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5,5′-DITHIOBIS-(2-NITROBENZOIC ACID) AS A PROBE FOR A NON-ESSENTIAL CYSTEINE RESIDUE AT THE MEDIUM CHAIN ACYL-COENZYME A DEHYDROGENASE BINDING SITE OF THE HUMAN 'ELECTRON TRANSFERRING FLAVOPROTEIN' (ETF)

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Human 'electron transferring flavoprotein' (ETF) was inactivated by the thiol-specific reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The kinetic profile showed the reaction followed pseudo-first-order kinetics during the initial phase of inactivation. Monitoring the release of 5-thio-2-nitrobenzoate (TNB) showed that modification of 1 cysteine residue was responsible for the loss of activity. The inactivation of ETF by DTNB could be reversed upon incubation with thiol-containing reagents. The loss of activity was prevented by the inclusion of medium chain acyl-CoA dehydrogenase (MCAD) and octanoyl-CoA. Cyanolysis of the DTNB modified-ETF with KCN led to the release of TNB accompanied presumably by the formation of the thio-cyano enzyme and with almost full recovery of activity. Conservation studies and the lack of 100% inactivation, however, suggested that this cysteine residue is not essential for the interaction with MCAD.

Keywords: Electron transferring flavoprotein; Human; Chemical modification; 5,5′-dithiobis-(2-nitrobenzoic acid)

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INTRODUCTION

The 'electron transferring flavoprotein' (ETF) is the physiological electron acceptor for at least 9 mitochondrial matrix dehydrogenases[1] including 4 straight-chain acyl-CoA dehydrogenases (short, medium, long and very long), involved in the β-oxidation pathway,[2,3] as well as several dehydrogenases involved in amino acid and choline catabolism.[4–7] Re-oxidation of reduced ETF is catalysed by the membrane-bound ETF : ubiquinone oxidoreductase (ETF-QO) which shuttles the electrons to the electron transport chain.[8] ETF deficiency results in the severe metabolic disorder glutaric aciduria type II,[9] reflecting the important physiological function of this protein.

Chemical modification studies of carboxylate groups and lysine residues in the porcine medium chain acyl-CoA dehydrogenase (MCAD)[10] and porcine ETF[11] have suggested the interaction between the two involves, in part, electrostatic forces. The interaction of ETF with 3 other flavoproteins, dimethylglycine dehydrogenase and sarcosine dehydrogenase and ETF-QO, also appears to be of an electrostatic nature (Ref. [12], Parker and Engel – unpublished data). The detailed determinants involved in the interaction of ETF with its dehydrogenase partners are, however, at present unknown.

Chemical modification is one of the main approaches available for probing the active-site residues of enzymes, and so, to investigate residues involved in binding MCAD, the cysteine-specific reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)[13–15] was used. This paper presents evidence for the presence of a non-essential cysteine residue located at the MCAD binding site.

METHODS AND MATERIALS

(A) Materials

2,6-Dichlorophenolindophenol (DCPIP), DTNB, dithiothreitol (DTT), β-mercaptoethanol, octanoyl-coenzyme A (octanoyl-CoA), phenazine methosulphate (PMS) and potassium cyanide (KCN) were purchased from Sigma Chemical Company UK. All other chemicals were of the highest grade available.

(B) Methods

Transformation and Expression of Cloned MCAD and ETF Genes

The hMCAD gene, in a pBluescript-based vector transformed into *E. coli* TG1 cells and the hETF gene, in a pBluescript-based vector, were generous gifts from Dr. Peter Bross (University of Aarhus, Denmark).

For transformation, the vector containing the hETF gene (40 ng) was added to 200 µl of competent *E. coli* TG1 cells and incubated on ice for 10 min. The cells were heat shocked at 42°C for 2 min. 600 µl of medium was added and the mixture was incubated at 37°C for 1 h. The mixture was grown on agar plates containing 100 µg/ml ampicillin for detection of transformants. The selected *E. coli* cells were grown at 37°C according to Bedzyk *et al.*[16] on a medium containing tryptone (16 g/l), yeast extract (16 g/l), NaCl (5 g/l), K_2HPO_4 (2.5 g/l), riboflavin (1 mg/l), adenosine (1 mg/l) and ampicillin (100 mg/l). The temperature for growth was 37°C. Isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM for ETF expression and 0.1 mM for MCAD, and the cultures were incubated at 37°C for a further 6 or 12 h respectively. The cells were pelleted by centrifugation and stored frozen at −20°C.

Purification of Human MCAD and ETF

Recombinant human MCAD was purified according to Thorpe[17] with slight modifications. The $A_{280}:A_{450}$ ratio was 5.6 and SDS polyacrylamide gel electrophoresis indicated > 95% purity with no visible contaminating bands of protein. Recombinant human ETF was purified by chromatography on DEAE-cellulose 52 and CM-Sephadex essentially as described by Husain and Steenkamp.[18] The $A_{280}:A_{450}$ ratio was 5.5 and SDS polyacrylamide gel electrophoresis indicated again > 95% purity with only the two ETF bands visible.

Assay of ETF Activity

The ETF assay was performed at 25°C in 50 mM potassium phosphate, pH 7.6 containing 0.3 mM EDTA and 5% glycerol. Enzyme activity was determined by following the reduction of DCPIP through measuring the decrease in absorbance at 600 nm. The assay mixture (0.8 ml) contained 30 µM octanoyl-CoA, 37.5 µM DCPIP and 158 nM hMCAD and was incubated at 25°C for 10 min before addition of ETF. For protection studies MCAD and octanoyl-CoA were omitted from the assay mixture as they would be transferred together with the ETF sample in suitable amounts for the activity measurement.

Assay of MCAD Activity

The MCAD assay was performed at 25°C in 50 mM potassium phosphate pH 7.6 containing 0.3 mM EDTA and 5% glycerol. Enzyme activity was determined by the rate of reduction of DCPIP at 600 nm. The assay mixture

(0.7 ml), containing 30 μM octanoyl-CoA, 37.5 μM DCPIP, 0.3 mM EDTA and 1.4 mM PMS, was incubated at 25°C before aliquots of MCAD were added. For the protection studies octanoyl-CoA was omitted from the assay mixture as it was present in the sample to be assayed.

Reaction of ETF and MCAD with DTNB

ETF (1.27 μM) and MCAD (12.7 μM) separately in 80 mM HEPES buffer, pH 8.0 at 25°C in the dark were incubated with specified concentrations of DTNB. Samples were assayed periodically as described. The release of thionitrobenzoate (TNB) was followed at 412 nm when 0.1–2 mM DTNB was added to ETF in 80 mM HEPES buffer pH 8.0. Quantitative measurements of DTNB incorporation per mole of ETF were made by taking the ratio of concentrations of released TNB and of native enzyme. The concentration of TNB was determined by using an absorbance coefficient of $13\,600\ M^{-1}\,cm^{-1}$ at 412 nm.[19] The molecular mass of ETF was taken as 60 kDa.[20] Controls containing no DTNB were run concurrently.

Measurement of Protein Concentration

The concentrations of ETF and MCAD were calculated by using the extinction coefficients of $13.5\ mM^{-1}\,cm^{-1}$ at 436 nm[18] and $15.4\ mM^{-1}\,cm^{-1}$ at 445 nm[17] respectively.

RESULTS AND DISCUSSION

Inactivation and Stoichiometry of ETF Activity with DTNB

Figure 1 shows that ETF modification by the thiol-specific reagent DTNB in 80 mM HEPES buffer, pH 8.0, led to a loss of activity towards MCAD, with 38% inactivation occurring after 32 min reaction with 2 mM DTNB. The reaction was dependent upon DTNB concentration. A linear relationship between time of incubation with DTNB and residual activity was obtained during the initial phase of the reaction, implying pseudo-first-order kinetics, but then progressed to completion. Using 0.5–2 mM DTNB the loss of ETF activity correlated well with the modification of 5 moles of cysteine residues per mole of ETF as determined from the increase in absorbance at 412 nm (Table I). No loss of activity towards MCAD was observed using 0.1 mM DTNB even though at this concentration 4 moles of cysteine residues per mole of ETF were still modified. It is noteworthy that porcine ETF was reported to contain no reactive cysteine residues.[21]

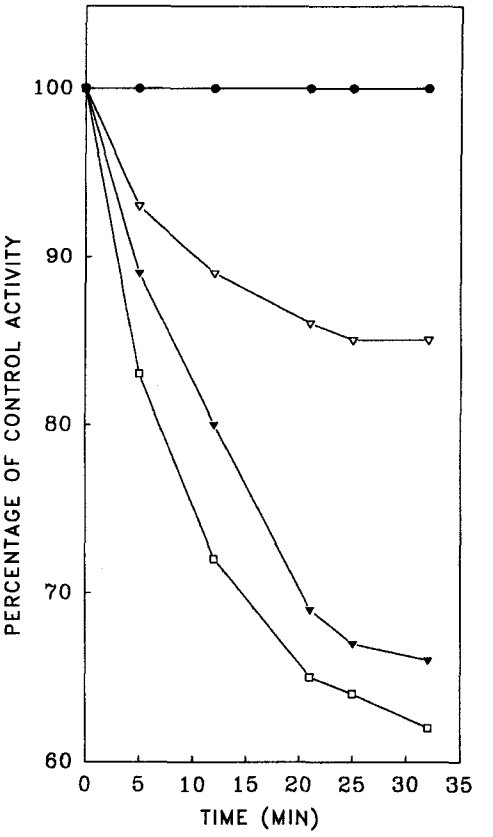

FIGURE 1 Time course of inactivation of ETF using DTNB. ETF (1.27 μM) in 80 mM HEPES buffer pH 8.0 was incubated with 0.1 mM (●), 0.5 mM (▽), 1 mM (▼) and 2 mM (□) DTNB at 25°C in the dark. Aliquots were withdrawn at indicated time intervals and the residual activity determined as described in "Methods".

TABLE I Number of cysteine residues modified per molecule ETF versus DTNB concentration

DTNB concentrations (mM)	*No. of cysteine residues modified*	*% Residual activity*
0.1	4	98
0.5	5	85
1.0	5	67
2.0	5	65

ETF (1.27 μM) was incubated in 80 mM HEPES buffer pH 8.0 with specified concentrations of DTNB at 25°C in the dark. Aliquots were withdrawn and assayed for residual activity as described. The release of TNB was followed at 412 nm when 0.1–2 mM DTNB was added to ETF (3.8 μM) in 80 mM HEPES buffer pH 8.0. Quantitative calculations were made by taking the ratio of concentrations of TNB released and of the native enzyme.

Effect of Thiol-Containing Reagents on DTNB-Inactivated ETF

The results of modification with the cysteine-specific reagent DTNB strongly implied the presence of several reactive cysteine residues in human ETF, of which one cysteine residue is either part of the active site or close enough that modification blocks or perturbs it. This was further supported by using the thiol-containing reagents β-mercaptoethanol and DTT (50 mM) which reversed the inactivation of DTNB-treated ETF (12.8 μM) as shown in Figure 2. Reactivation was rapid, with approx. 95% of the original activity restored after 3–6 min incubation. The results indicated that covalently bound TNB was displaced by an excess of smaller thiol-containing reagents as indicated by an increase in absorbance at 412 nm.

Protection of ETF Against Modification by DTNB

When treated with 1 mM DTNB for 32 min MCAD lost no activity (Figure 3) when assayed in the PMS and ETF system, in agreement with previous studies,[22] although 1 mole of cysteine was modified per mole of MCAD as indicated by an increase in absorbance at 412 nm. Although MCAD did not lose any activity when reacted with DTNB, for reaction with ETF, it was necessary to reduce it first with the inclusion of octanoyl-CoA. ETF (1.27 μM) was fully protected against inactivation by 1 mM DTNB when pre-incubated for 5 min with 12.8 μM MCAD and 0.3 mM

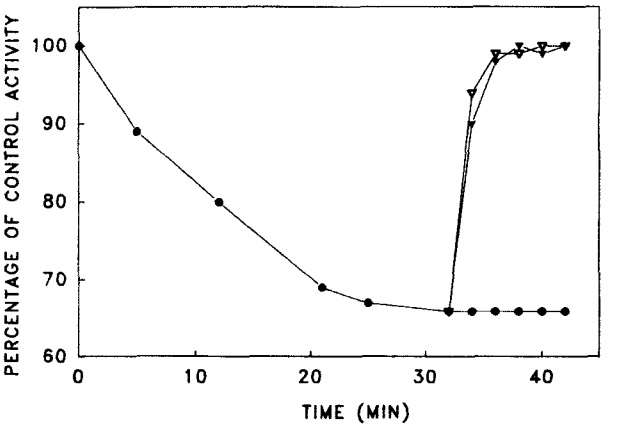

FIGURE 2 Reactivation of ETF by thiol-containing reagents. ETF (12.8 μM) was modified with 1 mM DTNB for 32 min (residual activity 68%) (●). To the solution were added 20 mM β-mercaptoethanol (▼) and DTT (▽). Aliquots were taken at the indicated time intervals and diluted 10-fold with 80 mM HEPES buffer pH 8.0. The ETF activity was determined as described in "Methods". A control without modifier was run concurrently.

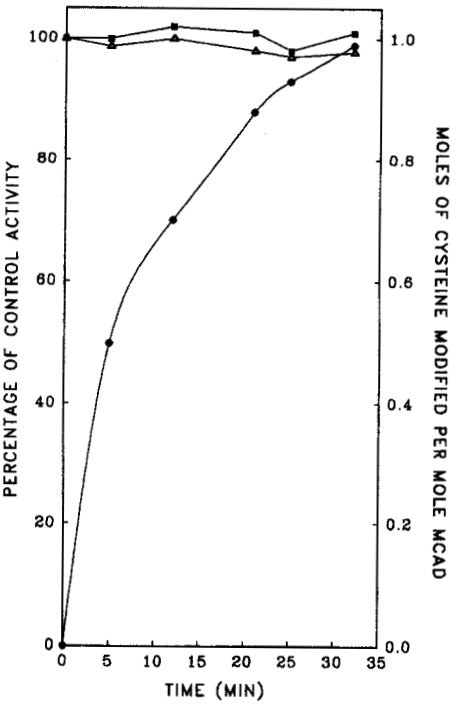

FIGURE 3 Modification of MCAD by DTNB. MCAD (12.8 μM) was incubated in 80 mM HEPES buffer pH 8.0 with 1 mM DTNB at 25°C in the dark. Aliquots were withdrawn and assayed for enzyme activity in the ETF (■) and PMS (△) assays as described. The release of TNB was followed at 412 nm and quantitative measurements of DTNB incorporation per mole of MCAD (●) was determined by taking the ratio of concentrations of TNB-release and the native enzyme.

octanoyl-CoA. This provided excellent evidence for the presence of a cysteine residue in or around the MCAD binding site of ETF.[23]

Investigation of Involvement of Cysteine Residue at the MCAD Binding Site of ETF

Chemical modifications of proteins using bulky reagents such as DTNB may in fact occur because of steric hindrance, thus implying that the cysteine residues modified are not genuinely essential in the sense of direct involvement in the binding of proteins or substrates.[13–15] Figure 4(a) shows the effect of cyanolysis (addition of 50 mM KCN) on the activity of DTNB-modified ETF (12.8 μM). Approx. 95% of the control activity was restored after 5–10 min. Monitoring the reaction at 412 nm showed the displacement of the TNB-moiety by the smaller functional group CN (Figure 4(b)).

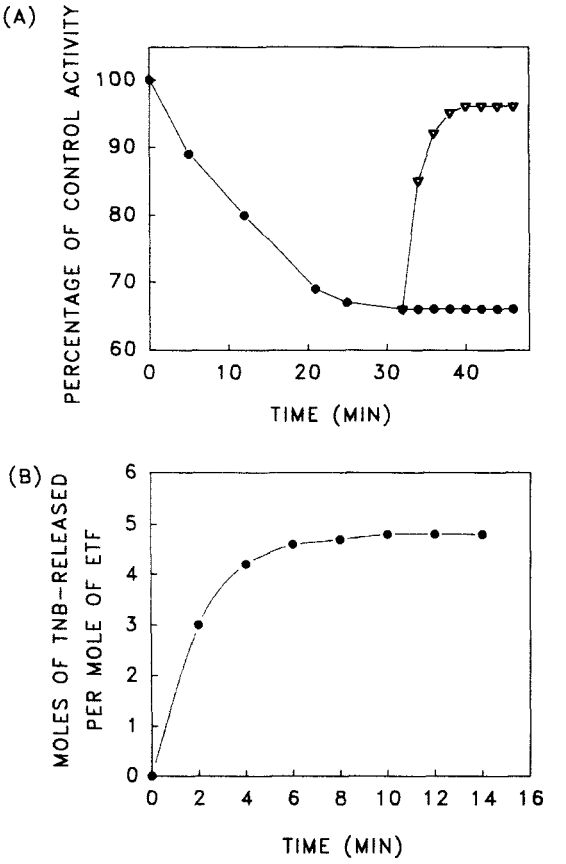

FIGURE 4 Effect of cyanolysis on DTNB-inactivated ETF. (A) ETF (12.8 μM) was modified with 1 mM DTNB for 32 min (residual activity 68%) (●). To the solution was added 50 mM KCN (▽). Aliquots were taken at the indicated time intervals and diluted 10-fold with 80 mM HEPES buffer pH 8.0. The ETF activity was determined as described in "Methods". A control without modifier was run concurrently. (B) ETF (12.8 μM) was modified with 1 mM DTNB for 32 min (residual activity 68%). To the solution were added 50 mM KCN and aliquots were taken at the indicated time intervals and the release of TNB followed at 412 nm. Quantitative measurements of TNB release per mole of ETF was determined by taking the ratio of concentrations of TNB release and the native enzyme as described in "Methods".

CONCLUSION

As mentioned, the determinants for the interaction of ETF and MCAD are unknown. 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. A study of

TABLE II Conservation of cysteine residues among various species of ETF*

	α-subunit		*β-subunit*		
	68	155	399	404	464
Human	C	C	C	C	C
M. elsdenii	—	A	M	A	T
P. denitrificans	A	Q	I	A	M
W3A1	—	V	V	V	Y
B. japonicum	A	Q	I	A	S
C. thermosacch	—	A	M	A	T
B. subtillus	—	E	V	A	S
N. menningtidus	—	—	L	P	A
Yeast	K	S	I	A	C

*Data according to Finocchiaro *et al.*[20]

the crystal structure of human ETF identified 5 exposed cysteine residues (Table II).[1,20] ETF sequence comparisons reveal that there is little conservation of the 5 cysteine residues in human ETF although the amino acid, at position 464 corresponding to cysteine, is also a cysteine residue in yeast.

These results using DTNB strongly indicate the presence of a cysteine residue in or around the MCAD binding site. However, the relatively low extent of ETF inactivation caused by this compound and lack of conservation from species to species when compared to the modification of arginine residues and lysine residues[11] suggest the role of the cysteine residue in the ETF : MCAD interaction is of a non-essential nature.

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