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ORGANIZATION IN THE MEMBRANE OF THE N-TERMINAL PROTON-TRANSLOCATING DOMAIN OF THE β SUBUNIT OF THE PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF *ESCHERICHIA COLI*

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SUMMARY: The proton-translocating transmembrane pyridine nucleotide transhydrogenase of *Escherichia coli* is composed of two types of subunits, α and β. The β subunit has several membrane-spanning segments in the N-terminal region followed by a cytosolic C-terminal domain bearing a binding site for NADP(H). The N-terminal region contains at least one residue involved in the process of transmembrane proton translocation. Using site-directed mutagenesis cysteine residues were introduced at selected sites into the N-terminal region of the β subunit. The pattern of labelling of these residues with 3-(N-maleimidyl propionyl)biocytin and other sulfhydryl reagents has shown that a model in which the N-terminal region of the β subunit spans the membrane in eight segments is more likely than a previously proposed six segment model (Holmberg et al. (1994) Biochemistry 33, 7691-7700). The preferred model accounts for the site of labelling of a glutamate residue (Glu124) in the N-terminal domain by N,N'-dicyclohexylcarbodiimide.

Pyridine nucleotide transhydrogenase, found in the cytoplasmic membrane of *Escherichia coli* and in the inner mitochondrial membrane, catalyzes transmembrane proton translocation coupled to transfer of a hydride ion equivalent between NAD and NADP [1-7]. Thus, this enzyme belongs to the bioenergetically important family of transmembrane proton pumps [8]. The relatively simple structure of the transhydrogenase makes it an attractive system in which to study the mechanism of proton pumping. In *E. coli* the enzyme is composed of two subunits, α (510 residues) and β (462 residues) organized as an $\alpha_2\beta_2$ tetramer [9,10]. The α subunit has a large N-terminal cytosolic domain followed by four

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CAPS, 3-cyclohexylamino-1-propane sulfonic acid; DCCD, N,N'-dicyclohexylcarbodiimide; IANBD, 4-[N-(iodoacetoxy)ethyl-N-methyl]amino-7-nitrobenzo-2-oxa-1,3-diazole; MIANS, 2[(4'-maleimidyl)anilino]naphthalene-6-sulfonic acid; MPB, 3-(N-maleimidylpropionyl) biocytin; NBT, nitro blue tetrazolium; PVDF, polyvinyl difluoride; SDM, stilbenedisulfonate maleimide; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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membrane-spanning hydrophobic domains [9,11]. The β subunit has six [12] or eight [11] membrane-spanning segments in the N-terminal region followed by a cytosolic C-terminal domain [9,11]. The catalytic sites for NAD(H) and NADP(H) lie within the cytosolic domains of the α and β subunits, respectively [10,13-17].

We have shown recently that His-91 in the transmembrane domain of the β subunit is involved in the mechanism of proton translocation [12,17]. To facilitate further investigation of the mechanism of proton pumping, it is important to establish the topology of this region. In this paper we have investigated the two potential models [11,12] for the arrangement of the amino acids in the N-terminal transmembrane domain of the β subunit shown in Fig. 1. Cysteine residues have been introduced by site-directed mutagenesis at selected points in this domain of the β subunit. The pattern of labelling of these residues with 3-(N-maleimidyl propionyl)biocytin (MPB) and other sulfhydryl reagents favours the eight membrane-spanning segment model to that with six segments (Fig. 1). Furthermore, the preferred model accounts for the site of labelling of a glutamate residue (Glu124) in the β subunit by N,N'-dicycohexylcarbodiimide.

MATERIALS AND METHODS

Materials.

PVDF membrane and streptavidin-alkaline phosphatase conjugate were from Bio-Rad. SDM, MPB, MIANS and IANBD were from Molecular Probes. NBT and BCIP were from Sigma. [^{14}C]DCCD (54 mCi/mmol) was from Amersham.

Isolation of washed membrane vesicles.

E coli JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ$^-$ Δ(lac-proAB) [F' traD36 proAB lacI^{q}ZΔM15]) [18] containing the multicopy plasmid pSA2 (or another mutant plasmid) [10] was grown for 16 hours in a medium of 1% bactotryptone, 0.5% yeast extract, 1% NaCl, and 0.1 mg/mL ampicillin. Site-directed mutants were isolated as described previously [10]. The cells were harvested by centrifugation and washed by resuspension in 0.9% NaCl. Cell pellets were resuspended in buffer A (50 mM Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA) at 1 g wet weight/5 mL. All subsequent steps were performed at 0-4°C. The cells were lysed by passage through an AMINCO French Pressure Cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12000 g for 10 minutes. The supernatant was centrifuged at 252000 g for 2 hours and the membrane pellet was suspended in buffer A at 1 g wet weight/5mL. This procedure gives inside-out membrane vesicles. The membrane vesicles were further purified as follows. Membrane vesicles (1.5 mL) were layered on a 6 mL sucrose cushion (45% sucrose (w:w) in buffer A) and centrifuged in a Beckman 65 fixed angle rotor at 40000 rpm (139000 g) for 1 hour. The outer membrane fraction pelleted to the bottom of the tube and was discarded. The inside-out membrane vesicles banded at the interface. They were diluted two-fold with buffer A. Triton X-100 was added to 1%, the suspension was stirred for 5 minutes, and then centrifuged at 218000 g for 1 hour. The pellet was resuspended in buffer A and sodium cholate was added to 50 mM. The suspension was stirred for 5 minutes and then centrifuged at 218000 g for 1 hour. The washed membrane pellet was suspended in buffer A.

Labelling of mutants with SDM and MPB.

Washed membranes of wild-type or mutant transhydrogenase were taken up in 50 mM Tris-HCl buffer, pH 7.8 at a concentration of 5 mg protein/mL. Membranes were treated with 2 mM SDM for 2 hours at room temperature. The reaction was terminated by centrifuging the SDM-labelled membranes through 1 mL columns of Sephadex G50 equilibrated in 50 mM Tris-HCl, pH 7.8. Untreated membranes were treated similarly. Both untreated and SDM-treated membranes were then labelled with 0.25 mM MPB (10 mM stock in dimethyl sulfoxide) for 30 minutes at room temperature. The labelling was stopped by adding 20 mM DTT. The membranes were then digested with trypsin (1:100 trypsin:transhydrogenase weight ratio) in the presence of 0.5 mM NADPH for 1 hour. Digestion was stopped by adding soybean trypsin inhibitor at a weight twice that of the trypsin. SDS-PAGE (12% (w/v) acrylamide) was performed as described previously [11]. The gels (10 μg protein/well) were either stained with Coomassie blue or electroblotted onto PVDF membrane (2 μg protein/well). In some cases the membranes were labelled with MPB in the presence of SDS. First, SDM labelling was performed as above. Excess SDM was then removed through columns of Sephadex G50 and the membranes were digested with trypsin in the presence of NADPH (as above). After digestion, SDS was added to 0.1% (w/v), followed by labelling with 0.25 mM MPB for 30 minutes at room temperature. The labelling was stopped with 20 mM DTT and SDS-PAGE was run as before.

Electroblotting and staining of PVDF membrane with streptavidin-alkaline phosphatase reagents.

Immediately after electrophoresis, gels to be blotted were soaked in blotting buffer (10 mM CAPS-NaOH buffer, pH 11, 10% methanol) for a few minutes and then electroblotted onto a PVDF membrane in a Bio-Rad transblot cell for 1 hour at a constant voltage of 100 volts. The PVDF membrane was soaked in TBS buffer (20 mM Tris-HCl pH 7.4, 0.9% NaCl) containing 0.5% (v/v) Tween-20 for at least 1 hour. The membrane was washed three times with TBS/0.2% Tween-20 and then incubated for 1 hour with a 1/2000 dilution of streptavidin-alkaline phosphatase conjugate (Bio-Rad) in TBS/0.2% Tween-20. The membrane was washed three times with TBS/0.2% Tween-20 and then equilibrated for 5 minutes in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The developing solution contained 132 μL of 61 mM NBT (in 70% N,N-dimethylformamide) and 66 μL of 135 mM BCIP (in H_2O) in 20 mL alkaline phosphatase buffer. The bands were developed for approximately 2 minutes. Development was stopped with 20 mM EDTA in TBS.

Labelling with MIANS and IANBD.

Wild-type or mutant washed membranes (2 mg protein/mL in 50 mM Tris-HCl, pH 7.8) were labelled with 1 mM MIANS or IANBD (50 mM stock solution in N,N-dimethylformamide) for 2 hours at room temperature. The reaction was stopped by centrifugation of the membrane suspension through 1 mL columns of Sephadex G50 equilibrated with 50 mM Tris-HCl, pH 7.8. The labelled transhydrogenase was then digested with trypsin in the presence of 0.5 mM NADPH for 30 minutes as above. SDS-PAGE was performed. The gels were fixed in 40% methanol/10% acetic acid and viewed under 254 nm UV light to detect labelled proteins before staining.

Labelling with [^{14}C]DCCD.

Washed membranes of wild-type or mutant transhydrogenases at 1 mg/mL in buffer A were labelled with 0.05 mM [^{14}C]DCCD (54 mCi/mmol) for 1 hour at room temperature. The DCCD was removed by centrifugation through Sephadex G50 in buffer A. The labelled transhydrogenase was either left undigested or was digested with trypsin in the presence of 0.5 mM NADPH for 30 min as described above. SDS-PAGE was performed on the samples. The Coomassie blue-stained gels were exposed to Kodak XAR5 film for 5 days.

RESULTS AND DISCUSSION

As discussed in the Introduction, the topology of the proton-translocating N-terminal domain of the β subunit of the transhydrogenase of *E. coli* is uncertain. Two models for this

region have been proposed [11,12] (Fig. 1). The following experiments have been performed to distinguish between them. The transhydrogenase in washed inside-out membrane vesicles was cleaved into distinct domains by trypsin in the presence of NADPH (Fig. 2). The 43, 29 and 16 kDa fragments originated from the α subunit cytoplasmic domain [11]. The α subunit transmembrane domain (13 kDa) could not be located on SDS-PAGE, although the site of trypsin cleavage has been found to be αLys390 (Glavas and Bragg, unpublished results). The β subunit was cleaved into the 30 kDa cytoplasmic domain and the 25 kDa transmembrane domain [11]. The trypsin cleavage site which generates the 25 kDa transmembrane fragment is shown in Fig. 1 and occurs on the cytoplasmic side of the membrane. The 25 kDa fragment has been N-terminally sequenced and corresponds to the N-terminal transmembrane domain of the β subunit (Glavas and Bragg, unpublished results).

Labelling with sulfhydryl reagents.

The topology of the potential transmembrane segments of the 25 kDa fragment was studied by labelling with reagents specific for cysteine residues. The β subunit contains only two cysteine residues, βCys147 and βCys260, both in the 25 kDa transmembrane domain.

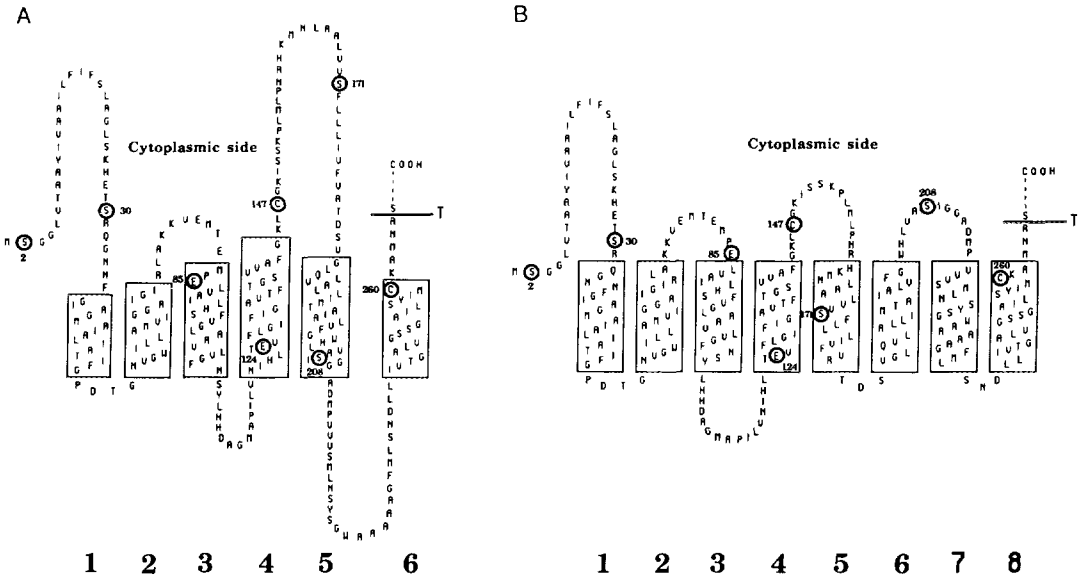

Fig. 1. Models of the topology of the N-terminal transmembrane domain of the β subunit of *E. coli* transhydrogenase. The relevant residues are circled and numbered. The position of the tryptic cleavage site (T) is indicated. A. Model of Holmberg et al. [12]. B. Based on the model of Tong et al. [11].

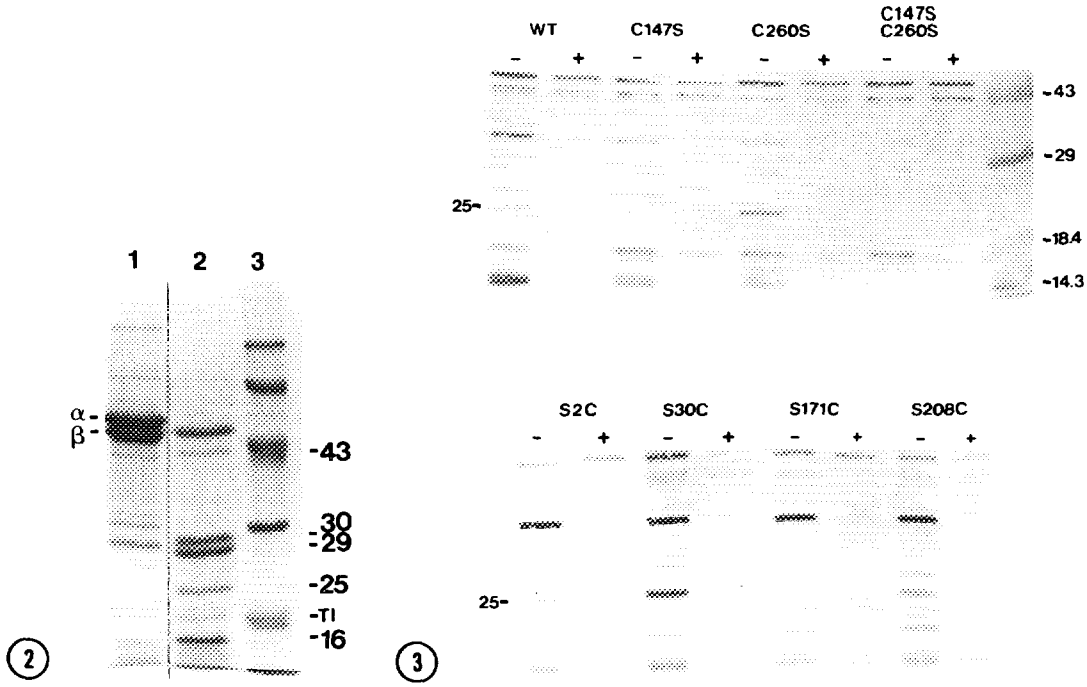

Fig. 2. Tryptic cleavage of transhydrogenase in washed membrane vesicles in the presence of NADPH. Lane 1, undigested transhydrogenase. Lane 2, tryptic digestion of transhydrogenase. Lane 3, Pharmacia low molecular weight standards, 94, 67, 43, 30, and 20.1 kDa. The α and β subunits, as well as the tryptic fragments (in kDa) in lanes 1 and 2, are indicated. TI, trypsin inhibitor.

Fig. 3. Labelling of mutant transhydrogenases with MPB with (+) or without (-) previous protection by SDM. Reaction conditions are as outlined in Materials and Methods. WT (wild-type) and the various mutant transhydrogenases are indicated. The far right lane of the upper panel contains GIBCO BRL prestained protein markers with the indicated molecular masses (kDa). The position of the 25 kDa transmembrane fragment of the β subunit is indicated. Note that the βS2C, βS30C, βS171C and βS208C mutations are in the βC147SC260S genetic background.

We have generated a series of cysteine mutants where the cysteine residue occurs in the positions shown in Fig. 1. βC147S contains one cysteine in the β subunit at position 260 in helix 6 (Fig. 1A) or helix 8 (Fig. 1B). βC260S contains one cysteine at position 147 in the cytoplasmic loop between helices 4 and 5 in both models. βC147SC260S has no cysteine residues in the β subunit. Using the mutant βC147SC260S, new cysteines were introduced into various positions in the N-terminal transmembrane domain of the β subunit (see Fig. 1). βS2C and βS30C are in the N-terminal cytoplasmic loop in both models. βS171C occurs between helices 4 and 5 of Fig. 1A or in helix 5 of Fig. 1B. βS208C occurs in helix 5 of Fig. 1A or in the cytoplasmic loop between helices 6 and 7 of Fig. 1B. All mutant transhydrogenases were incorporated at normal levels into the membrane and were active

(49-86% of wild-type activity), although βS30C had 26% of wild-type activity and was slightly less well expressed than the wild-type transhydrogenase. Washed membranes of the mutants were labelled with MPB, a biotin-maleimide reagent, with or without previous protection by SDM, stilbenedisulfonate maleimide (Fig. 3). SDM is a polar reagent with a charge of -2. It will not dissolve in the membrane and is therefore expected to label cysteine residues on the cytoplasmic side of the membrane only. After SDM protection, biotin-maleimide was used to label any unreacted cysteine residues. The pattern of labelling of the 25 kDa fragment shown in Fig. 3 supports the model proposed in Fig. 1B. (Note that the other bands shown in Figs. 3 and 4 represent cysteine containing fragments of the α subunit of the transhydrogenase as well as some impurities which are detected by the very sensitive streptavidin-alkaline phosphatase labelling procedure.) The 25 kDa fragments of the βC260S (i.e. having βCys147), βS2C, βS30C and βS208C mutants were labelled by biotin-maleimide and the extent of labelling was decreased by SDM. Therefore, these residues must occur on the cytoplasmic side of the membrane. As expected the cysteine-free 25 kDa fragment of the βC147SC260S mutant was not labelled. The 25 kDa fragments of the βC147S (i.e. having βCys260) and βS171C mutants were not labelled showing that these cysteine residues were inaccessible to the biotin-maleimide. However, βCys260 of the mutant βC147S became accessible to MPB labelling in the presence of SDS, although βS171C did not (Fig. 4). SDS presumably denatured the 25 kDa transmembrane domain exposing the βCys260 to labelling by biotin-maleimide. The labelling was not decreased by SDM pretreatment. These results suggest that βCys260 is located within the membrane. βCys171 in the βS171C mutant was

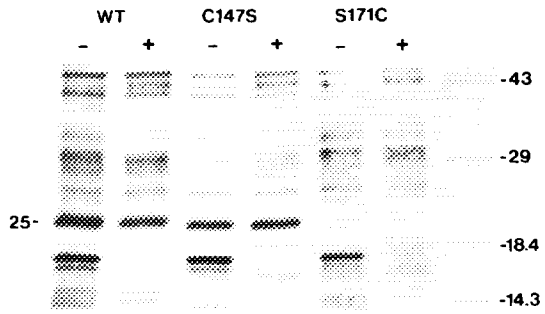

Fig. 4. Labelling of mutant transhydrogenases with MPB in the presence of SDS with (+) or without (-) previous protection by SDM. See legend to Fig. 3. The right-hand lane contains protein markers of the indicated molecular masses.

not labelled by biotin-maleimide even in the presence of SDS. This suggests that this residue must also be within the membrane but in a position where even the presence of SDS did not make it accessible to biotin-maleimide.

MIANS and IANBD are two reagents that will also label cysteine residues. MIANS is charged and is expected to label only those cysteine residues that are in a polar environment, while IANBD is more hydrophobic and should be able to dissolve in the membrane and label cysteines in a hydrophobic environment as well as those in a polar environment. Washed membranes of wild-type and mutants were treated with these reagents and labelling was observed by viewing the unstained gel under UV light. The results are summarized in Table 1. MIANS gave the same pattern of labelling as MPB, reacting with those residues which are on the cytoplasmic side of the membrane in model Fig. 1B. IANBD was able to label βCys260 in the mutant βC147S but not βS171C which again appears to be inaccessible.

DCCD labelling.

It has been previously shown that [^{14}C]N,N'-dicyclohexylcarbodiimide (DCCD) will label both α and β subunits of *E. coli* transhydrogenase. The α subunit labels near the NADH binding site at Asp232, Glu238 and Glu240 [14]. The β subunit was labelled strongly

Table 1: Labelling of the 25 kDa N-terminal transmembrane domain of the β subunit by MIANS and IANBD

Mutant	MIANS	IANBD
wild-type	+	+
βC147S	-	+
βC260S	+	+
βC147SC260S	-	-
βS2C	+	+
βS30C	+	+
βS171C	-	-
βS208C	+	+

Conditions are as indicated in Materials and Methods. Note that the βS2C, βS30C, βS171C and βS208C mutations are in the βC147SC260S genetic background. +, labelled; -, not labelled.

in the 25 kDa fragment although the site of labelling was not determined [17]. This transmembrane domain of the β subunit is extremely hydrophobic and attempts to separate and sequence labelled fragments of it were unsuccessful. Since DCCD reacts with aspartic and glutamic acid residues in hydrophobic environments, the site of DCCD labelling was examined by mutation of these residues to demonstrate lack of DCCD labelling in the 25 kDa fragment. The mutants βE82Q, βE82K, βD213N, βD213H [12], βE85Q, the triple mutant βE79QE82QE85Q, and βE124Q were examined for labelling by [^{14}C]DCCD. The transhydrogenase in all of these mutants was incorporated into the membrane, had activity and was able to translocate protons. Fig. 5 shows the [^{14}C]DCCD labelling pattern of the βE79QE82QE85Q and βE124Q mutants compared to wild-type transhydrogenase (other mutants not shown). The α and β subunits were labelled in all of the mutants except for the βE124Q mutant in which only the α subunit was labelled. Therefore the likely site of DCCD labelling in the β subunit is βGlu124. The position of DCCD labelling supports the model in Fig. 1B since in this model βGlu124 is the only acidic residue within the membrane whereas βGlu85 is also within the membrane in the model Fig. 1A. Since this residue is not conserved in the bovine transhydrogenase [18], and the mutant retains 31% of wild-type catalytic activity and 100% of wild-type proton pumping activity, it is not essential for transhydrogenase activity.

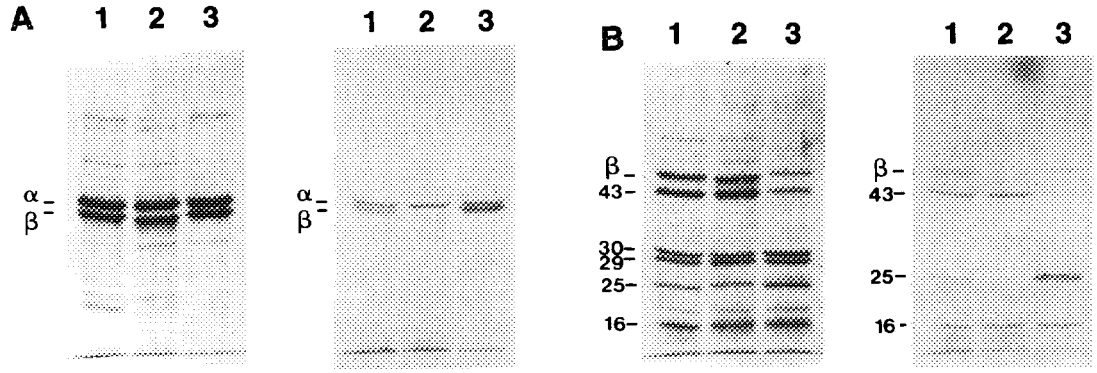

Fig. 5. [^{14}C]DCCD labelling of wild-type (1), βE124Q (2), and βE79QE82QE85Q (3) transhydrogenases in washed membrane vesicles. The experiment was carried out as described in Materials and Methods. A, uncleaved enzyme; B, trypsin-cleaved enzyme. The position of migration of the α and β subunits and of the fragments (in kDa) resulting from trypsin cleavage are indicated. Left-hand panels, Coomassie blue stained gels. Right-hand panels, autoradiographs of stained gels.

Conclusion.

The pattern of labelling of the 25 kDa N-terminal transmembrane domain of the β subunit with sulfhydryl reagents and DCCD is more consistent with the presence of eight transmembrane segments (Fig. 1B) than with the six transmembrane segment model (Fig. 1A).

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