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Oxidation–Reduction Midpoint Potentials of Mitochondrial Flavoproteins and Their Intramitochondrial Localization

Hilkka Voltti and I. E. Hassinen

Department of Medical Biochemistry, University of Oulu, SF-90220 Oulu 22, Finland

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Abstract

Spectrophotometric and fluorimetric substrate couple titrations and potentiometric spectrophotometric titrations were used to determine the oxidation–reduction potentials of components showing absorbance or fluorescence at the wavelengths attributable to the flavoproteins of mitochondria fractionated using digitonin together with sonication. A pure mitoplast fraction devoid of cytochrome b_5 contamination could be obtained using 230 μg digitonin/mg of mitochondrial protein. The digitonin-soluble fraction contained a species having $E_{m7.4} = -123$ mV and probably represents the outer membrane flavoproteins. The inner membrane-matrix fraction, treated with ultrasound, provided evidence of a flavoprotein species with low redox potential ($E_{m7.4} = -302$ mV) in the matrix fraction. The -302 mV component is probably lipoamide dehydrogenase. A high redox potential species with $E_{m7.4} = +19$ mV in titrations with the succinate fumarate couple was located in the inner membrane vesicles and is probably identical with succinate dehydrogenase. The electron-transferring flavoprotein (ETF) was isolated from bovine heart mitochondria and its $E_{m7.4} = -74$ mV determined. The component in the matrix fraction with an apparent $E_{m7.4} = -56$ mV probably represents ETF, and that in the inner membrane fraction with an apparent $E_{m7.4} = -43$ mV the NADH dehydrogenase flavoprotein. A component in an apparently low concentration with $E_{m7.4} = +30$ mV was detected in the inner membrane fraction. This probably represents the ETF-dehydrogenase flavoprotein. The origin of the flavoprotein fluorescence of mitochondria and intact tissues is discussed.

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Introduction

Considerable effort has been expended in determining the standard oxidation–reduction potentials of the enzymes of respiratory systems. The oxidation–reduction properties of the mitochondrial flavoproteins have a bearing on two important aspects. First, they could be used to determine the location and characteristics of one of the energy conservation sites, and second they could be used to identify the components responsible for the flavin absorbance and fluorescence signals of mitochondria, intact cells, and tissues. These signals have been employed in recent times as mitochondrial redox indicators in studies on energy metabolism [for references, see ref. 1].

Measurement of the flavoproteins of the mitochondrial respiratory chain has proved difficult due to spectral interference by the iron–sulphur proteins and cytochromes in the visible region. The reported midpoint potentials of the mitochondrial flavoprotein are mutually incompatible [2–5], and only a few reports exist on the redox properties of flavins of submitochondrial fractions which could be expected to be more useful in identifying the sources of the optical flavin signals of isolated preparations. Substrate couple titrations provide only a limited potential range in which the measurements can be deemed reliable. Continuous potentiometric titrations using spectrophotometric methods with appropriate mediators under strictly anaerobic conditions offer an opportunity of studying flavoproteins over a wide range of redox potentials, but this method is often hampered by the sluggishness of the mediators, the concentrations of which must be kept low to avoid spectrophotometric artifacts. Even so, spectrophotometry of mitochondria does not effectively discriminate between flavins and iron–sulphur proteins [6]. On the other hand simultaneous spectrofluorimetry may be useful to assertain the flavin nature of the component studied.

The aims of the present study were (1) to prepare pure mitoplasts devoid of outer membrane contamination, (2) to determine the oxidation–reduction midpoint potentials of the mitochondrial flavoproteins and their submitochondrial localization, and (3) to determine the redox potential of the isolated electron-transferring flavoprotein (ETF). Rat liver mitochondria were fractionated with digitonin into a digitonin-soluble fraction and mitoplast (inner membrane plus matrix) fraction, the latter being fractionated further with ultrasound into the inner membrane vesicles and matrix fraction. The redox potentials were determined (1) by spectrophotometry and potentiometric titrations under anaerobic conditions and by substrate couple titrations under anaerobic and aerobic conditions, (2) by fluorimetry and substrate couple titrations under aerobic conditions and in the case of

the electron-transferring flavoprotein by fluorimetry and potentiometric titrations in anaerobic conditions, to demonstrate the relative fluorescence intensities and flavin nature of the components titrated. Outer membrane contamination in the mitoplasts was studied using cytochrome b_5 as the marker.

Materials and Methods

Mediators. *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) Fluka AG, Buchs, Switzerland ($E_{m7.0} = +260$ mV), pyocyanine perchlorate, K&K Laboratories, Plainview, New York, ($E_{m7.0} = -34$ mV), 2-anthraquinone sulphonic acid, sodium salt, Eastman Kodak Co., ($E_{m7.0} = -225$ mV), dimethyl dipyridyl chloride (methyl viologen), K&K Laboratories, Plainview, New York, ($E_{m7.0} = -446$ mV), benzyl viologen, K&K Laboratories, Plainview, New York, ($E_{m7.0} = -359$ mV). The mediator $E_{m7.0}$ values reported by Dutton and Wilson [7] were used.

Enzymes. 3-Hydroxybutyrate dehydrogenase, 2 mg/ml, was obtained from Boehringer, Mannheim, and glutamate dehydrogenase from Sigma Chemical Co., St. Louis, Missouri.

Proteins. These were assayed by the method of Lowry et al. [8] using 1.5% Triton X-100 for protein solubilization [9].

The Cytochrome b_5 Content. This was measured in the intact mitochondria and in the digitonin particles in the presence of rotenone from difference spectra between oxidized and NADH or $Na_2S_2O_4$-reduced preparations at 424 nm and 405 nm and compared with the cytochrome *a* content.

Preparation and Fractionation of Rat Heart and Rat Liver Mitochondria: Male and female Long–Evans rats aged six to twelve months from the Department's own stock were used. The experiments were not preceded by a fasting period. The liver and heart mitochondria were isolated by conventional methods [10] in 0.225 M mannitol–0.075 M sucrose–0.05 mM EDTA, pH 7.4, and washed twice. The inner membrane-matrix fraction (mitoplasts) was prepared according to Chan et al. [11], except that the digitonin concentration was increased to 230 μg/mg of mitochondrial protein and mitochondria were incubated with digitonin for 30 min at 0°C. The mitoplasts were disrupted into the inner membrane and matrix fraction by sonication in 0.010 M Tris-HCl buffer using an MSE 60-watt 19-kHz ultrasonic power unit (Measuring & Scientific Equipment Ltd., London) for 24- to 30-sec periods at maximal output with 15-sec intervals for cooling in ice. The unbroken mitoplasts were removed by centrifugation at 9500*g* for 10 min. The broken mitoplasts were then centrifuged at 100,000*g* for 45 min

(Beckman L2 ultracentrifuge, SW 50 swinging-bucket rotor) to separate the matrix and inner membrane fractions.

Isolation of Electron-Transferring Flavoprotein (ETF) from Bovine Heart Mitochondria. Bovine heart mitochondria were isolated according to the method of Löw and Vallin [12] as modified by Hall et al. [13], kept frozen for at least 24 h and then sonicated in 25-ml aliquots. Crude ETF fraction was isolated according to Hall and Kamin [14], except that an Amicon PM-10 ultrafiltration membrane was used to concentrate the fraction. The fraction was purified further on a 1.6×40-cm DEAE-cellulose column by elution with 2.5 mM K_2HPO_4–0.1 mM EDTA, pH 7.9 [13]. The fractions containing the protein peak showing flavin fluorescence were combined and concentrated on an Amicon PM-10 ultrafiltration membrane for use in the redox titrations.

Titrations Employing Redox Substrate Couples. Titrations with β-OH-butyrate/acetoacetate $E_{m7.0} = -266$ mV [15], glutamate/NH_4 + α-keto-glutarate $E_{m7.0} = -220$ mV [16] and succinate/fumarate $E_{m7.0} = +30$ mV [17] were carried out under aerobic and anaerobic conditions using endogenous or added enzymes and coenzymes. The fluorimetric measurements included substrate couple titrations carried out using 475 nm as the excitation light wavelength and 520 nm as the emission wavelength in a Farrand MK-2 spectrofluorimeter.

The redox potential of a redox couple was calculated from equations 1 and 2.

$$E_h = E_{mi} + \frac{RT}{nF} \ln \frac{(\text{ox})}{(\text{red})} \tag{1}$$

$$E_h = E_o + \frac{RT}{nF} \ln \frac{\text{ox}}{\text{red}} - \frac{RTm}{nF} 2.303\ (\text{pH}) \tag{2}$$

where E_h = actual redox potential; E_{mi} = midpoint potential at specific pH, pH = i; E_o = standard redox potential; n = number of electrons transferred per molecule (2 for the flavoproteins and the substrate pairs used); m = number of protons involved per molecule (2 for the succinate/fumarate pair and 1 for the NAD-linked substrate couples).

Potentiometric Measurements. For the anaerobic spectrophotometric and fluorimetric measurements, the cuvette was fitted with a side-arm with a septum through which additions could be made. The cuvette was sealed with a stopper which also accommodated a platinum electrode, a salt bridge to a calomel electrode, and a gas inlet and outlet. The contents were maintained at room temperature under purified nitrogen. Small additions were made with 10-μl syringes through the septum. The cuvette was also equipped with a

magnetic stirrer. Readings of the mV meter were recorded with a two-pen recorder simultaneously with absorbance changes (475 minus 510 nm) in a dual wavelength spectrophotometer (Aminco DW-2) or spectrofluorimeter (Farrand MK-2) using 475 nm as the excitation light wavelength and 520 nm as the emission wavelength.

The vessel and contents were thoroughly flushed free of oxygen with a constant flow of purified nitrogen prior to each experiment, and small additions of neutralized ascorbate were made until anaerobiosis was denoted by a steady oxidation–reduction potential reading. The oxidation–reduction potentials were rendered more negative by additions of ascorbate (50–500 mM) up to −150 mV. Freshly prepared sodium dithionite was used to establish potentials lower than −150 mV. Oxidative titrations were carried out using additions of 100 mM potassium ferricyanide. Experiments conducted without mitochondria indicated that the mediators themselves contribute negligible absorbance.

Results and Discussion

Various approaches have been used in an attempt to separate the mitochondrial membranes [11, 18–27]. The method presented here is principally that described by Schnaitman and Greenawalt [24] as modified by Chan et al. [11], and utilizes digitonin to solubilize the outer mitochondrial membrane. The optimal ratio of digitonin per milligram of mitochondrial protein was found here to be 230 μg. The procedure resulted in a mitoplast fraction which is essentially devoid of outer membrane. Ragan and Garland [4] reported that most of the standard preparations of mitoplasts and submitochondrial particles contain cytochrome b_5. Full removal of the outer membrane of the mitochondria is possible, however, as shown in Fig. 1, since there is no evidence of the presence of cytochrome b_5 in the mitoplast fraction.

Spectrophotometric anaerobic potentiometric measurements of digitonin particles and rat heart mitochondria result in a curve with many inflection points when absorbance changes are plotted against the observed oxidation–reduction potential. Reductive and oxidative potentiometric titrations gave consistent results except in the few instances indicated below. The $E_{m7.4}$ values for the components observed are listed in Table I. A component with $E_{m7.4} = -43$ mV was located in the inner membrane fraction, while a component with $E_{m7.4} = -123$ mV (-172 mV by oxidative titration) was found by reductive titration in the digitonin-soluble fraction. This probably represents cytochrome b_5 reductase and cytochrome b_5, for which Ragan and Garland [4] reported a value more positive than −209 mV.

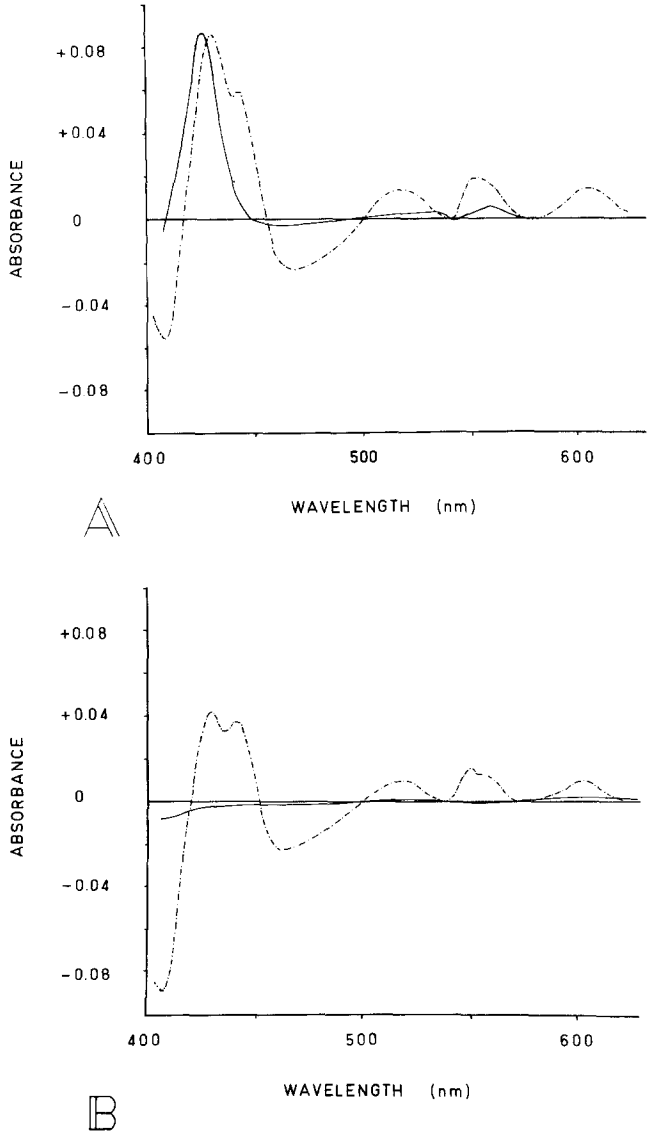

Figure 1. Absorbance spectra of (A) intact mitochondria and (B) mitoplasts. Conditions: After recording the baseline, the spectrum of NADH-reduced versus oxidized particles in the presence of 10 μM rotenone was recorded (—), after which sodium dithionite added to the sample cuvette and the spectrum recorded again (—.—). (A) Protein concentration, 2.8 mg/ml; final volume, 0.5 ml. (B) Protein concentration, 1.9 ml/mg; final volume, 1.0 ml.

TABLE I. Flavin Components Detectable in Anaerobic Spectrophotometric Potentiometric Titrations in Mitochondrial Fractions[a]

	Fraction			
Source	Mitoplast	Matrix	Inner membrane	Digitonin-soluble
Rat liver	−371	−308		
	−240	−223		
				−123
	−40	−56	−43	
	+34		+30	
		Fraction		
Rat heart	Mitochondria	Matrix	Submitochondrial particle	
	−290	−295		
	−160	−200	−175	
	−50		−55	
	+40		+40	

[a]The spectral changes were recorded at 475–510 nm, and the oxidation–reduction potentials corresponding to inflection points on the oxidation–reduction titration curve are listed. For other experimental conditions, see the text. The values are $E_{m7.4}$ (mV).

TABLE. II. Oxidation–reduction Potentials of Flavoproteins Detectable in Redox Substrate Couple Titrations in Subfractions of Rat Liver Mitochondria by Spectrophotometry[a]

Couple	Conditions	Mitoplasts	Matrix	Inner membrane
β-OHB/AcAc	aerobic	−310		n.d.
Glu/α-KG	aerobic	−247	−252	n.d.
	anaerobic	−244	−251	n.d.
Succinate/	aerobic	+13	n.d.	+10
fumarate	anaerobic	n.d.	n.d.	+9

[a]The values are $E_{m7.4}$ (mV). Experimental conditions as described under Materials and Methods.

The components listed in Table II could be demonstrated in substrate couple titrations under aerobic and anaerobic conditions. A component of redox potential $E_{m7.4} = +10$ mV was found in the inner membrane fraction by this technique.

Table III depicts the results of the fluorimetric aerobic substrate couple titrations. Three components were found in intact mitochondria. The low-potential, low-absorbing, high-fluorescence component of intact mitochondria, showing $E_{m7.4} = -294$ mV, is probably the same as that found in the matrix fraction here, having $E_{m7.4} = -302$ mV. Somewhat higher $E_{m7.4}$ values (−283 mV) were found by using the glutamate dehydrogenase equilibrium. A fluorescent high redox potential component $E_{m7.4} = +19$ mV is located in the inner membrane fraction. This is probably the same as the component titrating at +10 mV in spectrophotometric redox couple titration. Titrations with the succinate-fumarate couple may be interfered with by the malate-oxaloacetate couple due to the fumarase activity of intact mitochondria. Fumarase is a soluble enzyme located in the matrix fraction [28]. The succinate-fumarate couple was in any case used principally in titrations of the inner membrane fraction devoid of matrix enzymes, but the use of this technique also gave the same midpotentials for the flavoprotein components in the mitoplast fraction, indicating that fumarase does not seriously affect the titrations with the concentrations of the substrates used.

It is evident that the low-absorbance, high-fluorescence component titrating at −308 mV potentiometrically, at −304 mV fluorometrically and

TABLE III. Fluorimetric Aerobic Oxidation–Reduction Titrations of Flavoproteins in Rat Liver Mitochondrial Fractions Using Redox Substrate Couples[a]

Substrate couple	Intact mitochondria	Mitoplasts	Matrix	Inner membranes	Digitonin-soluble fraction
β-OHB/AcAc	−294±8 (n=5)	−304	−302±9 (n=3)	—	—
Glu/α-KG	−257	−261	−283	—	—
Succinate/ fumarate	+19	+19		+19	

[a]The fluorescence excitation wavelength was 475 nm and the fluorescence emission was measured at 520 nm. When titrating the matrix fraction with β-OHB/AcAc, 3-hydroxybutyrate dehydrogenase and NAD were added to final concentrations of 0.017 mg/ml and 0.2 μmol/ml, respectively. Other conditions as under Materials and Methods.

at −310 mV spectrophotometrically with the β-hydroxybutyrate dehydrogenase system is the same component as was observed by Hassinen and Chance [3] and tentatively identified as lipoate dehydrogenase. It should be noted that this component was not detected by Erecinska et al. [5] in rat liver mitochondria in spectrophotometric potentiometric titrations, probably because of its low concentration. The identity of this component is confirmed by the results presented in Fig. 2, which shows that the lipoamide-reducible flavoprotein contributes about 90% of the NAD-linked flavoprotein fluorescence of the matrix fraction, which is attributable to 17% of the total flavins of the fraction. The results show that the matrix fraction also contains NADP-linked flavoproteins. This species, which has a lower relative fluorescence, could be DT-diaphorase [29], an enzyme whose physiological role is largely unknown. The fluorescent component with an apparent $E_{m7.4} = -283$ mV when titrated with the glutamate dehydrogenase system is probably identical with the −310 mV species, the difference being due to shortcomings in the useful redox potential range of individual redox substrate couples.

Judging by the large absorbance changes, the flavoproteins of the fatty

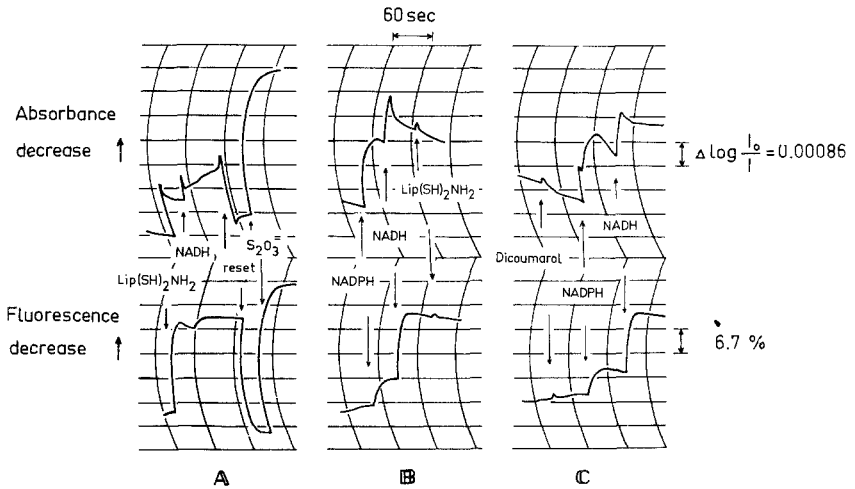

Figure 2. Fluorescence properties of flavoproteins in the sonic supernatant from rat liver mitochondria. Upper curves: flavin absorbance 475–510 nm. Lower curve: flavin fluorescence 436 nm→560 nm. Conditions: 30 mM potassium phosphate, 1 mM EDTA, pH 7.8, 4.3 mg protein/ml. 0.18 mM NADH, 0.18 mM NADPH, 0.18 mM reduced lipoamide [$Lip(SH)_2NH_4$] or 3.8 μM dicoumarol were added as indicated. The experiments A, B, and C were conducted under identical initial conditions. A combined spectrophotometer–fluorometer was used. For experimental details see refs. 2 and 3.

acid oxidizing system must account for a considerable proportion of the mitochondrial flavoproteins. Only one report exists [30] on the fluorescence and absorbance characteristics of the flavoproteins of the mitochondrial fatty acid oxidizing system, and nothing is known of their oxidation reduction properties. Knowledge of the latter is necessary, however, in order to explain the extensive quenching of flavoprotein fluorescence which occurs during fatty acid oxidation in intact tissues [1]. ETF is fluorescent, with emission at 495 nm 3.5 times as intense as that of its own free flavin at pH 7 [14]. Fluorescence is relatively rare in bound flavin, only lipoamide dehydrogenase [31] in mammalian systems showing brilliant fluorescence.

The present results demonstrate that ETF isolated according to Hall et al. [13] has an apparent $E_{m7.4}$ value of -88 mV in fluorimetric titration and -74 mV in spectrophotometric titration (Fig. 3). On the other hand,

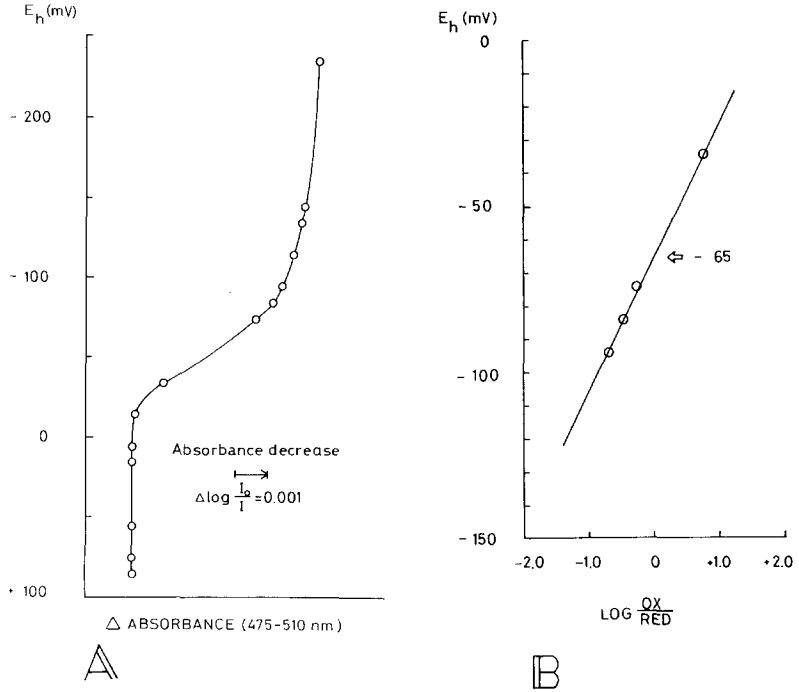

Figure 3. Potentiometric anaerobic spectrophotometric titration of electron-transferring flavoprotein (ETF). Conditions: 2.5 mM phosphate buffer, pH 7.25, in the presence of 8.4 μM TMPD, 3.4 μM pyocyanine, and 10.4 μM anthraquinone sulphonic acid at a concentration of 4.2 nmol flavin/mg of protein ($E = 11.3 \times 10^6$ cm^{-1} M^{-1}). (A) Titration curve. (B) Re-plot of the titration curve showing the midpoint potential of the ETF -65 mV at pH 7.25.

the $E_{m7.4}$ value of the couple butyryl-CoA/crotonyl-CoA, i.e., the substrate of the fatty-acid-oxidizing flavoprotein, is known, being −39 mV [32]. Therefore, a large concentration difference between the reduced and oxidized components would be needed in this couple in order to obtain the high degree of flavin reduction which is apparent upon the addition of fatty acids to mitochondria or perfused organs. It thus remains to be demonstrated whether flavin reduction during fatty acid oxidation is mainly due to ETF or to the NAD-linked flavins (i.e., lipoamide dehydrogenase).

The mitoplast and matrix fractions showed components with $E_{m7.4}$ values in the region of −40 to −56 mV. This is near the $E_{m7.4}$ of −74 mV of the ETF flavoprotein. Since there is a component of $E_{m7.4} = +10$ mV to +19 mV in the inner membrane fraction, it would be plausible to assume that the −40 to −56 mV component represents ETF and the +10 to +19 mV component the succinate dehydrogenase flavoprotein.

It is noteworthy that in the inner membrane and mitoplast fractions an inflection point was observed at approximately +30 mV in rat liver mitochondria and +40 mV in rat heart mitochondria. The concentration of this component was small, however, and represented only about 7% of the flavins of the inner membrane of rat liver mitochondria. This flavoprotein component is probably the same which is described by Ruzicka and Beinert [33] and identified as the ETF-dehydrogenase metalloflavoprotein showing an apparent $E_{m7.4}$ of +40 mV.

A component with $E_{m7.4} = -43$ mV (Fig. 4) in the inner membrane fraction could only be detected in potentiometric titrations. This E_h value is out of the usable titration range of the mitochondrial redox substrate couples. Erecinska et al. [5] found a low fluorescence component with $E_m = -45$ mV in submitochondrial particles and tentatively identified it as succinate dehydrogenase flavoprotein. In the present experiments fluorometric measurements with redox substrate couples revealed a high-potential flavoprotein with $E_{m7.4} = +19$ mV in the inner membrane, which perhaps represents the succinate dehydrogenase flavoprotein.

The flavoprotein titrating at +10 to +19 mV is also somewhat fluorescent (Tables II and III). The fluorescence of isolated succinate dehydrogenase is low [34], but succinate-induced fluorescence changes have been repeatedly shown even in matrix-free, rotenone-treated inner membrane fractions [for a discussion of succinate-linked substrate-reducible flavin fluorescence, see ref. 4]. The iron-sulphur center S-1 of succinate dehydrogenase has $E_{m7.4} = 0$ mV, and that is probably the species reacting with the flavin [35]. Therefore it would be more plausible for the +10 mV species to be the succinate dehydrogenase flavoprotein, and the species titrating at −45 mV in the experiments of Erecinska et al. [5] and suggested by them to

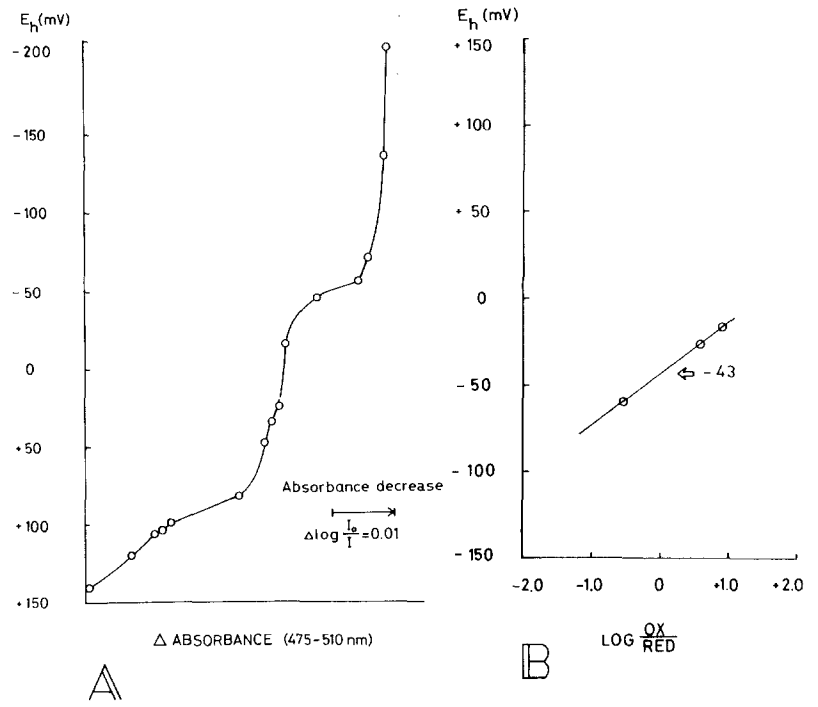

Figure 4. Potentiometric anaerobic spectrophotometric titration of the inner membrane fraction of rat liver mitochondria. Conditions: mitochondrial inner membranes in 0.225 M mannitol-0.075 M sucrose-0.05 mM EDTA, 16.7 μM TMPD, 6.7 μM pyocyanine, 20.8 μM anthraquinone sulphonic acid, 8.3 μM methylviologen, and 8.3 μM benzylviologen. (A) Titration curve. (B) Re-plot of the titration curve showing the midpoint potential of -43 mV at pH 7.4.

be the succinate dehydrogenase flavin, to be the same species as that titrating at -43 mV in the present experiments, probably the NADH–dehydrogenase flavin. The presence of a component with $E_{m7.4} = -160$ mV [5] could not be confirmed here (Fig. 4).

The present results demonstrate that the apparent discrepancies between the oxidation–reduction properties reported by various authors are probably due to methodological difficulties, especially the lack of suitable mediators for potentiometry below -200 mV. The results also show that outer membrane contamination of the mitoplasts can be effectively eliminated by careful adjustment of the digitonin concentration.

One of the main reasons for the need for accurate values for the redox properties, submitochondrial distribution, and relative fluorescence yields

of the mitochondrial flavoproteins is the potential use of these compounds as *mitochondrial redox indicators* both in vitro and in tissues in situ. This need is most obvious in the case of muscle tissue, in which the redox indicator substrate approach of Bücher and Klingenberg [36] is not easily applicable to mitochondria. On the other hand, the flavoprotein region of the mitochondrial respiratory chain is appreciated in much less detail than the other parts of the chain, and the potentiometric approach may prove useful in identifying the flavoprotein components involved. As has been shown neatly in recent times, this approach has been successful in the case of mitochondrial iron-sulphur proteins [6].

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