OXIDATION-REDUCTION MIDPOINT POTENTIALS OF THE MITOCHONDRIAL FLAVOPROTEINS

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SUMMARY

The potentiometric titrations of the mitochondrial flavoprotein reveal the presence of three absorbing and/or fluorescent species. The respective midpoint potentials are: -45 mV, -160 mV and about -220 mV.

The overlap of the absorbance maxima of the various flavoproteins with each other and with the cytochromes renders kinetic measurement of the individual flavoproteins very difficult. Improved selectivity may be obtained by measuring flavoprotein fluorescence (Chance, et al., 1967a) and by using an oxygen pulse technique (Chance, et al., 1967b) which makes it possible to resolve kinetically the flavoproteins from the faster cytochrome components. Since the kinetic data are a measure of the rate at which the oxidation-reduction components can approach equilibrium under specified conditions, determination of the actual oxidation-reduction equilibrium properties of the flavoproteins seems to be desirable in order to define more completely their catalytic role. Recently poising of the respiratory chain components by appropriate substrate couples (Hassinen and Chance, 1968, Ragan and Garland, 1969) allowed differentiation of the low potential lipoate dehydrogenase flavoprotein and the NADH dehydrogenase flavoprotein. Since substrate couples are limited by the enzymatic specificity of the respiratory chain dehydrogenases, appropriate mediators and potentiometric methods have been applied to the study of flavoproteins over a wide range of redox potentials. The methods closely follow

those previously employed for the measurement of redox potentials of cytochromes in mitochondrial membranes (Wilson and Dutton, 1970a,b). It has been also found useful to measure simultaneously, at the same wavelength pair, fluorescence and absorbance changes and employ the ratio of % fluorescence change to % of the absorbance change, which is related to the quantum efficiency of the individual flavoproteins, as an additional characteristic of these respiratory chain components. In intact pigeon heart mitochondria, three separate species with different efficiencies have been identified: a low fluorescence, absorbing flavoprotein of E_m= -45 mV (F/A~0.4), a fluorescent and absorbing flavoprotein of E_m= -165 mV (F/A~4), and a highly fluorescent (F/A> 50) flavoprotein with E_m of~-220 mV, tentatively identified with the lipoate dehydrogenase flavoprotein. The two former components appear to be present in the submitochondrial particles, though they do not exhibit any fluorescence changes upon oxidation-reduction reactions.

METHODS

Mitochondrial and submitochondrial particles were prepared according to the methods commonly used in the laboratory (Chance and Hagihara, 1963, Low and Vallin, 1963, Lee and Ernster, 1967). The oxidation-reduction potentials of flavoprotein components were measured as described by Dutton and coworkers (Dutton, 1970, Wilson and Dutton, 1970a,b) and the detailed experimental conditions are given in the legend of the Figure. Experiments in the absence of the mitochondria and experiments in which the dye concentration has been varied indicate that the mediators themselves contribute negligible fluorescence and absorbance. Simultaneous measurements of absorbance and fluorescence changes were carried out in a modified dual wavelength spectrophotometer in which the wavelengths employed for absorbance measurements (475-540 nm) were used to excite the fluorescence emission in the particle suspension. A time-shared gain control circuit equalized the amplitude of the signals obtained in the fluorescence detector from the light pulse at 475 nm and 540 nm. The resulting signal could be detected with high sensitivity with

the usual dual wavelength circuitry. Thus two readouts were obtained: one, dual wavelength absorbance changes and, the second, dual wavelength fluorescence signals.

Protein was determined by the biuret method.

RESULTS

When the logarithm of the ratio of the oxidized to reduced flavoproteins measured by absorbance and fluorescence changes is plotted against the observed oxidation-reduction potential (E_h), a sigmoidal curve typical of two components with differing midpoint potentials is obtained (Fig. 1). Resolution of the curve into two components indicates that each of them has an n value of 2 and that the respective midpoint potentials are -45 mV and -165 mV. The lower component (E_m = -165 mV) equilibrates rather sluggishly after addition of oxidant or reductant and the values obtained for its midpoint potentials are somewhat variable (between -120 mV and -165 mV). The midpoint potentials of the components measured are not changed by a three-fold increase in dye concentration and thus appear to be appropriately equilibrated with the mediators.

The two flavoproteins exhibit different fluorescence efficiencies expressed in terms of per cent fluorescence to per cent absorbance ratios (F/A value); the higher component (E_m = -45 mV) has a very low fluorescence (F/A = 0.38), while the component of lower midpoint potential has F/A value of approximately 4. In pigeon heart mitochondria about 40% of the absorbance changes can be attributed to the component of higher midpoint potential and the remaining 60% to the lower one. Similarly in beef heart mitochondria two species have been identified; most of the absorbance change is due to the -160 mV component and only about 10-20% is associated with the higher midpoint potential flavoprotein. The F/A values for both flavoproteins are significantly lower in beef heart mitochondria than in pigeon heart (approximately 0.1 and 1.0 for the two components, respectively). In beef heart submitochondrial particles (Fig. 1B) the respective midpoint potential values of the two components observed

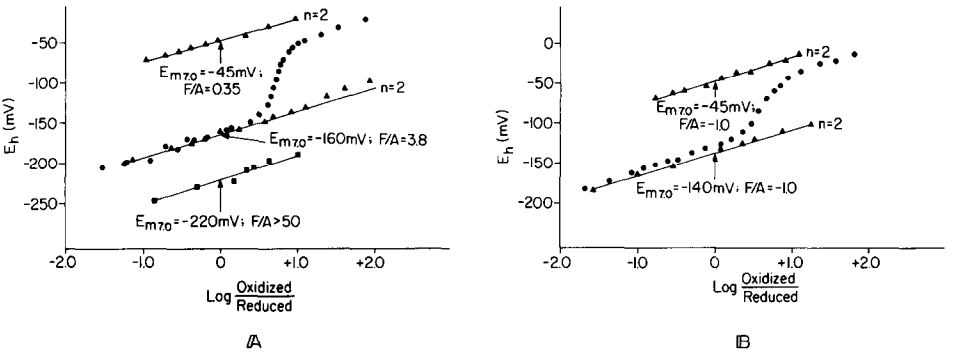

Figure 1. The oxidation-reduction potentials of the flavoproteins of intact pigeon heart mitochondria (A) and beef heart submitochondrial particles (B). The mitochondria (6 mg prot/ml) and submitochondrial particles (10 mg prot/ml) were suspended in 0.3M sucrose-0.05M potassium morpholinopropane sulphonate buffer pH 7.0. The redox mediators were: 10μM N, N, N', N'-tetramethyl-p-phenylenediamiane (TMPD), 15μM pyocyanine and 2μM antraquinone-β-sulphonate. The midpoint potentials of these mediators are: 250mV, -45mV and -225mV at pH 7.0 (E_0') and they are one, two and two electron acceptors (n values) respectively (Clark, 1960). Aliquots of dihydroascorbate were added until anaerobiosis was achieved as evidence by cytochrome reduction and an E_h of less than 300mV. Sodium dithionite was employed as reductant and potassium ferricyanide was used as oxidant. The same symbols (•) are used for the reductive and oxidative titrations. The logarithm of the oxidized and reduced form of the component was calculated (▲) assuming 40% of the absorbance change was from the high potential component and 60% was from the low potential component. (■) reductive and oxidative titration of the fluorescent component. The lines are for theoretical n=2.

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are found to be identical to those in intact mitochondria. However, the changes in fluorescence accompanying the absorbance changes occur in the opposite direction with F/A of about -1.0. This would be expected if the fluorescence changes recorded were due exclusively to the absorbance interference. Intact pigeon heart mitochondria possess in addition to the two components described above, a highly fluorescent flavoprotein (F/A> 50). Although this component equilibrates very sluggishly after the addition of oxidant or reductant, careful titrations gave a slightly sigmoidal curve with an n value of 2 and an overall midpoint potential of~ -220 mV. Therefore parallel experiments were carried out under identical experimental conditions using β-hydroxybutyrate-acetoacetate couple to determine the oxidation-reduction midpoint potential of the same

component. The sigmoidicity of the plot (data not shown) suggests the presence of more than one component in this region of the respiratory chain with midpoint potentials of -260 mV and -230 mV. The potential for half reduction of the complete pool of highly fluorescent flavoprotein is -245 mV.

DISCUSSION

Potentiometric titrations of the mitochondrial flavoprotein using simultaneous measurements of the absorbance and fluorescence changes as an identification tool reveal the presence of at least three and possibly four distinct flavoproteins with different midpoint potentials and fluorescence efficiencies.

On the substrate side of the rotenone block a highly fluorescent component with a potentiometrically determined midpoint potential of -220 mV can be identified with the lipoate dehydrogenase flavoprotein (FpL). This value is slightly higher that that reported previously (Hassinen and Chance, 1968; Ragan and Garland, 1969). The discrepancy might be attributed to the lack of appropriate mediators which results in a slow equilibration of the electrode after the addition of a reductant or oxidant and to the presence of another component (as suggested by a slight sigmoidicity of the titration curve) which would tend to change the overall midpoint potential. Titrations with substrate couple repeated under the same experimental conditions gave upon plotting a slightly sigmoidal curve with two components of E_m = -260 mV and -230 mV. The overall midpoint potential is -245 mV and is approximately 25 mV more negative than the potentiometrically determined value. The -230 mV component (NADH dehydrogenase flavoprotein - FpD_1?) accounted for not more than 25% of the total fluorescence changes.

On the oxygen side of the rotenone block, two components have been identified with E_m = -45 mV and -160 mV. Identical species have been found in submitochondrial particles and the isolated DPNH-cytochrome c reductase (complex I-III - unpublished) though in the latter two types of preparations no fluorescence associated with the absorbance changes could be detected. The low fluorescence,

high potential (-45 mV) component can be most probably identified with succinic dehydrogenase flavoprotein.

The identification of the other species (E_m = -160 mV) as a truly respiratory chain component encounters some difficulty; this negative midpoint potential value makes the reversible reducibility in the absence of energy a thermodynamically very unfavorable reaction. The relatively high fluorescence efficiency of this component might suggest that the so-called "fast fluorescent flavoprotein" (FpF) (Erecińska, 1970) and Fp_{D_2} represent respectively fluorescence and absorbance of the same species. High F/A value indicates that the component discussed is a flavin and not a non heme iron. Alternatively, it might be a flavoprotein of one of the mitochondrial dehydrogenases (e.g. choline, fatty acid) distinct from succinic and NADH, however its presence in the sonicated membrane fragments and complex I-III points to a rather tight association with the respiratory chain.

The method described uses simultaneous fluorescence and absorbance measurements for flavoprotein identification and, by controlling the potential values, eliminates the possibility of cytochrome interference. At the potential range used for flavoprotein titration, all the cytochromes except cytochrome b are being kept reduced and do not participate in oxidation-reduction reactions. Negligible absorbance and fluorescence changes have been noticed at the region of possible cytochrome b interference, which could be further resolved by a very clear n=1 value obtained for the latter carrier. Since however thermodynamics itself does not identify the actual reaction pathways, the combination of thermodynamic and kinetic methods is required to identify the functionality of the components of the respiratory chain. Such kinetic data obtained by oxygen pulse technique and flow flash technique are already available and will be reported elsewhere.

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