Roles of Individual Amino Acids in Helix 14 of the Membrane Domain of Proton-Translocating Transhydrogenase from *Escherichia coli* As Deduced from Cysteine Mutagenesis†

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ABSTRACT: Proton-translocating nicotinamide nucleotide transhydrogenase is a membrane-bound protein composed of three domains: the hydrophilic NAD(H)-binding domain, the hydrophilic NADP(H)-binding domain, and the hydrophobic membrane domain. The latter harbors the proton channel. In *Escherichia coli* transhydrogenase, the membrane domain is composed of 13 transmembrane α helices, of which especially helices 13 and 14 contain conserved residues. To characterize the roles of the individual residues βLeu240 to βSer260 in helix 14, these were mutated as single mutants to cysteines in the cysteine-free background, and in the case of βGly245, βGly249, and βGly252, also to leucines. In addition to the residues forming the helix, residues βAsn238 and βAsp239 were also mutated. Except for βI242C, all mutants were normally expressed, purified, and characterized with respect to, e.g., catalytic activities and proton pumping. The results show that mutation of the conserved glycines βGly245, βGly249, and βGly252, located on the same face of the helix, led to a general inhibition of all activities, especially in the case of βGly252, suggesting a role of these glycines in helix−helix interactions. In contrast, mutation of the conserved serines βSer250, βSer251, and βSer256 led to enhanced activities of all reactions, including the cyclic reaction which was mediated by bound NADP(H). Mutation of the remaining residues resulted in intermediate inhibitory effects. The results strongly support an important regulatory role of the membrane domain on the NADP(H)-binding site.

Proton-translocating nicotinamide nucleotide transhydrogenase (E.C. 1.6.1.2) is an integral membrane protein found in most organisms except some yeasts, plants, and certain bacteria. It is located in the inner membrane of mitochondria and in the plasma membrane of bacteria. The enzyme catalyzes the reversible reduction of $NADP^+$ by NADH which is linked to proton translocation across the membrane according to the reaction:

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

In the absence of an electrochemical proton gradient (Δp), the reaction from left to right (the forward reaction) is approximately 5-fold slower than the reverse reaction. In the absence of a Δp, the overall reaction proceeds to an equilibrium constant of close to 1. In the presence of a Δp, i.e., under more physiological conditions, the forward reaction is increased 5−10-fold and the apparent equilibrium constant is increased to about 500. The net effect of the transhydrogenase (TH)[1] reaction is probably to provide NADPH at the expense of NADH and Δp. This NADPH generation by TH suggests important roles of the enzyme in mitochondrial/cellular redox regulation including biosynthesis, detoxification (via the glutathione/thioredoxin systems), and apoptosis (*1*−*3*).

THs are structurally and functionally divided in three domains, the hydrophilic NAD(H)-binding domain (dI), the hydrophilic NADP(H)-binding domain (dIII), and the hydrophobic membrane domain (dII). The active forms are dimeric, in the case of the *Escherichia coli* enzyme a dimer of the α + β subunits, i.e., $\alpha_2\beta_2$, where dII is derived from the hydrophobic parts of both the α and β subunits (Figure 1). dI and dIII can be expressed separately and purified in an active state and, when combined, show catalytic activity (*4*, *5*). dI and dIII have been resolved structurally using X-ray crystallography (*6*−*10*) and NMR (*11*−*15*).

In contrast to intact TH, separately expressed dIII is purified in a so-called "occluded" state that involves tightly bound NADP(H) (*5*). The low rates of the forward and reverse reactions catalyzed by dIII in the presence of dI reflect a limiting release of the products NADP(H). The corresponding high rates catalyzed by the wild-type TH, especially in the presence of a Δp, demonstrate the essential role of dII in regulating the dissociation of NADP(H) from dIII and converting a high Δp into a high redox level of NADP(H). Despite its obvious importance for the function and energy coupling of TH, the structure and function of

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[1] Abbreviations: $AcPyAD^+$, 3-acetylpyridine−NAD^+; NEM, *N*-ethylmaleimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; wtTH, wild-type transhydrogenase; TH, nicotinamide nucleotide transhydrogenase; cfTH, cysteine-free transhydrogenase; dI, domain I of transhydrogenase; dII, domain II of transhydrogenase; dIII, domain III of transhydrogenase; rrI, the soluble domain I (PntAA) from *Rhodospirillum rubrum*; H14, transmembrane α helix 14.

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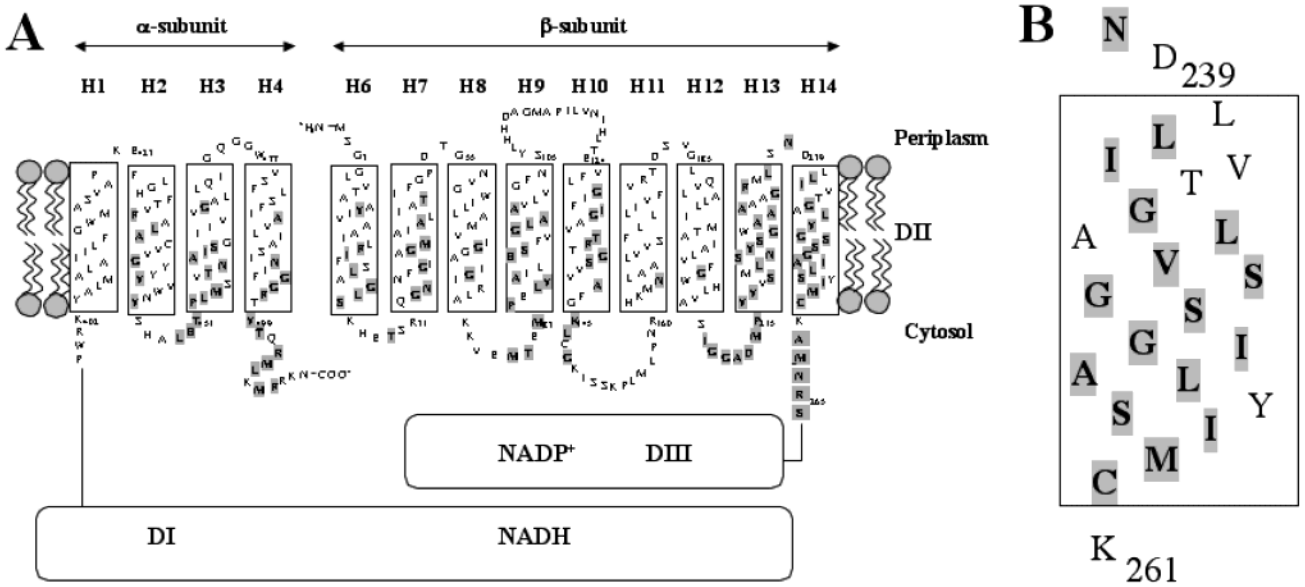

FIGURE 1: Membrane topology of *E. coli* transhydrogenase (A) and a close-up view of helix 14 (B). The assignments of helices were made according to Meuller et al. (*16*). Residues indicated in bold and gray background are at least 80% conserved between 61 THs from different species (*22*).

dII have as yet not received as much attention as dI and dIII. However, the membrane topology of the *E. coli* TH was shown to be composed of four transmembrane helices in the α subunit (denoted H1−H4) and nine transmembrane helices in the *β* subunit (denoted H6−H14) (Figure 1; cf. ref *16*). Several key observations indicate that the state of protonation of *β*His91 (*17*−*20*) and *β*Asn222 (*19*), and possibly also *β*Ser139 (*21*), as potential components of a proton channel, indirectly regulates the NADP(H)-binding properties of dIII through conformational changes. Indeed, on the basis of the "hinge" peptide connecting H14 in dII with dIII, a novel coupling mechanism has been proposed (*22*, *23*).

Two of the 13 α helices in the *E. coli* TH, i.e., H13 and H14, are essentially conserved between species, indicating that they are crucial for the function of the enzyme. Except for the participation of a potential salt bridge between *β*Arg265 and *β*Asp213 in the extramembraneous hinge region (*22*, *23*; cf. Figure 1), little information is available regarding the roles of the transmembranous α helices and their individual amino acids in general. In a first part of a systematic characterization of H13 and H14, using a cysteine-free TH constructed earlier with essentially wild-type properties (*24*), all residues of H14 in the intact *E. coli* TH were systematically mutated to cysteines and in case of the glycines also to leucines, and the resulting mutants were purified and characterized. The properties of some mutants of, e.g., glycines located on the same face of H14 and serines revealed unexpected dramatic effects on catalytic activities, which have implications for the coupling mechanism.

MATERIALS AND METHODS

Bacterial Strains. SCS110 is a *dam*-negative strain that was used in the cleavage steps with *Bcl*I and *Cla*I since the restriction enzymes are sensitive to methylation. Otherwise, the *E. coli* strain JM109 was used in the expression and purification of the protein.

Site-Directed Mutagenesis. Single cysteine and leucine mutants were introduced in the cysteine-free gene of transhydrogenase in pCHRS7. A total of 26 mutants were made, i.e., *β*N238C−*β*S260C and *β*G245L, *β*G249L, and *β*G252L (cf. Figure 1), with primers supplied from Invitrogen; mutants were made with the QuickChange site-directed mutagenesis kit (Stratagene). The entire gene was sequenced to check the ligated plasmid and the introduced cysteine mutant. Since only mutants of the *β* subunit are discussed in this report, the prefix "*β*" denoting the *β* subunit in, e.g., *β*N238C will be omitted in the following text. Also amino acid residues are denoted with three-letter codes and mutants with single-letter codes.

Plasmids. Bragg and co-workers constructed a plasmid, pCH93, which contains six unique and silent restriction enzyme sites in the cysteine-free transhydrogenase gene (*25*). The *Bcl*I site at residue Ile310 in pCH93 was deleted (GTGATC changed to GTTATC), leaving one *Bcl*I site at Asn285 (TGATC), resulting in the plasmid pCHRS7 which subsequently was used as a template for all mutants produced. The full length of the gene was sequenced, and the seven unique restriction enzyme sites were confirmed. The *Cla*I site at Ala207 (GCCTCCA changed to GCATCGA) and the *Bcl*I site at Asn285 were used to cut and ligate the plasmid.

When the introduced cysteine mutant had been sequenced, the gene was cut with *Cla*I (Ala207) and *Bcl*I (Asn285). The plasmid, pCHRS7, was cut into two fragments, 5.8 and 0.2 kb. On a 1% agarose gel the fragments were separated and purified with the QIAEXII agarose gel extraction kit. To ligate the 0.2 kb fragment, Ala207/Asn285, including the introduced mutation with cleaved plasmid, T4 ligase (Gibco) was used, and the fragments were added at a ratio of 1:1.

Expression and Purification of Enzymes. Cysteine-free transhydrogenase (cfTH) and all mutants were expressed and purified as described (*26*). As judged from SDS−PAGE, the purity was at least 90%.

Determination of Protein Concentrations. Protein concentrations were determined by the bicinchoninic acid assay (BCA) using BSA as standard (*27*).

Activity Assays. The activities of the reverse and cyclic reactions were measured, and the pH dependencies (pH 4.5−9.0) of the reactions were estimated for all of the mutants essentially as described (*23*). Both reactions were measured spectrophotometrically at 375 nm (25 °C, using the absorption coefficient 6100 M^{-1} cm^{-1}). The reverse reaction was assayed as reduction of 300 μM $AcPyAD^+$ by 300 μM NADPH in buffer A (20 mM Ches, 20 mM Mes, 20 mM Tris, 20 mM Hepes, 50 mM NaCl, and 0.1 mg/mL Brij-35, pH 7.5). To estimate K_m^{NADPH}, the reverse reaction was measured in the presence of 5−400 μM NADPH; K_m^{NADPH} was calculated by a Hanes plot. The cyclic reduction of 300 μM $AcPyAD^+$ by 150 μM NADH in the presence and absence of 300 μM $NADP^+$ was measured in buffer B (20 mM Ches, 20 mM Mes, 20 mM Tris, 20 mM Hepes, 50 mM NaCl, 0.1 mg/mL Brij-35, and 5 mM $MgCl_2$, pH 6.0).

Proton Pumping Activity. To estimate proton pumping activity in cytosolic vesicles, 50 mL of cultivated cells was centrifuged for 10 min at 5000 rpm in rotor JA-20. The pellet was resuspended in 10 mL of proton pumping buffer (20 mM K_2HPO_4, 50 mM KCl, and 5 mM $MgCl_2$, pH 7.2). The mixture was sonicated for 10 min (in 5 s intervals) to make inside-out vesicles and centrifuged for 10 min at 5000 rpm. The supernatant was transferred into an ultracentrifuge tube (75 μL of the supernatant was stored for estimating expression levels on SDS gels) and centrifuged for 1 h at 45000 rpm in a Beckman Ti70 rotor. The membrane pellet was resuspended in 400 μL of proton pumping buffer. Proton pumping driven by reduction of 400 μM $AcPyAD^+$ by 400 μM NADPH was assayed at excitation 415 nm and emission 475 nm as quenching of 2 μM ACMA (*23*). The activity of the reverse activity catalyzed by the vesicles identical to those used for proton pumping was also carried out in order to correlate directly the reverse activity to proton pumping.

Qualitative Determination of Bound NADP(H). To assay bound NADP(H), 10−30 μg of protein was incubated in 50 μL of 10 mM Tris-HCl (pH 9). A parallel incubation was made with 10 units of alkaline phosphatase added and was incubated for 15 min at 37 °C. Cyclic activity was subsequently measured with and without $NADP^+$ on the two sets of samples per mutant at pH 6.0 (cf. cyclic activity). This assay is based on the known ability of alkaline phosphatase to hydrolyze 2′-/3′-phosphate in 2′-NADP(H) and 3′-NADP(H) (*28*). Pyridine nucleotides were extracted as described by Klingenberg (*29*).

Inhibition by NEM. The thiol-specific NEM (200 μM) was incubated with the cysteine mutant protein (50−150 μg), in the presence and absence of 300 μM NADPH and 300 μM $NADP^+$, for 60 min at 25 °C and at pH 7.5. The reverse activity was measured after 0, 5, 30, and 60 min.

RESULTS

Mutagenesis, Expression, and Purification of Mutants. All residues in H14, i.e., Leu240−Ser260 (Figure 1B), were replaced as single mutants to cysteines in the cysteine-free background. In addition, G245L, G249L, and G252L and the N238C and D239C mutants in the loop connecting H13 and H14 were also made. Figure 2 shows a helical wheel representation of the positions of the mutated residues in H14. Except for I242C, all mutants were expressed essentially as cfTH.

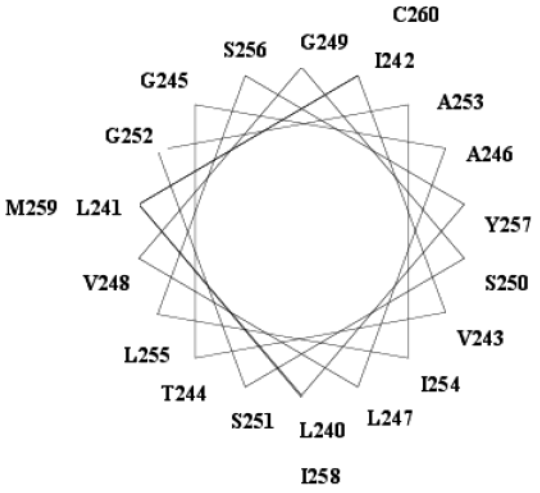

FIGURE 2: Helical wheel presentation of the residues in H14 of *E. coli* TH. The helix is viewed from the periplasmic side.

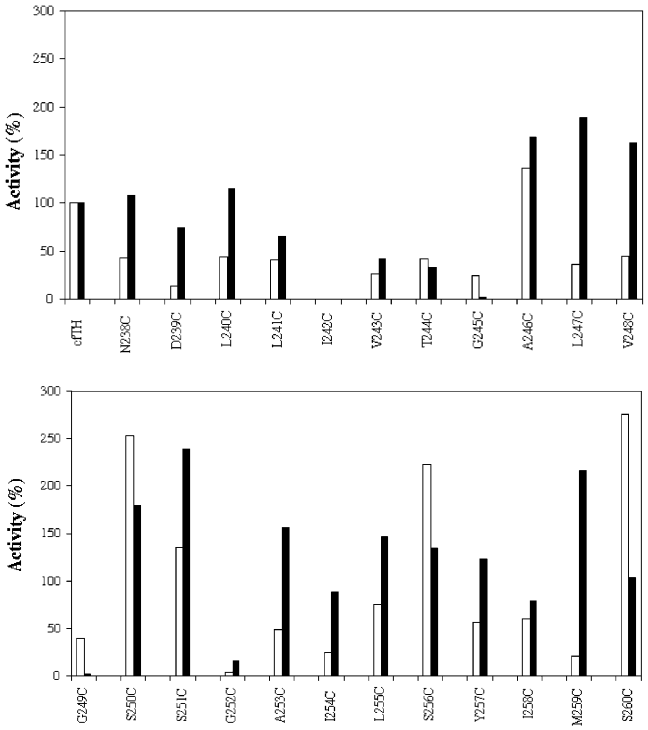

FIGURE 3: Activities of reverse and cyclic reactions catalyzed by the cysteine mutants in H14 of *E. coli* TH. The activity of the reverse and cyclic reactions catalyzed by cfTH was 3.6 and 31 μmol min^{-1} mg^{-1}, respectively. White (left) and black (right) bars denote the reverse and cyclic activity, respectively. Assays were as described in Materials and Methods.

Properties of Mutated Glycines. The G245C, G249C, and G252C mutants catalyzed reverse activities that were 24%, 40%, and <5%, respectively (Figure 3) of that of cfTH, with pH optima of 6.5, 8.0, and 7.5, respectively (not shown). Under the conditions used, cfTH had a pH optimum of 7.5. The corresponding cyclic activities were very low, i.e., <5%, <5%, and 16%, respectively (Figure 3), all with the pH optima of 5.5, which represents a downshift of 0.5 unit as compared to cfTH. As exemplified by the G249C mutant, the K_m^{NADPH} in the reverse reaction was determined to be 11 μM, i.e., close to that of cfTH, 7 μM. Thus, cysteine mutants of the conserved glycines generally showed low or very low activities, especially of the cyclic reaction, without

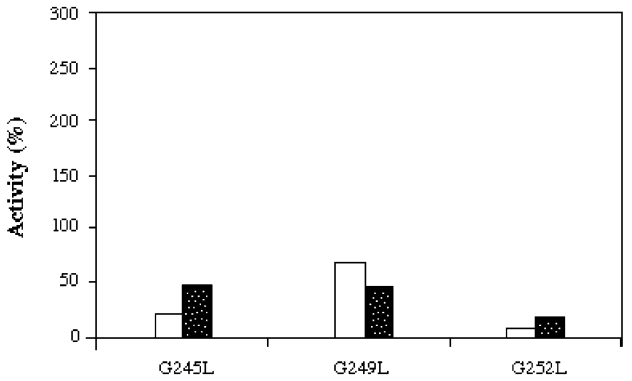

FIGURE 4: Activities of reverse and cyclic reactions catalyzed by the G245L, G249L, and G252L mutants of H14 of *E. coli* TH. Conditions were as in Figure 3. White (left) and black (right) bars denote the reverse and cyclic activity, respectively.

major changes in the K_m^{NADPH}. Separately expressed rrI has earlier been shown to be able to replace both functional and degraded dI in *E. coli* (*30*). Addition of rrI to the G245C, G249C, and G252C mutants increased the cyclic activities by 70%, 100%, and 55%, respectively, as compared to 30% for cfTH (not shown). However, it should be stressed that the activities of the mutants, even after addition of rrI, were still low.

The conserved glycines were also mutated to leucines, i.e., G245L, G249L, and G252L (Figure 4). Reverse activities of the G245L and G252L mutants were similar to those of the corresponding cysteine mutants, whereas the activity of G249L was higher, 70% of cfTH. Cyclic activities for the G245L, G249L, and G252L mutants were 52%, 48%, and 13%, respectively, i.e., higher for G245L and G249L than the corresponding cysteine mutants.

Properties of Mutated Serines. The S260C mutant represents a reversal to the wild-type residue leading to the expected activation of the reverse reaction with little or no effect on the cyclic rection (Figure 3; cf. ref *24*). Cysteine mutants of the conserved serines, i.e., S250C, S251C, and S256C, generally gave strongly increased reverse as well as cyclic activities. Reverse activities were 253%, 146%, and 223% of cfTH, and cyclic activities were 180%, 238%, and 135% of cfTH (Figure 3). As exemplified by the S251C mutant, the K_m^{NADPH} in the reverse reaction was determined to 6.2 μM, i.e., relatively unchanged compared to that of cfTH, 7.0 μM. The pH dependencies of the reverse reaction catalyzed by the mutants were shifted downward with 0.5 unit to pH 7.0, whereas those for the cyclic reaction were shifted downward with at most 0.5 unit to pH 5.5 (not shown). The cyclic reaction has previously been shown to occur even in the absence of $NADP^+$ or NADPH added to dIII (*31*). For this reaction to proceed either the enzyme must contain bound NADP(H) in dIII or the added NADH/$AcPyAD^+$ must interact unspecifically with dIII and catalyze hydride transfer with the substrate in dI. wtTH catalyzes this reaction but only to about 5% of the corresponding NADP(H)-dependent activity (*31*). However, when constructing cfTH, Meuller et al. (*24*) noticed that cfTH also catalyzed this cyclic reaction and at a rate substantially higher rate than that for wtTH.

The S250C, S251C, and S256C mutants all catalyzed the cyclic reaction in the absence of added $NADP^+$ with 3%, 10%, and 41%, respectively, of the $NADP^+$-dependent activity (Table 1). For cfTH the corresponding activity was 16%, in agreement with previous findings (*24*). Treatment of NADP(H) with alkaline phosphatase at pH 9 removes the 2′-phosphate, producing NAD(H) which normally has a very low affinity for the NADP(H)-binding site and which consequently disscociates from the binding site (cf. Materials and Methods). Treatment with alkaline phosphatase led to a decrease of this activity in cfTH to 8% (Table 1), whereas the activities of S250C, S251C, and S256C decreased to 3% or less (Table 1), indicating that the high cyclic activity of these mutants indeed was due to bound NADP(H). In contrast, M259C showed, surprisingly enough, only a minor change in activity following alkaline phosphatase treatment (Table 1). These qualitative determinations of bound NADP(H) obtained with phosphatase essentially agreed with those obtained enzymatically (not shown).

Table 1: Effect of Alkaline Phosphatase on the Cyclic Reaction Catalyzed by cfTH and the S250C, S251C, S256C, and M259C Mutants[a]

	activity (%)	
mutant	A −phosphatase	B +phosphatase
cfTH	16	8
S250C	3	1
S251C	10	1
S256C	41	3
M259C	74	60

[a] Values under "A" denote cyclic activity in the absence of added $NADP^+$ divided by the activity in the presence of $NADP^+$, expressed in percent, without treatment with alkaline phosphatase. Values under "B" denote the corresponding activities but following treatment with alkaline phosphatase. Treatment with alkaline phosphatase was carried out as described in Materials and Methods. Control activities were as shown in Figure 3. Values are averages of triplicates with an experimental error of less than 10%.

Properties of Remaining Residues in H14. The remaining mutants displayed relatively unchanged cyclic activities as compared to cfTH except for V243C and T244C, which were inhibited to approximately 60% (Figure 3). However, the reverse activities of all of these mutants were generally more inhibited than the cyclic activities. As compared to cfTH, the A246C, A253C, L255C, Y257C, and I258C mutants had between 50% and 70% reverse activity while the reverse activities of the N238C, D239C, L240C, L241C, V243C, T244C, L247C, V248C, L254C, and M259C mutants varied between 15% and 45% (Figure 3). None of the above mutants showed more than a 0.5 pH unit downshift of the pH optimum for the cyclic reaction, as compared to cfTH (not shown). In the case of the reverse reaction the majority of the mutants behaved like cfTH with pH optima around 7.5, except for the A246C, L255C, and Y257C mutants which had their pH optima at 6.5, 8.0, and 6.5, respectively (not shown). The M259C mutant catalyzed a low (21%) reverse reaction and an enhanced (215%) cyclic reaction, a picture reminiscent of other mutations in dII, e.g., H91K and N222R (*32*), that previously have been shown to increase the cyclic reaction and decrease the reverse reaction probably by limiting the release of NADP(H). Indeed, when determined separately, the K_m^{NADPH} was decreased from 7 μM for cfTH to 2.5 μM for the M259C mutant.

DISCUSSION

The present investigation constitutes part of an extensive effort to establish the role of the membrane domain (dII) in proton-translocating transhydrogenases. Attempts to deduce the structure of the intact *E. coli* TH by X-ray crystallography have so far been unsuccessful, and indirect approaches to study the structure−function of dII must therefore be used. A considerable amount of information regarding dII is now available, not least because of the ease by which mutants of the *E. coli* enzyme can be made in the wild-type and cysteine-free enzymes (cf. ref *4*) and because of the available 13-helix membrane topology of the enzyme (*16*).

Important experiments by Bragg and co-workers showed that dII and dIII communicate structurally (*32*−*34*). The key observation was that mutagenesis of His91 and Asn222 to postive residues, i.e., an arginine or a lysine, resulted in tightly bound NADP(H) in dIII, interpreted as the formation of the so-called occluded state through long-distance conformational changes (*4*). Except for the loop between H12 and H13, hydrophilic loops in dII are generally poorly conserved, suggesting that the functional communication occurs through the hinge region, i.e., the C-terminus of H14 and the loop connecting H12 and H13 to the N-terminus of dIII (*23*). Bragg and co-workers showed earlier that this peptide was strongly mobile and became trypsin-sensitive in the presence of NADP(H) (*35*). On the basis of a double mutant, D213C,R265C, Althage et al. (*23*) showed that these residues, of which Arg265 is located in the above peptide, possibly form a salt bridge involved in the communication between dII and dIII. On the basis of this observation, a new coupling mechanism was proposed (*22*) involving the hinge region described above (*23*).

Being highly conserved, and because it is associated with the hinge region, H14 was considered important for the function of dII and its communication with dIII (*22*). Polar residues that have been mutated previously in H14 are Ser250 (*20*, *21*), Ser251 (*20*, *21*), Ser256 (*20*, *21*), Tyr257 (*20*, *21*), and Ser260 (*24*). However, these mutants were not fully characterized, and the remaining 18 residues, including the conserved glycines, were excluded in these studies. Double S250A,S251A and S256A,Y257F mutants were first constructed by Bragg and co-workers, neither of which showed properties that deviated more than 30% from cfTH (*20*). In the case of single alanine mutants of serines, the activities of the S250A, S251A, and S256A mutants showed either a marginal inhibition of catalytic activities and proton pumping or a higher activity than control denoted as ">100%" (*21*); no further characterization of the properties was described. The same conclusion was reached for Y257F (*21*). A systematic cysteine scanning of all residues of H14 and a thorough characterization of the properties of the mutants were therefore initiated. Replacement of a residue with cysteine was used because it is generally a mild substitution and allows further cross-linking experiments and use of cysteine-specific probes. Expression of all mutant proteins was normal except for I242C. Several attempts were made to express this latter mutant but without success, suggesting that it was misfolded and/or degraded. It is possible that I242C, which is located on the same face in H14 as the conserved glycines, may interact with, e.g., Gly233 in H13 (Figure 1).

In current helix-packing models of the dII parts of the $\alpha + \beta$ unit, H14 is located peripherally with H9, H10, and H13 placed in the center and associated with the proton channel (M. Althage, J. Karlsson, and J. Rydström, unpublished results; *22*). Bragg and co-workers (*25*) suggested that an aqueous cavity exists between the cytosol and a region in the membrane corresponding to His91 (in H9) and Asn222 (in H13). This cavity may be associated with the proton channel and Glu124 (in H10) (*34*) and may also include Ser139 (in H10) (*21*). Thus, it is possible that H14 indirectly facilitates proton translocation by influencing the centrally located H9, H10, and H13, in which the proton channel is assumed to be located. Residues in H14 responsible for this effect could be Ser250, Ser251, and Ser256. It may then be argued that such an effect would uncouple or at least diminish coupling and proton translocation. However, a decreased efficiency of proton translocation may not have been deteced in the translocation assay presently used, but only when equilibrium is approached. Of course, the possibility that H14 contributes directly to the proton channel cannot be eliminated but is less likely at this point. In this context it should be noted that cysteine mutants that showed an increased inhibition by NEM in the presence of NADPH, i.e., L241C, L247C, V248C, S251C, L255C, and M259C, are all located on the same face of H14 (Figure 2); the G249C mutant was the only one that was much more inhibited by NEM in the presence of $NADP^+$ than NADPH (not shown). These results are consistent with the notion that $NADP^+$ and NADPH induce different conformational states of the hinge region (cf. ref *23*).

A helical wheel presentation of the residues in H14 shows that the conserved glycines Gly245, Gly249, and Gly252 are located along the same face (cf. Figure 2) and are likely to be involved in ridge−groove helix−helix interactions/packing, such as Ile-Gly (*36*). This assumption is consistent with the generally low or very low activities catalyzed by the G245C, G245L, G249C, G249L, G252C, and G252L mutants, especially regarding the cyclic activities of G245C, G249C, and G252L and the reverse activities of G252C and G252L, which were between 85% and 95% inhibited. Interestingly, the G245L and G249L mutants catalyzed higher cyclic activities than the G245C and G249C mutants, indicating that their side chains strongly affected the cyclic activity. It is actually uncommon that a transhydrogenase mutant, which is highly expressed and apparently properly membrane inserted, shows strongly diminished activities of *both* the reverse and cyclic activities with somewhat altered properties, e.g., pH dependence but with an unchanged $K_m^{NADP(H)}$. Thus, it is conceivable that the packing of the helices is disturbed, primarily affecting dIII since added rrI [which can replace dI (*30*)] did not alleviate the inhibition. In this context it should be stressed that all expressed mutants were also fully coupled and pumped protons at expected rates, i.e., at rates approximately related to those of the reverse reactions.

Mutants of the conserved serines S250C, S251C, and S256C generally showed much higher activities than cfTH, the increase being about 2−3-fold for *both* the reverse and cyclic reactions. In these mutants the pH dependencies were normal or only slightly altered. Again, a TH mutant showing pronounced increases in *both* reverse and cyclic activities is remarkable. A high cyclic activity in the absence of added

NADP(H), as those seen with the S250C, S251C, and S256C mutants, has previously been associated with tightly bound NADP(H) or nonspecific binding of NAD(H) in dIII (cf. refs *4*, *5*, and *23*). A novel and so far qualitative approach to determine the contribution of bound NADP(H) to the cyclic activity is by removing the 2′-phosphate by the action of alkaline phosphatase (*28*). Tightly bound NADP(H) in separately expressed and purified dIII has previously been shown to exchange rapidly with the surrounding medium (*37*). The cyclic activities of all cysteine mutants of the serines in the absence of added NADP(H) were generally reduced to between 1% and 3% by phosphatase treatment, indeed suggesting the presence of bound NADP(H). For example, of the maximal cyclic activity of S256C in the presence of $NADP^+$, 41% was $NADP^+$-independent, an activity which was reduced to 3% by phophatase. In this context it should be emphasized that the reverse activity but not the cyclic activity catalyzed by the particular S260C mutant was severalfold higher than that of the same mutant published previously (*23*). The reason for the low reverse activity of the previous mutant may have involved an unknown modification or truncation of the protein.

Since about 74% of the cyclic activity of M259C was independent of added $NADP^+$ but only reduced to 60% by phosphatase, this mutant stands out as particularly interesting. The only conceivable explanation for these properties is that the NADP(H)-binding site, which essentially lacks bound NADP(H), has become unspecific but with a retained high affinity for NAD(H) after phosphatase treatment. In general, the properties of this mutant otherwise resemble those of R265A in the hinge (*23*), with a low reverse activity and a high cyclic activity. Similarities between these two mutants are not surprising considering the short distance between Met259 and Arg265. This pattern is indeed expected if specific NADP(H) binding is decreased but still allowing NADH/$AcPyAD^+$ to interact unspecifically with the NADP(H)-binding site. It is indeed not inconceivable that these results may indicate a new coupling mechanism in which the Δp-dependent increased release of NADPH in the classical "energy-linked transhydrogenase reaction" is due to a lowered specificity in the NADP(H)-binding site, mediated by the hinge and the 2′-phosphate binding and substrate specificity-determining $K_{424}R_{425}S_{426}$ region in dIII (cf. refs *4* and *5*).

In conclusion, all residues of H14 in dII of *E. coli* transhydrogenase have been systematically mutated as single mutants to cysteines and in some cases leucines. The properties of the mutants suggest that conserved glycines are involved in helix−helix packing and therefore lose most activities when mutated. Conserved serines show considerably increased activities when mutated, possibly indicating a direct or indirect role in proton translocation. The results support the proposal (*21*, *22*) that dII exerts an essential and direct control of dIII and the NADP(H)-binding site, and possibly also its specificity, through long-range conformational changes mediated by the connecting hinge region.

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