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THE METHYLOTROPHIC BACTERIUM W3A1
ELECTRON TRANSFER FLAVOPROTEIN:
CLONING, EXPRESSION,
AND COFACTOR BINDING PROPERTIES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University

by

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* * * * *

The Ohio State University

1996

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To Xuehui and Yuwei
ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Professor Richard P. Swenson for his guidance, insight, and support throughout the research. His faith in my abilities and his friendship will never be forgotten. I also appreciate comments and suggestions from my dissertation committee members, Professors Edward J. Behrman, David H. Ives, and Smita Patel. Thanks go to Dr. Russul Hille for providing the W3A1 bacterium strain and proteins for initial N-terminus sequence analysis. Thanks also extend to current and previous coworkers in this laboratory for their friendship and helpful discussions: Fu-Chung Chang, Larry Druhan, Yucheng Feng, Larry R. Helms, David G. Myszka and Zhimin Zhou.

I will never forget the support and encouragement from my parents. To my daughter Yuwei, I love you and enjoy the happiness you have brought to me and I offer sincere apology for my frequent absences over the years. Finally, to my wife Xuehui, without your deepest understanding and sacrifice, this endeavor would have been impossible.
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Major Field: Biochemistry
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<th>FULL NAME</th>
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<tr>
<td>AMP</td>
<td>adenosine 5' monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5' diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5' triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-cyclohexylamino-1-propanesulfonic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETF</td>
<td>electron transfer flavoprotein</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<tr>
<td>hr</td>
<td>hour</td>
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<tr>
<td>IPTG</td>
<td>isopropyl thio-galactoside</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>M</td>
<td>molar concentration</td>
</tr>
<tr>
<td>m-</td>
<td>prefix milli-</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>8-\text{N}_3\text{-AMP}</td>
<td>8-azidoadenosine 5' monophosphate</td>
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<tr>
<td>8-\text{N}_3\text{-ATP}</td>
<td>8-azidoadenosine 5' triphosphate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>5'\text{-[}^32\text{p}]\text{AMP}</td>
<td>adenosine 5'[^32p] monophosphate</td>
</tr>
<tr>
<td>5'\text{-[}^32\text{p}]\text{ATP}</td>
<td>adenosine 5'[^\alpha-^32p] triphosphate</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>TMADH</td>
<td>trimethylamine dehydrogenase</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)-aminomethane</td>
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\[ \mu \]
\[ V_{\text{max}} \]

prefix micro-
maximum rate
CHAPTER I
INTRODUCTION

Electron transfer reactions occupy a position of central importance to the functioning of biological system. They are involved in many fundamental processes from photosynthesis to bioluminescence. Of especial significance is energy metabolism of the living system, in which the energy released by oxidation of metabolites is captured in useful form via the electron transport chain. Hundreds of proteins and enzymes catalyzing various electron transfer reactions have been identified and characterized. In almost all cases, small molecules called coenzymes or cofactors are required for the catalytic activity, since the functional groups of these proteins are unable to store the electrons which are released on oxidation. These cofactors offer chemical properties the enzyme does not have and act in cooperation with the enzyme, facilitating electron transfer from substrates to acceptor molecules. The most common cofactors involved in biological electron transfer are the nicotinamide coenzyme, flavin coenzyme, heme coenzyme, pterins, iron-sulfur clusters, and other transition metal centers.

Among the redox proteins and enzymes, an important group require flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as a cofactor and are therefore called flavoproteins. They catalyze various oxidation-reduction reactions in which the flavin
moiety of the cofactor accepts one or two electrons from the reducing substrate and donates one or two electrons to the oxidizing substrate or electron acceptor. Both FAD and FMN are the coenzymatic form of vitamin B₂, riboflavin. Riboflavin was first identified in 1926 by Smith and Hendrick as a heat-stable, water-soluble molecule. They called it B-factor. Its structure was subsequently determined by Kuhn et al. in 1933. The chemical structures of riboflavin, FMN, and FAD are shown in Figure 1. The chemical synthesis of riboflavin was completed in 1935 by Karrer et al. based on precursor studies of Kuhn et al. and the biochemical work of Warburg and Christian. Both FMN and FAD can be synthesized from riboflavin either chemically or enzymatically (Merrill and McCormick, 1980; Bacher, 1991). FMN is produced by phosphorylation of the ribityl C-5' hydroxyl group of riboflavin and FAD is formed by adenylylation of FMN.

Flavin takes its name from its yellow color when in the oxidized state, for the Latin word *flavus* means yellow. The yellow color is produced by absorbing visible light due to the conjugated double-bond system of the isoalloxazine ring in the oxidized state. The absorbance spectrum shows two absorption maxima in the visible region. The shape of the spectrum is little influenced by the N(10) substituent. Therefore, the absorbance spectra of riboflavin, FMN, and FAD are similar, especially those of riboflavin and FMN, as shown in Figure 2. The intensity and the first absorption maximum slightly differ in FAD due to the intramolecular complex formation between the adenine and the isoalloxazine moieties of FAD (Weber, 1950). The spectrum of the oxidized flavin also depends on the polarity of the solvent. The spectra are better resolved in apolar solvents.
Figure 1. Structures of riboflavin and the derived FMN and FAD coenzymes. The FAD can be looked at to contain a riboflavin portion and an adenosine diphosphate part (From Zubay G, 1988).
Figure 2. The absorption spectra of 1) Riboflavin, 2) FMN, and 3) FAD in the visible region (From Walsh, C., 1977)
In addition, the second transition in the spectrum shifted to shorter wavelength (Eweg et al., 1979).

Flavins can easily undergo reversible oxidation reduction reactions. The one-electron reduced state is called the flavin semiquinone radical. In free solution the flavin semiquinone radical ionizes with pKa ~8.5 (Land and Swallow, 1969). As a result, flavin semiquinone radical exists in an equilibrium between the neutral blue form and the anionic red form under physiological conditions. The two electron or fully reduced state is called flavin hydroquinone which is colorless due to interruption of the conjugation in the isoalloxazine system. Therefore, the three oxidation states are spectrally distinguishable, although spectra of the blue neutral or red anionic radicals for free flavin are relatively less easy to record due to their rapid disproportionation in free solution, with only a few percent of the total flavin being in the radical form at equilibrium (Ehrenberg et al., 1967). This fair degree of thermodynamic destabilization of the radical is reflected in the redox potentials of the two half reactions of the free flavin. The redox potential of the first electron transfer is -240 mV, being lower than that of the second electron transfer, which is -172 mM (Draper and Ingraham, 1968). Figure 3 shows the chemical structures of flavins in its three oxidation states. The spectra of flavin in its three oxidation states are shown in Figure 4 and 5.

Flavins are also fluorescent compounds. The oxidized free riboflavin, FMN, and FAD have similar emission spectra with an emission maximum at about 520 nm when excited at either 370 or 450 nm, which can be quenched by the presence of heavy metal
Figure 3. The three oxidation states of flavin coenzymes. They are the yellow oxidized form, the red or blue one-electron-reduced form, and the colorless two-electron-reduced form (Zubay, G., 1988)
Figure 4. The absorption spectra of FMN and FAD in the oxidized and fully reduced states (from Walsh, C., 1977).
Figure 5. The absorption spectra of flavin coenzyme in its oxidized and one-electron-reduced states. Both neutral and anion radicals are shown as illustrated with glucose oxidase (from Massey et al., 1969).
(Weber, 1950). The intensity of the fluorescence emission depends strongly on the solvent polarity, pH, and the presence of organic molecules. In aqueous solution of neutral pH, free FAD exists in an equilibrium between its fluorescent and non-fluorescent forms. The latter is due to internal quenching caused by a stacked conformation of the adenine moiety and the isalloxazine ring (Weber, 1950). As a result, the quantum yield of FAD fluorescence is several orders lower than those of riboflavin and FMN in polar solvent and physiological pH.

Despite these differences, FMN and FAD appear to be functionally equivalent as redox coenzymes (Walsh, 1977). In both FMN and FAD, the catalytically functional part is located in the flavin isalloxazine ring portion of the molecules. However, the phosphate group of FMN and the adenosine diphosphate part of FAD may function in cooperation with the bound apoprotein in affecting the redox properties of the isalloxazine ring system.

Flavins are very versatile coenzymes. A vast variety of oxidation-reduction reactions involving almost every metabolic pathway of the living systems require flavoproteins as catalysts. Their substrates include amines, amino acids, alcohols, sugars, hydroxy acids, dithiols, aldehydes, ketones, and acids (Walsh, 1980). Old yellow enzyme (OYE) from brewer’s yeast was the first discovered flavoprotein isolated by Warburg and Christian in 1933. The prosthetic group of the protein was identified as FMN by Theorell (1956). Since its discovery, more than 100 flavoproteins have been identified and isolated from various eukaryotic and prokaryotic organisms (Müller and Berkel, 1991). In most
cases, the flavin cofactor are tightly but noncovalently bound to the proteins. Dissociation constants of flavins in flavoproteins are usually in the range of $10^{-8}$ to $10^{-11}$ M. Since the pioneer work of Theorell, who removed the flavin from old yellow enzyme without denaturing the protein (1935), most flavoproteins have been successfully resolved into free flavins and apoproteins. The enzymatically inactive apoprotein could then be reconstituted to the catalytically functional holoprotein by incubation with the flavin originally present. Results of this type of reconstitution studies with FMN or FAD as well as flavin analogs modified at various positions in the isoalloxazine ring indicate that the principal factor that determines cofactor specificity appears to be the substituent at the N-10 position (Massey and Hemmerich, 1980). In some cases, the cofactor are covalently attached to the proteins through a sulfhydryl or imidazole group at the C-8a methyl group of the isoalloxazine ring. The biological significance of this type of covalent linkage is not completely understood.

Flavoproteins are often classified into oxidases, dehydrogenases, and oxygenases according the type of reaction they catalyze (Müller and Berkel, 1991), although other ways of classification also exist (Massey and Hemmerich, 1980). Flavoprotein oxidases catalyze the two-electron reduction of molecular oxygen to form hydrogen peroxide and an oxidized substrate. Flavoprotein dehydrogenases do not reduce molecular oxygen directly. Their physiological electron acceptors are cytochromes, nonheme iron proteins, quinones, oxidized pyridine nucleotides, or other flavoproteins in the electron transport chain. Flavoprotein oxygenases catalyze the incorporation of molecular oxygen into the
substrate to form an oxygenated product. In addition, there is another group of flavoproteins functioning solely as electron transferases. They mediate the transfer of reducing equivalents between the prosthetic groups of other redox proteins. Flavodoxins are the best studied within this group. They contain noncovalently bound FMN as the only redox center. They are called flavodoxins because of their functional interchangeability with ferredoxins (Meyhew and Ludwig, 1975). These small bacterial flavoproteins function as low potential electron carriers. Their redox properties differ significantly from those of the free FMN as a result of perturbation by the bound apoproteins. The redox potentials of the semiquinone/hydroquinone couple are in the range of ~ -450 mV (compared to -175 mV for free FMN) at neutral pH, and are the lowest among known flavoproteins. The redox potential of the oxidized/semiquinone couple are usually more positive than that for free FMN. The one-electron reduced semiquinone is unusually stable and is stabilized at the blue neutral flavin radical species. The two one-electron steps are well separated, suggesting that the flavodoxins act as one-electron carriers in vivo, shuttling between the hydroquinone and semiquinone states. Their structure and electron transferring mechanism have been studied extensively.

Since the same tricyclic isoalloxazine ring is used as electron sink during catalysis by a variety of flavoproteins with such a diverse catalytic and redox properties, it is obvious that the changes in redox potential and the catalytic specificities must be brought about by the active site environment provided by the apoprotein and the specific interaction between the apoprotein and the prosthetic group. Numerous studies on the redox
properties of the flavin using various substituted flavins indicated that the redox properties are sensitive to electronic perturbations induced by the substituents (Müller, 1991). Studies on structure and function of flavodoxin from *Desulfovibrio vulgaris* indicated that a group of acidic amino acid residues around the FMN binding site have dramatic effect on the redox potential of the FMN cofactor (Zhou and Swenson, 1995). Neutralization of these acidic residues caused increases in the midpoint potential for the semiquinone/hydroquinone couple. Other studies on various one-electron-carrier proteins, such as cytochromes, ferredoxins, and flavodoxins, suggest that increasing the number of positively charged groups on the protein usually leads to increasing stabilization of the more negatively charged reduced form of the cofactor, thus resulting in higher values of midpoint potential (Rees, 1985). Dielectric effects can also play significant roles in affecting the redox potential of the bound cofactor. If the two oxidation states have different charges, binding to an apolar region of the protein will energetically favor the less charged state. Based on site-directed mutagenesis studies on flavodoxin from *Desulfovibrio vulgaris*, Swenson and Krey proposed that the apolar aromatic amino acid residues adjacent to the flavin isoaalloxazine ring help to stabilize the neutral flavin semiquinone while destabilizing the anionic flavin hydroquinone (1994). Another well documented noncovalent interaction between flavin and apoprotein in affecting redox properties is hydrogen-bonding interaction. Donation of hydrogen bonds to the cofactor tends to preferentially stabilize the more electron-rich reduced form, thus raising the redox potential. In contrast, the presence of hydrogen bond acceptors preferentially stabilizes
the more positively charged oxidized form of the cofactor, thus lowering the redox potential (Yagi et al., 1980; Rees and Farrelly, 1990). Studies on sulphite reactivity of flavoproteins provide additional indirect evidence on how proteins control the redox properties of bound flavins. Free flavins have low thermodynamic affinity for formation of flavin N^6-sulphite adduct. Although this low affinity for sulphite is shared by most protein-bound flavins, a good correlation has been observed between the thermodynamic stabilization of red flavin radicals and the high thermodynamic stability of the sulphite adduct (Massey et al., 1969; Massey and Hemmerich, 1980). The diverse redox properties generated from flavin and apoprotein interactions are also, at least partially, due to steric effect caused by the structural nature of the cofactor (Walsh, 1980).

One unique feature of flavoproteins is their ability to stabilize the one-electron reduced free radical state of the bound flavin. In contrast to free flavin, radical stabilization in blue neutral or red anionic form is usually pH independent over the range of enzyme stability. This feature permits the proteins to transfer either one electron, cycling between the oxidized and semiquinone form or the semiquinone and hydroquinone form, or two electrons, cycling between the oxidized and the hydroquinone forms, during catalysis, allowing efficient mediation between donors and acceptors in the electron transport chain (Hemmerich and Massey, 1982). These chemical features "place flavin coenzymes squarely, and uniquely, at the crossroads of biological redox chemistry" (Walsh, 1980). They serve as bridges between the two-electron transfer processes, which predominate in cytosolic carbon metabolism, and the one-electron transfer process, which
predominate in membrane-associated electron transport chains. An understanding of the chemical nature of the specific interactions between the flavin cofactor and the apoflavoprotein will certainly lead to deeper insight into the fundamental biochemical properties of flavoproteins.

The electron transfer flavoprotein (ETF) represents a class of FAD-containing flavoproteins which, like the bacterial flavodoxins, function solely to mediate electron transfer between other redox proteins. They play crucial roles in coupling the oxidation of soluble substrates and the membrane-bound electron transport system. The first ETF was identified from the mitochondria of pig liver as a yellow component required to mediate the transfer of reducing equivalents from fatty acyl-CoA dehydrogenases to various physiological or non-physiological electron acceptors in the β-oxidation of fatty acids (Crane and Beinert, 1956). The pig liver ETF was purified by Hall and Kamin in 1975 and was found to be a dimer of identical subunits. In 1982, Steenkamp and Husain found, in their studies of the effect of tetrahydrofolate on the oxidative N-demethylation of sarcosine and dimethylglycine catalyzed by these dehydrogenases, that the pig liver ETF could be rapidly reduced to the anionic semiquinone state by sarcosine and dimethylglycine dehydrogenases. Husain and Steenkamp (1983) purified the pig liver ETF to homogeneity using a revised three-step procedure. The purified ETF exhibits an extinction coefficient for the bound FAD of 13,500 M⁻¹cm⁻¹ at 436 nm and an isoelectric point of 6.75. Results of SDS-gel electrophoresis indicated that the pig liver ETF was actually a dimer of non-identical subunits with molecular weights of 38,000 and 32,000. The apparent molecular
weight of the native ETF holoprotein was 68,000 as determined by gel filtration analysis. Flavin analysis indicated that only one FAD molecule was bound per dimer. A stable anionic flavin semiquinone intermediate could be produced upon anaerobic reduction by dithionite. The FAD was established to be the only redox center in the pig liver ETF protein. They suggested that the two subunits play distinct roles in the ETF-mediated electron transfer. Similar observations for the pig liver ETF were also reported by McKean et al. (1983). However, the molecular weights of the two subunits were 31,000 and 27,000, respectively, based on SDS-polyacrylamide gel electrophoresis in the presence of 8 M urea. These values are considerably smaller than those reported by Husain and Steenkamp. Peptide mapping and amino acid analyses of the purified subunits indicated that the two subunits have different primary structures (McKean et al., 1983).

In addition to the pig liver protein, similar ETF proteins have also been isolated from a number of different mammalian tissues, including rat liver (Furuta et al., 1981), pig kidney (Gorelik et al., 1982), and beef heart (Hall et al., 1976). Regardless of sources, all the mammalian ETFs are heterodimers with one non-covalently bound FAD per dimer. The molecular weight of the small subunit ranges from 25,000 to 30,000 and the molecular weight of the large subunit is from 31,000 to 35,000, as judged from results of SDS-polyacrylamide gel electrophoresis and HPLC gel filtration analyses. These mammalian ETFs have been reported to accept electrons from at least eight primary dehydrogenases in mitochondria (Figure 6). They are the short, medium, and long chain acyl-CoA dehydrogenases, which are involved in fatty acid β-oxidation (Gustafson et al.,
Figure 6. Mammalian ETFs accept electrons from several primary mitochondrial dehydrogenases. Mammalian ETFs accept electrons from at least eight mitochondrial dehydrogenases and transfer them to the membrane bound coenzyme Q through an iron-sulfur flavoprotein called ETF-ubiquinone oxidoreductase (from Thorpe C., 1991).
1986); isovaleryl-CoA (Ikeda and Tanaka, 1983), 2-methyl-branched chain acyl-CoA (Ikeda and Tanaka, 1983), and glutaryl-CoA (Leinich and Goodman, 1986) dehydrogenases, which involved in amino acids catabolism; dimethylglycine and sarcosine dehydrogenases (Frisell and MacKenzie, 1962), which involved in mitochondrial one carbon metabolism. The reduced ETF then transfers electrons to the membrane bound electron transport chain through a membrane-bound iron-sulfur flavoprotein called ETF-ubiquinone oxidoreductase (Ruzicka and Beinert, 1977), which can not only catalyze the re-oxidation of the reduced ETF but also facilitates the complete reduction of ETF to the hydroquinone state by disproportionation of the semiquinone radical (Ramsay et al., 1987). Defects in ETF or ETF-ubiquinone oxidoreductase in humans result in glutaric acidemia type II, a genetic metabolic disease which is often fatal (Loehr et al., 1990). Several mutations in ETF subunits resulting in either reduced activity or subunit instability have recently been identified and characterized (Colombo et al., 1992). The properties and electron-transferring mechanism of these mammalian ETFs have undergone extensive investigation recently.

Attempts to separate the subunits of the mammalian ETF by a variety of chaotrophs have been invariably associated with the loss of FAD and irreversible denaturation of the subunits (Thorpe, 1991). ApoETF from pig kidney could be prepared by an acid ammonium sulfate procedure. Photoaffinity labeling studies involving the reconstitution of the apoETF with 8-azido-FAD lead to the preferential labeling of the small subunit, providing indirect evidence that the isoalloxazine ring of the cofactor binds adjacent to or
within the small subunit (Gorelick and Thorpe, 1986). Studies on the effects of pH and ionic strength on the steady state kinetic parameters for reduction of pig liver ETF by general acyl-CoA dehydrogenase and chemical modification to remove the surface charges both resulted in an increase in the $K_m$ of the dehydrogenase (Beckmann and Frerman, 1983), suggesting the ETF interacts with the dehydrogenases in an electrostatic manner. Protein cross-linking studies using heterobifunctional reagents lead to cross-linking of the small subunit to both acyl-CoA dehydrogenase and ETF-ubiquinone oxidoreductase (Steenkamp, 1987), suggesting the small subunit may play a role interacting with both the electron donor and acceptor of ETF. No information concerning the function of the large subunit is currently available. Both subunits of mammalian ETF are encoded by nuclear genes and are transported to mitochondrial matrix after their synthesis in cytosol (Ikeda et al., 1986). The large subunit is synthesized as a precursor with a leader peptide which is cleaved during transport to mitochondria, while the small subunit seems not to be modified during mitochondrial transport. Whether the final binding of the cofactor happens before or after the transport is not known.

Sequences of cDNA encoding both the human ETF subunits (Finocchiaro et al., 1988; Finocchiaro et al., 1993) and the rat large subunit (Shinzawa et al., 1988) have been cloned. Comparison of the amino acid sequences deduced from the cloned nucleotide sequences between the large and small human subunits indicated no similarity. However, more than 90% sequence identity has been observed between the rat and human large subunits. In addition, the small and large human ETF subunits were found to share ~ 30%
sequence identities with those of the $\text{Fix A}$ and $\text{Fix B}$ gene products of the nitrogen-fixing bacterium *Azorhizobium caulinodans*, although no biochemical function has yet been assigned to these hypothetical gene products.

Dithionite titration and photochemical reduction of the mammalian ETFs yield the red anionic flavosemiquinone which absorb strongly at 375 nm. Subsequent reduction to the two-electron reduced hydroquinone state is very sluggish, suggesting that the quantitative accumulation of semiquinone at the midpoint is kinetically controlled. The midpoint potentials for the first and second electron transfer, determined by electrochemical experiments for the pig liver ETF at pH 7.5, were +4 and -50 mV, respectively (Husain, 1984). The overall midpoint potential for the two-electron couple was -23 mV. Similar values were reported by Gustafson *et al.* (1986). These values seem to be consistent with its function as electron acceptor for fatty acyl-CoA dehydrogenases. However, whether the mammalian ETF function as a one-, two-, or mixed-electron transferase *in vivo* is not clear. Mammalian ETFs have redox potentials considerably more positive than that of free FAD (-219 mV) (Clark, 1960), implying that the semiquinone and hydroquinone forms bind much more tightly than the oxidized form. In contrast, binding of the flavin to the protein in flavodoxins results in large decreases in oxidation-reduction potential. In addition, there is a large potential difference (-260 mV) between the first- and second-electron transfers in flavodoxins, resulting in stabilization of the blue neutral flavin radical (Mayhew and Massey, 1969). In marked contrast with mammalian
ETF, there is only a 54 mV separation between the midpoint potentials for the first- and second-electron transfers, and the red anionic flavin radical is only partially stabilized.

Results of kinetic studies for the association of FAD with apoETF from pig kidney suggest that the mammalian apoETF exists in an equilibrium between two different conformations. Only one conformation is capable of binding the FAD cofactor (Sato et al., 1991). Various halide ions induced the conversion from the inactive to the active conformation in terms of binding FAD (Sato et al., 1992). In addition to the natural FAD cofactor, adenine nucleotides were found to bind the pig kidney apoETF with the same kinetic reaction scheme as FAD and compete with FAD in binding (Sato et al., 1992). Both riboflavin and FMN failed to bind regardless of the presence or absence of other nucleotides, indicating that the dinucleotide portion of the FAD molecule is essential for the incorporation of the isoalloxazine ring in the pig kidney ETF (Sato et al., 1992). The affinity of FAD and the adenine nucleotides to the pig apoETF protein is $FAD > ADP > ATP > AMP$, as judged from the determined dissociation constants (Sato et al., 1992).

In addition to mammalian ETF proteins, a number of similar ETF-like proteins having functions analogous to the mammalian ETFs have also been identified and isolated from bacterial sources, including Paracoccus denitrificans (Husain and Steenkamp, 1985), Megasphaera elsdenii (Whitfield and Mayhew, 1974), and the obligate methylotrophic bacteria Methylophilus methylotrophus (Davidson et al., 1986) and W3A1 (Steenkamp and Gallup, 1978). All these ETF proteins contain FAD as the prosthetic group and seem to
function in linking the oxidation of soluble substrates by a variety of dehydrogenases to membrane-bound respiratory chains in mitochondria or bacteria (Husain, 1990; Thorpe, 1991). The properties and electron transferring mechanisms of these proteins are currently being studied.

ETF from *Paracoccus denitrificans*, together with glutaryl-CoA dehydrogenase, have been purified to homogeneity from cells grown with glutaric acid as the carbon source (Husain and Steenkamp, 1985). The ETF serves as the electron acceptor for glutaryl-CoA dehydrogenase during the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA catalyzed by this tetrameric flavoprotein. Like the mammalian system, the reoxidation of the reduced ETF by ubiquinone is catalyzed by ETF-ubiquinone oxidoreductase, an membrane-bound iron-sulfur flavoprotein (Husain and Steenkamp, 1985). The *Paracoccus* ETF has a visible spectrum identical to that of the pig liver ETF (Husain and Steenkamp, 1985). However, flavin fluorescence in the *Paracoccus* protein is only 15% that of the mammalian protein, suggesting some differences in the microenvironments of the bound flavin between the *Paracoccus* and mammalian ETFs (Watmough et al., 1992). Similar to the mammalian ETFs, it has subunits of 32,000 and 28,000 Dalton with one non-covalently bound FAD per dimer. Interestingly, the *Paracoccus* ETF can receive electrons from several mitochondrial dehydrogenases including the short-chain and medium chain fatty acyl-CoA dehydrogenases in addition to its natural electron donor, glutaryl-CoA dehydrogenase (Husain and Steenkamp, 1985). The steady state kinetic constants of the *Paracoccus* glutaryl-CoA dehydrogenase using
either the Paracoccus ETF or pig liver ETF as electron acceptor are very similar (Husain and Steenkamp, 1985). The ETF-Q oxidoreductase from Paracoccus could also accept electrons from either the bacterial or the pig liver ETF. However, the Paracoccus ETF does not receive electrons from pig dimethylglycine dehydrogenase nor does it reduce pig ETF-ubiquinone oxidoreductase (Husain and Steenkamp, 1985). An anti-pig ETF polyclonal antibody that crossreacts with the human ETF also crossreacts with the Paracoccus ETF (Watmough et al., 1992). Like the mammalian ETFs, enzymatic or photochemical reduction of Paracoccus ETF rapidly produces an anionic semiquinone. However, formation of the fully reduced flavin hydroquinone is very slow. The separation of the oxidation-reduction potentials of the flavin couples in the Paracoccus ETF is essentially identical to that in pig ETF as judged from the disproportionation equilibrium of the Paracoccus ETF flavin semiquinone, suggesting the redox potentials of the two ETFs must be very close (Watmough et al., 1992). The genes encoding the two subunits of Paracoccus ETF have been identified and cloned (Bedzyk et al., 1993). Comparison of the amino acid sequences deduced from the cloned nucleotide sequences with those of human indicate more than 50% sequence identity in both the subunits (Bedzyk et al., 1993). Based on these observations, it appears that Paracoccus ETF and mammalian ETFs are very similar proteins. The inability of the Paracoccus ETF to oxidize pig dimethylglycine dehydrogenase and reduce pig ETF-ubiquinone oxidoreductase may suggest slight variations in the electron donor and acceptor docking sites between the two ETF proteins.
ETF from the anaerobic bacterium *Megasphaera elsdenii* was first purified by Whitfield and Mayhew in 1974. It mediates electron transfer from NADH or D-lactate dehydrogenase to butyryl-CoA dehydrogenase (Brockman and Wood, 1975). Unlike the mammalian and *Paracoccus* ETF, *Megasphaera* ETF contains two molecules of FAD per heterodimer. Its visible spectrum shows an absorbance maximum at 450 nm rather than the strongly blue-shifted spectra shown by other ETFs (Whitfield and Mayhew, 1974). Dithionite titration suggests that the flavin is the only redox center of the protein. A midpoint potential of -259 mV at pH 7.1 was determined by electrochemical measurements (Pace and Stankovich, 1987). This value lies between that of NADH and butyryl-CoA dehydrogenase, consistent with the role of this ETF protein. Incubation of apoETF with 8-Cl- or 8-F-FAD led to the covalent attachment of the analog to ETF. SDS-polyacrylamide gel electrophoretic analysis indicated that only the small subunit was covalently modified, suggesting the possibility that both the FAD derivatives bind to the same subunit (Ó Nuallàin and Mayhew, 1987).

The growth of the obligate methylotrophic bacteria W3A1 and *Methylophilus methylotrophus* is supported only by a narrow range of one-carbon compounds, such as methanol, methyamine, dimethylamine, and trimethylamine (Davidson, 1985). They contain several unusual and interesting enzymes which oxidize methyl groups to formaldehyde as a first step in the utilization of methanol and methylated amines. The syntheses of ETF and trimethylamine dehydrogenase (TMADH) in the methylotrophic bacteria *M. Methylophilus* and W3A1 are induced when the organisms are cultured with
trimethylamine as the only carbon source (Kasprazak and Steenkamp, 1983). The ETF is thought to serve as the physiological electron acceptor to TMADH during the oxidative $N$-demethylation of trimethylamine by this iron-sulfur flavoprotein (Steenkamp and Mallinson, 1976; Davidson et al., 1986) (Figure 7). TMADH is a homodimeric complex flavoprotein of molecular weight of 166,000 (Kasprazak et al., 1983), containing one 4Fe-4S cluster (Hill et al., 1977) and one molecule of FMN covalently bound via a cystenyl thioether at the 6-position of the flavin per subunit (Steenkamp et al., 1978). A tightly bound adenosine diphosphate molecule of unknown function was recently detected in each subunit (Lim et al., 1988). A 2.4 Å X-ray structure has been deduced (Lim et al., 1986) and the gene encoding for this complex flavoprotein has been cloned and overexpressed in E. coli (Boyd et al., 1992). Although several lines of evidences support that the ETF is the natural electron acceptor of TMADH, the subsequent electron acceptor for the ETF is not known with certainty but may be a high-potential cytochrome (Cross and Anthony, 1980). The methylotrophic ETF was first isolated from W3A1 by Steenkamp and Gallup in 1978 and from M. Methylotrophus by Davidson et al. in 1986. Like ETFs from other sources, the methylotrophic ETFs are dimers of nonidentical subunits, containing one molecule of noncovalently bound FAD per dimer. The molecular weight of each subunit is 42,000 and 38,000, respectively, as determined from SDS-polyacrylamide gel electrophoresis. The bound prosthetic group exhibits a strongly blue-shifted spectrum with absorbance maximum at 438 nm. No significant flavin fluorescence was observed at least for the M. methylotrophus protein.
Figure 7. The methylotrophic ETF serves as electron acceptor for trimethylamine dehydrogenase during the oxidative $N$-demethylation of trimethylamine to produce dimethylamine and formaldehyde.
Although similar in physical and spectral properties to ETFs from other sources (Husain, 1990; Husain and Steenkamp, 1985; Gorelick et al., 1982), the methylotrophic ETFs exhibit several characteristics which make them unique among the ETF family and interesting flavoproteins in their own right. Unlike mammalian and the *Paracoccus* ETFs, the methylotrophic ETFs seem to be highly specific in their reduction by TMADH but not by other dehydrogenases which react with both mitochondrial and *Paracoccus* ETFs. The two methylotrophic ETFs even cannot receive electrons from methylamine dehydrogenase and methanol dehydrogenase isolated from W3A1 (Davidson et al., 1986). They cross-react with each other immunologically and enzymatically but not with mammalian and the *Paracoccus* ETFs. These ETFs are rapidly reduced to anionic semiquinone by TMADH in the presence of trimethylamine; however, the semiquinone is not reduced further by TMADH and is unusually stable to air oxidation (Husain, 1990; Byron et al., 1989). The W3A1 ETF apparently cannot be fully reduced to the hydroquinone state even under strong reducing conditions such as dithionite titration or deazaflavin-mediated photoreduction (Steenkamp and Gallup, 1978; Byron et al., 1989). However, the ETF from *M. methylotrophus* can be fully reduced by electrochemical methods utilizing methyl viologen as a very low-potential mediator although the reduction rates are extremely slow (Byron et al., 1989). The resistance of the methylotrophic ETFs to full reduction further differentiates these proteins from those from mammalian and *Paracoccus* sources (Davidison et al., 1986).
The midpoint potential for the oxidized/semiquinone couple of the *M. methylotrophus* ETF has been determined to be +196 mV, the most positive oxidation-reduction potential recorded for a FAD-containing protein, which shows no variation with pH. The midpoint potential for the semiquinone/hydroquinone couple which involves transfer of both electron and a proton, achieved during the prolonged potentiometric titration, is -197 mV, a reduction potential not unusually negative for a flavoprotein (Byron *et al.*, 1989). Thus, the two redox couples for the flavin in the methylotrophic ETF are well separated, which can account for the thermodynamic accumulation of the anionic semiquinone during chemical and enzymatic reductions. The very positive potential for the first electron transfer is consistent with their roles as single electron acceptors for the iron-sulfur cluster of trimethylamine dehydrogenase, whose midpoint potential is +79 mV (Byron *et al.*, 1989). In contrast, the midpoint potentials for the two couples of the pig liver and *M. elsdenii* ETFs are similar (Byron *et al.*, 1989). The semiquinone is not stabilized in the *M. elsdenii* ETF and is partially stabilized kinetically in the pig liver protein. The sluggish conversion of the methylotrophic ETF semiquinone to its fully reduced state seems to suggest a kinetic barrier towards reduction and may reflect an interesting conformational change during reduction (Byron *et al.*, 1989). These observations suggest that, at least for the methylotrophic proteins, the ETF cycles between the fully oxidized and semiquinone states (Steenkamp and Gallup, 1978). The mechanism of electron transfer between ETF and TMADH has not yet been elucidated.
Recent studies indicate that in addition to the FAD cofactor, the ETFs from pig liver and W3A1 contain a bound AMP molecule (Sato et al., 1993; DuPlessis et al., 1994). Both the AMP and FAD can be released from pig kidney ETF by guanidine hydrochloride denaturation, while only FAD can be released by KBr treatment of the protein (Sato et al., 1993). FAD can be added back to the pig apoETF by reconstitution regardless the presence or absence of AMP, although the reconstitution process can be accelerated by the presence of AMP (Sato et al., 1993). Both the reconstituted holoETF and the AMP-free ETF showed identical activity to the native ETF for electron transfer from substrate-reduced medium-chain acyl-CoA dehydrogenase to 2,6-dichlorophenolindophenol, although the reconstituted AMP-free ETF showed a slightly different pI value to the native ETF, indicating the bound AMP molecule in pig kidney ETF has no apparent influence on the electron transferring activity. The spectrum of the reconstituted holoETF was very similar to that of the native ETF. However, the highly resolved spectrum cannot be fully recovered for the AMP-free ETF. The spectrum of the AMP-free ETF was not altered by incubating with AMP after the reconstitution, suggesting a partial misfolding of the protein due to absence of AMP during the reconstitution process (Sato et al., 1993). The noncovalently bound AMP molecule in the methylotrophic bacterium W3A1 has been released by boiling or methanol denaturation (DuPlessis et al., 1994). Although a modest acceleration in the binding of FAD to pig kidney apoETF by the presence of AMP was demonstrated (Sato et al., 1993), the physiological role of this additional prosthetic group is not understood.
As a group of analogous function, ETF proteins from both mammalian and bacterial sources share many common physical and chemical properties. They are all isolated as heterodimers. The reported molecular weights are similar ranging from 31,000 to 42,000 for the large subunit and 25,000 to 38,000 for the small subunit. A single FAD cofactor is noncovalently bound per dimer in all ETFs except the *M. elsdenii* protein which binds two FAD molecules per heterodimer. The FAD represents the only oxidation-reduction center in these proteins (Husain, 1990; Thorpe, 1991). In almost all cases, the oxidized chromophore exhibits a characteristic highly resolved and strongly blue-shifted visible spectrum. All ETFs yield the red anionic flavin semiquinone radical on appropriate reduction. Despite all these similarities, the redox properties, the electron donor/acceptor specificities, the kinetic and thermodynamic stability of the one-electron reduced semiquinone radical differ considerably. The redox properties of each ETF appear to have uniquely evolved to match those of the molecules from which they accept electrons. Differences in redox property and free radical stabilization may result from differences in the noncovalent interactions between FAD cofactor and different apoETF proteins while differences in electron donor/acceptor specificity reflects different structural features at the donor/acceptor docking sites. Although there are documented examples of how hydrogen-bonding, electrostatic, and steric effects may affect the redox property of the bound flavin cofactor in flavoproteins, how ETF proteins control the redox property of their FAD cofactors and the mechanism of electron transfer are not yet thoroughly understood. Other questions remain to be answered include the structural features that
have been conserved for functions important for all ETFs and the features that vary to confer specificity for their respective electron donor and acceptor. The better understanding the structure, function, and redox potential relationships in ETF proteins will lead to deeper insights into the fundamental biochemical properties of this fascinating group of flavoproteins.

W3A1 ETF provides a good model system for researches aiming to answer the above questions because of its distinctive properties relative to the mammalian and Paracoccus proteins and because of the availability of the X-ray crystal structure of its electron donor, TMADH (Lim et al., 1988). This project has been designed as a first step for the in depth investigation of FAD/apoprotein interactions and electron transfer mechanism in ETF proteins. It includes cloning the genes encoding the two subunits of W3A1 ETF, over expressing the cloned ETF genes, and studying the cofactor binding property of the recombinant ETF protein. This work sets the foundation for the investigation of the structural basis for the unusual properties of the methylotrophic ETFs.
CHAPTER II
IDENTIFICATION, CLONING, AND SEQUENCE ANALYSIS OF
THE GENES ENCODING THE TWO DIFFERENT SUBUNITS
OF W3A1 ELECTRON TRANSFER FLAVOPROTEIN

A. INTRODUCTION

Cloning and characterizing genes encoding a protein of interest is important for studying the structure and function of the protein. The amino acid sequences deduced from the cloned nucleotide sequences will provide valuable structural information. The comparison of the deduced amino acid sequence with other previously characterized proteins usually leads to the identification of functional domains, cofactor binding motifs, and other regulatory sequences. More importantly, the cloned gene can be modified and overexpressed. Therefore, the structure and function of the mutant protein can be studied.

In order to clone a gene encoding a protein of interest, a library, either genomic, cDNA, or expression library, needs to be constructed. The library can then be screened by a radioactively labeled probe to identify the correct clone containing the gene of interest. The probe is most frequently a nucleic acid molecule containing part of the sequence of the gene being sought, although an antibody raised against the protein of interest can be used for screening an expression library. An oligonucleotide probe can be synthesized based on the
known sequences of the gene or closely related genes. However, in most cases, the sequence of the gene or closely related genes is not known. Therefore, the protein of interest must be purified and its amino acid sequence be partially determined by N-terminus amino acid sequencing. From this determined amino acid sequence, the corresponding nucleotide sequence can be deduced using the genetic code. DNA oligonucleotides can then be designed and chemically synthesized for use as probes. However, due to the degeneracy of the genetic code, many oligonucleotides must usually be synthesized to represent all possible ways to code for even a very short tract of amino acid sequence. Effort to reduce the degeneracy usually has to pay the trade-off of decreasing the length of the probe, which results in even lower specificity. The polymerase chain reaction (PCR) technique allows the DNA from a selected region of genome to be amplified by more than a million-fold, provided that the nucleotide sequences at the two ends of the region is known. Therefore, the determined amino acid sequence information can be used to design and synthesize PCR primers so that a molecular probe can be amplified by PCR. The PCR-amplified molecular probes have several advantages over the oligonucleotide probes designed directly from the amino acid sequence. The probe produced by successful PCR amplification has a unique sequence. The amplified sequence is usually long enough to confer high specificity on the probe. And more importantly, the incorrect amplification caused by the degeneracy within the two oligonucleotide pools can be largely eliminated through the selection for priming by the correct oligonucleotides during each cycle of PCR. Finally, The PCR amplified probe can be cloned directly into a vector and its sequence authenticity be confirmed by comparison with the determined amino acid sequence.
In this chapter, I describe the preparation of a highly specific molecular probe, the identification, cloning, and sequence determination of the structural genes encoding the two different subunits of the ETF protein from W3A1. Comparisons of the amino acid sequence of each subunit, as derived from the nucleotide sequences, to other known electron transfer flavoproteins are also summarized.

**B. EXPERIMENTAL PROCEDURES**

*Materials*—The W3A1 ETF initially used for NH₂-terminal amino acid sequence determination was kindly provided by Dr. C. Russell Hille of the Department of Medical Biochemistry, The Ohio State University. Wild-type W3A1 ETF holoprotein used in other aspects of this study was purified from bacterium W3A1 essentially as described previously (Husain, 1990). W3A1 ETF concentrations were determined using a molar extinction coefficient at 438 nm of 11,300 M⁻¹ cm⁻¹ (Steenkamp and Gallup, 1978). The trimethylamine dehydrogenase used for assaying the activity of recombinant W3A1 ETF was purified from W3A1 using the procedure described by McIntire (1990) except that the ammonium sulfate fractionation step was eliminated, chromatography on Sephadex G-200 resin was replaced by gel filtration on Sepharose 6B, and a HPLC anion exchange chromatographic step was added. The concentration of the purified trimethylamine dehydrogenase was determined using a molar extinction coefficient at 443 nm of 54,600 M⁻¹ cm⁻¹ (Kasprazak et al., 1983). Restriction endonucleases and other enzymes used in
cloning and sequence analysis were purchased from commercial sources as indicated. All other chemicals were analytical reagents or molecular biology grade.

Subunit Separation, Isolation, and NH\textsubscript{2}-terminal Sequencing—The two subunits from ETF purified from the methylotrophic bacterium W3A1 were separated by electrophoresis on a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). The subunit polypeptides were electrophoretically transferred to a ProBlott membrane (Applied Biosystems, Inc.) in CAPS transfer buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid, 10% methanol, pH 11) for 30 min at 170 mA using a Bio-Rad transblotting apparatus. The membrane was rinsed, stained briefly with Coomassie Brilliant Blue R-250 dye, and destained following the directions of the manufacturer. The bands corresponding to the two subunits of ETF were excised and sequenced using an Applied Biosystems, Inc. Model 470A gas-phase sequencer equipped with a Model 120A in-line phenylthiohydantoin amino acid analyzer using standard protocols.

Synthesis of Oligonucleotide Primers—The degenerate oligonucleotide primers used in the polymerase chain reaction (PCR) were synthesized by the Biochemical Instrument Center at The Ohio State University on an Applied Biosystems, Inc. Model 380B synthesizer using β-cyanoethyl phosphoramidite chemistry. The nucleotide sequences of the degenerate primers (designated as SC, SN, LC, and LN) were derived from the NH\textsubscript{2}-terminal amino acid sequences of the small and large subunits of W3A1 ETF as described in Figure 8.
Figure 8. NH₂-terminal amino acid sequences of W3A1 ETF subunits and the degenerate oligonucleotide primers designed for PCR. The nucleotide sequences of the SN and SC primers were derived from the amino acid sequence EEDFEI of the small subunit; the nucleotide sequences of the LN and LC primers were derived from the amino acid sequence VIAEHR of the large subunit.
Polymerase Chain Reactions (PCR)—PCR amplifications were carried out in a reaction mixture (100 µL) containing 0.5 µg of W3A1 genomic DNA template, 100 pmol each of the two degenerate primers described in Fig. 8 (SN with LC, SN with LN, or SC with LN), 2.5 unit of Taq DNA polymerase (BRL) in 10 mM Tris.HCl (pH 8.8), 0.2 mM each of the four dNTPs, 50 mM KCl, 2.0 mM MgCl₂, and 0.01% BSA. Thirty PCR cycles (94°C for 0.5 min, 42°C for 0.5 min, and 72°C for 2 min) were performed using a Thermak thermocycler (Ericomp, Inc.) and a portion of each reaction mixture was analyzed by 1% agarose gel electrophoresis. The amplified fragments were purified using the Geneclean II kit (Bio 101, Inc.) and subcloned into the "T-vector" constructed from pBluescript II KS⁺ by the procedure of Marchuk et al. (1991) and nucleotide sequences were established. The correctly amplified DNA fragment was confirmed by comparing the open reading frames within the nucleotide sequence with the NH₂-terminal amino acid sequences of the two ETF subunits.

Identification and Cloning of the ETF Subunit Genes—Unless otherwise specified, established DNA cloning techniques were carried out essentially as described by Sambrook et al. (1989). The DNA fragment amplified by PCR was used as a probe for Southern blotting analyses and colony hybridizations. This fragment was radiolabeled with [α-³²P]dATP as described by Feinberg and Vogelstein (1983) except that Sequenase Version 2.0DNA polymerase (United States Biochemical Corporation) was used in place of the Klenow fragment of E. coli DNA polymerase I. The reaction mixture, which included random hexanucleotide primers (Promega) and [α-³²P]dATP (3000 Ci/mmol), was allowed
to incubate at room temperature for 2 hr. W3A1 genomic DNA was isolated and purified as described (Schleif et al., 1981). The purified genomic DNA was digested with different pairs of restriction endonucleases to generate fragments that could be cloned into the multiple cloning site of pBluscript II KS' vector (Stratagene). The various restriction digests were electrophoresed on 0.8% agarose gels, DNA denatured under alkaline conditions, and transferred to GeneScreen Plus membrane (NEN Du Pont Inc.) according to the directions of the manufacturer. DNA fragments containing genes encoding the two ETF subunits were identified by direct hybridization of the blots with the radiolabeled probe. Restriction fragments corresponding to regions of strong hybridization were isolated from agarose gels using the Geneclean II kit and cloned into the pBluescript II KS' vector. This recombinant mixture was used to transform E. coli strain XLI-Blue to produce a partial genomic mini-library which was screened by the colony hybridization technique (Helms et al., 1990) using the same radiolabeled probe as above. Of 1000 colonies screened, 11 colonies were found to contain DNA fragments which hybridized to the probe. Plasmids isolated from these transformants were subjected to restriction mapping analysis and DNA sequencing.

**DNA Sequencing and Sequence Analysis**—A nested set of deletions was generated within the cloned W3A1 DNA fragment (in pBluescript) using the Erase-a-Base™ system (Promega) to facilitate sequencing the entire 2.2 kb fragment with the universal and reverse primers (Stratagene). Nucleotide sequences were established by the dideoxy chain termination method of Sanger et al. (1977) with [α-32P]dATP and Sequenase Version 2.0**
DNA polymerase using either single- or doubled-stranded template DNA. On some occasions, 7-deaza-dGTP and dITP were used to overcome various compressions. Both strands of the cloned fragment were sequenced. The nucleotide and deduced amino acid sequences for the W3A1 ETF subunits were compared to other related proteins using the Lipman-Pearson and Needleman-Wunsch algorithms within the DNAstar software program. Multiple alignments were established using the algorithm of Higgins and Sharp (1989) within the Clustal V program.

C. RESULTS AND DISCUSSION

*Identification, Cloning, and Sequence Analysis of the ETF Subunit Genes*—The general approach chosen at the outset of this study for the identification and cloning of the ETF subunit structural genes involved the highly efficient polymerase chain reaction technique to amplify a portion of the ETF coding region within the genomic DNA from the methylotrophic bacterium W3A1 for use as probe. The strategy used in the design of the three different PCR primer combinations required in this work was based on the following considerations. 1) The degenerate sequences of these primers could be derived from a limited amount of amino acid sequence generated by NH₂-terminal sequence analysis of each subunit protein. 2) In prokaryotic organisms, the genes encoding for functionally related peptides are usually clustered together in the genome and transcribed as a single polycistronic messenger RNA (Bächmann, 1983). 3) The relative physical
proximity and arrangement of the two ETF subunit genes within the W3A1 genome were not known when this study was initiated. 4) The coding regions for each subunit could be located in the same strand or in opposite ones of the DNA molecule. Several of the possible arrangements of the two ETF subunit genes in the genome and the primer combinations required for amplification in each case are represented in Figure 9. Other more unlikely arrangements, such as the large separation of the coding regions, would preclude the use of this approach. However, the successful amplification of the correct DNA fragment by one of the three combinations of primers should generate a highly specific probe for the identification and cloning of the complete structural genes encoding the two ETF subunit proteins.

As part of this study, the NH$_2$-terminal amino acid sequences of the two subunits of ETF purified from the methylotrophic bacterium W3A1 were established (Fig.8). Using this information, the four degenerate oligonucleotide PCR primers were designed and synthesized (Fig. 8). The SN and SC primers correspond to the sense and antisense strands of the amino acid sequences from Glu-14 to Ile-19 of the small subunit, and the LN and LC primers correspond to the sense and antisense strands of the amino acid sequences from Val-6 to Arg-11 of the large subunit. PCR amplifications were performed on W3A1 genomic DNA using either the SN/LC, SC/LN, or SN/LN primer pair.

Using this strategy, more than a dozen of DNA fragments ranging from 0.6 to 4.0 kb were amplified from genomic DNA using the three primer combinations. All the DNA fragments except one seem to be the results of nonspecific amplifications, since these DNA
Figure 9. Possible arrangements of W3A1 ETF subunit structural genes in the genome. Small arrows represent the position and polymerization direction of each pair of PCR primers (SC, SN, LC, LN) designed for the amplification of a portion of the genes in each of these possible arrangements.
fragments can even be amplified when only one of the two primers in the primer pair was present in the reaction mixture (Table 1). However, a 0.8 kb DNA fragment could only be amplified when both the LC and SN primers were present in the reaction mixture. This 0.8 kb DNA fragment was cloned into the T-vector constructed from the pBluescript KS' (Marchuk et al., 1991) and the nucleotide sequence was determined. The comparison of the nucleotide sequence of the 0.8 kb DNA fragment with the determined NH₂-terminal amino acid sequences of the two ETF subunits revealed that the genes encoding the two ETF subunits are arranged in tandem, with the small subunit gene preceding the large subunit gene (Fig. 9, arrangement 1). The relative position of this 0.8 kb fragment within the genomic sequence is included in Figure 11. For the isolation and cloning of the complete structural genes encoding the two ETF subunits, W3A1 genomic DNA was digested with various restriction endonucleases. A partial restriction map (Fig. 11) was constructed on the basis of the restriction sites found within the probe during nucleotide sequence analysis of the 0.8 kb DNA fragment and on results from Southern blots of the digested fragments using the radiolabeled 0.8 kb PCR fragment as a probe. Representative results from one such analysis are shown in Figure 10. Based on the molecular weight estimated for the two ETF subunits by SDS-polyacrylamide gel electrophoresis and information from the restriction map, size-selected BamHI-SalI fragments of about 2.2 kb were chosen and used to construct a partial genomic mini-library by cloning into the pBluescript KS' vector. The radiolabeled 0.8 kb PCR fragment was used to screen the library by colony hybridization. Of approximately 1,000 recombinant clones, eleven
Table 1. Analysis of the Polymerase Chain Reaction Products

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA Fragments Amplified (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN + SC</td>
<td>4.0, 2.5, 2.0, 1.3, 1.1, 0.9, 0.6</td>
</tr>
<tr>
<td>LN</td>
<td>4.0, 2.5, 2.0, 1.3, 1.1, 0.9, 0.6</td>
</tr>
<tr>
<td>SC</td>
<td></td>
</tr>
<tr>
<td>LC + SN</td>
<td>0.8</td>
</tr>
<tr>
<td>LC</td>
<td></td>
</tr>
<tr>
<td>SN</td>
<td></td>
</tr>
<tr>
<td>LN + SN</td>
<td>4.0, 2.5, 2.0, 1.3, 1.1, 0.9, 0.6</td>
</tr>
<tr>
<td>LN</td>
<td>4.0, 2.5, 2.0, 1.3, 1.1, 0.9, 0.6</td>
</tr>
<tr>
<td>SN</td>
<td></td>
</tr>
</tbody>
</table>
Figure 10. Southern blot analysis of W3A1 genomic DNA. The 0.8 kb DNA fragment amplified by PCR was radiolabeled and used as probe to analyze the genomic DNA digested with BamHI (lane 1), both BamHI and SalI (lane 2), and SalI (lane 3). A 2.2 kb DNA fragment, generated by the BamHI and SalI double digestion, was observed to hybridize strongly to the probe as shown in the autoradiogram. BamHI-SalI genomic DNA fragments of about 2.2 kb were size-selected and are used to construct a mini-library.
colonies were found to hybridize to the probe and to contain a plasmid with a 2.2 kb
BamHI-SalI insert (Fig. 10, lane 2).

The partial restriction map and the sequencing strategy used to establish the complete nucleotide sequence of this insert are included in Figure 11. The complete nucleotide sequence, shown in Figure 12, was established using data derived from both strands of DNA. Two consecutive open reading frames are found within this fragment, each having deduced NH₂-terminal amino acid sequences which match those of the two ETF subunits as determined by NH₂-terminal sequencing (see underlined amino acid sequences in Figure 12). Flanking sequences of 185 bp at the 5'-end and 250 bp at the 3'-end are present. The structural gene for the small subunit encodes a 28.9 kDa polypeptide and precedes the gene for the large subunit which encodes a 33.7 kDa polypeptide. There are only two bases between the TAG translation termination codon of the small subunit gene and the ATG translation initiation codon of the large subunit gene. The G + C contents of the small and large ETF subunit genes are 51.4% and 49.5%, respectively. Probable recognition sequences were searched for within the BamHI-SalI fragment and those observed are also included in Figure 12. Shine-Dalgarno sequences similar to those utilized in E. coli (Shine and Dalgarno, 1974) were found 5' to the ATG translation initiation codon of both the small subunit gene (8 bp upstream) and the large subunit gene (7 bp upstream). The ribosomal binding site preceding the large subunit gene is actually located within the coding region for the small subunit. A consensus promoter sequence similar to those found in E. coli (Rosenberg and Court, 1979; Hawley and McClure, 1983)
Figure 11. Partial restriction map of the cloned BamHI-Sall W3A1 genomic DNA fragment encoding the ETF subunits and the sequencing strategy. The locations of the open reading frames encoding the small and large subunits are indicated by $S$ and $L$, respectively. The arrows indicate the direction and the extent of the various sequencing reactions used to establish the nucleotide sequence of the entire 2.2 kb fragment. The physical position of the 0.8 kb probe generated by PCR and restriction sites for the BamHI, PstI, SacI, EcoRI, HindIII, EcoRV, and Sall restriction endonucleases are also shown.
Figure 12. Nucleotide sequence and the deduced amino acid sequences of W3A1 ETF subunits. The amino acid residues that were confirmed by NH2-terminal sequencing of the purified subunits and the PCR primer regions are underlined. Termination codons are indicated by an asterisk, and the potential Shine-Dalgarno sequences are boxed. Arrows locate potential stem-loop structures.
was not found within the 5'-flanking sequence of the cloned BamHI-Sall fragment. The consecutive arrangement of the two ETF genes suggests that they are part of the same operon and are co-transcribed during their expression; however, the separate translation recognition sites and the shifted reading frame for each coding region suggest that each subunit protein is translated separately rather than formed by proteolytic processing of a single polypeptide translation product.

The syntheses of TMADH and ETF in methylotrophic bacteria are induced by growth on trimethylamine as the sole carbon source (Kasprazak and Steenkamp, 1983). Coordinated induction might suggest that the genes encoding these two proteins are located in the same operon. Direct evidence of this arrangement has yet to be established; however, several observations related to the genomic organization of these genes can be made at this time. There are several potential stem-loop structures with stretches of T residues following the TAG translation stop codon of the large subunit gene. These stem-loop structures could be involved in the termination of transcription of the ETF genes, which are typical in prokaryotes (Platt, 1981; Ryan and Chamberlin, 1983). A potential stem-loop structure was also found following the translation stop codon of the W3A1 trimethylamine dehydrogenase gene (Boyd et al., 1992). Although not extensive in length, the 180 bp 5'- and 250 bp 3'-flanking sequences of ETF subunit genes do not overlap with the TMADH gene and its flanking sequences (Boyd et al., 1992). Similarly, overlap of the 2.2 kb fragment with the flanking sequences reported with the dehydrogenase gene was
not observed. These observations suggest that the TMADH gene and the ETF subunit
genes could be transcribed separately.

Protein Sequence Comparisons and Analysis—The deduced protein sequences of
W3A1 ETF subunits were compared to those of human (Finocchiaro et al., 1988; 1993),
rat (Shinzawa et al., 1988), and Paracoccus denitrificans (Bedzyk et al., 1993) ETF
subunits, and the hypothetical fixA and fixB gene products from Azorhizobium caulindans
(Arigoni et al., 1991) and Rhizobium meliloti (Earl et al., 1987). The results of these
comparisons are summarized in Tables 2 and 3. The multiple alignments of each of the
subunits are shown in Figure 13. Similar to the comparison results previously obtained
(Bedzyk et al., 1993), the Paracoccus ETF is observed to be from 52.3% to 59.1%
identical to the mammalian ETFs for the sequence information available for both subunits.
However, sequence identities of approximately one-half that level was noted in comparing
both the small and large subunits of the W3A1 ETF with the small and large subunits,
respectively, of the Paracoccus and mammalian ETFs (between 29 and 33%). (The
sequence for the rat small subunit has not been reported.) In general, then, the ETF from
the methylootrophic bacterium W3A1 is the least similar in amino acid sequence to the other
known ETF protein sequences. This conclusion is maintained even when conservative
replacements are included in the comparison. These sequence comparisons seem to be
consistent with the many relatively unique features of the methylootrophic ETFs and may
suggest that the structural aspects responsible for the electron donor/acceptor binding and
for cofactor interactions in the W3A1 ETF are different from that of the ETF proteins
Figure 13. Amino acid sequence alignment of W3A1 ETF with ETF proteins from other known sources. The small subunit sequence of W3A1 ETF was aligned with ETF small subunit sequences from *Paracoccus denitrificans*, human, and the *fixA* gene products from *Azorhizobium caulinodans* and *Rhizobium meliloti*. Regions containing three or more identical residues are highlighted. Dashes indicate gaps inserted to optimize the alignment.
Amino acid sequence of W3A1 ETF large subunit was aligned with ETF large subunit sequences from *Paracoccus denitrificans*, human, rat, and the *fixB* gene products from *Azorhizobium caulinodans* and *Rhizobium meliloti*. Regions containing three or more identical residues are highlighted. Dashes indicate gaps inserted to optimize the alignment.
Table 2. Sequence Comparison among ETF Small Subunits and Fix A Gene Products

<table>
<thead>
<tr>
<th>ETFs</th>
<th>W3A1</th>
<th>Paracoccus</th>
<th>Human</th>
<th>Rat</th>
<th>Fix A gene products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Azorhizobium</td>
</tr>
<tr>
<td>W3A1</td>
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<td>NDb</td>
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<tr>
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<td>Rat</td>
<td>-</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
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</tr>
<tr>
<td>Rhizobium</td>
<td>-</td>
<td></td>
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</tbody>
</table>

*aSequences were aligned using the Lipman-Pearson and Needleman-Wunch algorithm in DNAstar software. bBedzyk et al., 1993; cFinocchiaro et al., 1993; ddata not available; eArigoni et al., 1991; fEarl et al., 1987; gNumbers indicate percentage of identity between the two sequences compared. hNot determined.*
Table 3. Sequence Comparison among ETF Large Subunits and Fix B Gene Products

<table>
<thead>
<tr>
<th></th>
<th>ETFs</th>
<th></th>
<th>Fix B gene products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W3A1</td>
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<td>Human&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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<td>28.8</td>
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<tr>
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<td>Azorhizobium</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobium</td>
<td>-</td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Sequences were aligned using the Lipman-Pearson and Needleman-Wunsch algorithm in DNAstar software. <sup>b</sup>Bedzyk et al., 1993; <sup>c</sup>Finocchiaro et al., 1988; <sup>d</sup>Shinzawa et al., 1988; <sup>e</sup>Arigoni et al., 1991; <sup>f</sup>Earl et al., 1987. <sup>g</sup>Numbers indicate percentage of identity between the two sequences compared.
from other sources. For example, the Paracoccus ETF has been reported to be able to receive electrons from several mitochondrial dehydrogenases and have redox properties similar to mammalian ETF, while W3A1 ETF does not cross-react with the mitochondrial dehydrogenases and has a much more positive redox potential for the first electron transfer, which is different from the mammalian ETFs (Husain, 1984; Husain and Steenkamp, 1985; Byron et al., 1989; Watmough et al., 1992). So while the extent of sequence homology of the W3A1 ETF with the other proteins is within that expected for a family of structurally similar proteins, it may differ significantly in important functional regions, such as within the FAD binding site. Such differences should become evident as the three-dimensional structures of members of the ETF family are established.

Sequence similarities between many of the ETFs and the protein sequences deduced from the fixA and fixB genes characterized from A. caulinodans and R. meliloti have been noted (Arigoni et al., 1991; Earl et al., 1987; Bedzyk et al., 1993). Comparison of the amino acid sequences of both subunits of W3A1 ETF to the fixA and fixB gene products exhibits an identity of 27%, a level within the range observed in the comparison of the fixA, B proteins to the other ETFs (21-28%). Although no specific function has been assigned to these hypothetical gene products, the fixA, B genes are apparently required for symbiotic nitrogen fixation in R. meliloti and A. caulinodans (Earl et al., 1987; Kaminski et al., 1988). An electron-transferring capacity in the nitrogen fixation pathway has been suggested for these hypothetical gene products based on the sequence homologies to the mammalian ETFs (Arigoni et al., 1991).
Regions of identity among all the proteins analyzed are distributed nearly uniformly throughout the sequences of the small or β-subunits. However as a group, a distinct difference was noted in the degree of homology observed in the first two-thirds versus the COOH-terminal third of the ETF large subunit and fixB sequences (Figure 13). This difference was particularly dramatic in the comparison of the Paracoccus ETF large subunit to the fixB sequences. Sequence identities of only about 6% were noted in the NH₂-terminal two-thirds; however, identities of approximately 47% were noted in the COOH-terminal portion. This general pattern was found in the comparison of all large subunit sequences and suggests that the COOH-terminal region may be responsible for a functional aspect common to all these proteins. An obvious commonality is the binding of the FAD cofactor. Upon closer inspection, a region of amino acid sequence spanning residue 260 to 289 (numbering from the NH₂-terminus of the W3A1 ETF) was found to contain elements reminiscent of the glycine-rich sequences found in the dinucleotide-binding motif described by Wierenga et al. (1985). A comparison of all sequences to the general consensus βαβ motif summarized for FAD-binding proteins (McKie and Douglas, 1991) is shown in Figure 14. In this FAD binding consensus sequence, there are three highly conserved glycine residues flanked by small or hydrophobic residues. A highly conserved aspartate or glutamate residue is found at the COOH-terminus of this sequence. For the 13 conserved positions within the FAD binding consensus sequence as shown in Figure 14, ten positions are matched exactly by the ETF proteins and the fixB gene products. The first glycine residue has been substituted by an alanine in all the ETF
Figure 14. The comparison of the highly conserved region near the COOH-terminus of the large subunit of the ETF proteins and the fixB gene products with the dinucleotide binding consensus sequence for FAD binding proteins. The consensus sequence shown is from McKie and Douglas (1991). The highly conserved GxGxxG motif and acidic residue at the COOH-terminus of the sequence are highlighted with large bold letters. The corresponding degenerate sequences, comprised largely of hydrophobic residues, at various positions in the consensus sequence are underlined as they appear in each sequence. Regions constituting secondary structures within the consensus sequence are indicated with β representing for β-strand, α for α-helix, and L for loop or turn. Secondary structure predictions (see Discussion) suggest the conserved COOH-terminal regions of the ETF proteins may also form a similar βαβ motif as the consensus sequence.
proteins and the fixB gene products, which deviates from the more usual glycine residue. However, alanine can be regarded as similar to glycine and an alanine residue has also been noted at this position in the dinucleotide-binding region of alcohol dehydrogenase from yeast (Jörnvall, 1977) and malate dehydrogenase from pig heart (Joh et al., 1987). A tyrosine instead of the usual aliphatic residue was found in the hydrophobic region just upstream from the G(A)xGxxG motif, but this has also been noted in the FAD binding motif in glutathione reductase from human (Schulz et al., 1982). An acidic residue (Asp289 for W3A1 ETF) is completely conserved at the COOH-terminus of the consensus sequence in all the ETF proteins and the fixB gene product. The side chain of the acidic residue has been reported to form a hydrogen bond with the 2'-OH of the ADP-ribose, a common feature in dinucleotide and FAD binding proteins (Wierenga et al., 1986). Also, secondary structure predictions using both the algorithms of Robson (Garnier et al., 1978) and Chou and Fasman (1978) suggest that this same conserved region may form the classic βαβ fold (see Fig. 14). The predictions are quite strong for the W3A1 protein in this region. The second β-strand is predicted with relatively lower confidence in the mammalian ETF proteins. Collectively, these comparisons suggest, quite compellingly, that this COOH-terminal region in these proteins forms a portion of the binding site of the ADP moiety of the FAD cofactor. This binding motif has not been noted in previous sequence comparisons (Arigoni et al., 1991; Earl et al., 1987; Bedzyk et al., 1993). A putative glycine-rich region (KPGDLG) was previously identified in the β- (small) subunit of the human ETF and proposed as a possible nucleotide binding site (Finocchiaro et al.,
1993); however, the corresponding sequences within the other proteins from this analysis are not very homologous and the sequences conform rather weakly to the nucleotide consensus sequence. Thus, the weight of all of the aligned sequence information in the analysis reported here seems to more strongly favor the COOH-terminal region of the large subunit. Of course, it is not yet fully known whether the FAD cofactor is bound entirely by one subunit or whether both subunits contribute portions of the binding site. Photoaffinity labeling studies utilizing 8-azido-FAD bound to the porcine ETF protein provide evidence that at least a portion of the isoalloxazine ring of the cofactor may interact with the small subunit (Gorelick and Thorpe, 1986). Taken together, these observations may suggest that both subunits participate in binding the FAD cofactor. Alternatively, both the porcine and W3A1 ETF proteins apparently bind a separate AMP molecule in addition to the FAD cofactor (Sato et al., 1993; DuPlessis et al., 1994) making it possible that this COOH-terminal region may represent its binding site. However, it is not yet understood what function this AMP molecule might have or if it binds to the other ETF proteins or the putative fixB gene product. Further investigations may clarify its function and the nature of its binding site.
CHAPTER III

EXPRESSION OF THE W3A1 ETF GENES AND PURIFICATION

OF THE RECOMBINANT ETF PROTEIN

INTRODUCTION

The cloned W3A1 ETF genes provide valuable primary structural information. However, in order to produce fairly large amount of protein for structure-function studies, expression of the cloned genes in a heterologous cell system is needed. Due to the increased knowledge in microbial genetics, the ability to overexpress genes in *E. coli* has greatly improved. The synthesis of a functional protein depends on transcription of the gene encoding the protein, efficient translation of the mRNA, and in some cases, posttranslational processing of the nascent polypeptide. As for the ETF protein, the expressed subunits must be assembled to form the heterodimer and the FAD cofactor must be bound properly to form the holoETF protein. Moreover, the products of the transcription and translation must be stabilized as much as possible. While transcription of a cloned gene requires the presence of a promoter recognized by the host RNA polymerase, efficient translation requires that the mRNA bears a ribosome binding site and translational start codon. Since no promoter
sequences similar to those in *E. coli* were found within the cloned DNA fragment containing the W3A1 ETF genes, a suitable promoter must be provided for expression in *E. coli*.

Over the past several years, numerous vectors and host stains have been developed for expressing genes in *E. coli*. These vectors usually provide a promoter for transcription of the cloned, promoter-less genes. However, the number of restriction sites available in the expression vectors are often limited. In most cases, new restriction sites need to be introduced either into the vector or the gene to be expressed in order to insert the gene properly behind a promoter. Among the best characterized promoters are the bacteriophage λP*ₜ* promoter (Pirrotta, 1975), which is regulated by a temperature-sensitive repressor; bacteriophage T7 promoter (Tabor and Richardson, 1985), which requires T7 RNA polymerase for expression; the trp and lac promoters (Das, 1990), which is regulated by lac repressor; and the tac promoter (Amann *et al.*, 1983), which is a hybrid of the trp and lac promoters. Promoters regulated by the lac repressor require isopropyl thio-galactoside (IPTG) for induction to achieve maximum expression. A regulated promoter allows maximum expression of the gene to occur only when it is desired. It also limits the rate of expression. This is especially important when the expressed protein is deleterious to the host cell. Therefore, expression of the cloned promoter-less W3A1 ETF genes in *E. coli* involves inserting the genes properly into a suitable vector bearing one of the above promoters. The ribosome binding site can either be provided by the vector or by the cloned DNA sequence. The recombinant vector can then be used to transform a suitable host strain and the growth conditions adjusted for optimum expression. The results of the expression can be analyzed by electrophoresis of the cell extracts, *in situ* hybridization, or other functional assays.
Here, we describe the construction of a new expression vector, the expression of the cloned W3A1 ETF genes, the purification of the recombinant ETF protein, and its functional analysis.

**B. EXPERIMENTAL PROCEDURES**

*Expression, and Purification Of the Recombinant W3A1 ETF Holoprotein—*

Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to introduce an *EcoRI* site before the ATG translation initiation codon of the small subunit and a *XbaI* site following the TAG translation stop codon of the large subunit. The mutagenic oligonucleotides used for introducing the *EcoRI* and *XbaI* sites were 5'-CTAATATCTTCATGAATTCGTCTCCTTC-3' and 5'-CCTAAAGTCTAGACCACACTG-3', respectively. The endogenous *EcoRI* site near the COOH-terminus of the small subunit gene was eliminated by introducing a base change using the oligonucleotide 5'-TCCTTTGAACTCATTAATG-3' which did not change the amino acid sequence (Figure 15). This newly generated *EcoRI-XbaI* fragment contains the entire coding sequences for both the ETF subunits but lacks the 5' and 3' flanking sequences. This fragment was cloned behind the hybrid *tac* promoter (Amman *et al.*, 1983) of the plasmid expression vector pKK-GEM which had been constructed by subcloning the *EcoRI-HindIII* fragment containing the multiple cloning sites from pGEM-3Z plasmid (Promega) into the same sites in the pKK223-3 expression plasmid (Pharmacia) (Figure 15). The independent expression
Figure 15. Construction of the Expression Vector pKK-GEM and Subcloning the EcoRI-XbaI Fragment for Expression. PKK-GEM was constructed by subcloning the 55 bp multiple cloning sites from pGEM-3Z into the EcoRI-HindIII site of pKK223-3 so that the EcoRI-XbaI DNA fragment containing both the ETF subunit genes can be inserted behind the Tac promoter. The EcoRI and XbaI sites were created by site-directed mutagenesis within the cloned BamHI-SalI fragment while the endogenous EcoRI site was eliminated without changing the amino acid sequence.
of the two ETF subunits was achieved by subcloning DNA fragments containing each of
the subunit genes behind the tac promoter in the pKK-GEM expression vector as shown
in Figure 16. The ribosome binding sites were also provided by the vector. The presence
of a short NH₂-terminal region of the large subunit in the construction for the small subunit
expression (see panel A of figure 16) has no obvious effect on the expression of the small
subunit based on polyacrylamide gel analysis.

_E. coli_ cells (strain JM109) transformed with the expression system described above
were grown with aeration at various temperatures ranging from 25-37°C in a medium
similar to that described by Bedzyk et al. (1993) [tryptone (16 g), yeast extract (16 g),
NaCl (5 g), K₂HPO₄ (2.5 g), and ampicillin (100 µg) per liter of media]. In some
cultures, IPTG (0.5 mM) was added after the cells reached an OD₅₅₀ of approximately 0.7,
and growth was continued until the OD₅₅₀ reached 1.7. Expression of the ETF subunit
proteins was analyzed by electrophoresis on 12% SDS-polyacrylamide gel in the presence
or absence of 6 M urea using the Laemmli (1970) discontinuous buffer system. The
formation of holoETF in _E. coli_ was examined by total cellular flavin assay (Siegel, 1979).
10 ml cell culture with A₅₀₀ of ~ 1.7 was harvested by centrifugation at 4°C. The cell
pellet was resuspended in 400 µl of 1.0 N perchloric acid and incubated in dark for 20
min. The solution was centrifuged at 5000 g for 15 min. The supernatant was mixed with
800 µl of 2.0 M sodium phosphate and incubated on ice for 15 min. The precipitate was
removed by centrifugation and the absorbance spectrum of the supernatant was determined.
Figure 16. The Independent Expression of the Two ETF Subunit Genes. Panel A shows the original cloned BamHI-SalI fragment was digested with EcoRI and HindIII. A 1.0 kb fragment, which contains the small subunit gene and a short N-terminal region of the large subunit, was isolated and subcloned into the pKK-GEM vector for expressing the small subunit. Panel B shows the mutagenized BamHI-SalI DNA fragment (also see figure 15) was digested with EcoRI and SalI. A 1.2 kb DNA fragment containing the large subunit gene was isolated and subcloned into the pKK-GEM vector for expressing the large subunit.
The recombinant W3A1 ETF holoprotein was purified as follows. Transformed *E. coli* cells were grown in 2-liter flasks with aeration at 25-30°C for at least 40 hr to the stationary phase in the absence of IPTG. Recombinant W3A1 ETF was purified essentially by the procedure described by Husain (1990) with some modifications. The cells were harvested by centrifugation, re-suspended in 50 mM potassium phosphate buffer, pH 7.2, and lysed by a single passage through a French cell at 12000-15000 p.s.i. The cell lysate was centrifuged at 30,000 g for 30 min. The supernatant was filtered and loaded onto a DEAE-cellulose column (4 x 20 cm) equilibrated with the phosphate buffer. The column was then washed with the buffer until the effluent was colorless and eluted with a linear gradient formed with 600 ml of the phosphate buffer and 600 ml of the buffer containing 500 mM NaCl. An example of the elution profile is shown in Figure 17. Fractions with $A_{280}/A_{370}$ ratio smaller than 20 were pooled and diluted 2 times with the buffer. The diluted effluent was loaded onto a second DEAE-cellulose column of the same size and eluted under the same conditions as the first one. The yellow colored fractions with $A_{280}/A_{370}$ ratio about 15 were pooled, and concentrated by ultrafiltration using an Amicon PM 10 membrane (Amicon Corp., Danvers, MA). Just as with wild-type ETF, the recombinant holoprotein exists in its partially reduced state as isolated. The concentrated ETF sample was briefly treated with a slightly excess of potassium ferricyanide to reoxidize the protein before being loaded onto a Sephadex G-100 column (2.5 x 100 cm) equilibrated with the phosphate buffer. ETF was eluted with the same buffer at a flow rate of about 60 ml/hr. The potassium ferricyanide was removed during
Figure 17. Elution Pattern of DEAE Ion-exchange Chromatography in the Purification of Recombinant W3A1 ETF from *E. Coli*. The triangles represent absorbance at 280 nm; the circles represent absorbance at 370 nm, which has been added to 0.048 and multiplied by 10. The dashed line shows the NaCl gradient (mM/166.67). Fractions with $A_{280}/A_{370}$ ratios smaller than 20 were pooled.
Figure 18. Elution Pattern of Sephadex G-100 Gel Filtration Chromatography in the Purification of Recombinant W3A1 ETF Expressed in *E. coli*. The solid line represents absorbance at 370 nm, which has been multiplied by 5; the dashed line represents absorbance at 280 nm. Fractions with $A_{280}/A_{370}$ ratios smaller than 8 were pooled.
the chromatography. The elution pattern of the gel filtration column is shown in Figure 18. The peak fractions with $A_{280}/A_{438}$ ratio less than 8 were pooled and concentrated by ultrafiltration using a PM 10 Amicon membrane.

**Characterization of the recombinant W3A1 ETF**—The molecular weights of the ETF subunits were estimated by SDS-polyacrylamide gel electrophoresis in the presence or absence of 6 M urea. High resolution molecular mass determinations for each subunit were performed by matrix-assisted laser desorption mass spectrometry at the MSU-NIH mass spectrometry facility at Michigan State University. Sinapinic acid or cyano-4-hydroxycinnamic acid was used as the matrix. Similar results were obtained in each case. The error in these mass determinations, using internal calibration methods, was estimated to be $\leq 0.1\%$.

The steady-state kinetic parameters of trimethylamine dehydrogenase of W3A1 using the recombinant W3A1 ETF as the electron acceptor were determined in reaction mixtures containing 50 mM sodium pyrophosphate, pH 7.7, 245 $\mu$M trimethylamine hydrochloride, 2.5 nM trimethylamine dehydrogenase, and ETF as the varied substrate (Steenkamp and Gallup, 1978). Reaction rates were calculated using a difference molar extinction coefficient between the oxidized and one-electron reduced semiquinone forms of the ETF of 7830 M$^{-1}$ cm$^{-1}$ at 438 nm (Equation 3a and 3b). Kinetic constants and their

$$\Delta \varepsilon_{438} = \varepsilon_{438(\text{red})} - \left( \varepsilon_{438(\text{ox})} \times \frac{A_{438(\text{red})}}{A_{438(\text{ox})}} \right)$$

(where $\Delta \varepsilon_{438} = $ the difference extinction coefficient; $\varepsilon_{438(\text{ox})} = $ extinction coefficient

$$3a$$
of the oxidized ETF; $A_{438(ox)}$ = absorbance of the oxidized ETF at 438 nm; $A_{438(red)}$ = absorbance of the one-electron reduced ETF at 438 nm.)

\[
\text{Rate of Absorbance Change at 438 nm (min}^{-1}\text{)} = \frac{\text{Reaction Rate (v)}}{\Delta \epsilon_{438} / \text{Enzyme Concentration (mg/l)}}
\]

errors were determined by the weighted linear regression analysis described by Wilkinson (1961). All assays were conducted at 10°C.

C. RESULTS AND DISCUSSION

*Expression and Purification of the Recombinant W3A1 ETF—* As described above, potential Shine-Dalgarno sequences similar to those in *E. coli* are located 7 to 8 bases 5' of the ATG translation initiation codon of both subunit genes (Fig. 12), but a promoter region similar to the typical *E. coli* consensus sequences was not found in the 5'-flanking region. Therefore, an expression system utilizing the hybrid tac promoter was chosen for the efficient expression of the holoprotein in *E. coli*. The tac promoter was provided by the plasmid expression vector of pKK-GEM for the co-transcription of the two subunit genes. The endogenous Shine-Dalgarno ribosome binding site for the small subunit gene, which had been removed during cloning (see Experimental Procedures), was also provided by the expression vector. The endogenous ribosome binding site for the large subunit gene was left unchanged. Examination of *E. coli* extracts by SDS-polyacrylamide gel electrophoresis revealed the presence of two intense protein bands in cells transformed with
pKK-GEM plasmid construction containing the genes of the two ETF subunits (Figure 19, panel A, lane 4) but are absent in extracts of untransformed cells or cells transformed with the plasmid lacking the insert (Figure 19, panel A, lanes 2 and 3). The use of one promoter but two Shine-Dalgarno sequences and ATG translation initiation codons for the two ETF subunit genes is consistent with the co-transcription of the genes and independent translation of each subunit polypeptide.

The two subunits could also be expressed independently by subcloning each of the two subunit genes behind the tac promoter separately. However, the independently expressed subunits both form the inclusion bodies and have no cofactor binding activity. Solubilization of the inclusion body using the method of Sambrook et al. (1989) and reconstitution of the subunits in the presence of FAD and AMP resulted in yield of holoETF protein of less than 1%. Somewhat unexpectedly, the electrophoretic analysis of the two independently expressed subunits on 12% SDS-polyacrylamide gels using the Laemmli (1970) system revealed that the small subunit actually migrates more slowly than the large subunit (Figure 19, panel A, lanes 7 and 8). The more typical relative migrations of the two subunits were observed in 12% SDS-polyacrylamide gels containing 6M urea (Figure 19, panel B, lanes 3 and 4). The recombinant ETF and the wild type ETF isolated from W3A1 each behaved in the same way during electrophoresis (Figure 19, panel B, lane 2 and 5). Davidson et al. (1986) reported that the apparent molecular weights of the small and large W3A1 ETF subunits were 38,000 and 42,000 Daltons, respectively, based on SDS-polyacrylamide gel analysis. These molecular weight values are similar to the
Figure 19. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the expression of recombinant W3A1 ETF in transformed E. coli. Panel A, electrophoresis on a 12\% (w/v) polyacrylamide gel in the absence of urea. Lane 1, molecular weight standards expressed in kilodaltons; lane 2, extract of untransformed cells; lane 3, cells transformed with plasmid only; lane 4, cells transformed with the plasmid containing the genes of ETF subunits; lane 5, purified recombinant W3A1 ETF from E. coli; lane 6, purified wild type ETF from bacterium W3A1; lane 7, purified recombinant small subunit; lane 8, purified recombinant large subunit. Panel B, electrophoresis on 12\% (w/v) polyacrylamide gel containing 6 M urea. Lane 1, molecular weight standards expressed in kilodaltons; lane 2, purified recombinant W3A1 ETF; lane 3, recombinant small subunit; lane 4, recombinant large subunit; lane 5, wild-type W3A1 ETF. The Laemmli discontinuous buffer system was used. Samples were boiled for five minutes in sample buffer containing 2.5\% SDS and 0.4 mM β-mercaptoethanol before loading.
results for both the wild-type and recombinant ETF obtained in this study by SDS-polyacrylamide gel electrophoresis in the absence of urea (Figure 19, Panel A) but differ from those predicted from the cloned DNA sequences. However, molecular weight values of 28,970 and 33,672 Daltons for the small and large subunits, respectively, were obtained by matrix-assisted laser desorption mass spectrometric analysis of the subunits from the recombinant W3A1 holoprotein. These data agree very well with the molecular weights predicted from the cloned DNA sequences of the two subunit genes and largely preclude the possibility of any significant post-translational modification. Apparently the molecular weight of the small subunit has been over-estimated based on SDS-polyacrylamide gel electrophoresis due to its abnormal migration. Such anomalies may be caused by very rigid secondary or three dimensional structures leading to the abnormal binding of SDS.

It should be noted that the molecular weight of the native holoprotein was calculated to be 61,400 Da per single FAD residue from the amino acid analysis (Husain and Steenkamp, 1985) which agrees very well with that calculated from the deduced amino acid sequence and the mass spectrometric analysis (62,600 Da). The anomalous migration of the ETF small subunit on SDS-polyacrylamide gels demonstrated in this study may also occur with ETFs from other sources and could have some bearing on the interpretation of some of the molecular weight values obtained by this method and reported in the literature (Thorpe, 1991). The expression can also be achieved by subcloning the EcoRI-Xbal fragment containing the ETF genes behind the lac promoter. But the expression was not as efficient as when Tac promoter was used.
Initial attempts to purify the heterologously expressed recombinant W3A1 ETF holoprotein were not successful due to the formation of inclusion bodies. The ETF protein purified from the inclusion body fraction of cellular extracts appeared to composed of both subunits each with the same molecular weights as those of the wild-type ETF protein by SDS-polyacrylamide gel electrophoresis, but lacked the FAD cofactor and had no electron acceptor activity. Under modified growth conditions in which the IPTG induction was eliminated and the growth temperature was lowered to 25°C, about 50% of the expressed ETF protein was present in the soluble fraction of the cell extract. Approximately 25 mg of ETF holoprotein was purified per liter of cell culture grown to stationary phase. As estimated by SDS-polyacrylamide gel electrophoresis, more than 95% of purity was achieved using the purification method described in "Experimental Procedures". Impurities, if found in the final preparation, can be removed by HPLC anion-exchange chromatography. The $A_{272\text{ nm}}:A_{438\text{ nm}}$ ratio of the purified ETF in its fully oxidized state was $7.0 \pm 0.2$. The purified protein can be stored at -80°C in phosphate buffer up to six months without any effect on its spectral and catalytic properties. Slight spectral changes were occasionally observed for the protein preparations stored for more than six months.

The successful expression of the holoETF protein can be examined by flavin content analysis of the cell extracts. As shown in Figure 20, expression of holoETF coincides with a typical flavin spectrum of the cell extract, while cells with no expression or with the expressed protein ending in inclusion body when grown at high temperature shows no flavin spectrum. The identity of this protein as the recombinant W3A1 ETF
Figure 20. Total Cellular Flavin Content Assay to Determine the Expression of HoloETF Protein. *E. coli* cells was extracted with 1 N perchloric acid and analyzed by spectrophotomer as described in “Experimental Procedures”. Curve 1 is the spectrum of cell extract from *E. coli* transformed with plasmid containing the ETF gene insert. The *E. coli* cells have been grown at 25°C; curve 2 is the spectrum of extract from *E. coli* cells transformed with plasmid without ETF gene insert; curve 3 is the difference spectrum of curve 1 and curve 2. Only cells expressing the ETF genes and producing holoETF protein show the typical flavin spectrum.
protein was further established by NH$_2$-terminal sequence analysis of the two subunits from the purified protein which had been separated by ion exchange chromatography in 6 M urea. The NH$_2$-terminal sequence for the large recombinant subunit was determined to be: SKILVIAEHRRNDLRPVSLELIGAANGLKKSGEDK, while that observed for the small subunit was MKILVA V. These data agree completely with previous amino terminal sequencing results for the ETF subunits isolated from W3A1 and with the deduced amino terminal sequences based on the cloned DNA sequences (Fig. 12) with one exception. The terminal amino acids of both the wild-type large subunit isolated from W3A1 and the recombinant protein expressed in E. coli is serine instead of methionine as predicted from the DNA sequence. In contrast, the NH$_2$-terminal methionine residue is retained in both the wild-type small subunit isolated from W3A1 and the recombinant small subunit expressed in E. coli. These observations are consistent with what is known about the specificity of the E. coli methionine-specific aminopeptidase in that the terminal methionine residue is often removed when followed by serine but is retained when followed by lysine (Vogt, 1970; Sarimo and Pine, 1969; Waller, 1963). The similarities in the NH$_2$-terminal residues of both the wild-type ETF isolated from W3A1 and the recombinant protein obtained from E. coli suggests that the methylotroph contains an aminopeptidase with similar specificities. Amino terminal sequence analyses of the recombinant ETF subunits which had been transferred to ProBlott membranes also confirm the identities of the bands on SDS-polyacrylamide gel and the anomalous migration of the small subunit in the absence of urea.
Spectral and Catalytic Properties of the Recombinant ETF — Just as with wild-type ETF purified from W3A1 (Husain, 1990), the heterologously expressed recombinant ETF is isolated from *E. coli* as a mixture of fully oxidized and anionic semiquinone forms of the flavoprotein. Fully oxidized ETF can be obtained by addition of a slight excess of potassium ferricyanide followed by dialysis or gel filtration as reported for wild-type ETF (Husain, 1990). The recombinant W3A1 ETF is readily reduced to anionic semiquinone by trimethylamine dehydrogenase isolated from W3A1 in the presence of trimethylamine (Figure 21). The near ultraviolet-visible absorbance spectra (Figure 22) for the fully oxidized and red anionic semiquinone states of the recombinant ETF are essentially identical to those of wild-type W3A1 ETF and very similar to the ETF proteins from *Paracoccus denitrificans* and mammalian sources (Husain, 1990; Husain and Steenkamp, 1985; Watomough *et al.*, 1992). The visible spectrum of the fully oxidized ETF is highly resolved, exhibiting an absorbance maximum at 438 nm with a pronounced shoulder at 458 nm. Extended enzymatic reduction failed to reduce the recombinant ETF to its two-electron fully reduced state. The anionic semiquinone radical state showed a remarkable stability in air.

The \( K_m \) of the trimethylamine dehydrogenase for the recombinant W3A1 ETF expressed in *E. coli* was 7.4 ± 0.5 \( \mu \text{M} \), and the \( V_{\text{max}} \) was 4.5 ± 0.11 \( \mu \text{mol/min/mg} \), using trimethylamine hydrochloride as the electron donor (Figure 23). The \( K_m \) and \( V_{\text{max}} \) determined for the expressed ETF are comparable to those of wild-type ETF (\( K_m \) of 7.4 ± 0.33 \( \mu \text{M} \) and \( V_{\text{max}} \) of 6.1 ± 0.12 \( \mu \text{mol/min/mg} \)) assayed under the same conditions and
the results of Steenkamp and Gallup (1978) who determined the kinetic constants of the ETF purified from W3A1.
Figure 21. Reduction kinetics of recombinant W3A1 ETF (9.7 µM) by trimethylamine dehydrogenase (0.42 µg/ml) in the presence of trimethylamine hydrochloride (245 µM) at 10°C in 50 mM sodium pyrophosphate, pH 7.7. Spectra were recorded at regular intervals during the course of the reaction using a photodiode array spectrophotometer.
Figure 22. Ultraviolet-visible absorption spectra of oxidized (solid line) and reduced (dash line) recombinant W3A1 ETF holoprotein isolated from *E. coli* (in 50 mM sodium pyrophosphate buffer, pH 7.7). After recording the spectrum of the oxidized form, trimethylamine dehydrogenase was added to a final concentration of 0.42 μg/ml and the reaction started by addition of 0.245 μmol/ml of trimethylamine hydrochloride. The spectrum of the reduced ETF was recorded when the reaction had reached completion.
Figure 23. Double reciprocal plots of the dependence of rate of the absorbance decrease of the 438 nm absorption band of ETF on the concentration of recombinant ETF in the reaction mixture. All reactions were carried out using a fixed concentration of trimethylamine and trimethylamine dehydrogenase as in Panel A. Reaction rates were calculated using a difference molar extinction coefficient between the oxidized and one electron reduced semiquinone forms of the recombinant ETF of 7830 M$^{-1}$ cm$^{-1}$ at 438 nm.
CHAPTER IV
RECONSTITUTION AND PHOTOAFFINITY LABELING STUDIES

INTRODUCTION

Theorell in 1935 succeeded in dissociating the flavin moiety from the Old Yellow Enzyme on dialysis in acid solution. The colorless apoprotein could be reconstituted with FMN to regenerate the functional holoprotein. Since this important discovery, the dissociation of an enzyme into prosthetic group and apoprotein has been achieved in many flavoproteins. In most cases, the holoprotein can be reconstituted by adding the prosthetic group back under appropriate conditions.

Flavoproteins exhibit a great degree of variation in their ease of resolution into flavin and apoenzyme. The dissociation constants for most flavoprotein are in the range of 100 to 0.1 nM (Husain and Massey, 1979). The most common methods for resolving flavin from flavoprotein are the classical acid-ammonium sulfate treatment (Warburg and Christian, 1935) and potassium bromide treatment (Massey and Curti, 1966). The acid-ammonium sulfate method involves precipitating the protein using ammonium sulfate under acidic conditions while the potassium bromide method consists of dialysis of the protein solution against buffer containing 1 to 2 M potassium bromide. However, some proteins cannot be resolved by
potassium bromide in the neutral pH region. These methods have been successfully used for many flavoproteins, although the capacity of the prepared apoprotein for reconstitution differs considerably. The affinity of the apoprotein for flavin can also be decreased by the addition of denaturing agents such as urea or guanidine hydrochloride (Brady and Beychok, 1969). However, conditions have to be evaluated for different proteins to allow flavin to dissociate without losing the capacity for reconstitution. Calcium chloride treatment to resolve flavin from a complex flavoprotein such as xanthine oxidase was reported by Komai et al (1969).

Reconstitution of the holoprotein usually involves mixing apoprotein with the prosthetic group under appropriate conditions. The association of the prosthetic group to apoprotein can usually be monitored by absorbance or fluorescence changes. However, the mechanism of reconstitution may differ considerably for different flavoprotein (Husain and Massey, 1979).

Finding conditions for preparing apoflavoprotein and reconstituting holoproteins is important in the characterization of new flavoproteins. These investigations are useful for the identification of new prosthetic groups and the kinetic and thermodynamic properties of the interaction between the cofactor and the protein. More importantly, they form a prerequisite for studies where the natural prosthetic group is replaced by either chemically modified or isotopically labeled flavins. Reconstitution with cofactor analogs modified with functional groups capable of reacting covalently with many different amino acids can be used to identify the subunit, specific regions, or even the amino acid residues directly involved in cofactor binding.
In this chapter, the identification of a AMP molecule bound to the recombinant W3A1 ETF, the preparation of apoETF, reconstitution with natural cofactor and adenine nucleotides, and the photoaffinity labeling the FAD and AMP binding sites will be described.

**EXPERIMENTAL PROCEDURES**

*Materials*—Recombinant W3A1 ETF was purified to >95% homogeneity from *E. coli* as described (Chen and Swenson, 1994). FAD purchased from Sigma was further purified using a C18 reverse-phase HPLC column with a linear gradient from 100 mM potassium phosphate buffer, pH 5.3, containing 0% v/v methanol to 35% methanol. The adenine nucleotides, FMN, and riboflavin were purchased from Sigma and used without further purification. Adenosine 5' [α-32p] triphosphate (5'-[32p]ATP) was obtained from Dupont NEN. 8-Azidoadenosine 5'-[α32P] triphosphate was purchased from Research Products International Corp. Adenosine 5'-[32p]monophosphate (5'-[32 p]AMP) was prepared by hydrolyzing adenosine 5'-[α-32p]triphosphate using phosphodiesterase I (Worthington Biochemical Corporation). The hydrolysis reaction was carried out in 0.11 M Tris-HCl buffer, pH 8.8, containing 0.11 M NaCl, 15 mM MgCl2 at 37° C. The reaction mixture of 300 μl contained both 5'-[α-32p]ATP and ATP in a total concentration of 100 μM, and 0.05 u of enzyme. The completion of the reaction was monitored by analyzing the reaction mixture on a reverse-phase HPLC column, monitoring the radioactivity and the absorbance at 260 nm, as shown in Figure 24. All other chemicals
Figure 24. HPLC analysis of product generated from phosphodiesterase I digestion of adenine 5'[α-32p] triphosphate. A C18 reverse phase column was eluted with linear gradients formed by mixing 100 mM potassium phosphate buffer, pH 5.3, with the same buffer containing 35% methanol at a flow rate of 0.5 mL/min. Peak a and peak b show the chromatogram of 25 µl of 100 µM AMP and ATP, respectively; peak c shows the chromatogram of 25 µl of 100 µM adenosine; peak d shows the chromatogram of 25 µl of reaction mixture taken from a total volume of 300 µl of 5'[α-32p]ATP and ATP in a total concentration of 100 µM which has been digested by 0.05 u of enzyme for one hr at 37°C.
and enzymes were purchased from commercial sources as indicated. Buffers were made with reagent-grade chemicals using either distilled or deionized H₂O.

**General Methods**—Solution concentrations were determined spectrophotometrically using the following molar extinction coefficients: \( \epsilon_{438} = 12800 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) for holoETF (see "Results and Discussion"), \( \epsilon_{450} = 11300 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) for FAD, \( \epsilon_{445} = 12500 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) for FMN and riboflavin (Walsh *et al.*, 1978), and \( \epsilon_{260} = 15400 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) for ATP, ADP, and AMP (Bock *et al.*, 1956). The concentration of functional apoETF containing AMP was determined by titrating known concentration of FAD with the apoETF, monitoring flavin absorbance changes.

**Reverse-phase Chromatographic Analyses of ETF Extracts**—The recombinant W3A1 ETF samples (200-300 \( \mu \text{M} \) in 50 mM potassium phosphate buffer, pH 7.2) were extracted with 3 M guanidine hydrochloride in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA at 0°C, a procedure similar to the one described by Sato *et al.* (1993). Released nucleotides were separated from the protein by ultrafiltration using an Amicon Model 8200 stirred cell apparatus. The collected filtrates were analyzed by HPLC on a Vydac C-18 reverse-phase column (0.5x30 cm) using a 25-min linear gradient formed by mixing 100 mM potassium phosphate buffer, pH 5.3, with the same buffer containing 35% methanol at a flow rate of 0.5 mL/min. The column effluent was monitored at 260 nm. Released nucleotides were identified by their elution times relative to authentic standards and by their UV/visible absorbance properties.
ApoETF Preparation and Reconstitution Studies—FAD was removed from the ETF protein by extensive dialysis of the holoprotein sample against 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA and 2.0 M KBr or by several concentration/dilution cycles of the holoprotein in an Amicon ultrafiltration cell with the same buffer until all the yellow color disappeared, followed by dialysis to remove KBr. Unless otherwise specified, this ETF protein preparation which still retains the bound AMP molecule will be referred to "apoETF/AMP" throughout the following discussion. Both FAD and AMP were removed from ETF using a method similar to the one described by Sato et al. (1991). This preparation will be referred to as "apoETF" throughout.

HoloETF protein samples were extensively washed with 3 M guanidine hydrochloride in 50 mM phosphate buffer containing 0.3 mM EDTA and 1.0 mM DTT. Reconstitution of holoETF from apoETF/AMP was carried out by mixing this preparation with excess of FAD and incubating the mixture at room temperature for one hour. Excess ligand was removed by dialysis. Reconstitution of apoETF/AMP with either FMN, riboflavin, or adenine nucleotides was carried out under the same conditions using the same method. Reconstitution of holoETF from AMP-free apoETF was carried out using the procedure of Sato et al. (1993) except glycerol was omitted from the reconstitution mixture. In either case, the reconstituted protein was concentrated by ultrafiltration at 0-2°C using an Amicon stirred cell or Centricon 10 concentrators. The reconstituted ETF was extracted with 3M guanidine hydrochloride and the cofactor composition of the ETF extracts were analyzed on reverse-phase HPLC as described above. Electron-transferring activity of the
reconstituted ETF using the W3A1 trimethylamine dehydrogenase as the electron donor was assayed using the reaction conditions described previously (Steenkamp and Gallup, 1978; Chen and Swenson, 1994).

Photoaffinity Labeling Studies—Photoaffinity labeling of the ATP/FAD binding site was carried out in reaction mixtures containing 40 μg apoETF/AMP and 7 μM 8-azidoadenosine 5' [α32P]triphosphate in a total volume of 50 μl of 50 mM KH2PO4, buffer, pH 7.2, 0.3 mM EDTA. Samples were pre-incubated for 30 min at room temperature in the dark and were then irradiated at room temperature for 2 min at a distance of 5 cm from a short wavelength Mineralight lamp which produces maximal radiation at 354 nm with an intensity of 330 fW/cm² (distance, 15 cm). Competition experiments were performed under the same conditions except that the protein samples were pre-incubated with the photoaffinity reagent in the presence of different concentrations of FAD or ATP before photolysis. Photoaffinity labeling of the AMP binding site using 5'-[32p]AMP was carried out using a similar procedure described by Williams and Konigsberg (1991). ApoETF (with both FAD and AMP removed) was reconstituted with 5'-[32p]AMP in the presence or absence of FAD using the procedure as described above. The reconstituted ETF protein sample (~ 100 μl) was placed on a parafilm sheet and irradiated at 4°C for 30 min at a distance of 10 cm using the same Mineralight lamp as above. After photolysis, an equivalent volume of each protein sample was loaded on a 12% SDS-polyacrylamide gel and the unbound photoaffinity reagent was separated from covalently bound reagent by electrophoresis under denaturing conditions using the buffer systems described by Laemmli
(1970). The gels were stained with Coomassie Brilliant Blue and destained before exposure to X-ray film or quantitatively analyzed using PhosphoImager model 445 SI (Molecular Dynamics).

RESULTS AND DISCUSSION

Analysis of the Recombinant W3A1 ETF Extracts—To determine the cofactor composition of the recombinant W3A1 ETF heterologously expressed in *E. coli*, the non-covalently bound parts of the protein was extracted with 3 M guanidine hydrochloride and analyzed by reverse-phase HPLC. Figure 25, panel A shows a chromatogram of a mixture of equal amount of ATP (a), ADP (b), AMP (c), and FAD (d), the peaks being assigned by the separate chromatography of the individual components. Figure 25, panel B shows the chromatogram of the ETF extracts. The two distinctive peaks (c and d) were identified as AMP and FAD by comparison of their retention times with those of AMP and FAD and by their absorbance spectra. The molar ratio of the AMP and FAD in the ETF extracts was determined to be 1:1 by comparison of the area ratio under the two peaks with the area ratio of peaks of equal amount of AMP and FAD. These results indicate that the recombinant ETF expressed in *E. coli* contains a non-covalently bound AMP molecule per heterodimer in addition to the FAD cofactor. The mammalian ETF from pig kidney and the methylotrophic bacterium ETF isolated from W3A1 have recently been found to contain a non-covalently bound AMP molecule in addition to the FAD cofactor (Sato *et*
Figure 25. Reverse-phase HPLC analyses of recombinant W3A1 ETF extracts. Panel 1: Reverse phase (C18) HPLC analysis of standard mixtures of 100 μM of ATP (a), ADP (b), AMP (c), FAD (d). Panel 2: Analysis of guanidine HCl (3.0 M) extracts of the holoETF protein (in 50 mM potassium phosphate buffer, pH 7.2). Peaks designated x are buffer components, peaks c and d are AMP and FAD, respectively, as confirmed by spectrophotometry insets). Elution conditions: Linear gradient of 100 mM potassium phosphate, pH 5.3 to the same buffer containing 35% methanol over 25 min. The ratio of the peak areas for FAD and AMP are consistent with an equimolar amount of each nucleotide in the recombinant ETF holoprotein.
al., 1993; DuPlessis et al., 1994). The functional role of the bound AMP is not understood. Whether ETF proteins from other sources also contain the AMP molecule is not known. Interestingly, the physiological electron donor of the W3A1 ETF, trimethylamine dehydrogenase, which is a homodimeric iron-sulfur flavoprotein, contains a non-covalently bound ADP molecule per subunit in addition to the covalently-bound FMN cofactor and the iron-sulfur center (Lim et al., 1988). The functional role of the ADP molecule in this iron-sulfur flavoprotein is not yet understood. The recombinant holoETF protein was also extracted by repeatedly washing with 50 mM potassium phosphate buffer, pH 7.2, containing 2 M KBr in an ultrafiltration cell. The analysis of the KBr extracts by reverse-phase HPLC reveals the presence of only the FAD peak. ApoETF proteins generated by KBr treatment was further extracted with 3.0 M guanidine hydrochloride and the guanidine extract was analyzed by HPLC. The chromatogram of this guanidine extract contains only the AMP peak. These results indicate that treatment of holoETF protein with 2 M KBr only remove the FAD cofactor, generating the apoETF/AMP complex, while the guanidine hydrochloride treatment removes both the FAD and the AMP molecules, generating AMP-free apoETF protein (Scheme I and II).
**ApoETF Preparation and Reconstitution Studies**—Functional holoETF protein can be reconstituted from apoETF containing AMP by adding back the FAD cofactor (see experimental procedure) (Scheme III). The yield of the reconstituted holoprotein is about 50%. Figure 26 shows the effect of mixing excess apoETF/AMP on the visible absorption spectrum of a solution of FAD. The visible absorbance of FAD increased and \( \lambda_{\text{max}} \) shifted to a shorter wavelength. The absorbance change of FAD was due to the binding of FAD to the apoETF/AMP complex (Scheme III). The visible spectrum of reconstituted ETF was virtually identical to that of the native ETF protein. An extinction coefficient of 12,800 M\(^{-1}\)cm\(^{-1}\) was calculated based on the concentration of the starting FAD and the absorbance change upon complete binding using the following equation:

\[
\varepsilon_{438(\text{ETF})} = \frac{A_{438(\text{ETF})} \times \varepsilon_{438(\text{FAD})}}{A_{438(\text{FAD})} \times (V_f/V_i)}
\]

where \( \varepsilon_{438(\text{ETF})} \) is the extinction coefficient of the reconstituted holoETF, \( A_{438(\text{ETF})} \) is the absorption of the reconstituted ETF at 438 nm, \( \varepsilon_{438(\text{FAD})} \) is the extinction coefficient of FAD at 438 nm, \( A_{438(\text{FAD})} \) is the absorption of FAD at 438 nm before mixing with the apoETF, \( V_f \) is the volume of the FAD solution before mixing with apoETF, and \( V_i \) is the volume after mixing with apoETF. In addition to absorbance change, the fluorescence of the FAD
Figure 26: The absorbance spectrum change of FAD upon binding to W3A1 apoETF/AMP. 1: Spectrum of $1.06 \times 10^5$ M FAD. 2: Spectrum of $1.06 \times 10^5$ M FAD plus $1.5 \times 10^3$ M apoETF/AMP. FAD and apoETF/AMP solutions were mixed. The binding of FAD to apoETF/AMP results in the characteristic hyperchromic visible spectral changes to the cofactor. The spectrum was recorded after the absorbance changes complete. 3: Spectrum of $4.79 \times 10^6$ M apoETF/AMP. Buffer: 50 mM potassium phosphate, pH 7.2, 0.3 mM EDTA.
Figure 27. The Fluorescence Spectrum Change of FAD upon Binding to W3A1 ApoETF Containing AMP. Fluorescence of 1.8 \( \mu \)M FAD (circle) and 1.8 \( \mu \)M reconstituted holoETF (triangle). The holoETF was reconstituted by incubating apoETF containing AMP with excess of FAD followed by dialysis to remove excess of ligands. The excitation wavelength was 450 nm.
was almost completely quenched on binding to apoETF (Figure 27). The $K_m$ and $V_{max}$ of trimethylamine using the reconstituted ETF as electron acceptor were determined to be $6.8 \pm 0.5 \, \mu M$ and $5.4 \pm 0.3 \, \mu \text{mole/min/mg}$, respectively. These values are comparable to those determined for the native and recombinant ETF proteins (Chen and Swenson, 1994; Steenkamp and Gallup, 1978).

Functional holoETF protein can also be regenerated with AMP-free apoETF by adding back both the FAD and AMP using a reconstitution method similar to the one described by Sato et al. (1993) in the presence of DTT (see "Experimental Procedures") (Scheme IV). The yield of the reconstituted holoprotein starting with the AMP-free apoETF is ~25%. The apoETF preparation can also be reconstituted with AMP in the absence of FAD, generating the apoETF/AMP complex; however, this protein is nonfunctional as an electron acceptor. In marked contrast, the AMP-free apoETF can not be reconstituted with FAD in the absence of AMP (Scheme V). In addition, ATP and ADP also failed to bind to the AMP-free apoETF in the absence of AMP (Table IV), suggesting that neither ATP/ADP nor FAD can bind to the AMP site. The observation
Table 4. Reconstitution of ApoETF with FAD and Adenine Nucleotides

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<tr>
<td>FAD+AMP</td>
<td>holoETF</td>
<td>holoETF</td>
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<tr>
<td>ATP+AMP</td>
<td>apoETF/AMP/ATP</td>
<td>apoETF/AMP/ATP</td>
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<tr>
<td>ADT+AMP</td>
<td>apoETF/AMP/ADP</td>
<td>apoETF/AMP/ADP</td>
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1. ApoETF prepared using guanidine hydrochloride; 2. ApoETF prepared using KBr; 3. ApoETF containing AMP and ATP; 4. ApoETF containing AMP and ADP; 5. ApoETF containing AMP.
that functional holoprotein can only be regenerated from the apoETF preparation when both FAD and AMP are included in the reconstitution mixture is apparently different from that for mammalian ETF, since mammalian ETF holoprotein can be reconstituted in the absence of AMP (Sato et al., 1993). The reconstituted AMP-less mammalian protein exhibits electron transfer activity identical to the native protein, although the highly resolved visible absorption spectrum cannot be fully recovered. The AMP molecule in the mammalian ETF has been suggested to play a role in facilitating the correct folding of the protein (Sato et al., 1993).

Reconstitution of apoETF/AMP with FMN, riboflavin, as well as adenine nucleotides was also studied. As summarized in Table 4, incubation of apoETF/AMP with the adenine nucleotides resulted in the formation of the apoETF/AMP/ATP or the apoETF/AMP/ADP complex. The yield of the apoETF/AMP/ADP complex formation is relatively low compared to apoETF/AMP/ATP possibly due to weaker interactions between ADP and apoETF/AMP. Formation of both apoETF/AMP/ATP and apoETF/AMP/ADP complexes was effectively inhibited by the presence of equal molar amount of FAD in the reaction mixtures, suggesting the adenine nucleotides bind to the same site as FAD. In contrast, reconstitution of apoETF/AMP with riboflavin or FMN cannot be achieved regardless of the presence or absence of other adenine nucleotides in the reconstitution mixtures. Furthermore, the visible spectra of FMN and riboflavin were not affected when FMN or riboflavin solutions were mixed with apoETF/AMP, indicating that these two molecules do not bind strongly to apoETF/AMP. These results suggest that
the adenine nucleotide portion of the FAD cofactor is indispensable in its interaction with ETF proteins. These phenomena were also observed in the mammalian system by Sato et al. (1993), who reported that the pig kidney ETF could not be reconstituted with FMN and riboflavin. Reconstitutions of AMP-free apoETF with adenine nucleotides, FMN, and riboflavin were also carried out under the same conditions as the reconstitution with FAD. Similar results were obtained. Neither FMN nor riboflavin can reconstitute with apoETF protein regardless the presence or absence of other adenine nucleotides. However, the formation of apoETF/AMP/ATP(or ADP) complex can be observed when AMP was included in the reconstitution mixture. These results indicate that, like the FAD cofactor, the ATP and ADP molecules can only bind to ETF in the presence of AMP. The failure of FAD and ATP/ADP binding in the absence of AMP and the relative harsher conditions required for releasing the AMP molecule from the ETF suggest that the AMP molecule is deeply bound in the protein interior and is an integral part of the protein structure or is very tightly bound to a site having a strong influence on the holoprotein structure. It would be interesting to know how the AMP molecule affects the binding of FAD in the W3A1 ETF and whether it plays any role in regulating the redox properties of the bound flavin. The inhibition of the adenine nucleotide binding by FAD suggests the ATP and ADP bind to a protein site normally occupied by the adenosine diphosphate portion of FAD cofactor. The adenine nucleotides have been reported to bind to the pig kidney apoETF containing AMP with the same kinetic reaction scheme as FAD (Sato et al., 1992). However, the binding of adenine nucleotides and FAD to W3A1 apoETF/AMP
may follow a different mechanism. The binding of the isooalloxazine ring apparently not only depends on the presence of the adenyl nucleotide moiety but also a proper covalent linkage between the riboflavin and the ADP molecule is required, since both riboflavin and FMN failed to bind even in the presence of the adenine nucleotides. It seems possible that the W3A1 ETF has a pre-formed adenine nucleotide binding site. The binding of the adenyl nucleotide moiety at this site induces conformation changes in both the apoETF and the FAD molecule. These conformation changes lead to the formation of the binding site for the isooalloxazine ring portion of FAD.

*Photoaffinity Labeling Studies*—The amino acid sequences for each of the W3A1 ETF subunits have been deduced from the cloned nucleotide sequences (see Chapter II). Sequence comparisons show only ~30% sequence identity to ETF proteins from other known sources. However, a more highly homologous region of more than 50% sequence identity was noted near the COOH-terminus of the large subunits among the known ETF proteins. This highly conserved region contains a sequence that matches in several ways the ADP-binding motif of flavoprotein and other dinucleotide binding proteins. Whether this sequence represents a portion of FAD or AMP binding site in the ETF proteins is not known. As a first step to determine the cofactor binding site and the functional roles each of the two subunits might play in cofactor-protein interactions, the photosensitive analogs of ATP and AMP were used to label the FAD and AMP binding site. The photoaffinity analog of ATP, 8-azidoadenosine 5'-[α³²P] triphosphate (8-N₃-ATP), was incorporated into ETF by reconstitution of apoETF/AMP with the analog in the absence or presence of
various concentrations of FAD or ATP, as described in "Experimental Procedures". The results of covalent photoincorporation and protection studies are shown in Figure 28. Panel A represents the Coomassie Brilliant Blue-stained SDS-polyacrylamide gel analysis of the photoaffinity labeled protein sample; panel B represents the autoradiogram of the gel. Lane 1 shows the photoaffinity labeling experiment in the presence of 8-N₃-ATP only. The autoradiogram reveals that the small subunit of ETF, which migrates slower than the large subunit under these conditions (See Chapter III), was preferentially radiolabeled. Competitive labeling studies with FAD was done to determine if 8-N₃-ATP binds to the same site as the FAD cofactor. As shown in Figure 28, panel B lanes 2 through lane 4, the extent of labeling was greatly reduced by the presence of increasing amount of FAD, suggesting the 8-N₃-ATP and FAD compete for the same binding site. Competitive labeling in the presence of ATP was also carried out to further examine the specificity of 8-N₃-ATP as shown in panel B, lanes 5 through lane 7. The extent of labeling by 8-N₃-ATP was also reduced by the presence of increasing amount of ATP, although ATP was not as effective as FAD in protecting the protein from covalent labeling. This observation is consistent with ATP having a dissociation constant five times larger than that of FAD (see Chapter V). These results suggest that 8-N₃-ATP and ATP bind to the same site of ETF and compete with FAD in the same manner. The competition of ATP or 8-N₃-ATP with FAD is apparently due to the binding of these nucleotides to the ETF site normally occupied by the adenine nucleotide portion of FAD, suggesting the adenine moiety of FAD makes at least partial contact with the small subunit. The 8-N₃-
Figure 28: Photoaffinity labeling ATP/FAD binding site of ETF using 8-N$_3$-[³²p]ATP.
Reaction mixtures contained 0.8 mg/ml apoETF (prepared by KBr treatment) and 7 µM 8-N$_3$-[³²p]ATP in a total volume of 50 µl of 50 mM potassium phosphate, pH 7.2, containing 0.3 mM EDTA. Panel A represents the Coomassie Brilliant Blue-staining of each reaction mixture after SDS-polyacrylamide gel electrophoresis. Panel B is an autoradiogram of the gel. The molecular mass markers are expressed in kilodaltons. Lane 1 represents samples without FAD or ATP as the protecting compound in the reaction mixture; Lane 2: molar ratio of FAD to 8-N$_3$-[³²p]ATP = 1; Lane 3: FAD:8-N$_3$-[³²p]ATP = 3; Lane 4: FAD:8-N$_3$-[³²p]ATP = 5; Lane 5: ATP:8-N$_3$-[³²p]ATP = 1; Lane 6: ATP:8-N$_3$-[³²p]ATP = 3; Lane 7: ATP:8-N$_3$-[³²p]ATP = 5. The samples were incubated and photolyzed as described under “Experimental Procedures".
ATP labeling of yeast alcohol dehydrogenase and bovine albumin was also carried out under the same conditions. The extent of labeling for these two proteins remained at the background level similar to the ETF large subunit (not shown), further supporting the conclusion that covalent labeling of the ETF small subunit is specific and preferential. Photoaffinity labeling studies involving the reconstitution of the pig kidney ETF with 8-azido-FAD, where the azide group is located at the 8-position of the flavin isoalloxazine ring, led to the preferential labeling of the small subunit (Gorelick and Thorpe, 1986), suggesting the isoalloxazine ring of the FAD cofactor binds adjacent to or within the small subunit. Taken together, from these observations one would expect that both the isoalloxazine ring and the adenine moiety of the FAD cofactor have contact with the small subunit of ETF proteins. Of course, whether the FAD cofactors in mammalian ETF and W3A1 ETF are bound in the same manner is not known. FAD fluorescence is enhanced upon binding to the mammalian ETF (Gorelick et al., 1982), while the flavin fluorescence is almost completely quenched in W3A1 ETF, suggesting the microenvironments of the bound flavin between the mammalian and W3A1 ETFs may be different. The different fluorescence properties may also have resulted from different conformations adopted by the bound flavin, such as a stacked conformation of isoalloxazine ring and the adenine moiety of the FAD molecule may be stabilized by binding to W3A1 ETF. Future investigations using affinity labeling analogs with the reactive groups attached to different position of FAD or the adenine nucleotides may provide further information about the binding site.
Attempts at photoaffinity labeling the AMP binding site using the photosensitive analog of AMP, 8-azidoadenosine 5'-monophosphate (8-N₃-AMP), was unsuccessful, since 8-N₃-AMP fails to bind and cannot be incorporated into ETF by the reconstitution method described above. However, irradiation of protein-nucleic acid complexes with ultraviolet light has been demonstrated to produce covalent linkage between amino acid residues and nucleic acid bases (Shetlar, 1980). Although the reaction mechanisms of this photochemical process are not fully understood, several recent studies have shown that any amino acid residue can be induced to form a UV-induced covalent cross-link with any nucleotide residue of DNA or RNA (Hockensmith et al., 1991). Thus, apoETF was reconstituted with 5'-³²pAMP in the absence or presence of FAD. The reconstituted protein sample was irradiated with UV light to induce covalent modification of the ETF protein by the radioactive AMP as described in “Experimental Procedures”. The results are shown in Figure 29. Panel A represents the Coomassie Brilliant Blue stained polyacrylamide gel, while panel B shows the autoradiogram of the gel. Figure 29, lane 1 shows protein sample which has been reconstituted in a reaction mixture containing equal amount of AMP and FAD; lane 2 shows protein samples reconstituted in the absence of FAD. In either case, the radioactivity appears only at the position of the small subunit band. However, the intensity of the band in lane 2 is ~1.8 times of that in lane 1, indicating that more AMP was covalently incorporated into the ETF in the absence of FAD. This result suggests that the radioactivity was incorporated into the AMP binding site as well as the FAD binding site when FAD molecule is absent in the reaction mixture.
Figure 29 Photoaffinity labeling the AMP binding site using 5'-[32p]AMP. Lane 1: ETF samples reconstituted from AMP-free apoETF, FAD, and 5'-[32p]AMP; lane 2: ETF samples reconstituted in the absence of FAD. The samples were photolyzed and analyzed as described under "Experimental Procedures". Panel A represents the Coomassie Brilliant Blue-stained protein gel. The results of autoradiography of the gel are shown in Panel B. The molecular mass standards on the gel are expressed in kilodaltons.
These observations are consistent with results of competitive binding studies in which the binding of FAD to apoETF/AMP is effectively inhibited by the presence of AMP or other adenine nucleotides (see Chapter V for competitive binding studies). No labeling was detected in the absence of UV irradiation (data not shown). The dose of UV light used did not cause significant amounts of either intra- or interpeptide cross-links to the ETF protein, which would alter the protein mobility. These results, together with the results of 8-N3-ATP labeling, indicate that the small subunit of the W3A1 ETF comprises at least part of both the ATP/FAD and the AMP binding sites. Although the large subunit was not labeled by either analog, its involvement in cofactor/protein interaction cannot be excluded. Comparison of the tertiary protein and nucleotide structure of the FAD-binding domain of p-hydroxy-benzoate hydroxylase with the FAD and NADP-binding domains of glutathione reductase suggests that the consensus βαβ motifs mainly interact with the ribose moiety of the dinucleotides, and the 8 position of the bound adenine ring seems to point away from the consensus βαβ fold (Wierenga et al., 1983). These observations suggest that the consensus dinucleotide binding sequence identified in the large subunit of ETF proteins may still be involved in forming the binding site of FAD or AMP, although the large subunit was not labeled in our photoaffinity labeling studies. Independent expression of each of the two W3A1 ETF subunits in E. coli generated protein preparations lacking the FAD cofactor and electron transferring activity (Chen and Swenson, 1994); attempts to separate the mammalian ETF subunits by a variety of chaotrophes invariably resulted in the loss of FAD and irreversible denaturation of the
subunits (Thorpes, 1991), suggesting the correct folding of the protein and binding of cofactor require the presence of both subunits. Further studies using cofactor analogs bearing the azide or other reactive groups at different positions of the cofactor molecule will provide more information about the roles each subunit may play in interaction with the cofactors.
CHAPTER V

EQUILIBRIUM AND KINETIC STUDIES OF COFACTOR BINDING

INTRODUCTION

Our interest in electron transfer flavoproteins is primarily concerned with the interactions between apoETF proteins and the FAD cofactor and how these interactions affect the redox and chemical properties of the bound flavin. The interactions of proteins with ligand molecules invariably involve binding steps as one of the principal events. Therefore, the determination of the equilibrium binding constants and the study of the kinetics of complex formation and breakdown logically become the first stage in characterizing such molecular interactions. These types of studies can help to quantitatively determine the forces that stabilize the protein/cofactor complex, identify functionally important structural changes in the protein following binding, and other information on the nature of the binding site. Such quantitative measurements of the binding affinity and kinetic parameters can be achieved in many ways as long as there is a signal change, which can be monitored, upon binding. Spectrophotometric methods are usually the most convenient techniques to study cofactor apoprotein interactions in flavoproteins, since in most cases the absorption and fluorescence properties of flavins change upon binding to proteins. As we have demonstrated in Chapter
IV, the absorbance of FAD was increased on binding to W3A1 apoETF and the $\lambda_{\text{max}}$ shifted to a shorter wavelength. In addition, the fluorescence of FAD was quenched by binding to apoETF.

In this chapter, the results of equilibrium and kinetic studies on the binding of FAD cofactor to W3A1 apoETF containing AMP will be described.

**EXPERIMENTAL PROCEDURES**

*Equilibrium Studies for Binding of FAD Cofactor and Adenine Nucleotides*—Unless otherwise stated, all experiments were performed at 20°C in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA. UV-visible absorbance spectra were recorded on a Hewlett-Packard Model 8452A diode array spectrophotometer equipped with a temperature-controlled cuvette holder. FAD solutions of 5-10 $\mu$M were titrated with known concentrations of apoETF/AMP. The binding of FAD to apoETF/AMP was monitored by the flavin absorbance changes at 462 nm and 486 nm at equilibrium. The $K_d$ value for binding was determined by means of the Scatchard plot. Binding of the adenine nucleotides could not be determined directly because the absorbance and fluorescence changes associated with binding of the nucleotides or ETF apoprotein are very small or non-existent. Also, signal changes were not observed upon mixing apoETF/AMP complex with several of the common fluorescence analogs of the adenine nucleotides including 2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (Molecular
Probes), N-methylantraniloyl derivatives of ATP (synthesized as described by Hiratsuka, 1983a), and 1,6-ethenoadenosine 5'-triphosphate (Sigma). This may be due to the failure of these nucleotide analogs to bind to apoETF/AMP complex, or, less likely, due to a lack of fluorescence changes upon binding. Thus, the interaction of adenine nucleotides with apoETF/AMP complex was measured indirectly by competitive binding assays in which the flavin absorbance changes were monitored when FAD solutions of known concentrations were titrated with the apoETF/AMP complex in the presence of different concentrations of adenine nucleotides. The inhibition constants of the nucleotides to the binding of FAD were determined using the method described by Horovitz and Levitzki (1987)

Kinetic Studies of FAD Binding to ApoETF Containing AMP—The kinetics of FAD binding to apoETF/AMP were monitored by recording the absorbance spectrum of the flavin using a Hewlett Packard 8452A diode array UV-visible spectrophotometer equipped with a temperature-controlled cuvette holder. In a typical experiment, the binding was initiated by quickly mixing 10 to 100 microliters of apoETF solution with 1 ml of a 10 μM solution of FAD. The absorbance changes at 462 and 486 nm were plotted as a function of time and the rate constants determined by curve fitting to appropriate exponential equations using the Kaleidagraph software. Fluorescence changes of FAD upon binding were monitored using a model SF-61 Stopped-Flow spectrofluorimeter (Hi-Tech Scientific). The excitation wavelength was 450 nm and the emission wavelength was 520 nm. Stopped-flow kinetic time courses were analyzed using the nonlinear least-squares
fitting routines supplied with the SF-61 stopped-flow spectrofluorometer. Kinetic simulation and analysis were performed with the KINSIM kinetic simulation program (kindly provided by Dr. Smita Patel, Department of Biochemistry)(Barshop, et al., 1983).

_HPLC Gel Filtration—_The oligomeric and conformational states of the apoETF/AMP complex were determined by analytical gel permeation chromatography on a 30-cm Waters Protein Pak 300SW HPLC gel filtration column in 50 mM potassium phosphate, pH 7.2, 0.3 mM EDTA buffer with a flow rate of 0.5 ml/min. The effluent was monitored by Waters 484 absorbance detector at 280 nm. The following protein standards were used to calibrate the column: thyroglobulin (670,000); γ-globulin (150,000); bovine serum albumin (67,000); egg albumin (45,000), ovalbumin (43,000), bovine carbonic anhydrase (29,000), horse myoglobin (17,000); and vitamin B₁₂ (1,350). Standard curve was generated by plotting the retention times _versus_ log of molecular weights of the standard proteins.

**RESULTS AND DISCUSSION**

_Equilibrium Studies of FAD and Adenine Nucleotides Binding to ApoETF Containing AMP—_The interaction of the FAD cofactor and the adenine nucleotides with apoETF protein containing AMP was studied. To determine the model-independent macroscopic binding constant of FAD with apoETF/AMP, FAD solutions (in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA) was titrated with known
concentrations of apoETF/AMP. Substantial absorbance changes were observed consistent
with binding (Figure 30). The absorbance changes at 462 and 486 nm recorded in the
difference spectra were plotted against the apoETF/AMP concentrations (the peak to valley
absorbance changes were used to help reduce the effect of a small amount of light
scattering). The dissociation constant of the recombinant W3A1 ETF into apoETF/AMP
and FAD was determined to be $2.4 \times 10^8$ M using the Scatchard plot of data as shown in
Figure 30. Our initial equilibrium studies of the interactions between apoETF/AMP and
adenine nucleotides using several of the fluorescence analogues of the nucleotides were
unsuccessful due to the absence of either fluorescence or absorbance signal changes when
$2'(3')-O-(N$-Methylantraniloyl)adenosine 5'-triphosphate, $2'(3')-O-(2,4,6-$
trinitrophenyl)adenosine5'-triphosphate, or $1,N^6$-ethenoadenosine5'-triphosphate were
mixed with the apoETF/AMP protein. However, the interaction of adenine nucleotides
with the ETF protein can be observed indirectly using the competitive binding studies. As
shown in Figure 31, the absorbance changes were monitored during the titration of FAD
solutions with known concentration of apoETF/AMP in the presence of various
concentrations of ATP. The absorbance changes of FAD were monitored during the
titration. The results clearly indicated that the ATP molecules present in the solution
compete with FAD in binding to apoETF/AMP. An apparent inhibition constant of $1.3$
$\times 10^7$ M (Table 5) between ATP and apoETF/AMP was calculated by the method of
Horovitz and Levitzki (1987) (Figure 31, inset) using equation 5-1, where $[I]_t$ represents
total ATP concentration; $[L]_t$ represents total FAD concentration; $[E]_t$ represents total ETF
Figure 30. Determination of the dissociation constant for the complex of FAD and apoETF containing AMP. FAD solution (10.8 μM) was titrated with 120 μM apoETF/AMP at 20°C, in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA. Spectra have been corrected for dilution. The difference spectra between FAD and the spectra generated during titration (inset) indicates an absorbance increase at 462 nm and a decrease at 486 nm during the titration.
Absorbance changes at 462 nm and 486 nm were plotted against the [apoETF/AMP]. A dissociation constant of ~24 nM was determined from the experimental points which lie off the two linear parts of the curve, where alpha = [EL]/[E], as expressed in equation: E + L = EL (see inset). The triangle and circle represent data from two separate experiments.
Figure 31. Competitive binding of FAD and ATP to apoETF containing AMP. FAD (10.8 μM) was titrated with 115 μM apoETF/AMP in the presence of various concentrations of ATP. Absorbance changes at 462 nm were subtracted with those at 486 nm and were plotted against apoETF concentrations. Dilution due to volume changes has been corrected. Curve 1: absence of ATP; curve 2: molar ratio of ATP to FAD = 1; curve 3: ATP:FAD = 3; curve 4: ATP:FAD = 6. The dissociation constant between ATP and apoETF was calculated as shown in the inset, based on equation 5-1 (Horovitz and Levitzki, 1987).
Table 5. The Apparent Dissociation and Inhibition Constants of FAD, ATP, ADP, and AMP

<table>
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<tr>
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<th>FAD^a</th>
<th>ATP^b</th>
<th>ADP^b</th>
<th>AMP^b</th>
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</thead>
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<tr>
<td>(µM)</td>
<td></td>
<td>(µM)</td>
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<td>0.024 ± 0.003</td>
<td>0.130 ± 0.006</td>
<td>9.796 ± 0.430</td>
<td>19.840 ±0.632</td>
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a. Determined by equilibrium titration; b. Determined from competitive binding studies
concentration; $Y$ represents the degree of binding (e.g., $[\text{EL}]/[\text{E}]_t$, in which $[\text{EL}]$ is the ETF and FAD complex); $K_d$ represents the dissociation constant between FAD and apoETF; and $K_i$ represents the dissociation constant between ATP and apoETF. The

$$\frac{[\text{I}_t] \left( \frac{[\text{L}]_t}{[\text{E}]_t} - Y \right)}{(1 - Y) \frac{[\text{E}]_t}{K_d} \left( \frac{[\text{L}]_t}{[\text{E}]_t} - Y \right) - Y} = \frac{[\text{L}]_t}{[\text{E}]_t} - Y + K_d$$

apparent inhibition constants of ADP and AMP with the apoETF/AMP complex were calculated to be $9.8 \times 10^{-6}$ M and $2.0 \times 10^{-5}$ M (Table 5), respectively, using the same method. These results are consistent with previous observations in the reconstitution and photoaffinity labeling experiments. The lower yield of apoETF/AMP/ADP complex formation relative to apoETF/AMP/ATP during the reconstitution of apoETF/AMP with adenine nucleotides can be explained by the larger inhibition constant of ADP. The AMP molecule can bind to the ETF site normally bound by the adenyl nucleotide portion of the FAD as well as to the AMP binding site. The inhibition constant of ATP is only about five times larger than the dissociation constant between apoETF/AMP and FAD, indicating that the W3A1 apoETF/AMP complex has a well formed adenyl nucleotide binding subsite. The adenine nucleotides compete with FAD in binding by competing with the adenyl nucleotide portion of the FAD cofactor. Taken together, the observations that neither riboflavin nor FMN could bind even in the presence of other adenine nucleotides, it is reasonable to speculate that a specific conformation of the FAD and ETF must be formed before the binding can occur. The binding energy of the adenyl nucleotide and
apoETF/AMP may be used by the ETF protein and the FAD molecule to drive the conformation changes so that the binding of the isoalloxazine ring portion of the cofactor can be realized. The binding of the isoalloxazine ring in turn stabilizes the final apoETF/AMP/FAD complex to produce the functional holoETF protein. The phosphate groups of the adenine nucleotides are important for their binding to apoETF/AMP since adenosine failed to bind (data not shown) and the binding affinity decreases as the number of phosphate groups of the adenine nucleotides decreases.

**Kinetic Studies of FAD Binding to ApoETF Containing AMP**—To better understand how the W3A1 ETF protein interacts with the FAD cofactor and control its redox properties, kinetic studies for the association of FAD with apoETF/AMP was carried out. As shown in Figure 30, the visible absorbance of FAD increases and the $\lambda_{\text{max}}$ shifts to a shorter wavelength upon binding to ETF. In addition, the flavin fluorescence is quenched by protein upon binding. These optical and fluorescence changes provide convenient ways to monitor the rate of binding. The kinetics of FAD binding to apoETF/AMP was first studied by monitoring the absorbance change of FAD during binding. Our initial strategy was to determine the number of phases in the time course of binding and measure both the FAD and apoETF/AMP concentration dependence of each phase in order to determine a minimum number of intermediate states and the rate constants for their interconversion (Fersht, 1985). Figure 32 shows the time dependence of binding when apoETF/AMP was mixed with excess of FAD. The final concentration of apoETF/AMP was 6.0 $\mu$M and the final concentration of FAD was 64.8 $\mu$M. Two exponentials are required to fully
Figure 32. Kinetics of FAD binding to apoETF containing AMP after mixing apoETF/AMP with excess of FAD. The final FAD and apoETF/AMP concentration were 64.8 μM and 6.0 μM, respectively. The inset shows the dependence of the observed rate constants (k_{obs}) associated with the majority of the spectral changes on the concentration of FAD. The solid line is the best fit of the data to a simple hyperbola, with a plateau = 0.011 s\(^{-1}\). A limiting slope of 370 M\(^{-1}\)s\(^{-1}\) was calculated at low FAD concentration.
Figure 33. Kinetics of FAD binding to apoETF containing AMP after mixing FAD with excess of apoETF/AMP. The time trace has been fitted to double exponential. The final FAD and apoETF/AMP concentration are 3.6 μM and 19.8 μM, respectively. The inset shows the effect of apoETF/AMP concentration on the observed rate constants (kobs) associated with the majority of the spectral changes. An apparent second-order rate constant of 365 M⁻¹s⁻¹ was calculated from the slope of the plot.
describe the time course of the FAD absorbance change. There is a rapid major absorbance increase, with an observed rate constant \( k_{\text{obs,1}} = 0.01136 \text{ s}^{-1} \), followed by a slower minor absorbance decrease, with an observed rate constant \( k_{\text{obs,2}} = 0.00011 \text{ s}^{-1} \). Under low FAD concentrations (over the range of 7 to 18 \( \mu \text{M} \) FAD), \( k_{\text{obs,1}} \) seems to vary linearly with [FAD], yielding an apparent bimolecular association rate constant of 370 M\(^{-1} \) s\(^{-1} \). However, at higher FAD concentrations, \( k_{\text{obs,1}} \) becomes independent of [FAD], giving a limiting rate constant of 0.011 s\(^{-1} \) (Figure 32, inset). The observed rate constant of the slow phase, \( k_{\text{obs,2}} \), was independent of [FAD] over the range from 6 \( \mu \text{M} \) to 120 \( \mu \text{M} \). Figure 33 shows the absorbance change of FAD after mixing with excess of apoETF/AMP. The final FAD and apoETF/AMP concentrations are 3.6 \( \mu \text{M} \) and 19.8 \( \mu \text{M} \), respectively. The time course of the reaction shows a fast phase, with observed rate constant \( k_{\text{obs,1}} = 0.01409 \text{ s}^{-1} \), contributing the majority of the absorbance change, followed by a slow phase with observed rate constant \( k_{\text{obs,2}} = 0.00013 \text{ s}^{-1} \). The inset of Figure 33 shows that the observed rate constant of the fast phase varies linearly with the concentration of apoETF/AMP yielding an apparent bimolecular association rate constant of 365 M\(^{-1} \) s\(^{-1} \). The observed rate constant of the slow phase was independent of apoETF/AMP concentrations over the range from 6 \( \mu \text{M} \) to 45 \( \mu \text{M} \). These results suggest that the binding of FAD to W3A1 apoETF/AMP does not follow a simple second-order bimolecular process as described by scheme VI since a simple one-step binding mechanism would require that the approach to equilibrium be pseudo-first-order, e.g., the dependence of the observed rate constant on the concentration of either FAD or apoETF/AMP should
both be linear and the apparent bimolecular rate constants calculated from the concentration dependency when either FAD or apoETF in large excess should be equal, as defined by equation 5-2a and 5-2b. A common mechanism for ligand binding involves

$$\text{E} + \text{F} \xrightarrow{k_1} \text{EF}$$  \hspace{1cm} \text{Scheme VI}

$$k_{\text{obs}} = k_1 [\text{F}] + k_{-1}$$ \hspace{1cm} 5-2a

$$k_{\text{obs}} = k_1 [\text{E}] + k_{-1}$$ \hspace{1cm} 5-2b

fast binding followed by a slow ligand-induced protein conformation change as described by scheme VII. In this mechanism, if the two steps are well separated kinetically, the approach to equilibrium is a biphasic process under pseudo-first-order conditions. The concentration dependence of the observed rate constant of the fast phase will be linear as

$$\text{E} + \text{F} \xrightarrow{k_1 \text{E} + k_{-1}} \text{EF}$$  \hspace{1cm} \text{Scheme VII}

$$k_{\text{obs,1}} = k_1 [\text{F}] + k_1 + k_2 + k_2$$ \hspace{1cm} 5-3a

$$k_{\text{obs,2}} = \frac{k_1 [\text{F}](k_2 + k_2) + k_1 k_2}{k_1 [\text{F}] + k_1 + k_2 + k_2}$$ \hspace{1cm} 5-3b

defined by equation 5-3a (Johnson, 1992), and the concentration dependence of the observed rate constant of the slow phase should approximate a hyperbola as defined by
equation \( 5-3b \), with a limiting slope of the hyperbola at low ligand concentrations (Johnson, 1992). However, if the rate constants of the two steps, \( k_1 \) and \( k_2 \) are comparable, the two phases may not be well resolved. The determination of binding steps will totally rely on examination of the concentration dependence of the observed rates (Fersht, 1985). In either case, the dependence of the observed rate constants for binding, as described in scheme VII, on concentrations of either FAD or apoETF/AMP should behave in the same manner. Clearly, the data do not support the mechanism described by scheme VII. However, the data associated with the fast major absorbance change support a model involving a protein conformation change followed by ligand binding as described by scheme VIII. In this mechanism, if the two steps are well separated kinetically, the time dependence of the reaction may be biphasic and follow a double exponential. The concentration dependence of the fast and slow phases will be defined by equation \( 5-4a \) and \( 5-4b \), respectively (Johnson, 1992). The fast phase is due to the binding of F to E and the

\[
\frac{E^*}{k_1} = \frac{k_2[F]}{k_2} \frac{EF}{k_1} \quad \text{Scheme VIII}
\]

\[
k_{obs,1} = k_1 + k_{-1} + k_2 [F] + k_2 \quad 5-4a
\]

\[
k_{obs,2} = \frac{k_1(k_2 + k_2 [F]) + k_1k_2}{k_1 + k_{-1} + k_2 [F] + k_2} \quad 5-4b
\]

slow phase will be limited by the rate constant \( k_1 \) at high ligand concentrations; the apparent \( k_{on} \) is usually much lower than the diffusion limit which is typical for simple
bimolecular association processes (Johnson, 1992). Under certain circumstances, the two phases may not be well resolved if the rates of the two steps are comparable. Additional information regarding the mechanism of binding may be obtained by investigating the concentration dependence of the observed rates. A nonlinear behavior of the concentration dependence may suggest that more than one step is involved in the binding process (Fersht, 1985).

Although the data associated with the majority of the absorbance change support a protein conformation change before FAD binding, the very slow absorbance decrease associated with the second phase suggests an additional step. The observed rate of the second phase is independent of both FAD and apoETF/AMP concentrations, indicating a second conformation change after FAD binding. Based on these data, we have proposed a three-step mechanism for the binding of FAD to apoETF containing AMP, as described in scheme IX. Where E* and E represent two species of apoETF/AMP with different conformations, F represents FAD, EF and EF* represent holoETF proteins with different conformations. Based on this three-step mechanism, $k_1$ was calculated from the limiting rate achieved at high FAD concentrations as described in the inset of Figure 32. The rate of the second conformation change, $k_3$, was determined from the observed rates of the very slow absorbance change in the second phase, which depend on neither FAD nor
apoETF/AMP concentrations, consistent with a unimolecular process, e.g. a ligand induced isomerization step.

The second conformation change after FAD binding was confirmed by competitive binding studies as described in Figure 34. HoloETF protein was mixed with a large excess of ATP. The absorbance change of FAD with time was monitored. The high concentration of ATP inhibits the reassociation of FAD with ETF once dissociated. The time course of the absorbance change is biphasic and can be fitted to a double exponential. The rate of dissociation of FAD from holoETF, $k_2$, was determined to be $0.0002 \text{ s}^{-1}$ from the observed rate of the fast phase. The reverse rate of the second conformation change after binding, $k_3$, was determined to be $0.00004 \text{ s}^{-1}$ from the observed rate of the slow phase. The rates of both phases are independent of holoETF concentrations (Table 6), consistent with a unimolecular process. These results provided further evidence that the apoETF/AMP/FAD complex undergoes a conformation change after association, leading to the tighter binding. The nature of the conformation change needs to be further studied.

The determined rate constants, $k_1$, $k_3$, $k_2$, and $k_3$, can be fitted to the three-step mechanism described in scheme IX through computer simulation. The second-order binding constant, $k_2$, and the reverse rate constant of the first confirmation change before binding, $k_1$, were determined from the computer simulation when the simulated data match the experimental data, as shown in Figure 35 and Figure 36. All the rate constants, the equilibrium constants calculated from the determined rates, and the binding mechanism were summarized in Table 7.
$k_{obs,1} = 0.22 \pm 0.2(10^2 \text{s}^{-1})$

$k_{obs,2} = 0.04 \pm 0.01(10^2 \text{s}^{-1})$

Figure 34. Absorbance change of FAD after holoETF protein was mixed with large excess of ATP. The final concentration of holoETF protein and ATP was 25 $\mu$M and 0.4 mM, respectively. The absorbance change was monitored at 462 nm and 486 nm. The time course of the absorbance change is biphasic and can be fitted to double exponential. The fast phase reflects the dissociation of FAD and apoETF/AMP, with an observed rate of 0.00021 s$^{-1}$. The second slow phase may reflect a possible conformation change of ETF before the dissociation of FAD, with an observed rate of 0.00004 s$^{-1}$. 
Table 6. The Dissociation of FAD and ApoETF/AMP Is Independent of HoloETF Concentration

<table>
<thead>
<tr>
<th>[HoloETF] µM</th>
<th>[ATP] µM</th>
<th>$k_2$ $(10^{-4}s^{-1})$</th>
<th>$k_3$ $(10^{-3}s^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>160</td>
<td>2.2 ± 0.4</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>25</td>
<td>400</td>
<td>2.1 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>40</td>
<td>640</td>
<td>2.2 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 35. Computer simulation of kinetic data when FAD is in excess of apoETF/AMP. The simulation was carried out using the three-step mechanism described in Scheme IX and rate constants determined from experimental data. The squares represent the experimental data and the circles represent the simulated data. The concentration of FAD and apoETF/AMP was 77.6 and 6.0 μM, respectively.
Figure 36. Computer simulation of kinetic data when apoETF/AMP is in excess of FAD. The simulation was carried out using the three-step mechanism described in Scheme IX and rate constants determined from experimental data. The circles represent the experimental data and the squares represent the simulated data. The concentration of apoETF/AMP and FAD was 28.0 and 3.6 μM, respectively.
Table 7. Kinetic and Equilibrium Constants for Apparent Three-Step Binding of FAD to apoETF at 20°C

\[ E^* \rightleftharpoons E + F \rightleftharpoons EF \rightleftharpoons EF^* \]

<table>
<thead>
<tr>
<th></th>
<th>( k_1 ) (10^3 s(^{-1}))</th>
<th>( k_2 ) (10^3 ( \mu )M(^{-1})s(^{-1}))</th>
<th>( K_1 ) (10^3 ( \mu )M(^{-1}))</th>
<th>( k_2 ) (10^3 s(^{-1}))</th>
<th>( K_2 ) (( \mu )M(^{-1}))</th>
<th>( k_3 ) (10^3 s(^{-1}))</th>
<th>( K_3 ) (10^3 s(^{-1}))</th>
<th>( K_d ) (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.61 (±0.12)</td>
<td>2.20 (±0.44)</td>
<td>4.82 (±0.97)</td>
<td>1.65 (±0.07)</td>
<td>0.21 (±0.01)</td>
<td>7.86 (±0.50)</td>
<td>0.11 (±0.01)</td>
<td>0.04 (±0.01)</td>
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<td>2.75 (±0.73)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05 (±0.01)</td>
</tr>
</tbody>
</table>

a. Conditions: 50 mM KH\(_2\)PO\(_4\), pH 7.2, 0.3 mM EDTA. b. \( K_d \) was calculated from the rate constants \( k_2 \), \( k_3 \), and \( k_3 \). \( K_1 = k_1/k_2 \); \( K_2 = k_2/k_3 \); \( K_3 = k_3/k_3 \); \( K_d = 1/K_2 K_3 \).
The quenching of FAD fluorescence upon binding to apoETF/AMP was also studied by stopped-flow spectrofluorometry since additional steps may be resolved by using more than one physical probe (Fersht, 1985). In addition, we wanted to convince ourselves that no fast phases have been missed in the absorbance experiments when the stopped flow method was not used. Under conditions when FAD is in large excess over apoETF/AMP, the time-dependent quenching of FAD fluorescence during binding is biphasic and the data can be fitted to a double exponential as shown in Figure 37. The observed rate of the fast phase varies linearly with the FAD concentration as described in the inset. An apparent second-order rate constant of $1.64 \times 10^3 \text{M}^{-1}\text{s}^{-1}$ for binding was calculated from the slope of the line. The signal change of the slow phase is small and difficult to analyze. However, the observed rate constant of the slow phase seems to be independent of FAD concentration under these conditions. The dissociation rate, $k_2$, cannot be determined from the concentration dependence of the slow phase. Figure 38 shows the time dependence of FAD fluorescence quenching when FAD was mixed with large excess of apoETF/AMP. The time course has been fitted to a single exponential. The apoETF/AMP concentration dependence of the observed rate constant is linear as shown in the inset. An apparent bimolecular rate constant of $362 \text{M}^{-1}\text{s}^{-1}$ was calculated from the slope of the straight line. This value is consistent with the results from absorbance experiments. Apparently, under conditions when FAD is in large excess, the first conformation change and the binding step can be well resolved by monitoring the fluorescence change instead of the absorbance change. The second-order rate constant for
Figure 37. Kinetics of FAD fluorescence quenching upon mixing apoETF/AMP with excess of FAD. The final concentration of apoETF/AMP and FAD was 1.0 and 12.0 μM, respectively. The time dependence fluorescence quenching upon binding is biphasic under these conditions. The FAD concentration dependence of the observed rate constants of the fast phase defines a straight line as shown in the inset. A second-order rate constant of \(1.64 \times 10^3 \text{M}^{-1}\text{s}^{-1}\) for binding was calculated from the slope of the line. The signal change of the slow phase is too small to determine the rates accurately.
Figure 38. Kinetics of FAD fluorescence quenching upon mixing FAD with excess of apoETF/AMP. The final concentration of FAD and apoETF/AMP was 1.0 and 9.0 μM, respectively. The time dependence fluorescence quenching upon binding is monophasic under these conditions. The dependence of the observed rate constants on the concentration of apoETF was linear and shown in the inset. A second-order rate constant of 362 M$^{-1}$s$^{-1}$ for binding was calculated from the slope of the line.
binding, \( k_2 \), determined under such conditions was virtually identical to that calculated from the computer simulated data.

**HPLC analysis of ApoETF and HoloETF**—The conformations of apoETF containing AMP, holoETF, and the complex of apoETF with FAD or ATP were investigated using gel filtration HPLC analysis. The effluents were monitored by absorbance at 280 nm, as described in “Materials and Methods”. Figure 39A shows the elution profile of apoETF/AMP, which showed two major peaks with retention time of 15.5 min and 16.8 min, respectively. These two peaks cannot be explained by the dissociation of the two subunits of the apoETF/AMP since the two dissociated subunits should be eluted at longer retention times as estimated from the standard curve (Figure 40), assuming they are eluted normally in the column. However, the double peaks can be explained by that the apoETF/AMP exists in two different conformations, e.g. a loose conformation, with a retention time of 15.5 min and can be represented by E*, and a tight conformation with a retention time of 16.8 min and can be represented by E. The two minor peaks may be explained by partial denaturation of a small amount of the apoETF and possible dissociation of the two subunits, respectively. Figure 39B shows the elution profile of holoETF, which showed a major protein peak with a retention time of 16.8 min and a minor peak with a retention time of 26.9 min. The minor peak is contributed by a small amount of free FAD dissociated from ETF. Figure 39C is the elution profile of apoETF/AMP after incubating with FAD followed by dialysis. The elution profile shows a major protein peak with a retention time of the same as holoETF and a minor peak of
Figure 39. HPLC gel filtration analysis of apoETF/AMP. A Waters Protein Pak 300SW gel filtration column (30 cm) was used to analyze the conformation states of the apoETF/AMP. Gel filtration was carried out at 22-24°C in 50 mM potassium phosphate buffer, pH 7.2, 0.3 mM EDTA. The eluent was monitored by absorbance at 280 nm. Panel A: elution profile of apoETF containing AMP; Panel B: elution profile of holoETF; Panel C: elution profile of apoETF/AMP after incubating with an excess of FAD. Comparison of the elution patterns of apoETF/AMP and holoETF reveals that the apoETF/AMP exists in two major interconvertible conformations.
Figure 40. Standard curve of the HPLC gel filtration experiment. The molecular weight standards used are thyroglobulin (670,000), γ-globulin (150,000), BSA (67,000), egg albumin (45,000), ovalbumin (43,000), bovine carbonic anhydrase (29,000), horse myoglobin (17,000), and vitamin B₁₂ (1,350). Standard curve was generated by plotting the retention times versus log of molecular weights of the standard proteins (circle) and fitting to a straight line. The molecular weights of the two major species of apoETF/AMP (square) were estimated to be 65,000 and 95,000, respectively, with the first species close to the molecular weight of the holoETF determined by mass spectrometry and the deduced amino acid sequences.
FAD which has been dissociated from the protein. These results indicate that the apoETF/AMP protein exists in two major species, with one species having the same conformation as the holoETF, while the other species having a relatively "loose" conformation which resulted in a shorter retention time in the gel filtration chromatography. The species of loose conformation can be converted to the tight conformation by incubating with FAD (Figure 39C) or ATP (data not shown). These observations are consistent with our kinetic model. Only the species with the tight conformation can bind to FAD. After cofactor binding, the tight conformation was stabilized. And the binding of FAD drives the equilibrium to the direction which forms the tight conformation. These results also indicate that the association of the two ETF subunits does not require the binding of FAD. The nature of the two conformation states need to be further studied.

Although the mammalian and the W3A1 ETFs share only ~30% amino acid sequence identity, and the redox and fluorescence properties of the bound flavin cofactor are markedly different between these proteins, the results of our kinetic studies match in several ways the results obtained in the mammalian system. Two conformational states of pig kidney apoETF were also observed by Sato et al. in their studies of FAD binding to the apoETF by monitoring FAD fluorescence increase upon binding (1991). The rate constants for the binding was evaluated from the slopes of semilogarithmic, first-order rate plots. A two-step binding mechanism involving slow protein conformation change followed by FAD binding was proposed for this mammalian ETF protein based on the
kinetic data and HPLC gel filtration analysis of the apoETF protein. However, some differences exist between our observations. These include that an additional step of conformation change after binding needs to be introduced in the W3A1 ETF to explain the data adequately. In addition, the bimolecular rate constant in the mammalian ETF ($k_2 = 4 \times 10^4$ M$^{-1}$s$^{-1}$) seems to be much larger than that of W3A1 ETF although a much lower reaction temperature (7°C) was used in their studies. Future investigation on the structural basis of the conformation change will provide further information on the binding mechanism and on how the binding alters the redox property of the FAD cofactor.
CHAPTER VI

CONCLUSION AND PROPOSAL FOR FUTURE STUDIES

The structural genes encoding the two different subunits of the electron transfer flavoprotein from the methylotrophic bacterium W3A1 have been cloned, sequenced, and heterologously expressed in *E. coli*. The deduced amino acid sequences of W3A1 ETF have about 30% sequence identities with ETF proteins from *Paracoccus* and mammalian sources which are much more similar to one another and share more than 50% sequence identities. The less homologous W3A1 ETF seems to be consistent with the unique properties of this electron transfer flavoprotein. A strikingly high level of homology exists near the COOH-terminus of the large subunit of all the ETF proteins and the *fixB* putative gene products. Several features are very similar to the consensus dinucleotide-binding motif suggesting that this portion of the large subunit may be involved in FAD or AMP binding. The ETF subunits are expressed and assembled in *E. coli* to form the fully functional holoprotein heterodimer containing noncovalently bound FAD as an essential cofactor. The recombinant protein displays physical, spectral, and electron-accepting properties from TMADH that are very similar to the wild-type ETF isolated from W3A1. Independent expression of the two subunit genes form protein preparations which lack cofactor binding and electron transferring activities. The amino acid sequence information
for W3A1 ETF subunits and its heterologous expression should facilitate the interpretation of its crystal structure and provide for future structure/function and cofactor/protein interaction studies.

Like the mammalian and wild-type W3A1 ETF proteins, the recombinant W3A1 ETF expressed in *E. coli* contains a tightly bound AMP molecule in addition to the FAD cofactor. Both the FAD and the AMP cofactors can be removed from the recombinant ETF protein by guanidine hydrochloride treatment. Fully functional ETF holoprotein can be reconstituted by adding back both the FAD and the AMP. The yield of the reconstitution reaction is ~ 25%. However, reconstitution in the absence of AMP cannot be achieved, indicating the AMP molecule plays an important structural role and is an integral part of W3A1 ETF protein. The FAD cofactor can also be removed by potassium bromide treatment, producing apoETF retaining the AMP. Fully functional ETF can be reconstituted by adding back the FAD. The yield of the reconstitution reaction is ~ 50%. The dissociation constant between FAD cofactor and apoETF/AMP is determined to be ~ 10^{-8} M. The binding of FAD to apoETF/AMP is competitively inhibited by ATP, ADP and AMP in decreasing effectiveness. The apparent inhibition constants of ATP, ADP, and AMP were determined to be 1.3 \times 10^{-7} M, 9.8 \times 10^{-6} M, and 2.0 \times 10^{-5} M, respectively. The apparent inhibition constant of ATP is only 5-fold greater than that for FAD, indicating the FAD binding site contains a well formed dinucleotide subsite and that the adenyl nucleotide portion contributes the majority of the binding energy in FAD binding. This conclusion is supported by the weak or lack of binding of FMN. Photoaffinity
labeling studies involving the reconstitution of ETF using the photoaffinity ATP analog 8-N$_3$-ATP resulted in the preferential labeling of the small subunit. The labeling was effectively inhibited by the presence of FAD or ATP. UV-induced crosslinking of ETF reconstituted with 5'-[³²p]AMP also led to the radiolabeling of the small subunit. These results suggest that the small subunit of W3A1 ETF composes at least part of both the ATP/FAD and the AMP binding sites. Kinetic data for the association of FAD with apoETF/AMP are consistent with a three-step mechanism. There are slow conformation changes before and after the binding step. The rate constants for each step were determined and the equilibrium constants were calculated. Gel filtration analysis of apoETF/AMP suggests that the apoETF/AMP exists in an equilibrium between more than one conformations.

The cloning, expression of W3A1 ETF genes and the well defined reconstitution system have laid the foundation for structure and function studies of this interesting flavoprotein. Future studies of W3A1 ETF can be carried out in the following areas.

*Structural studies*— Large amounts of recombinant ETF proteins will assist in solving the X-ray crystal structure. The amino acid sequences deduced from the cloned W3A1 ETF genes will also aid in determination of the tertiary structure. The expression system has been successfully used to produce seleno-methionine substituted W3A1 ETF proteins, which will be helpful in solving the phasing problem during crystallographic studies. In the absence of specific structural information from crystallographic studies, a variety of biochemical, genetic, and computer graphic approaches may be used to develop
possible models for cofactor/protein interaction and electron transfer. Elucidation of the
dynamic aspect near the redox center and the possible conformation changes of FAD
cofactor during the redox reaction and the protein environment of the bound cofactor in
W3A1 ETF are crucial to understanding how the unusual redox potential is achieved and
how the semiquinone radical is stabilized. Conformational features of the cofactor at
different oxidation states and the protein environment can be studied using isotopic
substitutions within the cofactor as probes and NMR spectroscopy. Although the
isotopically labeled FAD cofactor can be obtained by combined chemical and enzymatic
synthesis, the yields of the products is very low and the purification procedure is very
tedious (Müller, 1991). The heterologous expression system of W3A1 ETF can be used
to produce large amount of pure isotopically labeled FAD. One just needs to supplement
the growth medium with simple isotope-labeled compound and let the *E. coli* do the
synthesis. The over expressed ETF, which has been labeled in both protein and cofactor,
is purified and the FAD cofactor is separated from the protein by potassium bromide
treatment. The isotopically labeled FAD can then be used to reconstitute with apoETF
protein without labeling. This reconstituted ETF, which contains the isotopically labeled
FAD cofactor, can then be studies by NMR spectroscopy to detect possible conformation
changes during its oxidation and reduction and to probe the nature of the environment
immediately around the protein-bound flavin.

Amino acid residues located in the cofactor binding site can be identified by
chemical modification and mutagenesis of the protein. ApoETF proteins can be
chemically modified using various reagent specific for different amino acid side chains. The reconstitution and binding of the modified apoETF with FAD cofactor can then be investigated. If the modification results in a loss of binding or altered binding affinity, then it is possible that the amino acid concerned is a component of the cofactor binding site. Of course, it is also possible that the loss of binding is due to a change in tertiary structure resulting from a modification to an amino acid residue not present at the cofactor binding site. However, the two situations may be distinguished by carrying out the modification in both apoETF and holoETF. The cofactor in holoETF may protect its binding site from being modified. Random and site-specific mutagenesis can be performed on the cloned W3A1 ETF genes. The site-specific mutagenesis will be directed to the highly homologous regions such as the C-terminal region of the large subunit. Mutant ETF proteins with altered cofactor binding affinity can be selected and the structural basis of the mutagenic effects on cofactor binding can be investigated. Amino acid residues directly interacting with the FAD and AMP cofactors can also be identified using affinity labeling combined with peptide mapping and amino acid sequencing. The 8-N₃-ATP labeled W3A1 ETF small subunit, as described in Chapter IV, can be isolated and digested with an appropriate protease such as trypsin. The peptide(s) containing the labeling can then be sequenced and the labeled amino acid residue may be identified. ATP and FAD analogs bearing the azide group or other reactive groups at various different positions of the cofactor molecule could be used to identify residues which make contact with the different part of the cofactor molecule. We have demonstrated that 8-N₃-AMP cannot bind
to W3A1 apoETF protein. Other affinity analogs of AMP, such as the ones with the reactive group attached to the ribose ring or the different position of the adenosine moiety, may be more effective in the labeling of the AMP binding site. Finding the AMP binding site and altering the interaction between AMP and ETF are significant since this type of study may answer such questions as whether the AMP molecule plays any role in regulating the redox potential of the FAD cofactor in addition to its more possible structural role.

Thermodynamics of electron transfer— After the FAD binding site has been identified, the availability of the cloned ETF genes and the heterologous expression system should make it possible to create various amino acid residue substitutions in the cofactor binding site. The well defined reconstitution system can be used to produce holoETF protein containing various FAD analogs modified on different positions on the isoalloxazine ring, such as deazaflavins. The effects of these modifications, either in cofactor or in the cofactor binding site, on the redox potential of the W3A1 ETF protein can then be investigated. Information obtained from these studies will lead to insight into the unusual redox properties of this interesting flavoprotein. The understanding of how apoETF protein exerts control over the redox and chemical properties of the bound flavin will make it possible to specifically alter reduction potentials by suitable modification of proteins or cofactor. The shift in redox potential arising from these modifications should be accurately predictable from theoretical calculations.
Kinetics of electron transfer—Although thermodynamic studies are fundamental in establishing the direction and equilibrium position of the electron transfer, kinetic studies are required to solve the mechanism of the reaction. The W3A1 ETF exhibits high specificity in its reduction by TMADH but not by other dehydrogenases which are thermodynamically suitable as its electron donors, suggesting the ETF protein may be designed to provide a high kinetic barrier against accepting electrons from the incorrect donors while simultaneously maintaining a low kinetic barrier for accepting electrons from the correct donor. In addition, the W3A1 ETF seems to cycle between the fully oxidized and the one-electron reduced semiquinone form during catalysis, although the redox potential for the second electron transfer is not unusually negative, suggesting a kinetic barrier for the second electron transfer. The nature of this kinetic barrier may be understood by studying the rate of association, dissociation, and electron transfer between TMADH and ETF, and by studying the structural features of the donor-acceptor complex. Factors, such as ionic strength and pH, on the rate of electron transfer between TMADH and ETF should also be studied. Most importantly, kinetic studies should be carried out using various modified ETF proteins generated by site-directed mutagenesis, ETF proteins reconstituted with various modified cofactors, as well as mutant TMADH proteins. The influence of these modifications on the rate of electron transfer will be investigated. These studies will help to establish the mechanism and pathways of electron transfer and to answer questions such as whether there are any well-defined electron conduction pathways
in the TMADH and ETF proteins and what is the structural basis of the kinetic barriers for the incorrect electron transfer.

**Specificity of electron transfer**— Discrimination between correct and incorrect redox partners appears to be mediated by complementary structural interactions that can be satisfied by a correct pair of proteins (Rees and Farrelly, 1990). These complementary structural features can be identified by random mutagenesis, deletion mutations. The energetics of association and disassociation between these mutant electron donor and acceptors can be evaluated. Since genes encoding ETF proteins with different electron donor and acceptor specificities are available, chimeric proteins can be designed and synthesized using recombinant DNA techniques. Chimeric ETF proteins with altered specificity can then be studied to identify functional domains involved in the donor or acceptor docking. Other approaches to identify functional domains interacting with electron donor and acceptor involve limited protease digestion of ETF protein and cross-linking studies using bifunctional reagents. Once domains containing the electron donor or acceptor docking site are identified, site-directed mutagenesis can then be performed to address issues of how changes in amino acid sequence in various domains alter the electron transfer specificity.

These studies will provide new insights into the roles of protein structure in controlling the redox properties of the bound flavin cofactor, molecular recognition, electron donor/acceptor specificity, and rates in the biological electron transfer processes. Knowledge obtained from these studies can be applied to designing novel electron transfer
proteins with new redox properties, developing more efficient solar cells, and designing molecular electronic devices. The ability to design novel electron transfer proteins will in turn provide the ultimate test of our understanding of both protein structure and electron transfer mechanisms.
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