Reduced Forms of the Iron-Containing Small Subunit of Ribonucleotide Reductase from *Escherichia coli*†

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ABSTRACT: The B2 subunit of ribonucleotide reductase from *Escherichia coli* contains a stable tyrosyl free radical and an antiferromagnetically coupled dimeric iron center with high-spin ferric ions. The tyrosyl radical is an oxidized form of tyrosine-122. This study shows that the B2 protein has a fully reduced state, denoted reduced B2, characterized by a normal nonradical tyrosine-122 residue and a dimeric ferrous iron center. Reduced B2 can be formed either from active B2 by a three-electron reduction in the presence of suitable mediators or from apoB2 by addition of two equimolar amounts of ferrous ions in the absence of oxygen. The oxidized tyrosyl radical and the ferric iron center can be generated from reduced B2 by the admission of air. The tyrosyl radical can be selectively reduced by one-electron reduction in the presence of a suitable mediator, yielding metB2, a form that seems identical with the form resulting from treatment of active B2 with hydroxyurea. ^{1}H NMR was used to characterize the paramagnetically shifted resonances associated with the reduced iron center. Prominent resonances were observed around 45 ppm (nonexchangeable with solvent) and 57 ppm (exchangeable with solvent) at 37 °C. From the temperature dependence of the chemical shifts of these resonances it was concluded that the ferrous ions in reduced B2 are only weakly, if at all, antiferromagnetically coupled. By comparison with data on the similar iron center of deoxyhemerythrin it is suggested that the 57 ppm resonance should be assigned to protons in histidine ligands of the iron center.

Ribonucleotide reductase from *Escherichia coli* consists of two nonidentical subunits, proteins B1 and B2. The active enzyme is a 1:1 complex of the two subunits. Protein B1 carries the redox-active dithiol groups and provides substrate binding sites, which accept all four ribonucleoside diphosphates. This subunit also contains two types of regulatory sites, which bind allosteric nucleoside triphosphates (Thelander & Reichard, 1979; Lammers & Follman, 1983). Protein B2 consists of two identical polypeptide chains, each with 375 amino acids. It carries a stable free radical on tyrosine-122 and a binuclear iron center, both of which are required for enzymatic activity (Atkin et al., 1973; Petersson et al., 1980; Larsson & Sjöberg, 1986). The radical is an oxidized form of the tyrosine residue with spectroscopic properties similar to that of a model phenoxy radical (Land et al., 1961). The iron center is an antiferromagnetically coupled pair of high-spin ferric ions connected by a μ-oxo bridge (Petersson et al., 1980). The two identical polypeptide chains of protein B2 together carry one binuclear iron center and one tyrosyl radical.

The tyrosyl radical and the iron center have been characterized by a variety of spectroscopic techniques (Atkin et al., 1973; Petersson et al., 1980; Gräslund et al., 1985; Sjöberg et al., 1982). So far, three different forms of protein B2 are known: (1) active B2, the originally isolated form with tyrosyl radical and iron center; (2) metB2, a nonradical form, with tyrosine-122 appearing as a normal tyrosine residue, but with an intact iron center (earlier denoted B2/HU, because it is easily obtained by treatment of active B2 with the radical scavenger hydroxyurea); and (3) apoB2, a form without both free radical and iron. The aim of the present study[1] was to find conditions to produce a fully reduced state of protein B2, to characterize this state, and to investigate possible ways to reoxidize the protein.

The biological importance of a reduced form of protein B2 is suggested from studies of an enzyme system in *E. coli* capable of reactivating the nonradical form of protein B2 (Eliasson et al., 1986; Fontecave et al., 1987). A NADPH–flavin oxidoreductase is part of the enzyme system, and the reaction may involve reduction of the iron center of metB2, which then spontaneously reacts with oxygen to generate the active iron center and the free radical. This reaction model indicates that a fully reduced state of protein B2 may be of importance for the regulation of ribonucleotide reductase activity.

The iron center of protein B2 is very similar to that found in the oxidized forms of hemerythrin, an oxygen-transporting protein in several marine invertebrates. Hemerythrins have been characterized in different redox states: fully oxidized (methemerythrins, oxyhemerythrin), half-reduced (semimethemerythrins), and fully reduced (deoxyhemerythrin). A variety of spectroscopic information is available which characterizes the iron center in these different forms of hemerythrin (Wilkins & Harrington, 1983; Elam et al., 1983; Armstrong et al., 1984; Shiemke et al., 1986). It is therefore a valuable model system for the iron center of protein B2, and we will make frequent comparisons between protein B2 and hemerythrin in the text.

Experimental Procedures

Materials. D_2O, 99.97% pure, was obtained from Studsvik Energiteknik AB, Sweden. Methylviologen hydrate was obtained from Janssen Chimica, benzylviologen from Sigma, phenosafranine from BDH Chemicals, and 2,6-dichloro-

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[1] A preliminary note on parts of this work has previously been presented at The Biophysical Society 32nd Annual Meeting, Phoenix, AZ (Gräslund et al., 1988).

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phenolindophenol (DCPIP)[2] from Fluka AG. Hydroxyurea was obtained from Calbiochem-Behring Corp. Potassium phosphate buffer in D_2O was prepared by freeze-drying a stock solution of 500 mM KP_i, pH 7.5, and dissolving it in D_2O. The freeze-drying procedure was repeated twice.

Protein Preparations. Active protein B2 was prepared from a B2-overproducing strain of *E. coli* as described earlier (Sjöberg et al., 1986). MetB2 was prepared by incubating active protein B2 (2.7 mM in protein) with 100 mM hydroxyurea for 15 min at ambient temperature. The protein was then diluted 10 times by addition of 50 mM Tris-HCl or KP_i buffer, pH 7.5, and concentrated by using a Centricon 30 filtration device (Amicon) at 4 °C. The dilution–concentration procedure was repeated four times to decrease the concentration of hydroxyurea to ≤ 10 μM. The final concentration of metB2 was 2.5 mM. ApoB2 was prepared as described by Atkin et al. (1973).

Buffer exchanges on protein preparations for NMR samples were performed by diluting the protein 8–10 times in 50 mM KP_i, pH 7.5, in H_2O or D_2O and then concentrating the samples by using the Centricon 30 filter device at 4 °C for 2 h. This procedure was repeated 4–5 times.

Anaerobic Reductive Titrations. Anaerobic titrations with dithionite and mediators were performed in EPR tubes fitted with airtight stopcocks. Argon gas was deoxygenated and humidified by passing through two consecutive gas wash bottles containing 2% dithionite in 0.1 M NaOH and 0.2% dithionite in 0.01 M NaOH, respectively. The latter also contained phenosafranine as an indicator of anaerobicity. The system was left running overnight to ensure argon saturation of the gas wash bottles. Argon-flushed EPR tubes were then filled with argon-flushed buffer prior to addition of protein and mediator. Additions of dithionite of known concentration were made by using a gas-tight argon-flushed Hamilton syringe with a 30-cm stainless steel needle. The dithionite (ca. 7 mM) was dissolved in an argon-flushed 5 mM NaOH/5 mM Na_2HPO_4 buffering solution. The actual concentration of dithionite was determined by titration against ferricyanide (Ehrenberg, 1962). The protein concentrations were typically 0.2 mM and the mediator concentrations in the range 0.02–0.06 mM.

Preparation of NMR Samples. NMR samples of reduced B2 were prepared in an anaerobic cell specifically designed for anaerobic treatment of small volumes (Petersson & Ehrenberg, 1985). ApoB2 was added to deoxygenated buffer, 50 mM KP_i, pH 7.5, contained in a small beaker inside the anaerobic cell. Deoxygenation at 4 °C was performed by flowing humidified argon gas over the sample surface for 2 h while stirring. Ferrous ions in the form $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, dissolved in anaerobic H_2O or D_2O, were added in equivalent amounts (i.e., two times the concentration of protein) to the protein, and the sample was incubated for 5–10 min. Then 100 μL of the sample was transferred anaerobically to an argon-filled microcell (Wilmad Glass Co.) by means of an argon-filled Hamilton syringe. The microcell was immediately sealed by melting the capillary. Anaerobicity of the samples was checked by recording optical spectra directly in the sealed microcells. The NMR samples with reduced B2 contained 1–8% active B2, i.e., oxidized protein. The lower limits of oxidized protein were achieved when dithionite (0.1 mM final concentration) was added to diminish the degree of oxidation during transfer of samples to the microcell. NMR samples of active B2 were generally not deoxygenated, since aerobically and anaerobically prepared samples show identical NMR spectra in the 15–80 ppm region. The NMR samples had a B2 concentration of ≥ 1.4 mM.

Light Absorption Spectra. Spectra were recorded on an Aminco DW-2 UV–vis spectrophotometer using an adapter to permit the use of EPR tubes as cuvettes.

Extinction Coefficients. To determine the concentration of iron and radical in active B2, as well as the iron content in metB2, the extinction coefficients determined by Petersson et al. (1980) were used. However, to determine the relative radical concentration during the reductive titrations, the peak height at 410 nm was measured relative to a fitted base line, i.e., a straight line between the absorbances at 400 and 420 nm.

EPR Measurements. EPR measurements were performed on a Varian E9 EPR system as described previously (Sahlin et al., 1987). Samples were frozen with liquid nitrogen, and spectra were recorded at 10 K, using an Oxford Instrument helium temperature control system.

NMR Measurements. ^1H NMR spectra (400 MHz) were recorded at different temperatures on a Jeol GX 400 spectrometer, using a super-WEFT pulse sequence (Inubushi & Becker, 1983) to suppress the water signal. A 80-kHz bandwidth and 16K data points were used; 50000 or more transients were collected. Since the paramagnetically shifted resonances studied have widths of typically >1000 Hz, we could improve the signal to noise ratio by using a broadening factor of 100 Hz without distorting the line. Chemical shifts are expressed in parts per million from the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; 0.2 mM) with water as a secondary standard. The microcells were used immersed in CCl_4. In order to obtain stable temperatures down to 10 °C, the dried cooling air was passed through a heat exchanger immersed into a cooling bath with ethanol kept at −16 °C and via a styrofoam-isolated stainless steel transfer tube fed into the standard Dewar insert of the NMR probe containing the heater and sensor system for fine regulation of the temperature.

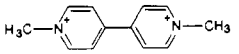
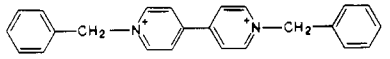
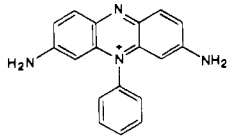
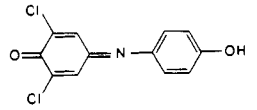
Results and Discussion

Spectroscopic Characteristics of Active B2 and MetB2. In active B2 both the iron center and the radical are in oxidized states. The binuclear iron center consists of two high-spin ferric ions linked by a μ-oxo bridge. The two ferric ions are antiferromagnetically coupled with $J = -108$ cm^{-1} (Petersson et al., 1980) and may thus not be directly observed with EPR. However, light absorption spectra in the near-UV and visible regions display characteristic features of the iron center, on which contributions from the tyrosyl radical are superimposed. The absorption spectrum of the iron center alone is obtained with metB2. It displays prominent bands at 370 and 325 nm, and it is very similar to that recorded for the oxidized forms of hemerythrins (Armstrong et al., 1984). Mössbauer spectra have shown that the two iron ions are in inequivalent environments (Atkin et al., 1973).

The light absorption spectrum of active B2 shows a sharp peak at 410 nm, which originates from the radical. The radical also contributes to the absorbance at 370 and 325 nm (Petersson et al., 1980). The radical is an oxidized form of tyrosine-122 which is a conserved amino acid residue in all iron-containing subunits of ribonucleoside diphosphate reductases (Larsson & Sjöberg, 1986; Slabaugh et al., 1988). EPR studies have also demonstrated that the tyrosyl radical is close enough to the iron center to exhibit enhanced relaxation

[2] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DT, dithionite; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; MCD, magnetic circular dichroism; Tris, tris(hydroxymethyl)aminomethane.

Table I: Properties of Chemicals Used in the Reduction Experiments of Ribonucleotide Reductase

compound	formula	midpoint potential $E_{1/2}$ (mV)	charge in reduced state	comment	reference
dithionite	$S_2O_4^{2-}$	−460	−2	at pH 7	Latimer, 1952
methylviologen		−446	+1	pH independent	Clark, 1960
benzylviologen		−359	+1	pH independent	Clark, 1960
phenosafranine		−252	0	at pH 7.0	Clark, 1960
DCPIP		217	0		Clark, 1960
ferricyanide	$Fe(CN)_6^{3-}$	360	−3	potential at zero ionic strength	Clark, 1960 (p 319)

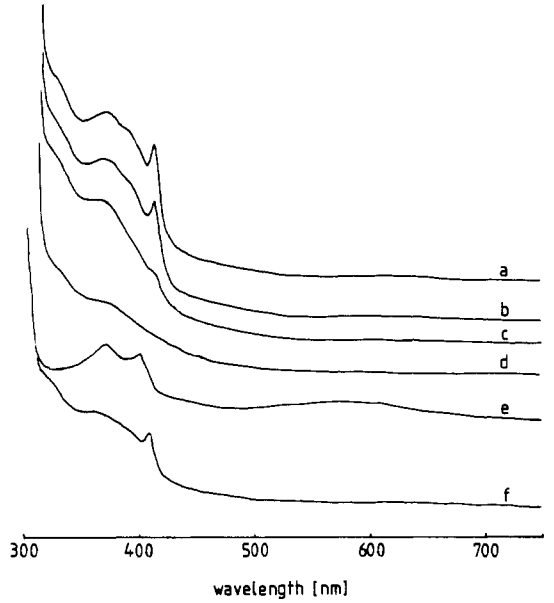


FIGURE 1: Reductive titration of active B2 with benzylviologen as a mediator. Protein B2 (30 nmol) with 4.9 nmol of benzylviologen was titrated with small amounts of 5.9 mM dithionite (DT): (a) before addition of DT; (b) after addition of 11.8 nmol of DT; (c) after addition of 29.5 nmol of DT; (d) after addition of 41.3 nmol of DT; (e) after addition of 90 nmol of DT; (f) after oxidation with 6 mL of air. The light absorption spectra are not compensated for dilution of the sample by additions of DT. The spectra were recorded under anaerobic conditions in closed EPR tubes with an innner diameter of 3.0 mm, and the absorption range was 0.5 OD units for the full scale. The spectra were normally registered 3 min after addition of DT.

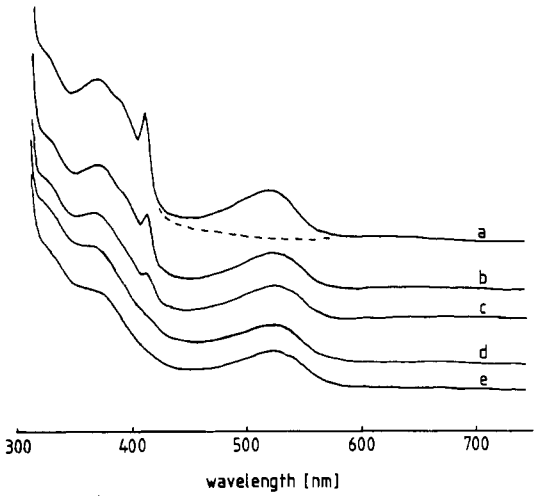


FIGURE 2: Reductive titration of active B2 with phenosafranine as a mediator. B2 (30 nmol) with 3 nmol phenosafranine was titrated with 2.7 mM dithionite (DT): (a) before addition of DT; the dashed line indicates the spectrum in absence of PS; (b) after addition of 10.8 nmol of DT; (c) after addition of 21.6 nmol of DT; (d) after addition of 27.0 nmol of DT; (e) after addition of 37.8 nmol of DT. Recording conditions were as in Figure 1, except that the spectra were normally registered 6–10 min after addition of DT.

rates as compared to an isolated tyrosyl radical (Sahlin et al., 1987). However, there is no indication from resonance Raman, EPR, or NMR spectroscopy that the tyrosyl radical should be a direct ligand to the iron center.

Chemical Reduction of Protein B2. Addition of dithionite in millimolar concentration to an anaerobic solution of active B2 (ca. 0.2 mM) produced no significant changes in the iron center or in the free radical, as judged from light absorption and EPR spectra. However, in the presence of a suitable mediator, reduction of both the free radical and the iron center took place. The structures and chemical properties of the mediators used are shown in Table I. Figure 1b–e shows a series of light absorption spectra obtained after successive additions of dithionite to protein B2 in the presence of benzylviologen as a mediator. Immediately upon addition of dithionite the light absorption spectra have weak contributions from the reduced mediator in the region where protein B2 absorbs. However, since the oxidized mediator lacks absorption bands in the studied region, these contributions disappear after a few minutes, when the mediator has delivered the electrons corresponding to the amount of dithionite added. In Figure 1d we have reached a stage where the light absorption of the free radical has disappeared and only a small fraction of the iron center contributes to the light absorption. Addition of excess dithionite at this point produced only the light absorption of reduced benzylviologen (Figure 1e). When finally air was introduced into the sample, a partial regeneration of both the iron center and the radical occurred (Figure 1f and Table II). It was not possible to reoxidize the system by addition of ferricyanide under anaerobic conditions.

The weaker reducing agent phenosafranine (Table I) is also active as a mediator in the reduction of protein B2 (Figure 2). The time needed to reduce the radical in the presence of phenosafranine is about three times longer than with benzylviologen. Several experiments with phenosafranine show that it is possible to obtain an intermediate state, in which the

Table II: Correlation between Air-Dependent Reconstitution of Tyrosyl Radical and Extent of Prior Reduction of the Iron Center in Protein B2

protein	mediator	abs of iron center after reduction and immediately before admission of air[a] (A)	abs after admission of air[a]		admission of air: ratio of abs of Tyr radical and increased abs of iron center[b] $[C/(B-A)]$
			iron center (B)	Tyr radical (C)	
active B2	benzylviologen	0[c]	0.8–0.9	0.54	0.6–0.7
		0.50	0.98	0.33	0.7
		0[c]	1.13[d]	0.76	0.7
active B2	phenosafranine	0.44	0.76	0.24	0.8
		0.56	0.96 (−1.2)	0.42	1.1
		0.84	≈0.93	0.08	0.9
		0.90[e]	0.95	0.02	(0.4)
metB2	benzylviologen	0[c]	1.17	0.71	0.6
		0.23	1.02	0.50	0.6
		0.40	0.95	0.40	0.7
metB2	phenosafranine	0.42	0.75	0.40	1.2

[a] Relative values. [b] $B - A$ denotes the extent of reduction of the iron center. [c] In the light absorption spectrum only contribution of the reduced benzylviologen spectrum was observed. [d] Initially reduced by phenosafranine, later benzylviologen added. [e] Compensated for extinction coefficient of metB2.

radical is entirely reduced while the iron center remains unchanged (Figure 2d). If the phenosafranine-dependent titration was interrupted when the ferric iron center was still largely intact and air was admitted to the sample at this point, practically no regeneration of the free radical occurred (Table II). Judging from the light absorption, the form of protein B2 obtained after a selective reduction of only the free radical (Figure 2d) should be equivalent to the metB2 form, which is obtained after treatment of active B2 with hydroxyurea.

A few preliminary reduction experiments were also performed using a thin electrochemical cell with a gold grid electrode (Heineman et al., 1978). The cell was fitted into the spectrophotometer so that the light absorption could be monitored directly during the reduction. In this experimental setup the mediators methylviologen and DCPIP were used (Table I). Protein B2 was readily reduced by methylviologen, but it was not possible to reduce the protein in the presence of DCPIP. Furthermore, it was not possible to reoxidize the reduced protein electrochemically under anaerobic conditions (data not shown). This is in analogy with the observation above that the reduced state was inert to direct oxidation by ferricyanide.

Quantitative Evaluation of the Chemical Reduction. In order to reach a quantitative estimate of the stoichiometry of the reduction, data from several experiments were compiled. Figure 3 shows a summary of the amounts of dithionite needed to reduce the free radical and iron center of active B2 in the presence of either benzylviologen or phenosafranine. It is obvious that addition of 1 equiv of dithionite reduces the free radical completely, whereas the iron center remains oxidized. An additional 2 equiv of dithionite are needed to reduce the iron center. Thus 3 equiv of dithionite are needed to completely reduce active B2, indicating that the oxidized radical-carrying tyrosine-122 has returned to its nonradical state and the two ferric ions have been reduced to their ferrous form.

The stoichiometry of the reduction was also studied with metB2 as the starting material. Figure 4b–d shows a series of benzylviologen-mediated reductive titrations of metB2. Similarly to what was observed in the reduction of active B2, a metB2 sample can, after complete reduction, be recovered to active B2 after the admission of oxygen (Figure 4e and Table II). Figure 5 shows a quantitative summary of reductive titrations of metB2 in the presence of benzylviologen or phenosafranine. In this case 2 equiv of dithionite is needed for full reduction.

The chemistry of dithionite is complex, and it has, e.g., been shown to function as a two-electron reductant with proteins like ferredoxin and metmyoglobin (Lambeth & Palmer, 1973).

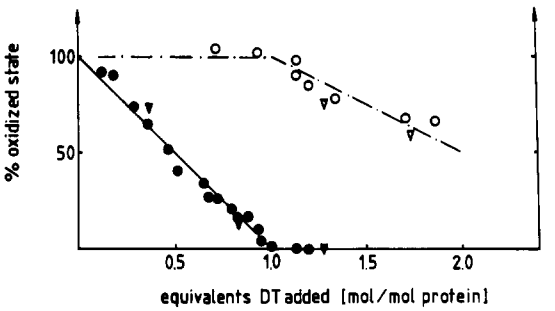


FIGURE 3: Quantitative results from mediated titrations of active B2 with dithionite (DT). Relative amounts (in percent) of oxidized radical (filled symbols) and iron center (open symbols) as a function of added equivalents of dithionite. (●, ○) denotes phenosafranine-mediated reduction, and (▼, ▽) denotes benzylviologen-mediated reduction, respectively. Data from four different experiments are summarized in the figure. Corrections have been made for the remaining dissolved oxygen at the beginning of the experiments, corresponding to 0–1.0% of dissolved oxygen at ambient temperature.

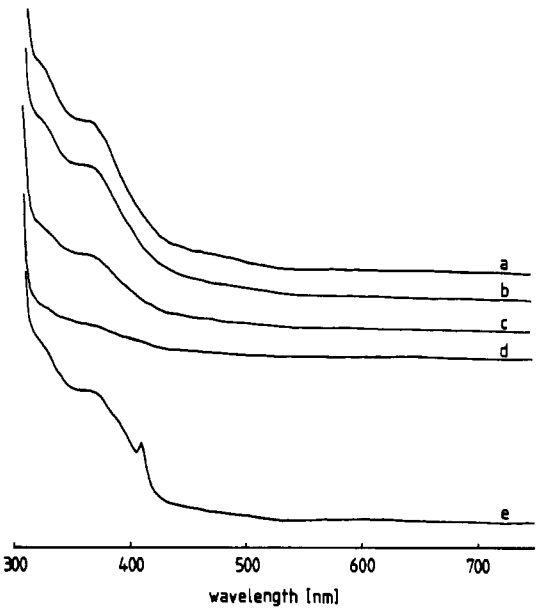


FIGURE 4: Reductive titration of metB2 with benzylviologen as a mediator. MetB2 (25 nmol) with 2.5 nmol of benzylviologen was titrated with 2.6 mM dithionite (DT): (a) before addition of DT; (b) after addition of 5 nmol of DT; (c) after addition of 27.5 nmol of DT; (d) after addition of 46 nmol of DT; (e) after oxidation with 0.75 mL of air. Recording conditions were as in Figure 1.

However, it has also been shown that hemerythrin is reduced to semimethemerythrin upon addition of 1 equiv of dithionite

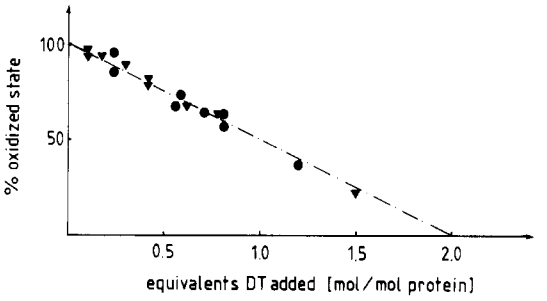


FIGURE 5: Quantitative results from mediated titrations of metB2 with dithionite (DT). Relative amounts (in percent) of oxidized iron center as a function of added equivalents of dithionite. (●) denotes phenosafranine-mediated reduction, and (▼) denotes benzylviologen-mediated reduction. Data from four different experiments are summarized in the figure. Corrections have been made for remaining dissolved oxygen at the beginning of the experiment, corresponding to 0–1.9% of dissolved oxygen at ambient temperature.

(Irwin et al., 1983), hence functioning as a one-electron reductant. Dithionite has also been found to function as a one-electron reductant in other systems (Husain et al., 1987). Given the knowledge of the chemical properties of the iron center and radical site of B2, and the fact that 1 equiv of dithionite reduces the tyrosyl radical and 2 equiv the iron center, we conclude that dithionite acts as a one-electron reductant in our experiments.

The reduction and reoxidation studies on protein B2 provide the first evidence for the existence of a stable, fully reduced form of protein B2. One important question concerns the fate of the iron center after reduction and whether ferrous iron is still liganded to the protein. One indication that reduced B2 contains a ferrous iron center came from an experiment where the metal chelator EDTA was present in the sample. There was no difference in the aerobic reoxidation of the iron center and the free radical in the presence or absence of EDTA (0.2 mM) (data not shown), indicating that the ferrous ions are neither in solution nor accessible to the chelator but must be bound to the protein.

What are the requirements for generation of the tyrosyl radical? The results presented in Table II summarize all reduction experiments, which have been followed by air oxidation. It is obvious that the extent of reconstitution of tyrosyl radical is directly correlated with the amount of ferrous iron produced prior to admission of air. Regardless of starting material (active B2 or metB2), mediator (benzylviologen or phenosafranine), and extent of reduction of the iron center ($B - A$ in Table II), approximately one tyrosyl radical is formed per reduced iron center upon air oxidation (Table II, last column). These results clearly demonstrate that a reduced iron center is a prerequisite for the generation of the tyrosyl radical via air-dependent oxidation. These results also corroborate indirect evidence obtained previously in reconstitution experiments with apoB2 and ferrous iron (Petersson et al., 1980), and in studies of the enzymatically catalyzed reactivation of metB2 (Fontecave et al., 1987).

Conditions Required for Reduction. In general the efficiency of a reducing agent or mediator to transfer electrons to a protein is governed by its redox potential as well as its ability to gain access to the site of reduction. The observations that methylviologen, benzylviologen, and phenosafranine are able to mediate the reduction of protein B2, whereas DCPIP is not, and dithionite itself without any mediator is inactive in spite of its low redox potential (cf. Table I) indicate that the reduction of B2 is not solely governed by the redox potential of the mediator. The fact that the negatively charged dithionite is inactive, whereas the positively charged mediators with considerably higher $E_{1/2}$ are active, would suggest that charge is a determining factor for accessibility and that the iron centers of active B2 and metB2 are negatively charged and/or surrounded by negative charges. The difference in rates between the benzylviologen- and phenosafranine-mediated reductions might originate from their redox potential difference, but it could as well depend on their difference in charge and/or geometrical topology (cf. Table I).

All the reduction experiments described here have to be considered as reduction titrations and not redox potential determinations, since the reduction process appears nonreversible under the anaerobic conditions so far explored. As far as is known, only oxygen reoxidizes the reduced iron center and regenerates the free radical. For these reasons no definite value can at this stage be estimated for any redox potential characterizing the iron center and the tyrosyl radical of protein B2, except possibly an approximate lower limit around −200 mV estimated from the highest redox potential among the active mediators. The apparent need for oxygen in the reoxidation reaction might indicate that its function is not only that of an oxidizing agent. In fact, oxygen may bind to the reduced iron center and after protonation give rise to a hydroxide ligand in active B2 and metB2. However, the presence of such a ligand has so far not been demonstrated (Backes et al., 1989).

^{1}H NMR Studies of the Reduced Form of Protein B2. The data described so far clearly show that the ferric iron center of protein B2 can be chemically reduced. The reconstitution experiments indicate that the reduced form of protein B2 contains a ferrous iron center. For a better understanding of the fully reduced state of protein B2 we have examined the system by ^{1}H NMR. In order to obtain a well-defined reduced B2, we started with a known molar amount of the apoB2 form and added twice the molar amount of ferrous ions under anaerobic conditions. If the ferrous iron is bound in a binuclear fashion, this method should result in one reduced binuclear iron center per B2 protein.

The ^{1}H NMR spectrum of an anaerobic mixture of apoB2 and Fe^{2+} exhibits resonances at 45 and 57 ppm at 37 °C (Figure 6a). As a comparison, NMR spectra of apoB2 show no resonances in the region 10–80 ppm (Figure 6e). Hence we conclude that the observed resonances in Figure 6a are paramagnetically shifted due to the presence of the ferrous irons in reduced B2. The intensity ratio of the peaks at 45 and 57 ppm appears to be approximately 1:1. The 45 ppm resonance is visible in the NMR spectra obtained with D_2O- as well as H_2O-containing buffers and therefore arises from one or more nonexchangeable protons (Figure 6c). The 57 ppm resonance exchanges with the aqueous solvent (Figure 6c).

The portions of the samples not transferred to the microcells were finally exposed to air, allowing normal reoxidation to occur. Light absorption spectra on these samples indicate a normal regeneration of the radical and the iron center with approximately one radical per B2. In one case the sealed NMR tube was opened at the end of the experiment, and the protein was withdrawn and mixed extensively with air to allow oxidation. After a brief centrifugation to remove a minor amount of precipitated material, the sample was again transferred to the same microcell and the same NMR tube, and an aerobic NMR spectrum was collected (Figure 6f). The oxidized sample lacked completely the 45 and 57 ppm resonances present before the exposure to air. Instead it exhibited the 19 and 24 ppm resonances characteristic of active B2 samples (Figure 6b,f).

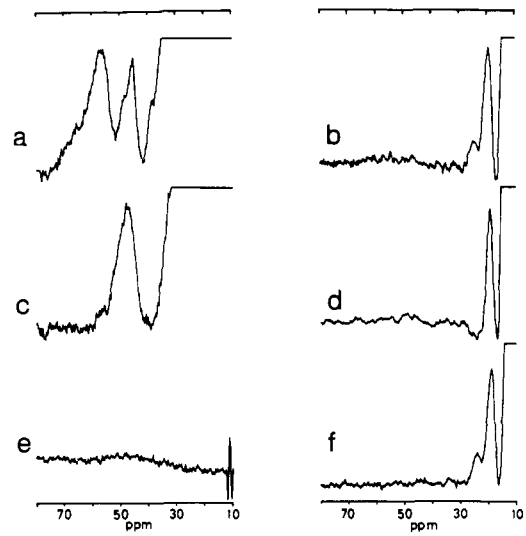


FIGURE 6: 400-MHz ^1H NMR spectra of protein B2 in different oxidation states: (a) 1.4 mM apoB2 + 2.8 mM Fe^{2+}, i.e., reduced B2, at 37 °C, H_2O, 50 000 transients; (b) 1.5 mM active B2 at 37 °C, H_2O, 50 000 transients; (c) 1.65 mM reduced B2 at 37 °C, D_2O, 50 000 transients; (d) 2.2 mM active B2 at 30 °C, D_2O, 50 000 transients; (e) 1.4 mM apoB2, at 25 °C, H_2O, 100 000 transients; (f) 1.4 mM reduced B2 after air oxidation, i.e., active B2, at 20 °C, H_2O, 50 000 transients. All samples were in 50 mM KP_i buffer, pH 7.5; H_2O samples contained 15% D_2O for the spectrometer lock.

Both paramagnetically shifted resonances of reduced B2 are in a region where one would expect to find resonances from protons in ligands of weakly coupled high-spin ferrous ions. However, the resonance at 57 ppm is in a region where one would also find mononuclear high-spin ferrous imidazole complexes (57–79 ppm) (Inubushi & Yonetani, 1983; Lauffer et al., 1983). In order to find out if the high-spin ferrous ions are indeed weakly antiferromagnetically coupled, we have made a temperature dependence study of the paramagnetically shifted resonances. The result is shown in Figure 7a for both the 45 and the 57 ppm resonances. The temperature variation for the resonances from active B2 is shown in Figure 7b. The lack of temperature dependence is consistent with the strong antiferromagnetic coupling of $J = -108$ cm^{-1} determined from susceptibility measurements (Petersson et al., 1980).

In the case of reduced B2 the observed temperature dependence of the 57 ppm resonance was modeled assuming the shift to be the sum of diamagnetic and contact shifts. If this resonance is assumed to originate from NH protons of the imidazole ring of histidine, then the diamagnetic shift should be around 8 ppm in analogy with NH resonances in random coil proteins (Wüthrich, 1986). The isotropic contact shift is due to hyperfine interaction between the proton(s) and the paramagnetic center. The temperature dependence of this shift originates only from the magnetic susceptibility of the center, provided that the hyperfine coupling is considered temperature independent. With the theoretical temperature dependence for the susceptibility of the coupled pair of high-spin ferrous ions calculated as outlined by Mabbs and Machin (1973), a best fit of the model to the experimental data was obtained for $J = -5$ cm^{-1} (Figure 7a). Due to the uncertainties in the measured points, the range of uncertainty for this value is estimated to be of the order of ± 5 cm^{-1}. However, it cannot be excluded that the interaction involves also a dipolar term, which would contribute a T^{-2} temperature dependence to the shift. As a consequence, the temperature dependence due to contact interaction would be less than assumed in our fitting procedure and correspond to a more negative value of J, i.e., a stronger antiferromagnetic interaction in the iron center.

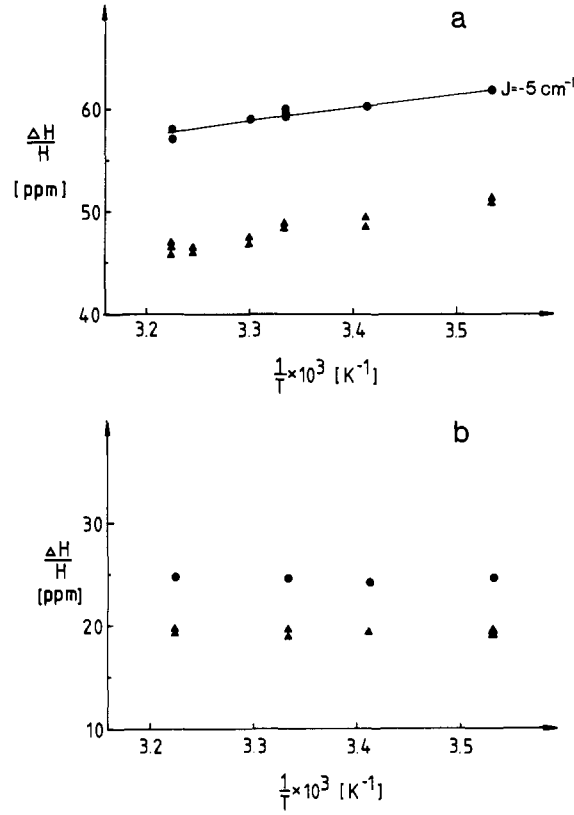


FIGURE 7: Temperature dependence of the observed paramagnetically shifted exchangeable (●) and nonexchangeable (▲) proton resonances in reduced B2. The full line represents the best fit between experimental and calculated values, for the exchangeable resonance, which was found for $J = -5$ cm^{-1} (see text). (b) Temperature dependence of the observed paramagnetically shifted exchangeable (●) and nonexchangeable (▲) proton resonances in active B2.

Since the resonance at 45 ppm has not been assigned to a specific amino acid side chain, no fitting of the model has been done in this case. In conclusion, our data indicate that the antiferromagnetic interaction in the iron center of fully reduced B2 is weak, if it at all exists. More conclusive evidence should be obtained from magnetic susceptibility studies.

Comparison of ^1H NMR Data Obtained with Protein B2 and Hemerythrin. We have previously shown that aerobic protein B2 exhibits two paramagnetically shifted resonances (Sahlin et al., 1986), one nonexchangeable at 19 ppm and one solvent exchangeable at 24 ppm (Figure 6b,d). The intensity ratio of these peaks is appoximately 3:1. The resonance at 24 ppm in active B2 and metB2 was tentatively assigned to one or more histidine residues (Sahlin et al., 1986).

The iron center of oxidized forms of hemerythrin was at an early stage found to be a useful model for the iron center of protein B2. The similarities in the binuclear iron centers were manifested in several of their spectroscopic characteristics (Armstrong et al., 1984; Petersson et al., 1980; Shiemke et al., 1984). From the crystal structure of methemerythrin it is known that its iron center has the following ligands: one μ-oxo bridge, two μ-carboxylate bridges, and five protein-derived nitrogen ligands from histidines. Since each polypeptide chain of hemerythrin binds one binuclear iron center, all five histidine ligands have slightly different surroundings in the tertiary structure. The crystal structure of protein B2 is not known, but a prominent difference to hemerythrin is that protein B2 consists of two identical polypeptide chains, which together bind one binuclear iron center. One might thus expect the iron center of protein B2 to be more symmetric than that of hemerythrin, but from Mössbauer measurements it is known

that the two irons are inequivalent, not only in active B2 but also in metB2 (Atkin et al., 1973).

The NMR spectra of oxy- and methemerythrin show three or four broad, poorly resolved peaks in the 12–25 ppm region associated with solvent-exchangeable protons, and a resonance at 11 ppm associated with nonexchangeable protons. The exchangeable resonances are assigned to the imidazole protons of the five histidine ligands; the nonexchangeable resonance is assigned to the methylene protons of the two carboxylate bridges (Maroney et al., 1984). In analogy with hemerythrin we previously assigned the exchangeable 24 ppm resonance in protein B2 to histidine protons. In contrast to the NMR spectrum of hemerythrin, the 24 ppm resonance appears as a single, symmetric peak in protein B2, which may reflect that protein B2 has a less complex pattern of histidine ligands. There is no prominent correspondence in the NMR spectrum of protein B2 to the nonexchangeable 11 ppm resonance observed in oxidized forms of hemerythrin. However, a possible 11 ppm resonance is considerably more difficult to detect in B2 than in hemerythrin, because of the much lower iron to protein ratio in B2 as compared to hemerythrin. There is no correspondence in hemerythrin to the nonexchangeable 19 ppm resonance observed in B2.

In the same way a methemerythrin is a good model system for the active and met forms of protein B2, we may consider deoxyhemerythrin as a possible model system for the fully reduced form of protein B2. The ^1H NMR spectra of deoxyhemerythrin contain three solvent-exchangeable resonances at 43, 46, and 62 ppm, assigned to protons in iron-coordinated histidines (Maroney et al., 1986). The resonance at 62 ppm is the most prominent one. In analogy with the results from deoxyhemerythrin, we assign the exchangeable 57 ppm resonance in the reduced protein B2 to protons of the histidine ligands of the iron center. There is no nonexchangeable resonance further out than 30–35 ppm in deoxyhemerythrin, and there is thus no obvious corresponding resonance in deoxyhemerythrin to the 45 ppm resonance in reduced B2.

Recent EXAFS studies on protein B2 give evidence for a relatively high number of oxygen ligands in protein B2 as compared to hemerythrin (Bunker et al., 1986, 1987; Scarrow et al., 1986, 1987). The number of nitrogen ligands in B2 may be as low as two per iron center (Bunker et al., 1987). There are at least two possible candidates for histidine ligands in the primary structure of protein B2 as judged from an alignment of the known amino acid sequences of members of the protein B2 family (Sjöberg et al., 1985). Close to the conserved histidines are several conserved carboxylic amino acid residues, which could provide additional ligands. It is possible that the 19 ppm resonance in active B2 and metB2 and the 45 ppm resonance in reduced B2 are related and originate from methylene protons of liganded nonbridging carboxylic amino acid residues.

For deoxyhemerythrin it was suggested, after temperature dependence studies of the paramagnetic shifts of the resonances from the histidine residues, that the antiferromagnetic coupling between the ferrous ions must be quite weak ($J \geq -20$ cm^{-1}), indicating a possible hydroxo-bridged ferrous iron center instead of the μ-oxo bridge in met- and oxyhemerythrin. These results were in agreement with MCD studies of deoxyhemerythrin indicating a $J = -13$ cm^{-1} (Reem & Solomon, 1984, 1987). The results obtained with reduced protein B2 (Figure 7a) are also compatible with a model where the two irons are connected by a μ-hydroxo bridge. However, in order to corroborate this and firmly establish a J value of the reduced B2, magnetic susceptibility and MCD results are needed.

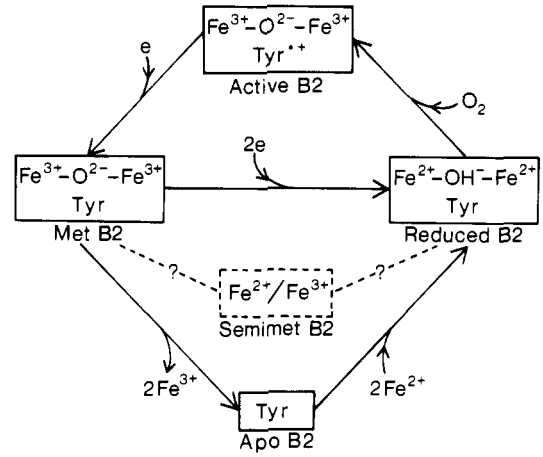


FIGURE 8: Summary of reduction–oxidation pathways of protein B2. Dotted lines indicate hypothetical pathways, suggested in the text. In active B2 the tyrosyl radical is an oxidized form of tyrosine-122. MetB2, reduced B2, and apoB2 contain a normal nonradical tyrosine-122. It is suggested that the irons in the reduced B2 are linked by a μ-hydroxo bridge. Also indicated in the figure is a semimet form of B2. This state has not yet been detected but is likely to exist in a transient state.

Structural and Mechanistic Considerations. The different oxidation states of protein B2 are depicted in Figure 8. Protein B2 of ribonucleotide reductase has a fully reduced state, denoted reduced B2, which is characterized by a normal nonradical tyrosine-122 residue and a binuclear ferrous iron center. Chemically reduced B2 can be formed from active B2 (via metB2) by a three-electron reduction, from metB2 by a two-electron reduction, or from apoB2 by addition of 2 equiv of ferrous iron under anaerobic conditions. The existence of a μ-hydroxo bridge in reduced B2, which is speculative at this point, is compatible with our data and would be in analogy with deoxyhemerythrin. Reduced B2 reacts spontaneously with oxygen to form active B2.

The tyrosyl radical of active B2 can be selectively reduced upon addition of 1 equiv of dithionite, or as shown earlier (Atkin et al., 1973) by treatment with the radical scavenger hydroxyurea, to generate metB2. The reaction pathway involving reduction of metB2 to reduced B2, followed by oxidation of reduced B2 to active B2, is formally equivalent to the enzymatic regenerating system described by Fontecave et al. (1987).

There are many spectroscopic similarities between the binuclear iron center of active B2 and metB2 and that of oxy- and methemerythrins. The physiological importance of the similarity may be fortuitous. However, the observation by Beinert [personal communication; cf. also Ehrenberg (1988)] of a free radical EPR signal from a sample of, supposedly, oxyhemerythrin suggests a possible functional relationship. In hemerythrin the normal reaction between the reduced iron center of deoxyhemerythrin and oxygen is the formation of oxyhemerythrin with a reversible di-ferric-peroxyl configuration (Shiemke et al., 1984). In protein B2 the reaction between the reduced iron center and molecular oxygen might lead to a similar di-ferric-peroxyl form, which, however, in this case, is not stable but immediately oxidizes the nearby tyrosine residue. A similar reaction could possibly occur in oxyhemerythrin under conditions of partial denaturation, e.g., at high or low pH.

The existence of a distinct reduced iron center suggests that also protein B2, like hemerythrin, might have a half-reduced iron center in the reaction sequence yielding a reduced state from an oxidized state and vice versa. During the course of

reductive titrations protein B2 samples were occasionally investigated by EPR close to liquid helium temperatures, to search for EPR signals arising from a possible transient half-reduced state of the iron center. So far we have not found any evidence for such a semimetB2 form. The semimet-like state may however be very short lived. It is possible that a positively charged mediator with a more positive midpoint potential than phenosafranine could prolong the time available to trap a half-reduced form of protein B2. Alternatively, the chances of trapping an intermediate state may be greater in an enzyme with a more open structure at the iron–radical center such as the mammalian enzyme (Kjøller Larsen et al., 1982).

In summary, the fully reduced state of protein B2 can in vitro be generated by two procedures: either by a reduction of active B2 or by anaerobic addition of ferrous iron to apoB2. Available evidence (i.e., observation in both cases of the direct regeneration of the ferric iron center and the tyrosyl radical by air) strongly suggests that both procedures give the same product. Further studies are required to characterize the detailed redox chemistry of the different forms of protein B2 and of their possible roles in the biochemical reaction catalyzed by ribonucleotide reductase.

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References

Armstrong, W. H., Spool, A., Papaefthymiou, G. C., Frankel, R. B., & Lippard, S. J. (1984) *J. Am. Chem. Soc. 106*, 3653–3667.

Atkin, C. L., Thelander, L., Reichard, P., & Lang, G. (1973) *J. Biol. Chem. 248*, 7464–7472.

Backes, G., Sahlin, M., Sjöberg, B.-M., Loehr, T. M., & Sanders-Loehr, J. (1989) *Biochemistry* (in press).

Bunker, G., Sjöberg, B.-M., Reichard, P., Petersson, L., Chance, B., Chance, M. (1986) *Fed. Proc. 45*, 1601.

Bunker, G., Petersson, L., Sjöberg, B.-M., Sahlin, M., Chance, M., Chance, B., & Ehrenberg, A. (1987) *Biochemistry 26*, 4708–4716.

Clark, W. M. (1960) *Oxidation–Reduction Potentials of Organic Systems*, Williams & Wilkins Co., Baltimore.

Ehrenberg, A. (1962) *Ark. Kemi 19*, 97–117.

Ehrenberg, A. (1988) *Chem. Scr. 28A*, 27–31.

Elam, W. T., Stern, E. A., McCallum, J. D., & Sanders-Loehr, J. (1983) *J. Am. Chem. Soc. 105*, 1919–1923.

Eliasson, R., Jörnvall, H., & Reichard, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2373–2377.

Fontecave, M., Eliasson, R., & Reichard, P. (1987) *J. Biol. Chem. 262*, 12325–12331.

Gräslund, A., Sahlin, M., & Sjöberg, B.-M. (1985) *Environ. Health Perspect. 64*, 139–149.

Gräslund, A., Sahlin, M., Sjöberg, B.-M., Petersson, L., & Ehrenberg, A. (1988) *Biophys. J. 53*, 69a.

Heineman, W. R. (1978) *Anal. Chem. 50*, 390–402.

Husain, M., Davidson, V. L., Gray, K. A., & Knaff, D. B. (1987) *Biochemistry 26*, 4139–4143.

Inubushi, T., & Becker, E. D. (1983) *J. Magn. Res. 51*, 128–133.

Inubushi, T., & Yonetani, T. (1983) *FEBS Lett. 160*, 287–290.

Irwin, M. J., Duff, L. L., Shriver, D. F., & Klotz, I. M. (1983) *Arch. Biochem. Biophys. 224*, 473–478.

Kjøller Larsen, I., Sjöberg, B.-M., & Thelander, L. (1982) *Eur. J. Biochem., 125*, 75–81.

Lambeth, D. O., & Palmer, G. (1973) *J. Biol. Chem. 248*, 6095–6103.

Lammers, M., & Follman, H. (1983) *Struct. Bonding (Berlin) 54*, 27–91.

Land, E. J., Porter, G., & Strachan, E. (1961) *Trans. Faraday Soc. 57*, 1885–1893.

Larsson, Å., & Sjöberg, B.-M. (1986) *EMBO J. 5*, 2037–2040.

Latimer, W. M. (1952) *Oxidation Potentials*, 2nd ed., Prentice Hall, New York.

Lauffer, R. B., Antanaitis, B. C., Aisen, P., & Que, L., Jr. (1983) *J. Biol. Chem. 258*, 14212–14218.

Mabbs, F. E., & Machin, D. J. (1973) *Magnetism and Transition Metal Complexes*, Chapman and Hall, London.

Maroney, M. J., Lauffer, R. B., Que, L., Jr., & Kurtz, D. M., Jr. (1984) *J. Am. Chem. Soc. 106*, 6445–6446.

Maroney, M. J., Kurtz, D. M., Jr., Nocek, J. M., Pearce, L. L., & Que, L., Jr. (1986) *J. Am. Chem. Soc. 108*, 6871–6879.

Petersson, L., & Ehrenberg, A. (1985) *Rev. Sci. Instrum. 56*, 575–580.

Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M., & Reichard, P. (1980) *J. Biol. Chem. 255*, 6706–6712.

Reem, R. C., & Solomon, E. I. (1984) *J. Am. Chem. Soc. 106*, 8323–8325.

Reem, R. C., & Solomon, E. I. (1987) *J. Am. Chem. Soc. 109*, 1216–1226.

Sahlin, M., Ehrenberg, A., Gräslund, A., & Sjöberg, B.-M. (1986) *J. Biol. Chem. 261*, 2778–2780.

Sahlin, M., Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M., & Thelander, L. (1987) *Biochemistry 26*, 5541–5548.

Scarrow, R. C., Maroney, M. J., Palmer, S. M., Que, L., Jr., Salowe, S. P., & Stubbe, J. (1986) *J. Am. Chem. Soc. 108*, 6832–6834.

Scarrow, R. C., Maroney, M. J., Palmer, S. M., Que, L., Jr., Roe, A. L., Salowe, S. P., & Stubbe, J. (1987) *J. Am. Chem. Soc. 109*, 7857–7864.

Shiemke, A. K., Loehr, T. M., & Sanders-Loehr, J. (1984) *J. Am. Chem. Soc. 106*, 4951–4956.

Shiemke, A. K., Loehr, T. M., & Sanders-Loehr, J. (1986) *J. Am. Chem. Soc. 108*, 2437–2443.

Sjöberg, B.-M., Loehr, T. M., & Sanders-Loehr, J. (1982) *Biochemistry 21*, 96–102.

Sjöberg, B.-M., Eklund, H., Fuchs, J. A., Carlson, J., Standart, N. M., Ruderman, J. V., Bray, S. J., & Hunt, T. (1985) *FEBS Lett. 183*, 99–102.

Sjöberg, B.-M., Hahne, S., Karlsson, M., Jörnvall, H., Göransson, M., & Uhlin, B. E. (1986) *J. Biol. Chem. 261*, 5658–5662.

Slabaugh, M., Roseman, N., Davis, R., & Mathews, C. (1988) *J. Virol. 62*, 519–527.

Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133–158.

Wilkins, R. G., & Harrington, P. C. (1983) *Adv. Inorg. Biochem. 5*, 52–85.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, pp 17–18, Wiley-Interscience, New York.