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Reduction of the Small Subunit of *Escherichia coli* Ribonucleotide Reductase by Hydrazines and Hydroxylamines

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ABSTRACT: Each polypeptide chain of protein R2, the small subunit of ribonucleotide reductase from *Escherichia coli*, contains a stable tyrosyl radical and an antiferromagnetically coupled diferric center. Recent crystallographic studies [Nordlund, P., Eklund, H., & Sjöberg, B.-M. (1990) *Nature 345*, 593-598] have shown that both the radical and the diiron site are deeply buried inside the protein and thus strongly support the hypothesis of long-range electron-transfer processes within protein R2. This study shows that monosubstituted hydrazines and hydroxylamines are able to reduce the tyrosyl radical and the ferric ions, under anaerobic conditions. It allows characterization of the site from which those compounds transfer their electrons to the iron/radical center. The efficiency of any given reducing agent is not solely governed by its redox potential but also by its size, its charge, and its hydrophobicity. We suggest, as a possible alternative to the long-range electron-transfer hypothesis, that conformational flexibility of the polypeptide chain might exist in solution and allow small molecules to penetrate the protein and react with the iron/radical center. This study also shows that two reduction mechanisms are possible, depending on which center, the radical or the metal, is reduced first. Full reduction of protein R2 yields reduced R2, characterized by a normal tyrosine residue and a diferrous center. Both the radical and the diferric center are regenerated from reduced R2 by reaction with oxygen, while only the diferric center is formed by reaction with hydrogen peroxide.

Ribonucleotide reductase is a key enzyme for all living organisms. It provides the deoxyribonucleotides required for the synthesis of deoxyribonucleic acid (DNA)[1] (Lammers & Follman, 1983; Reichard, 1988; Stubbe, 1990). The enzyme from *Escherichia coli* contains two homodimeric proteins that can be separated during purification. Each polypeptide chain of the small protein, named protein R2, contains a stable radical located on tyrosine-122 (Reichard & Ehrenberg, 1983; Larsson & Sjöberg et al., 1986) as well as a binuclear iron center, in which the Fe(III) ions are antiferromagnetically coupled by a μ-oxo bridge (Petersson et al., 1980; Sjöberg et al., 1982). Protein R2 has been crystallized, and a refined three-dimensional structure is now available (Nordlund et al., 1990).

The radical is absolutely required for enzyme activity and is thus believed to participate in the activation of the sugar moiety during ribonucleotide reduction (Stubbe, 1989). *Escherichia coli* contains an enzyme system that introduces the radical into the protein (Barlow et al., 1983; Fontecave et al., 1987a). It thus provides the cell with the ability to regulate the activity of ribonucleotide reductase through the amount of tyrosyl radical present in R2.

The radical is found buried within the protein (Nordlund et al., 1990). However, it is known to react with a large number of radical scavengers, antioxidants, and reductants (Reichard & Ehrenberg, 1983; Lammers & Follman, 1983). This has been the basis of numerous studies on the inhibition of ribonucleotide reductase. In particular, hydroxyurea and hydroxylamine are excellent scavengers of the tyrosyl radical (Ehrenberg & Reichard, 1972; Atkin et al., 1973). In contrast, the iron center was originally thought to be chemically inert. Iron is tightly bound to the polypeptide chain, and preparation of apoR2, the apoprotein, requires extensive dialysis against a very strong iron chelator (Atkin et al., 1973).

A few recent reports have shown that electrons could be transferred to the diferric site, which results in the formation of reduced R2 with a diferrous center + a normal tyrosine residue. Therefore, protein R2 can be prepared in three redox states: (i) as active R2, the fully oxidized state, with a binuclear Fe(III) center + a tyrosyl radical; (ii) as metR2, with a Fe(III) center + a normal tyrosine-122; (iii) as reduced R2. The reduction of the iron center can be chemically achieved by reaction with dithionite in the presence of catalytic amounts of a redox mediator such as viologens (Sahlin et al., 1989), with dithiothreitol at alkaline pH (Fontecave et al., 1990a), and with diimide (Gerez et al., 1991). The bacterial radical-introducing enzyme has also been shown to function as a protein R2–diferric reductase and might be the physiological reductant (Fontecave et al., 1987a, 1989). The formation of reduced R2 is a key step during enzymatic activation of protein R2 since the tyrosyl radical can only be generated during the reoxidation of the ferrous center by oxygen (Fontecave et al., 1987a,b).

In this study, we have tried to establish the stereoelectronic parameters that control the reactivity of the iron center of protein R2. Since the only reaction of ferric iron in ribo-

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[1] Abbreviations: DNA, deoxyribonucleic acid; DTT, dithiothreitol; EPR, electronic paramagnetic resonance; Tris, tris(hydroxymethyl)-aminomethane.

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nucleotide reductase known so far is its reduction, reducing agents were tested for their ability to transfer electrons to the iron center under anaerobic conditions. Herein, we report that various monosubstituted hydrazines and hydroxylamines are good reductants of the iron center. The results have been used to establish the relative reactivity of the metal and radical centers.

Experimental Procedures

Materials

Hydrazine monohydrate, methylhyrazine, benzoylhydrazine, hydroxylamine hydrochloride, and *N*-methylhydroxylamine hydrochloride were purchased from Janssen Chimica. Ethylhydrazine oxalate, propylhydrazine oxalate, *tert*-butylhydrazine hydrochloride, phenylhydrazine hydrochloride, benzylhydrazine oxalate, *N-tert*-butylhydroxylamine hydrochloride, and *N*-benzylhydroxylamine hydrochloride were obtained from Fluka. Phenylhydroxylamine was prepared from nitrobenzene and ammonium chloride, as described earlier (Vogel, 1967), and purified by recrystallization from toluene. The resulting pure compound has a melting point of 81 °C.

Active protein R2 was obtained from an overproducing strain of *E. coli* (Sjöberg et al., 1986). MetR2 was prepared from R2 by treatment with hydroxyurea (Barlow et al., 1983). ApoR2 was prepared by treatment of protein R2 with 8-hydroxyquinoline sulfonate and reconstituted with ferrous ammonium sulfate in the presence of sodium ascorbate, as previously described (Atkin et al., 1973). Protein concentrations were determined with the colorimetric assay of Bradford (1976).

Methods

All solutions were deaerated by flushing with argon in septum-capped vials. Optical spectra were recorded with an UVIKON 820 UV–visible spectrophotometer. Experiments were conducted in an anaerobic cuvette sealed with a rubber septum and thermoregulated at 40 °C. Additions were made through the septum with gas-tight syringes that had been thoroughly washed with deoxygenated water. EPR measurements were performed on a VARIAN E109, and spectra were recorded at low temperatures (from 4 to 10 K) with an Oxford helium temperature control system. The mixture preparation and all subsequent additions were made anaerobically inside a Miller Howe glovebox (O_2 <5 ppm; t = 30 °C).

Anaerobic Reduction of R2 and MetR2. (A) UV–Visible Spectroscopy. Under standard conditions, the spectroscopic cuvette was first filled with 500 μL of 0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM reductant (alkylhydroxylamine or hydrazine) and then deoxygenated by passage of humidified argon for at least 2 h. The optical spectrum of the solution was recorded between 300 and 600 nm and stored in the spectrophotometer memory for subtraction from all subsequent spectra. The reaction was started by addition of a small volume of R2 (or metR2), final concentration 0.5–1 mg/mL, that had been previously equilibrated with argon. A spectrum, recorded immediately, showed the characteristic features of R2 (or metR2): the broad absorbance band of the binuclear ferric center at 370 nm and the sharp peak of the tyrosyl radical at 410 nm. Iron and tyrosyl radical concentrations in active R2 as well as the iron content in metR2 were calculated from their molar extinction coefficients (Sahlin et al., 1990). The absorbance at 370 nm was noted (=OD_1). Further spectra between 300 and 600 nm were recorded at time intervals, and in each case, the absorbance at 370 nm was noted (=OD).

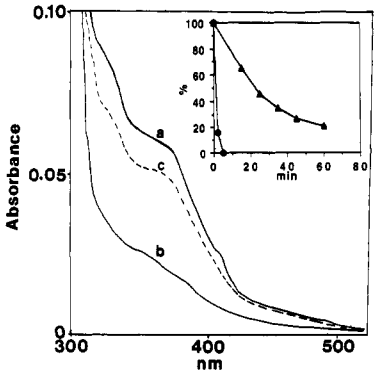

FIGURE 1: Reduction of active R2 by hydroxylamine. Protein R2 (1 mg/mL) was incubated with 5 mM hydroxylamine at pH 8.5 under anaerobic conditions. Spectra were recorded after 2 min (a) and after 80 min just before (b) and just after (c) admission of air. The inset shows the reduction kinetics of both redox centers of protein R2: (●) the tyrosyl radical and (▲) the iron center.

When no further changes in the spectra occurred (absorbance at 370 nm = OD_2), air was admitted into the cuvette, and a new spectrum was taken. In some experiments, hydrogen peroxide (1–5 mM) was added anaerobically to the cuvette before oxygen admission. the extent of reduction of the iron center at different time intervals was calculated from $(OD_1 - OD)/(OD_1 - OD_2)$, as described earlier (Fontecave et al., 1990a), and the relative radical concentration was determined from the peak height at 410 nm relative to a fitted base line, i.e., a straight line between the absorbances at 400 and 420 nm. Reduction with dithionite + methylviologen was carried as described earlier (Fontecave et al., 1990a).

(B) EPR Spectroscopy. Each solution used was first deoxygenated by passage of humidified argon for at least 2 h and then introduced into the glovebox. A reaction mixture that contained 0.25 mM R2 in 150 μL of 0.1 M Tris-HCl, pH 8.5, was prepared in an Eppendorf tube and transferred anaerobically with a gas-tight syringe to a 4-mm o.d. quartz tube. Once sealed with a rubber septum, the tube was quickly frozen in a liquid N_2 bath, and the EPR spectrum was recorded and used as a reference for subsequent spectra. After quick thawing, the tube was introduced into the glovebox, and NH_2–NH_2 (final concentration 20 mM) was added to the solution. At time intervals, the tube was frozen, analyzed by EPR spectroscopy, and thawed again before transfer to the box. At the end of the experiment, when the free radical signal had nearly disappeared, air was introduced into the tube, and a new spectrum was recorded. A similar experiment was performed but with 20 mM DTT and 20 mM $MgCl_2$.

Results

Reduction of Protein R2 by Hydroxylamine and Hydrazine. The anaerobic reduction of protein R2 can be monitored by UV–visible spectroscopy since the 370-nm absorption band and the 410-nm peak, characteristic of the iron center and the tyrosyl radical of protein R2, respectively, disappear during reduction. Reduced R2 has no absorption above 300 nm. Its presence can be demonstrated, however, by the instantaneous appearance of the characteristic spectrum of protein R2 after admission of oxygen.

When 1 mg/mL R2 was incubated with 5 mM hydroxylamine at pH 8.5, in an anaerobic cuvette, we observed the instantaneous disappearance of the 410-nm peak, followed by a much slower decay of the 370-nm band (Figure 1). This is consistent with the previous observation that NH_2–OH is a good scavenger of tyrosyl radicals (Ehrenberg & Reichard, 1972). It also shows that NH_2–OH is able to transfer electrons

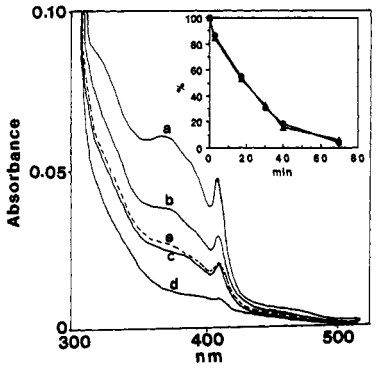

FIGURE 2: Reduction of active R2 by hydrazine. Protein R2 (1 mg/mL) was incubated with 10 mM hydrazine at pH 8.5 under anaerobic conditions. Spectra were recorded after 2 (a), 20 (b), 30 (c), and 70 min just before (d) and just after (e) admission of oxygen. The inset shows the reduction kinetics of both redox centers of protein R2: (●) the tyrosyl radical and (▲) the iron center.

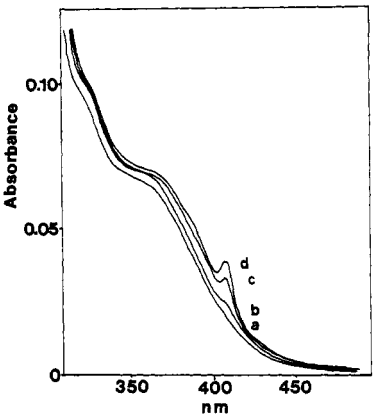

FIGURE 3: Aerobic activation of metR2 by hydrazine. Inactive protein metR2 (1 mg/mL) was incubated aerobically with 10 mM hydrazine in 0.1 M Tris-HCl, pH 8.5. Spectra were recorded after 2 (a), 5 (b), 10 (c), and 20 min (d).

to the iron center, albeit at a much slower rate. The kinetics of the reduction of both redox centers are shown in the inset of Figure 1. During this reaction, metR2 is an intermediate. A similar situation has previously been observed during enzymatic reduction of protein R2 by the flavin reductase system (Fontecave et al., 1989) and during a chemical reduction with dithionite + methylviologen (Sahlin et al., 1989).

Admission of air after 1 h of reaction resulted in the instantaneous reoxidation of iron, as shown by the reappearance of the 370-nm band. No 410-nm peak could be detected (Figure 1) since the newly formed tyrosyl radical was rapidly scavenged by the hydroxylamine present in high concentration in the reaction mixture. We have verified that the 410-nm peak instantaneously disappears when R2 is incubated aerobically with 5 mM hydroxylamine.

When 1 mg/mL active protein R2 was incubated with 10 mM hydrazine NH_2–NH_2, a completely different reduction process occurred (Figure 2). In this case, we observed a strictly parallel loss of the 410-nm and the 370-nm band. The reaction was complete after approximately 80 min, as shown in the inset of Figure 2. This suggests that the tyrosyl radical and the iron center are reduced concomitantly and that R2 is transformed all the way to reduced R2, without the intermediate formation of metR2. The rate of the reaction was more than 10-fold slower with 1 mM hydrazine and, significantly, decreased at lower pH (data not shown).

That reduced R2 was formed during the anaerobic incubation was checked by admitting oxygen into the spectroscopic cuvette. A new spectrum, characteristic of R2, was then produced. However, from the intensity of both the 410-nm peak and the 370-nm band, we estimated that only 30% of the original R2 was recovered (Figure 2). This is not due to reactions of reactivated R2 with hydrazine under aerobic conditions, since, in a separated experiment, 10 mM hydrazine had no effect on the R2 spectrum after 10 min of aerobic incubation. Moreover, this is not due to some irreversible damage to protein R2 during prolonged incubation with hydrazine since hydrazine-treated R2 could be fully reactived through one cycle of mobilization of iron with hydroxyquinoline/reconstitution with Fe^{2+} + ascorbate + O_2 (Atkin et al., 1973).

As shown in previous studies, compared with R2, reduction of metR2 was much faster (data not shown). This confirms that the absence of the tyrosyl radical makes the iron center more reactive (Fontecave et al., 1990a). Under aerobic conditions, hydrazine could transform metR2 to active R2 (Figure 3). This result indicates that the iron center of metR2 was reduced by NH_2–NH_2 even in the presence of oxygen. However, after 20-min incubation, the 410-nm peak stopped growing, probably because oxygen, in the bulk of the solution, was depleted, due to its reaction with hydrazine. Accordingly, vigorous reaeration of the cuvette resulted in an additional increase of the 410-nm peak (data not shown).

Hydrazine Does Not Mobilize Iron during Reduction. The low recovery of active R2 after reoxidation of hydrazine-reduced R2 might be due to dissociation and mobilization of iron, which would then no longer be available during the generation of the tyrosyl radical. Instability of ferrous iron in ribonucleotide reductase has previously been demonstrated (Fontecave et al., 1990a; Gerez et al., 1991). Two experiments were conducted to investigate this point. In the first, the anaerobic reduction of R2 was monitored by EPR spectroscopy. The iron center is EPR-silent since the ferric ions are antiferromagnetically coupled. Thus, the EPR spectrum of protein R2 displays only two signals: the first, at $g = 2.00$, is characteristic of the tyrosyl radical, and the second, at $g = 4.3$, indicates the presence of free adventitious ferric iron, unavoidable in standard preparations of protein R2. The liberation of iron can thus be quantitated from the increase of the intensity of the $g = 4.3$ signal.

21 mg/mL protein R2 was incubated anaerobically with 20 mM hydrazine inside an EPR tube at 30 °C. At time intervals, the solution was frozen, and the EPR spectrum was recorded at 10 K. While the intensity of the $g = 2.00$ signal decreased, indicating reduction of protein R2, a new, very weak signal was detected with all features at $g < 2.0$ (Figure 4). This signal was similar to that found during reduction of protein R2 by diimide (Gerez et al., 1991). It is characteristic of the mixed-valence state of the binuclear center, which indicates that a Fe(III)–Fe(II) complex is formed transiently during the reduction of protein R2 by hydrazine. The amount of this complex was quantitated and found to be very low (<5%). The $g = 4.3$ signal decreased, indicating that adventitious iron is also reduced.

When the tyrosyl radical was almost totally reduced, air was admitted into the EPR tube. This resulted in the complete disappearance of the $g < 2.0$ signal and in the increase of the free radical signal, which indicated reoxidation of the tyrosine residue. However, the intensity of the $g = 2.00$ signal was approximately 40% of that of the original signal, consistent with the spectrophotometric results described above. The same recovery was obtained when the incubation mixture was dialyzed anaerobically to remove the excess reductant just before admission of oxygen. On the other hand, the $g = 4.3$ signal

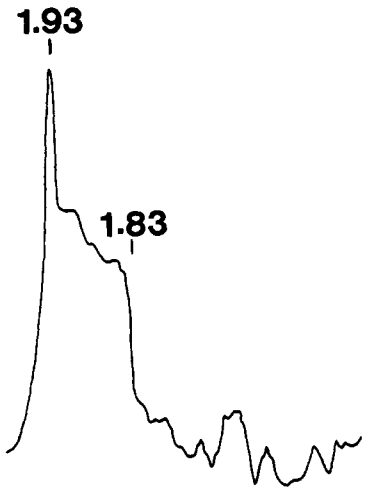

FIGURE 4: EPR signal of the mixed-valent (Fe^{II}–Fe^{III}) state of the R2 iron center. The signal was detected during anaerobic reduction of R2 with hydrazine under the conditions described under Experimental Procedures. It was maximal after 90-min reaction. EPR conditions: temperature = 10 K; microwave frequency = 9.228 GHz; microwave power = 30 mW; modulation amplitude = 12.5 G; gain amplitude = 2.5×10^3.

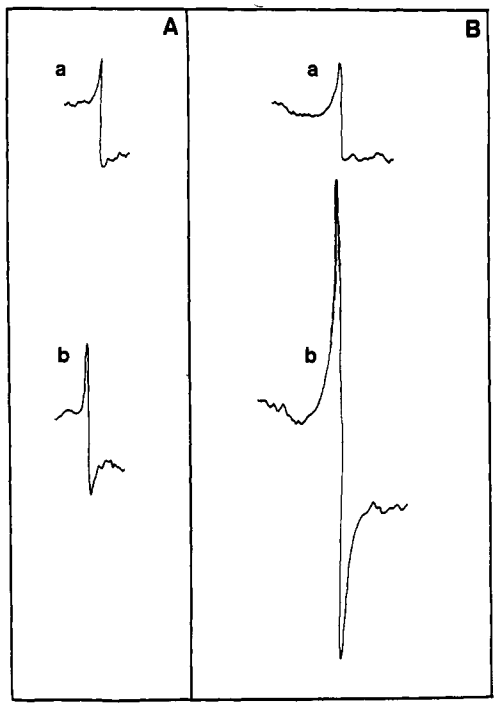

FIGURE 5: EPR quantitation of iron mobilization during one cycle of reduction–reoxidation of protein R2. Figure 5A shows the EPR signal at $g = 4.3$, characteristic of free iron: (a) in a sample of pure protein R2 (21 mg/mL; (b) in the same sample after a 90-min anaerobic incubation with hydrazine followed by admission of oxygen. Conditions are described under Experimental Procedures. Figure 5B shows the same signal at $g = 4.3$: (a) in a sample of pure protein R2 (21 mg/mL); (b) in the same sample after a 60-min incubation with 20 mM dithiothreitol followed by admission of oxygen. EPR parameters: temperature = 10 K; microwave frequency = 9.178 GHz; microwave power = 10 mW; modulation amplitude = 8 G; gain amplitude = 10^3.

increased only very slightly, when compared with that of the original spectrum (Figure 5A). This indicates that only a very small amount of iron (less than 5%) was removed from the protein after one cycle of reduction–reoxidation. A similar reaction carried out with 20 mM DTT as the reducing agent is also shown (Figure 5B). In this case, the large increase in the intensity of the $g = 4.3$ signal showed a significant loss of iron, as expected (Fontecave et al., 1990).

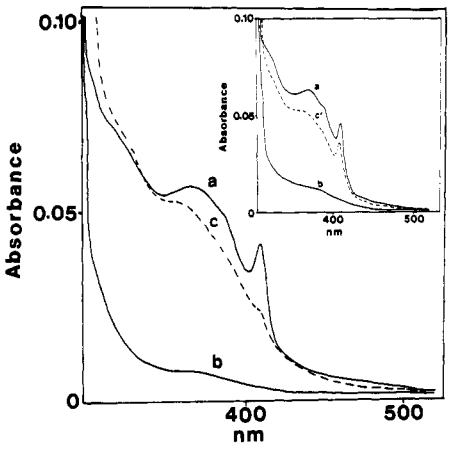

FIGURE 6: Oxidation of reduced R2 by hydrogen peroxide. 1 mg/mL protein R2 (spectrum a) was reduced by 100 μM sodium dithionite in the presence of 1 μM methylviologen under anaerobic conditions. After complete reduction, spectrum b, characteristic of reduced R2, was recorded. Addition of 1 mM hydrogen peroxide under anaerobic conditions instantaneously gave spectrum c. For comparison, the inset shows spectrum c′ obtained when air was added to reduced R2.

As shown below, we found that ferrous iron of reduced R2 reacted quantitatively with low concentrations of hydrogen peroxide. This provided us with a new chemical test for the presence of a full iron center. In Figure 6 is shown the UV–visible spectrum of reduced R2 obtained from reduction, under anaerobic conditions, of 1 mg/mL protein R2 with methylviologen in the presence of sodium dithionite. Reoxidation by oxygen led, as expected, to active R2 with the diferric center and the tyrosyl radical (inset of Figure 6). When, instead, 1–5 mM hydrogen peroxide was added anaerobically to reduced R2, iron was instantaneously oxidized, but the tyrosine residue remained reduced as shown by the spectrum of the solution which did contain the 370-nm band but lacked the 410-nm peak (Figure 6). Weak shoulders at 410 nm were sometimes observed when precautions to avoid air contamination were not sufficient. Subsequent introduction of oxygen had no effect on the spectrum. This experiment shows that hydrogen peroxide is capable of accepting electrons from the diferrous center of reduced R2. The product of the reaction is metR2 and not active R2. The intensity of the 370-nm band, shown in Figure 6, demonstrated that R2 had been quantitatively transformed to metR2 after the cycle of reduction by viologen/oxidation by H_2O_2. This in turn shows that iron had not been removed from the protein during reduction and that oxidation of ferrous iron by hydrogen peroxide was quantitative.

When 1 mg/mL protein R2 was fully reduced by hydrazine and then treated with hydrogen peroxide, again quantitative formation of metR2 was observed (data not shown). This indicates that iron had been totally transformed into ferrous iron and had not been mobilized during reduction of protein R2 by hydrazine.

Reduction of Protein R2 by Monosubstituted Hydrazines and Hydroxylamines. The reduction of the iron center and the tyrosyl radical of protein R2 (1 mg/mL) by various monosubstituted hydrazines and hydroxylamines was monitored by UV–visible spectroscopy to investigate the stereoelectronic parameters of the reaction. The concentrations were 1 and 10 mM. The rates of the reactions were analyzed in terms of $t_{1/2}$(Fe), the time required for the reduction of 50% of the iron centers, characterized by the 370-nm band, and of $t_{1/2}$(Tyr), the time required for the reduction of 50% of the tyrosyl radicals, characterized by the 410-nm peak. The results are shown in Table I.

Table I: Reduction of the Redox Centers of Ribonucleotide Reductase by Hydrazines and Hydroxylamines[a]

	R–NH–NH_2		R–NH–OH	
R	$t_{1/2}$(Fe)[b]	$t_{1/2}$(Tyr)[b]	$t_{1/2}$(Fe)[b]	$t_{1/2}$(Tyr)[b]
H (10 mM)	15	15	10	<0.25[c]
H (1 mM)	200	200	50	4
CH_3	75	5	13	<0.25[c]
C_2H_5	120	30		
C_3H_7	165	75		
$(CH_3)_2CH$			>240[d]	6
$(CH_3)_3C$	nr[e]	>240[d]	180	45
C_6H_5	?[f]	<0.25[c]	?[f]	<0.25[c]
C_6H_5–CH_2	nr[e]	70	45	3
C_6H_5–CO	nr[e]	45		

[a] R2 was incubated anaerobically with 10 mM reducing agent, and the reduction of the iron center and the tyrosyl radical was monitored spectroscopically as described under Experimental Procedures. [b] Time required (in minutes) for the reduction of 50% of the tyrosyl radicals [$t_{1/2}$(Tyr)] or the iron centers [$t_{1/2}$(Fe)] present in the reaction mixture. [c] The reaction is instantaneous, and $t_{1/2}$ cannot be defined. [d] The reduction is too slow, and $t_{1/2}$ cannot be defined. [e] No reduction. [f] Absorption of the phenyl derivative in the near-UV region prevents an accurate quantitation of the 370-nm band intensity.

Within each family of electron donors, increasing the size of the substituent generally led to a dramatic decrease in the rate of the reduction. For example, compare methyl-, ethyl-, propyl-, and *tert*-butylhydrazine (Table I). However, the reduction of the tyrosyl radical was much less sensitive to steric hindrance than the reduction of the iron center. With R–NH–NH_2 as the reducing agent, $t_{1/2}$(Fe) increased 11-fold from R = H to R = C_3H_7, while $t_{1/2}$(Tyr) only increased 5-fold. This was also clear from comparison of hydrazine and methylhydrazine: while the rate of reduction of the iron center was greatly lowered, that of the tyrosyl radical was approximately 3 times faster. This might originate from the hydrophobicity provided by the methyl group. In fact, hydrophobicity seems to be an important factor for the interaction of the electron donor with the tyrosyl radical, since phenylhydrazine was found to be the best hydrazine for reducing the radical.

Comparing hyroxylamines and hydrazines, we found that each R–NH–OH was much more reactive than the corresponding R–NH–NH_2. The difference is particularly clear-cut in the reduction of iron by NH_2–OH and by NH_2–NH_2 (Table I). From Table I, it appears, if one excludes just hydrazine, that reduction of the radical is much faster than that of the iron center. Extreme cases, such as hydroxylamine, methylhydroxylamine, methylhydrazine, and phenylhydrazine, involve, in the first step, a very fast electron transfer to the radical which results in the formation of metR2. In the second step, iron is slowly reduced.

Discussion

Protein R2 of ribonucleotide reductase from *E. coli* contains a binuclear non-heme ferric center as well as a tyrosyl radical, whose phenoxyl oxygen atom is found 5 Å from the closest iron atom (Nordlund et al., 1990). The function of iron is to generate and stabilize the radical (Barlow et al., 1983; Fontecave et al. 1987a). We have shown that various monosubstituted hydrazines and hydroxylamines are capable of transferring their reducing equivalents to both redox centers of protein R2. This has been monitored by the disappearance of the characteristic UV–visible absorptions of the tyrosyl radical and the diferric center.

The major implication of the recent structural information concerning R2 is that small molecules such as monosubstituted hydrazines and hydroxylamines should not be able to penetrate the protein and gain access to the tyrosyl radical and the diiron sites. Long-range electron-transfer processes between the surface and the interior of the protein have thus been suggested (Nordlund et al., 1990). Our data allow a characterization, in terms of electric charge, steric hindrance, and hydrophobic interactions, of the site where reducing agents interact with the polypeptide chain and from which they transfer electrons to the radical and to the ferric ions. This may help in identifying this electron donor binding site.

First, it is important to note that reducing agents with such very low redox potentials (inferior to −0.6 V) have been used in our study so as to be able to ignore the effect of the thermodynamic driving force on the rates of electron transfer to protein R2. Although the determination of the redox potential characterizing the iron center of protein R2 has not been possible so far, previous reduction titrations with redox mediators such as viologens led to an approximate lower value of about −200 mV (Sahlin et al., 1989). Thus, all the reducing agents used in this work satisfy the thermodynamic requirement. Hydroxylamines, however, have much lower redox potentials than the corresponding hydrazines. This, in part, could be at the origin of the greater reactivity of hydroxylamines (Table I). On the other hand, the redox potential of the tyrosyl radical is expected to be positive, approximately +0.6 to +0.9 V (Jovanovic et al., 1986; DeFelippis et al., 1989). It is therefore not surprising that in most cases (previous studies as well as this work) the radical is reduced first and that metR2 is an intermediate during reduction of protein R2. Hydrazine NH_2–NH_2, an exception, will be discussed later.

Charge has been previously shown to be a critical factor for efficient electron transfer to the iron/radical center. Negatively charged electron donors, such as dithionite, cannot reduce it while positively charged viologen radicals can (Sahlin et al., 1989). This could be due to the presence of negatively charged amino acid residues at the binding site. The electrical neutrality of hydrazines and hydroxylamines may thus also explain their reducing capacities.

Considering now the steric parameters that affect the reduction of protein R2, we observed that increasing the size of the reducing agent greatly decreases the rate of electron transfer to the iron center. On the other hand, the size effect is much less drastic for the reduction of the radical. This is rather surprising since the three-dimensional structure shows that the radical and the metal sites are not greatly separated from each other (Nordlund et al., 1990). For example, it has been shown that the rate of reduction of the tyrosyl radical by R–NH–NH_2 increased when R was changed from H to CH_3 and from CH_3 to C_6H_6, $t_{1/2}$(Tyr) changing from 15 to 5 min and from 5 to less than 0.25 min. Since the redox potentials of these three electron donors are in the order CH_3 < H < C_6H_6, this result confirms that the rate of electron transfer to protein R2 is not primarily controlled by the thermodynamic driving force.

This then leads us to suggest that the hydrophobicity of the reducing agent greatly favors electron transfer to the radical site. More precisely, the presence of a phenyl group makes the electron donor more active (note the reactivity of phenyl and benzyl derivatives). This might originate from specific interactions between the hydrophobic moiety of the reducing agent and hydrophobic residues at the binding site.

Whether this site is located at the surface of the protein or in close proximity to the iron/radical center cannot be concluded at this point. In fact, the characteristics of the site resemble those of the radical and iron sterically hindered close environments, revealed by the three-dimensional structure

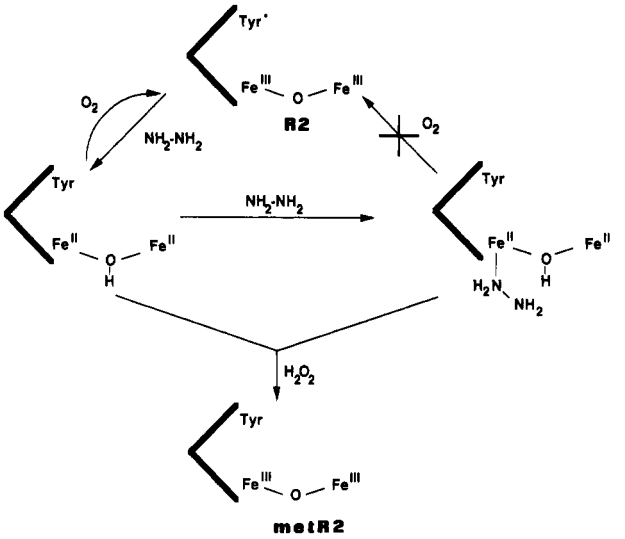

FIGURE 7: Scheme for the reduction of protein R2 by hydrazine. Hydrazine binds to the diferrous center and prevents it from oxygen binding and reoxidation. Oxidation by H_2O_2 quantitatively yields metR2.

(Nordlund et al., 1990). On one hand, Tyr-122 has been shown to be located in a pocket made of conserved hydrophobic residues (Phe-208, Phe-212, Ile-234, and Ser-211) which would be able to interact specifically with hydrophobic reducing agents. On the other hand, the large number of negatively charged glutamate and aspartate oxygen ligands around the ferric ions would explain the discrimination between positively and negatively charged electron donors. Moreover, the observed differences between the reactivities of the radical and the iron center would reflect the differences between their respective environments. We thus propose, as a possible alternative to the long-range electron-transfer hypothesis, that, in solution, protein R2 may have some conformational flexibility which allows small molecules to get, to some extent, into the interior of the protein. In fact, at least one channel might exist for dioxygen since it is assumed to covalently bind to the diferrous center in order to be activated (Nordlund et al., 1990; Sahlin et al., 1990; Fontecave et al., 1990). Hydrazine or diimide, for example, might be able to use that channel and react directly with the iron/radical center.

As mentioned above, reduction of protein R2 by hydrazine NH_2–NH_2 involves a completely different mechanism. In this case, the two redox centers are reduced simultaneously, and R2 is transformed directly to reduced R2. A likely interpretation is that iron is reduced first. This results in a strong destabilization of the radical. Thus, an intermediate species with a ferrous center and an intact tyrosyl radical in the presence of a reducing agent in excess could not be observed. Such a process has been observed previously with dithiothreitol at pH 8.5 (Fontecave et al., 1990a) or with diimide as a reducing agent (Gerez et al., 1991). It should be noted that there is not a single example of a reducing agent capable of transforming R2 into a stable ferrous–tyrosyl radical species. This suggests that an intact ferric, and not ferrous, center is required for stabilization of the tyrosyl radical.

We have shown that protein R2 was not irreversibly denatured by hydrazine. In particular, iron was not significantly removed from the polypeptide chain during reduction by hydrazine. However, a large part of this ferrous iron could not be reoxidized by oxygen (Figure 2). We suggest that this might originate from the coordination of NH_2–NH_2 to Fe(II), which would then prevent iron from oxygen binding and consequently inhibit the reactivation of protein R2. Iron–hydrazine complexes are well-documented (Hanstein et al., 1967; Battioni et al., 1983). In contrast, hydroxylamine is not a good iron ligand and thus does not prevent full reoxidation of reduced R2. The scheme shown in Figure 7 summarizes our current thinking on the mechanism of reduction of protein R2 by hydrazine. The product is postulated to be a mixture of the diferrous and diferrous–hydrazine forms. That the latter is reactive toward H_2O_2 and not O_2 probably means that the oxidation of Fe(II) does not require H_2O_2 binding. Note, in the scheme, that the oxygen bridge is protonated (one or two protons?) as a consequence of iron reduction: the rhombic EPR signal of the mixed-valence state, detected during this reduction (Figure 4), or during reduction of protein R2 by diimide (Gerez et al., 1991), closely resembles that of the mixed-valence state of another binuclear non-heme iron protein, hemerythrin, for which a hydroxo bridge has been demonstrated (Maroney et al., 1986). The involvement of a Fe(II)–hydrazine complex requires further investigation.

A new reaction of ferrous iron of reduced R2 has been demonstrated in this work: its oxidation by hydrogen peroxide which results in the formation of metR2 and not active R2 (Figure 6). This was shown by the UV–visible spectrum of the product, which displayed the band at 370 nm, thereby indicating that iron ions were oxidized and coupled by a μ-oxo bridge. That the reaction stopped at the level of metR2 is consistent with the two-electron oxidant character of hydrogen peroxide. Further oxidation to active R2 by hydrogen peroxide is possible but requires much higher concentrations of the oxidant and much longer incubation times (Sahlin et al., 1990; Fontecave et al., 1990b). Oxygen has to date been the only oxidant capable of accepting electrons from reduced R2; electrochemical oxidations of reduced R2 and ferricyanide have been unsuccessful (Sahlin et al., 1989). Again, the small size and electrical neutrality of hydrogen peroxide may explain its reactivity toward the iron center. A similar reaction has previously been observed with the diferrous center of deoxyhemerythrin, which is oxidized by H_2O_2 to give methemerythrin (Armstrong & Sykes, 1986).

Conclusion. In view of the limited accessibility of the redox centers of protein R2, it is difficult to understand how the monosubstituted hydrazines and hydroxylamines discussed above manage to transfer their electrons to the ferric ions and the tyrosyl radical. The refined structure does not show any obvious access routes from the surface to the buried redox sites and strongly supports electron transfers occurring over relatively long distances. On the other hand, the large variations observed in this study, in the kinetic accessibility of the redox sites for the different electron donors, may also be interpreted in terms of the differences in the extent of penetration of the redox agent into the protein. This would be possible only if considerable conformational freedom of the polypeptide chain might exist in solution. The discrimination between the two terms of the alternative requires further studies.

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Direct Evidence for Singlet–Singlet Energy Transfer in *Escherichia coli* DNA Photolyase†

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ABSTRACT: The active form of native *Escherichia coli* DNA photolyase contains 1,5-dihydro-FAD ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4Pte$(Glu)_n$]. Enzyme containing $FADH_2$ and/or 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) can be prepared in reconstitution experiments. Fluorescence quantum yield measurements at various wavelengths with native or reconstituted enzyme provide a simple method for detecting singlet–singlet energy transfer from pterin to $FADH_2$, a key step in the proposed catalytic mechanism. The data satisfy the following criteria: (1) Wavelength-independent quantum yield values are observed for 5,10-CH^+-H_4folate in the absence (0.434) or presence (3.57×10^{-2}) of $FADH_2$, for 5,10-CH^+-H_4Pte$(Glu)_n$ in the presence of $FADH_2$ (5.58×10^{-2}) and for $FADH_2$ in the absence of pterin (5.34×10^{-3}); (2) The observed decrease in pterin fluorescence quantum yield in the presence of $FADH_2$ can be used to estimate the efficiency of pterin fluorescence quenching (E_Q = 0.918 or 0.871 with 5,10-CH^+-H_4folate or 5,10-CH^+-H_4Pte$(Glu)_n$, respectively); (3) The fluorescence quantum yield of $FADH_2$ is increased in the presence of pterin and varies depending on the excitation wavelength, in agreement with the predicted effect of energy transfer on acceptor fluorescence quantum yield [$\Phi_{\text{acceptor (+ donor)}}/\Phi_{\text{acceptor (alone)}} = 1 + E_{ET}(\epsilon_{donor}/\epsilon_{acceptor})$, where E_{ET} is the efficiency of the energy transfer process]. With 5,10-CH^+-H_4Pte$(Glu)_n$ in native enzyme the value obtained for E_{ET} (0.92) is similar to E_Q, whereas with 5,10-CH^+-H_4folate in reconstituted enzyme the value obtained for E_{ET} (0.46) is 2-fold smaller than E_Q. The results indicate that the observed quenching of pterin fluorescence in native enzyme is entirely due to energy transfer to $FADH_2$.

DNA photolyase repairs pyrimidine dimers in UV-damaged DNA in a reaction which requires visible light. The active form of the enzyme from *Escherichia coli* contains 1,5-dihydro-FAD[1] ($FADH_2$) plus 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4Pte$(Glu)_n$, n = 3-6] (Wang et al., 1988; Wang & Jorns, 1989; Jorns et al., 1990; Johnson et al., 1988). [A reversible oxidation of $FADH_2$ to a blue neutral flavin radical ($FADH^\bullet$) occurs during enzyme

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^\bullet$, blue neutral FAD radical; $FADH_2$, 1,5-dihydro-FAD; 5,10-CH^+-H_4Pte$(Glu)_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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