Functional Split and Crosslinking of the Membrane Domain of the β Subunit of Proton-Translocating Transhydrogenase from *Escherichia coli*†

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Received April 8, 2003; Revised Manuscript Received June 26, 2003

ABSTRACT: Proton pumping nicotinamide nucleotide transhydrogenase from *Escherichia coli* contains an α subunit with the NAD(H)-binding domain I and a β subunit with the NADP(H)-binding domain III. The membrane domain (domain II) harbors the proton channel and is made up of the hydrophobic parts of the α and β subunits. The interface in domain II between the α and the β subunits has previously been investigated by cross-linking loops connecting the four transmembrane helices in the α subunit and loops connecting the nine transmembrane helices in the β subunit. However, to investigate the organization of the nine transmembrane helices in the β subunit, a split was introduced by creating a stop codon in the loop connecting transmembrane helices 9 and 10 by a single mutagenesis step, utilizing an existing downstream start codon. The resulting enzyme was composed of the wild-type α subunit and the two new peptides β1 and β2. As compared to other split membrane proteins, the new transhydrogenase was remarkably active and catalyzed activities for the reduction of 3-acetylpyridine-NAD^+ by NADPH, the cyclic reduction of 3-acetylpyridine-NAD^+ by NADH (mediated by bound NADP(H)), and proton pumping, amounting to about 50−107% of the corresponding wild-type activities. These high activities suggest that the α subunit was normally folded, followed by a concerted folding of β1 + β2. Cross-linking of a βS105C−βS237C double cysteine mutant in the functional split cysteine-free background, followed by SDS−PAGE analysis, showed that helices 9, 13, and 14 were in close proximity. This is the first time that cross-linking between helices in the same β subunit has been demonstrated.

Nicotinamide nucleotide transhydrogenase is a redox-driven proton pump that catalyzes the reduction of $NADP^+$ by NADH; the forward reaction is linked to the translocation of one proton across the mitochondrial inner membrane or the bacterial plasma membrane, from the intermembrane space to the matrix in mitochondria, and from the periplasmic space to the cytosol in bacteria. The opposite reaction, reduction of NAD^+ by NADPH, is called the reverse reaction. Proposed physiological roles of transhydrogenase include the generation of a high redox level of NADP(H), that is, a high NADPH/$NADP^+$ ratio for cellular regulation of NADP(H)-dependent reactions such as the detoxification of peroxides, the maintenance of reduced glutathione, and the regulation of the Krebs cycle (for reviews and recent reports, see refs *1*−*3*).

Proton-translocating transhydrogenases (TH) are dimeric proteins with two hydrophilic domains, the NAD(H)-binding domain I (dI) and the NADP(H)-binding domain III (dIII). The hydrophobic part of the enzyme containing the proton channel, denoted domain II (dII), is composed of a variable number of transmembrane helices depending on species. The *Escherichia coli* TH is composed of an α subunit and a β subunit, the former subunit containing dI and transmembrane helices 1−4 of dII and the latter subunit containing dIII and the transmembrane helices 6−14 of dII (Figure 1). Only the mitochondrial TH contains a fifth transmembrane helix. Both dI and dIII have been expressed as soluble and catalytically active proteins that have been structurally resolved by X-ray crystallography and NMR (for reviews see refs *4*−*6*).

Despite the obvious importance of dII in the overall TH reaction, this domain has generally received much less attention than dI and dIII (see, however, ref *5*). Even though the membrane topology of the dII of *E. coli* TH has been shown to be composed of 13 transmembrane α helices (*7*), little information is available regarding the packing of these helices (*5*). On the basis of the properties of the βH91R and βN222R mutants, located in helices 9 and 13, respectively, Bragg and Hou showed that these mutants contain tightly bound NADP(H) (ref *8* and references therein), suggesting that dII and dIII communicate through long-range conformational changes. Recently, a novel coupling mechanism was proposed involving the connecting hinge peptide between dII and dIII and a salt bridge between βAsp213 and βArg265 (*9*). Thus, it is likely that dII plays a much more important role in the regulation of dI and dIII than previously anticipated (*10*).

An understanding of helix packing in dII, structure and function of the proton channel, the role of individual helices, and specific amino acids are essential for establishing a molecular mechanism of action for TH. Since a 3-D structure

† This work was supported by the Swedish Research Council. J.K. gratefully acknowledges a stipend from the Lawski Foundation.

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10.1021/bi034560x CCC: $25.00
Published on Web 08/26/2003

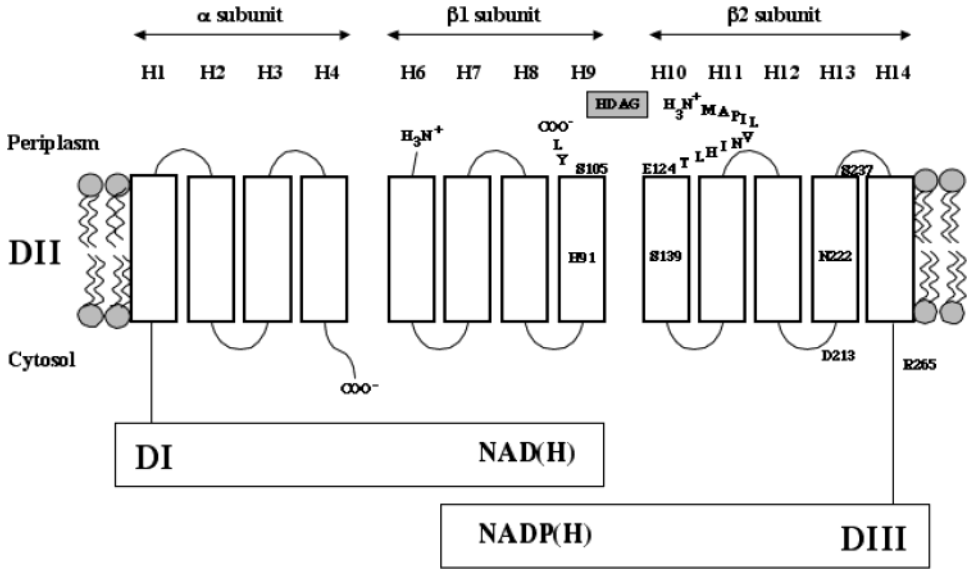

FIGURE 1: Membrane topology of *E. coli* TH. The loop between H9 and H10 is shown with the split and the HDAG tripeptide (in gray box) that was removed in the construct. The assignment of the helices was made according to Meuller and Rydström (*7*). Some essential residues are indicated.

determined by X-ray crystallography of the entire TH is lacking, one approach to achieve structural information of dII is cross-linking studies (i.e., cross-linking between cysteines introduced in a cysteine-free background (*11*, *12*)). However, cross-linking helices within the β subunit of *E. coli* TH has been hampered by difficulties in detecting these cross-links and the resulting minor structural changes of the β subunit on, for example, SDS−PAGE gels.

In the present investigation, a stop codon was introduced in the loop connecting transmembrane helices 9 and 10 in the β subunit of *E. coli* TH by a single mutagenesis step that, in combination with an already existing start codon (Met), resulted in three peptides (α, β1, and β2) instead of two (α and β) in the wild-type enzyme. Functionally split membrane proteins have been described earlier, but the resulting mutant proteins, if at all catalytically active, often showed an activity of less than 50% of the wild type (*13*−*19*). The properties of the functionally split TH were characterized and showed high catalytic and proton pumping activities. Using the βS105C−βS237C double mutant (Figure 1), the enzyme was successfully cross-linked between helix 9 and the loop between helix 13 and 14. This is the first time that cross-linking within the β subunit has been described, which now makes it possible to carry out more detailed studies of dII of the *E. coli* TH.

MATERIALS AND METHODS

Plasmids. The plasmid called pNHIS contains six histidines introduced in the N-terminus of the α subunit and encodes for the wild-type *E. coli* transhydrogenase (wtTH) (*7*). The second plasmid used in this investigation is denoted pCHRS7 derived from pCH93 and also contains a histidine tag (*20*). The difference between the cysteine-free *E. coli* transhydrogenase (cfTH) encoded by pCLNH (*7*) and pCHRS7 is that the latter contains seven silent unique restriction sites.

Site-Directed Mutagenesis. The plasmid pNHIS was used as a template both in the introduction of one and two factor Xa sites (see Results for more details) and in the construction of the plasmid named pNHIS(S) encoding for the mutant wtTH(S); (S) stands for split. WtTH(S) has a stop codon (TAA) introduced at βHis109 that, in combination with the existing βMet113 that constitutes a start codon, results in a split in the peptide chain and a loss of residues His-Asp-Ala-Gly (Figure 1). The same stop codon was introduced into pCHRS7 giving rise to the plasmid denoted pCHRS7-(S) encoding for the cysteine-free mutant cfTH(S). The plasmid pCHRS7(S) was then used as a template when introducing one cysteine at residue βSer105 and one at residue βSer237, resulting in the double mutant called βS105C−βS237C(S). These mutants were all produced using the Quikchange mutagenesis kit from Stratagene; the protocol provided with the kit was followed without any modifications. Primers used were all purchased from Medprobe. The full-length genes were sequenced to verify that no unwanted mutations had occurred. Since all mutations and other changes in this report exclusively concern the β subunit, the prefix β has been omitted in the forthcoming text. Also, transmembrane helix is replaced by H. Amino acid residues are described as three letter codes and mutants as single letter codes.

Expression and Purification. Expression and purification of all enzymes produced in this investigation were carried out in *E. coli* using the histidine tag followed by anion-exchange chromatography, according to Fjellström et al. (*21*) including purification of rrI,[1] that is, dI from *R. rubrum*. The enzymes were at least 90% pure, according to SDS−PAGE using 10−20% Tris-glycine gels from Novex.

Determination of Protein. The protein concentration was determined by using the bicinchoninic acid assay (BCA) with BSA as standard (*22*).

SDS−PAGE. The enzymes were diluted twice with 2× SDS sample buffer (Novex). Unless indicated otherwise, 10 μg of each enzyme was loaded on the 10−20% Tris-glycine gel (Novex) and run for 90 min at 125 V.

[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; AcPyAD$^+$, 3-acetyl pyridine NAD$^+$; rrI, dI from *R. rubrum*; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; wtTH, wild-type transhydrogenase; cfTH, cysteine-free transhydrogenase; wtTH(S), wild-type split transhydrogenase; cfTH(S), cysteine-free split transhydrogenase.

Conditions for Factor Xa Cleavage. The recommended conditions for factor Xa cleavage were, according to Novagen, 16 h at 20 °C in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 1 mM $CaCl_2$ (pH 8.0). When these conditions failed to produce cleavage, the following alternative conditions were used: pH (5.5, 6.5, 7.5, and 8.5), temperature (4, 25, and 37 °C), and time of incubation (2, 5, 15, 30, and 72 h); detergents were also added (i.e., Brij-35, Triton X-100, SDS, or octylglycoside).

Cross-Linking. The purified double cysteine mutant S105C−S237C (S) was mixed with 1 mM diamide (oxidizing agent) or 0.1 mM bifunctional maleimide cross-linker *o*-PDM (6 Å) in cross-linking buffer containing 30 mM sodium phosphate and 0.01% Brij-35 (pH 7.5). The reaction was terminated after 30 min at 4 °C by the addition of 5 mM NEM; 10 μg of the enzyme was run on SDS−PAGE. To reduce the disulfide bond, 5% β-mercaptoethanol was added prior to SDS−PAGE.

Assay of Reverse Activity and K_M^{NADPH}. The reverse activity (i.e., the reduction of 400 μM $AcPyAD^+$ by 400 μM NADPH, with the concomitant translocation of one proton through dII) was assayed in a buffer containing 20 mM Hepes, Mes, Tris-HCl, and Ches, 50 mM NaCl, and 0.01% Brij (pH 7.0). The reduction of $AcPyAD^+$ was monitored at 375 nm at 25 °C using the absorption coefficient 6100 M^{-1} cm^{-1}. By varying the concentration of NADPH between 0 and 500 μM, the K_M for NADPH was determined in a Hanes plot. Both the reverse and the cyclic reactions were also carried out in the presence of 1 mM DTT (incubated for 5 min prior to measurement) and 0.1 mM *o*-PDM as in the cross-linking experiments.

Assay of Cyclic Activity. The cyclic reaction involves hydride transfer between bound NADP(H) in dIII and $AcPyAD^+$/NADH in dI and does not involve proton translocation. This activity was estimated by the reduction of 400 μM $AcPyAD^+$ by 200 μM NADH in the presence or absence of 400 μM $NADP^+$ in a medium containing 20 mM Hepes, Mes, Tris-HCl, and Ches, 50 mM NaCl, 2 mM $MgCl_2$, and 0.01% Brij (pH 6.0). In addition, the cyclic activity was also assayed in the absence and in the presence of 5 μg of *rr*I as described (*23*).

Trypsin Digestion. When trypsin is added to TH, in the absence of NADP(H), dI is degraded, but the rest of the enzyme remains intact (*23*, *24*). Digestion was carried out at 10 °C with trypsin at a mass ratio of 1:30 of trypsin/enzyme. The reaction was terminated after 30 min by the addition of soybean trypsin inhibitor at a mass ratio of 1:2 of trypsin/inhibitor, resulting in a TH devoid of dI.

Preparation of Proteoliposomes. Preparation of liposomes and incorporation of the enzymes were performed as described in ref *9*. All enzymes were incorporated in the same batch of liposomes under identical conditions.

Preparation of Membrane Vesicles. A total of 50 mL of LB-medium per enzyme, supplemented with 5 mg of ampicillin, was inoculated in an incubator shaker with a 5 mL overnight culture. After 16 h at 37 °C, the pellets were harvested by centrifugation (5000 rpm for 10 min using rotor JA-20). Each pellet was resuspended in 10 mL of buffer, 20 mM K_2PO_4, 50 mM KCl, 5 mM $MgCl_2$ (pH 7.2), and sonicated with a rod-sonicator for 20 min in 5 s intervals. To separate the inside-out vesicles from the unbroken cells, the sonicated solution was centrifuged at 10 000 rpm for 10 min in a JA-20 rotor after which the supernatant was transferred to an ultracentrifuge (50 000 rpm for 1 h in a Ti-70 rotor). The pellet was resuspended in 300 μL of the same buffer as above.

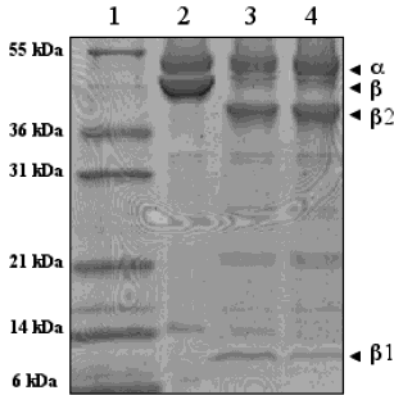

FIGURE 2: SDS−PAGE of wtTH and the functional wtTH(S) and cfTH(S). Lanes are as follows: 1, reference proteins; 2, wtTH; 3, wtTH(S); and 4, cfTH(S). To each well was added 10 μg of protein.

Proton Pumping. Proton pumping was estimated by the quenching of ACMA as described (*9*).

RESULTS

Expression, Purification, and Molecular Masses of the Split β Subunits. A modified version of the established membrane topology of *E. coli* TH is shown in Figure 1 (*7*). The loop connecting H9 and H10 in the β subunit has the sequence S_{105}YLH**H**DAG**M**APILVNIHLTE_{124} including a histidine and a methionine in positions 109 and 113 (bold), respectively. The **CAT** of *N*′-CTG CAT **CAT** GAC GCG GGA ATG GCA-C′ was mutated to a stop codon (**TAA**), generating the sequence *N*′-CTG CAT **TAA** GAC GCG GGA ATG GCA-C′. In the resulting TH mutants, this would generate a potential split β subunit with a C-terminal amino acid sequence of S_{105}YLH_{108} and an N-terminal amino acid sequence of M_{113}APILVNIHLTE_{124} (Figure 1). This means that the original peptide H_{109}DAG_{112} was removed from the loop. Indeed, expression and purification of the resulting wtTH(S), followed by analysis by SDS−PAGE, revealed a split TH with a wild-type α subunit of 54 kD, a β1 subunit of approximately 12 kD (H6−H9), and a β2 subunit of approximately 37 kD (H10−H14 and dIII) (Figure 2, lane 3). Thus, this is in agreement with the assumption that the native β subunit of 49 kD had been split into the β1 and β2 subunits. Expression and purification of the corresponding cysteine-free split cfTH(S) gave the same distribution of subunits as the wtTH(S) (Figure 2, lane 4). Both the purified wtTH(S) and the cfTH(S) contained an additional weak band with a slightly lower molecular mass than the α subunit; the identity of this band is as yet unknown but is probably an aggregation product of $\beta1 + \beta2$ (Figure 2). Otherwise, expression and purification of wtTH(S) and cfTH(S) with respect to yield and purity were essentially indistinguishable from those of wtTH (not shown). In addition, the preparations were highly stable as indicated by an unchanged catalytic activity following storage for at least 14 days at +4 °C.

Introduction of Potential Cleavage Sites for Factor Xa. To be able to carry out and analyze cross-linking between H6−H9 and H10−H14, attempts were made to introduce a single and a double cleavage site in the H9−H10

Table 1: Catalytic Activities of wtTH(S) and cfTH(S) as Compared to Those of wtTH[a]

enzyme	K_M^{NADPH} (μM)	reverse activity (%)	cyclic activity (%)	cyclic activity + *rr*I (%)	cyclic activity + trypsin + *rr*I (%)
wtTH	20	100 ± 5	100 ± 1	100 ± 3	100 ± 2
wtTH(S)	29	58 ± 3	49 ± 2	77 ± 6	107 ± 1
cfTH(S)	24	61 ± 5	55 ± 5	70 ± 5	102 ± 4

[a] Assays were carried out as described in the Materials and Methods. Wild-type reverse, cyclic, cyclic + *rr*I, and cyclic + trypsin + *rr*I activities were 8, 25, 40, and 22 μmol/min/mg, respectively; $n = 3$.

loop for factor Xa, a protease specific for the IEGR sequence in the cysteine-free background. The single and double cleavage site construct had the sequences S_{105}-YLHH**IEGR**APILVNIHLTE$_{124}$ and S_{105}YLHH**IEGR**AP-**IEGR**IHLTE$_{124}$, respectively, where bold indicates the introduced cleavage sites. As compared to that of cfTH, the reverse activity of the single and double factor Xa cleavage site constructs were 110 and 85%, respectively (not shown). However, neither construct resulted in cleavage by factor Xa using a number of different cleavage conditions (see the Materials and Methods). It is interesting to note that the H9−H10 loop thus can be mutated extensively without drastic effects on activity.

Catalytic and Proton Pumping Activities of wtTH(S) and cfTH(S). A somewhat surprising result was that the wtTH(S) as well as cfTH(S) showed high catalytic and proton pumping activities. As shown in Table 1, the activities of the reverse reaction catalyzed by wtTH(S) and cfTH(S) were 58 and 61%, respectively, of wtTH, whereas the corresponding cyclic activities were 49 and 55%, respectively, in the absence of added *rr*I and 77 and 70% in the presence of added *rr*I. Inactivation of the cyclic activities by trypsin due to the cleavage of dI was essentially restored by adding *rr*I, which has been shown to replace the function of dI in *E. coli* TH (*23*). All cyclic activities in the absence of NADP(H) were nil, suggesting that no NADP(H) was bound to dIII (*6*). Determination of K_m^{NADPH} for wtTH, wtTH(S), and cfTH(S) gave 20, 29, and 24 μM, respectively. The K_m^{NADPH} for the cfTH is known to be some 3-fold lower than that for wtTH (*9*). It is remarkable that this discrepancy was normalized by the introduction of the split. Catalytic activities as a function of pH were not significantly different from those for wtTH, wtTH(S), and cfTH(S) (not shown). As shown in Figure 3, assays of proton pumping activities in reconstituted vesicles were carried out as quenching of the fluorescent probe ACMA. Both initial rates and final maximal extents of quenching were similar for wtTH(S) and cfTH(S), both of which were lower as compared to wtTH. The differences were somewhat smaller than those shown for the reverse reactions shown in Table 1. However, it should be stressed that the ACMA assay is a semiqunatitative measure of proton pumping. Similar results were obtained with cytosolic vesicles (not shown).

Cross-Linking of the β1 and β2 Subunits. To test cfTH(S) as a tool in the cross-linking studies of dII, a double cysteine mutant was constructed in the cfTH(S) background. Of particular interest was the relationship between H9, H13, and H14, with His91 and Asn222 in H9 and H13, respectively, which are believed to form part of a proton channel (*5*), and H14 connecting dII with dIII through the hinge peptide (*9*).

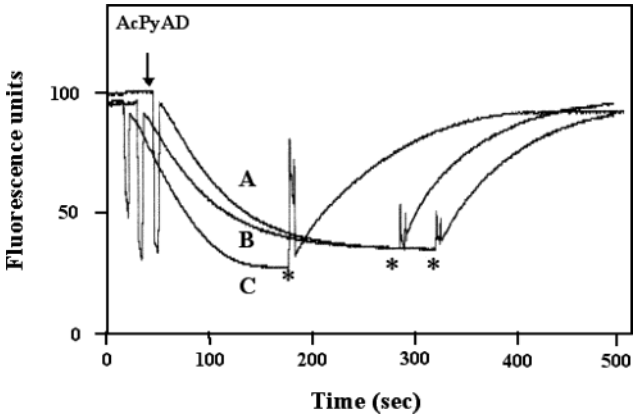

FIGURE 3: Proton pumping by purified wtTH, wtTH(S), and cfTH(S) in reconstituted vesicles. Traces are as follows: A, cfTH(S); B, wtTH(S); and C, wtTH. Medium contained $AcPyAD^+$ (400 μM), NADPH (400 μM), and proteoliposomes (10 μg of protein). The reaction was started by the addition of $AcPyAD^+$. After obtaining an essentially constant quenching of ACMA fluoresence, 1 μM CCCP (denoted by *) was added to abolish the electrochemical proton gradient and restore ACMA fluorescence.

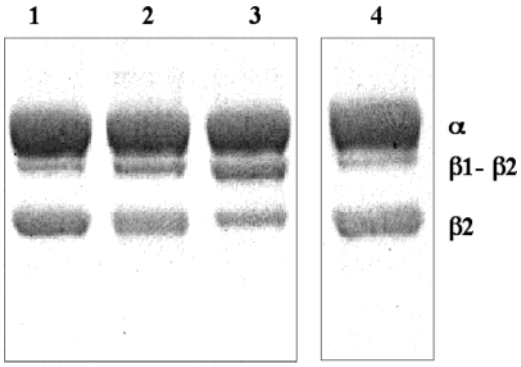

FIGURE 4: Cross-linking of the S105C−S237C(S) mutant with diamide and *o*-PDM. Lanes contained the following: lane 1, S105C−S237C(S); lane 2, S105C−S237C(S) incubated with diamide; lane 3, S105C−S237C(S) incubated with *o*-PDM; and lane 4, S105C−S237C(S) incubated with diamide followed by incubation with β-mercaptoethanol. To each well 10 μg of protein was added. For more details, see Materials and Methods.

Table 2: Catalytic Activities of the S105C−S237C(S) Mutant[a]

activity	addition: none (%)	+DTT (%)	+*o*-PDM (%)
reverse	52 ± 2	61 ± 5	48 ± 4
cyclic	120 ± 3	136 ± 6	125 ± 6

[a] Assays were carried out as described in the Materials and Methods. All activities are compared to cfTH(S), which had reverse and cyclic activities of 5 and 14 μmol/min/mg, respectively; $n = 3$.

The S105C−S237C(S) mutant was therefore constructed where the S105C resides in the C-terminal end of H9, and S237C resides in the short loop between H13 and H14 (Figure 1). This double mutant was properly folded as indicated by high reverse and cyclic activities both in the presence and in the absence of DTT and *o*-PDM, as compared to those of cfTH(S) (Table 2). Cross-linking between the S105C and the S237C residues was then carried out in the presence of the oxidizing agent diamide and the maleimide derivative *o*-PDM, of which the latter can span cysteines approximately 6 Å apart. As shown in Figure 4, cross-linking occurred already in the presence of the oxidizing agent diamide (lane 2) and increased further in the

presence of the maleimide derivative *o*-PDM (lane 3). Approximately 75% of the β1 and β2 subunits were cross-linked by *o*-PDM, whereas the cross-linked β1−β2 band induced by diamide was weaker than in the presence of *o*-PDM but significantly stronger than the weak background band found in all wtTH(S) and cfTH(S) preparations (Figure 2, lanes 3 and 4). No unspecific cross-linking of β1 and β2 occurred with cfTH(S) (not shown). Incubation with the cross-linker for more than 30 min resulted in approximately 90% cross-linking with unchanged activity (not shown), indicating that cross-linking caused little or no inhibition of the enzyme. In this context, it is important to stress that the formation of β1−β2 adducts, induced by diamide and *o*-PDM, was accompanied by the expected and proportional decrease in the β2 band (Figure 4). A corresponding decrease in the β1 band indeed also took place but was barely detectable under the SDS−PAGE conditions used (not shown). Other cross-links (i.e., β1−β1 or β2−β2) were not formed with the S105C−S237C(S) mutant, which demonstrates the specificity of the cross-linking. Addition of β-mercaptoethanol reduced the diamide-induced cross-linking and most of the weak background (cf. Figure 4, lane 4).

DISCUSSION

As shown in the pioneering work on lac-permease by Kaback and co-workers, and subseqently applied by several other research groups, site-directed and cysteine-scanning mutagenesis in a cysteine-free background is an efficient approach for probing the packing and roles of transmembrane helices in membrane proteins (*25*). Being one of a few membrane proteins that maintains the bulk of its catalytic activity in the cysteine-free state, and in the case of the *E. coli* TH also proton pumping (*11*), the latter enzyme is highly suitable for a similar approach. Thus, cross-linking between the α and the β subunit of *E. coli* TH was previously investigated on the basis of which a spatial relationship between some transmembrane helices was proposed (*12*). However, due to problems in analyzing cross-links between transmembrane helices in the β subunit, these results were not sufficient for proposing a packing model of the entire dII. The key problem was that, since structural differences between the native and the cross-linked β subunits were too small, cross-linking between transmembrane helices in the β subunit could not be readily detected by SDS−PAGE. As demonstrated with lac-permease, this problem may be overcome by the introduction of an extra protease cleavage site in a suitable helix−helix loop for, for example, factor Xa (*25*−*28*) or the expression of the protein as two contiguous nonoverlapping fragments dividing the transmembrane domain in at least two subdomains (*13*, *25*, *29*).

By changing two bases in the gene sequence for the long loop connecting H9 and H10 of the β subunit of *E. coli* TH (i.e., CAT (His109)) to TAA, transcription is stopped at TAA and reinitiated by the following existing ATG (Met113) (Figure 1). By introducing a stop-codon between H9 and H10 in the β subunit, an enzyme with remarkable wild-type-like properties was produced. It should be stressed that the β1 subunit contains the essential residue His91 (in H9) and that the β2 subunit contains Glu124 (in H10), Ser139 (in H10), Asn222 (in H13), and Asp213 (in the loop connecting H12 and H13). To obtain a tightly coupled proton pumping TH, it is reasonable to assume that all these residues must be in their correct position, which clearly is the case for the wtTH(S) and cfTH(S) enzymes. Both wtTH(S) and cfTH(S) could be solubilized and reconstituted in liposomes with wild-type-like proton pumping properties as judged by ACMA quenching (Figure 3). Similar activities were measured in crude membrane vesicles (not shown). In the case of the split enzymes, neither the pH-optimum for the cyclic nor the reverse reactions were significantly different from wtTH. After the removal of dI by trypsin, *rr*I restored wild-type-like cyclic activities indicating that dIII was not affected by the split (Table 1). The wild-type-like K_m^{NADPH} also supports that dIII was not altered (Table 1).

The theoretical possibility that the cross-linkers selectively react with denatured inactive protein is rendered unlikely due to the high extent of cross-linking, more than 75%, with essentially retained catalytic activity. That the purified preparation used in this investigation contains little or no inactive protein is also indicated by the use of affinity chromatography of a similar preparation on immobilized palmitoyl coenzyme A (*30*).

As compared to other proteins where disruption of a single coding gene produces an active enzyme, the functionally split *E. coli* TH has remarkably wild-type-like properties. Construction of the functionally split *E. coli* TH through a single mutagenesis step is also unique in its simplicity. It is conceivable that similar simple splits can be achieved in helix−helix loops of other membrane proteins containing methionine residues.

One approach to assay cross-linking between H6−H9 and H10−H14 was made by the introduction of hydrophilic factor Xa cleavage sites in the H9−H10 loop. However, these attempts were not successful but demonstrated that this loop is surprisingly insensitive to extensive mutagenesis.

Bragg and Clarke showed earlier that *E. coli* TH can assemble from the α and β subunits individually translated by different coexisting plasmids (*31*). However, truncations of the α subunit strongly affect folding of the β subunit and thus the entire enzyme, indicating that the folding of the two subunits is linked (*32*, *33*). The functionally split *E. coli* TH described in this paper was encoded by a dicistronic operon on a single plasmid. In fact, several active functionally split proteins required coexpression either on different operons in a single plasmid or a dicistronic operon on a single plasmid (*13*−*18*, *34*). It appears that the translation of segments from different plasmids in some cases leads to an extended assembly process and degradation (*34*, *35*). It is conceivable that the successful folding of three separate peptides of wtTH(S), of which two are artificial, is mediated by a mixed process in which folding of the α subunit is separate from and required for that of the β subunit (cf. refs *32* and *33*). However, it is unlikely that the small hydrophobic β1 peptide of 12 kD would exist separately and stably folded in the membrane. It is therefore suggested that β1 and β2 assemble as a short-lived complex prior to the folding in the membrane by the translocon. DII of *E. coli* TH largely obeys the positive-inside rule (*36*) (i.e., the bulk of the positive charges in dII are located on the cytosolic side).

The double cysteine mutant S105C−S237C(S) displayed essentially unchanged catalytic properties before as well as after cross-linking, suggesting a native fold. The cross-linking was specific and easily detectable on SDS−PAGE, and it was concluded that Ser105 is close to Ser237. This supports

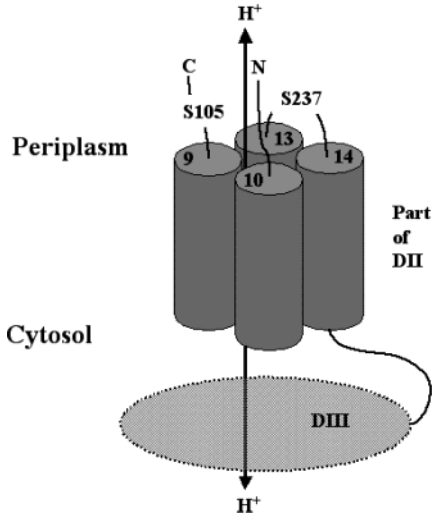

FIGURE 5: Helix packing model of part of the proton channel in dII. Residues mutated in the S105C−S237C(S) double mutant are indicated. The proton channel is positioned along H9, H10, and H13 in dII. Only four out of 13 transmembrane α helices are shown (cf. ref *7*).

the assumption that the proton channel is lined by H9 and H13. Obviously, the flexibility of both the C-terminal end of H9 and the H13/H14 loop is important in this context and may differ from that shown in Figure 1. However, it has been shown that S260 is in the membrane/medium interface and that D213 is exposed to the medium (*9*). Moreover, S183, S237, and D239 are exposed to the medium (periplasmic space in permeabilized *E. coli* cells) (*7*). On the basis of prediction (*7*), and in agreement with the composition of a normal transmembrane helix, H13 and H14 each contain 21 residues. A similar argumentation applies to S105 (periplasmic space), E85 (membrane interface), and T81 (cytosol), with H9 also containing 21 residues (*7*). Taken together, this leaves little room for an extended C-terminal of H9 and a larger H13/H14 loop. Indirect results also suggest that H10, and possibly also H14, are involved in the channel (*5*). With this new knowledge at hand, an approximate model of the proton channel has been constructed (Figure 5).

In conclusion, a functional split *E. coli* TH containing a β subunit divided in the β1 and β2 peptides has been constructed that retained largely wild-type-like activities, suggesting that it was folded with minor alterations in dII. The existence of stably folded hydrophobic β1 and β2 peptides generated by the split indicates a concerted folding process of the β1 and β2 subunits. By generating the S105C−S237C(S) mutant, cross-linking within the β subunit has been demonstrated for the first time, on the basis of which a model of the proton channel is presented. Using cfTH(S), a systematic cross-linking of dII of the β subunit has now become possible.

ACKNOWLEDGMENT

Dr. P. D. Bragg is gratefully acknowledged for the generous gift of the pCH93 plasmid. We would like to also thank Dr. Eva Arkblad for advice and discussions.

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BI034560X