Electron Spin Resonance of the Iron-containing Protein B2 from Ribonucleotide Reductase*

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SUMMARY

Protein B2 is an iron-containing subunit of ribonucleotide reductase. Its light absorption spectrum has similarities to that of oxyhemerythrin, but in addition shows a sharp peak at 410 nm. The electron spin resonance (ESR) spectrum recorded from 14-293 °K shows a slightly asymmetric doublet centered at \( g = 2.0047 \). Even though the integrated ESR absorption corresponded at the most to a spin concentration of only 30% of the total iron content, different lines of evidence strongly link the presence of the ESR signal (and the peak at 410 nm) to the enzymatic activity of protein B2. Several characteristics of the signal would suggest that it is due to a free radical. It is, however, dependent on the presence of iron in protein B2. A mononuclear or dinuclear iron complex is considered less likely as an explanation of the data.

Ribonucleotide reductase from Escherichia coli consists of two nonidentical subunits, proteins B1 and B2. The enzyme catalyzes the irreversible reduction of all four common ribonucleoside diphosphates to the corresponding deoxyribonucleotides. The hydrogen donor in the reaction is the reduced form of a small protein called thioredoxin. The reaction can be summarized as follows (1, 2):

\[
\text{Ribonucleoside diphosphate} + \text{thioredoxin}-(\text{SH})_2 + \text{Mg}^{2+} \rightarrow \text{protein B1 + B2} \rightarrow \text{deoxyribonucleoside diphosphate} + \text{thioredoxin-S}_2
\]

The process requires the simultaneous presence of both proteins B1 and B2. Each subunit alone is completely inactive and does not catalyze any known partial reaction. Protein B1 has been shown to bind allosteric effectors and is thus inter alia involved in the regulation of the enzyme.

Protein B2 is made up of two identical or very similar polypeptide chains, each with a molecular weight of about 40,000, and contains close to 2 atoms of iron (2). It has a characteristic light absorption spectrum with a very sharp peak at 410 nm, a broader peak around 360 nm, and a shoulder at 325 nm (2).

Iron can be removed reversibly from protein B2. This results in a reversible loss of enzyme activity and of the characteristic visible spectrum. However, several lines of evidence seemed to indicate that the peak at 410 nm was only indirectly connected with the presence of iron in the protein (2).

In the present communication we demonstrate that protein B2 gives rise to a unique electron spin resonance signal at \( g = 2.0047 \). The evidence also suggests that the ESR signal and the sharp peak at 410 nm are expressions of the same functional protein molecules.

MATERIALS AND METHODS

Protein B2 was prepared as described earlier (1). Chromatography on Sephadex G-200 was used as the final purification step. All materials, the methods used for the analyses of the enzyme, and the definition of enzyme activity were the same as those described in previous publications (1, 3). Tris-HCl buffer (pH 7.5, 0.05 M) was used as solvent in all experiments.

Optical spectra were recorded at room temperature in a Cary 14 spectrophotometer.

ESR was measured on frozen and liquid samples in a Varian V-4502 X-band (9.5 GHz) ESR spectrometer with a modified microwave bridge equipped with a circulator and a special crystal bias arm. Some ESR experiments were also carried out on a Varian E-9 X-band spectrometer or on a Varian V-4503 Q-band (35 GHz) spectrometer. (We are indebted to Drs. R. Assa and T. Vängård, Gothenburg, Sweden, for running the Q-band spectra.) The samples were contained in standard quartz tubes of 3- or 1-mm inner diameter, which were calibrated with solutions of CuEDTA whenever necessary. A variable temperature Dewar for N\(_2\) gas flow and a heater for temperature control was used to maintain the sample at the temperatures desired. A similar system without a gas heater was applied for flowing helium gas in order to obtain temperatures below 77 °K. Via a TMC Cat 1024 the derivative spectra were digitized and transferred to a punched tape, and later numerically doubly integrated on a computer. The magnetic field was measured with a proton resonance probe and a frequency counter.

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FIG. 1. Light absorption spectrum of protein B2 (30 nmoles of iron per mg of protein, specific activity = 19,800).

FIG. 2. Electron spin resonance spectrum of the same preparation of protein B2 as in Fig. 1. Temperature, 88 °K. The g value is 2.0047 at the zero passage indicated by the arrow. Cavity used: Varian V-4531, with gas flow Dewar insert. Field modulation frequency, 100 KHz, and amplitude, 2.4 gauss peak to peak; microwave frequency, 9.08 to 9.09 GHz, and power, 2 mwatts; field scanning rate, 25 gauss per min; time constant of detector, 1 s.

RESULTS AND DISCUSSION

Optical Spectrum of Protein B2—It will be shown below that different preparations of protein B2 with apparently the same degree of purity as judged from analytical gel electrophoresis and iron content showed widely differing specific enzyme activities. The spectrum of one of our most active preparations (specific activity = 19,800) is given in Fig. 1. It appears more detailed than the earlier published spectra (2) and shows two further peaks around 600 and 480 nm. Using the earlier determined molar extinction coefficient of $11.5 \times 10^4$ at 280 nm we find the following molar extinction coefficients: at 325 nm, $7.6 \times 10^3$; at 365 nm, $6.0 \times 10^3$; at 410 nm, $4.10 \times 10^3$; at 480 nm, $0.5 \times 10^3$; and at 600 nm, $0.3 \times 10^3$.

With the exception of the sharp peak at 410 nm, the spectrum of protein B2 shows striking similarities to the spectra of oxy- and methemerythrin (4). The similarities apply both to the position of the peaks and to the molar extinction coefficients.

ESR Properties of Protein B2—The ESR spectrum of Fig. 2 was recorded for the same preparation of protein B2 which was used for the visible spectrum given in Fig. 1. The main feature is a doublet structure with a splitting of 16.5 gauss and centered at $g = 2.0047$. The position of the zero passage indicated in Fig. 2 closely coincides with the center of gravity of the absorption, determined from the midpoint of the double integral. The doublet structure is slightly asymmetric suggesting some anisotropy of the g values. The absorption at $g = 2.0047$ was the only signal detected at 90 °K over the magnetic field range 500 gauss (g approximately 10) to 7000 gauss (g approximately 1).

The absorption was fairly easy to saturate by the microwave power as is seen in Fig. 3. In order to optimize the signal to noise ratio at temperatures above 77 °K we have worked at a power level of 2 mwatts which, however, caused a slight saturation of 5% or less. In the temperature range 14-77 °K it was necessary to work at 2 mwatts. These figures are valid for the Varian multipurpose cavity V-4531 with gas flow Dewar insert.

The derivative recordings at several temperatures have been doubly integrated. In order to obtain values for the concentrations of unpaired spins these data have been compared with the corresponding figures of a standard solution of CuEDTA (1 mM) obtained at the same temperatures. Correction was made for the g value dependence of the transition probabilities (5). It is clear from Fig. 5 that, within the experimental limits of error, the spin concentration detected is constant from 16.5-193 °K. Assuming a system with $S = 1/2$ this particular sample had a concentration of unpaired spins of 15.8 (± 3.5) µM. This sample had an iron concentration of 67 µM.

About 75% of the iron was removed from protein B2 by dialysis against 8-OH-quinoline which resulted in the loss of both the
Electron spin resonance recordings of protein B2 (2.4 mg per ml, specific activity = 15,000; 28 nmoles of iron per mg of protein) at different temperatures.

Frozen samples were in 3-mm sample tubes; liquid sample in 1-mm tube. Microwave power, 2 mwatts (except at 14 °K when 2 µwatts were used); field modulation, 2.4 G ptp (except at 298 °K when 12 G ptp were used); field scanning rate, 25 G per min (except at 14 °K when 50 G per min were used); time constant of detector, 1 s. Other conditions as given in Fig. 2.

After such treatment the ESR spectrum also disappeared. When the apoprotein was reactivated with Fe²⁺ the ESR spectrum reappeared. No major differences were found in the spectrum depending on whether ⁵⁷Fe or ⁵⁶Fe was used for reactivation; nor did substitution of D₂O for H₂O even during the iron-reactivation have any major effects except for some slight increase of resolvability of the substructure as observed at 88 °K.

From these experiments, it may be concluded first, that the ESR signal is directly dependent on the presence of iron in the enzyme; second, that there is no, or only very weak, hyperfine interaction with the iron nucleus; and third, that the hyperfine interaction with exchangeable protons has only line broadening effects.

The total width of the Q-band spectrum at 97 °K is only about 1.6 times larger than in the case of the X-band, and the main doublet splitting of about 20 G persists. This confirms that there is only a weak g anisotropy and that the splitting must be due to hyperfine interaction. From the total signal widths at the two microwave frequencies and reasonable assumptions of the widths of the individual hyperfine lines the g anisotropy may be estimated to be within the range Δg = 0.005 to 0.007.

**Correlation between Absorbance at 410 nm, ESR Spectrum, and Enzyme Activity**—Treatment of protein B2 with hydroxylamine or hydroxurea resulted in a time-dependent parallel disappearance of enzyme activity and the peak at 410 nm (2). Such treatment did not remove iron from the protein, nor did it change the rest of the optical spectrum of protein B2. It was now found that the ESR spectrum of protein B2 was sensitive to hydroxylamine to the same extent as the peak at 410 nm. This was our first observation indicating that the two parameters might be interrelated.

Further evidence for such a correlation came from experiments in which six essentially pure preparations of protein B2, prepared at different times, were compared with respect to enzyme activity and optical and ESR spectra. In spite of the fact that these preparations on analytical gel electrophoresis all gave rise to essentially a single protein band and contained 2.0 to 2.3 atoms of iron per molecule of protein, they varied in specific enzyme activity between 6,900 and 19,800 units per mg of protein. Furthermore, considerable variations were observed in the amplitudes of the ESR spectra and in the visible spectra between 400 and 420 nm. Fig. 6 compares the spectra between 325 and 500 nm for two preparations with specific activities of 6,900 and 19,800, respectively. The most striking difference concerns the height and
Fig. 7. Correlation between enzyme activity and optical and ESR spectra. Six different preparations of protein B2 were analyzed with respect to iron content, enzyme activity, optical spectrum, and ESR spectrum. The iron content varied between 25 and 30 nmoles per mg of protein. Specific activities and ESR amplitudes are corrected for the small differences in iron content.

(a) Correlation between specific enzyme activity and ratio of absorbances at 410 and 405 nm. B: correlation between amplitude of ESR spectrum and ratio of absorbances at 410 and 405 nm. The amplitudes are given for solutions containing 1 mg of protein B2 (= 26 nmoles of iron) per ml.

The sharpness of the peak at 410 nm for which the 410:405 ratio is a good measure.

In Fig. 7A this ratio is plotted against specific activity for the six preparations of protein B2. The points can be fitted well to a straight line with highest specific activity corresponding to the highest 410:405 ratio. Similarly, a plot of the 410:405 ratio against the amplitude of the ESR spectrum (Fig. 7B) gives a straight line.

In the samples investigated the ESR signals represent unpaired spin concentrations (assuming S = 1/2) corresponding to 15 to 30% of the iron content. Since there are 2 iron atoms per molecule, which could be independent or coupled, the ESR signal detected corresponds to a maximum of 30 to 60% of these iron centers.

Thus the magnitude of the spin signal corresponded to only a fraction of the total iron content or content of 2 iron centers of the enzyme and varied between different enzyme preparations. This raises the question whether the signal represents an artifact.

Three lines of evidence, however, closely connect the signal with enzyme activity. (a) Removal of iron resulted in an inactive apoprotein which had lost the ESR signal, but when iron was reintroduced, both enzyme activity and signal reappeared. (b) Treatment with hydroxylamine gave a time-dependent inactivation of the enzyme parallel to loss of the signal. (c) Different enzyme preparations showed a positive correlation between the size of the signal and specific enzyme activity.

Our results could tentatively be explained by assuming that preparations of protein B2 contain a mixture of two very closely related types of molecules. Both types have the same iron content, identical NH₂-and COOH-terminal amino acids, and have the ability to form complexes with protein B1. However, only one type shows the ESR signal and the sharp peak at 410 nm and this type is the enzymatically active form of protein B2. The molar extinction coefficient at 410 nm of this species would be about 5 × 10³. This calculation is based on the concentration determined by ESR and the fact that the specific light absorption at 410 nm is superimposed on the rest of the spectrum.

Some of the features of the ESR signal, its g value, its saturation behavior, its persistence in liquid aqueous solution, and the absence of temperature dependence of the spin concentrations determined from the integrated absorption intensity suggest that it could be due to a free radical. The value of g = 2.0047 is within the range expected for a semiquinone but could also be compatible with, for example, a sulfur-containing radical. The g-value dependence of the ESR signal on the presence of iron in the enzyme would then mean that the radical is formed by reaction of one or both of the iron atoms. Only the active nondenatured molecules would be able to give this reaction. The increase of the ESR line-width with increasing temperature means that there must be a temperature-dependent process that actively influences the spin relaxation. This could possibly take place either via magnetic dipolar interaction between the radical and the iron atom (atoms) or a complex of the two iron atoms, or via life-time shortening of the radical species, which would be in equilibrium with an iron oxidation-reduction system in the molecule. Both the iron of the active and that of the inactive molecules would remain undetected by ESR.

Another possibility is that the ESR signal is due to one or both of the iron atoms in a particular structure only formed by the active enzyme molecules. The present data could possibly be explained on the basis of either a mononuclear or a dinuclear iron complex. Again, the remaining iron in the former case and the iron of the nonactive molecules would not give a detectable ESR signal.

In order to reveal the nature of the iron and of the ESR-absorbing structure of protein B2 further information is needed. To this end measurements are in progress of the Mössbauer effect, of the electron nuclear double resonance, and of the paramagnetic susceptibility over a wide temperature range. Whatever the structure behind the signal might be, we have shown that its presence in the resting enzyme is essential for the activity of the protein in the enzyme system, and that it is very likely closely connected with the iron. Work is in progress to study possible changes of the ESR signal during the catalytic reaction.

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