Preferential cross-linking of the small subunit of the electron-transfer flavoprotein to general acyl-CoA dehydrogenase

Daniel J. STEENKAMP

Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, South Africa

The interaction between pig liver mitochondrial electron-transfer flavoprotein (ETF) and general acyl-CoA dehydrogenase (GAD) was investigated by means of the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate. Neither ETF or GAD contained reactive thiol groups. The substitution of 9.4 lysine residues/FAD group in GAD with pyridyl disulphide structures did not affect the catalytic activity of the enzyme. Thiol groups were introduced into ETF by thiolation with methyl 4-mercaptobutyrimidate. ETF containing 10.5 reactive thiol groups/FAD group showed undiminished electron-acceptor activity with respect to GAD. The reaction of thiolated ETF and GAD containing pyridyl disulphide structures resulted in a decreased staining intensity of the small subunit of ETF on SDS/polyacrylamide-gel electrophoresis. Preferential cross-linking of the smaller subunit of ETF to GAD did not take place when ETF was first treated with SDS, but was unaffected by reduction of GAD by octanoyl-CoA.

INTRODUCTION

The mitochondrial electron-transfer flavoprotein (ETF) transfers electrons from any one of eight soluble dehydrogenases to a membrane-bound iron-sulphur flavoprotein, ETF:ubiquinone oxidoreductase (Ruzicka & Beinert, 1977). The dehydrogenases can be divided into two categories. Six of the dehydrogenases catalyse the dehydrogenation of acyl-CoA derivatives and are homotetramers containing one FAD group per subunit (Beinert, 1963), whereas dimethylglycine dehydrogenase and sarcosine dehydrogenase are monomeric enzymes containing covalently bound flavin (Wittwer & Wagner, 1981). ETF is a heterodimer containing only one FAD group per molecule (Gorelick *et al.*, 1982; McKean *et al.*, 1983; Husain & Steenkamp, 1983). The most accurate estimate of the M_r values of the subunits of ETF is probably that of McKean *et al.* (1983), who reported values of 31 100 and 26 800 for the α - and β -subunits respectively. ETF is therefore larger than other proteins that function merely as electron carriers, such as the flavodoxins, ferredoxins or *b*- and *c*-type cytochromes. Thus far no attempt has been made to define the functions of the two subunits of ETF. Although the Michaelis constants of the dehydrogenases for ETF, obtained from steady-state kinetic studies, are in the micromolar range (McKean *et al.*, 1979), no direct evidence for the formation of a complex between the oxidized forms of ETF and any of the dehydrogenases has yet been presented.

This system is particularly well suited for an investigation of protein-protein interactions by cross-linking with heterobifunctional reagents. A very useful reagent for this purpose, *N*-succinimidyl 3-(2-pyridyldithio)propionate, was introduced by Carlsson *et al.* (1978). This reagent allows the incorporation of a reactive thiol-susceptible group into proteins by modification of lysine residues to pyridyl disulphide derivatives. To prevent cross-linking of the protein to itself, it is essential that it should not contain reactive thiol groups,

or that these, if present, can be blocked while retaining biological activity. Subsequent cross-linking is achieved by reaction of the pyridyl disulphide structures with thiol groups on another protein molecule.

The general acyl-CoA dehydrogenase (GAD) contains no reactive thiol groups, and the modification of amino groups was reported not to inactivate the enzyme (Frerman *et al.*, 1980). It therefore seemed likely that GAD can be activated for cross-linking to ETF by the introduction of pyridyl disulphide structures at lysine residues. Although ETF also does not contain reactive thiol groups, it seemed likely that these can be introduced by thiolation of lysine groups in the protein, since extensive amidination was found not to affect the electron-transfer activity of ETF (Beckmann & Frerman, 1982). In the present paper the preferential cross-linking of the β -subunit of ETF to GAD is described. Differential cross-linking of the subunits was dependent on the native conformation of the proteins and was little affected by prior reduction of GAD with octanoyl-CoA.

MATERIALS AND METHODS

Materials

Methyl 4-mercaptobutyrimidate, octanoyl-CoA, *N*-succinimidyl 3-(2-pyridyldithio)propionate and 4,4'-dithiodipyridine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Pig liver ETF was prepared as described previously (Husain & Steenkamp, 1983). Initial steps in the purification of GAD from pig liver were as described by McKean *et al.* (1979), but the $(\text{NH}_4)_2\text{SO}_4$ precipitation step was omitted. The preparation so obtained was impure, and was further subjected to chromatography on hydroxyapatite as described by Davidson & Schulz (1982). GAD was the last component to be eluted from the gradient.

Assays

The concentrations of ETF and GAD were determined by using molar absorption coefficients of

$13.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 436 nm (Husain & Steenkamp, 1983) and $15.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 445 nm (Thorpe *et al.*, 1979) respectively, and are expressed in terms of bound FAD. GAD was assayed in 50 mM-potassium phosphate buffer, pH 7.5, in the presence of 2 mM-phenazine methosulphate and 0.05 mM-2,6-dichlorophenol-indophenol at 30 °C. The turnover number per enzyme-bound flavin group, extrapolated to infinite octanoyl-CoA concentration, was 305 min^{-1} .

Thiol groups were determined as described by Grasetti & Murray (1967) at 15 °C. Pyridyl disulphide structures and the release of pyridine-2-thione as a measure of the cross-linking of thiolated ETF and GAD were determined at 15 °C as described by Carlsson *et al.* (1978).

Modification and cross-linking of ETF and GAD

ETF at a concentration of $36 \mu\text{M}$ in 75 mM-potassium phosphate buffer, pH 7.3, containing 10% (v/v) ethylene glycol was treated with 20 mM-methyl 4-mercaptobutyrimidate (added as the solid) for 5–7 h at 0 °C. GAD at a concentration of 7–26 μM in the same buffer was treated with 300 μM -*N*-succinimidyl 3-(2-pyridyldithio)propionate (added from a stock solution in ethanol) for 5–7 h at 0 °C. After modification, ETF and GAD were both separated from low- M_r components by chromatography on Sephadex G-25 (fine grade), with 20 mM-Hepes/NaOH buffer, pH 7.5, containing 10% (v/v) ethylene glycol as the eluent.

Thiolated ETF and modified GAD were mixed in various proportions in the presence and in the absence of

octanoyl-CoA, and the progress of the cross-linking reaction was monitored spectrophotometrically from the increase in absorbance at 343 nm due to the formation of pyridine-2-thione. Portions of the reaction mixture were removed at intervals and mixed with 2 mM-*N*-ethylmaleimide to quench the reaction. The apparent second-order rate constant for reaction of *N*-ethylmaleimide with the thiol groups of ETF was approx. 150 s^{-1} . The quenched samples were mixed in a 1:1 ratio with the digestion buffer described by Laemmli & Favre (1973), from which 2-mercaptoethanol had been omitted, and were immediately applied to a polyacrylamide gel that had been pre-run to remove persulphate. The compositions of the resolving gel and electrophoresis buffer were as described by Laemmli & Favre (1973), and the polyacrylamide concentration was 10% (w/v). In early experiments the stacking gel was omitted to avoid possible unwanted side reactions, which could have occurred in the time required for stacking of the proteins. Although the proteins were not as sharply resolved as during disc gel electrophoresis of samples that had been treated with 2-mercaptoethanol, the resolution was adequate to allow unambiguous interpretation of the results. Whenever the proteins were applied to a discontinuous gel electrophoresis system (Laemmli & Favre, 1973) the stacking gel was photopolymerized. The two methods gave similar results. The staining intensities of the bands were measured with a Beckman Appraise densitometer.

RESULTS

Effect of modification of lysine residues in GAD and ETF on catalytic activity

In preliminary experiments 7 μM -GAD was incubated overnight at 0 °C with 25–300 μM -*N*-succinimidyl 3-(2-pyridyldithio)propionate, and increasing degrees of modification of the enzyme up to a total of 9.4 lysine residues/enzyme FAD group were obtained. The modified enzyme preparations were assayed by using 18 μM -octanoyl-CoA and either 2 mM-phenazine methosulphate or 1.7 μM -ETF as the immediate electron acceptor. No significant decrease in activity was observed with either electron acceptor relative to the unmodified enzyme.

As reported by Mizzer & Thorpe (1980) for the pig kidney enzyme, the thiol groups of pig liver GAD reacted sluggishly with thiol-blocking reagents. When GAD was treated with a 12-fold excess of 4,4'-dithiodipyridine, only 0.66 thiol group/enzyme FAD group had reacted in 80 min, whereas 7.3 thiol groups/enzyme FAD group reacted in less than 2 min in the presence of 4 M-guanidinium chloride, in close agreement with the total number of half-cystine residues/enzyme FAD group determined by amino acid analysis of the pig kidney enzyme (Thorpe *et al.*, 1979). It is therefore evident that extensive modification of the lysine residues in GAD can be achieved without loss of catalytic activity, and that the enzyme does not contain reactive thiol groups that would generate extensive cross-linking of native GAD molecules to form homopolymers. During SDS/polyacrylamide-gel electrophoresis without pretreatment with 2-mercaptoethanol, GAD containing pyridyl disulphide structures migrated predominantly as an 80 kDa species, presumably the dimer (Fig. 2). Although dimerization could have

Fig. 1. Effect of thiolation on the spectral properties of ETF

ETF at a concentration of 9 μM in 75 mM-potassium phosphate buffer pH 7.3, containing 10% (v/v) ethylene glycol was treated with 20 mM-methyl 4-mercaptobutyrimidate (added as the solid). After various times at 0 °C portions were withdrawn and passed through Sephadex G-25 (fine grade) that had been equilibrated with 20 mM-Hepes/NaOH buffer, pH 7.5, containing 10% (v/v) ethylene glycol. The degree of thiolation of ETF was measured as described in the text. The curves represent the spectra of unsubstituted ETF (—) and ETF substituted with 3.6 (---) and 14.5 (— · —) reactive thiol groups/FAD group.

occurred by slow reaction of pyridyl disulphide structures with buried thiol groups in the native protein, it is likely that cross-linking took place upon unfolding the native structure in the presence of SDS in preparation for electrophoresis. Unmodified GAD migrated as a monomer in the same system.

The thiolation of ETF by treatment with methyl 4-mercaptobutyrimidate proceeded over a period of several hours at 0 °C. The thiol groups in thiolated ETF reacted in a biphasic manner with 0.2 mM-4,4'-dithiodipyridine. A rapid first phase reached completion within a minute ($k = 700 \text{ M}^{-1} \cdot \text{s}^{-1}$), whereas the slow phase proceeded for several hours. When unmodified ETF was left to react at 15 °C with 4,4'-dithiodipyridine, 5.24 thiol groups/FAD group reacted in 24 h, and this reaction probably corresponds to the slower phase seen in the reaction of the reagent with thiolated ETF. Spectral changes due to the thiolation of ETF were minor and appeared not to be isosbestic. The introduction of up to 10.5 reactive thiol groups/ETF FAD group resulted in an increased resolution of the absorbance band centred around 436 nm with no change in the molar absorption coefficient at this wavelength, but the introduction of 14.5 reactive thiol groups/ETF FAD group caused a slight decrease in the molar absorption coefficient from the previously estimated value of $13.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Husain & Steenkamp, 1983) to $13.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Fig. 1). As may be expected, oxidation of the thiol groups of thiolated ETF by 2,6-dichlorophenol-indophenol interfered with assays of the electron-acceptor activity of ETF, and the effect of thiolation of ETF was therefore not evaluated under conditions of turnover. Instead, the time courses of reduction of $4.07 \mu\text{M}$ -ETF and thiolated ETF by GAD in the presence of $36 \mu\text{M}$ -octanoyl-CoA were recorded at 436 nm under anaerobic conditions. Thiolated ETF used in this experiment contained 10.5 reactive thiol groups/ETF FAD group. From the initial rates of decrease in absorbance at 436 nm, turnover numbers of 58 and 60 min^{-1} were estimated for the reduction of ETF and thiolated ETF respectively. These values probably represent the rates of reduction of ETF and thiolated ETF to the semiquinone form. The total spectral change observed in the reduction of thiolated ETF by GAD was, moreover, similar to the change observed with native ETF. Similar results were obtained with thiolated ETF containing 21 reactive thiol groups/ETF FAD group. In early experiments it was found that the initial rate of reduction of thiolated ETF was somewhat lower than that of native ETF and did not go to completion. This effect was probably due to atmospheric oxidation of thiolated ETF during storage, since it could be eliminated by pretreatment with dithiothreitol. The present analysis therefore indicates that extensively thiolated ETF still serves as an electron acceptor from GAD and that its reduction proceeds at a rate not very different from that of the native protein. These results are essentially in agreement with those obtained by Beckmann & Frerman (1982), who found that acetimidation of ETF did not affect the rate of electron transfer between GAD and ETF. Thiolated ETF migrated as two separate subunits during SDS/polyacrylamide-gel electrophoresis without pretreatment with 2-mercaptoethanol (Fig. 2).

For the purpose of the present study it was important to establish that one of the two subunits of ETF is not preferentially modified by methyl 4-mercaptobutyrimidate.

Incubation of ETF for 24 h at 0 °C with 20 mM-methyl 4-mercaptobutyrimidate allowed the introduction of 22.3 reactive thiol groups/ETF FAD group. Disc gel electrophoresis of the highly substituted protein was performed in the system of Laemmli & Favre (1973), with the modifications that the stacking gel was photopolymerized and the sample digestion buffer did not contain 2-mercaptoethanol. The thiol groups in the separated subunits were detected by incubating the gel with 0.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid) in 100 mM-ammonium bicarbonate buffer, pH 8.5, for about 10 min. The gel was immediately examined by densitometry, and the ratio of the staining intensities of the β -subunit relative to the α -subunit was found to be 0.85:1.0. By comparison, amino acid analysis of the separated subunits (McKean *et al.*, 1983) indicated 23.7 and 19.1 lysine residues in the α - and β -subunit molecules respectively. ETF has been reported, variously, to contain 9.4 (McKean *et al.*, 1983) and 11.0 cysteine residues/molecule (Gorelick *et al.*, 1982). The ratio of the staining intensities of the β -subunit relative to the α -subunit for the unmodified protein was 1.14:1.0, which suggests that the β -subunit might contain six of a total of 11 cysteine residues/molecule.

Cross-linking of thiolated ETF and GAD containing pyridyl disulphide structures

The result of a preliminary cross-linking experiment in which thiolated ETF exceeded the amount of GAD by a factor of 1.35-fold is shown in Fig. 2. Cross-linking of thiolated ETF to GAD resulted in a decrease of the staining intensity of the β -subunit relative to the α -subunit, indicating preferential cross-linking of the β -subunit to GAD. This is further illustrated by the results of two-dimensional gel electrophoresis shown in Fig. 3. In the first dimension residual free ETF subunits that had not been cross-linked to GAD are separated from high-molecular-mass cross-linked complexes, a high proportion of which did not enter the resolving gel. Dissociation of the cross-linked complexes and electrophoresis in the second dimension showed that high-molecular-mass cross-linked components contained both subunits of ETF, but, whereas the α -subunit hardly entered the gel, the β -subunit was also present in components of intermediate molecular mass, as was GAD.

The reaction between thiolated ETF and GAD containing pyridyl disulphide structures was biphasic whether or not the dehydrogenase had first been reduced with octanoyl-CoA (Fig. 4). The rapid phase was somewhat more accentuated when octanoyl-CoA was omitted, and was almost completely absent when thiolated ETF was briefly pretreated at room temperature with 1% SDS. Electrophoretic analysis of samples withdrawn at the indicated times showed that the rapid phase coincided with a preferential disappearance of the β -subunit of ETF in one-dimensional gels (Fig. 5). This effect was observed whether or not GAD was first reduced with octanoyl-CoA, but was not observed when thiolated ETF was first treated with SDS. All samples showed a pronounced 80 kDa band, corresponding to the excess of GAD used in this experiment.

Consideration was given to the possibility that the GAD tetramer cannot accommodate four ETF molecules simultaneously because of steric hindrance. This would mean that specificity in the cross-linking of ETF and

Fig. 2. Cross-linking of thiolated ETF to GAD containing pyridyl disulphide structures

GAD containing 7.2 pyridyl disulphide structures/FAD group was reduced with octanoyl-CoA and then left to react with ETF containing 13.8 reactive thiol groups/FAD group at 15 °C. The final concentrations of the reactants were 3.64 μM -ETF, 2.73 μM -GAD and 36 μM -octanoyl-CoA. A sample was withdrawn after the release of 1.9 mol of pyridine-2-thione/mol of ETF FAD group, treated with 1 vol. of sample buffer not containing 2-mercaptoethanol and applied to gel electrophoresis. (lane 4). A further sample was withdrawn after the release of 2.3 mol of pyridine-2-thione/mol of ETF FAD group, treated with 1 vol. of sample buffer containing 10% (v/v) 2-mercaptoethanol and applied to the gel (lane 3). Thiolated ETF and GAD containing pyridyl disulphide structures were loaded in lanes 1 and 2 respectively.

GAD would only be obtained when GAD is in considerable excess over ETF in terms of FAD content. The amount of GAD relative to ETF in terms of enzyme-bound FAD was therefore varied in ratios of 1:1 to 4:1 in cross-linking experiments, but the results could not be unambiguously related to the stoichiometry in which ETF can maximally associated with GAD.

The specificity with which GAD was cross-linked to the β -subunit of ETF was dependent on the degree of substitution of the proteins. In the experiment documented in Table 1 GAD substituted to various extents with pyridyl disulphide structures was mixed with thiolated ETF after reduction of GAD with 36 μM -octanoyl-CoA (final concentration in the mixture). Samples were withdrawn at various times after mixing, quenched with

Fig. 3. Two-dimensional SDS/polyacrylamide-gel electrophoresis of cross-linked ETF and GAD

A gel strip containing separated cross-linked components from an experiment as described in the legend of Fig. 1 was incubated briefly in the sample digestion buffer of Laemmli & Favre (1973) and then subjected to electrophoresis in a second dimension in the discontinuous system described by those authors. The stacking gel consisted of 1% (w/v) agarose in 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Arrows labelled 1 and 2 indicate the directions of migration in the first and second dimensions.

Fig. 4. Effects of reduction of GAD with octanoyl-CoA and treatment of ETF with SDS on the cross-linking reaction

GAD containing 3.6 reactive pyridyl disulphide structures/FAD group was left to react at 15 °C with ETF containing 14.6 reactive thiol groups/FAD group. The final concentrations of the reactants were 2.74 μM -ETF and 7.13 μM -GAD (curve A). The reaction mixture for curve B contained in addition 55 μM -octanoyl-CoA, which was used to reduce GAD before the addition of ETF. In one case ETF was briefly treated with 1% SDS before it was mixed with GAD (curve C). The arrow indicates the time at which the reaction was initiated by the addition of 200 μl of ETF to 600 μl of GAD. Absorbance changes were recorded at 343 nm as a measure of the release of pyridine-2-thione.

Fig. 5. Electrophoretic analysis of the cross-linking of thiolated ETF and GAD containing pyridyl disulphide structures

ETF containing 10.5 reactive thiol groups/molecule was treated with GAD containing 6.7 pyridyl disulphide structures/molecule. The final concentrations of the reactants were $2.5 \mu\text{M}$ -ETF and $5.0 \mu\text{M}$ -GAD. Samples were withdrawn after 1.0 min (lanes a), 3 min (lanes b), 5 min (lanes c) and 8 min (lanes d) and subjected to electrophoretic analysis as described in the Materials and methods section. Lanes 1a–1d show samples that were withdrawn from a reaction mixture containing the oxidized proteins. In lanes 2a–2d GAD was reduced with octanoyl-CoA before the addition of ETF, and in lanes 3a–3d ETF was treated briefly at room temperature with 1% SDS before it was mixed with GAD.

Table 1. Dependence of the cross-linking of ETF and GAD on the degree of substitution of GAD with pyridyl disulphide structures

Thiolated ETF containing 10.5 reactive thiol groups/FAD group was mixed with GAD that had been substituted to various extents with pyridyl disulphide structures. The buffer was 20 mM-Hepes/NaOH, pH 7.5, containing 10% (v/v) ethylene glycol, and the temperature 15°C . Samples were withdrawn at the indicated times, quenched with *N*-ethylmaleimide and analysed electrophoretically as described in the Materials and methods section.

$$R = \frac{\text{staining intensity of } \beta\text{-subunit}}{\text{staining intensity of } \alpha\text{-subunit}}$$

$$x = \frac{\text{pyridine-2-thione released by cross-linking of } \beta\text{-subunit}}{\text{total pyridine-2-thione released}}$$

The quantity x was calculated as described in the text.

$$y = \text{pyridine-2-thione released/ETF FAD}$$

The concentrations of ETF and GAD are expressed in terms of FAD.

Concn. of ETF (μM)	Concn. of GAD (μM)	Pyridyl disulphide/ FAD	Time (min)	y	R	x
1.38	2.73	2.3	1	0.49	0.7	0.77
			3	0.67	0.61	0.74
			5	0.70	0.57	0.76
1.48	2.96	4.0	1	0.59	0.57	0.82
			3	0.89	0.435	0.75
			5	0.99	0.42	0.71
			8	1.09	0.324	0.71
2.2	4.4	5.8	1	0.69	0.43	0.88
			3	0.95	0.365	0.76
			5	1.06	0.346	0.72
			8	1.12	0.326	0.70

N-ethylmaleimide and subjected to electrophoretic analysis. As reported by Gorelick *et al.* (1982), the α - and β -subunits of ETF stain equally with Coomassie Brilliant Blue R250, a result that was confirmed also for thiolated ETF by densitometry of the separated subunits. The ratio of the integrated staining intensity of the α - and β -subunits, *R*, can then be expressed as:

$$R = \frac{\text{staining intensity of } \beta\text{-subunit}}{\text{staining intensity of } \alpha\text{-subunit}} = \frac{1-yx}{1-y+yx} \quad (1)$$

where *y* is the amount of pyridine-2-thione released per ETF FAD group as a measure of the cross-linking of ETF subunits to form higher-molecular-mass structures and *x* is the proportion of pyridine-2-thione released by reaction of the GAD-linked pyridyl disulphide structures with thiol groups on the β -subunit of ETF. In eqn. (1) the simplifying assumption was made that the release of pyridine-2-thione exactly parallels the cross-linking of an ETF subunit to produce a structure of higher molecular mass. It is, however, likely that some of the cross-linking events are superfluous, in that they involve ETF subunits that had already been cross-linked to GAD molecules. As cross-linking proceeds, GAD molecules will progressively become shielded from interaction with free ETF subunits, and superfluous cross-linking events between the excess reactive groups on GAD and ETF subunits that had already been cross-linked should predominate. Consequently, the experimental quantity, *y*, overestimates the number of effective cross-linking events. However, since

$$x = \frac{1-R+yR}{y(1+R)} \quad (2)$$

it is clear that assigning lower values to *y* would result in increasing values of *x*. The data presented in Table 1 provide a quantitative estimate of the specificity of cross-linking of GAD to either of the two subunits of ETF. Especially when highly substituted GAD was used, the results reflect the high specificity with which GAD was cross-linked to the β -subunit of ETF. After 1 min, which corresponds approximately to the end of the rapid phase of cross-linking, the value of *x* was 0.88 for the GAD sample containing 5.8 pyridyl disulphide structures/GAD FAD group. This indicates that during the first minute of cross-linking only 12% of all cross-linking events were between GAD and the α -subunit of ETF. The subsequent slower phase of cross-linking probably reflects non-specific interactions between ETF and GAD, including cross-linking events that do not lead to the incorporation of ETF subunits into higher-molecular-mass structures.

DISCUSSION

The ability of ETF to recognize eight different dehydrogenases as electron donors is noteworthy. Although the acyl-CoA dehydrogenases are similar in their quaternary structure, at least some of these proteins do not cross-react immunochemically (Ikeda & Tanaka, 1983; Ikeda *et al.*, 1985), and the basis of the recognition of the dehydrogenases by ETF is therefore of interest. The phenomenon whereby one protein can recognize a number of different proteins is of wider occurrence. The ability of thioredoxin to interact with several reductases, for instance, is well documented (Holmgren, 1985). In

order to develop methods by which the specific interactions between ETF and the various ETF-dependent dehydrogenases can be investigated, a study was undertaken to establish if these interactions can be localized to one of the two subunits of ETF. The results reported in the present paper indicate that GAD interacts specifically with the β -subunit of ETF and that this interaction depends on the native conformation of ETF. Moreover, the recognition of GAD by ETF does not depend significantly on the oxidation state of the dehydrogenase. The results cannot readily be explained by assuming a preferential thiolation of the β -subunit by methyl 4-mercaptobutyrimidate, since preferential cross-linking of the β -subunit was not observed when ETF was first treated with SDS. Amino acid analysis of the separated subunits by McKean *et al.* (1983) showed the presence of 24 and 19 lysine residues in the α - and β -subunits respectively, and these were found to be of similar reactivity towards 2,4,6-trinitrobenzenesulphonic acid. Moreover, I was unable to demonstrate a significant difference between the thiol contents of the two subunits of thiolated ETF.

The increase in the specificity with which the β -subunit of ETF is cross-linked to GAD at higher degrees of substitution of GAD with pyridyl disulphide structures (Table 1) probably reflects the increased availability of a pyridyl disulphide group suitably oriented for cross-linking when ETF and GAD combine in some preferred orientation.

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