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Binding of the Human "Electron Transferring Flavoprotein" (ETF) to the Medium Chain Acyl-CoA Dehydrogenase (MCAD) Involves an Arginine and Histidine Residue

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The interaction between the "electron transferring flavoprotein" (ETF) and medium chain acyl-CoA dehydrogenase (MCAD) enables successful flavin to flavin electron transfer, crucial for the β-oxidation of fatty acids. The exact biochemical determinants for ETF binding to MCAD are unknown. Here we show that binding of human ETF, to MCAD, was inhibited by 2,3-butanedione and diethylpyrocarbonate (DEPC) and reversed by incubation with free arginine and hydroxylamine respectively. Spectral analyses of native ETF *vs* modified ETF suggested that flavin binding was not affected and that the loss of ETF activity with MCAD involved modification of one ETF arginine residue and one ETF histidine residue respectively. MCAD and octanoyl-CoA protected ETF against inactivation by both 2,3-butanedione and DEPC indicating that the arginine and histidine residues are present in or around the MCAD binding site. Comparison of exposed arginine and histidine residues among different ETF species, however, indicates that arginine residues are highly conserved but that histidine residues are not. These results lead us to conclude that this single arginine residue is essential for the binding of ETF to MCAD, but that the single histidine residue, although involved, is not.

Keywords: Electron transferring flavoprotein; 2,3-Butanedione; Diethylpyrocarbonate; Chemical modification

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DEPC, diethylpyrocarbonate; ETF, electron transferring flavoprotein; ETF-QO, ETF ubiquinone oxidoreductase; GAD, general chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; TMADH, trimethylamine dehydrogenase; PMS, phenazine methosulphate

INTRODUCTION

The "electron transferring flavoprotein" (ETF) is localized to the mitochondrial matrix and is the physiological electron acceptor for at least nine mitochondrial matrix dehydrogenases involved in the β-oxidation pathway,[1–4] amino acid catabolism[5–7] and choline catabolism.[8] ETF:ubiquinone oxidoreductase (ETF-QO) catalyses the re-oxidation of reduced ETF and further transports the electrons to the electron transport chain.[9] ETFs are FAD-containing proteins that have been isolated from both mammalian and bacterial sources[10–16] and all are heterodimeric, with an α and β subunit, possessing considerable sequence and structural homology to one another.[17,18] All ETFs except the *Megasphaera elsdenii* protein possess one molecule of FAD per heterodimer, with the ETFs from human, pig, *Paracoccus denitrificans* and *Methylophilus methylotrophus* also reported to contain one molecule of AMP.[17–20] The physiological function of ETF is an important one, a deficiency of which results in the severe metabolic disorder glutaric aciduria type II (GAII).[9] A recent report has also suggested that ETF expression is increased in colorectal cancer.[21]

The precise biochemical requirements for the binding of ETF with the medium chain acyl-CoA dehydrogenase (MCAD) are still largely unknown, although previous reports have suggested some electrostatic bonding is involved. Trinitrophenylation

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of two porcine ETF lysine residues decreased binding with the general acyl-CoA dehydrogenase (GAD) by 75%[22] but did not increase the turnover number with ETF-QO,[23] suggesting that lysine residues are merely involved and probably not essential for the binding of ETF to GAD. On the other hand, modification of MCAD carboxyl groups, with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) in the presence of taurine and glycine ethyl esters, decreased its binding to porcine ETF by 90–96%.[24] Recently, a cysteine residue was located near or within the MCAD binding site on the ETF surface.[25]

Human *ETF* cDNA and *MCAD* cDNA have been cloned and the crystal structures of the recombinant proteins solved. Since successful flavin to flavin electron transfer must involve positioning of the flavins in relative close proximity, we hypothesized that amino acid residues surrounding the flavin moiety could be involved in the binding of human ETF to MCAD. Examination of the crystal structure has revealed approximately ten surface arginine residues and six surface histidine residues, several of which are in or around the flavin binding site. Furthermore, mutation of one of these surface arginine residues, βR164Q, is found in patients suffering from GAII.

Since arginine and histidine residues are important for protein–protein and protein–substrate interactions[26–30] and since lysine residues do not appear to be essential in the above ETF interaction with MCAD, but that retention of ETF positive charge was, we hypothesized that arginine and histidine residues may be essential for the interaction of ETF with MCAD. Using chemical modification, we present evidence to show that an exposed ETF arginine residue is essential for the interaction with MCAD but that an exposed histidine residue only contributes partially to the binding of ETF to MCAD. Using this information we propose a location of at least part of a putative MCAD binding site on ETF.

EXPERIMENTAL PROCEDURES

General Materials

2,3-Butanedione, 2,6-dichlorophenolindophenol (DCPIP), diethylpyrocarbonate (DEPC), octanoyl-coenzyme A, phenazine methosulphate (PMS) and riboflavin were purchased from Sigma Chemical Company, UK. Ampicillin and adenosine were supplied by Boehringer Mannheim, UK. Tryptone and yeast extract were obtained from Oxoid, Unipath Ltd., UK. Borate buffers were made according to Gomori.[31] All other chemicals were of the highest grade available.

Transformation and Expression of Human *ETF* and *MCAD* cDNAs in Bacteria

The human *MCAD* cDNA, in a pBluescript based vector (Stratagene, Cambridge, UK) and the human *ETF* cDNA, also in a pBluescript based vector, were generous gifts from Dr Peter Bross (University of Aarhus, Denmark). For transformation, *ETF* cDNA (10 ng) and *MCAD* cDNA (5 ng) were added to 50 μl of competent *Escherichia coli* TG1 cells (*supE hsdΔ5 thiΔ* (*lac-proAB*) *F'*[*traD36 proAB* + *lacI^q lacZ ΔM15*]) and incubated on ice for 10 min. The samples were then chemically transformed at 42°C for 2 min, incubated at 37°C for 1 h and then selected for on LB/ampicillin (100 μg/ml) agar plates. Selected colonies were grown at 37°C according to Bedzyk *et al.*[32] in LB medium supplemented with ampicillin (100 μg/ml), riboflavin (1 mg/l) and adenosine (1 mg/l). For induction of protein expression, iso-β-propylthiogalactoside (IPTG) was added to a final concentration of 1 mM for ETF expression and 0.1 mM for MCAD expression at an A600 of 0.4–0.8 OD units. The cultures were incubated for a further 6–12 h respectively and the cells harvested by centrifugation.

Purification of Human Recombinant ETF and MCAD Proteins

Recombinant human ETF was purified to homogeneity by chromatography on DEAE cellulose 52 and CM-Sephadex essentially as described by Husain and Steenkamp.[11] The ratio of absorbance at 280 nm to 450 nm (A280:A450) was 6.3 and SDS gel electrophoresis indicated approximately 99% purity with no visible contaminating bands of protein. Recombinant human MCAD protein was purified as described by Thorpe.[33] The A280:A450 ratio was 5.6 and SDS gel electrophoresis indicated >95% purity with no visible contaminating bands of protein.

Assay for ETF Activity with MCAD

ETF binding to MCAD was determined spectrophotometrically using a UVIKON 941 Dual Beam Spectrophotometer (Kontron Instruments, Herts, UK) to monitor the reduction of DCPIP ($\varepsilon_{600} = 21,000 \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$). The assay mixture for following changes in ETF activity during chemical modification contained 40 mM borate buffer, pH 7.6, 30 μM octanoyl-CoA, 37.5 μM DCPIP and 0.16 μM MCAD. The reaction mixtures were incubated at 25°C for 10 min before the addition of the ETF sample. Controls contained no chemical modification agent and no ETF. For protection studies MCAD and octanoyl-CoA were omitted from the assay solutions since the amounts used for protection with the ETF sample were adequate.

Assay for MCAD Activity

The activity of MCAD was measured using the dye-linked assay as previously reported.[33] The assay mixture for following MCAD modification contained 40 mM borate buffer, pH 7.6, 300 μM EDTA, 5% glycerol, 30 μM octanoyl-CoA, 37.5 μM DCPIP and 1.4 mM PMS. The reaction mixtures were incubated at 25°C for 10 min before the addition of the MCAD sample. Controls contained no chemical modification agent and no MCAD. For protection studies octanoyl-CoA was omitted from the assay solution.

Chemical Modification of ETF and MCAD by 2,3-butanedione and DEPC

ETF (0.9 μM) and MCAD (0.9 μM) in 160 mM borate buffer, pH 8.3, were incubated separately with specified concentrations of 2,3-butanedione at 25°C in the dark. Aliquots were withdrawn at intervals and assayed for appropriate activity as described. The control incubations contained no modification reagent. For modification with specified concentrations of DEPC the reaction took place in 160 mM borate buffer, pH 6.3, at 25°C in the dark. Timed aliquots were corrected for the rate of composition of DEPC (k', see below) according to the equation: Corrected time (t') $= [1 - \exp^{(k't)}]/k'$, where t is the time of withdrawal.[34]

Rate of DEPC Decomposition

Histidine (2.5 mM) and DEPC (2.5 mM) were incubated separately in 160 mM borate buffer, pH 6.3, in the dark at 25°C. At time intervals, 0.5 ml samples of each reactant were mixed together and the resulting absorbance at 242 nm was determined. From a plot of absorbance at 242 nm versus time the rate of decomposition of DEPC (k') was calculated.

Spectral Analyses of ETF Modification by 2,3-butanedione and DEPC

Spectrophotometric measurements were carried out using a UVIKON 941 Dual Beam Spectrophotometer. ETF (7.6 μM) in 160 mM borate buffer, pH 8.3, was incubated with and without 50 mM 2,3-butanedione for 70 min respectively. The reaction mixtures were dialyzed extensively against 160 mM borate buffer, pH 8.3, at 4°C before the spectra were recorded. For the formation of N-carbethoxyhistidine by modification with DEPC, ETF (4.3 μM) in 160 mM borate buffer, pH 6.3, was incubated with or without 10 mM DEPC for 30 min respectively. The rate of *N*-carbethoxyhistidine formation per mole of ETF was calculated using $\varepsilon_{242} = 3,200\,M^{-1}\,cm^{-1}$,[35] the concentration of ETF flavin $\varepsilon_{436} = 13,300\,M^{-1}\,cm^{-1}$ and the molecular mass of ETF was 60 kDa.[36] The absorption spectra of 2,3-butanedione-modified ETF and DEPC-modified ETF were compared to native unmodified ETF from 250–800 nm. In parallel experiments the residual ETF activity was determined as described in "Material and Methods Section".

Reversibility of 2,3-butanedione and DEPC Modified-human ETF Activity with MCAD

Reactivation of ETF (10 μM) activity after treatment with 50 mM 2,3-butanedione was attempted by 2-fold dilution of the ETF-2,3-butanedione mixture with 1 M arginine (pH adjusted to 8.3, in 160 mM borate buffer). For reactivation from DEPC inactivation, DEPC modified-ETF (1.9 μM), after treatment with 10 mM DEPC for 11 min, was incubated with 200 mM hydroxylamine (in 160 mM borate buffer, pH 7.0) for 4 h at 25°C. The reaction was then dialysed extensively against 160 mM borate buffer, pH 6.3. Controls without 2,3-butanedione or DEPC or reactivation reagents were run concurrently. For all reactions, aliquots were taken at specified time intervals and the ETF enzyme activity with MCAD was determined.

Substrate Protection of MCAD against Inactivation by 2,3-butanedione and DEPC

MCAD (3.8 μM) was pre-incubated for 5 min with 0.29–0.67 mM octanoyl-CoA in 160 mM borate buffer, pH 8.3 or pH 6.3, at 25°C in the dark before addition of 50 mM 2,3-butanedione or 10 mM DEPC respectively. Aliquots were taken at specified time intervals and the MCAD activity was determined as described. Controls contained MCAD in the absence of octanoyl-CoA and MCAD in the absence of inactivating agents.

Use of Substrate-protected MCAD to Protect ETF against Inactivation by 2,3-butanedione and DEPC

ETF (3.4 μM) was pre-incubated with MCAD (4.5 μM) and octanoyl-CoA (0.95–1.5 mM) in 160 mM borate buffer, pH 8.3 or pH 6.3, for 5 min before the addition of 50 mM 2,3-butanedione or 10 mM DEPC respectively. Aliquots were taken at specified time intervals and the enzyme activity was determined as described. Controls contained ETF in the absence of MCAD and octanoyl-CoA, and ETF in the absence of inactivating agents.

Native PAGE and Western Blot Analysis to Monitor ETF Conformational Changes

ETF (7.6 μM) in 160 mM borate buffer, pH 8.3, was incubated with and without 50 mM 2,3-butanedione for 70 min respectively and for DEPC modification,

ETF (4.3 μM) in 160 mM borate buffer, pH 6.3, was incubated with or without 10 mM DEPC for 30 min respectively. The reaction mixtures were dialyzed extensively against 160 mM borate buffer, pH 8.3, at 4°C before application to the native polyacrylamide gel. The electrophoresis of native ETF and modified ETF on non-denaturing polyacrylamide gels were performed according to Parker and Engel.[37] The gel resolved ETF samples were transferred onto nitrocellulose and detected using a rabbit anti-human ETF antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin.[37]

Measurement of Protein Concentrations

The concentrations of ETF and MCAD were determined by using $\varepsilon_{436} = 13,300\,M^{-1}\,cm^{-1}$ [11] and $\varepsilon_{445} = 15,400\,M^{-1}\,cm^{-1}$ [33] respectively.

Data Presentation

All results reported are the average of at least three independent determinations.

RESULTS

Inactivation of ETF Activity with MCAD by 2,3-butanedione and DEPC

ETF was incubated with various concentrations of 2,3-butanedione and DEPC. Figure 1 shows that maximal inactivation of ETF activity with MCAD was achieved by 50 mM 2,3-butanedione (compare lane A to B, $p < 0.002$ (paired t-test)) and 10 mM DEPC (compare lane A to C, $p < 0.01$ (paired t-test)) with 89% and 35% inactivation occurring after 70 min and 3 min respectively. No more activity was lost if increased concentrations of the reagents were used or if the reactions were incubated for longer periods of time. Activity of the ETF untreated control was stable under these conditions. Using both reagents, the initial reactions were approximately linear with respect to time implying both reactions followed pseudo-first-order kinetics but then progressed to completion.

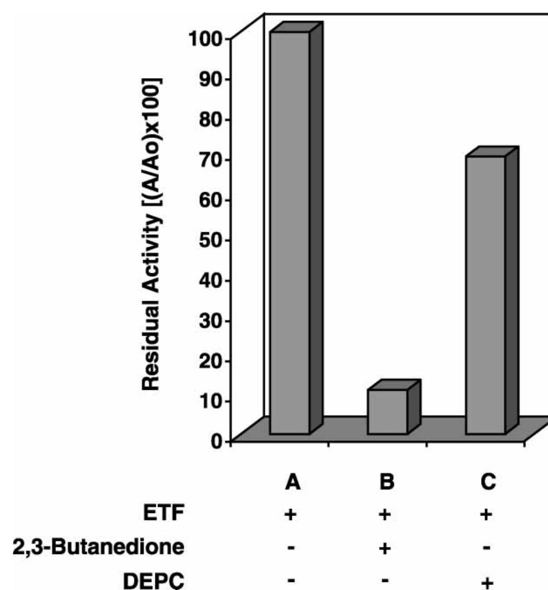

FIGURE 1 *Effect of arginine-specific and histidine-specific agents on ETF binding to MCAD*. ETF (0.9 μM) was routinely modified at 25°C, in the dark, in 160 mM borate buffer, pH 8.3, with 10–50 mM of 2,3-butanedione or in 160 mM borate buffer, pH 6.3, with 10 mM DEPC. Aliquots were withdrawn at intervals and assayed for ETF activity as described in "Experimental Procedures". The control incubations contained no modification reagent. Timed aliquots were corrected for the rate of composition of DEPC (k′) according to the equation: Corrected time $(t') = [1 - \exp^{(k't)}]/k'$, where t is the time of withdrawal.[34] The experiments were performed three times. The figure represents the result from one typical experiment and shows the final maximal percentage of ETF inactivation (residual activity $[(A/Ao) \times 100]$) alone (lane A), after treatment with 50 mM 2,3-butanedione (lane B) and after treatment with 10 mM DEPC (lane C).

Spectral Analyses of ETF Inactivation by 2,3-butanedione and DEPC

The absorbance spectrum of ETF treated with 50 mM 2,3-butanedione in 160 mM borate buffer, pH 8.3, is shown in Figure 2A. When ETF was treated with 50 mM 2,3-butanedione (for 70 min: 11% residual activity) the absorption spectra from 250–380 nm increased with the A280:A450 absorption ratio increasing from 6.3 to 8.8 (Figures 2A and 2B, $p < 0.03$ (paired t-test)). Modification of lysine residues by 2,3-butanedione involves an increase in absorbance at 530 nm, however, no absorbance change was observed at this wavelength (Figure 2B). There was no change of absorbance in the long-wavelength region or in the absorbance of the flavin environment at 450 nm implying that inactivation was probably due to modification of ETF at a site other then the flavin binding site. A similar increase in absorption at 280 nm was observed by Makinen *et al.*[38] using 100 mM 2,3-butanedione to photo-inactivate *Aeromonas* aminopeptidase. The difference in the spectra may be indicative of the formation of the dehydroxyimidazole derivative-borate complex.[39]

Modification of ETF with DEPC led to the characteristic formation of *N*-carbethoxyhistidine measured at 242 nm[34,35] (Figures 3A and 3B, $p < 0.001$ (paired t-test)). Although the ETF interaction with MCAD decreased 35% after 3 min incubation with DEPC, the formation of *N*-carbethoxyhistidine continued for up to 30 min. Modification of tyrosine residues by DEPC involves a change in absorbance at 280 nm, however, no absorbance change was observed at this wavelength (Figure 3B).

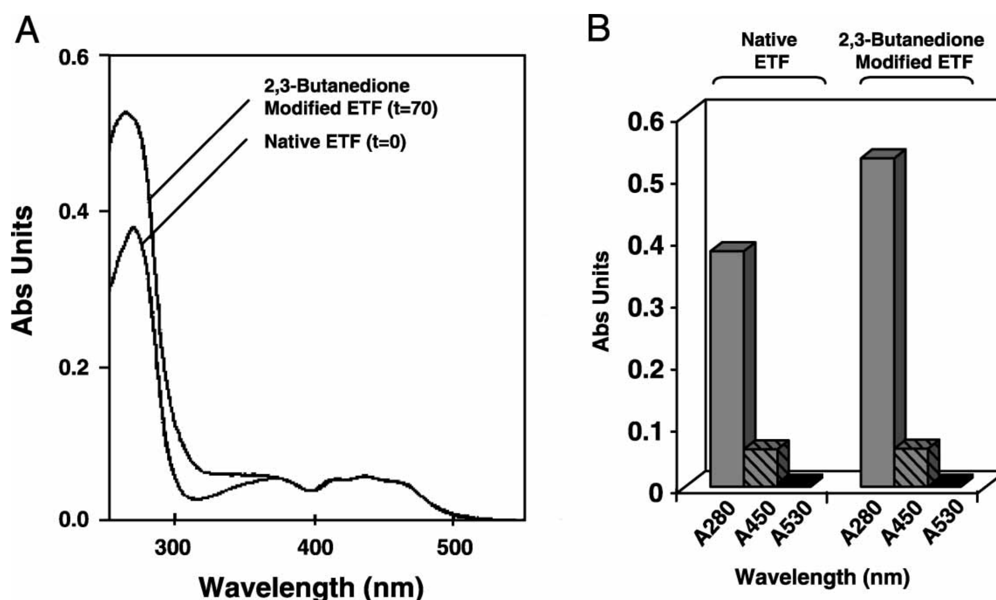

FIGURE 2 *Spectral analysis of ETF modification by 2,3-butanedione*. (A) ETF (7.6 μM) in 160 mM borate buffer, pH 8.3, was incubated with and without 50 mM 2,3-butanedione for 70 min respectively. The reaction mixtures were dialysed extensively against 160 mM borate buffer, pH 8.3, at 4°C before the spectra were recorded. In a parallel experiment the residual ETF activity was determined. The absorption spectra of 2,3-butanedione-modified ETF was compared to native ETF from 250–800 nm. The experiments were performed three times and the figure represents one typical experiment. (B) Graph to represent data obtained from spectral analysis of 2,3-butanedione modified ETF versus native ETF.

Inhibition of ETF Binding to MCAD, by Modification, does not Involve any ETF Conformational Change

The spectral data suggests that the mechanism of modification does not involve perturbation of the ETF flavin. Modification of an ETF arginine and ETF histidine may however cause adverse conformational changes in the ETF protein and thus could account for the lack of binding to MCAD. In fact the pathogenic mutation αG116R has been shown to effect folding of the ETF polypeptides.[41] Modification of ETF with either 2,3-butanedione or DEPC did not affect the mobility of the protein on a non-denaturing gel, when compared to the mobility of the wild-type protein, suggesting that there is no overall adverse conformational changes to the ETF protein during either modification procedures (data not shown).

Stoichiometry of 2,3-butanedione and DEPC Mediated ETF Inactivation

From a semilogarithmic plot of percentage residual activity remaining (A/Ao) versus time, k_{inact} values for different 2,3-butanedione concentrations (10–50 mM) were calculated[40] (Figure 4). The pseudo-first-order rate constants were approximately proportional to 2,3-butanedione concentrations resulting in a linear logarithmic–logarithmic plot (Figure 4: inset) with a slope $(n) = 0.85$. This strongly suggested that inactivation of ETF binding to MCAD by 2,3-butanedione involved modification of one arginine residue per MCAD binding site active on ETF. This does not of course preclude further reactions at other sites that were not involved in activity.

As shown from the spectral analysis, the production of *N*-carbethoxyhistidine was monitored at 242 nm (Figure 3A). After 11 min of incubation with 10 mM DEPC, there was no further loss of ETF activity and 4 moles of *N*-carbethoxyhistidine per mole of ETF histidine residues were formed (Figure 5). However, it can be clearly seen that after 1 min the vast majority of ETF activity with MCAD was lost corresponding to the modification of one histidine residue per ETF heterodimer.

ETF Inactivation by 2,3-butanedione and DEPC is Reversible

Modification with the arginine-specific reagent 2,3-butanedione and histidine-specific reagent DEPC strongly implied the presence of a reactive arginine residue and histidine residue on the human ETF surface which are part of the MCAD binding site or close enough to the site so that modification blocks or perturbs it. This was further supported by using 1 M arginine (in 160 mM borate buffer, pH adjusted to 8.3) to reverse the inactivation of 2,3-butanedione-modified ETF (11% residual activity, 70 min) as shown in Figure 6. Approximately 87% of the original activity was restored after 25 min incubation

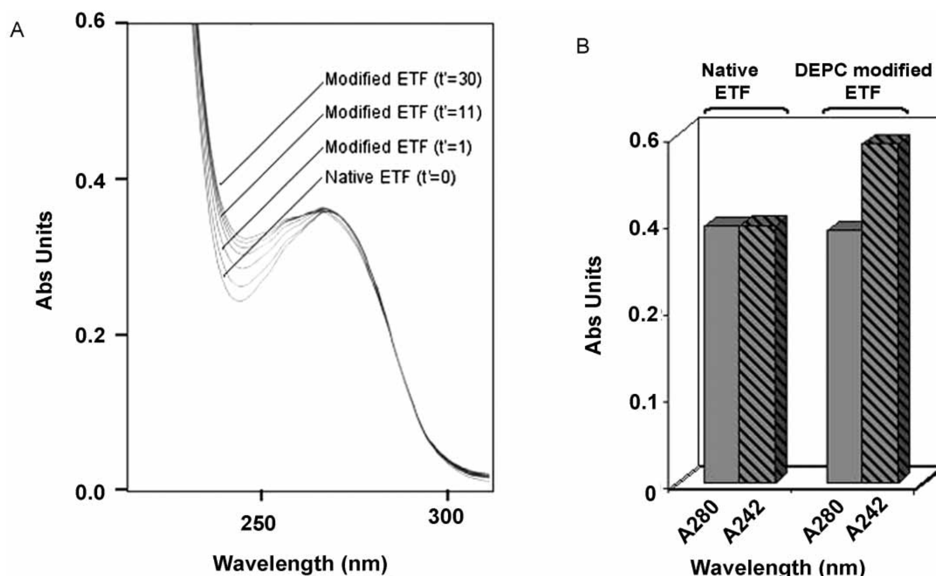

FIGURE 3 *Spectral analysis of ETF modification by DEPC*. (A) ETF (4.3 μM) in 160 mM borate buffer, pH 6.3, was incubated with and without 10 mM DEPC for 30 min respectively. In a parallel experiment the residual ETF activity was determined. The absorption spectra of DEPC-modified ETF was compared to native ETF from 250–800 nm. The experiments were performed three times and the figure represents one typical experiment. (B) Graph to represent data obtained from spectral analysis of DEPC modified ETF versus native ETF.

(compare lane A to B, $p < 0.02$ (paired t-test)). Figure 6 also shows that after treatment of DEPC-modified ETF (65% residual activity after 11 min incubation) with 200 mM hydroxylamine for four hours, ETF activity was restored to almost 80% of its original activity (compare lane C to D, $p < 0.009$ (paired t-test)). With both reactions, increasing the concentration of reactivating reagents or time of incubation did not increase the amount of reactivation.

Protection of MCAD and ETF against Modification by 2,3-butanedione and DEPC

If 2,3-butanedione and DEPC modify an arginine and histidine residue involved in the ETF recognition site for MCAD then it should be possible to protect against the modification by including MCAD in the reaction mixture. These experiments are complicated, however, by the facts that, (i) MCAD is involved in the subsequent assay for residual ETF activity, and (ii) that porcine MCAD itself is susceptible to inactivation by modification of an arginine residue[42] and may also be susceptible to DEPC inactivation. Accordingly it was necessary to protect MCAD by inclusion of octanoyl-CoA.

After 40 min incubation with 50 mM 2,3-butanedione, 75% inactivation of MCAD was achieved (Figure 7A, compare lane A to B, $p < 0.02$ (paired t-test)). Pre-incubation with octanoyl-CoA (0.29 mM) strongly protected MCAD from inactivation (Figure 7A, compare lane B to C, $p < 0.02$ (paired t-test)). Similarly, MCAD was inactivated after incubation with DEPC (50% inactivation after 70 min) and pre-incubation with octanoyl-CoA (0.67 mM) fully protected MCAD from DEPC inactivation (Figure 7A, compare lane D to E, $p < 0.02$, and lane E to F, $p < 0.01$ (paired t-test)). No further loss of activities was observed when incubated for longer periods of time.

Protection of ETF against modification by 2,3-butanedione and DEPC was achieved by pre-incubation with MCAD and octanoyl-CoA (1.5 mM for 2,3-butanedione and 0.95 mM for DEPC) (Figure 7B, compare lane A to B and lane B to C, $p < 0.02$, and compare lane D to E and lane E to F, $p < 0.04$ (paired t-test)). No protection was achieved using octanoyl-CoA alone without MCAD (data not shown). Again this provided excellent evidence for the presence of an arginine residue and histidine residue in or around the MCAD binding site.

DISCUSSION

Summary of Results with 2,3-butanedione

ETF binding to MCAD decreased 89% upon incubation with 50 mM 2,3-butanedione indicating that arginine residues were involved in the interaction. Since 2,3-butanedione is highly specific for arginine modification[43] and due to its size, steric hindrance is probably not an issue, it was assumed that the loss of ETF activity with MCAD correlated with specific modification of ETF arginine residues only. This is further supported by, (1) the absence of any significant lysine modification indicated by the lack of absorption increase at 530 nm (Figure 2B),

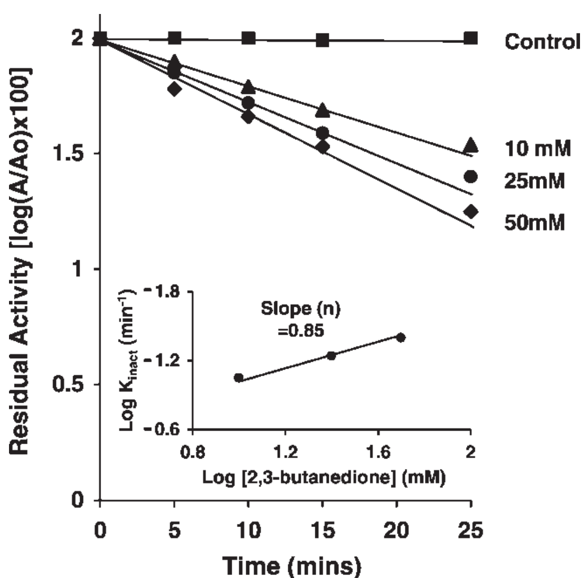

FIGURE 4 *Kinetics of ETF inactivation by 2,3-butanedione*. ETF (3.4 μM) was incubated in 160 mM borate buffer, pH 8.3, at 25°C, in the dark, with 10–50 mM 2,3-butanedione. Aliquots were taken and assayed for residual ETF activity [(A/Ao) × 100] with MCAD as described in "Experimental Procedures". Inset: plot of logarithmic observed pseudo-first-order-rate constants (k_{inact}) versus logarithmic 2,3-butanedione concentrations. Slope (n) equals the number of arginine residues modified. The experiments were performed three times and the figure represents one typical experiment.

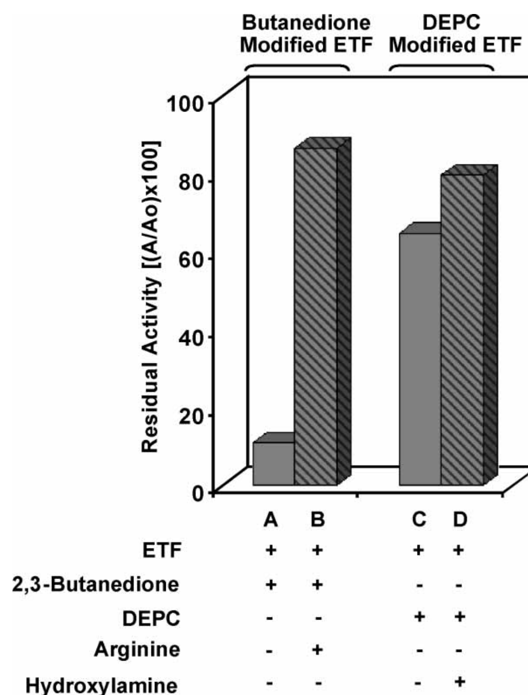

FIGURE 6 *Reversibility of ETF inactivation*. Reactivation of ETF (10 μM) after treatment with 50 mM 2,3-butanedione at 25°C in the dark (70 min, 11% residual activity) was attempted by 2-fold dilution of the ETF-2,3-butanedione mixture with 1 M arginine (pH adjusted to 8.3, in 160 mM borate buffer). For reactivation from DEPC (10 mM) inactivation, DEPC modified ETF (1.9 μM, 65% residual activity after 11 min) was incubated with 200 mM hydroxylamine, pH 7.0, for 4 h at 25°C. The reaction was then dialyzed extensively versus 160 mM borate buffer, pH 6.3. Controls without 2,3-butanedione, DEPC or reactivation reagents were run concurrently. Aliquots were taken and assayed for residual ETF activity [(A/Ao) × 100] with MCAD as described in "Experimental Procedures". The experiments were performed three times and the figure represents the final percentages of residual ETF activities remaining from one typical experiment.

(2) that the activity of modified-ETF was almost completely reversed with exogenous arginine suggesting that mostly arginine residues were modified (Figure 6), (3) that spectral analyses of 2,3-butanedione-modified ETF showed increased absorption from 250–380 nm only, suggesting the specific formation of the arginine derivative dehydroxyimidazole-borate,[38] and (4) no overall adverse conformational changes in modified ETF were detected by native PAGE. Initial inactivation of ETF by 2,3-butanedione obeyed pseudo-first-order kinetics and indicated that modification of one essential arginine residue was responsible for the loss of 89% of ETF activity with MCAD. Upon incubation with octanoyl-CoA protected MCAD, ETF was fully protected from modification by 2,3-butanedione providing excellent evidence for the presence of this single essential arginine residue in or around the binding site for MCAD.

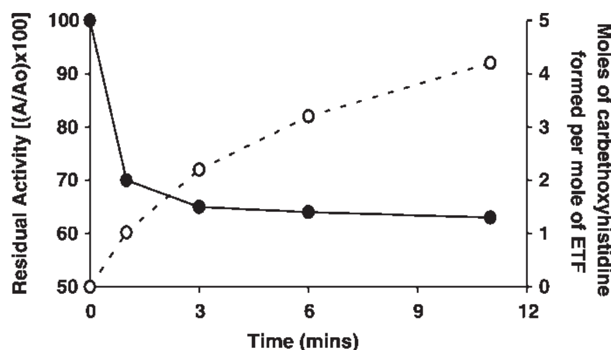

FIGURE 5 *Correlation of ETF inactivation by DEPC with increased N-carbethoxyhistidine formation*. ETF (4.3 μM) in 160 mM borate buffer, pH 6.3, was incubated with 10 mM DEPC for 11 min. The absorption spectrum of DEPC-modified ETF was compared to native ETF at 242 nm as described above. At time intervals the residual ETF activity was also determined. The experiments were performed three times and the figure represents one typical experiment.

Summary of Results with DEPC

Incubation with 10 mM DEPC resulted in the maximum amount of ETF-histidine modification leading to the maximal amount of inhibition of ETF binding with MCAD. DEPC has been reported to modify lysine, tyrosine and cysteine residues but at near slightly acidic and neutral pH, the reaction of DEPC is relatively specific for histidine.[35] Four further observations strongly indicated that the modification was specific for histidine residues only: (1) Modification of cysteine and lysine residues with DEPC is not reversible with hydroxylamine but

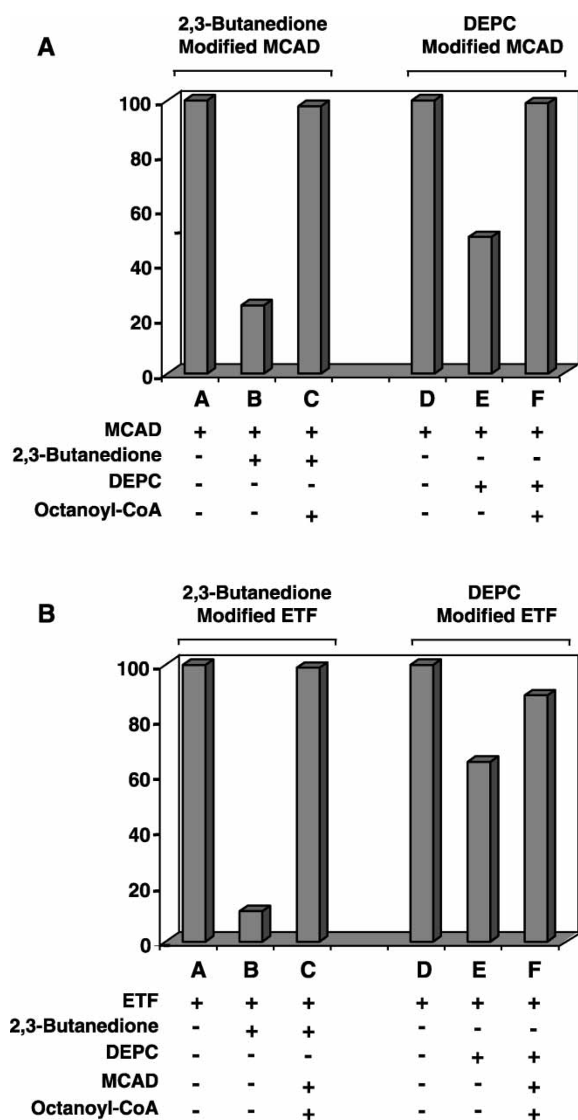

FIGURE 7 *ETF and MCAD inactivation by 2,3-butanedione and DEPC can be protected*. (A) MCAD (3.8 μM) was pre-incubated for 5 min with 0.29 mM octanoyl-CoA or 0.67 mM octanoyl-CoA respectively in 160 mM borate buffer, pH 8.3 or pH 6.3, 25°C in the dark before addition of 50 mM 2,3-butanedione or 10 mM DEPC. Aliquots were taken at specified time intervals and the MCAD activity was determined as described in "Experimental Procedures Section". The experiments were performed three times and the figure represents the final percentage of residual MCAD activity remaining from one typical experiment. Lane A, no 2,3-butanedione; Lane B, with 50 mM 2,3-butanedione; Lane C, pre-incubation with 0.29 mM octanoyl-CoA before addition of 50 mM 2,3-butanedione; Lane D, no DEPC; Lane E, with 10 mM DEPC; Lane F, pre-incubation with 0.67 mM octanoyl-CoA before addition of 10 mM DEPC. (B) ETF (3.4 μM) was pre-incubated alone (lane A); with 50 mM 2,3-butanedione (lane B); with 4.5 μM MCAD and 1.5 mM octanoyl-CoA before the addition of 2,3-butanedione (lane C); alone (lane D); with 10 mM DEPC (lane E) or with 4.5 μM MCAD and 0.95 mM octanoyl-CoA before the addition of 10 mM DEPC (lane F). Aliquots were taken at specified time intervals and the enzyme activity was determined as described in "Experimental Procedures Section". The experiments were performed three times and the figure represents the final percentages of residual ETF activities remaining from one typical experiment.

in this study partial reactivation of DEPC modified ETF was achieved with hydroxylamine (Figure 6), (2) the increase in absorbance at 242 nm over the first 3 min of the reaction, indicative of formation of *N*-carbethoxyhistidine, correlated well with the decrease in ETF activity with MCAD (Figure 5), (3) the absence of any absorbance change at 280 nm indicates no *0*-carbethoxytyrosine was formed (Figure 3B), and (4) there appeared to be no overall adverse conformational changes in modified ETF as detected by native PAGE. The lack of total recovery of ETF activity by hydroxylamine treatment could have been due to the reaction of two molecules of DEPC per histidine residue followed by a ring opening that irreversibly destroys the imidazole ring.[35] The majority of ETF inactivation by DEPC involves modification of one histidine residue and of course does not preclude reaction at other sites with three more histidines modified after 11 min that are not required for activity. Similar to arginine modification, ETF was protected from modification by DEPC, by pre-incubation with octanoyl-CoA protected MCAD, providing excellent evidence for the presence of the single histidine residue in or around the binding site for MCAD. It is also possible that the histidine (and arginine) residues are involved in an induced conformational change that is needed for electron transfer or directly involved in electron transfer.

Identification of Potential Arginine Residues

The interaction of arginine with 2,3-butanedione requires that the guanidino group must be exposed. Using the crystal structure we have identified approximately ten arginine residues exposed on the ETF surface (Table I, Parker, A.R. and Engel, P.C.– Unpublished data). All ten are highly conserved among several different species of ETF. Two of the arginine residues lie in the FAD binding domain, αR223 and αR249. αR249 has been proposed to stabilize the anionic semiquinone and may function in the electron transfer pathway.[18] This is homologous to αR237 in *M. methylotrophus* which if mutated to alanine results in impaired assembly of the electron transfer complex with TMADH.[44] The absorption spectra for the mutant αR237A, however, is different to that of wildtype ETF with a shift in the flavin region (438 nm to 446 nm). The absorbance spectra of 2,3-butanedione modified human ETF (Figure 2A) only shows an increase in the UV region, indicative of protein modification, suggesting that arginine modification may not occur with arginine residues involved in FAD binding.

TABLE I Conservation of 10 exposed arginine residues amongst various species of ETF

	α-subunit					β-subunit				
	122	146	169	223	249	21	76	164	191	233
Human	R	R	R	R	R	R	R	R	R	R
M. elsdenii	R	R	R	R	R	K	R	Q	R	R
P. denitrificans	R	R	R	R	R	K	R	R	R	R
W3A1	R	R	R	R	R	E	R	R	R	G
B. japonicum	R	R	R	R	R	R	R	R	R	R
C. thermosacch	R	R	R	R	R	–	R	R	R	R
B. subtillus	K	R	R	R	R	K	R	R	R	K
N. menningtidus	–	–	–	–	–	R	R	R	R	R

The interaction of ETF and MCAD has been modelled[18] allowing electrons to pass from MCAD at the *si* side of the FAD ring since kinetic studies have also demonstrated that the enoyl-CoA product is present, "blocking" the *re* side, when MCAD binds ETF.[45] There are at least thirteen salt bridges and several hydrogen bonds formed between one ETF molecule and the MCAD dimer and for efficient electron transfer, the most likely ETF:MCAD complex would be an assembly that minimizes the distance between the two isoalloxazine rings of the flavin cofactors. This interaction would involve mainly domains II (β-subunit) and domain III (C-terminal of α-subunit/small C-terminal portion of β-subunit) and probably not domain I (N-terminal of α-subunit).[18] This would therefore suggest that αR122, αR146 and αR169 are probably not involved in the binding of ETF to MCAD. One other arginine residue that appears exposed on the ETF surface is βR164 which also resides at the α/β subunit interface and is frequently mutated in patients suffering from GAII. A good candidate could also be βR21 which is near to the FAD binding site of which βY16 is a part of.[18]

Identification of Potential Histidine Residues

There are seven histidine residues in ETF, six of which are exposed on the ETF surface (Table II). As is the case with arginine modification, DEPC modification of ETF does not disturb any absorbance surrounding the flavin environment suggesting that may be αH286 is not modified. The histidine residues αH28 and αH81 are present in domain II of ETF and similar to several arginine residues may therefore be non-essential for MCAD docking.[18] As with βR21 above, βH36 may be a good candidate as it is close to the FAD binding site but not directly involved in FAD binding. It is also close to a residue, βF41, that is involved in FAD binding. Using previous results from our laboratory,[25] it is tempting to speculate that βH36 or βH88, βR21 or βR76, and βC66 or βC71 may be part of the MCAD binding site on ETF. It must also be borne in mind that conformational changes could occur upon binding (like *M. methylotrophus* W3A1), which could decrease the distance between the two FADs further, and would possibly increase the number of interactions between the two molecules.

TABLE II Lack of conservation of 6 exposed histidine residues amongst various species of ETF

	α-subunit				β-subunit	
	28	81	88	286	36	88
Human	H	H	H	H	H	H
M. elsdenii	Q	D	G	P	N	L
P. denitrificans	V	D	G	H	M	L
W3A1	H	G	G	H	Y	R
B. japonicum	H	G	G	H	M	L
C. thermosacch	Q	N	G	H	S	L
B. subtillus	I	D	G	H	W	L

It might be expected that important residues required for successful docking of ETF and MCAD would be conserved from species to species or at least that the charge at such positions would be maintained. Comparison of corresponding histidine residues in six other ETF species show, that with the exception of αH286 which resides in the FAD site, that there is little conservation between histidine residues. Infact, αH81 and αH242 on the α-subunit and βH36 and βH88 on the β-subunit are not even histidine residues in six other ETF species. When comparing the protein sequence from other species of ETF it is noteworthy that the interaction of ETF with its dehydrogenase in W3A1 may not be electrostatic thus charge may not be as important[46] and that ETF from *M. elsdenii* possesses an acidic pI with more aspartic and glutamic acid residues than mammalian ETF. Nevertheless, it can be concluded that histidine residues are probably non-essential in the binding of ETF to MCAD and that maintenance of the positive charge would therefore only involve substitution of an arginine residue with a lysine residue.

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