Cross-Linking and *N*-(1-Pyrenyl)maleimide Labeling of Cysteine Mutants of Proton-Pumping Pyridine Nucleotide Transhydrogenase of *Escherichia coli*†

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ABSTRACT: The pyridine nucleotide transhydrogenase of *Escherichia coli* is a proton pump composed of two subunits (α and *β*) organized as an $\alpha_2\beta_2$ tetramer. The enzyme contains seven cysteine residues, five in the α-subunit and two in the *β*-subunit. The reaction of these residues with the cross-linking agent cupric 1,10-phenanthrolinate and with the fluorescent thiol reagent *N*-(1-pyrenyl)maleimide was investigated in mutants in which one or more of these cysteine residues had been mutated to serine or threonine residues. Mutation of αCys395 and αCys397 prevented disulfide bond formation to give the cross-linked α_2 dimer. We concluded that the two α-subunits of the holoenzyme interface in the region of these two cysteine residues. Pyrenylmaleimide reacted with detergent-washed cytoplasmic membrane vesicles containing high levels of transhydrogenase protein to show characteristic fluorescence emission bands at 378−379, 397−398, and 419−420 nm. At higher ratios of pyrenylmaleimide:transhydrogenase (>5:1) and longer times of reaction, an eximer band at 470 nm was formed. This was attributed to interaction between noncovalently bound molecules of pyrenylmaleimide. The cysteine residues of the *β*-subunit (*β*Cys147 and *β*Cys260) were covalently modified by pyrenylmaleimide. *β*Cys147 reacted more strongly than *β*Cys260 with the fluorophore, and the pyrene derivative of *β*Cys147 was more accessible to quenching by 5-doxylstearate, suggesting a proximity to the surface of the membrane. Covalent modification of *β*Cys260 resulted in inhibition of enzyme activity. The inhibition was attributed to the introduction of the bulky pyrene group into the enzyme.

Pyridine nucleotide transhydrogenase is a proton pump which catalyzes the reversible reaction

$$H^+_{out} + NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ + H^+_{in}$$

where reduction of $NADP^+$ by NADH is linked to an inward translocation of protons from the periplasm (in bacteria) or cytosol (mammalian cells) into the cytosol or mitochondrial matrix, respectively. In the presence of an electrochemical proton gradient generated by another proton pump, the apparent equilibrium of the reaction is shifted strongly toward formation of NADPH and NAD^+, and the rate of the reduction of $NADP^+$ by NADH is increased 10-fold. The properties of transhydrogenases have been reviewed recently by Jackson (1991), Hatefi and Yamaguchi (1992, 1996), and Olausson et al. (1995).

The transhydrogenase of *Escherichia coli* is composed of two subunits, α (510 residues) and *β* (462 residues), organized as an $\alpha_2\beta_2$ tetramer (Clarke & Bragg, 1985; Clarke et al., 1986; Ahmad et al., 1992; Hou et al., 1990). The relative simplicity of this structure makes the enzyme an excellent system in which to study the mechanism of proton pumping. Three domains are recognized in transhydrogenases (Jackson et al., 1993; Williams et al., 1994; Hatefi & Yamaguchi, 1996; Olausson et al., 1995). Domains I and III are extramembrane domains carrying the NAD(H)- and NADP(H)-binding sites, respectively. Domain II is inserted in the cell or mitochondrial membrane.

In *E. coli*, domain II is composed of the C-terminal 100 residues of the α-subunit and the N-terminal 260 residues of the *β*-subunit. This region of the α-subunit is organized as four transmembrane α-helices. The *β*-subunit region contains six or eight transmembrane α-helices (Clarke et al., 1986; Tong et al., 1991; Holmberg et al., 1994; Glavas et al., 1995b) (Figure 1). Domains I and III can be readily released from the membrane as 43 and 30 kDa proteins by digestion with trypsin (Tong et al., 1991). Cleavage of the *β*-subunit to yield the 30 kDa protein occurs only in the presence of substrate NADP(H). This suggests that binding or release of NADP(H) induces a conformational change in the enzyme which is important in the mechanism of proton translocation (Tong et al., 1991).

The three-dimensional structures of relatively few membrane enzymes have been determined, e.g., the photosynthetic reaction center, bacteriorhodopsin, and cytochrome *c* oxidase (Deisenhofer et al., 1985; Henderson et al., 1990; Iwata et al., 1995; Tusukihara et al., 1996). Other methods have to be employed to provide structural information which will lead to an understanding of the mechanism of membrane enzymes. Methods have been developed which take advantage of the reactivity of the thiol groups of cysteine residues in the enzyme. Thus, we have previously induced the formation of intersubunit disulfide bonds between cysteine residues of subunits of the *E. coli* transhydrogenase to study the subunit structure of the holoenzyme (Hou et al., 1990).

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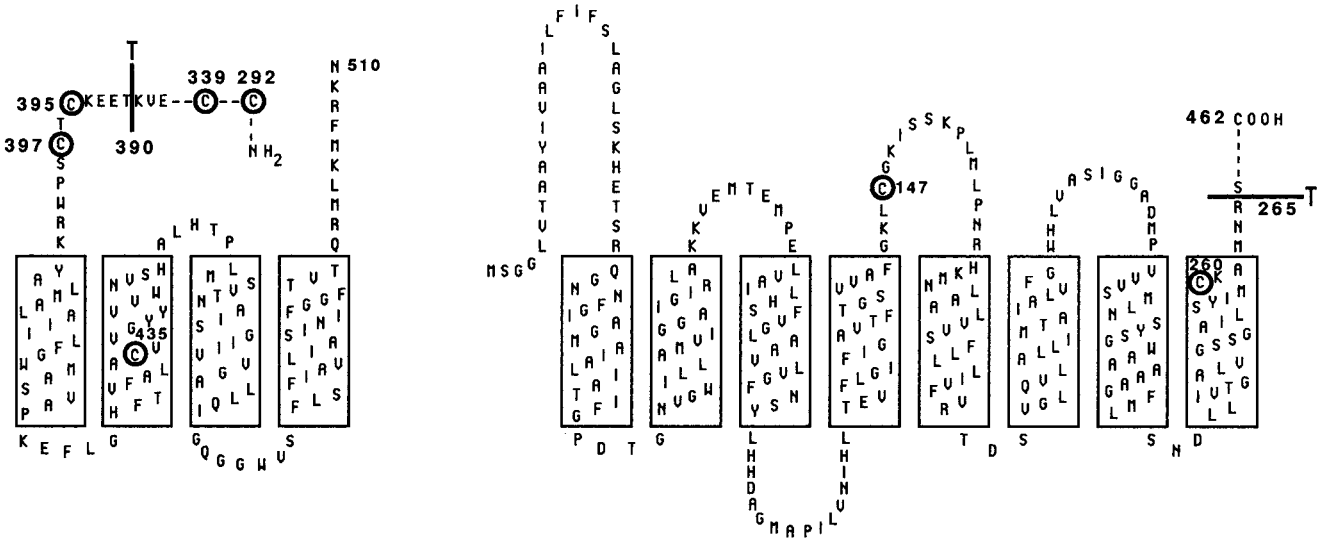

FIGURE 1: Model of the topology of the transmembrane domains of the α- and β-subunits of the *E. coli* transhydrogenase. Cysteine residues are circled and numbered. The positions of tryptic cleavage sites (T) are indicated.

A variety of thiol-directed fluorescent labels have been developed to study structure and conformational changes occurring during the reaction cycle of enzymes (Haugland, 1996). One such reagent is *N*-(1-pyrenyl)maleimide (Haugland, 1970; Weltman et al., 1973). Pyrenylmaleimide is essentially nonfluorescent in aqueous systems until it has reacted with thiols. Pyrene at high concentrations in organic solvents can form excited state dimers (eximers) (Förster & Kaspar, 1955). Pyrenylmaleimide forms eximers after reaction with thiols such as 1-butanethiol (Weltman et al., 1973) or membrane proteins such as the ATPase of sarcoplasmic reticulum (Lüdi & Hasselbach, 1983) and the *E. coli* lactose permease (Jung et al., 1993). Eximers are formed if the pyrene ring systems are within 0.35 nm and in the correct orientation. Thus, the proximity of cysteine residues in pyrenylmaleimide-treated proteins can be determined by the appearance of an eximer band.

The transhydrogenase of *E. coli* contains seven cysteine residues, five in the α-subunit and two in the β-subunit (Ahmad et al., 1992). Previously, we have generated single-site and multiple-site replacements of these cysteine residues, as well as a cysteine-free enzyme (Meuller et al., 1997). In this paper, we describe the use of these mutants in determining which cysteine residues are involved in the formation of disulfide cross-linkages between the two α-subunits. The sites of action of pyrenylmaleimide with the transhydrogenase were determined, as well as the site of substitution which leads to inhibition of enzyme activity. The formation of eximers in the absence of reaction of pyrenylmaleimide with thiol groups was demonstrated as a complicating factor in studies of the action of this reagent on proteins in membranes.

MATERIALS AND METHODS

Materials. Materials were obtained from the following suppliers: Sigma, diphenyl carbamyl chloride-treated trypsin (bovine pancreatic) and soybean trypsin inhibitior; Bio-Rad, electrophoresis reagents; Pharmacia, Sephadex G50 Fine and electrophoresis low-molecular weight standards; and Molecular Probes, 5-doxylstearic acid, 12-doxylstearic acid, and *N*-(1-pyrenyl)maleimide. All other chemicals were of reagent grade purity and were obtained from commercial sources.

Bacterial Strains and Plasmid. The *pnt* gene was introduced into the pGEM-7Zf(+) plasmid, resulting in the construct denoted pSA2. pSA2 was subsequently used to transform the *E. coli* K12 strain JM109 and used as a source of nonmutant and mutant transhydrogenase (Ahmad et al., 1992).

Mutagenesis. *E. coli* transhydrogenase contains seven cysteine residues, i.e., 292, 339, 395, 397, and 435 in the α-subunit and 147 and 260 in the β-subunit. Mutants in the α-subunit were made by PCR mutagenesis (Meuller et al., 1997) using a *Sal*I/*Bst*EII cassette to avoid amplification of the whole gene, in which the cysteine residues were replaced with Ala, Thr, and/or Ser. The most active substitutions were chosen for further manipulations.

The β-subunit cysteines were replaced with Ser as described before (Ahmad et al., 1992). The construct αC292T, αC339T, αC395S, αC397T, αC435S, βC147S, βC260S was made by replacing a *Bst*BI/*Bss*HII restriction fragment of pSA2 lacking α-cysteines, with the corresponding fragment from pSA2 lacking β-cysteines. This construct codes for a transhydrogenase without cysteines, i.e., cysteine-free transhydrogenase (Meuller et al., 1997). Gene sequences of mutants were verified by sequencing the manipulated subfragment of the transhydrogenase gene as well as the whole gene of the final construct.

Preparation of Everted Cytoplasmic Membrane Vesicles Containing Transhydrogenase. Detergent-washed cytoplasmic membrane vesicles were prepared from *E. coli* strain JM109 containing the multicopy mutant plasmids as previously described (Glavas et al., 1995a).

Cross-Linking. Cupric 1,10-phenanthrolinate cross-linking of transhydrogenase was performed as previously described (Bragg & Hou, 1980). Washed membranes were diluted in 50 mM triethanolamine (pH 7.5). Stock solutions of $CuSO_4$ and 1,10-phenanthroline were combined to give a final concentration of 62.5 μM $CuSO_4$ and 125 μM 1,10-phenanthroline in the reaction mixture. Cross-linking was terminated after 3 h at room temperature by addition of 4 mM EDTA and 0.8 mg/mL *N*-ethylmaleimide.

Trypsin Digestion of Transhydrogenase. Washed membranes (3.33 mg/mL) in 50 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$ were digested with 60 units of diphenyl carbamyl chloride-treated trypsin for 1 h at room temperature. The reaction was stopped by addition of soybean trypsin inhibitor at twice the weight of trypsin. The buffer was 0.1 M Mes at pH 6 where indicated.

Fluorescence Spectra of Transhydrogenase Labeled with Pyrenylmaleimide. Washed membranes (5 mg/mL) in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, or 50 mM potassium phosphate buffer (pH 7.5) were labeled with pyrenylmaleimide at molar ratios of probe:protein as indicated in the figure legends. Fluorescence spectra were obtained at room temperature with an SLM Aminco SPF-500C spectrofluorometer at intervals indicated in the figure legends. An excitation wavelength of 338 nm was used.

Labeling of Transhydrogenase with Pyrenylmaleimide for Gel Electrophoresis. Washed membranes (3.33 mg/mL) in 50 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$ were labeled with 0.5 mM pyrenylmaleimide overnight at room temperature or for shorter times as specified in the figure legends. Excess label was removed by centrifugation of the sample through a 1 mL column of Sephadex G50 equilibrated with buffer. Aliquots of the eluted sample were diluted with equal volumes of sample buffer and subjected to SDS−PAGE by the method of Laemmli (1970) using a Bio-Rad mini-protean II gel apparatus. The separating gel concentration was 7.5%, unless indicated otherwise. The gels were photographed under UV irradiation using a UVP Inc. (Cambridge, England) Gel Documentation System prior to staining with Coomassie blue (Fairbanks et al., 1971).

Quenching of Pyrenylmaleimide Fluorescence with Spin-Labels. Washed membranes (0.1 mg/mL) were labeled with pyrenylmaleimide at a molar ratio of 1:1 at room temperature until a maximum fluorescence was achieved. Aliquots of the spin labels 5- or 12-doxylstearic acid (in ethanol) were added sequentially to give the concentrations indicated in the figure legends. Spectra were recorded 1 min after each addition of the spin-label.

Assay of Transhydrogenase Activity. Transhydrogenase activity was measured as the reduction of 3-acetylpyridine NAD^+ by NADPH as described in Glavas et al. (1995a).

RESULTS

Detergent-washed everted cytoplasmic membrane vesicles were used in this study. Proton translocation is efficiently coupled to hydride transfer in these vesicles (Glavas et al., 1995a). Furthermore, the very high levels of enzyme expression from the plasmids encoding the transhydrogenase make this enzyme the predominant protein in the vesicles (Ahmad et al., 1992).

Cross-Linking of Subunits of Transhydrogenase. We showed previously that treatment of membrane-bound transhydrogenase with cupric 1,10-phenanthrolinate catalyzed the formation of intersubunit dimers between α- and β-subunits. Cross-linked dimers α_2, $\alpha\beta$, and β_2 were recognized (Hou et al., 1990). The major cross-link was formed between α-subunits. This cross-linked product formed spontaneously if the membrane vesicles were prepared in the absence of a reducing agent such as dithiothreitol.

Detergent-washed everted cytoplasmic membrane vesicles were prepared from plasmid-containing strains encoding transhydrogenase in which one or more cysteine residues had been replaced by serine or threonine residues. Enzymes with the following mutations were treated with the cross-linking agent cupric 1,10-phenanthrolinate: αC339T, αC435S, αC292TαC339T, αC395SαC397T, αC395SαC397TαC435S, αC292TαC339TαC395SαC397TαC435S, βC147SβC260S, βC147S, βC260S, and cysteine-free. Examination of the cross-linked material on SDS−polyacrylamide gels revealed that the α_2 product (verified by examination on a second-dimension gel following cleavage of the cross-link with 2-mercaptoethanol) (gels not shown) was formed only when both αCys395 and αCys397 were present (Figure 2, lanes 1, 2, 5, and 6). The α_2 dimer was not formed when both of these cysteine residues were mutated (Figure 2, lanes 3, 4, and 7). (Although not shown in Figure 2, but consistent with these data, the α_2 dimer was formed by the mutant enzymes αC339T, αC435S, αC292TαC339T, βC147S-βC260S, βC147S, and βC260S.) It was not formed by mutant enzymes αC395SαC397TαC435S and αC292T-

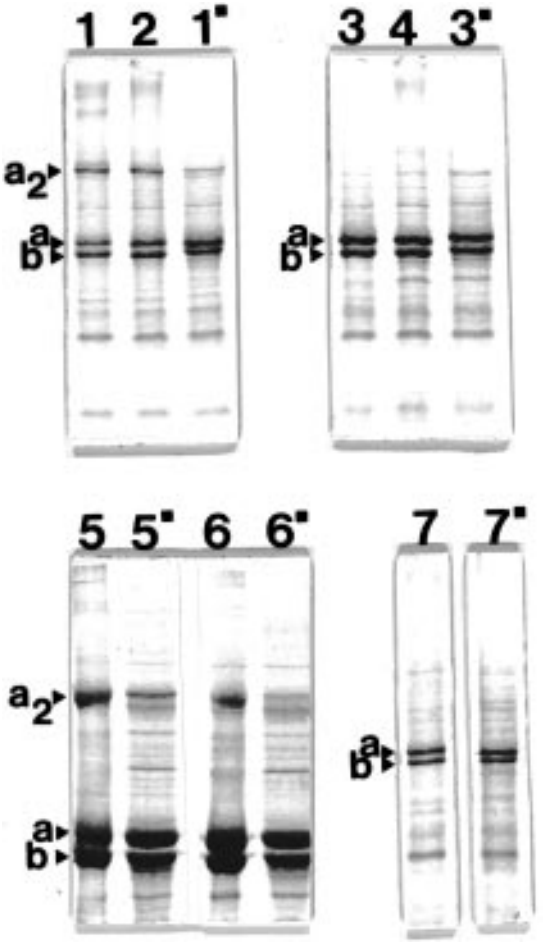

FIGURE 2: Cross-linking of α- and β-subunits of transhydrogenase by cupric 1,10-phenanthrolinate. Cross-linking was carried out as described in Materials and Methods. The samples were separated by SDS gel electrophoresis on a 7.5% (w/v) polyacrylamide gel: lane 1, nonmutant membranes treated with cross-linking reagents; lane 2, nonmutant membranes prepared in the absence of reducing agents; lane 3, cysteine-free membranes treated with cross-linking reagent; lane 4, cysteine-free membranes prepared in the absence of reducing agents; lanes 5−7, membranes of cysteine mutants treated with cross-linking reagent; lane 5, αC395S; lane 6, αC397T; and lane 7, αC395SαC397T. Samples numbered with a superscript black square were run in the presence of 2-mercaptoethanol to reverse cross-linking: a, α-subunit; b, β-subunit; and a_2, α-subunit cross-linked dimer.

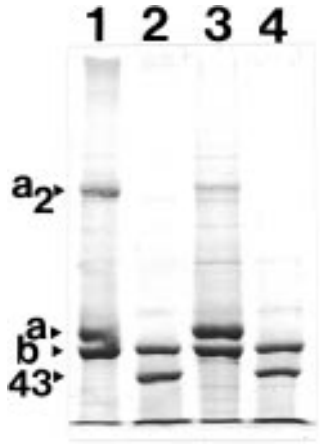

FIGURE 3: Trypsin treatment of cross-linked nonmutant membranes. Cross-linked nonmutant membranes (1 mg/mL) were treated with trypsin at pH 6 as described in Materials and Methods and the samples separated by electrophoresis on SDS−7.5% (w/v) polyacrylamide gels: lanes 1 and 3, nontreated membranes; and lanes 2 and 4, trypsin-treated membranes. The samples in lanes 3 and 4 were treated with 2-mercaptoethanol before electrophoresis: a, α-subunit; b, β-subunit; a_2, α-subunit cross-linked dimer; and 43, 43 kDa tryptic cleavage fragment of α-subunit.

αC339TαC395SαC397TαC435S. The dimer is formed in the single αC395S and αC397T mutants (Figure 2, lanes 5 and 6). These two cysteine residues are in proximity in the amino acid sequence. It is likely that at the interface between the two α-subunits cross-links can be formed between two αCys395, two αCys397, and/or αCys395 and αCys397 residues on different α-subunits. We conclude that the two α-subunits interact in the 395−397 region. This region is close to, but not within, the transmembrane domain of the α-subunit (Figure 1). Trypsin treatment of transhydrogenase releases the extrinsic amino-terminal region of the α-subunit as a 43 kDa fragment (Tong et al., 1991). This fragment contains αCys292 and αCys339. The gel shown in Figure 3 verified that α_2 cross-linking did not involve these residues. Trypsin cleavage did not release a dimer of the 43 kDa fragment. Only the non-cross-linked 43 kDa protein was observed. The carboxyl-terminal fragment of the α-subunit, which should be cross-linked as a dimer, was not detected on the gels. Possibly because of its highly nonpolar nature, we have never been able to reliably detect this tryptic fragment using several different staining methods (Tong et al., 1991).

Reaction of Transhydrogenase with N-(1-Pyrenyl)maleimide. Reaction of pyrenylmaleimide with transhydrogenase was examined by fluorescence spectroscopy. The fluorescence emission spectra for the reaction of pyrenylmaleimide with the nonmutant enzyme showed maxima at 378−379, 397−398, and 419−420 nm (Figure 4, spectra 1−4). At longer times of reaction and with higher ratios of pyrenylmaleimide:transhydrogenase (5:1 and 10:1 molar ratios), a broad emission peak at about 470 nm, typical of the eximer, was formed. The spectroscopic behavior is consistent with the reaction of pyrenylmaleimide with at least two cysteine residues in close proximity. This conclusion was thrown into doubt by the finding that similar peaks were formed by

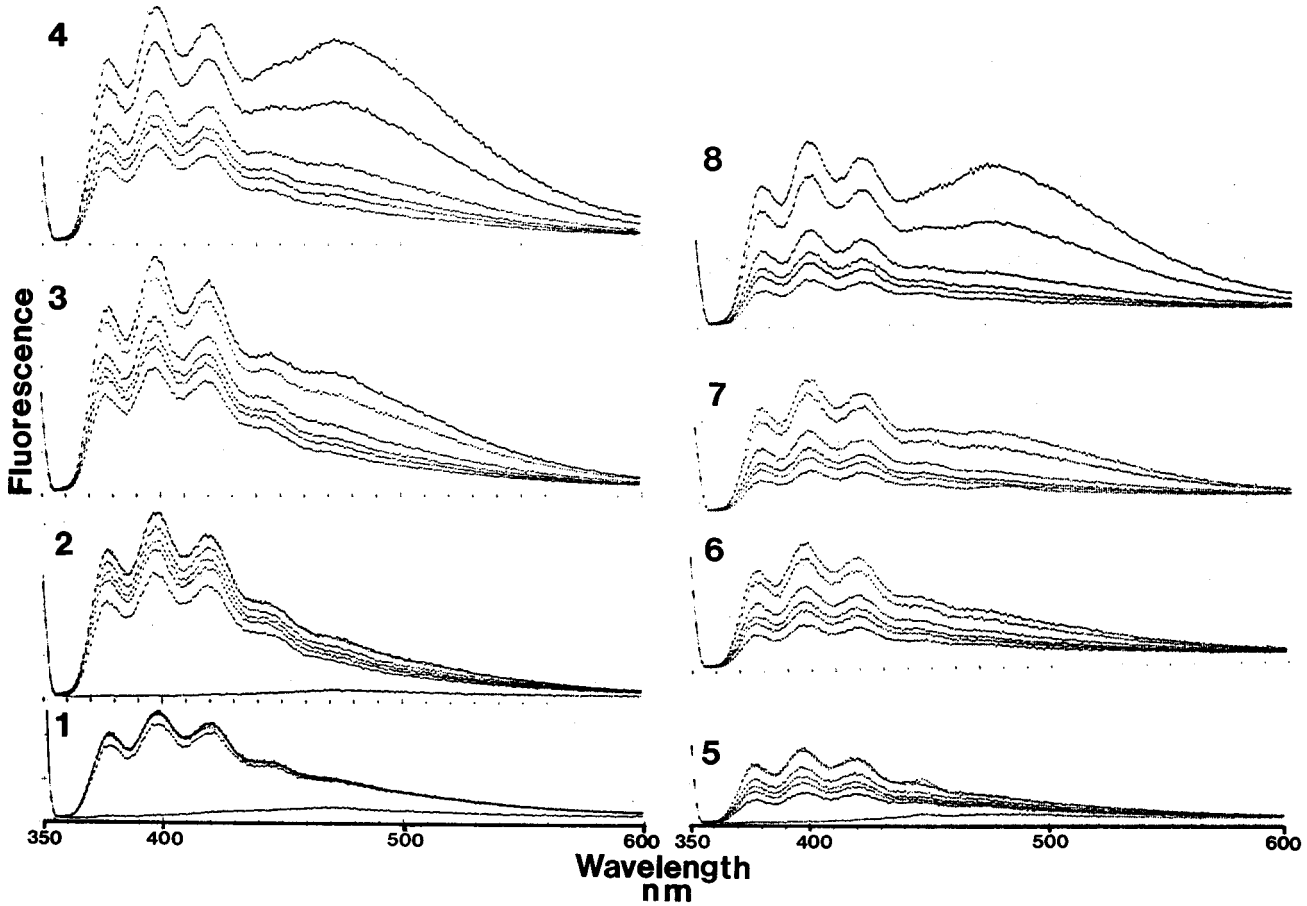

FIGURE 4: Fluorescence emission spectra of pyrenylmaleimide-treated membranes containing nonmutant (panels 1−4) or cysteine-free (panels 5−8) transhydrogenase. The experiment was carried out as described in Materials and Methods. The curves in each panel were recorded 1, 3, 5, 10, 30, and 60 min after addition of pyrenylmaleimide. The molar ratios of pyrenylmaleimide:transhydrogenase were as follows: panels 1 and 5, 1:1; panels 2 and 6, 2.5:1; panels 3 and 7, 5:1; and panels 4 and 8, 10:1.

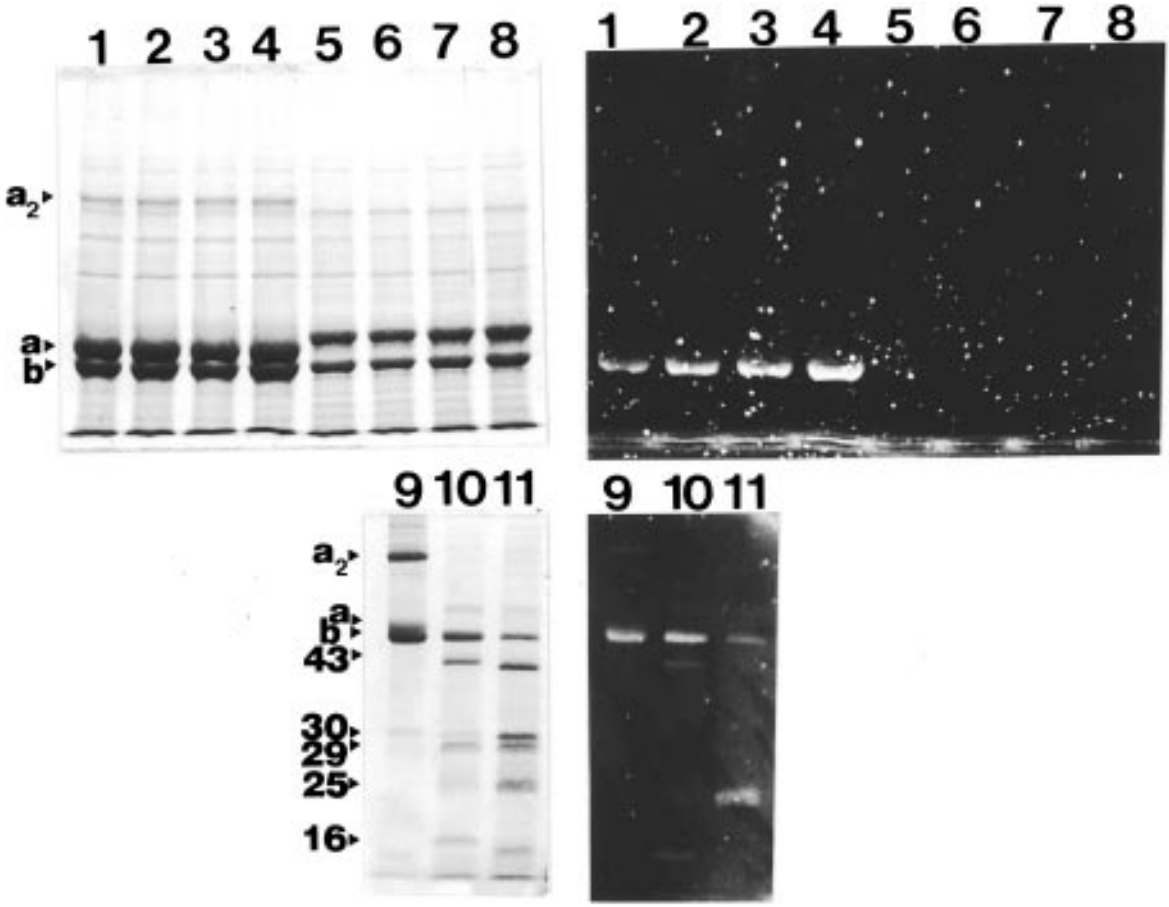

FIGURE 5: SDS−polyacrylamide gels of membranes containing nonmutant and cysteine-free transhydrogenase treated with pyrenylmaleimide. Membrane vesicles containing nonmutant (lanes 1−4) or cysteine-free transhydrogenase (lanes 5−8) were treated with pyrenylmaleimide for 0.5 (lanes 1 and 5), 1 (lanes 2 and 6), 3 (lanes 3 and 7), or 5 (lanes 4 and 8) h as described in Materials and Methods. Excess reagent was removed, and the samples were examined by SDS−polyacrylamide gel electrophoresis. The gels in the left-hand panels were stained with Coomassie blue. In the right-hand panel are the same gels examined under ultraviolet light before staining: lane 9, nonmutant enzyme prepared in the absence of reducing agents; lane 10, nonmutant enzyme prepared in the absence of reducing agents, treated with trypsin, and then with pyrenylmaleimide overnight; lane 11, pyrenylmaleimide-labeled nonmutant enzyme prepared in the absence of reducing agents treated with trypsin in the presence of 0.5 mM NADPH; a, α-subunit; b, β-subunit; a_2, α-subunit cross-linked dimer; and 16−43, tryptic digestion fragments of transhydrogenase. The number indicates the mass of the fragment in kilodaltons. Samples 9−11 were separated on a 12% (w/v) polyacrylamide gel.

the cysteine-free enzyme (Figure 4, spectra 5−8). The latter peaks were formed more slowly than with nonmutant enzyme. This was particularly evident at the lowest molar ratio (1:1) of pyrenylmaleimide:transhydrogenase used. The formation of peaks with the nonmutant transhydrogenase was almost complete within 1 min, whereas that of the cysteine-free enzyme continued over 30 min. The most likely explanation for these data is the fact that the spectra in panel 1 of Figure 4 represent the rapid reaction of pyrenylmaleimide with cysteine residues. The slower development of peaks in panel 5 is probably due to the partitioning of the lipophilic reagent into the membrane lipids or nonpolar domains of the protein. This phenomenon contributes little to the spectra in panel 1 since the available pyrenylmaleimide is rapidly consumed by reaction with thiol groups. However, at higher ratios of pyrenylmaleimide:transhydrogenase, the excess reagent binds noncovalently in the membrane lipid and/or protein. Thus, there is a slow increase in the size of the peaks and the development of the eximer band. Consistent with the explanation above, the cysteine-free enzyme into which βCys147, the residue reacting most readily with pyrenylmaleimide (see later), had been introduced behaved like the nonmutant enzyme with rapid formation of the maxima at 378−379, 397−398, and 419−420 nm (results not shown). The presence of the eximer band in the cysteine-free enzyme is a clear indication that it must originate from the noncovalently bound pyrenylmaleimide dimers. Eximer band formation has been observed at high concentrations of pyrene in organic solvents (Förster & Kaspar, 1955). The reaction of pyrenylmaleimide with vesicles of egg phosphatidylcholine and of synthetic dioleoylphosphatidylcholine was examined. Fluorescence emission maxima were observed at 378, 398, and 420 nm, but there was no formation of an eximer band. Since amino groups are modified by maleimides at alkaline pH values (Means & Feeney, 1971), the reaction of lysine with pyrenylmaleimide at pH 9 was examined. Emission peaks were observed at 391, 410, 432, and 457 nm. The positions of these peaks are different from those obtained with nonmutant or cysteine-free transhydrogenases, phosphatidylcholines, or the glutathione monomer. It is unlikely, therefore, that reaction of pyrenylmaleimide with amino groups accounts for the fluorescence spectra of the cysteine-free enzyme.

Reaction of Pyrenylmaleimide with Transhydrogenase Subunits. Pyrenylmaleimide was reacted with membrane vesicles containing nonmutant and cysteine-free transhydrogenases, and the products of the reaction were examined by SDS−polyacrylamide gel electrophoresis. The presence of

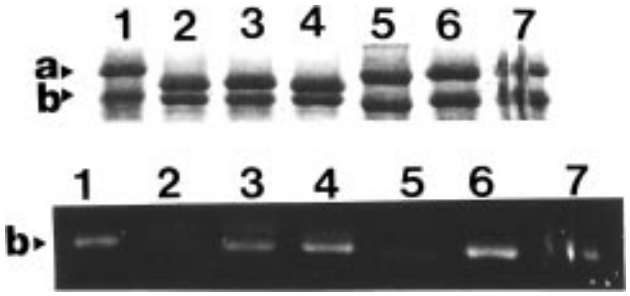

FIGURE 6: Fluorescent labeling of β-subunits of cysteine mutants. The mutant membranes were labeled with pyrenylmaleimide for 3 h as described in Materials and Methods and then submitted to SDS−polyacrylamide gel electrophoresis. The gels were examined under ultraviolet light (lower panel) and then stained with Coomassie blue (upper panel): lane 1, αC292TαC339TαC395SαC397TαC435S; lane 2, βC147SβC260S; lane 3, βC147S; lane 4, βC260S; lane 5, cysteine-free enzyme; lane 6, cysteine-free enzyme with βCys147; lane 7, cysteine-free enzyme with βCys260; a, α-subunit; and b, β-subunit.

the pyrenyl group was detected by examination of the gels under ultraviolet light. As shown in Figure 5 (lanes 1−4), the β-subunit of the nonmutant enzyme was labeled by pyrenylmaleimide. The cysteine-free transhydrogenase was not labeled (Figure 5, lanes 5−8). This result supports the studies described above which suggested that pyrenylmaleimide could also be incorporated noncovalently into membranes. The region of the β-subunit reacting with pyrenylmaleimide in the nonmutant enzyme was determined by cleaving the transhydrogenase with trypsin. Trypsin in the presence of NADP(H) cleaves the β-subunit into a 25 kDa N-terminal transmembrane fragment and a 30 kDa C-terminal extrinsic fragment (Figure 1) (Tong et al., 1991). Cleavage of pyrenylmaleimide-treated enzyme showed that only the 25 kDa transmembrane region was labeled (Figure 5, lane 11). This region contains two cysteine residues (βCys147 and βCys 260) (Figure 1). The cysteine-free 30 kDa region was not labeled. If the enzyme was first cleaved with trypsin and then reacted with pyrenylmaleimide, then the 43 kDa fragment of the α-subunit was labeled, as well as the 16 kDa further cleavage product (Figure 5, lane 10). Presumably, the cysteine residues (αCys292 and αCys339) in the trypsin-cleaved α-subunit were now accessible to pyrenylmaleimide. These cysteine residues would be present in the 16 kDa fragment (Tong et al., 1991).

The preferential reaction of the cysteine residues of the β-subunit with pyrenylmaleimide was confirmed by the use of mutants (Figure 6). The β-subunit was not labeled in mutants in which βCys147 and βCys260 were both absent (lanes 2 and 5). The β-subunit was labeled if either of these cysteine residues was present (lanes 3, 4, 6, and 7). Pyrenylmaleimide appeared to react more readily with βCys147 than with βCys260, as indicated by the intensity of the fluorescent band.

In Figures 5 and 6, there are differences between the mutants in the rate of migration of the α-subunit on SDS−polyacrylamide gels. By examination of a number of mutants (data not shown), we have concluded that the αC435S mutation is responsible for the retardation in the rate of migration of the α-subunit. This difference is not seen if the gels are run in the presence of 3 M urea and 2-mercaptoethanol (J. Mueller and J. Rydström, unpublished data). Thus, it is likely that the αC435S mutation prevents complete unfolding of the α-subunit in SDS.

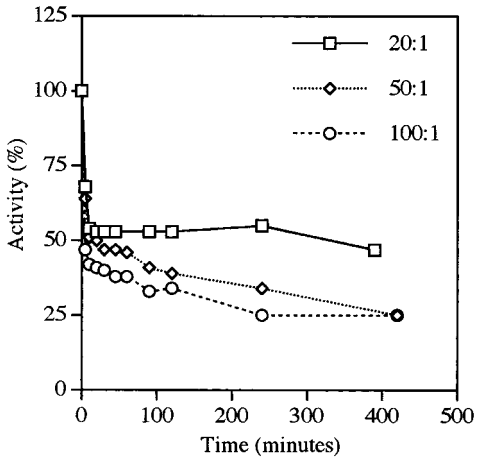

FIGURE 7: Inhibition of nonmutant transhydrogenase activity in membranes treated with various molar ratios (pyrenylmaleimide: transhydrogenase) of pyrenylmaleimide.

Table 1: Inhibition of Mutant Transhydrogenase Activity by Pyrenylmaleimide[a]

mutant	activity (% control value)
Cys-free α	51
βC147S	63
βC260S	95
Cys-free	111
Cys-free/βCys147	96
Cys-free/βCys260	66

[a] The mutant transhydrogenases in membranes were treated with a 100:1 molar ratio of pyrenylmaleimide:transhydrogenase. The activity of the enzyme after 45 min of treatment is expressed as a percentage of the activity of untreated enzyme. The specific activities (micromoles per minute per milligram of protein) of the enzymes in untreated membranes were as follows: Cys-free alpha, 1.1; βC147S, 6.0; βC260S, 3.6; Cys-free, 8.7; Cys-free/βCys147, 9.9; and Cys-free/βCys260, 8.8. "Cys-free α", αC292SαC339TαC395SαC397TαC435S. "Cys-free", αC292SαC339TαC395SαC397TαC435SβC147SβC260S. "Cys-free/βCys147" and "Cys-free/βCys260", enzymes in which all cysteine residues had been replaced by serine or threonine residues with the exception of βCys147 or βCys260, respectively.

Inhibition of Transhydrogenase Activity by Pyrenylmaleimide. The reduction of 3-acetylpyridine NAD^+ by NADPH in membrane vesicles of nonmutant transhydrogenase was inhibited by pyrenylmaleimide. Although 50% of the activity was lost readily, higher concentrations of pyrenylmaleimide were required for further inhibition (Figure 7). The cysteine-free enzyme was not inhibited (Table 1), suggesting that inhibition of activity was due to covalent modification of cysteine residues. Similar results were obtained previously with *N*-ethylmaleimide (Meuller et al., 1997). Enzyme sensitivity to inhibition by pyrenylmaleimide was retained by transhydrogenase in which all cysteine residues of the α-subunit had been mutated (Table 1), indicating that modification of a cysteine residue of the β-subunit was responsible for inhibition. This residue was shown to be βCys260 since mutation of this cysteine to a serine residue gave transhydrogenase which was resistant to inhibition by pyrenylmaleimide. Furthermore, introduction of βCys260 into the cysteine-free transhydrogenase restored sensitivity to the inhibitor (Table 1). Mutation of βCys147 did not provide resistance to inhibition of transhydrogenase activity by pyrenylmaleimide.

Quenching of the Fluorescence of Pyrene-Labeled Transhydrogenase by Spin-Labeled Fatty Acids. Fluorescence quenching by spin-labeled fatty acids has been used to

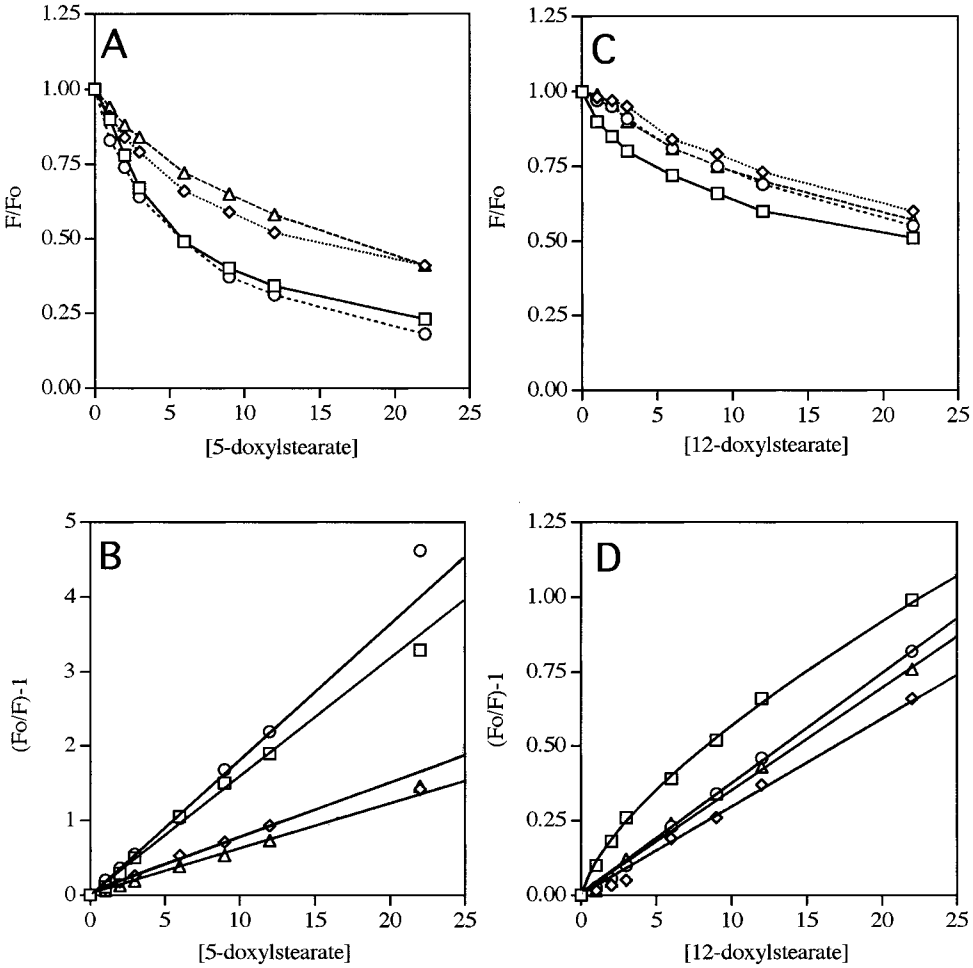

FIGURE 8: Quenching of the fluorescence of pyrenylmaleimide-treated membranes containing nonmutant and mutant transhydrogenases by 5- and 10-doxylstearic acids. The experiments were carried out as described in Materials and Methods: F_o, fluorescence intensity before addition of the quencher; F, fluorescence intensity after addition of the quencher; □, nonmutant enzyme; ◇, cysteine-free enzyme; ○, cysteine-free enzyme with βCys147; △, cysteine-free enzyme with βCys260. The concentration of the quencher is expressed in micromolar.

estimate the depth of a fluorophore within the membrane bilayer (Mitra & Hammes, 1990). 5- and 12-doxylstearic acids are stearic acid molecules in which the doxyl spin-label is linked at the indicated region of the chain. Thus, these spin-labels probe the lipid bilayer at different depths from the membrane surface.

Stern−Volmer or dynamic collisional quenching obeys the equation

$$F_o/F = 1 + K[Q]$$

where F_o and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration of the quencher, and K is the Stern−Volmer quenching constant (Lacowicz, 1983). Figure 8(panels A and C) shows the extent of quenching by 5- and 12-doxylstearic acid of pyrene-labeled nonmutant, cysteine-free, and cysteine-free transhydrogenases containing either βCys147 or βCys260. The fluorescence of membrane vesicles containing the cysteine-free enzyme was quenched by both doxylstearates. In this case, the fluorophore was pyrenylmaleimide which was dissolved in the lipid phase and/or noncovalently bound to protein. Cysteine-free transhydrogenase containing βCys260 showed behavior similar to that of the cysteine-free enzyme. This suggests that pyrene-labeled βCys260 was not accessible to the quenchers and that its apparent response was due to quenching of noncovalently bound pyrenylmaleimide. By contrast, nonmutant transhydrogenase and cysteine-free transhydrogenase containing βCys147 showed a greater degree of quenching by 5-doxylstearate. This was attributed to pyrene-labeled βCys147. The small response of the enzymes to 12-doxylstearate (Figure 8C) was consistent with the pyrene group attached to βCys147 being close to the membrane surface. The data of Figure 8A,C were plotted to determine Stern−Volmer constants (Figure 8B,D). Linear plots were obtained with both 5- and 12-doxylstearate with the exception being that 12-doxylstearate quenching of the fluorescence of pyrene-labeled nonmutant enzyme gave a curved plot (Figure 8D). This indicated that a fraction of the fluorophore was inaccessible to the quencher. The data for this case were replotted according to the equation

$$F_o/(F_o - F) = 1/(f_a K[Q]) + 1/f_a$$

where K is the quenching constant of the accessible fraction and f_a is the fraction of the initial fluorescence accessible to quencher (Lacowicz, 1983). A straight line was obtained for f_a. The quenching constants (μM^{-1}) for 5-doxylstearic acid (values for 12-doxylstearic acid are in parentheses) were as follows: nonmutant enzyme, 0.16 (0.17); cysteine-free enzyme, 0.072 (0.030); cysteine-free enzyme with βCys147, 0.18 (0.037); and cysteine-free enzyme with βCys260, 0.061 (0.035).

DISCUSSION

The pyridine nucleotide transhydrogenase of *E. coli* contains seven cysteine residues (Figure 1). We have used mutants in which one or more cysteine residues of the transhydrogenase were replaced by serine or threonine residues (Meuller et al., 1997) to study the structure and mechanism of the enzyme. Transhydrogenase exists as an $\alpha_2\beta_2$ tetramer (Hou et al., 1990). Mutation of αCys395 and αCys397 together prevented the formation of disulfide-linked α-subunit dimers on cross-linking with cupric 1,10-phenanthrolinate or spontaneously in the absence of a reducing agent such as dithiothreitol. This result is consistent with there being an interface between two α-subunits in the αCys395−αCys397 region. We have not at present identified the cysteine residues which contribute to the formation of disulfide-linked $\alpha\beta$ and β_2 species in the presence of cupric 1,10-phenanthrolinate.

N-(1-Pyrenyl)maleimide has been used extensively to study the structure and mechanism of various proteins (Haugland, 1996). It can be used as an inhibitor to monitor conformational changes as shown by changes in the fluorescence of pyrene-labeled proteins, and for structural studies. The presence of eximer bands in emission spectra of pyrenylmaleimide-treated proteins has been taken as evidence that two pyrene groups are within 0.35 nm. This implies that the cysteine residues to which these pyrene groups are attached are in proximity (Betcher-Lange & Lehrer, 1978; Ishii & Lehrer, 1987; Lüdi & Hasselbach, 1988; Sen & Chakrabarti, 1990; Wang et al., 1992; Jung et al., 1993). When we attempted to apply this procedure to the membrane-bound pyridine nucleotide transhydrogenase of *E. coli*, it became apparent that any eximer band which was formed as a consequence of the modification of two neighboring cysteine residues would be obscured by an eximer band arising from noncovalently bound pyrenylmaleimide. The location of the molecules giving rise to the latter band was not established with certainty. Pyrenylmaleimide equilibrates into phospholipid vesicles but does not give significant eximer formation. Eximers could be a consequence of pyrenylmaleimide molecules adsorbing within nonpolar regions of the transhydrogenase protein or at the protein/membrane lipid interface. Lux and Gerard (1981) observed that bovine serum albumin adsorbed significant amounts of pyrenylmaleimide [2−8 mol/(mol of protein)]. An eximer band was not observed. However, serum albumin is not a "typical" protein in the sense that its biological role is to adsorb and transport nonpolar substances. Pyrenylmaleimide was likely bound to these binding sites.

Covalent labeling of transhydrogenase primarily involved the two cysteine residues of the β-subunit (βCys147 and βCys260). Reaction of pyrenylmaleimide with βCys260, but not with βCys147, resulted in the inhibition of transhydrogenase activity. Only a few other sites where covalent modification of transhydrogenases leads to inhibition of enzyme activity have been identified. Thus, covalent modification of tyrosine residues in the beef-heart mitochondrial enzyme, equivalent to αTyr226 and βTyr431 of the *E. coli* transhydrogenase, with 5′-(*p*-fluorosulfonylbenzoyl)-adenosine (Yamaguchi et al., 1988; Wakabayashi & Hatefi, 1987) and of βTyr431 by 8-azidoadenosine 5′-monophosphate (Hu et al., 1992) caused inhibition. Treatment of the *E. coli* transhdyrogenase with *N*,*N*′-dicyclohexylcarbodiimide resulted in covalent modification of αAsp232, αGlu238, and αGlu240, and inhibition of activity. All of these residues likely form part of the binding sites for NAD(H) on the α-subunit and NADP(H) on the β-subunit (Olausson et al., 1993; Fjellström et al., 1995). βCys260 is unlikely to be part of a substrate-binding site. Mutation of this residue to a serine residue has a marginal effect on activity (Holmberg et al., 1994). Thus, loss of activity in the pyrenylmaleimide-treated enzyme must be due to the presence of the bulky pyrene/maleimide group.

Pyrenylmaleimide reacted more strongly with βCys147 than with βCys260, as judged from the intensity of fluorescence labeling of the β-subunit on SDS−polyacrylamide gels. This may indicate that βCys147 is more accessible than βCys260 to the reagent. This would agree with our previous data (Glavas et al., 1995b) which showed that βCys147 could react with 3-(*N*-maleimidylpropionyl)biocytin whereas βCys260 was nonreactive. Furthermore, the fluorescence of pyrene-labeled βCys147 was more extensively quenched by 5-doxylstearate than was that of pyrene-labeled βCys260. Thus, it is likely that βCys260, in contrast to βCys147, is not exposed to the surrounding membrane phase. This notion is supported also by the highly conserved region associated with βCys260 which is not typical for membrane-facing residues. In the folding model for the N-terminal region of the β-subunit shown in Figure 1, βCys147 is located on an interhelical loop. It is conceivable that folding of this loop could bring the pyrenyl substituent of βCys147 sufficiently close to the membrane surface to permit insertion of the pyrene into the membrane where it could interact with 5-doxylstearate. Alternatively, the transmembrane helix (β4) might extend to include this residue. This seems less likely due to the presence of a lysine residue (βLys145) close to βCys147. Lysine residues are known to terminate transmembrane α-helices (von Heijne, 1986, 1992). The lack of significant reaction of the cysteine residues of the α-subunit with pyrenylmaleimide is likely due to their inaccessibility to this reagent. This is surprising since αCys435 is in a nonpolar environment. However, αCys395 and αCys397 are probably protected from reaction at the α/α interface. αCys292 and αCys339 only react with the reagent following the release of 43 and 16 kDa fragments of the transhydrogenase by trypsin.

In summary, the two α-subunits in the $\alpha_2\beta_2$ structure of the transhydrogenase interface in the region of αCys395−αCys397. Pyrenylmaleimide covalently modifies βCys147 and βCys260 of the seven cysteine residues of the transhdyrogenase. βCys147 is modified more readily than βCys260, but reaction with the latter results in inhibition of enzyme activity. The formation of an eximer band in the emission spectrum of pyrenylmaleimide-treated membrane-bound transhydrogenase cannot be used to determine the proximity of cysteine residues within the transhydrogenase molecule.

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