Iron and Free Radical in Ribonucleotide Reductase

EXCHANGE OF IRON AND MöSSBAUER SPECTROSCOPY OF THE PROTEIN B2 SUBUNIT OF THE
ESCHERICHIA COLI ENZYME*

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SUMMARY

Ribonucleotide reductase from Escherichia coli consists of two nonidentical subunits (Protein B1 and Protein B2), both required for activity. Protein B2 contains 2 iron atoms and a lesser, variable amount of a paramagnetic species, characterized by g = 2.0047 EPR and 410 nm electronic absorptions. Except for the 410 nm band, Protein B2 much resembles oxy- and methemerythrins in electronic spectra. The paramagnetic species is probably organic free radical rather than metal, is dependent upon the presence of iron in Protein B2, is directly correlated with enzymatic activity, and is destroyed by NH₂OH and hydroxyurea (EHRENBERG, A., and REICHARD, P. (1972) J. Biol. Chem. 247, 3485-3488).

We here describe complete removal of iron from Protein B2, reconstitution to active metalloenzyme, and Mössbauer spectroscopy of [¹⁷Fe]Protein B2. Addition of excess Fe²⁺ to metal-free protein yielded reconstituted/reactivated protein with 2:1 iron:subunit stoichiometry and a hemerythrin-like electronic spectrum, but with 120 to 390% of the original specific enzymatic activity and correspondingly increased 410 nm and EPR absorptions. NH₂OH- or hydroxyurea-inactivated protein was also converted to highly active enzyme subunit by the same iron substitution procedures.

Mössbauer and electronic spectra suggested that Protein B2 contains 2 nonidentical high spin Fe(III) ions in an antiferromagnetically coupled binuclear complex that resembles both methyldioxohemerythrin and oxyhemerythrin. Below 105°K the iron in Protein B2 was completely diamagnetic, in confirmation of the previous assignment of the 410 nm- and EPR-absorbing species as stable organic free radical of unknown structure.

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We propose that the function of iron in Protein B2 is the initial generation of radical from a protein-bound group, and that stability of the radical depends upon some continuing interaction with the iron center. The radical is apparently less stable than is metalloprotein structure; this would account for the variability of radical content in native protein and for the > 100% yield of radical obtainable by substitution of the iron. We further propose that the free radical participates in the reduction of ribonucleotides by the enzymatically active Protein B1-Protein B2 complex.

Ribonucleoside diphosphate reductase from Escherichia coli uses the reduced form of thioredoxin, a small protein, as the electron donor for the irreversible reduction of the four common ribonucleoside diphosphates (1, 2). The ribonucleotide reductase system consists of the enzyme plus thioredoxin and thioredoxin reductase, as shown in Fig. 1. The enzymatically active form of ribonucleotide reductase is a 1:1 complex of two different subunits, called Proteins B1 and B2 (3–5). Either protein possesses no known enzymatic activity in the absence of the other subunit (3).

Per molecule of 78,000 daltons, Protein B2 contains two identical or nearly identical polypeptide chains that do not dissociate except under denaturing conditions, and 2 iron atoms (5, 6). Except for a sharp 410 nm band of widely variable absorbance (in different near-homogeneous preparations; see Refs. 6 and 7), the electronic spectrum of Protein B2 at >320 nm much resembles spectra of oxy- and met-forms of hemerythrin,3 intracellular O₂-carrying proteins from certain species in four primitive animal phyla (8, 9).

In addition to iron, Protein B2 contains a variable amount of a paramagnetic species that gives a characteristic EPR ab-
sorption centered at $g = 2.0047$; double integration of the EPR signal and assumption of spin one-half yielded $\pm 0.6$ unpaired electron per subunit molecule (7). The EPR and 410 nm absorptions and the specific enzymatic activity of Protein B2 were linearly correlated properties, and treatment of the protein with hydroxyurea or hydroxylamines concurrently destroyed all of these properties (6, 7, 10). From characteristics of the EPR signal, the 410 nm and EPR species was tentatively assigned as stable organic free radical rather than as metal (7).

We here report Mössbauer studies that, along with complementary magnetic studies to be reported elsewhere, confirm the assignment of the paramagnetic species in Protein B2 as organic free radical. For convenience we shall henceforth refer to the EPR- and 410 nm-absorbing species as "radical."

**EXPERIMENTAL PROCEDURE**

**Materials**—Buffer and precipitating solutions for protein were made from low iron reagents: Schwarz-Mann ultrapure grade ($\text{NH}_{4})_{2}\text{SO}_{4}$ and Tris base (both $<0.00005\%$ iron); Baker analytical $\text{Na}_{2}\text{citrate} \cdot 2\text{H}_{2}\text{O}$ ($<0.0001\%$ iron); and citric acid ($<0.0001\%$ iron), British Drug Houses, Ltd. Other chemicals and sources were: sodium ascorbate, 8-hydroxyquinoline-5-sulfonic acid dihydrate; and thiorhodanine for better sensitivity and trichloroacetic acid was a special low iron product. Blair and Dicli gave $\epsilon_{280} = 22,110$ $\text{m}^{-1}\text{cm}^{-1}$ for the absorption maximum of the Fe(II) complex of this chelator (13). However, the order of mixing precluded cuvette corrections before addition of iron, and we therefore measured $A_{280} = 1.4$ (ng per ml)$^{-1}\text{cm}^{-1}$ was used for assays of metal-free Protein B2 (see Fig. 4).

**Iron Assays**—The colorimetric ferroin method of Massey (12) was scaled down to final assay volumes of 125 $\mu$l. $p$-Phenanthroline was replaced by an equivalent concentration of sodium bathophenanthroline-sulfonate for greater sensitivity and trichloroacetic acid was a special low iron product. Blair and Dicli gave $\epsilon_{280} - \epsilon_{260} = 21,700$ $\text{m}^{-1}\text{cm}^{-1}$. Protein B2 with exactly 2 iron atoms per subunit contains 0.1435% iron or 25.6 $\mu$g atoms iron per $\mu$g, according to Thelander’s molecular weight data (5).

**Saturated Lithium 8-Hydroxyquinoline-5-Sulfonate Solution for Removal of Iron from Protein B2**—A suspension of 100 g of 8-hydroxyquinoline-5-sulfonic acid dihydrate in 700 ml of H$_2$O was dissolved and neutralized by addition of approximately 80 ml of saturated aqueous LiOH and warming to 60°C. The solution was filtered, cooled, and repeatedly extracted first with CHCl$_3$ and then with Et$_2$O to remove odiferous impurities and dark residue at the phase interface. The aqueous phase was vacuum-evaporated to 300 to 400 ml and residual ether was removed by boiling. The resulting solution was supersaturated at room temperature but required generous seeding and long standing (2 1 week) to precipitate yellow crystals at equilibrium with clear dark orange saturated solution of the salt at pH 7.0. The saturated solution at 25°C 0.47 m in lithium 8-hydroxyquinoline 5 sulfonate (by dry weight). The stock mixture of crystals and solution was stored protected from light. The method developed for removal of iron from Protein B2 (see "Results") employed a 10-fold dilution of the stock saturated solution of chelator.

**Mössbauer Spectroscopy**—$^{57}$Fe metal powder was dissolved in aqueous HCl. [$^{57}$Fe]Protein B2 was prepared and precipitated as described under "Results", the precipitate was evenly suspended in part of the supernatant solution and the resulting thick suspension (0.70 ml total volume) and a blank sample (0.70 ml of the supernatant solution) were frozen with powdered solid CO$_2$ in polystyrene Mössbauer cells (14) that gave 3.2 mm sample thicknesses in the γ-beam direction. These samples, prepared in Stockholm, were packed in solid CO$_2$ and transported immediately to Harwell for Mössbauer studies.

The blank, the [$^{57}$Fe]Protein B2 sample, and smaller fractions subsequently derived from the latter (see "Results") and frozen with liquid N$_2$ in Mössbauer cells were individually masked with lead foil and maintained at $\pm 195\text{K}$ during spectroscopy or storage. Mössbauer spectra were obtained with 256-channel constant acceleration spectrometers designed by Cranshaw (15).
Counter, other equipment, and sample handling technique have been described in detail (14, 16). The y-ray sources were 57Co diffused in palladium foils. Velocity calibrations were made with 57Fe-enriched iron foil absorbers at room temperature, and velocities herein are specified relative to the center of symmetry of their spectra (17). The background rate was determined at the start of each run by insertion of an aluminum filter in the y-ray beam (14); the absorption scales on Fig. 5 are background corrected. Most of the runs were made with the samples in low transverse magnetic fields from permanent magnets (cf. Refs. 14 and 18); these low fields in fact had negligible effect since iron in the present case turned out to be diamagnetic. High transverse magnetic field (30 kG) was obtained with a superconducting solenoid. Low field spectra were run for ~48 hours at each temperature, and the high field spectrum required ~72 hours running time. This gave about 6 × 106 and 4 × 106 counts per channel for spectra at low and high applied magnetic field, respectively. Least squares computer fits to low field data were made using a program written by B. Window. The high field spectra were calculated using a program which computed the Mössbauer spectrum averaged over all possible orientations of the molecule relative to applied field and y-beam directions.

RESULTS

Stability of Iron and Radical in Protein B2—Protein B2 was insensitive to a number of chemical treatments that effect distinct oxidation-reduction or ligation changes in other iron proteins. Thus, neither the enzymatic activity nor the ultraviolet spectrum (see later) of Protein B2 in 0.5 m Tris-HCl, pH 7.6, was affected by the following: deaeration-oxygenation cycling; treatment at 25° for 1 hour with 1 atm CO in the dark, or with the reductants mercaptoethanol, diethylenetriamine-pentaacetate, NaBH4, or Na2S2O4; treatment for 2 days at 0 to 4°C with 1 M KF, 0.1 M KCN, 0.01 M NaN3, or with a variety of 0.001 M siderochrome polyhydroxamate chelators (19) and ferrozine chelators (20) with or without ascorbate or diethylenetriaminepentaacetate reductant. Higher concentrations of NaN3 caused inactivation of an unknown sort, but observed gradual losses of Protein B2 absorption at >320 nm excluded the presence of anything resembling metazido- or hydroxamate chelators (19) and ferroin chelators (20) with or without ascorbate or diethylenetriaminepentaacetate reductant. Higher concentrations of NaN3 caused inactivation of an unknown sort, but observed gradual losses of Protein B2 absorption at >320 nm excluded the presence of anything resembling metazido- or hydroxamate chelators (19) and ferroin chelators (20) with or without ascorbate or diethylenetriaminepentaacetate reductant. However, the presence of NaN3 caused inactivation of a form of Protein B2 with little or no enzymatic activity, which was destroyed by treatment with Protein B1, thioredoxin-(SH)2, or -S or any other components or combination of protein components, substrates, and allosteric effectors of the complete ribonucleotide reductase system.

The radical and associated enzymatic activity decayed spontaneously with a half-life of roughly 2 weeks for concentrated Protein B2 (≥10 mg per ml) stored at 4°C in 50 mm Tris-HCl, pH 7.6; decay was much faster at higher temperatures and dilutions. Aged Protein B2 resembled partially NH2OH-inactivated protein in ultraviolet spectra and in capacity to be reactivated (see later).

Removal of Iron from Protein B2—Two previous procedures resulted only in partial iron depletion and caused loss of denatured, insoluble protein (6). We sought conditions to cause rapid dialysis of 57Fe label from 57Fe-Protein B2 (6) to yield metal-free protein that could be reconstituted. Three hours dialysis at 4°C against a freshly prepared solution containing 50 mm lithium 8-hydroxyquinoline-5-sulfonate, 1 m imidazole-HCl, pH 7.0, and 30 mm NH2OH-HCl achieved this purpose.

In a typical experiment 50 to 200 μl of Protein B2 solution (10 mg per ml) were pipetted into the tip of a collodion vacuum dialysis bag, and the bag was then dipped to the level of the internal liquid into 10 ml stirred chelator solution. With the 57Fe-Protein B2, radioactivity outside the bag approached a constant level after 3 hours dialysis. Dialyzed protein could be vacuum-concentrated at least 2-fold directly within the bag before filtration through a small column of Sephadex G-25 equilibrated with 50 mm Tris-HCl, pH 7.6. This method gave, in 80 to 100% yield, protein with less than 2% of the original iron content. From here on this metal-free Protein B2 will also be called Apoprotein B2.

Imidazole and 8-hydroxyquinoline-5-sulfonate were both essential to the procedure. Rather than serving as an iron ligand, imidazole more likely induced conformational changes that exposed iron to chelator (see Ref. 21). NH2OH was not essential but gave marginally faster removal of iron.

Properties of Apoprotein B2—Metal-free protein obtained by the above procedure had no enzyme activity, showed great loss of absorption above 320 nm as compared to native protein (see later), and gave no EPR signal; similar properties were reported for partially iron-depleted protein (7). Apoprotein prepared from NH2OH- or hydroxurea-inactivated Protein B2 (cf. Refs. 6, 7, and 10) appeared identical with apoprotein prepared from native Protein B2 (with omission of NH2OH in the foregoing procedure) and could be stoichiometrically reconstituted to highly active metalloenzyme.

By the criterion of reconstitution to active enzyme (see below), apoprotein was as stable as native Protein B2. Aliquots of apoprotein taken over a 4-day period from a solution (5 mg per ml) stored at 4°C gave reconstituted fractions with identical ultraviolet absorbance ratios and specific enzymatic activities. Apoprotein could be precipitated with 3.2 m (NH4)2SO4 frozen and stored in liquid N2, thawed, and recovered in desalted solution with the same good efficiency as could native protein (3). Apoprotein resembled native protein in several other respects. Both forms showed the same mobility during (nonstacking) disc gel electrophoresis at pH 8.7 (3) and in sucrose density gradient centrifugation.6 One difference between apoprotein and native protein, however, appeared upon reaction of sulfhydryl groups with excess 5,5'-dithiobis(2-nitrobenzoate) under nondenaturing conditions. Fig. 2 shows that only two such groups were oxidized per molecule of native protein, while additional —SH groups were slowly titrated in apoprotein. The same experiment with the addition of 8 m guanidine HCl (not shown) gave 8.4 and 7.2 rapidly titrating sulfhydryl equivalents per mole of apoprotein and native protein, respectively, approaching the value of 10 half-cystines found by amino acid analysis (5).

Reconstitution to Active Metalloenzyme—Reconstitution and reactivation were achieved simultaneously by treatment of Apoprotein B2 with Fe(II) ascorbate. We modified the original reconstitution procedure of Brown et al. (6) to further minimize air oxidation of Fe(II). A solution of 100 mm sodium...
ascorbate and 10 mM Fe(III)-ascorbate mixture was freshly prepared in deaerated 50 mM Tris-HCl, pH 7.6, from weighed-out salts and left standing several minutes to permit reduction of any residual Fe(III). The solution turned deep purple due to formation of an Fe(II)-oxidized ascorbate complex (22). When *Fe or *Fe in HCl solution was the source of 10 mM iron, a similar procedure was used but buffer concentration was increased to yield a final pH near 7.6, and ascorbate was increased to 150 mM. A volume of Fe(II)-ascorbate mixture, containing 50 to 100% excess iron, was added to Apoprotein B2 solution and after 5 min at 25° the mixture was filtered immediately at 4° through a column of Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.6. In a typical experiment, 10 μl of Fe(II)-ascorbate mixture were added to 100 μl of protein solution (10 mg per ml) and filtered through a gel column of 1-ml bed volume. Sealing the experiment up by a factor of 10 or more worked equally well. Protein yields after the gel filtration approached 90%. Specific activities directly proportional to iron content (not shown). In light of our assignment of much of the electronic spectrum—notably A325— to a binuclear iron center (see “Discussion”), it appeared that only binuclear and no mononuclear iron centers were formed in partially reconstituted protein. If activity were indeed associated only with a binuclear iron structure in Protein B2, random rather than pairwise binding of iron ions to apoprotein would have given activity proportional to the square of iron content (dotted line in Fig. 3). In experiments not shown, reconstitution in the presence of 10-fold excess iron gave active protein with ~110% of the stoichiometric iron content.

Comparison of Electronic Spectra of Native, NH2OH-inactivated, Metal-free (“apo”-), and Reactivated Forms of Protein B2—As isolated, Protein B2 has a highly characteristic spectrum between 320 and 540 nm, with a sharp peak at 410 nm and broader peaks or shoulders at ~390, 350, and ~480 nm (6, 7); a very weak, broad band near 680 nm—similar to that in oxy- and deoxyhemerythrin (9)—may be present but was near our limit of detection (ε = 200 μM cm⁻¹). Spectra of native Protein B2 in phosphate or Tris buffers at pH 6.5 to 9.0 did not differ significantly. Inactivation of Protein B2 by hydroxylamine or hydroxypyruvate—which does not result in the loss of iron—caused loss of the 410 nm band and smaller losses in absorption near 380 nm and elsewhere (6, 7, 10). Fig. 4A gives a spectrum of native Protein B2. NH2OH-inactivated protein is shown in Fig. 4B and a difference spectrum between native and inactivated protein is given in Fig. 4C. Fig. 4, A and B, also includes studies of reconstituted protein that showed negligible g = 4.3 iron and magnetic susceptibility corresponding to that expected from radical content of the protein according to EPR.

Treatment of apoprotein with limiting amounts of *Fe gave partially reconstituted fractions with specific enzymatic activities directly proportional to iron content (Fig. 3). Chemical and radioactivity assays for iron agreed. Specific absorbances at 365 and 410 nm, as well as the rest of the characteristic spectrum of active Protein B2 at >320 nm, also increased proportionately with iron content (not shown). In light of our assignment of much of the iron binding to a binuclear iron center (see “Discussion”), it appeared that only binuclear and no mononuclear iron centers were formed in partially reconstituted protein. If activity were indeed associated only with a binuclear iron structure in Protein B2, random rather than pairwise binding of iron ions to apoprotein would have given activity proportional to the square of iron content (dotted line in Fig. 3). In experiments not shown, reconstitution in the presence of 10-fold excess iron gave active protein with ~110% of the stoichiometric iron content.

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\begin{array}{c|c|c|c|c}
\text{Form of Protein B2} & \text{Iron/mg}^a & \text{Enzymatic activity} & A_{365}/A_{410} & A_{410}/A_{400} \\
\hline
\text{Native} & 22.6 & 12 & 0.023 & 1.10 \\
\text{NH2OH-inactivated} & 22.6 & 12 & 0.014 & 0.90 \\
\text{Metal-free} & 3.0 & 0 & 0.005 & 1.30 \\
\text{Reconstituted} & 23.0 & 20 & 0.044 & 1.30 \\
\text{Native} & 10^\circ & & & \\
\text{Metal-free} & 0.6 & 0 & & \\
\text{Hydroxypyruvate-inactivated} & 25.0 & 19 & 0.034 & 1.33 \\
\text{Metal-free} & 25.0 & 0 & 0.012 & 0.81 \\
\end{array}
\]

* Exactly 2 atoms of iron per protein molecule of 78,000 daltons (5) correspond to 75.6 mg atoms of iron per mg.

| Arbitrary batch No. | Form of Protein B2 | Iron/mg | Enzymatic activity | A₃₆₅/A₄₁₀ | A₄₁₀/A₄₀₀ |
|---------------------|-------------------|---------|--------------------|------------|------------|
| 1                   | Native             | 22.6    | 12                 | 0.023      | 1.10       |
|                     | NH₂OH-inactivated  | 22.6    | 12                 | 0.014      | 0.90       |
|                     | Metal-free         | 3.0     | 0                  | 0.005      | 1.30       |
|                     | Reconstituted      | 23.0    | 20                 | 0.044      | 1.30       |
|                     | Native             | 10°     |                    |            |            |
|                     | Metal-free         | 0.6     | 0                  |            |            |
|                     | Hydroxypyruvate-inactivated | 25.0 | 19             | 0.034      | 1.33       |
|                     | Metal-free         | 25.0    | 0                  | 0.012      | 0.81       |

Average of five preparations combined to make *Fe-Protein B2.

Inactivated sample used for magnetic susceptibility measurements.
ADDED IRON IN RECONSTITUTION MIXTURE, g atoms per mole apoprotein B2

FIG. 3. Partial reconstitution of Protein B2. Portions of apoprotein B2 were treated as described under "Results" with <2 moles of iron per mole of protein or with excess iron from a 57Fe(II)-ascorbate mixture. Iron content values from radioactivity and chemical assay (---) and specific enzymatic activities (---) are shown for gel-filtered, partially and fully reconstituted fractions. The corresponding values for the native Protein B2 starting material were 12,000 units per mg and 1.89 atoms of bound iron per protein molecule. The dotted line shows expected enzymatic activity for the hypothetical case of enzymatic activity proportional to the square of protein iron content (see "Results").

Spectra of apoprotein B2 and reconstituted protein. The apoprotein had lost most absorption above 320 nm, as compared with native protein. Reconstituted/reactivated protein gave the characteristic spectrum of native protein but with increased A_380 and other absorbances that we attribute to radical (see "Discussion").

Mössbauer Spectroscopy of [57Fe]Protein B2—Apoprotein (37 mg) was prepared from several batches of Protein B2 and treated with 57Fe(II)-ascorbate as described above except that the reconstituted protein was precipitated (after the 5 min incubation with iron ascorbate) with 3.5 volumes of ice-cold saturated (NH_4)_2SO_4 solution. The precipitate was collected immediately, dissolved in 1.5 ml of 10 mM sodium ascorbate-50 mM Tris citrate, pH 7.6, and desalted on a column of Sephadex G-25 equilibrated with 50 mM Tris citrate, pH 7.6. Analyses of the resultant [57Fe]Protein B2 (28.4 mg in 4 ml of buffer) and of its metal-free precursor are given in Table I, Batch 2.

At this point protein could not be concentrated with (NH_4)_2SO_4 because high-Z atoms such as S parasitically absorb the 14.4 keV γ-rays of the Mössbauer experiment (14). [57Fe]Protein B2 (26.6 mg) was instead precipitated by addition of 3.5 volumes of saturated NaOH-ascorbate-50 mM Tris citrate, pH 7.6, and prepared for Mössbauer spectroscopy as described under "Experimental Procedure." Assuming ≥84% yield of protein precipitated from the solution (from pilot experiments) and no exchangeability of iron, we estimated that the Mössbauer cell contained 0.53 to

FIG. 4. Comparison of electronic spectra of various forms of Protein B2 and hemerythrin, with specific absorbance in terms of Protein B2 or hemerythrin monomer concentration. Other properties of the Protein B2 fractions shown are described under Batch 1, Table I. Spectra A, enzymatically active forms of Protein B2 in 50 mM Tris-HCl, pH 7.6, with absorbance normalized to ε_280 = 117,000 M^{-1} cm^{-1} (from Thelander's physical data, (5)): native (---) and reconstituted (-----). Spectra B, enzymatically inactive forms of Protein B2 in 50 mM Tris-HCl, pH 7.6: NH_3OH-inactivated protein (---, same as native) and metal-free or "apo" protein (----, ε_280 8% less than native according to amino acid analyses). Spectrum C, NH_3OH-sensitive portions of native Protein B2 spectrum, assigned (see "Discussion") as spectrum of the nonstoichiometric free radical in Protein B2 [note different ordinate scale, here normalized to A_380 = 117,000 M^{-1} cm^{-1}]; correlation with EPR gave ε_380 ≈ 5000 M^{-1} cm^{-1} based on concentration of free radical (7)]. Spectra D, Glosfusia (Phascolosoma) gouldii hemerythrin, replotted from Klotz (8) and Garbett et al. (9) with permission: methyldihydroxohemerythrin (--.--), oxyhemerythrin (-----, ε_280 interpolated from Love (23)); and deoxy- and metal-free hemerythrins (----).
of the intensity ratio from unity and its variation with temperature indicated different recoil-free fractions, hence different vibration amplitudes for the two classes of iron nuclei.

The high field spectrum of Fig. 5Ae provided a test for integral unpaired electron spin on the iron as well as a measure of the sign and symmetry of the electric field gradient interaction. Visual inspection suggested that only the direct effect of applied magnetic field on the iron nuclei was present, i.e., that the iron sites were diamagnetic. Assuming such diamagnetism, we made a least squares fit with the program described under "Experimental Procedure." Quadrupole splittings, line widths, and isomer shifts were taken from the low field spectrum at 4.2°K. The electric field gradient was taken as negative (i.e. \( V_{zz} < 0 \)) at each site, and the asymmetry parameters (\( \eta \) values) were allowed to vary. The best fit (curve in Fig. 5Ae) corresponded to \( \eta \) of -0.5 and +0.6 for outer and inner doublets, respectively.

The mean square difference between this theoretical spectrum and the data points was only 1.3 times the value expected from counting statistics, so we regarded the fit as satisfactory. The \( \eta \) values, however, were not well determined: setting both to zero increased the mean square error by only 1%. Calculated individual contributions of the two types of iron are shown in Fig. 5A, f and g.

Two features of the spectra further support the above evidence that iron in Protein B2 is diamagnetic at low temperatures. First, we estimate that a minority fraction of at most 20% of the iron in the sample could in principle have yielded a paramagnetic spectrum that was lost in the noise level of low field spectra, provided that it behaved as do known dilute ferric compounds. We would, however, have detected any paramagnetic iron at the concentration of the "radical" (\(-0.55\) unpaired electron per protein molecule) as determined by EPR. Second, the following theoretical estimate of absolute absorption strength shows that much or all of the iron in the sample contributed to the observed absorptions. The fractional absorption of thin absorbers is given by the product of surface density of \( ^{57}\text{Fe} \), peak absorption cross-section \( \sigma \), the product i.e. source and absorber recoil-free fractions, and two factors depending on line widths and structures of emission and absorption spectra. Surface density was 1.4 to 1.7 \( \times 10^{20} \) nuclei per cm\(^2\) (from \( ^{57}\text{Fe} \) concentration estimate above and 3% mm sample thickness). We take \( \sigma = 2 \times 10^{29} \) cm\(^2\) and use the rough estimate \( i.e. = 0.5 \) which is typical of hemoglobin at low temperature.

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Footnote:

7 See reviews by Lang (14) and Debrunner (24) for general discussions of Mössbauer spectroscopy of iron proteins.


TABLE II

Mössbauer spectroscopic parameters

| Iron protein         | Temperature | Isomer shift δ* | Quadrupole splitting ΔE\(^0\) | Line width 2\(^1\) | Intensity ratio outer/inner |
|----------------------|-------------|-----------------|-------------------------------|-------------------|-----------------------------|
| \[^{57}\text{Fe}\]Protein B2 | 105 K       | +0.48 mm s\(^{-1}\) | -1.65 mm s\(^{-1}\) | 0.32 mm s\(^{-1}\) | 0.84                       |
|                      | 77 K        | +0.53 mm s\(^{-1}\) | -1.66 mm s\(^{-1}\) | 0.26 mm s\(^{-1}\) | 0.93                       |
|                      | 4.2         | +0.53 mm s\(^{-1}\) | -1.05 mm s\(^{-1}\) | 0.28 mm s\(^{-1}\) | 0.98                       |
| Oxyhemerythrin       | 77          | +0.48 mm s\(^{-1}\) | ±1.00 mm s\(^{-1}\) | 1 ± 0.1           |                             |

a Relative to iron foil standard absorber (see "Experimental Procedure").
b Definite signs given only as determined for signs of the principal electric field gradients (\(V_{zz}\) values) by simulations of Mössbauer spectra in high applied magnetic fields.

discussion

Protein B1, one of the two subunits of ribonucleotide reductase, binds at distinct sites both substrates\(^{4}\) ([equiv.

If all lines were of natural width (0.19 mm s\(^{-1}\)) the next factor would be 0.6; we reduce this to 0.1 since the lines were slightly wider than natural. Because the absorption is divided into four lines the last factor is \(1/4\). The predicted maximal absorption is thus 1.4 to 1.7% where the spread does not include the uncertainty in \(E\). The maximal observed absorption (Fig. 54d) was about 1.4%, well within the expected range.

Diamagnetism in a mononuclear iron complex would normally signify low spin Fe(II). However, when a pair is present we have the possibility that 2 metal atoms of equal spin will couple antiferromagnetically to yield zero net spin. If such coupling occurs in Protein B2, it is unlikely that the iron is either low spin Fe(III) or high spin Fe(II) because these are characterized by low lying excited orbital states and resulting temperature-dependent quadrupole splittings. It might be argued that the present case does correspond to one of the above, and the temperature dependence is extremely small because the highly distorted environment has given rise to an unusually large orbital splitting. Such a splitting would, however, suppress the mixing of orbital levels by spin-orbit coupling, and we would expect to see a quadrupole splitting characteristic of a pure crystal field orbital, approximately 3.5 mm per s. Thus, the temperature independence of the quadrupole splitting combined with the fact that it is so small tends to argue against a low spin Fe(III) or high spin Fe(II) assignment. The high spin ferrous assignment is also unlikely in view of the isomer shift which is exhibited by B2. Most high spin ferrous isomer shifts are greater than 0.8 mm s\(^{-1}\). The smallest of which we are aware is the ferrous component of spinach ferredoxin, which has an isomer shift of 0.54 mm per s at 4.2°K (28, 29) probably associated with the fact that the ferrous atom is closely coupled to a ferric neighbor. The corresponding values for the two B2 components are 0.53 and 0.45 mm per s. The observation of net zero spin requires that both B2 irons have the same spin, so we cannot allow just one of them to be high spin ferrous. Isomer shifts alone do not allow us to choose between the remaining alternatives—low spin Fe(II) or spin-coupled binuclear high spin Fe(III)—while the large quadrupole splittings are unusual but not unheard of for either state. The splittings and negative \(V_{zz}\) values imply strong asymmetric bonding that produces a planar defect (or axial excess) of electron charge relative to the zero order symmetrical distribution which characterizes both low spin Fe(II) and high spin Fe(III). In magnitude of quadrupole splittings Protein B2 resembles oxyhemerythrin (Table II), methemerythrin, and oxo-bridged six-coordinate binuclear high spin Fe(III) complexes wherein antiferromagnetic coupling was convincingly demonstrated by the appearance of paramagnetism at high temperatures (25-27, 30). Although the \(V_{zz}\) signs have not been determined for oxyhemerythrin, they were found to be negative in metisothiocyanatohemerythrin and the model compound (Fe(salen)Cl\(_2\)) (25, 27). It must be recognized that the concept of valence state is an idealization and seldom applies to a real chemical compound without at least some qualification. In the present situation the spin coupling has even confused the usually distinct division between integral and half-integral spin. By describing the B2 system in terms of a coupled high spin ferric binuclear complex we imply that a low lying excitation exists corresponding to the breaking of this coupling. This has not as yet been observed in B2 although, as mentioned above, the closely similar hemerythrim have exhibited it.

Mössbauer Spectra of Inactivated Protein B2—The protein suspension used for spectra depicted in Fig. 5A was divided into three portions. One portion (0.4 ml) was immediately refrozen in liquid N\(_2\) and served as a control for freezing artifacts and radiation damage; its spectrum (Fig. 5Bc) was unchanged as compared to the low field spectrum taken earlier at 4.2°K (Fig. 54d). This sample was then maintained at \(\leq 195°\)K and later used for magnetic studies.\(^{4}\) Protein precipitates from the other two portions (0.15 ml each) were separately dissolved in 50 mm Tris-citrate, pH 7.6, and inactivated for 30 min at 4° with 10 mm NH\(_2\)OH and 20 mm hydroxyurea, respectively. These manipulations involved about 3-fold dilution of the original volume. Mössbauer spectra of frozen solutions of the two treated samples are given in Fig. 5A, a and b. Even though these spectra are quite weak it is clear that their general features are the same as those of the untreated control. EPR measurements and recordings of the 410 nm absorptions of the two inactivated samples demonstrated that the NH\(_2\)OH treatment had nearly completely destroyed the radical, while the hydroxyurea-treated sample was only about 50% inactivated. These experiments further demonstrate that the radical had no effect on Mössbauer spectra of \[^{57}\text{Fe}\] in Protein B2.

\(^{4}\) Sulfur-bridged binuclear high spin Fe(III) complexes in oxidized ferredoxins tend to show quadrupole splittings of \(<0.5 mm s^{-1}\) (28, 29). Note that Protein B2 lacks acid-labile inorganic sulfide (6).
The site of interaction between enzyme and the
electron donor (=thioredoxin) is unknown. An obvious
question is whether the second subunit, Protein B2, is an electron
carrier and interacts with thioredoxin. Protein B2 contains 2
atoms of iron (6) and a lesser, variable amount of a paramag-
netic species that from EPR studies was presumed to be an
organic free radical (7). The main purpose of the present work
was to define further the structure of iron and its relationship
to the paramagnetic species.

The results of Mössbauer spectra of [57Fe]Protein B2 together
with magnetic susceptibility studies clearly establish that the
iron is diamagnetic and distinct from the paramagnetic entity,
thus confirming the previous assignment (7) of the paramagnetic
species as stable organic free radical. These and previous re-

sults (8, 9) also show that the enzymatic activity of Protein
B2 depends upon the presence of radical and that the radical in
turn depends upon the presence of iron. Removal of iron from
Protein B2 yielded diamagnetic, enzymatically inactive "Ap-
protein B2." Treatment of apoprotein with excess Fe+3 gave
stoichiometrically reconstituted metalloprotein in which activity
and radical had been regenerated with more than 100% over-all
yield.

The mechanism of inactivation of Protein B2 by NH2OH
and hydroxyurea clearly involves destruction of radical: hy-
droxyurea was recently found to be a powerful radical scavenger
in another system (31). Iron is not removed nor apparently
affected in any way by treatment of Protein B2 with these
agents. Similarly, aging preparations of native protein showed
spontaneous gradual losses of activity and radical with no
losses of or apparent changes in iron. Reactivation of pro-
tein was achieved only by removal of iron followed by reconsti-

tution. Other components of the enzyme system, oxidation-
reduction agents, or treatment of inactivated protein with Fe+2-
ascorbate showed no effect.

At present, the radical is characterized only by its EPR
and magnetic susceptibility properties (7) and by its electronic
spectrum (Fig. 4C), which last we assign from the portions of
the spectrum of active Protein B2 that are sensitive to NH2OH
or hydroxyurea.

The electronic spectrum of Protein B2 after destruction of the
radical with NH2OH (Fig. 4B) showed absorption bands at 320
and 365 nm in close resemblance to the spectrum of methyldih-
hydroxymetährin (Fig. 4D). We assign absorption of inactivated
Protein B2 at >320 nm directly to the iron center. The studies
of Williams' and Klots' groups on hemerythins and model
binuclear high spin Fe(III) complexes (9, cf. Ref. 25) have pro-

vided strong evidence for binuclear iron centers in oxy- and
methemerythins, and they have assigned different absorption
bands to specific bridging ligands. The similarity of electronic
spectra of Protein B2 and hemerythins suggests the presence of
a binuclear iron center also in our case, but we have no independ-
ent evidence for the presence or nature of any bridging ligands
for iron in Protein B2. \Delta v_{	ext{iso}} \approx 9000 \text{cm}^{-1} \text{absorbance differ-
ence between metal-free and native Protein B2 (Fig. 4) is}
consistent with loss of intense absorption bands in this region
from a binuclear iron center (see Ref. 9).

Our Mössbauer results were again very similar to those for
oxidized forms of hemerythrin (cf. Refs. 25-27) and were con-
sistent only with two alternative structures: (a) two low spin
Fe(II) complexes in two sites per Protein B2 molecule; or (b)
one antiferromagnetically coupled binuclear high spin Fe(III)
complex per Protein B2 molecule. Because of the similarities of

Protein B2 and oxidized forms of hemerythrin in both electronic
and Mössbauer spectra, we strongly favor a binuclear high spin
Fe(III) structure for iron in Protein B2.

There is one important known difference between hemerythins
and Protein B2: in hemerythrin one subunit consists of a single
polyepitide chain with 2 bound iron atoms (8) whereas in Protein
B2 one subunit contains two apparently identical polyepitide
chains (5) and 2 iron atoms. It appears likely to us that in the
latter case each iron is bound to a separate polyepitide chain
but we have no evidence on this point. Our Mössbauer spectra
showed the presence of two equal populations of iron. We
therefore assume that, as in the proposed oxihemerythrin struc-
ture (27), each binuclear iron complex in Protein B2 is intrin-

sically asymmetric and contains 1 iron ion of each class. Radical
content clearly had no effect on the distribution into two iron
populations.

What is the function of iron in Protein B2? The metal does
not appear to participate as an electron carrier in the enzyme
reaction. The electronic spectrum of Protein B2 was not affected
by addition of thioredoxin or any other components—alone or in
combination—of the ribonucleotide reductase system. Fur-
thermore, the part of the electronic spectrum due to iron was not
influenced by a variety of oxidizing or reducing agents.

Our results also argue against a major structural role for iron
in Protein B2. Thus, the apoenzyme and the native protein
had identical electrophoretic and ultracentrifugal properties
and both formed 1:1 complexes with Protein B1 in the presence
of Mg2+ (4, 5). \^ The apoprotein had, however, more exposed
—SH groups than did Protein B2.

Instead, we suggest the hypothesis that the function of iron is
to generate an organic radical from a protein-bound group as
the metal binds to apoprotein and to stabilize that radical in
the metalloprotein by some continued interaction. Radical for-
mation might involve iron-catalyzed 1-electron aerobic oxidiza-
tion of the radical precursor, or a 1-electron reduction of the
precursor by Fe(II) to leave Fe(III) in the protein.2 According
to this hypothesis the organic radical is the functional con-
tribution of Protein B2 to the enzymatically active Protein B1-
Protein B2 complex and the reduction of ribonucleoside di-
phosphates by the E. coli enzyme would thus involve the par-

ticipation of radical intermediates. The identification of the
chemical nature of radical in Protein B2 is now an important
task.

In this connection we would like to recall that Blakley et al.
recently postulated the involvement of transient free radical
intermediates in the reduction of ribonucleoside triphosphates
by the reductase from Lactobacillus leichmannii (32, 33). In
this case cob(II)alamin coenzyme and not iron is part of the
enzyme system and radical formation is thought to involve a
homolytic scission of the cobalt-carbon bond of the coenzyme.

Both the E. coli and the L. leichmannii enzymes catalyze the
stereospecific replacement of the OH group of a secondary
alcohol, i.e. reduction with retention of configuration (1, 34-37).
In organic chemistry there are to our knowledge no general
methods for the direct reduction of alcohols. However, catalytic
hydrogenolyses reduces substituted benzyl alcohols with retention
of configuration and might be a model reaction (37-39). The
active reductant in catalytic hydrogenolyses is likely to be
echemosorbed hydrogen atom radicals (40).

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