Measurement of the oxidation-reduction potentials for one-electron and two-electron reduction of electron-transfer flavoprotein from pig liver

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Potentiometric titrations of pig liver electron-transfer flavoprotein (ETF) were performed at pH 7.5 and 4°C, both in the reductive and oxidative directions. Reduction of ETF to the hydroquinone form required a total of two reducing equivalents/mol of ETF with the formation of sub-stoichiometric amounts of anionic semiquinone as an intermediate. The oxidation–reduction potentials for the two one-electron couples, oxidized ETF/ETF semiquinone and ETF semiquinone/fully reduced ETF, are +4 mV and –50 mV respectively. The overall midpoint potential for the two-electron couple (oxidized ETF/fully reduced ETF) is –23 mV.

The electron-transfer flavoprotein (ETF) is a key component of mitochondrial oxidations of fatty acyl-CoA species (Crane *et al.*, 1956; Hauge *et al.*, 1956), branched-chain-fatty acyl-CoA species (Noda *et al.*, 1980) and glutaryl-CoA (Rhead *et al.*, 1980), as well as sarcosine and dimethylglycine (Frisell & MacKenzie, 1962). It serves to link the oxidation of these substrates to the respiratory chain by mediating the transfer of reducing equivalents from the various primary flavoprotein dehydrogenases to the membrane-bound iron–sulphur flavoprotein ETF:ubiquinone oxidoreductase (Ruzicka & Beinert, 1977). First isolated by Crane & Beinert (1956) from pig liver mitochondria, ETF was believed until recently to be a dimer (M_r 58 000) of identical subunits (M_r 27 500), each containing one FAD molecule (Hall & Kamin, 1975). More recently, close scrutiny by three different laboratories (Gorelick *et al.*, 1982;

McKean *et al.*, 1983; Husain & Steenkamp, 1983) has revealed that mammalian ETF is actually a heterodimer (M_r 27 000–32 000, 31 000–38 000) with only one FAD molecule per dimer.

Since ETF accepts electrons from a number of primary flavoprotein dehydrogenases and since it does not appear to exist in mitochondria in overwhelming excess to the dehydrogenases, it is of considerable importance to understand how the transfer of reducing equivalents from these dehydrogenases to ETF and subsequently to ETF:ubiquinone oxidoreductase is regulated. As suggested by Beckmann *et al.* (1981), one possible mechanism of regulation may be through relative affinities of various dehydrogenases for ETF. Attempts to demonstrate directly the formation of complexes between general fatty acyl-CoA dehydrogenase and ETF by monitoring changes in absorption (Crane & Beinert, 1956) or fluorescence (McKean *et al.*, 1979) have been unsuccessful. Although mammalian ETF has, in recent years, been the subject of many papers, a detailed study on the oxidation–reduction potential, a property central to the physiological functions of ETF, has not yet been reported.

In the present paper we describe the characterization of pig liver ETF with respect to the oxidation–reduction potentials of its two redox couples. These results, it is hoped, when combined with the measurements obtained with other ETF-

Abbreviations used: ETF, electron-transfer flavoprotein; ETF_{ox}, oxidized ETF; ETF^{•-}, one-electron-reduced ETF; ETF_{red}.H⁻, two-electron reduced ETF; E_1^0 , first-electron reduction potential; E_2^0 , second-electron reduction potential; E_m , midpoint potential; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PMS, phenazine methosulphate; PYC, pyocyanine; DHNQ, 2-hydroxy-1,4-naphthoquinone; LFA, lumiflavin acetate; MV²⁺, oxidized Methyl Viologen; DCIP, 2,6-di-chloroindophenol.

linked dehydrogenases, will add to our understanding of the control of ETF-linked mitochondrial oxidations.

Experimental

Purified ETF was prepared as described by Husain & Steenkamp (1983).

Special and electrochemical equipment and general methods for performing the spectroelectrochemical experiments were as previously reported (Stankovich, 1980; Stankovich & Fox, 1983a,b). All determinations were made at 4°C in a medium consisting of 10 mM-Hepes, pH 7.5, and 10% (v/v) ethylene glycol. Lumiflavin acetate (the gift of Dr. V. Massey) and pyocyanine [synthesized as described by McIlwain (1937)] were used as redox indicators to measure redox potentials. The potential of the Ag/AgCl reference electrode was checked before and after each experiment with 5 mM-ferricyanide/5 mM-ferrocyanide in 0.1 M-phosphate buffer, pH 7.0 at 25°C (O'Reilly, 1973). As reported previously (Stankovich & Fox, 1983a), the potential of the reference electrode changes +0.004 V as a result of the difference between the calibration temperature and the experimental temperature. All potentials reported in the present paper have been corrected for this change and all are reported against the standard hydrogen electrode. The concentrations of ETF species present during potentiometric experiments were calculated by simultaneous solution of a three-variable matrix of coefficients by means of a computer program adapted from that described by Cooper (1981). Computer calculations and figure plotting were done with an Apple II+ PASCAL system.

Results and discussion

Reductive titrations of ETF with dithionite as the reductant have been published (Gorelick *et al.*, 1982; Husain & Steenkamp, 1983). Reduction proceeds in two phases, requiring a total of two electron equivalents/mol of ETF, with essentially quantitative formation of anionic semiquinone in the first phase. Molar absorptivities for all three ETF species at different wavelengths were calculated from similar dithionite titrations and are presented in the legend to Fig. 2 (below).

Enzymic reduction of ETF by sarcosine and dimethylglycine dehydrogenases (Steenkamp & Husain, 1982) and by general acyl-CoA dehydrogenase (Hall & Lambeth, 1980; Reinsch *et al.*, 1980) also results in the rapid formation of anionic semiquinone shown to be catalytically active (Hall & Lambeth, 1980), further reduction to fully reduced ETF occurring only sluggishly.

Potentiometric titrations were performed to measure E^0 values for the two redox couples of

ETF ($\text{ETF}_{\text{ox.}}/\text{ETF}^{\cdot-}$ and $\text{ETF}^{\cdot-}/\text{ETF}_{\text{red.}}\text{H}^-$). Initially, a group of redox indicators covering a range of potentials from +0.08 V to -0.45 V was used to reduce ETF electrochemically. This potentiometric reduction of ETF, in the presence of PMS, PYC, DHNQ, LFA and MV^{2+} , gave a midpoint potential (E_m^0) of -0.03 V and also revealed that only 55% of the anionic semiquinone was formed in the course of reduction with MV^{2+} as the reductive mediator. This is in contrast with reduction with dithionite, which, as noted above, gives rise to 100% anionic semiquinone, presumably because of kinetic stabilization (Stankovich & Fox, 1983b). The generation of 55% anionic semiquinone observed here suggested that the E^0 values for the two redox couples of ETF were less than 65 mV apart (Clark, 1960). On this basis, a series of experiments was performed with PYC as the redox indicator, and MV^{2+} and DCIP as the reductive and oxidative redox mediators respectively.

A Nernst plot for both electron transfers to ETF is shown in Fig. 1. The plotted lines represent those

Fig. 1. Nernst plots for two one-electron transfers to ETF. For the first-electron transfer ($\square$), $E_1^0 = +0.004$ V, Nernst slope = 0.050 V, and for the second-electron transfer ($\circ$), $E_2^0 = -0.050$ V, Nernst slope = 0.056 V.

calculated by the least-squares method from data collected in both oxidative and reductive directions in four separate experiments. For the first- and second-electron transfers, the data are consistent with the thermodynamic relationship shown in eqns. (1) and (2) respectively.

$$\begin{aligned} \text{ETF}_{\text{ox.}} + e^- &= \text{ETF}^{\cdot-} \\ E_1^{0'} &= +0.004 \pm 0.015 \text{ V}, \\ \text{Nernst slope} &= 0.05 \text{ V} \end{aligned} \quad (1)$$

$$\begin{aligned} \text{ETF}^{\cdot-} + e^- + \text{H}^+ &= \text{ETF}_{\text{red.}}\text{H}^- \\ E_2^{0'} &= -0.05 \pm 0.016 \text{ V}, \\ \text{Nernst slope} &= 0.056 \text{ V} \end{aligned} \quad (2)$$

For the first-electron transfer, the $E_1^{0'}$ values for the reductive and oxidative directions differ by 20 mV. At this time the reason for this difference is not known.

For transfer of the second electron, the redox-potential values obtained in the oxidative and reductive directions differed by only 3 mV; the Nernst slopes were the ideal value of 55 mV. Thus the second-electron transfer is ideally reversible. The amount of semiquinone formed during an oxidative titration was comparable with that formed in the reductive titration. This is different from the behaviour of fully reduced ETF reported by Gorelick *et al.* (1982) on oxidation with ferricyanide; they observed no semiquinone accumulation.

The $E^{0'}$ values reported here for ETF give a potential separation of 0.054 V and a midpoint potential ($E_m^{0'}$) of -0.023 V. Feinberg & Gustafson (1982), on the basis of preliminary potentiometric titrations with pyocyanine as the redox mediator, reported the midpoint potential of ETF to be in the region of 0 to -25 mV. The titration data were not presented, however, and the amount of semiquinone generated and the $E^{0'}$ values for the individual half-couples were likewise not reported.

The environment of the flavin in ETF thus increases the potential by 196 mV, since the oxidation-reduction potential for free FAD is -219 mV (Clark, 1960). In contrast, binding of the flavin to the protein in flavodoxins, which serve as electron carriers between redox proteins as does ETF, results in large decreases in oxidation-reduction potential. For example, in the flavodoxins of *Anacystis nidulans* (Van Lin & Bothe, 1972) and *Escherichia coli* (Vetter & Knappe, 1971), the midpoint potential for the two-electron couple of FMN is -3255 mV. In addition, there is a large potential difference (-260 mV) between the first- and second-electron transfers in flavodoxins. This results in thermodynamic stabilization of 95% of the neutral flavin radical (Mayhew & Massey, 1969). This is in marked contrast with ETF, which has only a 54 mV separation between the two-

electron transfers and stabilizes only 56% of the anionic radical.

The value of -23 mV for the midpoint potential of ETF determined in the present work is intermediate between the midpoint potentials reported for general fatty acyl-CoA dehydrogenase (-150 mV) (Feinberg, 1982) and ETF:ubiquinone oxidoreductase (-15 mV and $+40$ mV for the flavin and iron-sulphur groups respectively) (Ruzicka & Beinert, 1977) and thus consistent with its role as a mediator.

Since less radical was produced during reduction in the dye-mediated system than during dithionite titrations, it was desirable to perform a coulometric reductive titration of ETF with MV^{2+} as the only redox mediator. Since MV^{2+} does not absorb in the visible region, reduction of ETF by MV^{2+} will result in spectra corresponding to different species of ETF. Fig. 2 shows spectral changes observed during such a reductive titration of ETF. Note that, for clarity, intermediate spectra in the second half of the titration have been omitted. The dotted line represents the spectrum that would have been seen had 100% anionic semiquinone been produced.

The amount of semiquinone observed is only 54% of the maximum value. In seven experiments we have observed $56 \pm 3\%$ semiquinone, in good agreement with a value of 61% predicted on the basis of a potential separation of 0.054 V between the two ETF couples observed in this work (Clark, 1960). Like the dithionite titration, the coulometric titrations confirms FAD to be the only redox-active site in ETF. The overall current efficiency for the two-electron transfers to ETF was 93%, where current efficiency is the percentage of total number of electrons transferred that go to the species of interest. The inset in Fig. 2. shows the absorbance values obtained at three wavelengths plotted against n (where n is the number of electrons transferred/mol of ETF). The potential separation measured in the potentiometric titration can also be used to calculate the concentration of each enzyme species present throughout the course of the reductive titration (Clark, 1960). Since the molar absorptivity of each ETF species is known, the concentrations can be used to create a simulation of the A -versus- n plot for the given potential separation. The curves drawn in the inset represent the theoretical A -versus- n line for 4°C and 0.054 V potential separation. The close match of the observed values of absorbance with the theoretical curves further supports the validity of the potential separation measured.

As noted previously, nearly quantitative amounts of semiquinone were stabilized in dithionite titrations of ETF (Gorelick *et al.*, 1982; Husain & Steenkamp, 1983), much more than

Fig. 2. Coulometric reduction of $18.3 \mu\text{M-ETF}$ in the presence of $100 \mu\text{M-MV}^{2+}$

The dotted line represents the spectrum that would correspond to formation of 100% anionic semiquinone. Absorption coefficients ($\text{mm}^{-1}\cdot\text{cm}^{-1}$) used in this paper for 500, 436, and 374 nm respectively are: ETF_{ox} , 0.75, 13.5, 12.1; $\text{ETF}^{\cdot-}$, 2.98, 4.14, 17.16; $\text{ETF}_{\text{red}}\cdot\text{H}^-$, 1.88, 2.37, 7.38 (Husain & Steenkamp, 1983). Curve 1, oxidized ETF; 2, $n = 0.08$ electron/molecule of FAD; 3, $n = 0.40$; 4, $n = 0.72$; 5, $n = 1.05$; 6, $n = 1.36$; 7, $n = 2.05$. The inset shows plots of A against n at 374 (■), 436 (▲), and 500 (●) nm observed during a coulometric titration of ETF. The continuous lines represent the theoretical value for an A -against- n plot for 4°C and 0.054 V potential separation.

predicted from the redox equilibria. Similar observations have been reported for the flavoproteins glucose oxidase (Stankovich *et al.*, 1978) and lactate oxidase (Stankovich & Fox, 1983). In glucose oxidase, this stabilization was interpreted as being due to a kinetic barrier against the transfer of the second electron by a negatively

charged reducing agent. This barrier was overcome when the positively charged MV^{2+} was added to the system. Similarly, EDTA/light-reduced flavin, which is believed to be negatively charged, failed to transfer the second electron to lactate oxidase (Choong & Massey, 1980). However, when Methyl Viologen was added to the

EDTA/light-reduced-flavin system, the second electron was easily transferred to lactate oxidase.

In the case of ETF, experimental observations support the interpretation given above for glucose oxidase and lactate oxidase. As reported previously (Gorelick *et al.*, 1982; Husain & Steenkamp, 1983), addition of the first reducing equivalent to produce anionic semiquinone is a rapid process, whereas further reduction to fully reduced ETF proceeds sluggishly, requiring about 1 h between each addition of dithionite. This observation, together with the fact that disproportionation ($2 \text{ ETF semiquinone} \rightleftharpoons \text{Oxidized ETF} + \text{fully reduced ETF}$) is attained very slowly (Gorelick *et al.*, 1982), provides an explanation for the unexpectedly high yields of radical during dithionite titrations.

Reoxidation of fully reduced ETF by molecular oxygen or by ferricyanide, as reported by Gorelick *et al.* (1982), occurs without detectable accumulation of semiquinone, in contrast with reoxidation during potentiometric titration (with DCIP as mediator), which generated 56% radical, as in the reductive titration. The lack of observable semiquinone in the oxygen reaction may again be explained on a kinetic basis, since the semiquinone reacts with oxygen 90 times faster than does fully reduced ETF (Gorelick *et al.*, 1982), and thus will not accumulate in significant amounts during the reaction. The lack of semiquinone formation during reoxidation by ferricyanide presumably also involves a similar differential reactivity of the fully reduced and semiquinone forms of ETF.

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