Cross-linking of the electron-transfer flavoprotein to electron-transfer flavoprotein–ubiquinone oxidoreductase with heterobifunctional reagents

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The mitochondrial electron-transfer flavoprotein (ETF) is a heterodimer containing only one FAD. In previous work on the structure–function relationships of ETF, its interaction with the general acyl-CoA dehydrogenase (GAD) was studied by chemical cross-linking with heterobifunctional reagents [D. J. Steenkamp (1987) *Biochem. J.* **243**, 519–524]. GAD whose lysine residues were substituted with 3-(2-pyridyldithio)propionyl groups was preferentially cross-linked to the small subunit of ETF, the lysine residues of which had been substituted with 4-mercaptobutyramidine (MBA) groups. This work was extended to the interaction of ETF with ETF–ubiquinone oxidoreductase (ETF–Q ox). ETF–Q ox was partially inactivated by modification with *N*-succinimidyl 3-(2-pyridyldithio)propionate to introduce pyridyl disulphide structures. A similar modification of ETF caused a large increase in the apparent Michaelis constant of ETF–Q ox for modified ETF owing to the loss of positive charge on some critical lysines of ETF. When ETF–Q ox was modified with 2-iminothiolane to introduce 4-mercaptobutyramidine groups, only a minor effect on the activity of the enzyme was observed. To retain the positive charges on the lysine residues of ETF, pyridyl disulphide structures were introduced by treating ETF with 2-iminothiolane in the presence of 2,2'-dithiodipyridyl. The electron-transfer activity of the resultant ETF preparation containing 4-(2-pyridyldithio)butyramidine (PDBA) groups was only slightly affected. When ETF–Q ox substituted with MBA groups was mixed with ETF bearing PDBA groups, at least 70 % of the cross-links formed between the two proteins were between the small subunit of ETF and ETF–Q ox. ETF–Q ox, therefore, interacts predominantly with the same subunit of ETF as GAD. Variables which affect the selectivity of ETF–Q ox cross-linking to the subunits of ETF are considered.

INTRODUCTION

The electron-transfer flavoprotein (ETF) of mammalian mitochondria transfers electrons from any of eight different dehydrogenases to a membrane-bound iron–sulphur flavoprotein, ETF:ubiquinone oxidoreductase (ETF–Q ox) and, therefore, plays a central role in several catabolic pathways [1–4]. Six of the enzymes which use ETF as electron acceptor catalyse the dehydrogenation of fatty acyl-CoA esters and are homotetramers containing one FAD group per subunit [5,6]. Despite their similarity in structure, recent evidence suggests that the different acyl-CoA dehydrogenases do not cross-react immunochemically [5]. In addition, ETF accepts electrons from the sarcosine and dimethylglycine dehydrogenases, which are structurally unrelated to the acyl-CoA dehydrogenases [4,7]. Apart from the relatively large number of proteins which interact with ETF *in vivo*, other interesting properties of ETF and ETF–Q ox have recently come to light. It was first reported by Furuta *et al.* [8], and later confirmed in several other laboratories [9–11], that ETF is a heterodimer containing only one FAD. The physiological significance of this finding is not clear. To gain a deeper insight into the structure–function relationships of this protein, it seems important to

establish whether different functional roles can be assigned to its two subunits. The ribonucleotide reductases [12] and protein kinases [13] are striking examples of such a functional differentiation between subunits. At present two lines of evidence suggest that the smaller subunit of ETF may be directly involved in electron transfer. Gorelick & Thorpe [14] presented evidence on the photoaffinity labelling of ETF, the apoprotein of which had been reconstituted with [³H]azido–FAD. Photolysis resulted in the incorporation of a low level of radioactivity into ETF, at least 74 % of the label being recovered in the smaller β -subunit of ETF. However, as pointed out by these authors, this result cannot be regarded as conclusive evidence for the location of the flavin-binding site of mammalian ETF on the smaller subunit. Studies on the reconstitution of apo-ETF with flavin analogues indicated that the 8-position of the flavin is exposed to solvent. The possibility that FAD binds to the larger α -subunit of ETF with the 8-position on the benzene ring in close proximity to residues belonging to the β -subunit cannot, therefore, be ignored. Further evidence that the β -subunit of ETF is involved in electron transfer was, however, obtained when it was shown that general acyl-CoA dehydrogenase (GAD) preferentially cross-links to the β -subunit after compli-

Abbreviations used: EDC, 1-ethyl-3-[3(dimethylamino)propyl]carbodi-imide hydrochloride; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; PDP, 3-(2-pyridyldithio)propionyl; PDBA, 4-(2-pyridyldithio)butyramidine; MBA, 4-mercaptobutyramidine; Q₁, ubiquinone 1; APDP, 3-[(4-azidophenyl)dithio]propionic acid; GAD, general acyl-CoA dehydrogenase; ETF, electron-transfer flavoprotein; ETF–Q ox, ETF:ubiquinone oxidoreductase; TNBS, trinitrobenzenesulphonic acid; NEM, *N*-ethylmaleimide.

mentary activation of lysine residues on the two proteins with heterobifunctional reagents [15]. If, however, ETF and GAD interacted in such a manner that unequal numbers of thiolated lysines on the two subunits of ETF were in proximity to pyridyl disulphide structures on GAD, this would result in preferential cross-linking of one of the subunits, irrespective of the location of the binding site for GAD. The result obtained may, therefore, merely reflect the precise arrangement of activated lysine residues on ETF and GAD, even though it was shown that the lysine residues substituted with 4-mercapto-butylamide (MBA) moieties were approximately equally distributed between the two subunits of ETF. Although both studies, therefore, suggest that electron transfer is mediated by the β -subunit of ETF, the available data are not unambiguous and it was hoped that further experimentation would add to the weight of evidence concerning the respective functions of the two subunits of ETF.

In this paper, the importance of charge retention in studies of the cross-linking of proteins with heterobifunctional reagents is stressed. The effect of the substitution of ETF and ETF-Q ox with 4-(2-pyridyldithio)butylamide (PDBA) and MBA groups, respectively, on the catalytic activities of these proteins is described. Thiolated ETF-Q ox was preferentially cross-linked to the β -subunit of the derivatized ETF.

MATERIALS AND METHODS

Materials

2-Iminothiolane was obtained from Pierce Chemical Company, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), 2,2'-dithiodipyridyl, 4,4'-dithiodipyridyl, octanoyl-CoA and Hepes buffer from Sigma Chemical Company, St. Louis, MO, U.S.A., and trinitrobenzenesulphonic acid (TNBS) from Fluka AG, Buchs, Switzerland. Ubiquinone 1 (Q_1) was a generous gift from Esai Company, Tokyo, Japan. GAD and ETF were isolated from pig liver mitochondria by published procedures [11,15]. ETF-Q ox was isolated as described by Ruzicka & Beinert [1] and was further subjected to chromatography on hydroxyapatite [16].

Enzyme assays

ETF-Q ox was assayed as described previously [17]. Assay mixtures contained 0.279 nmol of acyl-CoA dehydrogenase, 1.09 nmol of ETF, 80 nmol of Q_1 , 40 nmol of octanoyl-CoA and 20 μ mol of K^+ /Hepes, pH 7.8, in a final volume of 0.8 ml. The temperature was 30 °C. Reactions were started by the addition of ETF-Q ox and followed spectrophotometrically by recording the absorbance change at 275 nm. The difference molar absorption coefficient at 275 nm for the conversion of octanoyl-CoA to octenoyl-CoA was determined as described for the formation of crotonyl-CoA from glutaryl-CoA [17], and was subtracted from the difference molar absorption coefficient for the reduction of Q_1 to give a value of 7.5 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ for use in activity calculations. The concentrations of the proteins were estimated spectrophotometrically assuming molar absorption coefficients of 13.5 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ at 436 nm for ETF [11], 15.4 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ at 448 nm for GAD [18] and 26 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ at 380 nm for ETF-Q ox [16]. Some chemically-modified ETF-Q ox preparations were re-

covered at concentrations of 1 μM or less after gel chromatography to remove low- M_r substances. In such cases the concentration of the enzyme was estimated by fluorimetric determination of the FAD content as riboflavin [19].

Modification of ETF-Q₁ ox

Modification with TNBS. ETF-Q ox which had been dialysed for 3 h against 25 mM- Na^+ /Hepes, pH 8.4, containing 10% (v/v) ethylene glycol, was allowed to react with 1 mM-TNBS at 15 °C. The reaction was followed spectrophotometrically at 367 nm and the number of trinitrophenyl groups introduced estimated using a millimolar absorption coefficient of 10.8 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ [20]. Aliquots of the reaction mixtures were withdrawn at intervals and added to 1/10th vol. of 100 mM-lysine to stop modification of the enzyme.

Modification of ETF-Q ox with SPDP. SPDP was added to ETF-Q ox from a 10 mM stock solution in ethanol to obtain final concentrations of 100 μM - or 200 μM -SPDP. The mixtures were incubated at 0 °C for 3 h and then passed through Sephadex G-25 equilibrated with 25 mM- Na^+ /Hepes, pH 7.4, containing 10% (v/v) ethylene glycol. The number of 3-(2-pyridyldithio)propionyl (PDP) groups introduced was quantified by reduction with 1 mM-dithiothreitol as described previously [21].

Thiolation of ETF-Q ox with 2-iminothiolane. ETF-Q ox which had been dialysed against 25 mM- Na^+ /Hepes, pH 7.4, was added to a mixture of solid 2-iminothiolane and 1 M- K_2HPO_4 to obtain final concentrations of 30 mM-2-iminothiolane and 30 mM potassium phosphate, pH 7.4. The mixture was incubated at 0 °C for various times after which the reaction was terminated by removing low- M_r reactants by chromatography on Sephadex G-25. The number of thiol groups introduced was quantified by reaction with 4,4'-dithiodipyridyl as described previously [22]. To determine the effect of thiolation on the activity of the enzyme, the thiol groups introduced were blocked by incubation with 1 mM-*N*-ethylmaleimide (NEM) for 10 min at 0 °C, followed by chromatography on Sephadex G-25. Thiol groups were found to react rapidly with Q_1 .

Modification of ETF

ETF was thiolated by reaction with 2-iminothiolane as described previously [15]. Two procedures were used to introduce pyridyl disulphide structures at the lysine residues of ETF. In the first of these, the reagent SPDP was used to introduce PDP groups into ETF. This results in the conversion of positively-charged lysine residues to neutral amides. In the alternative procedure, PDBA groups were introduced with retention of the positive charge on the lysine residues by incubating ETF with 50 mM-2-iminothiolane in the presence of 5 mM-2,2'-dithiodipyridyl. To prevent a downwards shift in pH, 1 M- K_2HPO_4 was added to a final concentration of 50 mM. In either case the buffer was 25 mM- Na^+ /Hepes, pH 7.4, containing 10% (v/v) ethylene glycol and the temperature was 0 °C. The reaction was terminated by removing low- M_r reactants by chromatography on Sephadex G-25, which had been equilibrated with the same buffer. Times of incubation and the concentration

of SPDP used in particular experiments are described in the text or Figure legends. The number of pyridyl disulphide structures in the modified ETF samples was determined as described [21].

Cross-linking of ETF and ETF-Q ox

The cross-linking of ETF and ETF-Q ox was attempted in two ways. As in earlier work [15] in which thiolated ETF was cross-linked to general acyl-CoA dehydrogenase containing pyridyl disulphide structures, ETF was thiolated with 2-iminothiolane and mixed with ETF-Q ox, lysine residues of which had been substituted with PDP groups. In an alternative procedure ETF-Q ox was thiolated using 2-iminothiolane and mixed with ETF, lysine residues of which had been substituted with PDBA moieties. In either case, the resultant absorbance change at 343 nm was recorded as a measure of the release of pyridine-2-thione. Aliquots of reaction mixtures were withdrawn at time intervals and mixed with 1/25th volume of an ethanolic solution of 100 mM-NEM. Cross-linked samples were electrophoresed as described [23], but 2-mercaptoethanol was omitted from the sample digestion buffer. The relative staining intensities of the larger (α) and smaller (β) subunits of ETF were measured using a Beckman Appraise densitometer.

RESULTS

Effect of the derivitization of lysine residues on the activity of ETF-Q ox

As was reported by Beckmann & Frerman [24], trinitrophenylation of lysine residues in ETF-Q ox had little effect on the activity of the enzyme. Upon incubating

Fig. 1. The effect of chemical modification of lysine residues of ETF-Q ox on its activity

ETF-Q ox was dialysed for 3 h against 25 mM-Na⁺/Hepes, pH 7.4, and was subsequently modified by treatment with 2-iminothiolane or SPDP as described in the Materials and methods section. The activity of enzyme samples containing 11.5 MBA groups/FAD group as *N*-ethylmaleimide derivatives (●), and 6.7 (◆) or 11.8 PDP groups/FAD group (○) were measured in the standard assay described in the Materials and methods section, but with ETF variable. A control sample was dialysed and then passed through Sephadex G-25 equilibrated with 25 mM-Na⁺/Hepes, pH 7.4 (▲).

ETF-Q ox for 66 min at 15 °C with 1 mM-TNBS, 9.43 lysine residues were derivatized and the enzyme activity measured in a single point assay was 90 % of that of a control. It therefore seems that the positive charge on the lysine residues is not important, either for binding of ETF or for subsequent electron-transfer steps to take place. The substitution of lysine residues with PDP groups, however, decreased activity of the enzyme. ETF-Q ox samples, which had been modified to various extents, had the same apparent Michaelis constant for ETF, but a decreased apparent maximal velocity (Fig. 1), which indicates that substitution with PDP groups inactivates the enzyme. This effect is most probably due to a slow reaction of the pyridyl disulphide substituent with thiol groups in the enzyme. ETF-Q ox contained

Fig. 2. The effect of chemical modification of lysine residues of ETF on its electron-transfer activity

ETF was passed through a Sephadex G-25 column equilibrated with 25 mM-Na⁺/Hepes, pH 7.4, containing 10 % (v/v) ethylene glycol. PDBA groups were then introduced and quantified as described in the Materials and methods section. ETF was also incubated with 150 μM- or 300 μM-SPDP at 0 °C to introduce either 3.25 or 6.7 PDP groups/FAD group. The ETF-Q ox assay, in which various levels of chemically-modified ETF were substituted for native ETF, was used as a measure of the effect of derivatization on the electron-transfer activity of ETF. Chemically-modified ETF samples contained either 3.25 PDP groups (▲) or 8.9 PDPA groups/FAD group (◆) and are shown relative to those obtained with native ETF (●). An ETF sample containing 6.7 PDP groups/FAD group gave no measurable rate in the assay.

Scheme 1. Possible reactions of thiolated ETF with 2,2'-dithiodipyridyl

no reactive thiol groups. When the enzyme was incubated with dithiodipyridyl only 1.5 thiols had reacted after 2 h at 15 °C. It is possible that the sensitivity of the enzyme to thiol reagents is conferred by the derivatization of lysine residues, as suggested by the results of a few trial experiments. ETF-Q ox, which had been thiolated with 2-iminothiolane to the extent of 10.8 thiols per mol of the enzyme, was completely inactivated by treatment with 1 mM-NEM for 18 h at 0 °C, whereas the unmodified enzyme, upon similar treatment, retained 75 % of the original activity and 86 % of the activity of a control incubated without NEM. Moreover, when thiolated ETF-Q ox, prepared by reacting the enzyme with 2-iminothiolane, was briefly treated with NEM to block the reactive thiol groups only a minor effect on the activity of the enzyme, relative to the native enzyme, was observed (Fig. 1).

Effect of chemical modification of amino groups on the electron-transfer activity of ETF

Because of the commercial availability of SPDP as a heterobifunctional reagent, the effect of modifying ETF with this reagent was investigated. The substitution of 3.25 lysine residues with PDP groups had a marked effect on the ability of ETF to function as a substrate of ETF-Q ox (Fig. 2) and no activity could be detected after the introduction of 6.7 PDP groups. This dramatic effect of chemical modification of ETF with reagents which neutralize the charge on some critical lysine residues was previously described by Beckmann & Frerman [24]. The feasibility of introducing pyridyl disulphide groups into ETF with retention of charge was therefore investigated. This can be achieved by allowing ETF to react first with 2-iminothiolane to introduce thiol groups and then with 2,2'-dithiodipyridyl to convert the thiol groups to pyridyl disulphides. The reaction of thiol groups with aromatic disulphides, such as Ellman's reagent [25] or 2,2'-dithiodipyridyl (D. J. Steenkamp, unpublished observa-

tions), however, gives rise both to a mixed disulphide of the protein-bound thiol group and the thiol reagent, and to cross-linking of the protein thiols to disulphides by thiol-disulphide exchange reactions. These possibilities are shown for the reaction of thiolated ETF with 2,2'-dithiodipyridyl in Scheme 1. Fortunately, it is possible to

Fig. 3. The release of pyridine-2-thione upon mixing ETF containing PDPA groups with ETF-Q ox containing MBA groups

ETF containing 12.5 PDPA groups/FAD group was allowed to react with ETF-Q ox containing 9.4 MBA groups/enzyme. Final concentrations of the proteins were 2.2 µM-ETF and 2.7 µM-ETF-Q ox. The change in absorbance at 343 nm was recorded as a measure of the release of pyridine-2-thione.

minimize the disulphide exchange reactions [reaction (c) in Scheme 1] by appropriate choice of reaction conditions.

It was previously shown [26] that 2-iminothiolane does not contain free thiol groups, but that these become available for reaction upon administration of amino groups with the reagent. Consequently, the disulphide exchange reactions, which would lead to a cross-linking of amino groups by the sequence of reactions shown in Scheme 1, can be minimized by continual blocking of nascent thiol groups by reaction with 2,2'-dithiodipyridyl. When ETF was thiolated with 2-iminothiolane in the presence of an excess of the thiol reagent, 2,2'-dithiodipyridyl, an efficient substitution of lysine residues with PDBA moieties was achieved. This was borne out by the following experiment. ETF was converted to the PDBA derivative as described in the Materials and methods section and the number of pyridyl disulphide structures was estimated by reduction with dithiothreitol. The remainder of the derivatized sample was then reduced with 20 mM-dithiothreitol to convert all available disulphide groups, including symmetric disulphides which could have arisen by disulphide bond formation between two MBA moieties, to free thiols. The reduced sample was passed through Sephadex G-25 and the number of thiol groups immediately quantified. The number of thiol groups found corresponded with the

number of pyridyl disulphide structures detected before reduction and, therefore, lysine residues which reacted with 2-iminothiolane were, for all practical purposes, quantitatively converted to PDBA derivatives. Even though PDBA is a relatively bulky substituent, the change in the electron-transfer activity of ETF substituted with 8.9 PDBA groups was relatively minor compared with the pronounced effect of substitution with PDP groups (Fig. 2).

Cross-linking of thiolated ETF-Q ox and ETF containing pyridyl disulphide structures

The results of an experiment in which thiolated ETF-Q ox was mixed with ETF, the amino groups of which had been substituted with PDBA moieties, are shown in Figs. 3 and 4. The release of pyridine-2-thione is biphasic. Even during the initial, faster phase more than a stoichiometric amount of pyridine-2-thione per ETF is released, indicating the formation of more than one cross-link per ETF molecule. The ratio, pyridine-2-thione released:ETF-FAD as a function of time was unaltered by changes in the cross-linking conditions described in the legend to Fig. 4. These involved increasing the amount of ETF-Q ox relative to ETF or introducing conditions of turnover by the addition of GAD and octanoyl-CoA. While the subunits of ETF

Fig. 4. Cross-linking of ETF containing PDBA groups to ETF-Q ox containing MBA groups

The chemically-modified ETF and ETF-Q ox samples were as described in the legend to Fig. 3 and lanes (a)1–(a)4 show electrophoresis patterns of aliquots which were withdrawn 1, 2, 4 and 6 min after mixing the two proteins. These aliquots were mixed with 1/25th volume of an ethanolic solution of 100 mM-NEM. Lanes (b)1–(b)4 show a similar time course for an experiment in which modified ETF and ETF-Q ox were mixed to obtain final concentrations of 1.8 μ M-ETF and 2.9 μ M-ETF-Q ox. The concentrations of modified proteins in the reaction mixture analysed in lanes (c)1–(c)4 were similar to those in lanes (a)1–(a)4, but the mixture contained in addition 0.13 μ M-GAD (expressed in terms of FAD content) and 0.1 mM-octanoyl-CoA, which was added to ETF-Q ox immediately before the addition of ETF.

Fig. 5. Attempted cross-linking of thiolated ETF to ETF-Q ox containing PDP groups

ETF was thiolated by treatment with 50 mM-2-iminothiolane, neutralized by the addition of 50 mM-dipotassium phosphate, at 0 °C for 75 min, and contained 20.2 thiols/FAD group. ETF-Q ox was modified with 100 μ M-SPDP for 4 h and contained 7.7 PDP groups. The chemically-modified proteins were mixed at 15 °C to obtain final concentrations of 1.9 μ M-ETF-Q ox and 2.1 μ M-ETF. The samples applied were: ETF-Q ox containing 7.7 PDP groups (lane 1); ETF containing 20.2 MBA groups/FAD group (lane 2); and aliquots of the reaction mixture withdrawn 1 min (lane 3), 2 min (lane 4) and 3 min (lane 5) after mixing the proteins.

stain equally with Coomassie Brilliant Blue [9,15], a decreased staining intensity of the β -subunit relative to the α -subunit is apparent in one-dimensional gels (Fig. 4). This was more apparent when ETF-Q ox was used in excess of ETF and the ratio of the staining intensities of the α -: β -subunit is about 2.7:1 in lanes (b)2–(b)4 of Fig. 4. Very-high-molecular-mass complexes were formed, part of which was still in the stacking gel when the run was terminated. This result may be compared with the one-dimensional electrophoretic analysis of an experiment in which thiolated ETF was mixed with ETF-Q ox containing PDP groups. In this case the functional groups required for cross-linking had been introduced, but, as judged from activity assays (Fig. 1), ETF-Q ox was inactivated in the process. Cross-linking is inefficient (Fig. 5) and no preferential cross-linking of one of the ETF subunits is observed.

The results of two-dimensional gel electrophoresis in which the cross-linked ETF-ETF-Q ox complexes were

dissociated with 2-mercaptoethanol before migration in the second dimension are shown in Figs. 6 and 7 and confirm that thiolated ETF-Q ox cross-links predominantly to the β -subunit of ETF. Because complexes of differing molecular size are formed, the bands corresponding to the ETF subunits, which were present in high-molecular-mass complexes, appear as two smears in the second dimension. The ratio in which the α - and β -subunits were recovered by dissociation of higher-molecular-mass complexes was determined by scanning perpendicularly across these smears in a densitometer. The results of two such scans in which the β -subunit represented about 70 % of the integrated staining intensity of the two subunits are shown in Fig. 7.

DISCUSSION

Chemical cross-linking has been used extensively in studies of the nearest-neighbour relationships of proteins, especially when these are present in a stable structural relationship, as is the case for the respiratory chain complexes [27,28] or ribosomes [29]. In such cases both homo- and heterobifunctional reagents have been applied with good effect [29,30]. A different approach is required when the problem concerns the identification of the subunits which participate in the protein-protein interaction of two multimeric proteins, which associate transiently. Such a problem cannot be addressed by means of homobifunctional reagents, that predominantly produce intramolecular cross-links between the subunits. A very useful approach, developed by Carlsson *et al.* [21], is to derivatize the two proteins with different heterobifunctional reagents, such that substituted functional groups on one protein will be cross-linked to complementarily-modified groups on the other protein rather than intramolecularly to functional groups of an adjacent subunit. However, when this technique is used to cross-link two proteins in the orientation in which the unmodified proteins would interact, such that the subunits involved in the interaction can be identified, additional aspects must be taken into consideration. It is essential that the inherent affinity of the two proteins for one another is conserved in the modified proteins. The functional group most commonly derivatized in cross-linking studies is the amino group. Amino groups have good accessibility, are good nucleophiles for chemical modification and are relatively abundant. Unfortunately, the presence of charged amino groups in at least one of a pair of interacting proteins often plays an important role in their electrostatic interaction [24,31–33]. Modification of both proteins with a hydroxysuccinimide ester, such as SPDP, which neutralizes the positive charge on the amino groups, can, therefore, have a profound effect on their mutual affinity for one another. In the present paper this problem was circumvented by basing the modification of both ETF and ETF-Q ox on the cyclic thioimide, 2-iminothiolane, which allows the introduction of the requisite functional groups with retention of a positive charge. An additional problem, which is less readily compensated for, is that carboxyl, rather than amino, groups provide the matching negatively-charged groups in one of the pair of interacting proteins. Consequently, amino groups bearing a reactive substituent on the negatively-charged member of the pair may not be appropriately positioned to allow the cross-linking reaction to take place. The likelihood that a

Fig. 6. Two-dimensional-gel electrophoresis of cross-linked ETF and ETF-Q ox

A gel strip containing separated cross-linked components from an experiment such as in Fig. 4 was incubated for 10 min in the sample digestion buffer of Laemmli & Favre [23] and then subjected to electrophoresis in the second dimension in the discontinuous system described by these 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 1 and 2 indicate the directions of migration in the first and second dimensions, respectively.

suitably positioned, reactive substituent will be available can be improved by increasing the degree of complementary substitution on the two proteins. Unfortunately, however, proteins highly substituted with pyridyl disulphide structures have poor solubility properties [21], and solutions of ETF bearing more than 12 pyridyl disulphide structures tend to develop turbidity. It is likely that these variables limited the extent to which differential cross-linking of the subunits of ETF to ETF-Q ox could be achieved.

Two alternative cross-linking procedures were therefore explored. In view of the low recovery of the enzyme, sufficient ETF-Q ox was not available for these exploratory experiments and it was instead decided to investigate the effectiveness of the watersoluble carbodi-imide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide hydrochloride (EDC), in the cross-linking of ETF to GAD. EDC has been extensively used as a zero-length cross-linker between proteins which enter into electrostatic association by juxtapositioning of amino and carboxyl groups [32,34]. In these experiments GAD was first acetamidylated with ethyl acetimidate to prevent the formation of intramolecular cross-links to itself, and

subsequently allowed to interact with ETF in the presence of EDC under more than one set of conditions. Although treatment of GAD with EDC caused a pronounced decrease in its activity, as was earlier reported [35], no cross-linking of ETF to GAD could be demonstrated. An attempt was also made to cross-link ETF and GAD by means of the photoactivable heterobifunctional reagent *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionic acid (NHS-APDP). While substitution of GAD with APDP did not adversely affect its activity, no cross-linking of GAD to ETF was observed upon subsequent irradiation of a mixture of the proteins. In view of these discouraging results the experiments were not extended to the interaction between ETF and ETF-Q ox. It seems, however, that the cross-linking of two interacting proteins by means of the thiol-pyridyl-disulphide exchange reaction is a very efficient process not easily realized in alternative methodologies. The results presented here indicate that ETF-Q ox, like GAD [15], interacts predominantly with the β -subunit of ETF. The results cannot readily be explained by assuming that the distribution of reactive lysine favours the small subunit of ETF, since this had previously been shown

Fig. 7. Quantification of the α - and β -subunits of ETF which were cross-linked to ETF-Q ox

The tracings represent two densitometer scans taken in different positions perpendicularly across the α - and β -subunit bands derived from dissociation of the high-molecular-mass complexes of ETF and ETF-Q ox. Scan (b) was taken in a position where the bands are less intense, hence the higher noise level.

not to be the case [15], and therefore, provides a third line of evidence that this subunit is involved in the redox reactions of ETF. Further studies are being undertaken to establish whether the interaction of GAD and ETF-Q ox with ETF involves the same group of residues on the ETF protein.

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