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Reversible Red-Ox Reactions of the Diiron Site in the Mouse Ribonucleotide Reductase R2 Protein

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The red-ox reactions of the dinuclear iron center of mouse R2 protein upon interaction with different reductants (dithionite alone and with mediators) and oxidants (PES, methylene blue, hydrogen peroxide and para-benzoquinone) have been studied by EPR and optical spectroscopy. The obtained results indicate that the transitions between Fe(III)Fe(III), Fe(II)Fe(III) and Fe(II)Fe(II) states of the dinuclear iron center are reversible and the μ-oxo-bridge may be formed upon oxidation by non-oxygen oxidants. In contrast to the case for the *E. coli* R2 protein, dithionite alone reduces the tyrosyl radical and diiron center in mouse R2 protein.

Ribonucleotide reductase catalyses the reduction of ribonucleotides to the corresponding deoxyribonucleotides. Mammalian and *E. coli* ribonucleotide reductases belong to the class I ribonucleotide reductases (1). In this class the enzyme has two components, proteins R1 and R2, both of which are homodimers. The larger component, protein R1, provides substrate binding sites for the ribonucleotide diphosphates and contains the red-ox active dithiol groups for the reduction of the ribose moiety. The small subunit, protein R2, contains in each of its two polypeptide chains an antiferromagnetically coupled diferric center and a site for the tyrosyl free radical. Both the iron center and the radical are needed for enzymatic activity (2,3). The function of the dinuclear iron center appears to be generation and likely stabilization of the catalytically essential tyrosyl radical. Binuclear iron clusters in R2 proteins can exist in one of the three red-ox states, the fully oxidized (Fe(III)Fe(III)), the mixed-valent (Fe(II)Fe(III)) or the fully reduced (Fe(II)Fe(II)) state. The mixed-valent form of mouse R2 protein is stable under aerobic conditions and exhibits a rhombic $S = 1/2$ EPR signal with $g = 1.93$, 1.73, and 1.60 (4). In contrast, the mixed-valent form of the *E. coli* R2 protein is unstable at room temperature. The mixed-valent state in *E. coli* R2 can be produced by radiolytic reduction at 77K (5) or by chemical reduction with hydrazine (6).

The three-dimensional structures for Fe(III)Fe(III) and Fe(II)Fe(II) forms of *E. coli* R2 protein have been reported (3,7). In the oxidized form the ferric ions are linked by one μ-oxo- and one μ-carboxylato bridge (3,7). The diiron center in the fully reduced R2 protein has two carboxylate bridges but no oxo-bridge (7), as was also found for the Mn^{2+}-containing R2 which should mimic the fully reduced iron form (8). The EPR properties of the Fe(II)Fe(III) center in R2 proteins have been shown to be close to those of other hydroxo-bridged Fe(II)Fe(III) species (9). The results of various spectroscopic and genetic studies (10) indicate that the diiron center in mouse R2 protein is structurally close to that of the *E. coli* protein.

The oxo-bridge in the diferric center of R2 proteins is formed in a reaction where molecular oxygen oxidizes the Fe(II)Fe(II) cluster. In *E. coli* R2 the oxo-bridge originates from the molecular oxygen (11) but then exchanges relatively rapidly with water (12). The aim of this work was to study the reversibility of the red-ox transitions in the binuclear iron cluster in the mouse R2 protein where all three red-ox states are known to be stable. A new question is whether the μ-oxo-bridge might form upon oxidation of the reduced form of R2 protein by other non-oxygen chemical oxidants.

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MATERIALS AND METHODS

Recombinant mouse protein R2 was prepared according to (13) and reconstituted by addition of 6 Fe(II) per R2 and oxygen as described in (14). The protein was studied in 50mM TRIS-HCl, 100 mM KCl, pH 7.6, containing 5–10% of glycerol. Protein R2 concentration is given as homodimer molarity (ϵ_{280} = 124000 M^{-1} cm^{-1}, (13)).

The chemicals used are as follows: methyl viologen (MV) (Sigma), sodium hydrosulfite (dithionite) (Merck), N-ethyldibenzopyrazine ethyl sulfate salt (PES) (Sigma), hydrogen peroxide (Merck), 4-butoxyphenol (Aldrich), methylene blue (Riedel de Haën), para-benzoquinone (Hopkin & Williams), glycerol (Merck), ammonium ferrous sulfate (Mohr's salt) (Merck), 4,7-diphenyl-1, 10-phenanthrolinedisulfonic acid (bathophenanthroline) (Sigma).

EPR spectra were recorded on a Bruker ESP300 spectrometer coupled with an Oxford ESP900 helium flow cryostat and a Bruker Model ER 4116 DM dual mode cavity to allow both perpendicular ($B_1 \perp B_0$) mode and parallel ($B_1 B_0$) mode measurements. The quantitations of the mixed-valent signal and estimations of tyrosyl free radical concentration were done by double integration of the first derivative of EPR spectra in comparison to a frozen solution of 1mM $Cu(ClO_4)_2$ as standard. The standard Bruker ESP300 software was used for double integration. The estimation of tyrosyl free radical concentration after the reactivation resulted in 1.2 ± 0.05 radical per protein R2. The iron quantitation was carried out spectrophotometrically using bathophenanthroline as a chelator according to Fish et al (15) and gave a result of 5.5 ± 0.2 iron atoms per protein R2. Similar iron contents were found upon full reduction of the protein using dithionite and methyl viologen as mediator in the presence of bathophenan-throline. The theoretical iron content with full occupation of the iron sites would be 4 Fe/R2, and the excess iron is expected to be bound non-specifically (14).

Light absorption spectra were recorded on a CARY 4 Varian spectrophotometer in rectangular anaerobic cuvets with a path length 1 cm and volume 600 μl.

Anaerobic samples both for EPR and light absorption measurements were made by repeated cycles of evacuation and flushing with O_2 free argon gas. The fully reduced form of mouse protein R2 was prepared under anaerobic conditions by addition of Fe(II) to apoprotein R2 in a ratio 6:1 or by reduction of active mouse R2 protein by 5 red-ox equivalents of dithionite in the presence of 100 μM MV as red-ox mediator. The samples for EPR measurements contained 150 μl of 0.1 mM protein R2. All the experiments on the red-ox titration of the protein were performed at 0°C under anaerobic conditions by adding small volumes (5–15 μl) of oxygen free solutions of the red-ox agents to the anaerobic protein solution by a gastight Hamilton syringe. The samples were incubated at 0°C for 5–30 min to reach chemical equilibrium. For EPR measurements, the solutions were studied directly under anaerobic conditions unless otherwise described. For optical measurements, the solution of the protein oxidized under anaerobic conditions was passed through an aerobic Sephadex G25 column (volume = 2 ml) equilibrated with buffer to remove low molecular weight compounds prior to measurements. The dithionite concentration was quantified by titration with potassium ferric cyanide (ϵ_{420} = 1.03 mM^{-1} cm^{-1}).

RESULTS AND DISCUSSIONS

1. Reduction of Mouse R2 Protein

Dithionite and mediators as reductants. Fig. 1a shows the EPR spectrum of a solution of 0.1 mM mouse R2 protein at 4 K. The spectrum displays a strong tyrosyl radical signal centered at g = 2.0 and a weak signal at g = 4.3 due to adventitiously bound Fe(III) ions. When 0.5 mM PES reduced by 0.25 mM dithionite was added to the anaerobic protein solution the tyrosyl radical signal disappeared very rapidly and a rhombic EPR signal with g = 1.92, 1.73 and 1.60 characteristic for the mixed-valent species (4) appeared (Fig. 1b). The latter reached a maximal magnitude after incubation for about 30 min at 0°C. The quantitation of the mixed-valent signal of Fig. 1b accounted for 25 ± 5% of the diiron centers (assuming two diiron centers per R2 dimer). A similar mixed-valent EPR signal was observed upon reduction of the R2 protein by 0.5 mM methylene blue reduced by 0.25 mM dithionite. In the latter case the mixed-valent species yield was not above 15% (data not shown). 0.25 mM dithionite in the presence of 0.1 mM methyl viologen as electron mediator rapidly reduced (within 1 min at 0°C) both the tyrosyl radical and the diferric center to the fully reduced Fe(II)Fe(II) state in the mouse R2 protein. The EPR spectrum of the fully reduced protein R2 which is shown in Fig. 1c contains no signals from either the tyrosyl radical, the Fe(II)Fe(III) or unspecifically bound Fe(III) centers. The g = 17 signal in parallel mode is characteristic of integer spin (S = 2.0) Fe(II) sites (16).

Dithionite alone as reductent. It was also observed that dithionite by itself in the absence of a mediator is able to reduce the radical as well as the diferric centers in mouse R2 protein. Figs. 1d and 2 show the changes in the EPR and light absorption spectra of mouse R2 protein during the

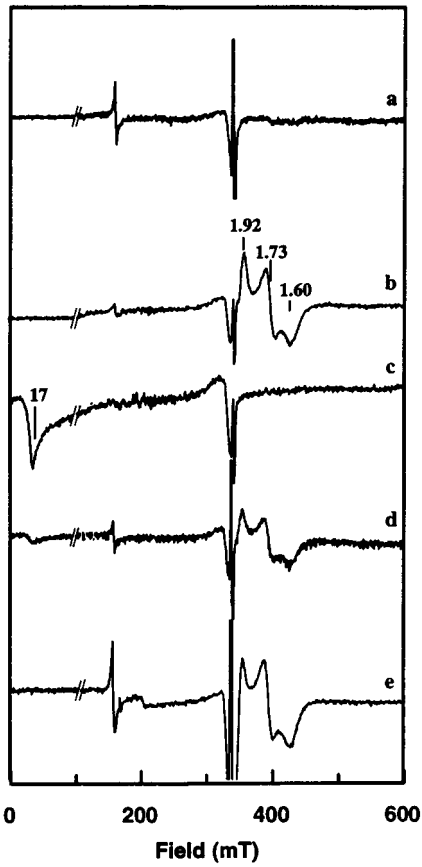

FIG. 1. EPR spectra of (a) 100 μM of active mouse R2 protein; (b) 100 μM of mouse R2 after 30 min of incubation with 0.5 mM PES and 0.25 mM of dithionite under anaerobic conditions at 0°C; (c) 100 μM of mouse R2 after about 1 min treatment by 0.25 mM dithionite with 0.1 mM of methyl viologen under anaerobic conditions; (d) 100 μM of mouse R2 after 10 min of incubation with 0.5 mM of dithionite under anaerobic conditions at 20°C; (e) 15 min of anaerobic incubation at 0°C after addition of 0.5 mM of anaerobic but oxidized PES to (c). The part of spectra from 0 to 100 mT was measured in parallel mode and the rest in perpendicular mode (conditions are the following: 9.62 GHz (perpendicular mode) or 9.35 GHz (parallel mode), 100 kHz, 0.5 mT modulation of amplitude, 2.4 mW, 4K).

reaction with dithionite alone in the absence of mediator. In the presence of dithionite the signal from the tyrosyl radical disappears slowly and the transient $S = 1/2$ mixed-valent signal and $g = 17$ integer spin signal from the iron site appear (Fig. 1d). As observed in Fig. 2 the loss of the 410 nm absorption from the radical and 370 nm absorption mainly from the iron center occur with similar kinetics, i.e. demonstrating very similar rates for the reduction of the tyrosyl radical and diiron center by dithionite. At 20°C, in the presence of 0.1 mM dithionite, the kinetics of reduction of diiron center and tyrosyl radical is characterized by $t_{1/2} = 1340$ s. The reactivation by molecular oxygen of mouse R2 protein reduced by dithionite is dependent on the protein and reductant concentrations used. We have found that mouse R2 protein is completely reactivated (to about 50% of the potential tyrosyl radical sites, like in normal preparations) upon admission of O_2 protein R2 which had been fully reduced under the conditions of Fig. 2. (0.1 mM dithionite, 94 min). The tyrosyl radical content in the reactivated protein decreases markedly when higher dithionite concentrations are used. In protein reduced by 0.4 mM or 1 mM of dithionite, only 50% or 10%, respectively, of the normal yields of tyrosyl radical were found after O_2 oxidation. With a protein concentration lower than 2 μM, the tyrosyl radical disappeared very rapidly upon addition of 0.1

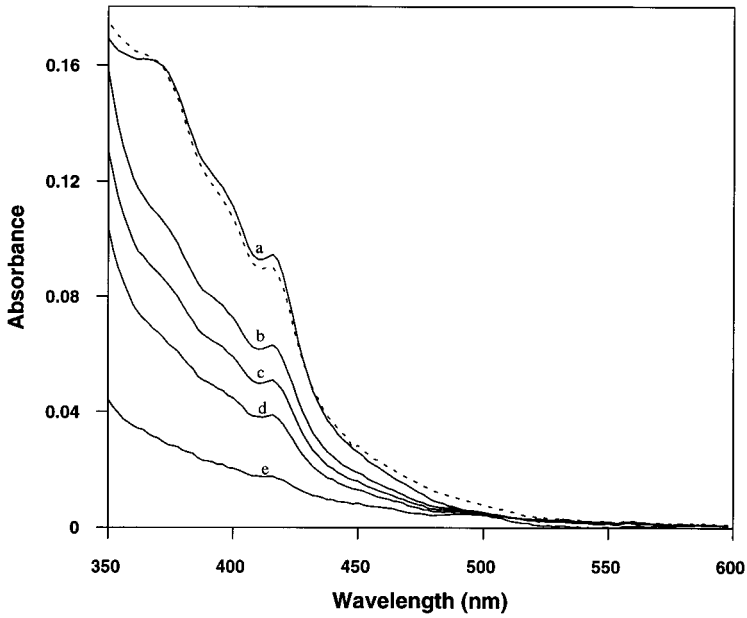

FIG. 2. Light absorption spectra of (a) 14.9 μM of active mouse R2 protein; (b) after 13 min of anaerobic incubation with 100 μM of dithionite at 20°C; (c) after 25 min; (d) after 39 min; (e) after 94 min; (--) immediately after admission of air to (e).

mM dithionite, but the radical could not be regenerated by O_2 addition (data not shown). A hypothetical explanation is that the polypeptide dimer may dissociate under these conditions, and that single polypeptides are not able to react with O_2 to form active protein. Another possible explanation is gradual protein denaturation.

2. Oxidation of the Reduced Forms of R2 Protein

Phenazine ethosulfate and methylene blue as oxidants. Upon addition of 2–3 fold excess of an anaerobic solution of oxidized PES (midpoint potential $E_{1/2}$ = +108 mV (17)) to an anaerobic solution of the fully reduced R2 protein with EPR spectrum as in Fig. 1c, the g = 17 EPR signal of the diferrous center vanished and a mixed-valent signal similar to that of Fig. 1b appeared (Fig. 1e). The conditions for the reduction of the sample had been chosen so that no excess of reducing equivalents from dithionite remained. Under the conditions of Fig. 1e the maximum yield of the mixed-valent species was about 20% of the diiron centers (assuming two diiron centers per R2 dimer). This demonstrates that PES is able to oxidize a considerable part of the Fe(II)Fe(II) centers to the mixed-valent state and also further to the diferric state. No tyrosyl radical signal was observed upon the oxidation of the reduced protein by PES. It should be noted that a twofold growth of the g = 4.3 EPR signal due to adventitiously bound Fe(III) was observed in the samples oxidized by 3-fold excess of PES and this effect increases with the oxidant concentration. Methylene blue (E° = +11 mW) (18) in concentrations lower than 1 mM could also oxidize the reduced protein, but only incompletely. In the presence of 1 mM of methylene blue the g = 17 signal decreased by 60% and a small mixed-valent signal corresponding to 10% of the diiron center content appeared (data not shown).

H_2O_2 as oxidant. Fig. 3b demonstrates the light absorption spectrum of a met form of mouse protein R2 which is formed upon interaction of reduced R2 with H_2O_2 with subsequent desalting by passing the protein through a G25 column. There is no trace of any tyrosyl radical contribution in this spectra, indicating that no reduced iron center remained in the sample when air was

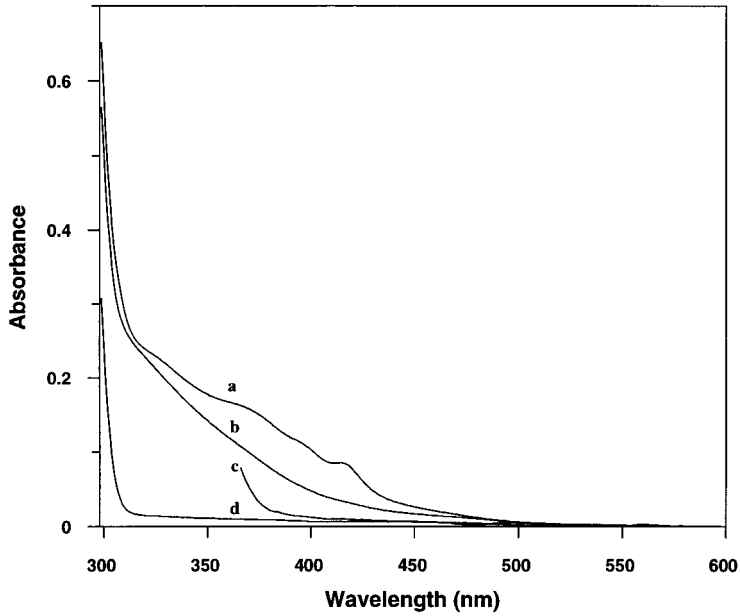

FIG. 3. Light absorption spectra of (a) 14.2 μM of active mouse R2 protein; (b) the met form of mouse protein obtained after anaerobic oxidation of 14.2 μM apoR2 and 90 μM Mohr's salt with 200 μM H_2O_2 and aerobic desalting of the protein, (c) the reduced form of mouse R2 obtained from 14.2 μM of apo R2 protein in the presence of 100 μM dithionite and 90 μM Mohr's salt (dithionite absorption edge appears around 375 nm), (d) 14.2 μM of apoR2 protein.

admitted. Upon anaerobic reduction by 0.5 mM dithionite or (reduced) PES (0.25mM dithionite and 0.5mM PES as redox mediator) of the met form of 0.1mM mouse R2 protein obtained by H_2O_2 oxidation and subsequent desalting we observed the formation of the mixed-valent and fully reduced species, by EPR spectroscopy identical to those obtained from the active protein (data not shown). Up to 60% of active protein could be reconstituted by complete reduction using 0.5mM dithionite and reactivation with oxygen from 0.1mM met mouse R2 protein produced by anaerobic oxidation with hydrogen peroxide as described above.

Identical spectral shapes but with lower intensity were obtained upon oxidation of the fully reduced state by para-benzoquinone (data not shown). The spectrum of Fig. 3b also proved to be identical to that observed for a radical free met form of mouse protein R2 obtained by scavenging the tyrosyl radical in the active protein by equimolar amounts of 4-butoxyphenol under aerobic conditions (data not shown). We conclude that a presumably diferric met form of mouse R2 can be stabilized and that it has a rather featureless light absorption spectrum in the 310–450 nm region (Fig. 3b). It must however be mentioned that similar featureless spectra are observed in proteins (e.g. bovine serum albumin) containing non-specifically bound iron. Also for the *E. coli* protein R2 it has been reported (6) that the diferrous form could be oxidized by a 2–3 fold excess of H_2O_2 to the diferric form. The light absorption spectrum of the H_2O_2 oxidized *E. coli* protein was found to be identical to the one reported for the radical free met form obtained by scavenging radical by hydroxyurea (19). According to our observations para-benzoquinone does not oxidize the reduced *E. coli* protein R2.

CONCLUSIONS

The results reported here show that the reduction-oxidation transitions between the oxidized and reduced forms of the diiron cluster in mouse R2 protein are reversible. The μ-oxo-bridge absent in the diferrous form must therefore be reformed even when the oxidation does not involve molecular

oxygen. It should be noted however that the low stability of the reduced forms of the mouse protein (20) in general and particularly in the presence of low molecular red-ox reactants at relatively high concentrations makes it difficult to carry out quantitative studies to determine the thermodynamic parameters of the process.

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