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Effect of truncation and mutation of the carboxyl-terminal region of the β subunit on membrane assembly and activity of the pyridine nucleotide transhydrogenase of *Escherichia coli*

Philip D. Bragg *, Cynthia Hou

Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada

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Abstract

The pyridine nucleotide transhydrogenase of *Escherichia coli* is a proton pump composed of two different subunits (α and β) assembled as a tetramer ($\alpha_2\beta_2$) in the cytoplasmic membrane. A series of mutants was generated in which the carboxyl-terminal region of the β subunit was progressively truncated. Removal of the two carboxyl-terminal amino acid residues prevented incorporation of the enzyme into the cytoplasmic membrane. Deletion of the carboxyl-terminal amino acid allowed incorporation of the α subunit to near normal levels, but the amount of the β subunit was much decreased. It is concluded that, although the α subunit can be incorporated into the cytoplasmic membrane without the β subunit, the carboxyl-terminal region of the β subunit is involved in determining the correct conformation of the α subunit for assembly. The carboxyl-terminal amino acid of the β subunit, βLeu462, and the penultimate residue, βAla461, were individually mutated and the effect on two transhydrogenase activities determined. The reduction of 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$) by NADPH, and by NADH in the presence of $NADP^+$, was decreased maximally by about 60%. The reduction of $AcPyAD^+$ by NADH in the absence of $NADP^+$ was decreased to a greater extent. Most mutants of βLeu462 showed at least an 80% reduction in activity as well as abnormal kinetics. The abnormal kinetics were explored in the βA461P mutant and were attributed to tighter binding of the product AcPyADH. This compound competed with $NADP^+$ at the NADP(H)-binding site. It is concluded that the carboxyl-terminal region of the β subunit contributes to the NADP(H)-binding site on this subunit

Keywords: Transhydrogenase; Proton pumping; Pyridine nucleotide transhydrogenase; Subunit truncation; Enzyme assembly; Cyclic transhydrogenation

1. Introduction

In *Escherichia coli*, the pyridine nucleotide transhydrogenase is an integral cytoplasmic membrane protein that catalyzes the transfer of a hydride ion equivalent between NAD(H) and NADP(H) and is coupled to proton translocation from the cytosolic side of the membrane to the periplasm [1–3]. The enzyme is composed of two different subunits (α,

Abbreviations: $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

* Corresponding author. Fax: +1 (604) 822-5227; E-mail: pbragg@unixg.ubc.ca

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510 residues; β, 462 residues) assembled as an $\alpha_2\beta_2$ tetramer [4–7]. All membrane pyridine nucleotide transhydrogenases are organized into three domains [2,3,8] (Fig. 1). Domain 1 is a cytosolic domain which contains the binding site for NAD(H) and is the amino-terminal region of the α subunit. Domain 3 is a cytosolic domain found at the carboxyl-terminus of the β subunit. It contains the NADP(H)-binding site [2,3,6,9]. Domain 2 is the transmembranous domain of the enzyme. In *E. coli*, it consists of two parts [5,10]. The α subunit of this organism terminates in four transmembrane α-helices and a short (10 residues) hydrophilic 'tail'. The second portion of domain 2 is at the amino-terminus of the β subunit and consists of eight transmembrane α-helices [10,11].

In previous work [12], we studied the effect of deleting portions of the α and β subunits on the assembly of the tetramer in the membrane. Deletions in domain 1 prevented assembly of the enzyme. Removal of the first two transmembrane helices of the α subunit did not prevent incorporation of the tetramer into the membrane, although the enzyme was non-functional. The last two transmembrane helices of the α-subunit were indispensable. Deletions in the domain 2 region of the β subunit allowed incorporation of a truncated form of the tetramer into the membrane. Deletions in domain 3 prevented incorporation of the truncated β subunit into the membrane enzyme; however, the α_2 portion of the tetramer was incorporated into the membrane. We concluded that the assembly of the tetrameric $\alpha_2\beta_2$ transhydrogenase required preassembly of the α_2 moiety with subsequent addition of the two β subunits or of a β_2 dimer. In a subsequent paper, we showed that the 10-residue hydrophilic 'tail' at the carboxyl-terminus of the α subunit had a critical role in both the activity and assembly of the transhydrogenase tetramer [13]. Removal of the six carboxyl-terminal residues resulted in abolition of enzyme activity. The conformation of the β subunit was perturbed, although α and β subunits were incorporated into the membrane. Systematic truncation and site-directed mutagenesis revealed that at least one positively-charged amino acid in the tail region was required for assembly of the subunits into a functional enzyme, while a phenylalanine residue (αPhe^{507}) was essential for activity.

The essentiality of such a small region of the molecule for the proper conformation of the subunits, assembly into the membrane, and the activity of the enzyme prompted the present study of the role of the carboxyl-terminal region of the β subunit in the assembly and activity of the *E. coli* transhydrogenase. In our previous study [12], deletions in the β subunit had not extended beyond residue 394. In the present paper, we describe the effect of systematically truncating the β subunit from the carboxyl terminus. We show that the two carboxyl-terminal amino acids are critical for the successful assembly of the transhydrogenase in the inner membrane of *E. coli* and for the activity of the enzyme.

2. Materials and methods

2.1. Bacterial strain, plasmids and mutagenesis

E. coli JM109 cells containing wild-type (pSA2) or mutant plasmids were grown overnight at 37°C in LB broth. The medium was shaken at 250 rpm in a New Brunswick Scientific Controlled Environment Incubator Shaker. Plasmid pSA2 contains the *pnt* genes of the pyridine nucleotide transhydrogenase of *E. coli* introduced into the pGEM-7Zf(+) plasmid [6].

Plasmid pSA2 was used to isolate single-stranded phagemid DNA. Site-directed mutagenesis to convert selected residues was performed by the method of Taylor et al. [14] using degenerate primers. The reagents and protocols as outlined in the Amersham and the BioRad Muta-Gene Phagemid In Vitro mutagenesis kits were followed, except that competent *E. coli* JM109 cells were used for transformation. Plasmid DNA was prepared from individual colonies, and the mutants were identified by double-stranded DNA sequencing. The entire coding region of the *pnt* genes from each mutant was completely resequenced.

The carboxyl-terminus deletion mutants of the β subunit were generated by the introduction of TAA or TAG termination codons at selected sites.

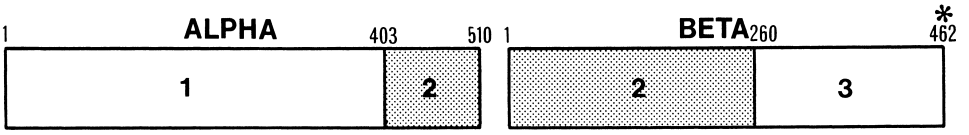

Fig. 1. The domain structure of the *E. coli* transhydrogenase. Transmembrane domain 2 is composed of the carboxyl-terminal region of the α subunit and the amino-terminal region of the β subunit. The asterisk indicates the site of mutations used in this study.

2.2. Preparation of membrane vesicles containing transhydrogenase

The cell cultures were harvested by centrifugation at 4400×*g* for 20 min. The cell pellets were washed by resuspension in 0.9% NaCl followed by centrifugation at 12 000×*g* for 15 min. Cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 7.8, 1 mM DTT, 1 mM EDTA) at 1 g wet weight/5 ml. All steps were performed at 0–4°C. The cells were lysed by passage through an AMINCO French Pressure Cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12 000×*g* for 10 min. The supernatant was centrifuged at 252 000×*g* for 2 h, and the membrane pellet containing everted membrane vesicles was suspended in buffer A at 1 g wet weight/5 ml. Membrane vesicles (1.5 ml) were layered on a 6 ml sucrose cushion (45% sucrose (w/w) in buffer A) and centrifuged in a Beckman Type 65 fixed angle rotor at 40 000 rpm (139 000×*g*) for 1 h. The outer membrane fraction pelleted to the bottom of the tube and was discarded while the everted membrane vesicles banded at the interface and were removed by a syringe. The vesicles were diluted with buffer A then centrifuged at 252 000×*g* for 3 h. The washed membrane pellet was suspended in 50 mM HEPES-KOH, pH 8, 5 mM magnesium acetate for use.

2.3. Measurement of transhydrogenation activities

Transhydrogenation of AcPyAD$^+$ by NADPH was measured as described previously [15]. An appropriate amount of washed membrane (20–100 μg protein) was added to 1 ml of 50 mM sodium phosphate buffer (pH 7), 0.5 mM EDTA, 2 mM DTT, 0.01% Brij 35 containing AcPyAD$^+$ and NADPH at 0.5 mM. Reduction of AcPyAD$^+$ was followed at 375 nm using a Perkin–Elmer Lambda 3A UV/VIS spectrophotometer. For assay of transhydrogenase activities at pH 6, the buffer was 50 mM MES-KOH (pH 6.0) containing 0.5 mM EDTA, 2 mM DTT and 0.01% Brij 35. For transhydrogenation of AcPyAD$^+$ by NADH, 0.5 mM NADH was used instead of NADPH. For transhydrogenation of AcPyAD$^+$ in the presence of NADP$^+$ ('cyclic reaction'), 0.5 mM NADP$^+$ was added. Protein concentration was determined by the method of Lowry et al. [16].

2.4. SDS-PAGE

Membrane vesicles were examined by SDS-polyacrylamide gel electrophoresis [17] and stained with Coomassie blue [18]. The level of incorporation of the α and β subunits was determined by scanning stained gels dried on a transparent plastic sheet with a LKB UltroScan XL laser densitometer.

2.5. Materials

All biochemicals including NADH, AcPyAD$^+$,

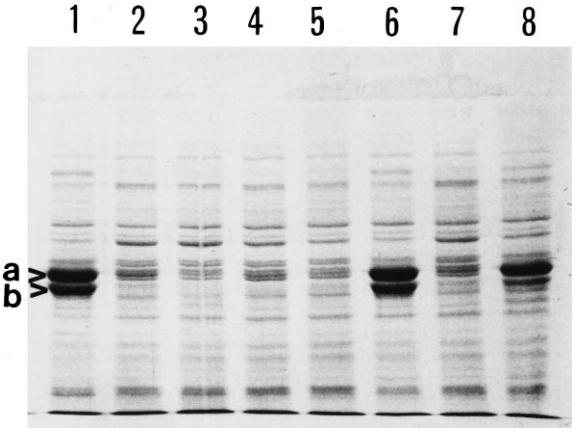

Fig. 2. SDS-PAGE of cytoplasmic membrane vesicles from β subunit carboxyl-terminal truncation mutants. Lane 1, non-mutant; lane 2, 21 residues deleted; lane 3, 15 residues deleted; lane 4, 7 residues deleted; lane 5, 3 residues deleted; lane 6, βK460Q mutant; lane 7, 2 residues deleted; lane 8, 1 residue deleted. a, b denote α and β subunits of the transhydrogenase. The concentration of acrylamide was 9% (w/v).

Fig. 3. Carboxyl-terminal sequence of β subunit. The residues whose codon was converted to a termination codon are starred.

AcPyADH, $NADP^+$ and NADPH were obtained from Sigma. LB broth was supplied by Difco.

3. Results

Everted cytoplasmic membrane vesicles were used in this study. The very high level of enzyme expression from the plasmids encoding the transhydrogenase makes this enzyme the predominant protein in non-mutant vesicles (Fig. 2).

3.1. Effect of truncation of the β subunit on enzyme activity and incorporation into the cytoplasmic membrane

Termination codons were introduced at selected sites in the plasmid DNA encoding the β subunit of the transhydrogenase. A series of mutants was generated in which the β subunit was shortened from the carboxyl end by 1–32 residues (Fig. 3). The transhydrogenase activities of everted cytoplasmic membrane vesicles prepared from these mutants were measured. The rates of reduction of $AcPyAD^+$ by NADPH, or by NADH in the presence of added $NADP^+$, were determined. In the first reaction, $AcPyAD^+$ is used as an analog of NAD^+ since the absorption maximum of the product AcPyADH (375 nm) is sufficiently displaced from that of the substrate NADPH (340 nm) to permit transhydrogenation to be followed spectrophotometrically. The latter reaction (the 'cyclic reaction') probably involves reduction by NADH of bound $NADP^+$ at the NADP(H)-binding site followed by reoxidation of the NADPH by reduction of $AcPyAD^+$. NADH and $AcPyAD^+$ alternately occupy the NAD(H)-binding site on the α subunit of the transhydrogenase [19–22]. Table 1 shows that removal of two or more amino acid residues from the carboxyl end of the β subunit results in almost complete loss of transhydrogenase activities. Removal of only the carboxyl-terminal amino acid reduced transhydrogenase activities by over 70%. The effect of the mutations on incorporation of the transhydrogenase into the cytoplasmic membrane is shown in Table 2 and Fig. 2. Little enzyme was detected in the membranes from mutants in which two or more residues had been removed from the carboxyl end of the β subunit. Deletion of the carboxyl-terminal amino acid gave only a small reduction in the incorporation of the α subunit. Incorporation of the β subunit was reduced by about 60%. Thus, the effect of truncation of the β subunit on transhydrogenase activities mirrors the extent of incorporation of the enzyme into

Table 1
Transhydrogenase activities of β subunit termination mutants

Number of amino acid residues in β subunit	Specific activity for the reduction of $AcPyAD^+$ by:	
	NADPH	NADH (+$NADP^+$)
462	9.9	32.8
461	2.8	8.8
460	0.26	0.72
459	0.07	0.14
455	0.09	0.19
447	0.13	0.38
441	0.75	2.3
430	0.04	0.03

The activities were assayed as described in Section 2. Specific activity is expressed as μmol $AcPyAD^+$ reduced/min/mg protein.

Table 2
Extent of incorporation of transhydrogenase subunits into inner membrane vesicles of β subunit termination mutants

Number of amino acid residues in β subunit	Incorporation (%)	
	α subunit	β subunit
462	100	100
461	82	39
460	0	0
459	0	0
455	0	0
447	0	0
441	0	0
430	0	0

Incorporation of α and β subunits was measured by scanning stained SDS-polyacrylamide electrophoresis gels.

the membrane. The activity remaining in the mutant membranes is likely due to chromosomally encoded transhydrogenase.

3.2. *Transhydrogenase activities of βLys460, βAla461 and βLeu462 mutants*

Since removal of one or two carboxyl-terminal amino acids from the β subunit had a significant effect on transhydrogenase activity and incorporation, mutants of βLys460, βAla461 and βLeu462 were isolated. The transhydrogenase activities of everted cytoplasmic membrane vesicles of these mutants are shown in Table 3. In addition to the assays previously described, the reduction of $AcPyAD^+$ by NADH, in the absence of added NADP(H), was measured. The mechanism of this reaction is unclear. It may involve binding of NADH or $AcPyAD^+$ at the NADP(H)-binding site, not normally occupied by these substrates [23,24], or more likely by NAD^+ participating in a cyclic mechanism analogous to that with added $NADP^+$ [25]. The reduction of $AcPyAD^+$ by NADPH, or by NADH in the presence of $NADP^+$, was affected to a limited extent (maximally 60%) in the mutants examined (Table 3). The effect of the mutations on the reduction of $AcPyAD^+$ by NADH, in the absence of $NADP^+$, was more interesting. Mutants of βAla461 were relatively little affected except that the activity in cytoplasmic membrane vesicles of βA461L was enhanced and that of βA461P was decreased by 85%. However, mutants of the carboxyl-terminal amino acid, βLeu462, with exception of the mutant βL462G, showed activities reduced by 80–97%. Moreover, the activities were abnormal, rapidly declining from the initial rate to approach zero activity. This was true also of βL462G, which had a very high activity initially. The mutant transhydrogenase in cytoplasmic membrane vesicles of βA461P behaved similarly to the βLeu462 mutants. In Fig. 4, the abnormal reduction of $AcPyAD^+$ by NADH in cytoplasmic membrane vesicles of βA461P is compared with that in vesicles of βA461L, which do not show the decrease in rate of reduction of $AcPyAD^+$. The reduction of $AcPyAD^+$ by NADH, in the presence of

Table 3
Transhydrogenase activities of βLeu462 and βAla461 mutants

Mutant	Specific activity for reduction of $AcPyAD^+$ by:		
	NADPH	NADH	NADH (+$NADP^+$)
Non-mutant	11.5	5.0	44
βL462A	4.4	0.13[a]	24
βL462E	5.4	0.80[a]	31
βL462G	6.7	7.3[a]	38
βL462P	4.3	1.1[a]	23
βL462R	4.3	0.81[a]	20
βL462T	7.1	1.1[a]	45
βA461C	5.3	4.9	25
βA461F	4.8	7.3	31
βA461P	5.5	0.77[a]	26
βA461R	9.9	5.2	52
βA461S	7.9	4.4	41
βA461L	7.8	17	38
βK460Q	6.6	3.6	27

The activities were assayed as described in Section 2. Specific activity is expressed as μmol $AcPyAD^+$ reduced/min/mg protein.

[a]Initial rate of a curved plot going to zero rate.

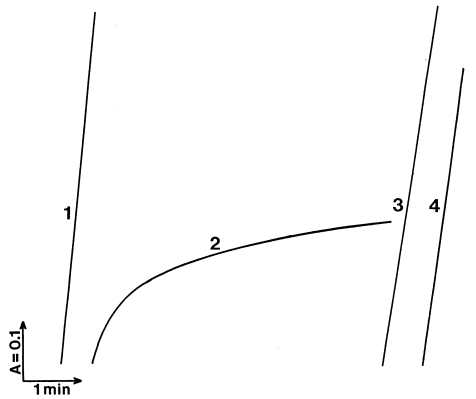

Fig. 4. Change in absorbance (*A*) at 375 nm with time following addition of membrane vesicles (1, 13.5 μg βA461L; 2, 15 μg βA461P; 3, 2.7 μg βA461L; 3.0 μg βA461P) to 1 ml of a buffer containing 50 mM MES-NaOH, 2 mM DTT, 0.5 mM EDTA, 0.01% (w/v) Brij-35, 0.5 mM $AcPyAD^+$ and 0.5 mM NADH at pH 6.0. The cuvette also contained 0.5 mM $NADP^+$ for the experiments giving traces 3 and 4.

$NADP^+$, was similar in both strains and did not show the decrease in rate.

The possible role of bound NADP(H) in the abnormal reduction of $AcPyAD^+$ by NADH was examined. As shown in Table 4, cytoplasmic membrane vesicles from the non-mutant strain, as well as selected mutants of βAla461 and βLeu462, contained 0.066 or less mol $NADP^+$/mol tetrameric transhydrogenase. NADPH was absent. Since these mutants showed a variety of kinetic properties, it is unlikely that the abnormal of reduction of AcPyAD by NADH in some of them was due to the presence of inhibitory $NADP^+$ or NADPH. Furthermore, the high activities of the βA461L or βA462F enzymes in this reaction, in comparison with that of the βA461P enzyme, cannot be due to different levels of bound NADP(H). A similar comparison can be made between the enzymes from the βL462G and βL462A mutants (Tables 3 and 4). The amount of bound $NADP^+$ in the non-mutant enzyme (0.028 mol/transhydrogenase tetramer) is in reasonable agreement with the highest value (0.02 mol/transhydrogenase tetramer) previously reported [23] (Note that in [23], the value was reported as 0.01 mol $NADP^+$/transhydrogenase dimer.)

Calculation of the amount of AcPyADH which had been formed when near-zero reduction of AcPyAD had been achieved gave the data shown in Table 5. The amounts formed by the different vesicle preparations related to their activities in reducing $AcPyAD^+$ by NADH. Thus, a larger amount of AcPyADH was formed by vesicles of the βL462G strain, and a lesser amount by the βL462A strain, in comparison with the other strains (Tables 3 and 5). These results suggested that the difference in the kinetic behavior of the various mutants in the reduction of $AcPyAD^+$ by NADH reflects the affinity of the enzyme for the product AcPyADH. This hypothesis was examined using membrane vesicles from βA461L and βA461P mutants. The specific activities for the reduction of $AcPyAD^+$ by NADPH (7.8 vs. 5.5) and by NADH in the presence of $NADP^+$ (38 vs. 26) differed less than the activities for the reduction of $AcPyAD^+$ by NADH in the absence of $NADP^+$ (17 vs. 0.77) (Table 3). Moreover, the rate of reduction of $AcPyAD^+$ in the last reaction declined only with membrane vesicles from the βA461P mutant.

Table 4
NADP(H) content of membrane-bound transhydrogenases

Mutant	mol nucleotide/mol transhydrogenase tetramer	
	$NADP^+$	NADPH
Non-mutant	0.028	0
βA461C	0.049	0
βA461F	0.033	0
βA461L	0.064	0
βA461P	0.041	0
βA461R	0.053	0
βA461S	0.066	0
βL462A	0.022	0
βL462G	0.060	0

Nucleotides were analyzed as described in [23]. The values are the mean of analyses with two different membrane preparations.

Table 5
Extent of formation of AcPyADH when the reduction of $AcPyAD^+$ by NADH had reached a near-zero rate

Mutant	mol AcPyADH/mol transhydrogenase tetramer
βL462A	72
βL462E	266
βL462G	1607
βL462P	235
βL462R	322
βL462T	346
βA461P	274

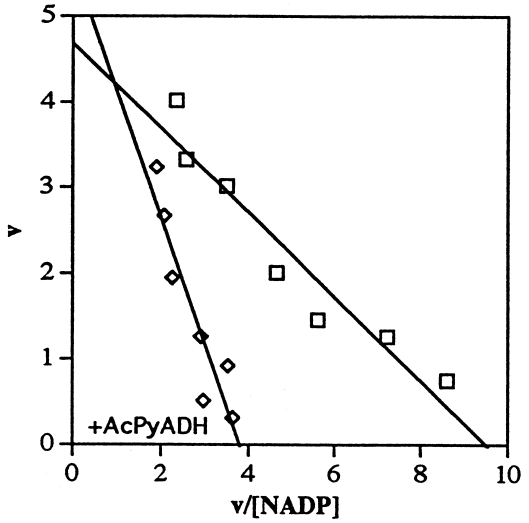

Fig. 5. Inhibition by AcPyADH of the cyclic reduction of AcPyAD$^+$ by NADH. The concentration of AcPyADH was 0.16 mM. v is expressed as μmol AcPyAD$^+$ reduced/min/mg protein. The concentration of NADP$^+$ is given as μM.

Using these two mutants, K_m values for NADH, AcPyAD$^+$ and NADPH were determined from Eadie–Hofstee kinetic plots for the various transhydrogenase reactions (Table 6). In addition, the $K_m$for NADP$^+$for the reduction of AcPyAD$^+$by NADH, in the presence of NADP$^+$, was measured as well as the inhibitor constant (K_i) for the product AcPyADH. Although there were some differences between the strains in $K_m$values for NADH, AcPyAD$^+$and NADPH, larger differences between the two strains occurred with $K_m^{NADP}$and $K_i^{AcPyADH}$. $K_i^{AcPyADH}$was significantly smaller with the βA461P enzyme than with the βA461L transhydrogenase (Table 6). This indicates that the product AcPyADH binds more strongly to the βA461P than to the βA461L enzyme. AcPyADH is competitive with respect to NADP$^+$in the cyclic reaction as shown by the converging lines in Fig. 5. Thus, it is possible that tighter binding of AcPyADH at the NADP(H)-binding site was responsible for the progressive decrease in the rate of reduction of AcPyAD$^+$by NADH which occurred with the βA461P enzyme.

4. Discussion

Two main points emerge from the experiments described in this paper. First, even small deletions from the carboxyl-terminus of the β subunit have a profound effect on the assembly of the transhydrogenase in the membrane. Second, mutations in this region affect transhydrogenase activity.

Removal of only the carboxyl-terminal alanine and leucine residues (βAla461 and βLeu462) (Fig. 3) resulted in complete lack of incorporation of the transhydrogenase into the cytoplasmic membrane. Deletion of only the carboxyl-terminal leucine residue drastically decreased the incorporation of the β subunit, although incorporation of the α subunit was little impaired. We have encountered this situation previously [12] when deletions in domain 3 allowed insertion into the membrane of the α subunit but not of the truncated β subunit. Several deletions in the β subunit from residue 236 to residue 394 were effective in this respect. Since these deletions removed relatively large segments of the protein, the smallest deletion being of 46 amino acid residues, it was not surprising to find that the β subunit could not be properly assembled in the enzyme. Therefore the effect of deleting one or two carboxyl-terminal residues, as described here, is more dramatic and indicates the important role of this region of the β subunit in the assembly of the transhydrogenase and even in the incorporation of the α dimer into the cytoplasmic membrane. Presumably, interaction with the carboxyl-terminus of the β subunit is required to establish the conformation of the α subunit appropriate for assembly and incorporation into the membrane. In the previously studied deletion mu-

Table 6
K_m values for the reduction of AcPyAD$^+$ by NADPH, NADH and NADH in presence of NADP$^+$

Mutant	NADPH		NADH		NADH+NADP$^+$			
	K_m^{NADPH} (μM)	K_m^{AcPyAD} (μM)	K_m^{NADH} (μM)	K_m^{AcPyAD} (μM)	K_m^{NADH} (μM)	K_m^{AcPyAD} (μM)	K_m^{NADP} (μM)	$K_i^{AcPyADH}$ (μM)
βA461L	42	69	104	121	14	223	0.39	419
βA461P	35	60	110	159	21	191	0.79	177

tants this region of the β subunit was still present. Therefore, assembly of the α dimer and incorporation into the membrane could occur, but the interactions were not sufficiently strong to permit the incorporation of the β subunit also. In the present study, removal of the carboxyl-terminal leucine residue was not sufficient to prevent incorporation of the α subunit, and some truncated β subunit was assembled also, although in reduced amount. The carboxyl-terminal residues (alanine and leucine) are both non-polar. However, this does not appear to be the major determining factor in their role in assembly since replacement of one or the other by a variety of polar or charged residues has a relatively limited effect on enzyme activity. For example, replacement of the carboxyl-terminal leucine residue (βLeu462) by glutamic acid or arginine residues diminished the reduction of $AcPyAD^+$ by NADPH by 50–60%, and replacement of βAla461 by an arginine residue decreased activity by 14%.

Although reduction of $AcPyAD^+$ by NADPH, and by NADH in the presence of $NADP^+$ ('cyclic' reaction), were relatively tolerant of amino acid replacements in βLeu462 and βAla461, the reduction of $AcPyAD^+$ by NADH alone was more affected. In the case of βLeu462, most of the amino acid replacements drastically affected this reaction. This was most evident as a lower than control rate declining rapidly to zero. Although, most of the mutations in βAla461 did not greatly affect the reduction of $AcPyAD^+$ by NADH alone, the βA461P mutant had lowered activity and behaved similarly to the βLeu462 mutants. As discussed in the Section 3, we attribute the abnormal reduction of AcPyAD by NADH to tighter binding of product AcPyADH to the enzyme.

Residue βTyr431 is equivalent to Tyr^{1006} of the mitochondrial transhydrogenase. The latter is covalently labeled by the nucleotide substrate analogs 5′-(*p*-fluorosulfonylbenzoyl)adenosine and 8-azidoAMP [26–28]. Although βTyr431 is not an essential residue, these results, nonetheless, suggest that the region about this residue is part of the NADP(H)-binding site on the β subunit, which also encompasses the sequence from residues 315 to 350 [9]. Thus, it seems likely that βAla461 and βLeu462 are part of this binding site. In the mutants of these two amino acids showing abnormal kinetics for the reduction of $AcPyAD^+$ by NADH (in the absence of $NADP^+$), it is possible that the inhibitory AcPyADH binds to the NADP(H)-binding site on the β subunit. The kinetic evidence that $NADP^+$ and AcPyADH are competitive in the cyclic reaction is consistent with this suggestion.

Acknowledgements

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References

[1] J.B. Jackson, J. Bioerg. Biomemb. 23 (1991) 715–741.
[2] T. Olausson, O. Fjellström, J. Mueller, J. Rydström, Biochim. Biophys. Acta 1231 (1995) 1–19.
[3] Y. Hatefi, M. Yamaguchi, FASEB J. 10 (1996) 444–452.
[4] D.M. Clarke, P.D. Bragg, J. Bacteriol. 162 (1985) 367–373.
[5] D.M. Clarke, T.W. Loo, S. Gillam, P.D. Bragg, Eur. J. Biochem. 158 (1986) 647–653.
[6] S. Ahmad, N.A. Glavas, P.D. Bragg, Eur. J. Biochem. 207 (1992) 733–739.
[7] C. Hou, M. Potier, P.D. Bragg, Biochim. Biophys. Acta 1018 (1990) 61–66.
[8] J.B. Jackson, N.P.J. Cotton, R. Williams, T. Bizouarn, M.N. Hutton, L.A. Sazanov, C.M. Thomas, Biochem. Soc. Trans. 21 (1993) 1010–1013.
[9] P.D. Bragg, N.A. Glavas, C. Hou, Arch. Biochem. Biophys. 338 (1997) 57–66.
[10] R.C.W. Tong, N.A. Glavas, P.D. Bragg, Biochim. Biophys. Acta 1080 (1991) 19–28.
[11] N.A. Glavas, C. Hou, P.D. Bragg, Biochem. Biophys. Res. Commun. 214 (1995) 230–238.
[12] S. Ahmad, N.A. Glavas, P.D. Bragg, J. Biol. Chem. 267 (1992) 7007–7012.
[13] S. Ahmad, N.A. Glavas, P.D. Bragg, J. Mol. Biol. 234 (1993) 8–13.
[14] J.W. Taylor, J. Ott, F. Eckstein, Nucleic Acids Res. 13 (1985) 8764–8785.
[15] D.M. Clarke, P.D. Bragg, Eur. J. Biochem. 149 (1985) 517–523.
[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr, A.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[17] U.K. Laemmli, Nature 227 (1970) 680–685.
[18] G. Fairbanks, T.L. Steck, D.F. Wallach, Biochemistry 10 (1971) 2506–2617.
[19] M. Hutton, J.M. Day, T. Bizouarn, J.B. Jackson, Eur. J. Biochem. 219 (1994) 1041–1052.
[20] L.N.Y. Wu, S.R. Earle, R.R. Fisher, J. Biol. Chem. 256 (1981) 7401–7408.

[21] T. Bizouarn, R.L. Grimley, N.P.J. Cotton, S.N. Stilwell, M. Hutton, J.B. Jackson, Biochim. Biophys. Acta 1229 (1995) 49–58.
[22] L.A. Sazanov, J.B. Jackson, Biochim. Biophys. Acta 1231 (1995) 304–312.
[23] N.A. Glavas, P.D. Bragg, Biochim. Biophys. Acta 1231 (1995) 297–303.
[24] P.D. Bragg, FEBS Lett. 397 (1996) 93–96.
[25] S.N. Stilwell, T. Bizouarn, J.B. Jackson, Biochim. Biophys. Acta 1320 (1997) 83–94.
[26] S. Wakabayashi, Y. Hatefi, Biochem. Int. 15 (1987) 915–924.
[27] M. Yamaguchi, Y. Hatefi, K. Trach, J.A. Hoch, J. Biol. Chem. 263 (1988) 2761–2767.
[28] P.-S. Hu, B. Persson, J.-O. Höög, H. Jörnvall, A.F. Hartog, J.A. Berden, E. Holmberg, J. Rydström, Biochim. Biophys. Acta 1102 (1992) 19–29.