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Properties of a proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli* with α and β subunits linked through fused transmembrane helices

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Abstract

Proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli* is composed of an α and a β subunit, whereas the homologues mitochondrial enzyme contains a single polypeptide. As compared to the latter transhydrogenase, using a 14-helix model for its membrane topology, the point of fusion is between the transmembrane helices 4 and 6 where the fusion linker provides the extra transmembrane helix 5. In order to clarify the potential role of this extra helix/linker, the α and the β subunits were fused using three connecting peptides of different lengths, one (pAX9) involving essentially a direct coupling, a second (pKM) with a linking peptide of 18 residues, and a third (pKMII) with a linking peptide of 32 residues, as compared to the mitochondrial extra peptide of 27 residues. The results demonstrate that the plasma membrane-bound and purified pAX9 enzyme with the short linker was partly misfolded and strongly inhibited with regard to both catalytic activities and proton translocation, whereas the properties of pKM and pKMII with longer linkers were similar to those of wild-type *E. coli* transhydrogenase but partly different from those of the mitochondrial enzyme although pKMII generally gave higher activities. It is concluded that a mitochondrial-like linking peptide is required for proper folding and activity of the *E. coli* fused transhydrogenase, and that differences between the catalytic properties of the *E. coli* and the mitochondrial enzymes are unrelated to the linking peptide. This is the first time that larger subunits of a membrane protein with multiple transmembrane helices have been fused with retained activity. © 2001 Published by Elsevier Science B.V.

Keywords: Transhydrogenase; Nicotinamide adenine dinucleotide; Nicotinamide adenine dinucleotide, reduced; Proton pump; Membrane protein

1. Introduction

The proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli* is a member of a larger family of some 20 transhydrogenases, the cDNAs of which have been fully or partly sequenced. H^+-Transhydrogenases catalyze a reversible reduction of $NADP^+$ by NADH linked to proton trans-

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location across the bacterial plasma membrane or the mitochondrial inner membrane:

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

where 'out' and 'in' denote the periplasmic space and cytosol in bacteria, respectively, and the intermembrane space and matrix in mitochondria, respectively. Physiologically, the role of H^+-transhydrogenases has been proposed to be the maintenance of a high redox level of NADP(H) for, e.g., reduction of glutathione and associated detoxication purposes [1] and regulation of intermediary metabolism at the level of the NADP(H)-dependent isocitrate dehydrogenase [2]. The gene for *E. coli* H^+-transhydrogenase was the first to be cloned and expressed, and the *E. coli* transhydrogenase has since been extensively studied, but also the genes and enzymes from *Rhodospirillum rubrum* and beef heart, have been studied in depth regarding structure–function relationships using, e.g., site-directed mutagenesis, kinetics, and resolution–reconstitution approaches (for reviews, see [3–6]).

H^+-Transhydrogenases have approximately the same molecular mass in their active forms, i.e., about 220 kDa. However, whereas all known H^+-transhydrogenases are homodimers, their subunit compositions differ. In contrast to the mitochondrial H^+-transhydrogenases characterized thus far, which are composed of a single polypeptide, the bacterial and parasitic ones are composed of 2–3 subunits. The *E. coli* enzyme has two subunits, the α and β subunits with the amino acid sequences α1–α510 and β1–β462. The *Rhodospirillum rubrum* enzyme consists of three subunits of 384,139 and 464 amino acids corresponding to the N-terminal and C-terminal regions of the *E. coli* α subunit and the *E. coli* β subunit, respectively. Of all bacterial H^+-transhydrogenase subunits only the *R. rubrum* α1 is soluble, the remaining subunits containing transmembrane helices [3–5].

All H^+-transhydrogenases are composed of three major domains, the hydrophilic domain I (dI) containing the binding site for NAD(H), the hydrophilic domain II (dIII) containing the NADP(H)-binding site, and the hydrophobic domain II (dII) containing 13–14 transmembrane α helices and the assumed proton-transport pathway [7]. DI and dIII can be expressed separately and are inactive individually but catalyze hydride transfer when combined, suggesting that dII is not directly involved in hydride transfer (for a review see [6]). Fig. 1 shows a model of the *E. coli* transhydrogenase with dI (+NAD(H)) interacting with dIII (+NADP(H)); for clarity the helix bundle and hydrophobic dII is shown separated from dI+dIII.

Of the 13 transmembrane α helices suggested to form dII of *E. coli* H^+-transhydrogenase, four belong to the α subunit and nine to the β subunit [7]. As compared to the *E. coli* H^+-transhydrogenase, the mitochondrial enzyme possesses a 25-residues-long extra peptide between the α and β subunits. The reason for this discrepancy is intriguing and was the subject of the present study. To this end the α and β subunits of the *E. coli* H^+-transhydrogenase were fused at the gene level with linkers of varying lengths, the longest being comparable to the mitochondrial extra 25-residues-long peptide, and the properties of the resulting fusion enzymes investigated. The results show that the fusion enzymes were active to various extents, suggesting that the fusion region of the transmembrane dII is important for correct folding of the consecutive transmembrane α helices of dII, and important but not essential for activity.

2. Materials and methods

2.1. Strains and plasmids

The *pnt* gene was introduced into the pGEM-7Zf(+) plasmid resulting in the construct denoted pSA2 [8]. The *E. coli* K12 strain JM109 was used for protein expression.

2.2. Computational analysis

The sequences for *E. coli* and bovine transhydrogenases were aligned using ClustalW [9]. Bovine transhydrogenase was examined for the location of transmembrane helices using TopPredII ([10]; see also http://www.biokemi.su.se/~server/toppred2). Sequence alignments indicate that the gap between the α and β-subunit of the *E. coli* transhydrogenase corresponds to the bovine sequence PPEYNYLYLL-

PAGTFVGGYLASLYSGY. The transmembrane prediction suggests that this sequence constitutes a membrane spanning helix. The score for this helix is in the same range as for helices 2 and 10 and far higher than those for helices 11 and 13 (cf. Fig. 2A).

2.3. Construction of mutants

Three mutants in which the α and β subunits were fused with a peptide linker of variable lengths were constructed. The fusion mutant pAX9, containing a short linker, was constructed from pSA2 with the Amersham In vitro mutagenesis system based on the method of Taylor et al. [11]. The TAA stop codon of the α subunit was mutated to TCA and the 10-bases-long noncoding region between the subunits were changed by deleting a C three bases upstream of the α subunit initiating codon. The resulting construct contained the protein sequence SGVN inserted in-frame between the C-terminus of the α subunit and the N-terminus of the α subunit.

The fusion mutant pKM, containing a longer linker, was constructed from pSA2 with an insertional polymerase chain reaction (PCR) mutagenesis technique according to Ho et al. [12]. The entire noncoding sequence between the α and β subunit including the last residue of the α subunit and the first residue of the β subunit was replaced with a new coding sequence which also served to phase the reading frames of the two subunits. The resulting construct contained the protein sequence PTDPPEYNYLYLLPAGTF inserted between αK509 and βG3. A third fusion mutant, pKMII, contained the amino acid sequence PTDPPEYNYLYLLPAGTSVGGYLASLYSGYN inserted between αK509 and βL5.

All mutants were verified by sequencing of the entire gene or of the DNA fragments that had been manipulated by PCR and/or restriction digests.

2.4. Purification of mutant transhydrogenases

The mutants pAX9, pKM and pKMII were purified essentially as described [13].

2.5. Trypsin digestion of mutant transhydrogenases

Trypsin treatment of transhydrogenases was carried out as described [14].

2.6. Assays

Transhydrogenase activities were measured spectrophotometrically at 375 nm using an absorption coefficient of 6100 $M^{-1}\,cm^{-1}$ essentially as described [13]. The reverse reaction was estimated as reduction of 200 μM $AcPyAD^+$ by 200 μM NADPH in a buffer composed of 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM DTT and 0.01% Brij 35, the forward reaction as reduction of 500 μM thio-$NADP^+$ by 100 μM NADH in the same buffer as for the reverse reaction, and the cyclic reaction as reduction of 200 μM $AcPyAD^+$ by 100 μM NADH in the presence of 100 μM NADP(H) in a buffer composed of 50 mM MES–KOH (pH 6.0) and 0.01 Brij 35. Assays were carried out at room temperature.

Initial relative rates of the reverse reaction and the associated proton pumping catalyzed by freshly prepared plasma membrane vesicles obtained from transformed JM109 cells were assayed spectrophotometrically as described [15] and fluorimetrically as quenching of quinacrine fluorescence, using excitation at 430 nm and emission at 505 nm [15]. Standard deviation of catalytic and proton pumping assays was less than 10%.

2.7. Protein

Protein concentration was estimated by the Bicinchoninic procedure using bovine serum albumin as standard [16].

2.8. Chemicals

All biochemicals were of analytical grade and purchased from Sigma or Boehringer.

3. Results and discussion

In the fusion mutant pAX9 containing the short linker, the noncoding region between the genes for the *E. coli* transhydrogenase α and β subunits was mutated in order to change the α subunit stop codon to a serine codon and to delete one base in order to phase the reading frames of the α and β subunits. The resulting fusion mutant consists of a single poly-

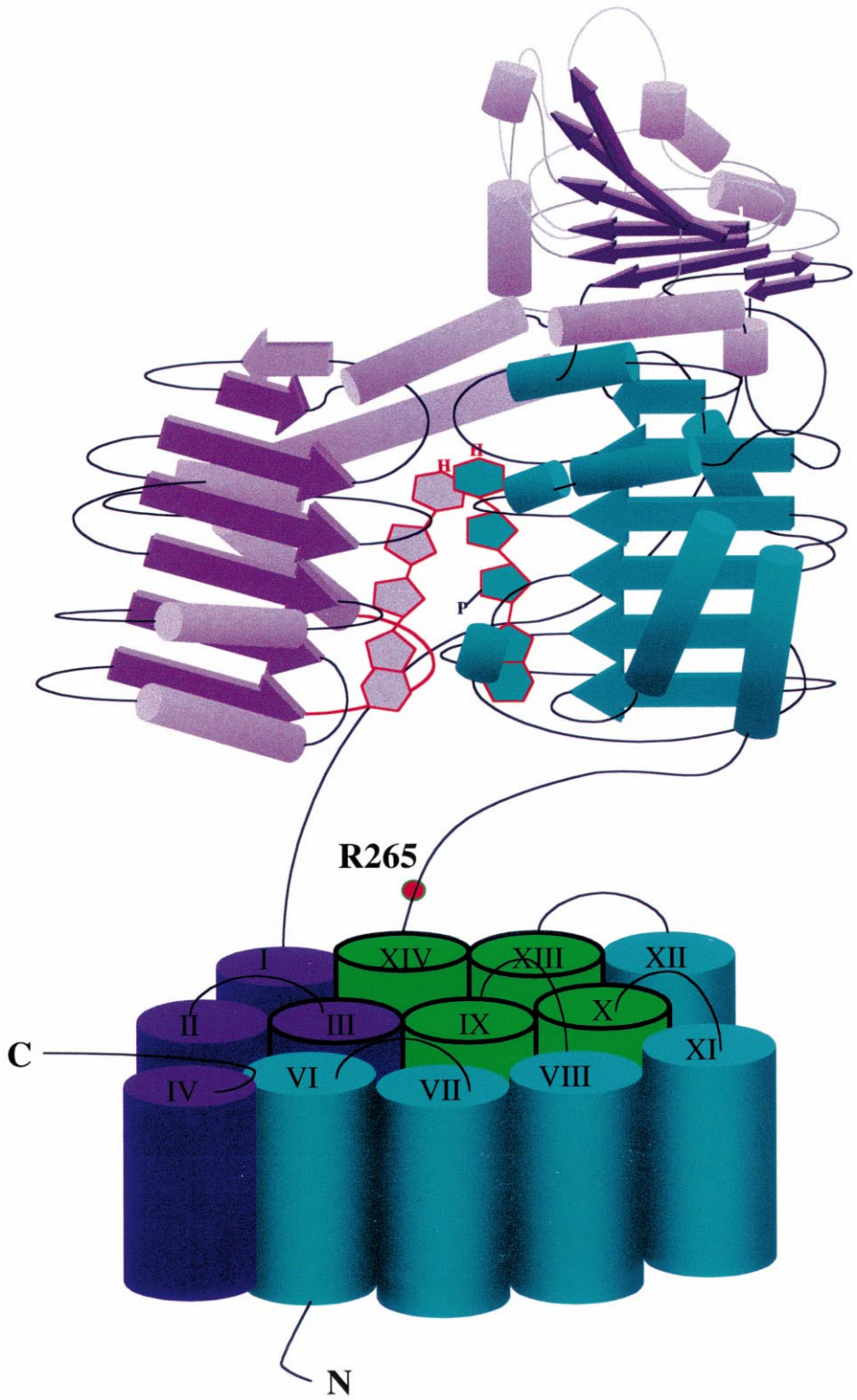

Fig. 1. Model of the *E. coli* H^+ transhydrogenase. DI with bound NAD(H) and dIII with bound NADP(H) are placed to the left and to the right, respectively, and are detached from dII for clarity. Subunit α and subunit β are in blue and green, respectively. Helices 9, 10, 13 and 14 form a tentative proton channel. The C-terminal in helix 4 of the α subunit, the N-terminal in helix 6 of the β subunit, and the hinge peptide connecting dII and dIII and containing the trypsin-sensitive R265 (red) are indicated. Cytoplasmic and periplasmic side are up and down, respectively. The model shows only the α+β subunits and not the active $\alpha_2\beta_2$ dimer, and is mainly based on data reviewed in [7,23], and results from [17].

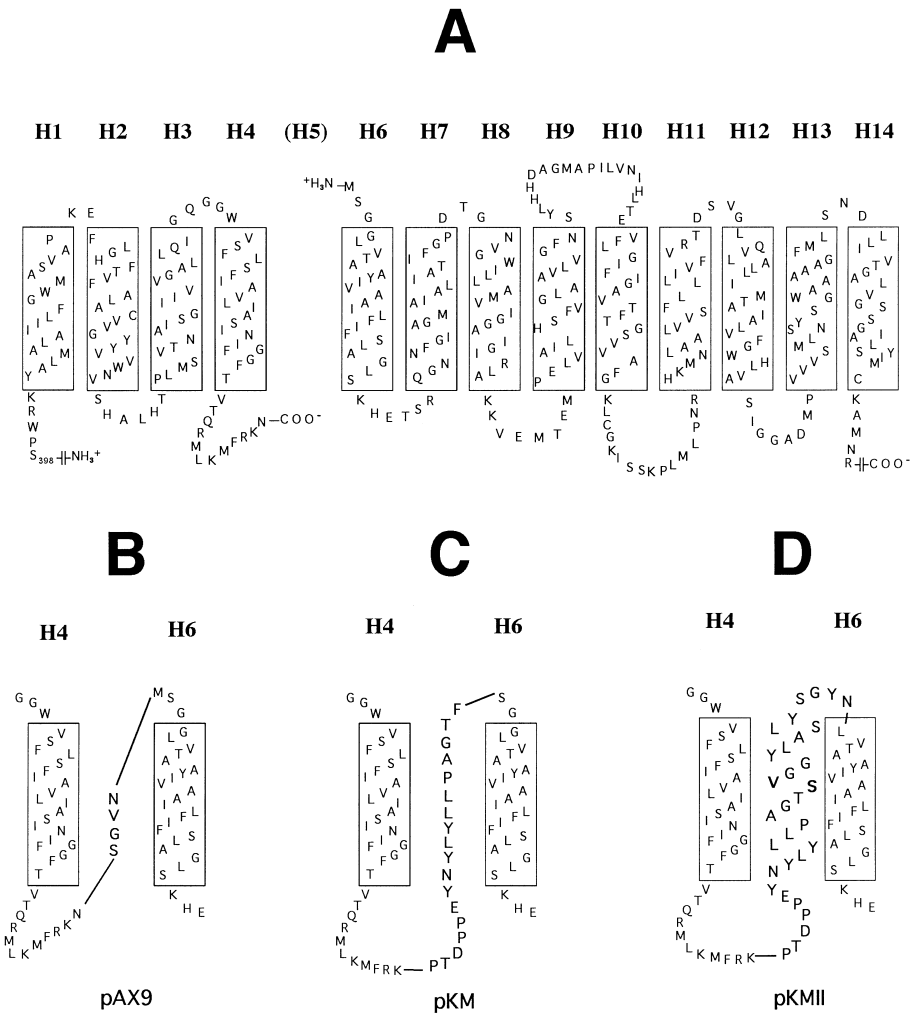

Fig. 2. Membrane topology models of the H^+-transhydrogenase encoded by the pSA2 (wild-type), pAX9, pKM and pKMII plasmids. Only dII is shown with dI and dIII being omitted The numbering of the transmembrane helices follows a previously suggested 14-helix model of the mitochondrial enzyme (cf. [7]). Note that the sidedness is altered as compared to Fig. 1, i.e., cytoplasmic side and periplasmic space are down and up, respectively.

peptide with a molecular mass of 104 kDa in which the protein sequence SGVN is inserted in-frame between the C-terminus of the α subunit and the N-terminus of the β subunit (Fig. 2B).

In the fusion mutants pKM and pKMII containing the longer linkers, the α and β subunits of *E. coli* transhydrogenase were fused by replacing the non-coding sequence with a new in-frame coding sequence (Fig. 2C,D). In addition, the last residue of the α subunit was changed from Asn (N) to Pro (P) in order to further resemble the bovine enzyme. The fusion mutant pKM consisted of a single polypeptide with a molecular mass of 105 kDa in which the protein sequence PTDPPEYNYLYLLPAGTSV was inserted between αK509 and βG3. The fusion mutant pKMII with the longest fusion peptide contained the amino acid sequence PTDPPEYNYLYYPAGTSV-GGYLASLYSGYN inserted between αK509 and βL5, and has a molecular mass of 106 kDa.

When assayed in plasma membrane vesicles as reduction of $AcPyAD^+$ by NADPH (reverse reaction), the pAX9, pKM and pKMII fusion mutants showed catalytic activities of 0.1, 0.3 and 0.6 μmol/min/mg protein, i.e., 7%, 21% and 41%, respectively, as compared to the wild-type activity of 1.4 μmol/min per mg protein (Table 1). Using the same plasma membrane preparations, the mutants pAX9, pKM and pKMII showed proton pumping activities that were

Table 1
Catalytic and proton-pumping activities of membrane-bound wild-type and mutant *E. coli* H^+-transhydrogenases

Mutant	Reverse reaction		Proton pumping	
	μmol/min/mg	%	Fluor units/min	%
Wild-type	1.4	100	6.6	100
pAX9	0.1	7	0.7	11
pKM	0.3	21	1.8	27
pKMII	0.6	42	4.4	66

Proton pumping is expressed as change in arbitrary fluorescence units per minute.

approximately 11%, 27% and 66%, respectively, of the wild-type activity (Table 1). These results clearly demonstrate that the fusion proteins are catalytically active and that the length of the peptide connecting the α and β subunits is important for both catalytic and proton pumping activities. In addition, the activities of the reverse reaction and proton pumping were quantitatively similar, a relationship that is often observed with especially the wild-type enzyme (cf. [3]).

The pAX9, pKM and pKMII fusion mutants were further characterized following purification since some activities, e.g., the cyclic reaction, are difficult to assay in plasma membranes. Whereas the pAX9 mutant was unstable and tended to denature as indicated by its increased susceptibility to proteolysis (not shown), pKM was wild-type-like with regard to stability during purification (not shown). As shown in Table 2, the purified pAX9, pKM and pKMII mutants catalyzed forward and reverse activities that were of the same order as those catalyzed by plasma membranes, i.e., about 8–11% for pAX9, 44–46% for pKM and 58–86% for pKMII of the wild-type activities. The cyclic activity of the pAX9 enzyme was 11% of the wild-type. In contrast, the rate of the cyclic reaction catalyzed by the pKM mutant was 73% of the wild-type, i.e., proportionally much higher than the activity of the forward and reverse reactions catalyzed by the same mutant; pKMII showed a similar cyclic activity. Thus, the introduction of a fusion peptide in *E. coli* H^+-transhydrogenase similar to the corresponding mitochondrial sequence, led to a largely retained capacity for all transhydrogenase reactions.

The trypsin sensitivity of *E. coli* H^+-transhydrogenase in the absence and presence of NADP(H) is a sensitive assay of a proper folding of the enzyme as well as NADP(H)-induced conformational changes in domain III, especially the region around the main cleavage site βR265 that connects dII and dIII ([14]; cf Fig. 1). In a series of trypsin digestion experiments, wild-type (pSA2), pAX9, pKM and pKMII H^+-transhydrogenases were subjected to trypsin proteolysis in the absence and presence of different substrates. As shown in Fig. 3A, the α subunit of wild-type H^+-transhydrogenase in pSA2 plasma membranes was cleaved by trypsin in the absence of NADPH (lane 2) to yield 29 and 16 kDa fragments. These originated from cleavage of dI. The β subunit was uncleaved unless NADPH was present in which case 30 kDa (dIII) and 25 kDa fragments were formed (lane 3) in addition to those derived from the α subunit. In the absence of NADPH, the H^+-transhydrogenase encoded by pKM yielded 63, 29 and 16 kDa fragments (lane 5). The 63 kDa fragment originated from the removal of dI from the

Table 2
Forward, reverse and cyclic activities of purified wild-type, pAX9, pKM and pKMII *E. coli* H^+-transhydrogenases

Mutant	Forward		Reverse		Cyclic	
	μmol/min/mg	%	μmol/min/mg	%	μmol/min/mg	%
Wild-type	0.9	100	9.6	100	63	100
pAX9	0.1	11	0.8	8.3	7.0	11
pKM	0.4	44	3.9	41	46	73
pKMII	0.7	86	5.5	58	42	67

The cyclic reaction was assayed in the presence of 100 μM NADPH.

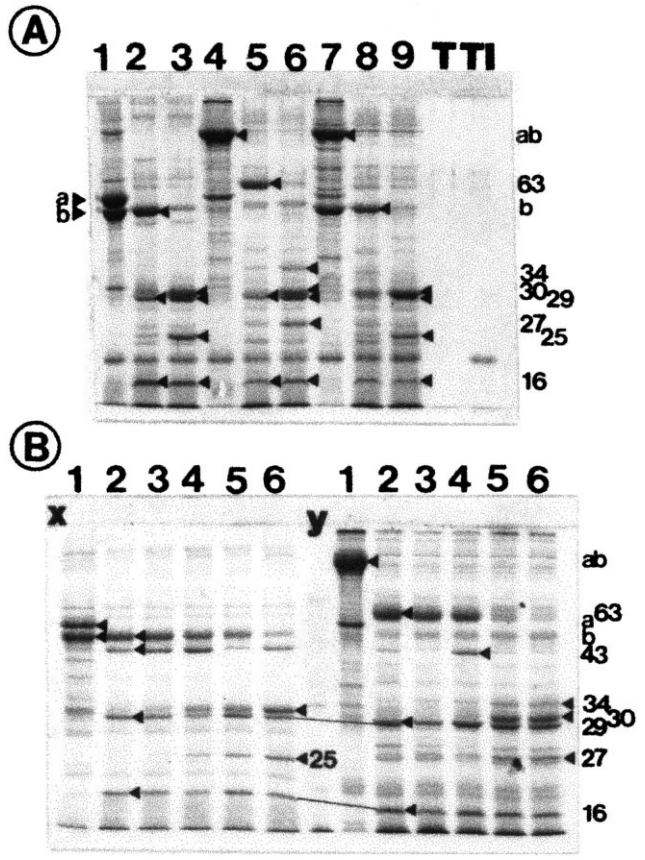

Fig. 3. Trypsin digestion of purified soluble H^+-transhydrogenase encoded by plasmids PAX9 and pKM, and of the wild-type H^+-transhydrogenase encoded by plasmid pSA2 in purified inner membranes. (A) The H^+-transhydrogenases were digested in the absence (lanes 2,5,8) or presence (lanes 3,6,9) of 0.5 mM NADPH. Untreated H^+-transhydrogenase are in lanes 1,4,7. Lanes 1–3 are for the enzyme from pSA2, lanes 4–6 for pKM and lanes 7–9 for pAX9. T denotes trypsin and TI, trypsin inhibitor. (B) H^+-Transhydrogenases encoded by pSA2 (x) and pKM (y) were treated with trypsin in the absence of nucleotides (lanes 2) or in the presence of 0.5 mM NAD^+ (lanes 3), NADH (lanes 4), $NADP^+$ (lanes 5) and NADPH (lanes 6). Untreated H^+-transhydrogenases are in lanes 1. The positions of migration of the α (a) and the β (b) subunits and of the proteolytic cleavage fragments (in kDa) are indicated; ab, uncleaved linked α and β subunits encoded by plasmids pKM and pAX9. The gel was an 11% polyacrylamide gel.

fused enzyme and was composed of the β subunit attached to the fusion peptide and the C-terminal 100 residues of the α subunit. In the presence of NADPH (lane 6), the 63 kDa fragment was further cleaved to 34, 30 (dIII) and 27 kDa polypeptides. The 34 kDa polypeptide was essentially dII with the fusion peptide. The 27 kDa polypeptide originated by cleavage near the C-terminus of the α subunit and was composed of the fusion peptide attached to the N-terminal 25 kDa fragment of the β subunit. The H^+-transhydrogenase encoded by pAX9 was readily cleaved even in the absence of NADPH (lane 8) to yield the β subunit and 30, 29, 25 and 16 kDa polypeptides. Further cleavage of the β subunit occurred in the presence of NADPH (lane 9). Trypsin digestion of pKMII resulted in a pattern indistinguishable from that of pKM (not shown).

Fig. 3B shows a further characterization of the trypsin cleavage patterns of the transhydrogenases of pSA2 (X) as compared with those of pKM (Y). In the absence of nucleotides (lanes 2), or in the presence of NAD(H) (lanes 3 and 4), both enzymes were mainly cleaved in the α subunit with removal of dI as a 43 kDa polypeptide, followed by further cleavage of dI to 30 and 16 kDa fragments. The β subunit of pSA2 and the 63 kDa polypeptide of pKM were cleaved to 30 and 25 kDa fragments (pSA2) or to 30, 34 and 27 kDa fragments (pKM) in the presence of NADP(H) (lanes 5 and 6).

These trypsin experiments show that pKM and pKMII H^+-transhydrogenases were properly folded and that their dIII still underwent the conformational change in the presence of NADPH typical of the wild-type enzyme. In contrast, the pAX9 enzyme did not appear to be folded or incorporated in the membrane properly since the enzyme was readily cleaved even in the absence of NADP(H). Presumably, this reflected the introduction of a too short and hydrophilic linker which could not form a proper transmembrane helix, which led to an inappropriate assembly of the connection between the α and β subunits, and an exposure of the βR265 cleavage site. Therefore, no stable dI-free fragment like the 63 kDa fragment of pKM was detected with pAX9. The results also explain the difficulties in purifying the pAX9 mutant.

Fig. 2 shows membrane topology models of the H^+-transhydrogenases encoded by wild-type pSA2 (Fig. 2A), the pAX9 (Fig. 2B), the pKM (Fig. 2C) and the pKMII (Fig. 2D) plasmids The wild-type model (Fig. 2A) was recently suggested on the basis of cysteine scanning of all potential loops using thiol-reactive fluorescent probes [7]. It is obvious from the results in Tables 1 and 2 and Fig. 2B that the short sequence introduced in the pAX9 enzyme probably

caused a constraint in the packing of helices 4 and 6. It is also likely that the sequence, together with the C-terminus of the α subunit and the N-terminus of the β subunit, was too hydrophilic to form a proper α helix. In contrast, the pKM (Fig. 2C) and pKMII (Fig. 2D) enzymes with their extended and somewhat more hydrophobic linking peptides, are suggested to have relatively slight effects on the packing of helices 4 and 6, and may even, especially in the case of the pKMII enzyme (Fig. 2D), form an extra membrane helix (helix 5) similar to that in the mitochondrial enzyme. Thus, these results strongly support the recently proposed 13-helix topology of the *E. coli* transhydrogenase in Fig. 2A, where the C-terminus of the α subunit resides on cytosolic side whereas the N-terminus of the β subunit resides on the periplasmic side [7]. This topology was recently independently supported by cysteine mutations followed by labelling with thiol reagents [17]. If the 13-helix topology model would be incorrect, it is less likely that a fusion of the C-terminus of the α subunit and the N-terminus of the β subunit with a short peptide, in that case on the same side of the membrane, would give a misfolded protein and a low activity.

The higher activities of the pKM and pKMII enzymes, especially the cyclic activity, as compared to both forward, reverse and cyclic activities of the pAX9 enzyme, may suggest that the presumably disturbed helix packing in helices 4 and 6 in the pAX9 H^+-transhydrogenase is detrimental for all activities, whereas the pKM/pKMII H^+-transhydrogenases essentially show wild-type behavior. This agrees with the previous finding that the α subunit precedes and is essential for the folding of the β subunit [18]. It is also interesting to note that the cyclic reaction is consistently more active than the reverse reaction with both the pSA2 and pKM/pKMII enzymes, possibly indicating that the intactness of domain II is less important for this activity. However, it should be stressed that, unlike the bacterial H^+-transhydrogenases, the purified mitochondrial enzyme only catalyzes the cyclic reaction in the presence of an electrochemical proton gradient in a reconstituted liposome system [19,20]. Thus, in this respect the pKM/pKMII H^+-transhydrogenases are still similar to the bacterial H^+-transhydrogenase suggesting that other factor(s) are responsible for the discrepancy between the mitochondrial and *E. coli*/*R. rubrum* enzymes.

It is indeed remarkable that, despite the considerable distortion of helices 4 and 6 as indicated by the altered trypsin sensitivity, pAX9 is still possible to catalyze, e.g., proton translocation as well as the cyclic reaction although at low but significant rates. This suggests that, in pAX9, the transmission of hydride transfer events to the proton translocation system in domain II is affected but not fully inhibited by the distortion of helices 4 an 6.

To our knowledge this is the first time that larger subunits of a membrane protein have been fused with retained activity. However, F_0F_1-ATPase was recently demonstrated to retain activity with genetically fused dimers and trimers of subunit c [21]. In related studies Hatefi and coworkers [22] have earlier shown that a cut of the mitochondrial domain II in the region corresponding to the loop between helices 9 and 10 does not affect transhydrogenase activities. Likewise, Kaback and coworkers [23] showed earlier that the lac permease could be expressed as separate domains with retained activity. These results indicate that the helix packing and helix–helix recognition is largely unaffected by alterations in the loops connecting membrane helices.

In conclusion, the α and β subunits were linked through three peptides of different lengths, the longer one corresponding essentially to the mitochondrial peptide, producing fusion enzymes with varying activities. The fusion enzyme with the mitochondrial-like peptide, but not that with a short linkage, showed wild-type like trypsin sensitivity and activities, demonstrating the importance of a proper interface between the C-terminus of the α subunit and the N-terminal of the β subunit for the folding of the β subunit.

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