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Cross-linking of transmembrane helices in proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli*: implications for the structure and function of the membrane domain

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Abstract

Proton-pumping nicotinamide nucleotide transhydrogenase from *Escherichia coli* contains an α and a β subunit of 54 and 49 kDa, respectively, and is made up of three domains. Domain I (dI) and III (dIII) are hydrophilic and contain the NAD(H)- and NADP(H)-binding sites, respectively, whereas the hydrophobic domain II (dII) contains 13 transmembrane α-helices and harbours the proton channel. Using a cysteine-free transhydrogenase, the organization of dII and helix–helix distances were investigated by the introduction of one or two cysteines in helix–helix loops on the periplasmic side. Mutants were subsequently cross-linked in the absence and presence of diamide and the bifunctional maleimide cross-linker *o*-PDM (6 Å), and visualized by SDS-PAGE.

In the $\alpha_2\beta_2$ tetramer, αβ cross-links were obtained with the αG476C-βS2C, αG476C-βT54C and αG476C-βS183C double mutants. Significant αα cross-links were obtained with the αG476C single mutant in the loop connecting helix 3 and 4, whereas ββ cross-links were obtained with the βS2C, βT54C and βS183C single mutants in the beginning of helix 6, the loop between helix 7 and 8 and the loop connecting helix 11 and 12, respectively. In a model based on 13 mutants, the interface between the α and β subunits in the dimer is lined along an axis formed by helices 3 and 4 from the α subunit and helices 6, 7 and 8 from the β subunit. In addition, helices 2 and 4 in the α subunit together with helices 6 and 12 in the β subunit interact with their counterparts in the $\alpha_2\beta_2$ tetramer. Each β subunit in the $\alpha_2\beta_2$ tetramer was concluded to contain a proton channel composed of the highly conserved helices 9, 10, 13 and 14.

Keywords: Transhydrogenase; Proton-pumping; NAD; NADP; Membrane protein; Transmembrane helix; Helix–helix interaction

1. Introduction

In mitochondria and most bacteria, transfer of hydride ions between the NAD and NADP pools is carried out mainly by the proton-translocating nicotinamide nucleotide transhydrogenase (TH) according to the reaction

$$H^+_{out} + NADH + NADP^+ \rightleftharpoons H^+_{in} + NAD^+ + NADPH \quad (1)$$

where "out" and "in" denote the mitochondrial intermembrane space (or periplasmic space in bacteria) and matrix (or cytosol in bacteria), respectively. All proton-translocating transhydrogenases are composed of three domains, the

Abbreviations: AcPyAD$^+$, 3-acetyl-pyridine-NAD$^+$; MIANS, 2-((4′-maleimidyl)anilio)naphthalene-6-sulfonic acid; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; NEM, *N*-ethylmaleimide; Diamide, azodicarboxylic acid bis[dimethylamide]; cfTH, cysteine-free transhydrogenase; TH, transhydrogenase; dI, domain I of transhydrogenase; dII, domain II of transhydrogenase; dIII, domain III of transhydrogenase

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hydrophilic domain I (dI) and domain III (dIII) that bind NAD(H) and NADP(H), respectively, and the hydrophobic membrane domain II (dII) which harbours the proton channel. Whereas all known proton-translocating THs are homodimers, their subunit compositions differ. The *Escherichia coli* enzyme contains an α subunit with residues α1–α510 and a β subunit with residues β1–β462, with an active form of $\alpha_2\beta_2$. Thus, the α subunit contains dI and part of dII, whereas the β subunit contains dIII and the remaining part of dII. Especially the bovine and *E. coli* THs have been purified and reconstituted in liposomes and characterized extensively. Both dI from *E. coli* and *R. rubrum* and dIII from bovine heart, human, *E. coli* and *R. rubrum* have also been separately expressed and characterized functionally by, e.g., mutagenesis and structurally by X-ray crystallography and NMR (for reviews see Refs. [1–3]). Recently, the structure of a dI–dIII complex from *R. rubrum* TH was determined [4]. Although the maintenance of a highly reduced mitochondrial/cellular redox level of NADP(H) for, e.g., biosynthesis, reduction of oxidised glutathione and detoxication of toxic metabolites, was proposed at an early stage [5], other alternative physiological functions have subsequently been proposed, such as a regulation of the citric acid cycle [6].

Despite the extensive structure–function information available for dI and dIII, the corresponding information for dII is limited (for a review see Ref. [7]). Structurally, the membrane topology of the *E. coli* enzyme has been established to be composed of 13 transmembrane helices, four in the α subunit and nine in the β subunit [8], where helices 13 and 14 are almost completely conserved and helices 7, 9 and 10 partly conserved (Fig. 1). The proton channel was suggested to form an aqueous cavity lined by helices 9, 10, 13 and 14 [7,9,10].

The membrane topology [8], recent cross-linking/thiol reactivity, site-directed mutagenesis experiments [7,9–13], and sequence alignment of 61 different transhydrogenases [7], together with the fusion between the subunits in the *E. coli* enzyme [14], have contributed to the understanding of the structure of dII. Recently, using a split *E. coli* TH and cross-linking, helices 9, 10, 13 and 14 were shown for the first time to be located close to each other [15]. In addition, a mechanism for the communication between dII and dIII in the *E. coli* enzyme involving Arg265 and Asp213 and the "hinge" region, i.e., the connecting peptide between dII and dIII, was recently proposed [16]. Thus, although some significant and valuable information has been provided, especially regarding the putative proton channel and its regulation, the organization of the transmembrane helices in dII as a whole remains largely unknown [cf. 7].

As one of the four to five available active cysteine-free membrane proteins, cfTH (cysteine-free *E. coli* TH) was previously shown to be at least 50% active as compared to wild type, suggesting that the former enzyme behaved essentially wild type-like [17]. In the present investigation, single or double cysteine mutations were introduced in most helix–helix loops on the periplasmic side of the purified and solubilized cfTH (Fig. 1). In the case of double mutations, one cysteine residue was always introduced in the loop connecting helix 3 and 4 in the α subunit, together with one cysteine in each of the periplasmic loops in the β subunit. All mutants were studied by cross-linking in the absence or in the presence of the oxidizing agent diamide, or a chemical cross-linker, *o*-PDM (6 Å). Combined with a predicted propensity for helix–helix interactions, mainly within the β subunit, the results provided approximate distances between helices, and thus allowed, for the first time, the design of an experimentally based model of the membrane organization of the entire transhydrogenase in the active $\alpha_2\beta_2$ form. Based on these results, the interaction surface between the dII parts of the α and β subunits, as well as that between the α+β subunits in the active $\alpha_2\beta_2$ tetramer, was identified. A

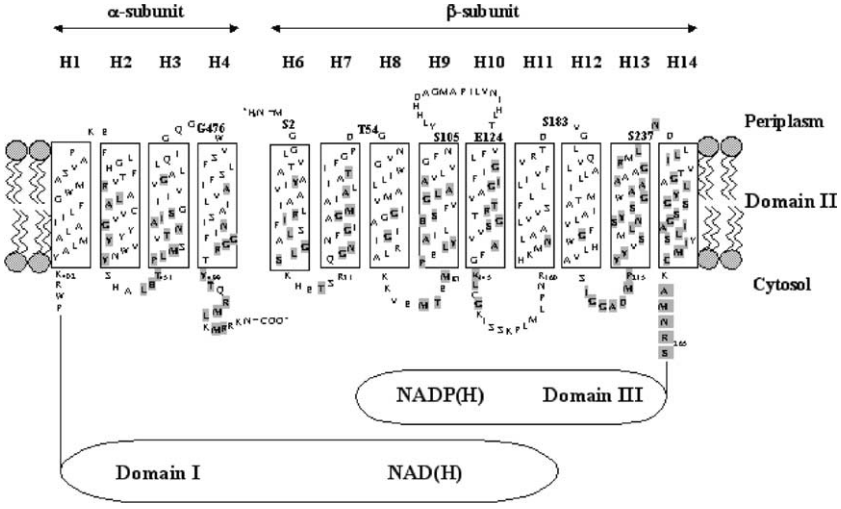

Fig. 1. Transmembrane helix topology and conserved residues in *E. coli* transhydrogenase. The model is based on the proposal of Meuller and Rydström [8]. The hydrophilic dI and dIII are indicated below dII. Shaded and bold residues are at least 80% conserved among 61 transhydrogenase sequences [7]. Mutated residues are indicated in bold. The numbering of the helices and the location of the α and β subunits are indicated above the sequence.

consequence of the model is that the active $\alpha_2\beta_2$ tetramer contains two separate proton channels.

2. Materials and methods

2.1. Site-directed mutagenesis

The plasmid pCLNH contains the gene coding for cfTH to which a six-residue-long histidine tag was attached to the N-terminal of the α subunit [13]. This plasmid served as a template in the construction of all single and double mutants, i.e., αG476C, βS2C, βT54C, βS105C, βE124C, βS183C, βS237C, αG476C-βS2C, αG476C-βT54C, αG476C-βS105C, αG476C-βE124C, αG476C-βS183C and αG476C-βS237C. The Quikchange mutagenesis kit (Stratagene), which was strictly followed according to the protocol, together with primers from Medprobe (Norway), was used to produce these mutants. The full-length genes were sequenced in order to verify the mutants and to make sure that no unwanted mutations had occurred.

In describing a mutant below, the prefixes "α" and "β" will be omitted, e.g., αG476C-βS237C is denoted G476C-S237C. Also, transmembrane helices will be denoted as "H", e.g., helix 6 is H6.

2.2. Expression and purification

The enzymes were expressed and purified as described in Ref. [17] with the only exception that Thesit was omitted from all buffers. All mutants were expressed and purified like cfTH, i.e., there were no significant differences in final yield. All mutants were analyzed by SDS-PAGE using 10–20% tris-glycine gels from NOVEX or Bio-Rad. The purity of the mutants was at least 90%.

2.3. MIANS labeling

2-((4′ -Maleimidyl)anilio)naphthalene-6-sulfonic acid (MIANS) is a sulfhydryl-specific probe, which becomes fluorescent upon reaction with a thiol, e.g., cysteine. This property, together with the fact that this molecule is large and hydrophilic, makes MIANS suitable to examine the accessibility of the cysteines in single mutants. The sample preparation was carried out as described in Ref. [8], and MIANS labeling of the enzyme (10 μg) was analyzed by fluorescence after SDS-PAGE.

2.4. Reverse reaction

The activity of the reverse reaction catalyzed by the purified mutant proteins was assayed by measuring the rate of reduction of 400 μM 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$) by 400 μM NADPH in buffer A (20 mM Mes, Ches, Tris–HCl and HEPES, 50 mM NaCl and 0.01% Brij-35 (pH 7.5)). The reverse reaction involves hydride transfer between $AcPyAD^+$ and NADPH and a concomitant proton translocation from the cytosolic to the periplasmic side of the enzyme. The measurements were carried out in the absence and presence of 2 mM DTT or 100 μM *o*-PDM. Due to interactions with the substrates, the reverse reaction was not possible to be assayed in the presence of diamide.

2.5. Cyclic reaction

The activity of the cyclic reaction catalyzed by the purified mutant proteins was assayed by measuring the rate of reduction of 300 μM $AcPyAD^+$ by 150 μM NADH in the presence of 300 μM $NADP^+$ in buffer B (20 mM Mes, Ches, Tris–HCl and HEPES, 50 mM NaCl, 5 mM $MgCl_2$ and 0.01% Brij-35 (pH 6.0)). The cyclic reaction involves hydride transfer between NADH/$AcPyAD^+$ and bound NADP(H) without a net transfer of protons [18]. Measurements were carried out in the absence and presence of 2 mM DTT or 100 μM *o*-PDM. Due to interactions with the substrates, the cyclic reaction was not possible to be assayed in the presence of diamide.

2.6. Cross-linking

The solubilized and purified mutated enzymes were incubated with the oxidizing agent diamide or the thiol-specific and bifunctional maleimide-cross-linker *o*-PDM that can cross-link two cysteines up to 6 Å apart. Cross-linking experiments were performed in buffer C (30 mM sodium phosphate and 0.01% Brij 35, (pH 7.5)). The purified mutants were mixed in buffer C with 100 μM *o*-PDM or 1 mM diamide and after incubation, for 30 min at 4 °C, 5 mM NEM was added to terminate the reaction. Each enzyme was analyzed by SDS-PAGE. Formation of disulfide bonds in the absence of the *o*-PDM was reversed in the presence of 5% β-mercaptoethanol.

2.7. SDS-PAGE

The enzyme was diluted twice with 2× SDS sample buffer (NOVEX). Unless indicated otherwise, 10 μg enzyme was loaded on the gel and the electrophoresis was run for 60 min at 150V, 90 min at 120V, or 90 min at 150V, on 7.5% tris-glycine (Bio-Rad), 8% tris-glycine (NOVEX), or 10–20% tris-glycine (NOVEX) gels, respectively. To reduce disulfides, 5% β-mercaptoethanol was included in the 2× SDS sample buffer. After staining with Coomassie blue, the extent of cross-linking was estimated by densitometric scanning and denoted (−), (+), (++) and (+++) if cross-linking was estimated to <5%, 5–10%, 10–20%, or >20%, respectively, of total subunit protein.

2.8. Chemicals

o-PDM, diamide and NEM were obtained from Sigma: MIANS was obtained from Molecular Probes and protease

inhibitor tablets were purchased from Boehringer. All other chemicals were of analytical purity and purchased from Sigma or Boehringer.

3. Results

3.1. Mutagenesis and cross-linking

In order to determine the position of the transmembrane helices in the intact purified and detergent-dispersed cfTH, single or double cysteine residues were introduced in all of the loops on the periplasmic side, except that between H1 and H2, in the cysteine-free background. Cross-linking was achieved by introducing cysteine mutations, followed by purification of the mutant and subsequent analysis by SDS-PAGE of the cross-linking products obtained after incubation in buffer C in the absence and presence of the oxidizing agent diamide. In case no spontaneous or diamide-induced cross-linking was observed, the chemical cross-linker *o*-PDM was also used. Except for the loop connecting helix 9 and 10, all periplasmic loops are short (three to five amino acids). Due to the longer loop between helices 9 and 10, the cysteines were introduced in the beginning and the end of the loop, respectively, in order to avoid an uncertain positioning of the helices (see Fig. 1). All cysteines introduced were accessible to the fluorescent thiol-specific agent MIANS (Fig. 2). The apparently low accessibility of some G476C mutants, especially the G476C single mutant, was due to their high tendency for spontaneous disulfide formation (cf. Tables 2 and 4), resulting in a decreased number of MIANS-reactive thiols. MIANS (molecular mass 416 Da) is considerably larger than both diamide (molecular mass 172 Da) and *o*-PDM (molecular mass 268 Da). It was therefore concluded that all introduced cysteines were freely accessible to the cross-linking agents. Cross-linking with diamide was also fully reversible as indicated by the effect of β-mercaptoethanol which restored the original α and β subunits (Fig. 3).

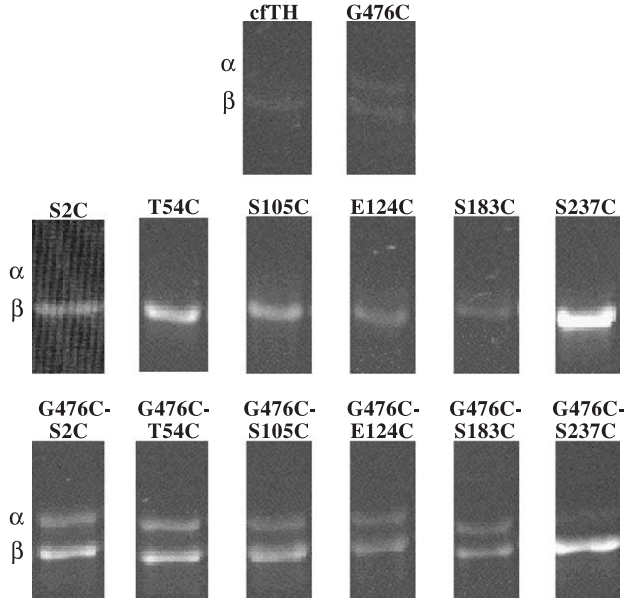

Fig. 2. SDS-PAGE of purified and cross-linked periplasmic single and double *E. coli* transhydrogenase mutants. Qualitative assay of thiol accessibility by MIANS fluorescence. Purified enzymes were incubated for 10 min with 10 μM MIANS, after which the reaction was terminated by the addition of 100 μM NEM. Subsequently, 10 μg of each enzyme was added to each well and the fluorescence determined under UV light using a video recorder; "α" and "β" denote the α and the β subunit, respectively.

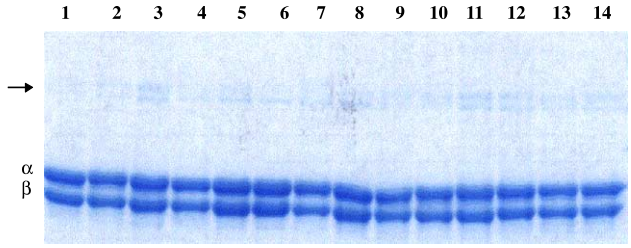

Fig. 3. Reversal of diamide-induced cross-linking by β-mercaptoethanol. Following incubation with 1 mM diamide for 30 min, all enzymes were mixed with 5% β-mercaptoethanol. Each enzyme (10 μg) was then loaded on a well (see Materials and methods for details). Arrow indicates the approximate location where cross-links appear in the absence of β-mercaptoethanol; "α" and "β" denote the α and β subunits, respectively. Lanes contained: 1, cfTH; 2, G476C; 3, S2C; 4, T54C; 5, S105C; 6, E124C; 7, S183C; 8, S237C; 9, G476C-S2C; 10, G476C-T54C; 11, G476C-S105C; 12, G476C-E124C; 13, G476C-S183C; 14, G476C-S237C. (For colour see online version).

Introduction of one or two cysteines may theoretically lead to major structural and functional alterations in the protein reflected as changes in specific activities and/or pH dependencies. However, except for the E124C, all mutants showed activities between 37% and 100% of cfTH (Tables 1 and 3) and pH optima that deviated less than 0.5 unit from that of cfTH (not shown). Both the single E124C mutant and the double G476C-E124C mutants showed activities of about 15% of cfTH (Tables 1 and 3) and slightly altered pH dependences (Fig. 4).

Table 1
Reverse and cyclic activities catalyzed by single cysteine mutations in the transmembrane domain of transhydrogenase

Mutant	Position	Reverse activity (%)			Cyclic activity (%)		
		−DTT	+DTT	+*o*-PDM	−DTT	+DTT	+*o*-PDM
G476C	H3/H4	93	88	80	81	78	78
S2C	H6	>100	98	85	95	99	>100
T54C	H7/H8	50	40	31	65	50	>100
S105C	H9	37	42	38	85	69	68
E124C	H10	15	14	9	>100	91	>100
S183C	H11/H12	50	49	35	65	53	83
S237C	H13/H14	81	79	60	>100	>100	>100

Conditions were as described in Materials and methods. The reverse activity of cfTH in the absence of 2 mM DTT, in the presence of 2 mM DTT and in the presence of 100 μM *o*-PDM was 4.5, 5.5 and 6.1 μmol/min/mg, respectively. The cyclic activity of cfTH in the absence of 2 mM DTT, in the presence of 2 mM DTT and in the presence of 100 μM *o*-PDM was 29, 34 and 34 μmol/min/mg, respectively. Values are expressed in % of the activity of cfTH and are averages of duplicate determinations. Experimental error was less than 10%.

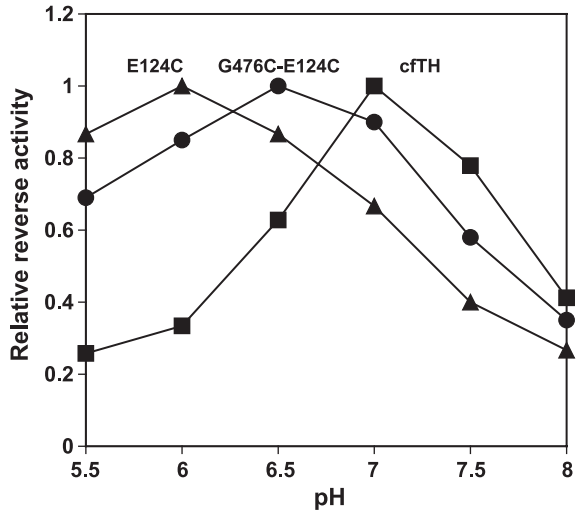

Fig. 4. pH dependencies of the reverse reaction catalyzed by cfTH, the E124C and G476C-E124C mutants. Assays were carried out as described in Materials and methods.

3.2. Catalytic properties and cross-linking of single cysteine mutants

In the first part of this investigation, cross-linking between the dII components of the two β subunits or the two α subunits in the $\alpha_2\beta_2$ tetramer was achieved by single cysteine mutations on the periplasmic side of dII. Single periplasmic loop mutants produced were (with the loop involved indicated) G476C (H3/H4), S2C (H6), T54C (H7/H8), S105C (H9), E124C (H10), S183C (H11/H12) and S237C (H13/H14) (Table 1 and Fig. 1).

The only mutant in the α subunit, G476C, displayed cfTH-like activities (Table 1) despite the occurrence of extensive αα cross-linking (Fig. 5, lanes B1 and B2; Table 2), even in the absence of diamide. This was interpreted to indicate that αα cross-linking occurred spontaneously in the host bacterium or during the isolation of the protein. The same conclusion was drawn for the S2C mutant because of its high activities (Table 1) and the high extent of spontaneous cross-linking (Fig. 5, lanes C1 and C2; Table 2). In terms of activities (Table 1) and cross-linking (Table 2), the T54C (Fig. 5, lanes D1 and D2) and S183C (Fig. 5, lanes G1 and G2) mutants behaved very similar to each other. These mutants retained about 50% and 65% activity relative to the cfTH reverse and cyclic activity, respectively, and required diamide to produce significant cross-linking. Residue S105 is located in the N-terminal of H9, which contains the essential residue His91 (Fig. 1). The S105C mutation lowered the reverse activity to about 37% but the cyclic activity was essentially unaffected (Table 1). Cross-linking between two S105C mutants in the tetramer could not be detected unless *o*-PDM was present, indicating a relatively large distance between the pairs of S105C (Fig. 5, lanes E1–E3; Table 2). The same cross-linking pattern was observed for the S237C mutant (Fig. 5, lanes H1–H3; Table 2). The reverse and cyclic activities of the S237C mutant were 81% and 100% of cfTH, respectively (Table 1). H10 contains the S139 residue, which recently has been suggested to be important for proton-translocation [10]. The E124C mutant could not be cross-linked in the tetramer by diamide, and was the only mutant that did not cross-link significantly above a weak background even in the presence of *o*-PDM (Fig. 5, lanes F1–F3; Table 2). The cyclic activity of the E124C mutant was cfTH like, but the reverse activity was inhibited approximately 80%. Incubation with DTT or *o*-PDM did not significantly alter the catalytic activities (Table 1).

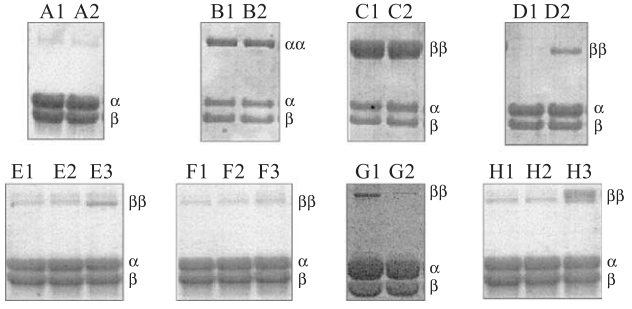

Fig. 5. Cross-linking between single cysteine mutants in dII of *E. coli* TH. Cross-linking between two single cysteines in the functional $\alpha_2\beta_2$ tetramer gives "αα" or "ββ", where "α" and "β" denote the α and β subunits, respectively. Following incubation with no addition (none), diamide (di) or *o*-PDM, each mutant enzyme (10 μg) was loaded in each well for SDS-PAGE (see Materials and methods). Lanes contained: A1–A2, cfTH (none), cfTH (di); B1–B2, G476C (none), G476C (di); C1–C2, S2C (none), S2C (di); D1–D2, T54C (none), T54C (di); E1–E3, S105C (none), S105C (di), S105C (*o*-PDM); F1–F3, E124C (none), E124C (di), E124C (*o*-PDM); G1–G2, S183C (di), S183C (none); H1–H3, S237C (none), S237C (di), and S237C (*o*-PDM).

Table 2
Cross-linking between single cysteine mutations in the transmembrane domain of transhydrogenase

Mutant	Position	Additions		
		None	Diamide	*o*-PDM (6 Å)
G476C	H3/H4	αα+++	αα+++	n.d.
S2C	H6	ββ+++	ββ+++	n.d.
T54C	H7/H8	–	ββ++	n.d.
S105C	H9	–	–	ββ+
E124C	H10	–	–	–
S183C	H11/H12	–	ββ+	n.d.
S237C	H13/H14	–	–	ββ++

αα and ββ correspond to cross-linking between two α subunits or two β subunits, respectively, in the functional tetramer ($\alpha_2\beta_2$). Cross-linking was estimated by densitometric scanning to <5%, 5–10%, 10–20%, or >20% of total monomer content, denoted (–), (+), (++) or (+++), respectively. When cross-linking was detected in the presence of diamide, cross-linking by *o*-PDM was not examined (n.d., not determined).

3.3. Catalytic properties and cross-linking of double cysteine mutants

Cross-linking between loops of the α and β subunits on the periplasmic side of the dII components was achieved by combining the G476C mutation in the loop connecting

Table 3
Reverse and cyclic activities catalyzed by double cysteine mutations in the transmembrane domain of transhydrogenase

Mutant	Position	Reverse activity (%)			Cyclic activity (%)		
		−DTT	+DTT	+*o*-PDM	−DTT	+DTT	+*o*-PDM
G476C-S2C	H3/H4–H6	75	78	57	75	71	85
G476C-T54C	H3/H4–H7/H8	85	79	64	83	69	>100
G476C-S105C	H3/H4–H9	44	55	43	93	91	97
G476C-E124C	H3/H4–H10	16	15	10	95	85	87
G476C-S183C	H3/H4–H11/H12	80	79	63	71	63	79
G476C-S237C	H3/H4–H13/H14	70	68	52	77	60	75

Conditions were as described in Materials and methods. The reverse activity of cfTH in the absence of 2 mM DTT, in the presence of 2 mM DTT and in the presence of 100 μM *o*-PDM was 4.5, 5.5 and 6.1 μmol/min/mg, respectively. The cyclic activity of cfTH in the absence of 2 mM DTT, in the presence of 2 mM DTT and in the presence of 100 μM *o*-PDM was 29, 34 and 34 μmol/min/mg, respectively. Values are expressed in % of the activity of cfTH, and are averages of duplicate determinations. Experimental error was less than 10%.

H3 and H4 with a second cysteine mutation in the periplasmic loops connecting the transmembrane helices of the β subunit. Mutants produced were G476C-S2C (H3/H4–H6), G476C-T54C (H3/H4–H7/H8), G476C-S105C (H3/H4–H9), G476C-E124C (H3/H4–H10), G476C-S183C (H3/H4–H11/H12) and G476C-S237C (H3/H4–H13/H14)

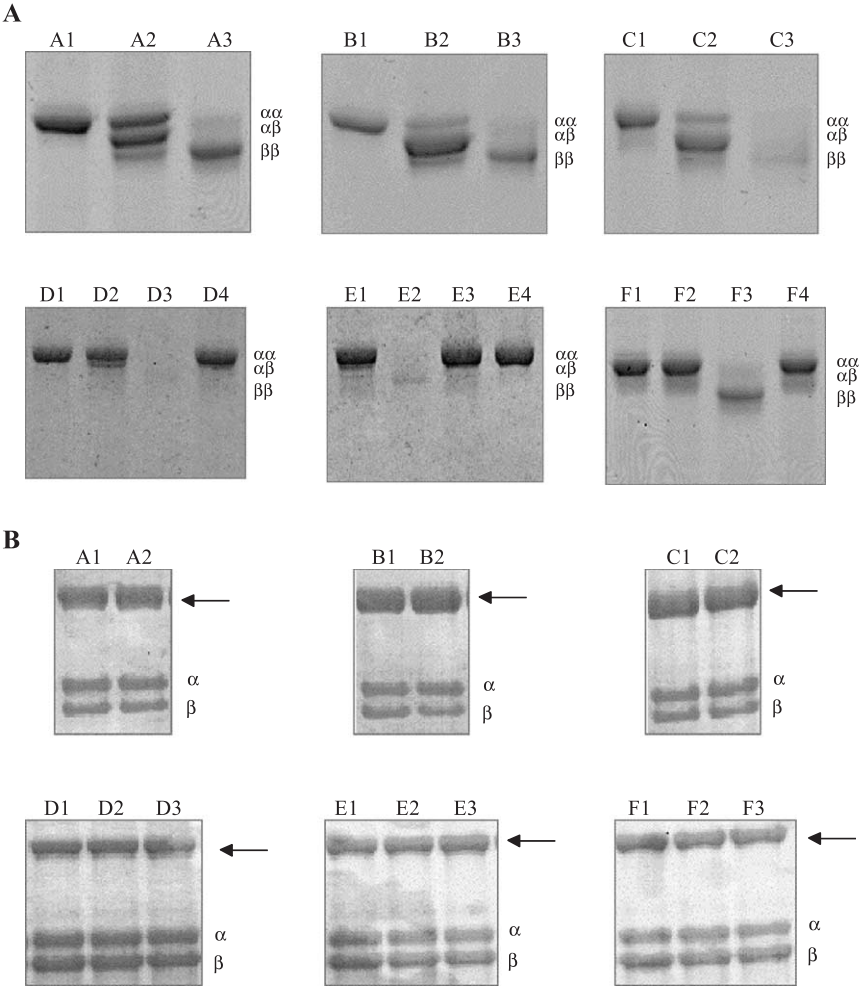

Fig. 6. Cross-linking between double cysteine mutants in dII of *E. coli* TH. (A). The single mutants G476C and T54C were used as references for cross-linking between the two α subunits and the two β subunits, respectively; "αα", "ββ" and "αβ" denote cross-links between respective subunits in the functional $\alpha_2\beta_2$ tetramer. Following incubation with no addition (none), diamide (di) or *o*-PDM, each mutant enzyme (10 μg, unless otherwise indicated) was loaded in each well for SDS-PAGE (see Materials and methods). Lanes contained: A1–A3, G476C (di), G476C-2SC (di), T54C (di); B1–B3, G476C (di), G476C-T54C (di), T54C (di); C1–C3, G476C (di), G476C-S183C (di), T54C (5 μg, di); D1–D4, G476C-S105C (di), G476C-S105C (*o*-PDM), T54C (5 μg, di), G476C (di); E1–E4, G476C (di), T54C (5 μg, di), G476C-E124C (di), G476C-E124C (*o*-PDM); F1–F4, G476C-S237C (di), G476C-S237C (*o*-PDM), T54C (di), and G476C (di). (B). SDS-PAGE conditions emphasized separation of α and β subunits rather than cross-links. All other conditions and labeling were as in (A). Arrows indicate cross-linked products.

(Fig. 1 and Table 3). In order to separate αα, αβ and ββ cross-links, SDS-PAGE conditions had to be chosen under which the separation of the α and β subunits was not optimal (cf. Fig. 6). Because of the similar sizes of the αα (108 kDa), αβ (103 kDa) and ββ (98 kDa) cross-links, spontaneously cross-linked G476C (αα) and T54C (ββ) were used as internal size standards.

Except for the G476C-S105C and the G476C-E124C mutants, the reverse activities of which were 44% and 16%, respectively, of cfTH (Table 3); the reverse activity was not markedly affected by any of these double mutations. The corresponding cyclic activities were essentially unaffected (Table 3). Like the single mutants, incubation with DTT or *o*-PDM did not significantly alter the catalytic activities (Table 3).

Among the double mutants, G476C-S2C, G476C-S105C, G476C-E124C and G476C-S237C gave the expected spontaneous αα dimer formation due to G476C with no increase in the presence of added cross-linkers (Fig. 6A, lanes A2, D3–D4, E3–E4, F1–F2; Table 4). In addition, G476C-S2C also gave a spontaneous but minor ββ dimer (Fig. 6A, lane A2; Table 4). Weak cross-linking between two β subunits was also detected in both the G476C-T54C (Fig. 6A, lane B2; Table 4) and the G476C-S183C (Fig. 6A, lane C2; Table 4) mutants, but only in the presence of diamide. In addition, the two latter together with the G476C-S2C mutant gave extensive αβ cross-linking both in the absence and presence of diamide (Table 4). It should be stressed that this latter αβ cross-linking with the G476C-T54C and G476C-S183C, and to a lesser extent G476C-S2C, occurred at the expense of the extensive αα cross-linking with G476C seen with, e.g., G476C-S183C and the single G476C mutants (Table 4). This indicates that the "affinity" of G476C for especially T54C and S183C, presumably in the same α+β unit, was higher than that between the two G476C in different α subunits. The G476C-S105C mutant (Fig. 6A, lane D2) gave a weak αβ band but only in the presence of *o*-PDM. The two remaining double mutants, G476C-E124C (Fig. 6, lanes E3–E4); Table 4) and G476C-S237C (Fig. 6A, lanes F1–F2; Table 4) did not show any αβ or ββ cross-linking under the conditions used in this investigation. SDS-PAGE conditions in Fig. 6A did not allow a separation of α and β subunits. Fig. 6B shows cross-linking of the double cysteine mutants under conditions identical to those in Fig. 6A but using SDS-PAGE conditions that allowed a separation of α and β subunits but not of cross-linking products. Thus, Fig. 6B demonstrates the overall extent of cross-linking of the different mutants.

Table 4
Cross-linking between double cysteine mutations in the transmembrane domain of transhydrogenase

Mutant	Position	Additions		
		None	Diamide	*o*-PDM (6 Å)
G476C-S2C	H3/H4–H6	αα++	αα++	n.d.
		αβ+++	αβ+++	
		ββ+	ββ++	
G476C-T54C	H3/H4–H7/H8	–	αα+	n.d.
		αβ+++	αβ+++	
		–	ββ+	
G476C-S105C	H3/H4–H9	αα+++	αα+++	αα+++
		–	–	αβ+
		–	–	–
G476C-E124C	H3/H4–H10	αα+++	αα+++	αα+++
		–	–	–
		–	–	–
G476C-S183C	H3/H4–H11/H12	–	αα++	n.d.
		αβ+++	αβ+++	
		–	ββ+	
G476C-S237C	H3/H4–H13/H14	αα+++	αα+++	αα+++
		–	–	–
		–	–	–

αα, ββ and αβ correspond to cross-linking between the two α subunits, the two β subunits or one α and one β subunit, respectively, in the functional tetramer ($\alpha_2\beta_2$). Cross-linking was estimated by densitometric scanning to <5%, 5–10%, 10–20%, or >20% of total monomer content, denoted (–), (+), (++) or (+++). When cross-linking was detected in the presence of diamide, cross-linking by *o*-PDM was not examined (n.d., not determined).

4. Discussion

By using cfTH [8,17], single and double cysteine mutations were made in all predicted loops on the periplasmic side of the membrane, except in the loop connecting helix 1 and 2 (Fig. 1), and investigated by cysteine cross-linking. Single and double cysteine mutants were also constructed in the cytosolic loops, but due to a low accessibility presumably caused by dI and dIII, and a high flexibility in the long loops, no reliable cross-linking results were obtained (Althage and Rydström, unpublished). In principle, the approach was the same as that used in the pioneering work of Kaback et al. [19] with *lac-permease* which, as revealed by the recent X-ray structure of the permease [20], proved to provide a surprisingly accurate model of the relative positions of the transmembrane α helices. However, even though the predicted model based on cross-linking [19] and the X-ray structure [20] of lac-permease were surprisingly similar, some distances were significantly underestimated up 10–15 Å. The main reason for these differences appears to be the fact that cross-linking was carried out with a permease that was alternating between inward- and outward-facing conformations, whereas the crystal structure was locked by a substrate analogue in the inward facing conformation. Given the extensive efforts spent on indirect structural studies on the *lac-permease*, this transporter therefore constitutes a unique reference system where the accuracy of cross-linking can be evaluated [21]. Briefly, it was concluded that cross-linkers/thiols tend to trap thiols at the shortest distance, and average distances therefore tend to be underestimated in flexible proteins [22]. Spontaneous cross-linking in the absence of added cross-linker is

therefore taken as evidence that the SH groups of the two cysteines are closer than 3–4 Å. The oxidizing agent diamide provides a more efficient cross-linking with the same or slightly larger distance. The backbone diameter of a transmembrane α-helix is approximately 5 Å, the diameter of an α-helix approximately 7–15 Å, and the center-to-center distance between two helices 10 Å for cysteines or larger depending on the amino acid composition. In the presence of *o*-PDM, cross-linking would indicate a thiol distance of up to 9 Å and a helix center-to-center distance of 19 Å. As emphasized above, these constitute minimal distances between loops of varying lengths connecting helices that may be inherently flexible and/or where the helices may adopt different positions due to possible different conformational states.

In the present cross-linking studies of cfTH, it is obviously essential to eliminate the possibility that thiols of different $\alpha_2\beta_2$ tetramers react with each other, i.e., intermolecular cross-linking. In the present work, this was ascertained by varying the protein concentration, or by adding SDS, during cross-linking. As expected, an increased protein concentration gave no difference, and SDS inhibited cross-linking (not shown). Intermolecular cross-links would have resulted in an increased cross-linking with increased protein concentration, and should not be sensitive to SDS. Also, cross-linking was time-dependent and led to the expected disappearance of the native α and/or β subunits.

The expression and purity of the mutants were similar to that of cfTH. Except for E124C, all mutants showed a reverse activity of 37–100% of cfTH, indicating that the basic structure of the mutant proteins had not been affected by the mutation. This was supported by little or no effects on the pH dependencies and the cyclic activities of all mutants. Bragg and Hou [23] have suggested that E124 may be involved in proton translocation. In the present investigation, introduction of a cysteine in this position in the enzyme affected both the reverse activity and its pH dependence, but not the cyclic activity, stability, expression levels or yield of purified enzyme, suggesting that the structure of the enzyme was not changed.

Using *E. coli* transhydrogenase in cytosolic membranes and cupric 1,10-phenanthrolinate as an oxidizing agent, Bragg and Hou [11,12] concluded that, in the α+β dimer, H2 and H4 are close to H6 and that H3 and the C-terminal of the α subunit are close to H7 [10]. In the $\alpha_2\beta_2$ tetramer, H2, H4 and H6 were suggested to be close to the corresponding helices in the second α+β dimer. Moreover, cross-links were obtained between β subunits, suggesting that A114C (in the middle of the 20 amino acids long loop between H9 and H10) and S183C (in the loop between H11 and H12) and A262C (in the C-terminal of H14) were close to the same residue in the other β subunit. The A262C mutant (in the C-terminus of H14) produced ββ cross-links in the presence of *o*-PDM. Despite this information, a packing model was not proposed [11,12].

Based on previous information and the present results, a packing structure of dII was modelled (Fig. 7). The discussion below is separately focussed on the single mutants that generate cross-links between two α subunits (αα) or two β subunits (ββ) in the $\alpha_2\beta_2$ tetramer, and on the double mutants that in addition generate cross-links between one α and one β subunit (αβ) in the $\alpha_2\beta_2$ tetramer.

4.1. Cross-linking of single mutants

1. The G476C in the H3/H4 loops as well as the S2C residue in the N-termini of the β subunits (H6) gave spontaneous and extensive αα and ββ cross-linking, demonstrating that these must be close to their counterparts in the $\alpha_2\beta_2$ tetramer (Table 2).
2. In the presence of diamide, T54C in the H7/H8 and S183C in the H11/H12 loops gave weak to intermediate cross-links to their counterparts in the $\alpha_2\beta_2$ tetramer (Table 2). Thus, both loops are pairwise relatively distant but close enough to cross-link. Bragg and Hou [11] concluded that the loops connecting H7 and H8 are not close in the $\alpha_2\beta_2$ tetramer. In this context it should be stressed that Bragg and Hou [11,12] used non-purified and membrane-bound TH and the stronger oxidizing agent cupric-1,10-phenatrolinate instead of diamide.
3. The two single cysteine mutants, S105C in H9 and S237C in H13/H14, did not produce ββ cross-links unless *o*-PDM was added, suggesting a thiol distance of at least 9 Å (Table 2).
4. The only single cysteine mutant that did not cross-link to its counterpart even in the presence of *o*-PDM was E124C (H10) (Table 2). The most likely explanation is that the two H10s are quite distant from each other in the $\alpha_2\beta_2$ tetramer.

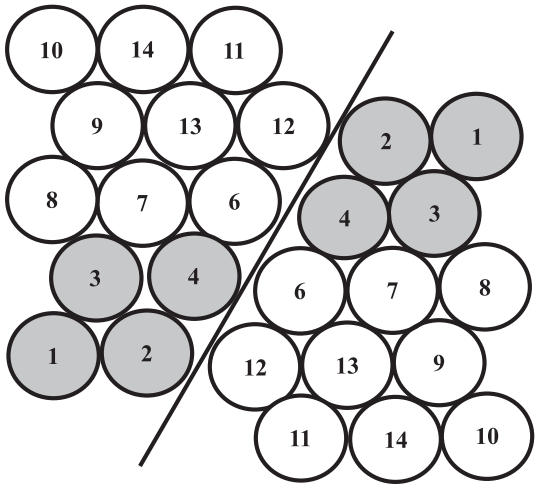

Fig. 7. Model of the helix organization of the transmembrane domain of *E. coli* TH. H1–H4 (shaded circles) belong to the α subunit and H6–H14 belong to the β subunit. The two α+β dimers are separated by a line. The model is viewed from the periplasmic side.

4.2. Cross-linking of double mutants

1. A prerequisite within the α+β unit is that H4 and H6 are close since these can be fused with a peptide corresponding to that in the mitochondrial single polypeptide transhydrogenase [14]. This was confirmed by extensive αβ cross-links formed by the G476C-S2C double mutant (Table 4). Note that the αβ cross-links were formed partly at the expense of αα cross-links, which were extensively formed in the single G476C mutant and, e.g., the G476C-S237C double mutant, indicating that the distance between H6 and H3/H4 is shorter than that between the two H3/H4.
2. Extensive αβ cross-links formed with the G476C-T54C double mutant indicate that H3/H4 and H7/H8 are close, most likely in the same α+β dimer. This is in agreement with Bragg and Hou [11,12] who showed that H3 and the C-terminus of the α subunit were close to H7. In this case, no H3/H4 cross-links were observed, strongly indicating that the distance between H3/H4 and H7/H8 is much shorter than that between the two H3/H4.
3. Extensive αβ cross-links formed with the G476C-S183C double mutant indicate that the H3/H4 loop is close to the H11/H12 loop. Again, no H3/H4 cross-links were formed, indicating that the distance between H3/H4 and H11/H12 is shorter than that between the two H3/H4. Presumably, H3/H4 in one α+β dimer is therefore close to H11/H12 in the second α+β dimer.
4. The mutant G476C-S105C formed weak αβ cross-links but only in the presence of *o*-PDM, indicating that H3/H4 is relatively distant from H9.
5. The only double mutants that failed to form αβ cross-links were G476C-E124C (H3/H4–H10) and G476C-S237C (H3/H4–H13/H14). This supports the distant position of H3 and H4 relative to H10, H13 and H14 in the model, both within the same α+β dimer as well as between different α+β dimers.

The central core of the model in Fig. 7 is primarily based on strongly cross-linking loops in Tables 2 and 4 (denoted with "+++" in Tables 2 and 4) as well as previous information regarding roles and relative positions of certain helices, e.g., H4 and H6, discussed above (cf. Ref. [7]). More peripheral helices/loops giving little or no cross-linking are obviously more difficult to position. Suggested distances larger than about 20 Å or more between weakly interacting helices, i.e., the two H11/H12, the two H13/H14, the two H7/H8, H3/H4 and H9, and the two H9, despite a helix center-to-center cross-linking distance of about 19 Å, may be explained by flexible loops and/or helices as discussed above for *lac-permease* [21]. However, except for the "hinge" region involving H13 and H14 [16], it is not known whether global conformational changes similar to those that have been demonstrated for *lac*-permease [21] occur in TH, but it appears to be unlikely. Peripheral helices may also be more flexible than those in the core of dII. Attempts to demonstrate an altered cross-linking pattern, indicative of different conformational states, by substrates and site-specific inhibitors are currently being carried out. In the absence of such agents, i.e., conditions that were used in the present investigation, TH is assumed to adopt a stable "resting" conformation (cf. Ref. [1]).

In the model shown in Fig. 7, residues important for catalytic activity and proton pumping, i.e., His91 (H9), Asn222 (H13) and Ser139 (H10), are assumed to form a proton proton channel [7,9,10,15], one in each α+β dimer, where each set of H9–H10–H13–H14 forms a tight unit [15] closely associated with the hinge region for communication with dIII [16]. This conclusion is mainly based on the relatively large distance between these units in the $\alpha_2\beta_2$ tetramer, and is indeed consistent with previous proposals [7] as well as the alternating site binding change mechanism recently proposed by Jackson et al. [2], in which two proton channels are envisaged. Likewise, the two dI peptides of the α subunits in the $\alpha_2\beta_2$ tetramer are tightly bound, suggesting that the remaining parts of the α subunits, i.e., the two sets of H1–H4, are also close (cf. Fig. 1), and apparently remain close after removal of the dIs by trypsin [24].

Like all crystallized dimeric membrane proteins, e.g., cytochrome *c* oxidase [25], the two α+β units in transhydrogenase are assumed to be positioned "face-to-face", i.e., the second α+β unit is rotated 180° in the same plane relative to the first unit. Indeed, a calculation of the aggregated kPROT score [26] for each transmembrane region, which is related to the extent of exposure of the helix side chains to the surrounding lipid phase, shows that especially H7, H9 and H13 are buried, whereas H11 and H14 are more exposed (J. Mullins, M. Althage and J. Rydström, unpublished), in agreement with the model. In this context, it is interesting to note that there are several conserved glycine residues, especially in H7, H9, H10, H13 and H14, that may be involved in helix–helix, i.e., groove–ridge, interactions through GxxxG motifs and neighbouring branched amino acid side-chains of, e.g., Ile and Val [27].

In conclusion, systematic single and double cysteine mutagenesis of loops on the periplasmic side between predicted transmembrane α helices in the *E. coli* transhydrogenase has been carried out. Based on cross-linking of the purified mutants, a helix packing model of the entire membrane domain is proposed for the first time. An important consequence of the model is that the active $\alpha_2\beta_2$ tetramer is suggested to have two separate proton channels.

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