Reaction of Electron-Transfer Flavoprotein with Electron-Transfer Flavoprotein-Ubiquinone Oxidoreductase†

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ABSTRACT: The oxidative half-reaction of electron-transfer flavoprotein (ETF), electron transfer from ETF to electron-transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO), is dependent on complementary surface charges on the two proteins. ETF is the positively charged member of the redox pair. The evidence is based on the pH and ionic strength dependencies of the comproportionation of oxidized ETF and ETF hydroquinone catalyzed by ETF-QO and on the effects of chemical modification of ETF on the comproportionation reaction. Acetylation of one and five ϵ-amino groups of lysyl residues results in 3- and 13-fold increases, respectively, in the K_m of ETF-QO for ETF but no change in V_{max}. Amidination, which maintains positive charge at modified loci, has no effect on steady-state kinetic constants. These chemical modifications have no effect on the equilibrium constant for equilibration of ETF redox states. The K_m of ETF-QO for ETF is pH dependent above pH 8.5, suggesting titration of lysyl residues as previously observed in studies of the reductive half-reaction of ETF [Beckmann, J. D., & Frerman, F. E. (1983) *J. Biol. Chem. 258*, 7563–7569]. The ionic strength dependence of TN/K_m^{ETF} for the reaction follows the limiting Brønsted equation $\ln (TN/K_m) = \ln k_0 + 2\alpha Z_1 Z_2 I^{1/2}$, and Z_1Z_2, the product of charges on the reacting proteins, is similar to the value of Z_1Z_2 for the reductive half-reaction of ETF by the general acyl-CoA dehydrogenase. The ETF-QO-catalyzed comproportionation reaction exhibits a primary deuterium isotope effect in D_2O, perhaps indicating the participation of solvent water in the electron-transfer reaction.

In previous work, we have shown that the reductive half-reaction of electron-transfer flavoprotein (ETF)[1] by the general acyl-CoA dehydrogenase (G-AD) is dependent upon complementary surface charges on the two proteins (Frerman et al., 1980; Beckmann & Frerman, 1983). There are 14 chemically reactive, exposed lysyl residues in ETF. Some of these lysyl residues at the binding domain for electron donors are important in the reductive half-reaction (Beckmann & Frerman, 1983). As pointed out by Koppenol & Margoliash (1982) in the case of cytochrome *c*, the total distribution of lysyl groups may also serve to orient a redox protein with respect to its anionic reductants and anionic oxidants. Cytochrome *c* apparently acts as a diffusible electron shuttle between complex III and complex IV (Koppenol & Margoliash, 1982). ETF may function in a similar fashion, shuttling electrons between anionic reductants, soluble flavoprotein dehydrogenases (Ikeda et al., 1983; Frisell & Mackenzie, 1962), and the anionic oxidant electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) (Ruzicka & Beinert, 1977; Beckmann & Frerman, 1985). In contrast to cytochrome *c*, ETF is a heterodimer (Gorelick et al., 1982; Husain & Steenkamp, 1983; McKean et al., 1983), which suggests the possibility of separate sites for reduction by primary flavoprotein dehydrogenases and oxidation by ETF-QO. It is not known whether the ϵ-amino groups of lysyl residues in ETF have a role in the oxidative half-reaction, and no role can be inferred since the protein is a heterodimer.

In this paper, we present evidence to show that the exposed lysyl residues of ETF also play a role in the reaction with ETF-QO and that the interaction between the two proteins involves electrostatic forces. The evidence is based upon the effects of pH, ionic strength, and chemical modifications on the ETF-QO-catalyzed comproportionation of oxidized ETF and ETF hydroquinone. In addition, a D_2O solvent isotope effect was observed on the turnover number of the catalyzed comproportionation reaction, suggesting that solvent water participates in the rate-limiting step in this reaction.

Experimental Procedures

Materials and Enzymes. The sources of reagents (Beckmann & Frerman, 1983) and the purification of ETF and ETF-QO have been described previously (Beckmann & Frerman, 1985). Deterium oxide, 99.8 atom %, was purchased from Sigma.

Methods. The steady-state fluorometric comproportionation assay has been described (Beckmann & Frerman, 1985). In all steady-state kinetic experiments, 20 mM buffers were used, with the pH and ionic strength adjusted with HCl and KCl, respectively. Final reaction pH values were measured with a calibrated combination glass electrode. Both ETF and ETF-QO were found to be stable for at least 2 h within the range of pH investigated. Initial reaction velocities were determined by the method of Foster & Niemann (1953). This analysis was facilitated by a program for an IBM personal computer, which was written with the assistance of David Schowalter in this laboratory. For the determination of steady-state kinetic parameters, initial velocities were measured at a minimum of four substrate concentrations in the vicinity of K_m. Values of TN and K_m and their standard errors were obtained as described by Wilkinson (1961).

Chemical covalent modifications of ETF with acetic anhydride and methyl acetimidate were performed essentially

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[1] Abbreviations: ETF, electron-transfer flavoprotein; ETF-QO, electron-transfer flavoprotein-ubiquinone oxidoreductase; TN, turnover number; AMPD, 2-amino-2-methyl-1,3-propanediol; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TNBS, trinitrobenzenesulfonate; GAD, general acyl-CoA dehydrogenase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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as described (Beckmann & Frerman, 1983). Acetylation was accomplished with a 4–8-fold molar excess of acetic anhydride over ETF flavin in 50 mM sodium pyrophosphate buffer, pH 8.5, containing 5% glycerol. Samples were then passed through a column (1 × 12 cm) of Sephadex G-50 equilibrated with 2.5 mM dibasic potassium phosphate containing 1% glycerol. Amidination of ETF was carried out by reaction of approximately 25 μM ETF with two to four additions of fresh 20 mM methyl acetimidate at 15-min intervals. After 1–2.5 h of reaction at 4 °C, the samples were either dialyzed or passed through a column of Sephadex G-50 as described above. In all cases, the chemically modified species were characterized by visible and near-UV absorption spectra, polyacrylamide gel electrophoresis of the native proteins (Davis, 1964), and quantitation of remaining amino groups with trinitrobenzenesulfonate (TNBS) (Beckmann & Frerman, 1983). In all experiments, an unmodified sample of ETF served as the relative standard for amino group determinations. Neither acetylation nor amidination altered the typically resolved absorption spectrum of the ETF flavin (Beckmann & Frerman, 1983). Acetylation resulted in the formation of a heterogeneous mixture of at least six charge isomers (Beckmann & Frerman, 1983). However, amidination resulted in no significant change in the electrophoretic mobility of ETF. Trinitrophenylated ETF (Beckmann & Frerman, 1983) was not used in the experiments presented here because the trinitrophenyl chromophore would interfer with the fluorescence assay.

As previously noted, there are approximately 14 lysine residues in ETF that are exposed and easily chemically modified (Beckmann & Frerman, 1983). The chemically modified species used in this work that contain less than 14 modified lysine residues per mole of ETF flavin are mixtures of modified species with the average number of modifications per flavin indicated. The mixtures were not resolved because of the lability of the flavin and because even highly purified modified ETF derivatives are not homogeneous with respect to the specific lysine residues modified (Beckmann & Frerman, 1983).

Results

Steady-State Reaction Mechanism. Since the redox states of the ETF reactants and products in the equilibration reaction are known, it is possible to discuss a steady-state reaction mechanism. The two simplest kinetic mechanisms that could fully describe the reaction are the sequential Bi Bi (Segel, 1975a) and Ping-Pong Bi-Bi mechanisms (Segal, 1975b). A major difference between these two mechanisms is that the sequential Bi-Bi predicts a ternary complex between ETF-QO and two ETF molecules. In contrast, the Ping-Pong Bi-Bi system involves only binary complexes. The simplest method to distinguish between these two types of mechanisms is to vary both substrates in a constant ratio (Segel, 1975a,b) and plot the dependence of initial velocity as a function of substrate concentration in a double-reciprocal fashion. Ternary complex mechanisms yield a nonlinear double-reciprocal plot, whereas the binary complex mechanism yields a linear plot.

The data from such an experiment in which the ratio of ETF hydroquinone to oxidized ETF was held constant at 7 are shown in Figure 1; the data are consistent with a binary complex mechanism. Thus, the most tenable reaction scheme is the Ping-Pong Bi-Bi mechanism, which infers a single ETF binding site on ETF-QO.

Effects of pH on Reaction Kinetics. To optimize assay conditions for subsequent kinetic–ionic strength and chemical modification studies, the effects of pH upon the steady-state

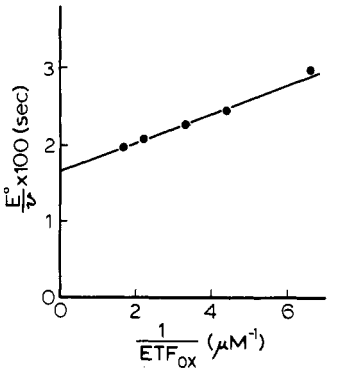

FIGURE 1: Effect of varied oxidized ETF and ETF hydroquinone concentrations on comproportionation velocity. The ratio of ETF hydroquinone to oxidized ETF was maintained at 7.7. Each point is the average of duplicte assays conducted at 25 °C in 20 mM Tris-HCl, pH 7.4.

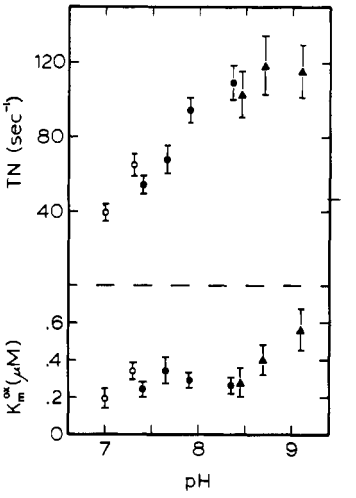

FIGURE 2: Effect of pH on the steady-state kinetic parameters for comproportionation. ETF hydroquinone concentration and ionic strength were held constant at 1.5 μM and 17 mM, respectively. The buffers used were (●) 20 mM Tris-HCl, (○) 20 mM Bis-Tris-HCl and (▲) 20 mM 2-amino-2-methyl-1,3-propanediol hydrochloride.

kinetic parameters for comproportionation of oxidized and hydroquinone ETF were investigated. Figure 2 shows the effect of pH upon the reaction turnover number (TN) and upon the K_m of ETF-QO for oxidized ETF. In these experiments, the ionic strength was maintained at 17 mM with KCl, and the concentration of ETF hydroquinone was held constant at 1.5 μM, which is 5-fold greater than the K_m for this substrate. The TN is pH dependent and increases about 3-fold between pH 7.0 and pH 8.5. The very low reaction velocities below pH 7 have thus far prevented determination of kinetic parameters below this pH. Although not shown, the pH dependence of TN can be fit to the titration curve for a single essential titratable group with a pK_a of 7.3. However, this pH dependence may also reflect the equilibrium constant for the reaction, which is pH dependnet (Beckmann & Frerman, 1985). That is, as the pH is raised, the ETF semiquinone becomes thermodynamicaly stabilized, so that ETF semiquinone formation is favorable. The K_m of ETF-QO for oxidized ETF is relatively constant at about 0.3 μM from pH 7 to pH 8.5. However, above pH 8.5 the K_m value increases. Initial velocities at and above pH 9.4 were very slow, probably due to decreased saturation of ETF-QO with ETF hydroquinone (not shown). This result suggests titration of surface lysyl groups on one of the two proteins. Thus, the effect of pH on the K_m of the ETF-QO for ETF is very similar to the pH dependence for the reductive half-reaction of ETF by the

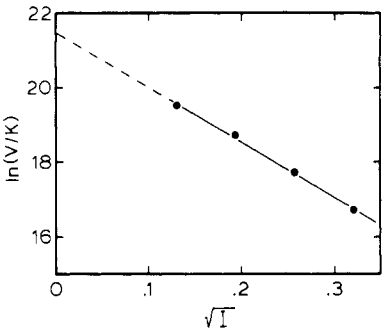

FIGURE 3: Effect of ionic strength on the apparent V/K_m value for the comproportionation of oxidized and hydroquinone ETF. The reaction was conducted in 20 mM 2-amino-2-methyl-1,3-propanediol, pH 8.7, with the ionic strength adjusted from 0.017 to 0.102 M with KCl. Units of V/K are M^{-1} s^{-1}. The line represents a simple linear least-squares fit and indicates $-Z_1Z_2$ and k_0 to be 6.3 and 2.2×10^9 M^{-1} s^{-1}, respectively.

Table I: Effect of Chemical Modifications on the Steady-State Kinetic Parameters for ETF Comproportionation

ETF	assay pH	N^a	$K_m \pm$ SE (μM)	TN ± SE (s^{-1})
native	7.4[b]	0	0.32 ± 0.13	78 ± 18
acetylated	7.4[b]	5.5	4.2 ± 0.7	90 ± 15
native	8.7[c]	0	0.41 ± 0.09	99 ± 12
amidinated	8.7[c]	18.7	0.24 ± 0.04	70 ± 6
acetylated	8.7[c]	1	0.77 ± 0.04	82 ± 3

[a] Number of lysine residues modified determined by difference with TNBS, with unmodified ETF as the standard. [b] Assays used 2.1 μM native ETF hydroquinone as cosubstrate, in 20 mM Tris-HCl. [c] Assays used 1.5 μM native ETF hydroquinone as cosubstrate, in 20 mM 2-amino-2-methyl-1,3-propanediol, made to 17 mM ionic strength with KCl.

general acyl-CoA dehydrogenase (Beckmann & Frerman, 1983). In the reductive half-reaction of ETF, the pH dependence of K_m for ETF can be attributed to titration of ϵ-amino groups of lysyl residues in ETF.

Kinetic–Ionic Strength Effect. Since the comproportionation reaction procedes via a binary complex mechanism, the apparent V/K_m value, obtained when ETF hydroquinone is subsaturating, is equivalent to the true TN/K_m value. TN/K_m provides a minimal estimate of the second-order rate constant for the reaction between ETF and ETF-QO. Consequently, it is possible to study the effect of ionic strength on TN/K_m, with only one ETF hydroquionone concentration (1.5 μM). The concentration of oxidized ETF was varied, and the apparent TN/K_m values were obtained at several values of ionic strength at pH 8.7. Figure 3 shows that TN/K_m follows the limiting Brønsted equation $\ln k = \ln k_0 + 2\alpha Z_1Z_2I^{1/2}$, where $\alpha = 1.17$ at 25 °C, Z_1 and Z_2 are the total charges on the reactants, and k_0 is the second-order rate constant at zero ionic strength. The data indicate k_0 to be 2.2×10^9 M^{-1} s^{-1} and $-Z_1Z_2$ to be 6.3.

Chemical Modification Studies. The kinetic–ionic strength experiments clearly indicate an electrostatic interaction between ETF and ETF-QO. However, those result do not distinguish the protein that carriers the required positive charges. Therefore, amino groups in ETF were acetylated, and the reduction of the oxidized acetylated ETF was observed in the comproportionation assay, with native ETF hydroquinone as the cosubstrate. Table I summarizes several experiments, including results obtained with extensively amidinated ETF. Acetylation of ETF to the extent of 5.5 modifications per flavin results in a 13-fold increase in K_m with no appreciable effect on reaction turnover number when assayed at pH 7.4. Amidination, which maintains positive charge at modified loci, has no appreciable effect on the steady-state reaction parameters. These effects on the K_m for ETF semiquinone are also observed when unmodified and acetylated ETF semiquinone are catalytically disproportionated at pH 7.4 (data not shown). In these experiments, the K_m for modified ETF semiquinone (4.5 lysines modified per FAD) was increased 4-fold, and there was no effect on TN.

Table II: Disproportionation Equilibrium Constants of Native and Chemically Modified ETF Species[a]

sample	N^b	$K_{app} \pm$ SD (n)[c]
native	0	0.66 ± 0.29 (7)
acetylated	4.7	0.74 ± 0.22 (4)
amidinated	14.5	0.72 ± 0.06 (5)

[a] Number of lysine residues modified determined in 20 mM Tris-HCl, pH 7.4, at 15 °C. ETF flavin concentrations ranged from 3 to 20 μM. [b] Number of lysine residues modified determined by difference using TNBS, with unmodified ETF as the standard. [c] The number of determinations are placed in parentheses.

A possible explanation of the results is that the covalent modifications alter the thermodynamic properties of the ETF flavin. For example, acetylation of a lysyl residue near the N-1 region of the flavin could prevent stabilization of the anionic semiquinone or hydroquinone (Massey & Hemmerich, 1980). If this were the case, acetylation would alter the equilibrium constant of the reaction. Therefore, native, amidinated, and acetylated ETF semiquinone species were catalytically disproportionated with ETF-QO as previously described (Beckmann & Frerman, 1985). Table II summarizes the results and clearly indicates that the chemical modifications do not affect the relative oxidation–reduction potentials of the ETF flavin couples. Thus, the data can be interpreted in terms of the effects on the protein–protein interaction.

Although surface positive charges on ETF are important for the interaction with ETF-QO, the possibility was considered whether exposed lysyl groups of ETF-QO play a role in the reaction with ETF. ETF-QO has 13 exposed lysyl groups that are reactive with TNBS (data not shown); however, no decrease in enzymatic activity of ETF-QO was observed after modification of nine lysine residues. It thus appears that the surface lysyl groups on ETF-QO play no direct role in the interaction with ETF.

Solvent Isotope Effect. Electron double nuclear resonance spectroscopy of ETF semiquinone indicated that the water in the immediate vicinity of the flavin N-5/C-4a region is not exchangeable with bulk solvent (McKean et al., 1982). In addition, ETF apparently binds a proton upon reduction from the anionic semiquinone to the anionic hydroquinone, and ETF-QO binds one to two protons upon two-electron reduction (Beckmann & Frerman, 1984). It has been suggested that electron transfer between redox centers can occur via a bridging water molecule (Lambeth & Kamin, 1979; Lambeth, 1982). Therefore, we examined the possiblity that ETF sequesters one or more water molecules for purposes of electron transfer. One test of this hypothesis was to conduct the comproportionation assay in D_2O. A primary solvent isotope effect would be expected if a rate-limiting step involves breaking or joining of a bond with solvent, or solvent-exchangeable, hydrogen. The experiments were conducted at relatively basic pH to avoid the pH dependence of the turnover number shown in Figure 2. Three experiments in 10 mM Tris-HCl, pH 8.7, using saturating oxidized and hydroquinone forms of ETF, gave V_{H_2O}/V_{D_2O} of 1.5–2.1 (1.8 average of the three experiments). A more thorough analysis is shown in Figure 4. In this experiment, $TN_{H_2O} = 118 \pm 17$ s^{-1}, and $TN_{D_2O} = 68 \pm 9$ s^{-1}; K_m^{ETF} in $H_2O = 0.40 \pm 0.09$ μM, and K_m^{ETF} in $D_2O = 0.30 \pm 0.08$ μM. In one set of experiments,

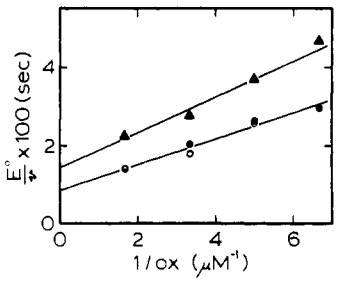

FIGURE 4: Effect of D_2O on the steady-state comproportionation reaction. Asays were conducted in 20 mM 2-amino-2-methyl-1,3-propanediol, pH 8.7, made to 0.017 M ionic strength with KCl. The solid circles (●) were obtained by using 1.5 μM ETF hydroquinone, prepared in H_2O, with the reactions also in H_2O. The open circles (○) were obtained from assays conducted in H_2O, except that ETF hydroquinone was prepared in D_2O. The closed triangles (▲) were obtained from reactions conducted in 76% D_2O. Each point represents the average of assays conducted in triplicate.

the ETF hydroquinone was generated in D_2O, and the reaction was carried out in H_2O. Under these conditions, no isotope effect was observed (Figure 4).

Discussion

The interaction between ETF and ETF-QO was investigated by examining the effects of pH, ionic strength, and chemical modifications on the steady-state kinetic constants of the ETF-QO-catalyzed comproportionation of oxidized ETF and ETF hydroquinone. The nature of the interaction between the proteins is comparable to that observed between ETF and the G-AD (Beckmann & Frerman, 1983). That is, the surface positive charges of ETF provided by ϵ-amino groups of lysyl residues play a crucial role in directing the reaction of the protein with both the flavoprotein reductant, the acyl-CoA dehydrogenase, and its oxidant, ETF-QO. The effects of chemical modification on the reductive and oxidative reactions of ETF are also very comparable. Modification of three lysine residues with TNBS results in an 11-fold increase of the K_m of G-AD for ETF (Beckmann & Frerman, 1983). Acetylation of five lysine residues increases the K_m of ETF-QO for ETF 13-fold. Using the limiting Bronsted equation for a relative quantitation, G-AD and ETF exhibit $-Z_1Z_2 = 7$ at pH 8. ETF and ETF-QO exhibit $-Z_1Z_2 = 6.3$ at pH 8.7. The simplest explanation for the similarity of results of the pH, ionic strength, and chemical modification effects on the oxidative and reductive half-reactions of ETF is that ETF has a single redox active site. This site is presumably surrounded by a positively charged protein domain, which is required for specific interaction with both reductants and the oxidant, ETF-QO. If this is correct, then ETF would necessarily function as a diffusable electron shuttle, in similar fashion to cytochrome *c* of the respiratory chain (Koppenol & Margoliash, 1982). In contrast to cytochrome *c*, it has not been possible to demonstrate a stable binary complex between ETF and either a primary dehydrogenase Crane & Beinert, 1956; McKean et al., 1979) or ETF-QO. However, the data do not explicitly demonstrate that ETF must function as a diffusable electron shuttle or that only one redox active site exists.

The actual mechanisms of electron transfer between ETF and its reductants and oxidant has never been addressed. It is no simple matter to demonstrate, for example, a direct interaction between the flavin prosthetic groups. One interesting mechanism for electron transfer has been proposed for the reduction of adrenodoxin, a 2Fe-2S protein, by the adrenodoxin reductase flavin hydroquinone (Lambeth & Kamin, 1979; Lambeth, 1982). In that system, the rate-limiting step is the electron transfer from the reductase flavin to the iron–sulfur cluster of adrenodoxin, and a solvent isotope effect of about 1.8 ± 0.3 was observed when the reduction was carried out in D_2O. Lambeth & Kamin (1979) have proposed a mechanism for electron transfer involving a bridging water molecule. ETF sequesters H_2O upon reduction and binds at least one proton upon reduction (Beckmann & Frerman, 1985); therefore, it is reasonable to propose a similar mechanism for electron transfer between ETF and ETF-QO. The mechanism cannot be identical with the adrenodoxin system because a neutral ETF semiquinone intermediate is predicted, which, if a free species, would be unstable. The solvent isotope effect could be explained by effects of D_2O on dissociation of the protein–protein complex if it is rate limiting or by rearrangement of solvent water in an activated complex (Lambeth & Kamin, 1979). However, the solvent isotope effect cannot be due to entrapped, nonexchangeable water in ETF hydroquinone because after chemical reduction of ETF to the hydroquinone in D_2O, no isotope effect is observed when the reaction is run in H_2O.

Registry No. ETF-QO, 86551-03-3; deuterium, 7782-39-0; L-lysine, 56-87-1.

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