydroxy acid dehydratase [4], 6-phosphogluconate dehydratase [5], aconitase [6,7] and fumarase A, B [7]. Here we report the first evidence that $O_2^{\bullet-}$ can also inactivate ribonucleotide reductase, the enzyme which, in all living organisms, provides the deoxyribonucleotides required for DNA synthesis [8]. Protein R2, the small component of the enzyme, containing an iron center and an essential tyrosyl radical located at position 122 [9], is irreversibly converted into an inactive form lacking the radical, during reaction with $O_2^{\bullet-}$. A mechanism for the inactivation reaction is proposed.

2. Materials and methods

2.1. Materials

Protein R2 [10], NAD(P)H:flavin oxidoreductase [11], and protein R1 [12] were purified from overproducing strains of *E. coli* according to previously described procedures. MetR2 was prepared from R2 by treatment with hydroxyurea [13]. ApoR2 was prepared from R2 by treatment with 8-hydroxyquinoline sulfonate [13].

2.2. Enzymatic assays

Ribonucleotide reductase activity was measured as described in [14]. Flavin reductase activity was determined as described in [11].

2.3. Inactivation of protein R2 by $O_2^{\bullet-}$

The reaction mixture contained 1.25 mM NADPH, 50 μM FMN, 50 mM glucose 6-phosphate, 40 μg/ml glucose-6-phosphate dehydrogenase, 15 μg/ml catalase, variable amounts of flavin reductase and 1 mg/ml (11 μM) protein R2, in 200 μl of 60 mM HEPES buffer, pH 7.4. The reaction was initiated by the addition of NADPH. The reaction mixture was slowly shaken under a stream of oxygen at room temperature. After incubation, the solution was transferred to an EPR tube and frozen in liquid N_2. The EPR spectrum of the solution was recorded on a Varian 102 (band X) EPR spectrometer at 77 K. Spin concentrations in the protein samples were determined by calibrating double integrations of the EPR signal centred at $g = 2.00$, characteristic of the tyrosyl radical, with a Cu-EDTA standard sample at the same temperature. Recording conditions were: microwave power, 10 mW, modulation amplitude, 3.2 G.

2.4. Activation of metR2

Chemical activation of metR2 requires incubation with 8 mM dithiothreitol (DTT) at alkaline pH in the presence of 8 mM Mg^{2+} in 0.1 M Tris-HCl, pH 9, at 37°C [15]. Enzymatic activation is achieved during incubation of metR2 for 1 h with *E. coli* soluble extracts in the presence of 1 mM NADPH, 10 μM FMN, 8 mM DTT and 10 mM Mg^{2+} at pH 7.4 [16].

2.5. Assay for catechol

After 40 min incubation of 1 mg/ml protein R2 with the enzymatic generator of superoxide radicals described above, sodium dodecyl sulfate-polyacrylamide gel (12%, 1 mm thick) electrophoresis was run according to Laemmli [17]. The protein was blotted onto a nitrocellulose membrane (0.45 μm Bio-Rad) at 100 V for 1 h using a blotting buffer containing 25 mM Tris-HCl, pH 8.3, 102 mM glycine, 20% methanol. Detection of protein catechol were performed according to Paz et al. [18] by immersing the filter in 0.24 mM nitroblue tetrazolium and 2 M potassium glycinate, pH 10, in the dark for 45 min at room temperature.

2.6. Crystallographic data

The inactivated protein R2 was crystallized in the same space group ($P2_12_12_1$) and under the same conditions as native R2 [19]. Data were collected at room temperature on a RAXIS-II image plate from a rotating anode X-ray source. The protein crystal was translated along the spindle axis after 48° of oscillation when the diffraction became weak, allowing one complete data set to be scaled together from one crystal. Data were indexed, processed and scaled with Denzo and ScalePack [20]. A few rounds of positional and B-factor refinement was performed with XPLOR [21] before a difference map was calculated between the inactive protein R2. No positive or negative density was found around Tyr-122.

2.7. Mass spectrometry

Mass spectra were obtained on a Perkin-Elmer Sciex API III+ triple quadrupole mass spectrometer equipped with a nebulizer-assisted elec-

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trospray source (ionspray) operating at atmospheric pressure. A 5 kV voltage was applied to the electrospray needle. The mass spectrometer was scanned from *m/z* 1200 to 2400, with steps of 0.5 *m/z* unit and at a declustering potential (orifice voltage) from 90 to 120 V. The dwell time was 1.8 ms and the resolution was one mass unit. For each mass spectrum, roughly 5 scans were averaged. Each molecular species produced a series of multiply charged protonated molecular ions. The reconstructed molecular mass profiles were determined by using a deconvolution algorithm (PE/Sciex). In flow injection analyses, samples (about 100 pmol) were introduced by means of a Harvard 22 syringe pump at a flow rate of 5 μl/min on a Valco C6W injector equipped with a 1 μl internal loop. To promote protonation of the proteins the solvent contained 25% methanol and 1% acetic acid in water.

3. Results

3.1. Destruction of the tyrosyl radical of protein R2 by superoxide radicals

Protein R2 (1 mg/ml) was incubated at room temperature in HEPES buffer pH 7.4 in the presence of a superoxide-generating NAD(P)H:flavin oxidoreductase system [22,23]. The flavin reductase system consists of 1.25 mM NADPH, 50 μM FMN and varying amounts of the NAD(P)H: flavin oxidoreductase from *E. coli*. The enzyme catalyzes the reduction of FMN by NADPH. In aerated solutions, reduced FMN transfers electrons to O_2, giving rise to a continuous flux of superoxide radicals [23]. Considering the specific activity of the enzyme, the rate of $O_2^{\bullet-}$ generation was estimated at 0.6 μM s^{-1} while the steady-state concentration was about 3 μM, if one takes into account the non-catalyzed dismutation of $O_2^{\bullet-}$ [24]. As shown in Fig. 1, the EPR signal centered at $g = 2.00$, characteristic of the tyrosyl radical of protein R2, slowly decayed during incubation, indicating that the radical was destroyed. After about 30 min, no radical could be detected. At that point, protein R2 was no longer active during CDP reduction assayed under standard procedures [14]. The reaction was dependent on the flavin reductase system since no destruction of the radical could be observed in the absence of NADPH and since increasing the concentration of the flavin reductase resulted in increased rates of radical destruction (Fig. 1). The complete inhibition of the reaction by SOD (Fig. 1) demonstrated that the superoxide radicals generated by the flavin reductase system were responsible for the inactivation of protein R2. The protective effects of SOD were not a protein effect since the addition of bovine serum albumin (BSA) or boiled SOD did not provide any protection of the tyrosyl radical. Addition of 15 μg/ml catalase had no effect on the rate and extent of radical destruction, indicating that H_2O_2 was not involved in the reaction.

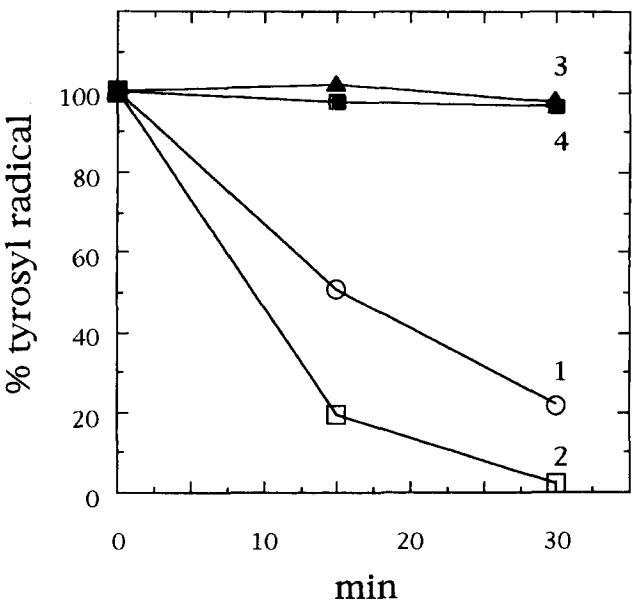


Fig. 1. Superoxide-dependent scavenging of the tyrosyl radical of protein R2, the small component of ribonucleotide reductase of *E. coli*. The reaction mixture contained 1.25 mM NADPH, 50 μM FMN, 50 mM glucose 6-phosphate, 40 μg/ml glucose-6-phosphate dehydrogenase, 1.8 μg/ml (○, 1) or 4 μg/ml (□, 2) flavin reductase and 1 mg/ml (11 μM) protein R2, in 200 μl of 60 mM Hepes buffer, pH 7.4. In some experiments with 1.8 μg/ml flavin reductase, NADPH was omitted (▲, 3), or 0.15 mg/ml superoxide dismutase (■, 4) was added. The amount of radical was determined by EPR spectroscopy at 77 K. Recording conditions were: microwave power, 10 mW; modulation amplitude, 3.2 G.

3.2. Inactivation of protein R2 is irreversible

MetR2 is an inactive protein that differs from R2 only in having a normal Tyr-122 residue. The tyrosyl radical can be regenerated and thus metR2 fully reactivated either chemically [15], or enzymatically [16]. Both the chemical and enzymatic systems failed to regenerate the tyrosyl radical from the $O_2^{\bullet-}$-inactivated protein R2, thus excluding metR2 as being the product of the inactivation reaction. Furthermore, the tyrosyl radical could be fully restored from $O_2^{\bullet-}$-treated metR2 by the chemical and enzymatic systems described above. In addition, apoR2, the inactive iron- and radical-free form, was totally transformed into active R2 during the conventional reaction with Fe^{2+} and ascorbate in the presence of oxygen [13] even after treatment with $O_2^{\bullet-}$. The radical-free forms, metR2 and apoR2, thus do not lose their ability to generate the radical upon treatment with fluxes of $O_2^{\bullet-}$. This clearly showed that protein R2 was irreversibly altered by $O_2^{\bullet-}$ only because it carries a tyrosyl radical.

3.3. Protein R2 is oxidized by superoxide radicals

Identification of the protein reaction product(s) is not trivial. However, we reasoned that the reaction of the superoxide radical or its protonated form with the tyrosyl radical could generate a tyrosine peroxide and its reduction product, 3,4-dihydroxyphenylalanine, dopa, as depicted in Fig. 2. Recent studies have shown that the superoxide radical reacts with protein-free tyrosyl radicals by addition rather than by electron transfer, thus generating tyrosine peroxide and derivatives as major products [25,26]. Our working hypothesis can be tested by assaying for the presence of a catechol group in $O_2^{\bullet-}$-inactivated protein R2. This is usually made with a colorimetric assay in which the protein is first denatured, separated from low molecular weight molecules on a SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and stained after reaction with nitroblue tetrazolium, according to Paz et al. [18]. As shown in Fig. 2, $O_2^{\bullet-}$-inactivated protein R2 stained positively for catechol (or its quinone oxidation product), while the wild type R2 control was completely negative. Neither $O_2^{\bullet-}$-treated metR2 nor apoR2 stained positively for catechol. This strongly suggested that the reaction of $O_2^{\bullet-}$ with protein R2 led to a catechol-containing protein.

The inactivated protein was separated from the various components of the reaction mixture and analyzed by electro-

spray mass spectrometry. This showed that the molecular band ($M = 43\,387$ Da) decayed as a function of incubation time and that two new bands, at larger masses $M = 43\,402$ Da and $M = 43\,417$ Da, increased (data not shown). Considering the 4–5 Da uncertainty for such an heavy protein, the new masses fitted quite well with binding of one and two oxygen atoms, respectively.

We first assumed that oxidation was on Tyr-122. Well-diffracting crystals of $O_2^{\bullet-}$-inactivated protein R2 were obtained and could be studied by X-ray crystallography. The resolution range was 20.0–2.2 Å and the R factor 0.228. Difference electron density maps, however, unambiguously established that Tyr-122 was not modified and could thus not be the oxidation site (data not shown).

4 Discussion

Direct deleterious but generally reversible effects of $O_2^{\bullet-}$ have been demonstrated in a limited number of situations [4–7]. In most cases, $O_2^{\bullet-}$ plays a role as an oxidant, for example converting an iron-sulfur center into an oxidized inactive form. Now we demonstrate that, in vitro, ribonucleotide reductase is irreversibly inactivated by $O_2^{\bullet-}$. Ribonucleotide reductase is a key enzyme for DNA synthesis and inactivation of such an enzyme is lethal for any living organism. Our present observation thus provides a new insight into the mechanisms for toxicity of superoxide radicals, identifying a possible link between oxidative stress and DNA synthesis. Of course, whether it is physiologically relevant and whether aerobic organisms have evolved SOD in part to protect ribonucleotide reductase and DNA synthesis need to be investigated in biological and genetic studies. It should be noted that superoxide dismutase was rediscovered, in *E. coli*, as an essential component of a multienzyme system which participates in the introduction of the tyrosyl radical into protein R2 [22]. Furthermore, *E. coli sod* mutants lacking both Fe- and Mn-superoxide dismutase were much less efficient than the wild type strain during activation of ribonucleotide reductase [22].

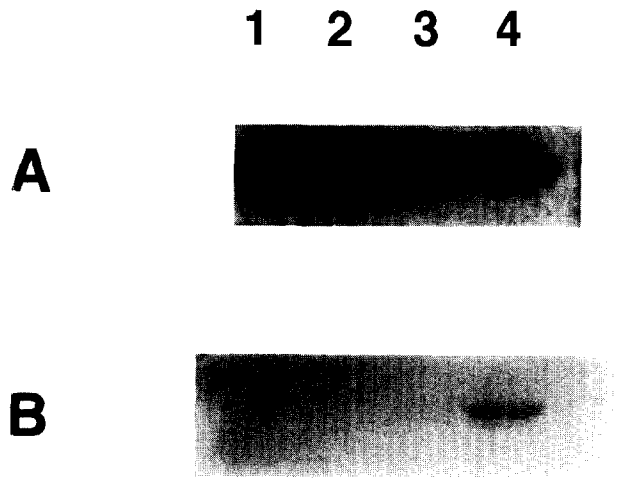
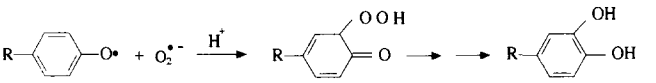


Fig. 2. Catechol staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis electroblot with protein R2 and superoxide-treated R2, metR2 and apoR2. (A) Coomassie stained sodium dodecyl sulfate-polyacrylamide gel with 2 μg loading of the following proteins; lanes: 1, wild type R2; 2, superoxide-treated metR2; 3, superoxide-treated apoR2; 4, superoxide-treated R2. (B) Catechol stained nitrocellulose filter with 5 μg protein; lanes 1–4, as in A.



Scheme 1. Postulated mechanism for the inactivation of protein R2. Tyrosine peroxide results from the coupling of a tyrosyl radical with the superoxide radical. It is then reduced into 3,4-dihydroxyphenylalanine.

The mechanism of the inactivation is still unclear even though we have clearly established that inactivation occurs because of the presence of an organic radical in the polypeptide chain. The reaction could be described as a radical-radical coupling between $O_2^{\bullet-}$ and protein R2. As a matter of fact, the organic radical of protein R2 was scavenged during the reaction, as shown by EPR spectroscopy, and one or two oxygen atoms were fixed to the polypeptide chain, as shown by mass spectrometry. It was thus tempting to suggest that the coupling reaction and the oxidation of the protein took place at Tyr-122, where the tyrosyl radical would be converted to tyrosine peroxide and dopa (Scheme 1). However, Tyr-122 can be ruled out on the basis of X-ray diffraction studies. It should be noted that the structure determination may not allow detection of modifications if these modifications occur at several side chains.

Thus, the site(s) of oxidation remain(s) to be identified. The tyrosyl radical is deeply buried within the polypeptide chain. Moreover, negatively charged species cannot gain access to the radical site. This may explain why Tyr-122 is not significantly modified. However, protein R2 also contains a few surface residues, such as the conserved Trp-48 and Tyr-356, which play key roles during long range electron transfer to the tyrosyl radical [27-29]. Since these residues are electronically connected to the tyrosyl radical, they may display some radical character. One should recall that the radical density has been suggested to be delocalized much further away to Cys-439 of protein R1, the large component of ribonucleotide reductase, within an active R1-R2 holoenzyme [30]. These surface residues in protein R2 are thus good candidates for a coupling reaction with superoxide radicals. The mechanism in Fig. 2 would then be valid for Tyr-356, for example. A modification at Tyr-356 would, moreover, be consistent with the observation that the radical cannot be reintroduced at Tyr-122. As a matter of fact, generation of the tyrosyl radical in a mutant Y356A metR2 form proved to be extremely difficult [29]. Unfortunately, Tyr-356 is not visible by X-ray crystallography as it belongs to the highly flexible C-terminal tail [31]. Further studies are needed to clarify this point, in particular with the help of site-directed mutants.

It should be noted that, in chemical systems, there are exceedingly few examples to date of coupling between $O_2^{\bullet-}$ and an organic free radical [32]; in biological systems, the only known example is the reaction between flavin radicals and $O_2^{\bullet-}$ in flavin-dependent monooxygenases [33]. It is tempting to generalize the concept that proteins carrying free radicals, such as tyrosine [34], tryptophan [35,36] or glycine [37,38] radicals, might be critical targets of $O_2^{\bullet-}$. This sheds new light on biological mechanisms of $O_2^{\bullet-}$ toxicity.

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