TETRANUCLEAR AND BINUCLEAR IRON-SULFUR CLUSTERS IN SUCCINATE DEHYDROGENASE: A METHOD OF IRON QUANTITATION BY FORMATION OF PARAMAGNETIC COMPLEXES

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SUMMARY

Soluble succinate dehydrogenase contains 8 atoms of iron, 8 atoms of acid labile sulfur and one covalently bound FAD per molecule; however, the distribution of iron and sulfur has not been well established. An iron counting method was devised in which electron spin resonance detectable complexes containing one iron each were formed with NO and cysteine and complex formation was measured during the gradual dissociation of the iron-sulfur clusters. In addition, a method described by Cammack was used to provide independent evidence Both methods point to the existence of two binuclear clusters and one tetranuclear iron-sulfur cluster in the succinate dehydrogenase molecule.

INTRODUCTION

It is well established that the reconstitutively active soluble succinate dehydrogenase (1, 2) contains 8 atoms of iron, 8 mol of acid labile sulfide and one covalently bound FAD per molecule (2-4). The distribution of the iron and sulfide in binuclear or tetranuclear clusters, however, has not been completely established. EPR signals from two distinct iron-sulfur clusters are visible in the dithionite reduced reconstitutively active enzyme, one at temperatures near 77°K (5, 6) and the other at colder temperatures (7, 8). Quantitation of the EPR signals in the fully reduced enzyme yields nearly two spins per flavin, and the spectra show evidence of spin-spin interaction (8); the iron-sulfur clusters responsible for these absorptions must therefore be distinct and have been designated Centers S-1 and S-2 (7, 8). A third resonance

has been detected in the oxidized enzyme (9, 10) characteristic of iron-sulfur clusters of the bacterial high potential iron-sulfur protein type (11); such clusters are known to contain 4 atoms of iron and 4 mol of acid labile sulfide.

We previously proposed that the enzyme contained two binuclear clusters of the spinach ferredoxin type and one tetranuclear cluster of paramagnetic in the oxidized state; the iron-sulfur cluster responsible for the third resonance has been designated S-3 (10). Our reasons for assigning these resonances to three separate centers have been discussed in a previous publication (8), although we were unable to exclude the possibility that two tetranuclear clusters are instead present. The work of Cammack (12) and Sweeney et al (13) supports the hypothesis of Carter et al (14) that tetranuclear clusters of this type in other proteins can undergo two sequential oxidation-reduction reactions with widely separated midpoint potentials. This has led Beinert et al (9) to point out in a recent publication the possibility that EPR signals attributed to 'Center S-2' could be due to a super-reduced form of 'Center S-3.'

In order to distinguish between these possibilities, an iron counting method was developed in which the iron-sulfur clusters were disrupted and each iron was bound in a paramagnetic complex; the number of iron atoms bound was correlated with the disappearance of the EPR signals from the iron-sulfur clusters. In addition, a method described by Cammack was used to provide corroboration. Both methods point to the existence of two binuclear clusters and one tetra-nuclear cluster in the succinate dehydrogenase.

MATERIALS AND METHODS

A paramagnetic complex described by Woolum et al (15) consisting of two cysteine, one iron and two NO moieties was prepared by adding excess sodium nitrite to a solution containing a large excess of cysteine with either ascorbate or dithionite as reductant and iron was added as either ferrous sulphate or ferric chloride. The formation of the complex was found to be quantitative with respect to iron either in aqueous solution or in 50% dimethyl sulfoxide at iron concentrations up to 3 mM, by double integration of EPR spectra under non-saturating condition against a copper II-EDTA standard.

Soluble succinate dehydrogenase (BS-SDH) was prepared as described in reference (8). EPR samples containing the paramagnetic complex described above were prepared by adding the enzyme to the solution, incubating, and freezing in a cold isopentane and methyl cyclohexane mixture. Dimethyl sulfoxide was used

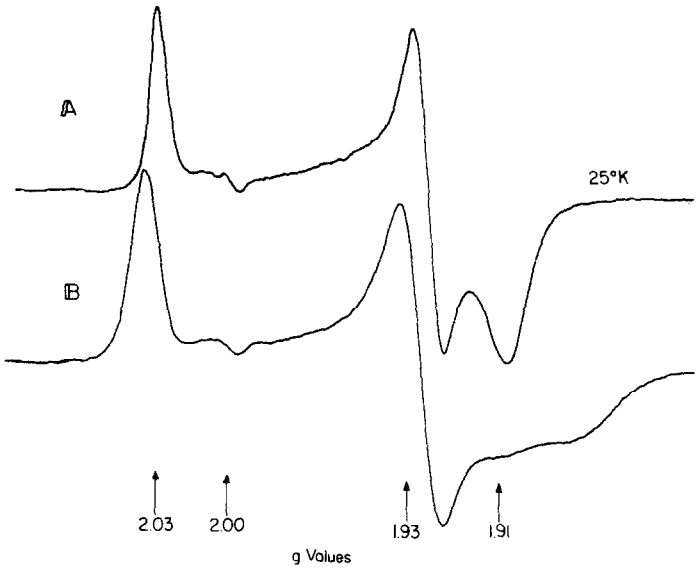

Figure 1. EPR spectra of dithionite reduced reconstitutively active succinate dehydrogenase; both samples at 16.7 mg. protein per ml. of solution, 4.1 nmoles flavin/mg protein. EPR operating conditions were: modulation frequency, 100 KHz; modulation amplitude, 5 gauss; microwave frequency, 9.12 GHz; time constant, 0.3 sec; scanning rate, 200 gauss/min; microwave power, 1 mW; temperature, 25°K.

A) enzyme in Tris maleate buffer at pH 7.0
B) enzyme in Tris maleate buffer at pH 6.2, followed by addition of dimethyl sulfoxide to a final concentration of 50%

as a solvent because it promoted more complete formation of the complex, possibly by affecting protein conformation in such a way that some of the iron was more accessible.

The method of Cammack (16) was applied to succinate dehydrogenase to attempt to distinguish between tetranuclear and binuclear clusters by an alternate method.

EPR spectra were obtained on a Varian E-4 EPR spectrometer. Temperature control was provided by an Air Products cryostat.

Integrations of spectra were performed on a Nicolet Instrument Computer. Spin concentrations were obtained as in reference (9), except that the revised method of Aasa and Vaangard (17) was used to correct for transition probability. Both Copper II-EDTA[a] and a series of standards prepared from iron salts were used as standards.

Iron and flavin were assayed as described in reference (18).

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[a] EDTA, Ethylenediamine tetraacetic Acid Tetrasodium Salt

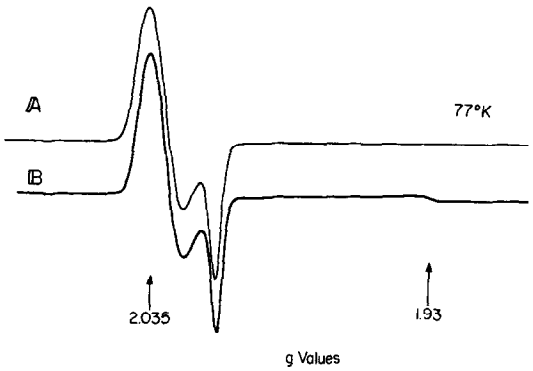

Figure 2. EPR spectra of cysteine-iron-NO complexes. EPR operating conditions were as in Figure 1 except: modulation amplitude, 1 gauss; microwave power, 200 μW, temperature, 77°K.

A) complex formed by addition of nitrite to a solution of ascorbate, cysteine (30 mg/ml) and iron salts (Ferrous sulphate, 1.5 mM) in 50% dimethyl sulfoxide

B) as in A)except succinate dehydrogenase, 16.7 mg protein per ml solution instead of iron salts; 4.1 nmoles flavin per mg protein, 8.1 atoms iron per mol flavin

RESULTS AND DISCUSSION

Soluble succinate dehydrogenase in 170 mM Tris maleate buffer at pH 6.2 containing excess ascorbate was added to an equal volume of dimethyl sulfoxide. The EPR spectrum at 25°K of the dithionite reduced enzyme in this solvent is shown in Fig. 1b; the spectrum of the dithionite reduced enzyme in Tris maleate buffer alone at neutral pH is shown in Fig. 1a. Quantitation of paramagnetic species yielded 1.7 spins per flavin even at 25°K for the enzyme in 50% dimethyl sulfoxide; thus signals arising from more than one cluster can be detected.

When excess sodium nitrite was added to the solution described above and a small amount of solid dithionite was used as a reductant, a paramagnetic complex was formed showing an identical EPR spectrum to that produced with simple iron salts as described. Comparison of these spectra, shown in figure 2, makes quantitation of iron bound in this complex possible.

Three or four iron atoms per flavin could be incorporated into cysteine-iron-NO complexes without significant decrease in the 'g = 1.94' signal from

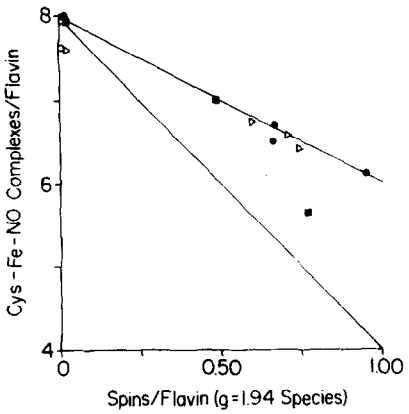

Figure 3. Plot of increase in number of iron atoms/flavin present as paramagnetic cysteine-iron-NO complex (vertical axis) against the number of spins/flavin present as 'g = 1.94' species (horizontal axis). The slanted lines are the upper limits possible if the g = 1.94 species is a tetranuclear (lower lines) or binuclear (upper line) iron sulfur cluster, the limiting condition being that the total number of iron atoms per flavin is eight. Data were obtained from three batches of enzyme.

iron-sulfur clusters. An additional two iron atoms were incorporated as the concentration of the species absorbing at g = 1.94 decreased from 1.7 to 1 per flavin. The time scale of these changes was too rapid for the formation of the complex to be quantitative, however.

On further incubation, the number of iron atoms per flavin present in the cysteine-iron-NO complex increased from 6 to 8 as the number of spins in the species absorbing at 'g = 1.94' decreased from one to zero per flavin as shown in figure 3. Further incubation or addition of nitrite, cysteine or reductant resulted in no further change in the spectrum.

The absorption at g = 1.94 must therefore be due to a species containing only two irons per cluster. Under conditions resulting in six iron atoms per flavin being present in the form of the paramagnetic cysteine-iron-NO complexes, one spin per flavin is present as the 'g = 1.94' species. If a tetranuclear cluster were responsible for this species, a total of ten iron atoms per flavin would have to be present in the enzyme. A binuclear cluster would yield a total

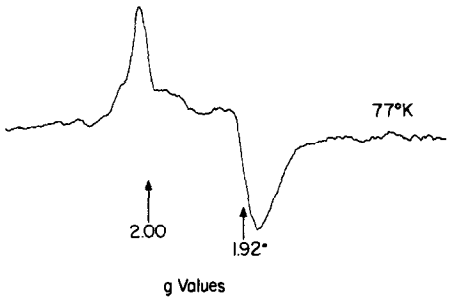

Figure 4. EPR spectrum of dithionite reduced enzyme in 70% dimethyl sulfoxide. EPR conditions were as in Figure 1 except: time constant, 1 sec; scanning rate, 250 gauss/min; temperature, 77°K; final protein concentrations as in Figure 1.

of eight irons per flavin, in agreement with chemical analysis on these samples and with the literature (2-4). Although we cannot distinguish between Centers S-1 and S-2 under the conditions of complex formation, the assumptions that either or any combination of both account for the remaining g = 1.94 signal observed after nitrite treatment leads to identical conclusions.

A method described by Cammack (16) to distinguish between binuclear and tetranuclear iron-sulfur clusters was applied to succinate dehydrogenase. Figure 4 shows the EPR spectrum, recorded at 77°K, of the dithionite reduced enzyme in 70% dimethyl sulfoxide. The signal is readily saturated at 25°K and at 15°K is heavily saturated at all microwave powers accessible with our equipment (<200 μW). This spectrum is nearly identical to the spectrum published by Cammack of adrenodoxin in 80% dimethyl sulfoxide. This strongly suggests that a binuclear iron-sulfur cluster is responsible for the absorption. Tetranuclear clusters in 70% dimethyl sulfoxide were shown to have quite different spectra and were neither detectable at 77°K nor readily saturable until below 15°K (16).

We were able to detect only 0.7 spins per flavin in the soluble enzyme in 70% dimethyl sulfoxide. We also were unable to detect any EPR spectra of the tetranuclear type. These observations are consistent with the observations of Cammack (16) on the extreme lability of iron-sulfur centers in dimethyl sul-

foxide at high concentrations and with the reported extreme lability of Center S-3 in soluble preparations (10).

We conclude from the results of these two methods that succinate dehydrogenase contains at least two binuclear iron-sulfur clusters. The possibility for the presence of two tetranuclear clusters in the molecule (giving rise to three distinct EPR spectra, S-1, S-2, and S-3) is completely ruled out. Up to now there is no known binuclear iron-sulfur cluster which is paramagnetic in the oxidized state. The EPR spectrum of the oxidized enzyme is most likely due to a single tetranuclear cluster, and the arrangement in the best agreement with available data is the presence of two binculear and one tetranuclear iron-sulfur clusters per flavin, in line with our earlier proposal (8).

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