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CHARACTERIZATION OF THE IRON-SULFUR PROTEIN OF THE MITOCHONDRIAL OUTER MEMBRANE PARTIALLY PURIFIED FROM BEEF KIDNEY CORTEX

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Summary

The iron-sulfur protein present in the mitochondrial outer membrane has been partially purified from beef kidney cortex mitochondria be means of selective solubilization followed by DEAE-cellulose chromatography. The EPR spectrum of the iron-sulfur protein with *g*-values at 2.01, 1.94 and 1.89 was well resolved up to 200 K which is unusual for an iron-sulfur protein. Analyses confirmed a center with two iron and two labile sulfur atoms in the protein. By measuring the effect of oxidation-reduction potential on the EPR signal amplitude, midpoint potentials at pH 7.2 were determined both for the purified iron-sulfur protein, +75 (±5) mV, and in prepared mitochondrial outer membrane, +62 (±6) mV. At pH 8.2 slightly lower values were indicated, +62 and 52 mV, respectively. The oxidation-reduction equilibrium involved a one electron transfer. A functional relationship to the rotenone-insensitive NADH-cytochrome *c* oxidoreductase in the mitochondrial outer membrane is suggested. Both this activity and the iron-sulfur center were sensitive to acidities slightly below pH 7 in contrast to the iron-sulfur centers of the inner membrane.

Introduction

From studies of rat kidney cortex microsomes by EPR spectroscopy a new absorption showing *g*-values characteristic for an iron-sulfur protein was discovered [1]. The signal appeared after reduction of the microsomes with NADH or NADPH. As the generally known iron-sulfur proteins in the animal cells are found in the inner membrane of the mitochondria, comparisons

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between microsomes and submitochondrial particles were made. The signal was not obtained by reduction with succinate, and the activity of NADH-oxidase in the microsomal preparation was so low that the possibility that the signal should originate from the inner membrane impurities could be ruled out. Furthermore, the iron-sulfur protein was reduced by NADH or NADPH whether the respiratory chain inhibitors rotenone or tenoyltrifluoroacetone were added or not. Thus a function of the iron-sulfur protein in the respiratory chain after the rotenone inhibitor site and with NADH as substrate seemed unlikely. The most distinct difference from the inner membrane iron-sulfur proteins, however, was the characteristic *g*-values and temperature dependence. Subsequently closer examination of different subcellular fractions revealed that a high intensity of the new iron-sulfur signal followed the mitochondrial fraction and could be traced to the outer membrane [2]. This has also been confirmed by other groups [3,4]. Reduction of the outer membrane resulted in a well resolved EPR spectrum at 77 K with *g*-values 2.01, 1.94 and 1.89. Double integration of the EPR spectrum and acid-labile sulfur analysis indicated that the iron-sulfur center contains 2 iron and 2 acid-labile sulfur atoms for each unpaired spin observed. This is similar to what is known about several other iron-sulfur proteins containing 2Fe-2S centers [5]. In this communication partial purification and some chemical and physicochemical properties of the iron-sulfur protein are described.

Materials and Methods

Chemicals were obtained from the following sources: cytochrome *c* type III, L-kynurenine sulfate, β-NADH grade III, and NADPH type I from Sigma Chemical Co.; Triton X-100 from Rohm and Haas Co.; DEAE-cellulose DE-32 from Whatman Biochemicals Ltd.; Sephadex G-200 from Pharmacia Fine Chemicals; benzylamine from E. Merck, Darmstadt, was converted into its hydrochloride form and recrystallized from ethanol before use. All other chemicals were of reagent grade. Solutions were made with deionized, glass distilled water.

For the preparation of beef kidney cortex mitochondria the procedure used by Löw and Vallin [6] for beef heart mitochondria has been followed with some slight modifications: (i) Before the first centrifugation the disintegrated material was homogenized in a 200 ml Potter-Elvehjem type glass homogenizer by two complete strokes with a loosely fitting teflon pestle (r = 23 mm) rotating at 345 rev/min. (ii) The centrifugations to collect and wash the mitochondria were done at 6000 × g for 20 min. The preparation of crude outer membrane was carried out as described for rat liver mitochondria [7], omitting the last step.

Rotenone-insensitive NADH and NADPH-cytochrome *c* oxidoreductase activities were assayed essentially according to Sottocasa et al. [8], measuring the reduction of cytochrome *c* at 550—540 nm and using $\Delta A = 19.1\ cm^{-1} \cdot mM^{-1}$ [9]. The enzymatic activity was inhibited by Triton X-100. The inhibition was about 50% at 0.1% Triton X-100 (w/v) and remained almost constant at higher concentration of the detergent. Since the various fractions examined contained different concentrations of Triton X-100 all the measurements were

carried out in presence of 0.1% Triton X-100 in the basic reaction mixture. Monoamine oxidase (EC 1.4.3.4) activity [10] was measured using benzylamine as substrate and observing the absorbance increase at 250—225 nm. Kynurenine hydroxylase (EC 1.14.1.2) activity was measured according to Bandlow [11], and NADH-coenzyme Q reductase according to Sanadi et al. [12] with coenzyme Q_6 as electron acceptor.

Protein content was determined by the biuret method [13] and in some cases checked by the Lowry method [14]. Bovine serum albumin was used as standard. Total and acid-extractable flavin content was measured spectrophotometrically according to Rao et al. [15]. The FAD to FMN ratio of the extractable flavin was determined fluorimetrically according to Faeder and Siegel [16] on an instrument constructed in this laboratory (Astlind, T. and Ehrenberg, A., to be published elsewhere). Acid-labile sulfide was determined by the method of Colbeck and San Pietro [17]. For spectrophotometric determination of iron the samples were ashed with H_2SO_4 by addition of H_2O_2, reduced by sodium ascorbate, neutralized with sodium acetate, and measured at 535 nm in presence of 4,7-diphenyl-1,10-phenanthrolinesulfonic acid disodium salt. Disc gel electrophoresis and "dodecyl sulfate" gel electrophoresis were carried out according to Gabriel [18] and Weber and Osborn [19], respectively. EPR spectra were obtained as described in the figure legends using a Varian V-4502 spectrometer with 100 kHz field modulation, and concentration of the iron-sulfur protein was determined by double integration and *g*-value correction [20] using 1 mM Cu^{2+} in 10 mM EDTA (pH 8.3) as standard. Light absorption spectra were obtained with an Aminco DW-2 UV-VIS spectrophotometer.

The oxidation reduction potentials were measured potentiometrically according to the method of Dutton [21] with some slight modifications. The reaction vessel, with a thermostatically controlled water jacket (25° C), was sealed with a stopper of butyl rubber which also accomodated the electrodes and a gas inlet and outlet. The oxidation-reduction potential was measured with a pH and mV meter type PHM 26 (Radiometer, Copenhagen) using a platinum electrode (Radiometer type P1312) and a standard calomel electrode (Radiometer type K4112). The vessel content was maintained under an argon atmosphere introduced through the stopper via a stainless steel tube, internal diameter 0.7 mm. The argon was obtained almost oxygen-free by conveying it to the vessel through a series of two gas-scrubbing bottles containing 2 g $Na_2S_2O_4$ per 100 ml 0.1 M NaOH. All gas flow connections were made of butyl rubber tubings and were as short as possible. The anaerobic condition of the apparatus was checked occasionally by bubbling the argon through a vessel with 100 μM phenosafranin at pH 7.0 reduced with $Na_2S_2O_4$ to the titration point. The solution remained colourless for several hours indicating that the oxygen concentration of the argon stream was <1 ppm. Small additions to the reaction vessel of reductant and oxidant were made with 5-μl syringes (Hamilton type 7105N). Each sample for EPR measurements was withdrawn in a 0.5 ml gas-tight syringe (Hamilton type 1750) flushed before use with argon. The sample was immediately transferred into a 3 mm quarts EPR tube previously flushed with argon and rapidly frozen by immersion into liquid nitrogen.

In all redox titrations the following mediators were used: *N,N,N',N'*-

tetramethyl-*p*-phenylenediamine (Eastman Organic Chemicals; E'_0 = +260 mV at pH 7), 2,6-dichlorophenolindophenol (Sigma; E'_0 = +217 mV), phenazine methosulfate (Calbiochem; E'_0 = +80 mV), duroquinone (Fluka; E'_0 = +7 mV), resorufin (Eastman Organic Chemicals; E'_0 = —50 mV). Results did not vary with the concentrations of these mediators or if two other mediators, phenazine ethosulfat (Sigma Chemical Co.; E'_0 = +55 mV) and/or 2-hydroxy-1,4-naphthoquinone (Eastman Organic Chemicals; E'_0 = —145 mV) were added or not.

The oxidation reduction potential was adjusted by small additions of argon-bubbled solutions of 100 mM $K_3Fe(CN)_6$ for higher potentials and $Na_2S_2O_4$ in equimolar NaOH for lower potentials. In all the experiments samples were taken 5 min after adjustment of the oxidation-reduction potential, first in the reductive phase and then in the oxidative phase of the titration to establish that the system was reversible. The potential E_h relative to the standard hydrogen electrode was obtained by adding the value for the calomel electrode, +244.4 mV, to the measured values.

The equipment and experimental procedure were tested using saturated solutions of quinhydrone at different pH values and further by measuring the mid-point potentials of phenosafranin and myoglobin spectrophotometrically at 552 nm and by EPR at 77 K, respectively. The E'_0 values thus determined at pH 7 agreed within 1—4 mV with reported values [22].

Anaerobic experiments in order to study reactions of the iron-sulfur protein with reduced pyridine nucleotides and cytochrome *c* were carried out by using the same apparatus as used for the redox measurements. The iron-sulfur protein (0.3 ml), dialyzed against 50 mM Tris · HCl/0.1% Triton X-100 (pH 7.5) was transferred to EPR tubes containing 10 µl reactant, or water as reference, and frozen immediately, i.e. within 10 s after mixing. In the oxidation experiment the iron-sulfur protein was adjusted by dithionite to approx. —160 mV before the transfer to EPR tubes.

Results

Partial purification of the iron-sulfur protein

The iron-sulfur protein was prepared from beef kidney cortex mitochondria by a procedure including selective solubilization of the mitochondria and anion-exchange chromatography of the solubilized materials as follows:

(1) Mitochondria with a protein content of 35—50 mg/ml were diluted with an equal volume of a solution containing 20 mM glycine/NaOH, 1 mM EDTA and 1 mM 2-mercaptoethanol, pH 9.6 (buffer A). The pH drop was compensated by adjustment to pH 9.6 with 0.1 M NaOH under continuous stirring. Solid KCl to a final concentration of 1 M was then added. The mixture was kept at room temperature, slowly stirred for 30 min and then centrifuged at 105 000 × *g* for 1 h at 4°C. All further work was carried out in the cold room at 4°C.

(2) The pellet was suspended with the buffer A with 0.1 mg Triton X-100 added per mg of original mitochondrial protein. After 20 min incubation the sample was again centrifuged as in step 1.

(3) The pellet was suspended with buffer A in presence of 0.5 mg Triton X-100 per mg of original mitochondrial protein and treated as in the previous

TABLE I

PARTIAL PURIFICATION OF THE OUTER MITOCHONDRIAL MEMBRANE IRON-SULFUR PROTEIN FROM BEEF KIDNEY CORTEX

About 1 kg of fresh beef kidney tissue was used as starting material. The purification was carried out as described in the text.

	Protein (mg)	Iron-sulfur protein (g = 1.89)		
		nmol	Purification (-fold)	Yield (%)
Mitochondria	7100	570	1	100
Solubilized supernatant (step 3)	1200	460	5	81
DEAE-cellulose eluate (iron-sulfur fraction, step 4)	370	370	12	65

step. The main part (approx. 80%) of the iron-sulfur protein was solubilized in this step and after the centrifugation recovered in the supernatant.

(4) The protein was further purified by fractionation on a DEAE-cellulose column equilibrated with buffer A containing 0.01% (w/v) Triton X-100. The iron-sulfur protein was eluted with the equilibrium solution. The eluate containing the iron-sulfur protein was concentrated approximately three times using the Diaflo ultrafilter XM50 (Amicon).

(5) Finally the sample was dialyzed against the EDTA-containing buffer A to remove iron impurities with EPR absorption at $g = 4.3$.

Results of a typical preparation are summarized in Table I. By this preparation procedure contamination from proteins of the mitochondrial inner membrane was effectively eliminated. Only traces of cytochromes could be present according to light absorption and no other iron-sulfur proteins could be detected by EPR in the iron-sulfur fraction from the DEAE-cellulose column (cf. next section). It was found that most of the cytochrome oxidase remained unsolubilized in step 3 but could be released in a further solubilization step (1—2 mg Triton X-100 per mg of original mitochondrial protein).

Disc gel electrophoresis of the DEAE-cellulose eluate resulted in one intense high molecular weight band and one weak molecular weight band. The iron-sulfur protein was found in the void volume of a Sephadex G-200 column, indicating that it belongs to the high molecular weight component. Dodecyl sulfate gel electrophoresis gave rise to several bands.

Analytical and spectral data

Fig. 1 shows the EPR spectrum at 28 K of the iron-sulfur protein as prepared (A) and after reduction with $Na_2S_2O_4$ (B—D) recorded at three temperatures. Spectrum A shows only a weak cavity background and no signal of an iron-sulfur protein of high potential type could be detected. The g-values of the reduced form of the iron-sulfur protein are 2.01, 1.94 and 1.89. The composition of the iron-sulfur center was examined by double integration of spectrum B and chemical analysis of the same sample with respect to iron and acid-labile sulfide. The results were 1.1 ± 0.2 nequiv. spin $\frac{1}{2}$/mg protein, 2.5 ± 0.5 ng atoms iron/mg protein, and 2.3 ± 0.3 ng atoms labile sulfur/mg protein. These data

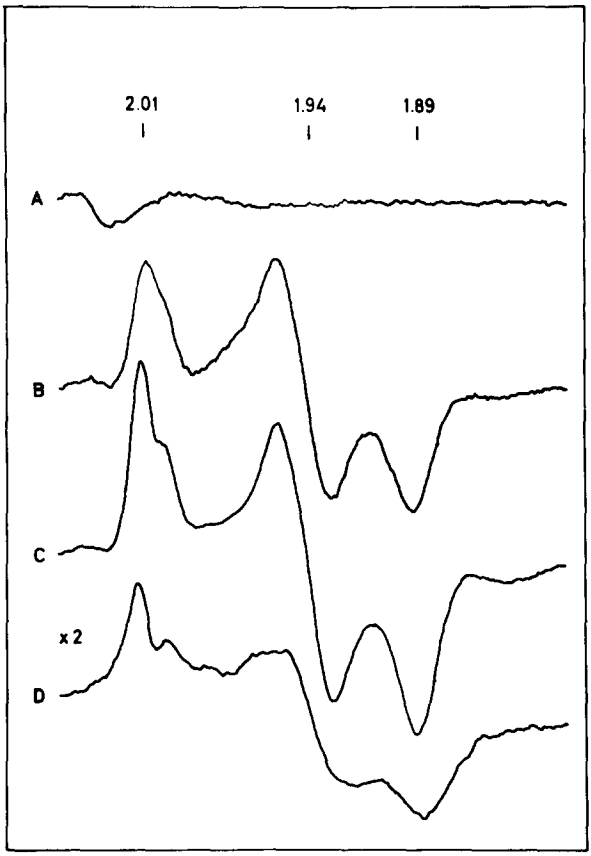

Fig. 1. EPR spectra of iron-sulfur protein fraction (10 mg protein/ml) eluted from DEAE-cellulose column: (A) As prepared. (B—D) After reduction with $Na_2S_2O_4$ recorded at 28 K (A,B), 94 K (C) and 203 K (D). EPR conditions were: microwave frequency, 9.2 GHz; scanning rate, 100 G/min; time constant, 1 s; modulation amplitude, 12 G (A,B) or 15 G (C,D); microwave power, 5 mW (A,B) or 24 mW (C,D).

indicate a stoichiometry of one unpaired spin for two iron and two labile sulfur atoms. The same sample had before the dialysis in the final step a total iron content of 4.6 ng atoms/mg protein and showed an EPR absorption at $g = 4.3$. By comparison with a standard solution of Fe^{3+} in EDTA buffer, accounting for a small difference in amplitudes by the equation: (intensity) = (amplitude) × (width)^2, the amount of contaminating ferric iron was estimated to be 2.3 ng atoms/mg protein. The difference, 2.3 ng atoms/mg protein, represents the iron-sulfur protein in good agreement with the analysis of total Fe after the dialysis.

Although some broadening of the EPR lines had occurred the iron-sulfur protein could be observed at high temperatures (>200 K) where most known iron-sulfur proteins are not detectable (Fig. 2). The $g = 2.01$ signal was overlapped by a free radical component, the amplitude of which was relatively more pronounced at higher temperatures because of microwave saturation at low temperatures.

The radical signal was absent if the iron-sulfur protein was reduced with a

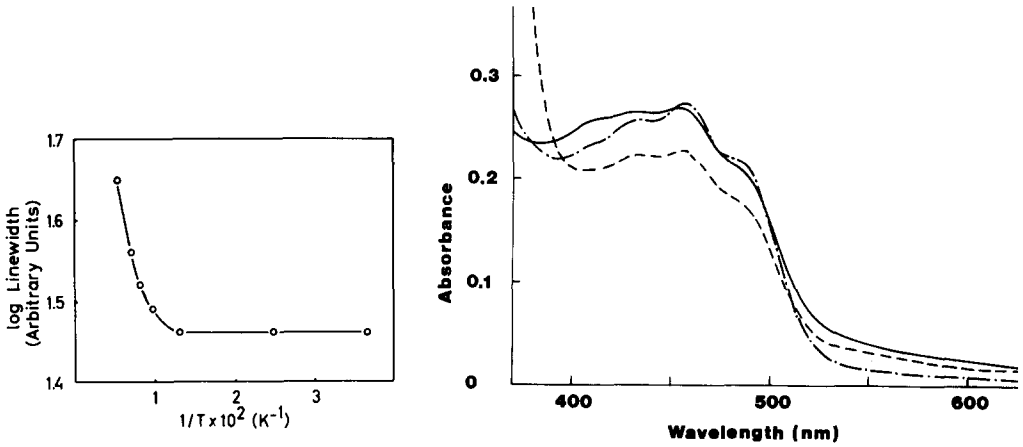

Fig. 2. Temperature dependence of the linewidth of the iron-sulfur protein measured at the half height of the $g = 1.89$ signal. Vertical axis, log linewidth; horizontal axis, the inverse of the temperature. EPR conditions as in Fig. 1.

Fig. 3. Light absorption spectra of DEAE-cellulose eluted iron-sulfur protein (2.5 μM), untreated (———) and after reduction with $Na_2S_2O_4$ (- - - - -); and of acetone extract of the same fraction (— · — · —). The acetone extract was obtained in the following way: The iron-sulfur fraction in 25% glycerol was added to 9 volumes of cold acetone (—70°C). After 30 min continuous stirring at —70°C the solution was centrifuged at 10 000 × g for 10 min at —20°C. The acetone was then evaporated from the supernatant. The remaining glycerol/water solution, the optical spectrum of which is presented, did not show any iron-sulfur EPR signal upon reduction.

small amount of $Na_2S_2O_4$ to approx. —160 mV but was developed after addition of excess of $Na_2S_2O_4$. In the iron-sulfur fraction no other iron-sulfur protein EPR signals were detected from 4 to 77 K.

When the sample was reduced by NADH or NADPH an EPR absorption typical of complexed Cu^{2+} was observed. This could be shown to originate from an impurity in the reduced pyridine nucleotides, amounting approx. 0.5% on a molar basis in the samples used.

The preparations contained different amounts of flavin. The samples of Fig. 1 also analyzed for iron and labile sulfide was found to contain 1.7 nmol flavin/mg protein of which 0.4 were covalently bound and 1.3 were acid extractable. Of the latter 1.1 nmol/mg protein were found to be FAD, which hence was the only flavin species of this preparation present in equivalent amounts to the iron-sulfur center. In another sample FAD was still the most abundant species but corresponded on a molar basis to only 50% of the iron-sulfur centers.

Light absorption spectra of the iron-sulfur fraction in the visible region are shown in Fig. 3. The solid line refers to the oxidized and the dashed line to $Na_2S_2O_4$-reduced protein. The general decrease of absorbance above 400 nm upon reduction is a normal behaviour for iron-sulfur proteins [23]. From the light absorption changes in the Soret region it may be concluded that any cytochrome contaminants must be less then 4% on a molar basis. Reduction of flavin could also contribute to the observed decrease in absorbance around 450 nm. Making account for the flavin content, estimation of a difference extinction coefficient of the iron-sulfur protein could be made. Thus a

ΔA_{450nm} of approx. 4.2 $cm^{-1} \cdot mM^{-1}$ was found. This value is comparable with the corresponding value 5.1 $cm^{-1} \cdot mM^{-1}$ calculated from data reported for spinach ferredoxin [24]. The structure found in the light absorption spectrum could to a major part be recovered in an acetone extract of the iron-sulfur protein, the spectrum of which is also shown in Fig. 3. There was no iron-sulfur protein in this fraction and no change in absorbance upon $Na_2S_2O_4$ addition. After acetone extraction the iron-sulfur protein remained completely in the residue. A similarly structured spectrum, characteristic for carotenoid-like compound(s) has also been found in acetone extract from mitochondria [25,26]. Based on an absorbance of 2500 for 1% solution and 1 cm optical path length [25] a rough estimate of the concentration of carotenoid in the iron-sulfur fraction was 0.6 mg/g protein, i.e. in the equimolar range with respect to the iron-sulfur protein concentration.

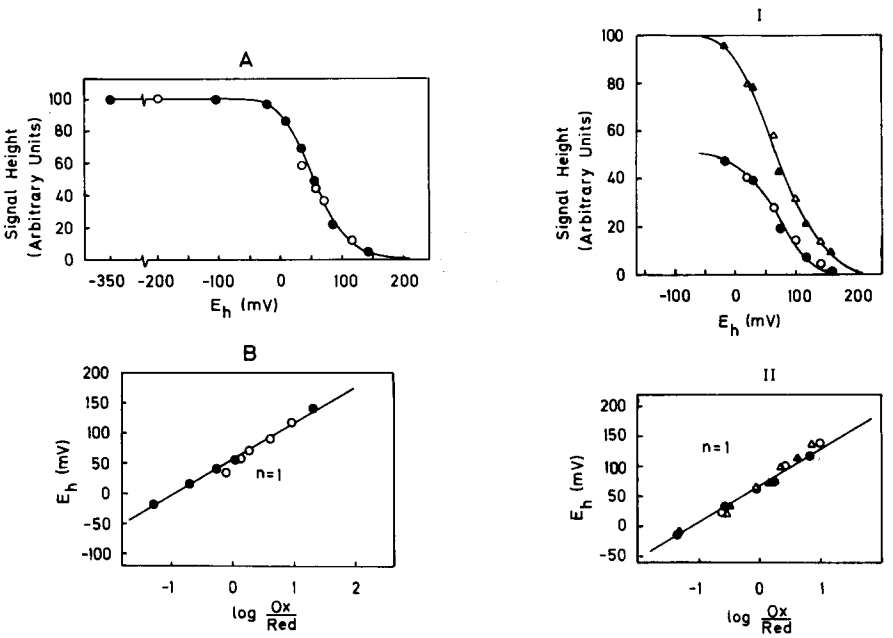

Fig. 4. Results of EPR measurements at varied oxidation-reduction potentials of the iron-sulfur protein in mitochondrial outer membrane (21 mg protein/ml) from beef kidney cortex. The sample was buffered with 40 mM morpholinopropane sulfonate and the following oxidation-reduction mediators were added: 80 μM *N,N,N',N'*-tetramethyl-phenylenediamine, 80 μM 2,6-dichlorophenolindophenol, 40 μM phenazine methosulfate, 50 μM duroquinone, 6 μM resorufin, 33 μM 2-hydroxy-1,4-naphthoquinone. Final pH, 7.2; temperature, 25°C. (A) Peak height of $g = 1.89$ signal as a function of E_h. (B) E_h as a function of the logarithm of the concentration ratio of the oxidized and reduced form of the iron-sulfur protein. The line is drawn for a theoretical one electron acceptor. Reductive (●) and oxidative (○) titrations were made with $Na_2S_2O_4$ and $K_3[Fe(CN)_6]$, respectively. The EPR operating conditions were the same as in Fig. 1C except for microwave power, 30 mW, and the sample temperature, 77 K. For further details of the titration procedure, see Materials and Methods.

Fig. 5. Results of EPR measurements at varied oxidation-reduction potential of the purified iron-sulfur protein from the mitochondrial outer membrane. The iron-sulfur fraction (3 mg protein/ml) was titrated and measured at the same conditions as in Fig. 4. Reductive titration for $g = 1.94$ (▲) and $g = 1.89$ (●) signals. Oxidative titration for $g = 1.94$ (△) and $g = 1.89$ (○) signals.

Oxidation-reduction potential

The effect of oxidation-reduction potential on the EPR amplitude of the $g = 1.89$ iron-sulfur signal in the mitochondrial outer membrane (Fig. 4A) as well as on the amplitude of the $g = 1.89$ and 1.94 signals in the purified protein (Fig. 5A) was measured. The midpoint potential, E'_0, for the iron-sulfur protein was obtained from plots of the logarithm of the concentration ratio of the oxidized to reduced form of the iron-sulfur protein versus the oxidation-reduction potential in the sample. From three experimental series at pH 7.2 using mitochondrial outer membrane (one of which is illustrated in Fig. 4) $E'_0 = 62$ (± 6) mV was determined. In the same way from two experimental series at pH 7.2 using the purified protein (Fig. 5) $E'_0 = 75$ (± 5) mV was found. At pH 8.2 slightly lower E'_0 values for the iron-sulfur protein were obtained, 52 and 62 mV in the outer membrane and in the purified protein, respectively.

In all experiments carried out the fit of the points in the logarithmic plot was very close to the slope of the theoretical line for an oxidation-reduction process involving one electron transfer ($n = 1$).

The small change in E'_0 values going from pH 7.2 to 8.2 shows that no proton transfer is involved in the redox reaction in this pH range. Redox measurements of the iron-sulfur protein could not be made at pH 6.5 or lower because of the instability and denaturation of the protein observed in this pH region (as shown below).

Enzymatic activity

The specific rotenone-insensitive NADH-cytochrome *c* oxidoreductase activity increased 8-fold with the iron-sulfur protein enrichment during the purification and was about 0.1 unit/mg protein in the final sample. The protein was however unstable and the activity decreased progressively even during the storage of the iron-sulfur protein at −80° C. The NADPH-cytochrome *c* oxidoreductase was enriched with a factor of 6 with a final activity of approx. 0.03 unit/mg protein. Enzymatic activities of monoamine oxidase, kynurenine hydroxylase of NADH-coenzyme Q reductase could not be detected in the iron-sulfur fraction.

In order to study reactions of the iron-sulfur protein with reduced pyridine nucleotide and with ferric cytochrome *c* anaerobic experiments were carried out. In the reduction experiments 300 µl of 9 µM oxidized iron-sulfur protein was mixed anaerobically with 10 µl NADH or NADPH to a final concentration of 77 µM and frozen. In either case complete reduction was observed. In the oxidation experiments the reduced iron-sulfur protein, 4 µM, was mixed anaerobically with ferric cytochrome *c*, final concentration 29 µM, and frozen. Complete oxidation was observed.

Some samples of the iron-sulfur protein that had been stored for several months at −80° C could not be reduced by NADH or NADPH although addition of $Na_2S_2O_4$ still produced the EPR signal. Reducibility by NADH could be restored by addition of small amounts of freshly prepared microsomes or whole mitochondria.

The iron-sulfur signal was found to be very sensitive to pH values slightly below neutrality. Fig. 6A shows the EPR signal at 77 K of intact beef kidney cortex mitochondria at pH 7.5 reduced by $Na_2S_2O_4$. In addition of the outer mem-

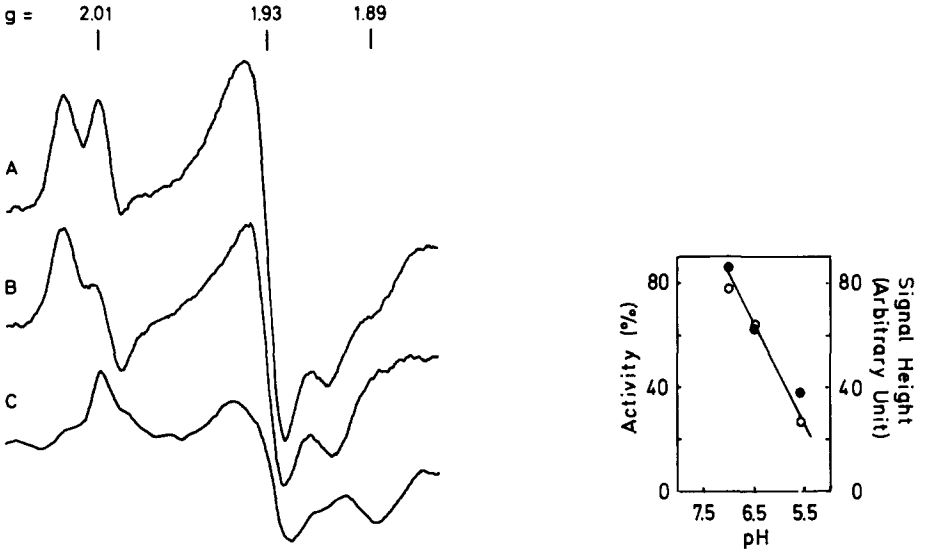

Fig. 6. Denaturation by low pH of the iron-sulfur center with $g = 1.89$ in beef kidney cortex mitochondria recorded by EPR. (A) Mitochondria in 35 mM Tris · HCl, pH 7.5, reduced by $Na_2S_2O_4$. (B) Mitochondria titrated with 1 M HCl to pH 6.0 and kept for 1 h at 23°C. Before adding $Na_2S_2O_4$, a final pH of 7.5 in the samples was obtained by adding 35 mM Tris · HCl, pH 7.5. (C) The difference spectra A—B. EPR conditions as in Fig. 4 except for modulation amplitude 19 G.

Fig. 7 . Effect of pH on $g = 1.89$ signal height (○) and on rotenone-insensitive NADH-cytochrome *c* oxidoreductase activity (●) of purified iron-sulfur protein. Samples were incubated at each pH for 1 h at 23°C. The experimental procedure and EPR conditions were those described in the legend to Fig. 6. The enzymatic activity was measured as described in Materials and Methods.

brane iron-sulfur protein signal ($g = 2.01$, 1.94 and 1.89), inner membrane signals from succinate dehydrogenase ($g = 2.03$, 1.93 and 1.91) [27] and NADH dehydrogenase ($g = 2.02$, 1.94 and 1.92) [28] are visible. Incubation of the mitochondria for 1 h at pH 6.0 and readjustment to pH 7.5 before reduction resulted in the disappearance of the $g = 2.01$ and 1.89 signals and a decrease of the $g = 1.93$—1.94 signal, whereas the signals at $g = 2.02$—2.03 and 1.91 were unaffected (Fig. 6B). The difference of the two spectra, shown in Fig. 6C (A minus B), resulted in a spectrum practically identical with that of the purified iron-sulfur protein (see Fig. 1).

The above results clearly show that among the iron-sulfur centers of whole mitochondria revealed by EPR at 77 K the one in the outer membrane is selectively affected by treatment at low pH. In these experiments a close correlation between the decrease of the iron-sulfur EPR signal and a decrease of the rotenone-insensitive NADH-cytochrome *c* oxidoreductase activity was also found. On the other hand, the activities of kynurenine hydroxylase, monoamine oxidase and NADPH-cytochrome *c* oxidoreductase did not show comparable decrease. All enzyme assays were performed under normal pH conditions.

The same pH sensitivity was found for the isolated protein. By lowering the incubation pH, as shown in Fig. 7, the $g = 1.89$ signal decreased together with a corresponding and proportional inhibition of the NADH-cytochrome *c* oxidoreductase activity. The time course of the enzymatic inactivation and the loss of the EPR signal at pH 6.0 also gave rise to mutually similar curves.

Discussion

The iron-sulfur protein from beef kidney cortex mitochondria now characterized has been found to be present also in rat kidney cortex [1], rat liver [2] and beef heart [29]. Partial purification has been performed from these organs with results similar to those presented above (Bäckström, D., unpublished). Kidney was preferred in the present study because the outer membrane iron-sulfur protein was found to be relatively more abundant in this organ with respect to the inner membrane iron-sulfur proteins as observed in fresh tissue pieces by EPR at 77 K.

The type of purification procedure presented was also practiced starting from previously prepared mitochondrial outer membrane instead of whole mitochondria. However, more than 90% of the wanted material was generally lost in the step for preparing the outer membrane from mitochondria, and the final product was not of higher purity. Furthermore, time-consuming preparation procedures could result in an altered EPR spectrum as shown in Fig. 8, where new signals at 2.03 and 1.85 have appeared simultaneously as the signals at 2.01, 1.94 and 1.89 have decreased.

The stepwise solubilization procedure used was found to be a simple and useful way to selectively extract certain proteins from the mitochondrial membranes. The high salt concentration and EDTA at pH 9.6 was used to detach the external proteins before the integral membrane proteins. By the gradual increase in detergent concentration during extraction the iron-sulfur protein from the outer membrane could be solubilized before the cytochrome oxidase and NADH dehydrogenase from the inner membrane. Most of the unrecovered iron-sulfur protein was found in other fractions, which indicates that the solubilization procedure did not denature the protein. The proteins in the solubilized fraction were mainly retained in the DEAE-cellulose column and the iron-sulfur protein eluted first at low ionic strength was free from cytochromes as well as inner membrane iron-sulfur proteins, as observed by EPR and light absorption spectroscopy. The presence of 2-mercaptoethanol during preparation was found to stabilize the iron-sulfur center leading to higher yields.

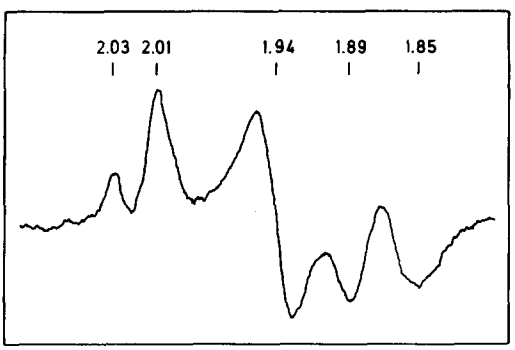

Fig. 8. EPR spectrum of the sample shown in Fig. 1 after 2 weeks storage at 4°C and reduced with $Na_2S_2O_4$. EPR conditions were as in Fig. 1A.

Several observations support that the iron-sulfur protein isolated is an integral membrane protein: (i) it was not released from the mitochondrial membrane by trypsin treatment (10 min, 25°C, pH 7.4); (ii) it was not solubilized by several hours' treatment of mitochondria of outer membranes with 1 M KCl and 1 mM EDTA at pH 9.6, even in the presence of low concentration of detergent (0.1 mg Triton X-100/mg mitochondrial protein); (iii) when the Triton concentration was raised to 0.5 mg/mg protein, a high molecular weight complex was liberated containing the iron-sulfur protein and rotenone insensitive NADH-cytochrome *c* oxidoreductase; (iv) methods used for detergent removal such as phase separation [30], extensive dialysis or acetone extration caused precipitation of the solubilized protein.

That the prepared protein actually was the iron-sulfur protein earlier localized to the outer membrane [2], was verified by the characteristic EPR spectrum and its saturation and temperature dependence. The analytical data show that the iron-sulfur protein has a center with two iron atoms and two acid-labile sulfur atoms, which upon reduction gives an $S = \frac{1}{2}$ system by EPR.

The partially purified protein was found to contain variable amounts of flavin in molar concentrations of the same order as the iron-sulfur center. Probably a certain fraction was converted to the flavosemiquinone form after reduction, and gave rise to the free radical component observed at $g = 2.00$. At present it cannot be decided whether the flavin is a constituent of a reductase for the iron-sulfur centre or originates from a non-related protein.

The midpoint potential found (60—75 mV) is different from a value (—270 mV) reported for an EPR spectrally similar iron-sulfur protein detected in rat liver microsomes [31]. However, the latter value was obtained from measurements on a quite weak $g = 1.93$ signal seriously overlapped by the signal from cytochrome *P*-450 in a nine-fold concentration excess.

A close correlation between the iron-sulfur protein and the rotenone-insensitive NADH-cytochrome *c* oxidoreductase activity is possible from the following findings: (i) The enzymatic activity was enriched in the same fraction as the iron-sulfur protein at both the selective solubilization as well as the DEAE-cellulose chromatographic step; (ii) the iron-sulfur center could be reduced by NADH or NADPH in mitochondria and also in the purified protein. However, it could not be reduced by the pyridine nucleotides when the oxidoreductase was inactivated by storage; (iii) the reduced iron-sulfur protein was oxidized by ferric cytochrome *c*; (iv) the enzymatic activity and EPR signal after low pH treatment decreased in proportion (Fig. 7); (v) the midpoint potential found, 60—75 mV, is compatible with an intermediate role in transfer of electrons from NADH to cytochrome *c*. Since the iron-sulfur protein could be reduced by NADPH and a weak NADPH-cytochrome *c* oxidoreductase activity was detected we cannot exclude the presence of some microsomal impurities in the preparation.

At low pH the iron-sulfur center was destroyed and the oxidoreductase activity lost. This is in contrast to iron-sulfur centers of the mitochondrial inner membrane which were not affected by similar treatment as measured at 77 K.

The iron-sulfur center in samples inactivated by storage could be reduced by $Na_2S_2O_4$ but not by NADH or NADPH, and was readily reoxidized by cytochrome *c*. Thus the effect of storage seemed to be a specific denaturation of

the nucleotide reacting site of the iron-sulfur protein or of an intermediate reductase. The reactivation by added microsomes or mitochondria corroborates this conclusion.

The finding in this work that the iron-sulfur protein is involved in or in close oxidation-reduction contact with the rotenone-insensitive NADH-cytochrome *c* oxidoreductase should lead to more detailed kinetic studies. Also the role of the observed flavin and carotenoid should be investigated.

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