ISOLATION AND PROPERTIES OF AN IRON-PROTEIN FROM THE (REDUCED COENZYME Q)-CYTOCHROME C REDUCTASE COMPLEX OF THE RESPIRATORY CHAIN[1]

John S. Rieske,[2] David H. MacLennan,[3] and Roger Coleman[3]
Institute for Enzyme Research, University of Wisconsin
Madison 6, Wisconsin

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The existence of at least three entities in beef heart mitochondria giving electron paramagnetic resonance (EPR) signals in the g = 1.94 region have been described by Beinert and his associates (Beinert and Lee, 1961; Beinert et al., 1962). These signals have been tentatively ascribed to a specific type of iron-protein compound (Beinert et al., 1962). Fractionation of beef heart mitochondria has concentrated one of these entities, giving an EPR signal at g = 1.90, together with the (reduced coenzyme Q)-cytochrome c reductase complex (Complex III) (Rieske et al., a, in preparation). Also, on cleavage of Complex III most of the nonheme iron present was precipitated together with an insoluble protein of low cytochrome content; a large fraction of the residual g = 1.90 EPR signal was concentrated along with the iron in this material (Rieske et al., b, in preparation). This communication deals with the isolation from Complex III of a succinyl derivative of the iron-protein which contains the g = 1.90 EPR signal. In addition, some of the pertinent properties of this protein are described.

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[3] Postdoctoral trainees, Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin. 53706

Separation of the Crude Iron Protein from Complex III - A solution of complex III (10-20 mg/ml) in a buffer which was 0.67 M in sucrose, 0.05 M in Tris-chloride, pH 8, and mM in histidine, was treated with antimycin A in a molar amount equal to, or in excess of, the cytochrome c_1 content. This antimycin-treated preparation was incubated in the presence of sodium taurocholate (2 to 5 per cent) and saturated (0°) ammonium sulfate (0.25 to 0.30 volumes), whereupon a brown precipitate of crude iron-protein formed. This reaction was essentially complete after 30 minutes at 25° or after several hours at 0°. Without pretreatment with antimycin A most of the cytochrome b in Complex III coprecipitated with the crude iron protein (Rieske and Zaugg, 1962).

Succinylation of the Crude Iron Protein - Without modification the crude iron protein was too insoluble in water to permit further purification. Although 0.5% sodium lauryl sulfate readily brought the protein into solution, all attempts at further purification in the presence of this reagent failed; also, the g = 1.90 EPR signal was rapidly lost. However, after treatment with succinic anhydride the protein became completely soluble with no alteration or diminution of the g = 1.90 EPR signal. The procedure used for succinylation was similar to that employed by Klotz and Keresztes-Nagy, 1963, except that the reaction with succinic anhydride was carried out at 0°. A suspension of the crude iron-protein in 0.05 - 0.1 M phosphate, pH 7.5, was treated with succinic anhydride in excess over that required for "solubilization" of the protein, the pH being maintained between 7 and 8 during the reaction by addition of M KOH. The solution was allowed to stand until the excess of reagent was hydrolyzed as indicated by no further change in pH.

Purification of the Succinylated Iron-Protein - The clear, brownish-pink solution of crude, succinylated, iron-protein was placed on a Sephadex G-100 column (2.5 x 120 cm, equilibrated with 0.05 M phosphate, pH 7.5) the same buffer being used for elution. Two bands containing

protein were eluted from the column — a fast moving major band, followed by a slower minor band. The protein from the pooled fractions containing the slow moving component was concentrated by ammonium sulfate precipitation (0.70 saturated at 0°). About 20 per cent of the protein and about 40 per cent of the nonheme iron in the crude iron-protein preparation were recovered in this purified iron-protein fraction.

Composition and Properties of the Purified, Succinylated Iron-Protein - Table I gives the composition and some of the properties of this iron-protein. As recovered from the Sephadex column the protein contained 50-65 mμmoles of iron per mg of protein. A second passage of the purified protein through the Sephadex G-100 column yielded a preparation with an iron-to-protein ratio (mμmoles/mg) as high as 85. The higher values for the iron content indicated a minimal molecular weight of the succinylated protein of about 12,000. The purified, succinylated iron-protein maintained a single homogeneous boundary during sedimentation in the ultracentrifuge. Based on the s/D value (not extrapolated to infinite dilution) as determined by the Archibald approach to equilibrium method and the assumption of a $\bar{V}$ of 0.75, the estimated molecular weight is 26,000. Therefore, it appears that there are two atoms of iron per molecule of protein. At most, only trace amounts of heme were found as a contaminant in the purified protein, as evidenced by the virtual absence of a Soret band in the spectrum upon reduction with dithionite. No flavin contamination could be detected. By the use of C^{14} succinic anhydride in the succinylation step it was determined that approximately 3 per cent by weight of the purified protein was due to the added succinyl groups. On the basis of a molecular weight of 26,000 it was computed that 7-8 succinyl residues per molecule are present in the iron-protein.

Absorption spectra - Absorption spectra in the visible range are given in Fig. 1. The spectrum of the oxidized form displayed absorption peaks at 575 mμ and 460 mμ, and a shoulder at 315 mμ. Treatment with ascorbate reduced the absorbancy throughout the visible range, yielding

TABLE I

Composition and Properties of the Succinyl Derivative of the Iron-protein Prepared from Complex III

Property	Value
Iron content[1]	60-80 mμmoles/mg protein
Heme content	< 0.07 mμmoles/mg protein
Flavin content	not detectable
Succinyl content	3% by weight
Labile sulfide[2]	0.7-0.8 moles/gram atom of Fe
Molecular weight (minimal)	$12\text{-}16 \times 10^3$
Sedimentation coefficient, $S_{20,w}$	2.0 S
s/D (not extrapolated to infinite dilution)	2.8×10^{-7}
Molecular weight (estimated)[3]	26×10^3

[1] Iron content is reported on freshly isolated fractions.
[2] Determined by the method of Fogo and Popowsky, 1949.
[3] Molecular weight as determined with an assumed $\bar{V} = 0.75$.

a spectrum with small maxima at 380 mμ, 420 mμ, and 515 mμ; also, the shoulder at 315 mμ disappeared. A difference spectrum (oxidized minus reduced, insert in Fig. 1) displayed prominent absorbancy changes at 335 mμ, 465 mμ, and 570 mμ. Dithionite, added to the protein after reduction with ascorbate, caused additional bleaching without further change in the spectral pattern. Oxidation with ferricyanide of the dithionite-reduced protein has caused the typical oxidized spectrum to reappear; however, in this case the loss in absorbance specifically caused by dithionite was not reversed.

EPR spectra - The electron paramagnetic resonance (EPR) spectra of this protein in the g = 2.0 region are given in Fig. 2. For comparison, the EPR spectra of intact Complex III are also shown. Both the intact complex and the purified iron-protein showed a prominent g = 1.90 signal upon reduction either with ascorbate or with dithionite. However, in the intact Complex III a definite g = 1.94 signal appeared after the addition of dithionite. This signal is attributable to either one or both of the g = 1.94 EPR components found in the DPNH dehydrogenase and the succinic dehydrogenase flavoproteins (Beinert et al., 1962). The absence of this

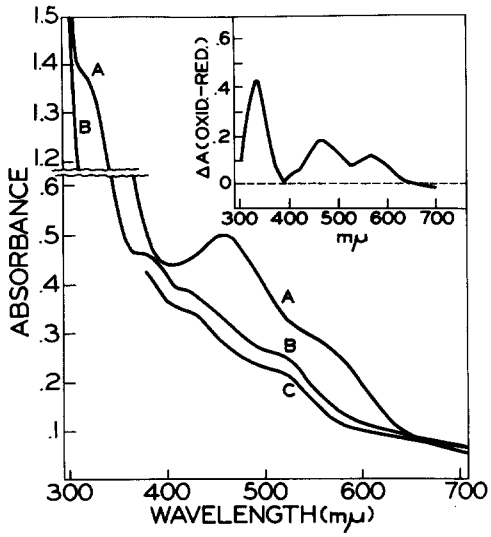

Fig. 1. Absorption spectra of the purified, succinylated, iron-protein. Spectra were taken on a solution of the iron-protein containing 3.1 mg of protein/ml (Lowry et al., 1951) and 48 mμmoles of iron/mg of protein. The protein was oxidized with a small excess of potassium ferricyanide, then precipitated at 0.70 saturation with ammonium sulfate at 0° and collected by sedimentation. The protein pellet was thoroughly drained and dissolved in 0.05 M phosphate, pH 7.5. The absorption spectra were taken immediately at 0° with a Beckman DK-2 spectrophotometer: A, oxidized form; B, reduced by the addition of solid potassium ascorbate; C, dithionite added to the cuvette treated as in B.

signal in the purified iron-protein is a good indication that it is free of these other components. Although the essential characteristics of the EPR signal of the intact complex are preserved in the purified iron protein, some broadening appears to have taken place. It may be significant that this type of broadening often occurred in the intact complex after addition of dithionite (Fig. 2, curve B). That these two effects are related is indicated by the absence of any further broadening in the EPR signal of the purified iron-protein after the addition of dithionite. However, dithionite did cause the disappearance of the small contaminant copper signal that was evident both in the spectrum of the oxidized form and in the spectrum produced by reduction with ascorbate. No alteration in intensity, form, or stability of the signal

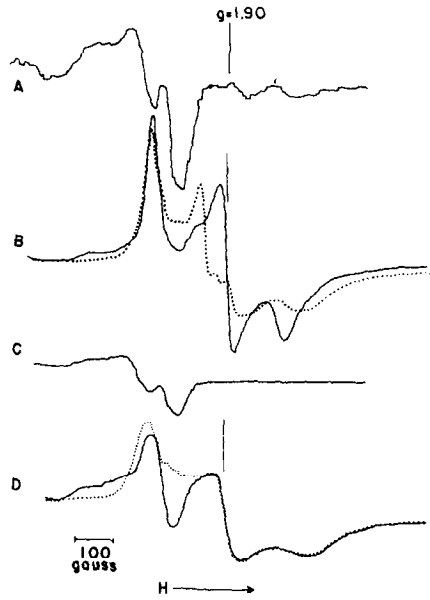

Fig. 2. Electron paramagnetic resonance (EPR) spectra of the intact Complex III and the succinylated iron-protein derived from Complex III. A, oxidized Complex III (29 mg of protein/ml), amplitude X 4; B, same as A but treated with ascorbate (solid line), then with dithionite (dotted line), amplitude X 1; C, oxidized iron-protein (5.0 mg of protein/ml, amplitude X 1.3; D, same as C but treated with ascorbate (solid line), then with dithionite (dotted line), amplitude X 1.3. All spectra were recorded at -176° with a Varian V 4500-10A spectrometer set at a modulation amplitude of 18 gauss.

To conform to accepted convention the recorded spectra are rotated 180° with respect to spectra previously reported from this laboratory.

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occurred as a result of the succinylation process used to "solubilize" the crude iron-protein.

Further studies of this iron-protein are reported in a companion communication (Coleman et al., 1963). A more detailed report of these and additional studies will be published elsewhere.

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