FOR THE RECORD

Crystallization and preliminary X-ray analysis of electron transfer flavoproteins from human and *Paracoccus denitrificans*

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Abstract: Mammalian electron transfer flavoprotein (ETF) is a soluble, heterodimeric flavoprotein responsible for the oxidation of at least nine primary matrix flavoprotein dehydrogenases. Crystals have been obtained for the recombinant human electron transfer flavoprotein (ETF^{hum}) by the sitting-drop vapor diffusion technique using polyethylene glycol (PEG) 1500 at pH 7.0 as the precipitating agent. ETF^{hum} crystallizes in the monoclinic space group P2$_1$, with unit cell parameters $a = 47.46$ Å, $b = 104.10$ Å, $c = 63.79$ Å, and $\beta = 110.02°$. Based on the assumption of one $\alpha\beta$ dimer per asymmetric unit, the V_m value is 2.69 Å^3/Da. A native data set has been collected to 2.1 Å resolution. One heavy-atom derivative has also been obtained by soaking a preformed crystal of ETF^{hum} in 2 mM thimerosal solution for 2 h at 19 °C. Patterson analysis indicates one major site. The analogous electron transfer flavoprotein from *Paracoccus denitrificans* (ETF^{par}) has also been crystallized using PEG 8000 at pH 5.5 as the precipitating agent. ETF^{par} crystallizes in the orthorhombic space group P2$_1$2$_1$2$_1$, with unit cell parameters $a = 79.98$ Å, $b = 182.90$ Å, and $c = 70.07$ Å. The V_m value of 2.33 Å^3/Da is consistent with two $\alpha\beta$ dimers per asymmetric unit. A native data set has been collected to 2.5 Å resolution.

Keywords: crystallization; electron transfer; electron transfer flavoprotein

Mammalian electron transfer flavoprotein (ETF) is located in the mitochondrial matrix and serves as the sole shuttle of electrons between the primary flavoprotein dehydrogenases and the membrane bound ETF-ubiquinone oxidoreductase (ETF-QO). ETF is a heterodimer comprised of α (M_r of 33 kDa) and β (M_r of 28 kDa) subunits and contains a single FAD moiety per $\alpha\beta$ dimer (McKean et al., 1983; Frerman & Goodman, 1985). ETF

is the electron acceptor for at least nine different flavoprotein dehydrogenases, including the four chain-length-specific acyl-CoA dehydrogenases of fatty acid β-oxidation (Frerman, 1988; Izai et al., 1992). ETF is oxidized by ETF-QO in a pathway that links the primary dehydrogenases with ubiquinone–cytochrome c reductase in the main respiratory chain (Frerman, 1987; Beckmann & Frerman, 1985). Recessively inherited defects in ETF or ETF-QO result in glutaric acidemia type II (GA2) (Loehr et al., 1990), an often fatal metabolic disease. The genes encoding the human ETF have been cloned and sequenced (Finocchiaro et al., 1988, 1993), and several mutations that occur in patients with GA2 have been identified (Loehr et al., 1990; Freneaux et al., 1992).

ETFs have been isolated from a variety of sources (Thorpe, 1991), and subunit sequences have been deduced from human (Finocchiaro et al., 1988, 1993) and rat (Shinzawa et al., 1988) cDNAs and the genes of several bacteria (Arigoni et al., 1991; Bedzyk et al., 1993; Chen & Swenson, 1994). Human and *Paracoccus denitrificans* ETF subunits exhibit high sequence identity/similarity (60%/72%), consistent with similarity of flavin redox potentials and the capacity of *P. denitrificans* ETF to substitute for human ETF in several redox reactions (Watmough et al., 1992; Herrick et al., 1994). In contrast, ETF subunits from the methylotrophic bacterium W3A1 have much lower overall sequence identity (~29%) with human ETF subunits (Chen & Swenson, 1994). The redox potential for the flavin semiquinone/oxidized couple for W3A1 is approximately 220 mV greater than the corresponding value for mammalian ETFs (Byron et al., 1989; Herrick et al., 1994). Further, the methylotroph ETF will not substitute for mammalian ETF as an electron acceptor for primary flavoprotein dehydrogenases (Husain et al., 1984). The deduced gene products of the *fixB* and *fixA* genes of symbiotic nitrogen-fixing bacteria correspond to human α and β subunits, respectively, and exhibit about 24% sequence identity with the human subunits (Chen & Swenson, 1994). Among the six ETFs for which amino acid sequences have been deduced, sequence identities in the β subunit appear uniformly spaced throughout the subunit. Sequence similarities in the α subunits are marked
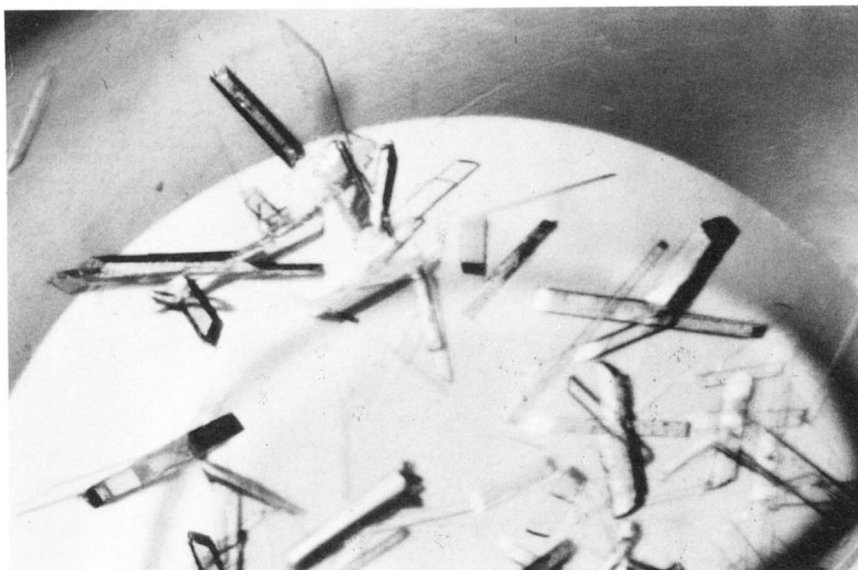
Fig. 1. Crystals of human ETF. Crystals of ETF[hum] were grown by first equilibrating equal volumes of ETF[hum] and crystallization buffer (15% PEG 1500, 50 mM Hepes, pH 7.0) against crystallization buffer overnight in sitting-drop trays. Macroseeds were then introduced and continued to grow at 19 °C. Diffraction quality crystals typically reached dimensions of 0.2 mm × 0.3 mm × 0.5 mm within 3–5 days.

in the carboxyl-terminal third of the subunit, which contains a potential nucleotide binding domain (Chen & Swenson, 1994). This domain might be involved in binding the nucleotide moiety of the FAD or noncovalently bound AMP that has been identified in W3A1 (DuPlessis et al., 1994), porcine (Sato et al., 1994), and recombinant human ETF (T. Dwyer, K.J. Griffin, & F.E. Frerman, unpubl. data).

We now report the crystallization of the human and *P. denitrificans* ETF proteins in order to determine their three-dimensional structures, which will undoubtedly aid in understanding much of the complex physical and biochemical data that have been accumulated to date. Crystals of the human ETF protein have been produced, and one native data set to 2.1 Å has been collected. In order to solve the phasing problem, we have begun the search for heavy-atom derivatives. One mercury derivative has been obtained, and a complete data set has been collected to 3.0 Å. Crystals have also been obtained for the ETF protein isolated from *P. denitrificans* and a native data set has been collected to 2.5 Å.

Results and discussion: *Crystallization of human ETF:* Initial crystallization of ETF[hum] involved using a variety of precipitants along with different salts and pHs. Of all the conditions screened, only one condition using 30% PEG 1500 gave crystals that showed promise. These crystals were "flower-like" in shape and were not consistently reproducible. However, using these crystals, the streak seeding method was used to yield small single crystals that could be used for macroseeding. At this stage, the PEG 1500 concentration was lowered to 15%, Hepes buffer (50 mM, pH 7.0) was added, and macroseeding was performed. Only then were reproducible, diffraction-quality crystals obtained (Fig. 1). Addition of FAD, MgCl$_2$, or NaCl retarded crystal growth, whereas addition of AMP enhanced crystal nucleation. Precession photographs indicate that the ETF[hum] crystals belong to the monoclinic space group P2$_1$, with unit cell parameters $a = 47.46$ Å, $b = 104.10$ Å, $c = 63.79$ Å, and $\beta = 110.02°$. Based on the assumption of one $\alpha\beta$ dimer of ETF per asymmetric unit, the V_m value was calculated to be 2.69 Å^3/Da (Matthews, 1968). One native data set has been collected to

2.1 Å from a single ETF[hum] crystal, and statistics are given in Table 1. These crystals were stable to radiation, with only 10% decay after exposure for 30 h at 4 °C.

Heavy-atom derivatization of human ETF: There are 10 cysteine residues found in the complete human ETF sequence, so the first derivative used was a mercury compound. Crystals of ETF[hum] were derivatized using the bulky mercurial compound, thimerosal. The mercury-containing crystal was more sensitive to ex-

Table 1. *Data collection statistics for ETF^{par}, ETF^{hum}, and ETF^{hum} derivatives*

	ETF^{par}	ETF^{hum}	ETF^{hum} + thimerosal
Space group	P2$_1$2$_1$2$_1$	P2$_1$	P2$_1$
Cell dimensions			
a (Å)	79.98	47.46	47.41
b (Å)	182.90	104.10	104.06
c (Å)	70.07	63.79	63.69
β (°)	—	110.02	110.07
Total observations[a]	64,813	63,481	25,050
Unique reflections	28,645	28,126	11,296
Resolution (Å)	2.5	2.1	3.0
R_{merge} (%)[b]	7.14	6.60	8.40
Completeness (%)	76.7	80.1	92.4
Soaking condition	—	—	2 mM/2 h
R_{iso} (%)[c] (based on F^2)	—	—	16.2
Number of sites	—	—	1

[a] Total observations are based upon $I/\sigma(I) > 1$.

[b] $R_{merge} = \sum_h [(\sum_i |I_i - \langle I \rangle|)/\sum_i I_i]$, where h values are unique reflection indices and i indicates symmetry equivalent indices.

$$^c R_{iso} = \frac{\sum_h \|F_P^h|^2 - |F_{PH}^h|^2|}{\sum_h |F_P^h|^2}.$$

posure to X-rays than the native crystals, and one complete data set was collected to 3.0 Å (Table 1). Patterson analysis shows the presence of one major site (23% of the origin peak). We are presently searching for additional derivatives to complete the phasing analysis.

Crystallization of Paracoccus ETF: In addition to crystallization of the human ETF protein, we have also begun crystallization of the ETF protein from *P. denitrificans*. An initial screening of ETF^{par} identified that one condition, 50 mM KH_2PO_4 with 20% PEG 8000, gave small, clustered crystals. Slight modification of the crystallization conditions showed that the addition of 100 mM NaCl along with 5 mM $MgCl_2$ led to the formation of larger, more defined crystals. During dialysis, it was noted that the protein had lost associated FAD, indicated by a loss of yellow color over time. ETF^{par} had been previously shown to be readily reconstituted; therefore, subsequent crystallizations were performed in the presence of $MgCl_2$ along with the further addition of stoichiometric levels of FAD. Indeed, large crystals of ETF^{par} suitable for diffraction analysis were obtained using this condition. Precession photographs were taken and it was determined that crystals of ETF^{par} belong to the space group $P2_12_12_1$, with unit cell parameters $a = 79.98$ Å, $b = 182.90$ Å, and $c = 70.07$ Å. Based on the assumption of two $\alpha\beta$ dimers per asymmetric unit, the V_m value was calculated to be 2.33 Å^3/Da. These crystals were quite stable to radiation, resulting in only a 20% decay after exposure for 24 h at 24 °C. One native data set has been collected to 2.5 Å (Table 1).

Although ETF^{hum} is capable of transferring electrons to the *P. denitrificans* ETF-QO, ETF^{par} cannot be oxidized by mammalian ETF-QO, indicating that there are subtle differences between the two proteins in the interactions with their respective ETF-QO. Studies using heterobifunctional crosslinking reagents have indicated that the β subunit of ETF harbors the docking site for ETF-QO as well as medium-chain acyl-CoA dehydrogenase, but definitive location of the docking site was not determined (Steenkamp, 1987, 1988). A chimeric ETF protein was recently studied in which the amino-terminal third of the β subunit of the ETF^{hum} protein was replaced with the corresponding domain from ETF^{par} (Herrick et al., 1994). Comparison of kinetic constants of ETF-QO and of several primary dehydrogenases for the chimeric and wild-type ETFs showed increased K_m's and decreased maximal velocities for the chimeric ETF. These data support the previous hypothesis that the β subunit plays an important role in the interactions between ETF and its physiological redox partners. Further studies trying to locate the subunit responsible for FAD and AMP binding have indicated that both subunits are necessary for binding of FAD and AMP (Sato et al., 1994). Structural studies on ETF^{hum} and ETF^{par} will locate the FAD and AMP moieties and comparison of these two ETF structures will help to determine regions responsible for the interactions with its physiological redox partners.

Materials and methods: *Cloning and purification of ETF proteins:* Human ETF was expressed in *Escherichia coli* JM83 from pJR46-1 as described (Herrick et al., 1994). pJR46-1 contains the entire coding sequence of the human β subunit, which overlaps the initiation codon of the presumed mature human α subunit by one nucleotide. The amino-terminus of the expressed α subunit is Gln 20. Human ETF was purified by chromatography on DEAE BioGel and CM-Sepharose fast-flow and had an

A_{270nm}/A_{436nm} ratio of 5.6. *P. denitrificans* ETF was expressed in *E. coli* DH5α and purified as described previously (Bedzyk et al., 1993). The A_{270nm}/A_{436nm} ratio of the purified protein used for crystallization was 5.8.

Crystallization of recombinant human ETF: The initial crystallization of ETF^{hum} was developed using the hanging-drop vapor diffusion technique in 24-well Linbro culture plates (McPherson, 1990). ETF^{hum} was dialyzed and concentrated against 50 mM Hepes, pH 7.0, to a final concentration of 12 mg/mL as determined using $\epsilon_{436nm} = 13.3$ mM^{-1}. An incomplete factorial screen was performed (Hampton Crystal Screen 1) using equal volumes of precipitant to protein. From this initial screen, relatively poor crystals were obtained using 30% PEG 1500 as the precipitating agent. Refinement of the crystallization conditions was accomplished using the macroseeding technique. Sitting drops containing an equal volume of ETF^{hum} and precipitating solution (50 mM Hepes, pH 7.0, 13% PEG 1500) were pre-equilibrated against 0.5 mL of precipitating solution for 16 h at 19 °C prior to seeding. After seeding, crystals routinely grew to 0.2 × 0.3 × 0.5 mm within 3 days.

Crystallization of P. denitrificans ETF: ETF^{par} was first dialyzed and concentrated against 10 mM Tris, pH 7.4, to a final concentration of 12 mg/mL. The initial crystallization of ETF^{par} was carried out following the same procedure as ETF^{hum}, and crystals were obtained within 2 weeks in 50 mM KH_2PO_4 with 20% PEG 8000 as the precipitating agent at 4 °C. Refinement of the condition involved addition of $MgCl_2$ and FAD. The final crystallization involved mixing equal volumes of ETF^{par} (12 mg/mL) with crystallization solution (25 mM KH_2PO_4, 100 μM FAD, 5 mM $MgCl_2$, and 18% PEG 8000, final pH = 5.8) using the sitting-drop technique in 24-well plates equilibrating against 0.5 mL crystallization solution.

X-ray diffraction data collection and analysis: All data collection was performed using CuK$_\alpha$ radiation from a Rigaku Rotaflex rotating anode generator (RU-200)operating at 50 kV and 100 mA with a 0.3-mm collimator. Crystals suitable for diffraction analysis were mounted in thin-walled glass capillary tubes and still photographs were taken with an R-AXIS image plate detection system. Space group assignment was assisted by indexing and cell reduction using the R-AXIS program (Higashi, 1990) in addition to precession photography. For ETF^{hum}, diffraction data were collected using a crystal-to-detector distance of 100 mm with $2\theta = 0°$ at 4 °C. The oscillation method was used for data collection, and 120 frames were collected using 1.0° oscillation per frame. For ETF^{par}, diffraction data were collected at 24 °C using a crystal-to-detector distance of 200 mm with $2\theta = 9°$. Data reduction for all crystals was carried out using the R-AXIS software. Details about the data collection and refinement are summarized in Table 1.

Heavy-atom derivatization of human ETF and preliminary phasing analysis: Crystals of ETF^{hum} were prepared as described above and washed in artificial mother liquor prior to soaking. Thimerosal was added to a final concentration of 2 mM and the crystal was soaked for 2 h at 19 °C. The crystal was then mounted and a data set was collected as described above. The R_{iso} between the thimerosal derivative and the native data set was 16.2% based on F^2. All data set statistics are summarized

in Table 1. Patterson analysis was performed using the PHASES package (Furey, 1995).

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