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Rapid report

Identification of an aspartic acid residue in the β subunit which is essential for catalysis and proton pumping by transhydrogenase from *Escherichia coli*

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Abstract

Based on the alignment of 7 known amino acid sequences, including the recently determined sequences for the mouse and human enzymes, a highly conserved acidic domain was identified which in the *Escherichia coli* enzyme is located close to the C-terminal end of the predicted NADP(H)-binding site of the β subunit. The effect of replacing the four conserved acidic residues, βE361, βE374, βD383 and βD392, in this domain on catalytic and proton-pumping activity was tested by site-directed mutagenesis. In addition, βE371, which is not conserved but located in the same domain, was also mutated. Of these residues, βAsp 392 proved to be the only residue which is essential for both activities. However, two βAsp 392 mutants were still partly active in catalyzing the cyclic reduction of 3-acetylpyridine-NAD^+ by NADH in the presence of NADPH, suggesting that the mutations did not cause a global change but rather a subtle local change influencing the dissociation of NADP(H). It is proposed that βAsp 392 together with the previously identified βHis91 form part of a proton wire in transhydrogenase.

Keywords: Transhydrogenase; NAD; NADP; Proton pump; Membrane protein

Nicotinamide nucleotide transhydrogenase is a redox-driven proton pump localized in the inner membrane of mitochondria and the plasma membrane of certain bacteria, which catalyzes the reversible reduction of $NADP^+$ by NADH and the concomitant translocation of 1 proton according to Eq. 1

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

where 'out' and 'in' denote the intermembrane space and matrix, respectively, in mitochondria and the periplasmic space and cytosol, respectively, in bacteria. In *Escherichia coli* the enzyme is composed of an α subunit (54 kDa) and a β subunit (48 kDa), the active form being $\alpha_2\beta_2$. The gene of the enzyme has been cloned and its DNA sequenced, and the catalytic and proton-pumping properties of the purified and reconstituted enzyme have been characterized extensively. Likewise, other transhydrogenases and their genes from, for example, bovine heart and *Rhodospirillum rubrum* have also been characterized. Fig. 1 shows a schematic model of the *E. coli* transhydrogenase (Fig. 1A) and the three major domains (Fig. 1B) of this enzyme (for reviews see [1,2]).

Based on prediction of the membrane structure of the bovine and *E. coli* enzymes and subsequent mutagenesis of conserved charged residues in the latter transhydrogenase, a single basic residue, βHis91, located in the middle of a membrane α helix, was found to be important but not essential for catalysis and proton pumping [3,4]. Thus, of several mutants, βH91K, βH91N, βH91T and βH91S as well as acidic mutants, were partially active in both catalysis and proton pumping [4]. It was therefore concluded that, even though βHis91 probably constitutes part of a proton-conducting wire, its function may be partially replaced by other charged or hydrophilic residues, Most other proton pumps have two or more hydrophilic/charged, usually negative, residues that are assumed to form part of a proton wire [5,6]. At least one of these residues, e.g., Asp96 in bacteriorhodopsin [5] and Asp135 in cytochrome *bo* [6], is located in a more hydrophilic domain close to the membrane surface. The possible existence of a similar residue in transhydrogenases has not been considered or demonstrated previously.

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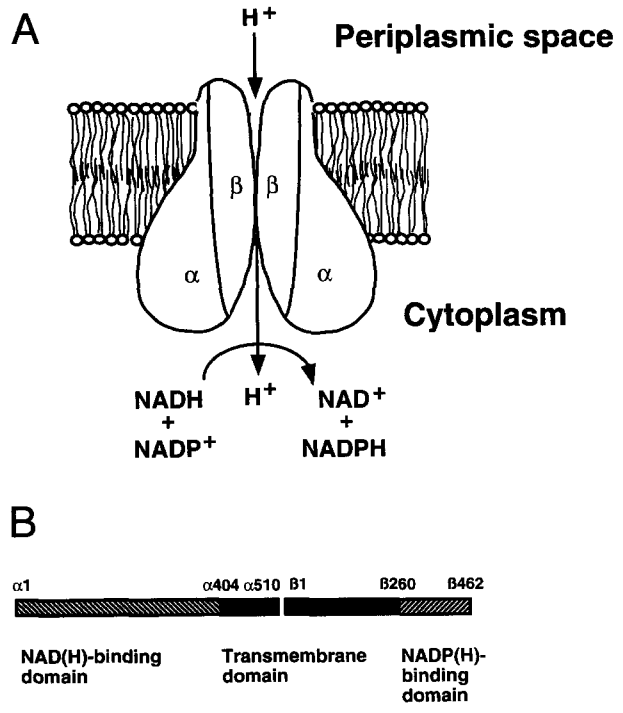

Fig. 1. Schematic presentation of the proton-pumping *E. coli* transhydrogenase (A) and the three major domains of the enzyme (B).

The recent cloning and sequencing of the mouse and human transhydrogenase genes have provided important additions to the existing transhydrogenase sequence information and identification of possible conserved residues essential for catalysis and proton pumping. Alignment of all seven known amino acid sequences of transhydrogenases from different organisms/species show that the overall identify is relatively low (not shown). However, in addition to the substrate-binding domains [1], other domains show a considerable identity. One of these domains comprises residues β351–420 (Fig. 2), which are located immediately downstream the proposed NADP(H)-binding site in the sequence β314–340. This domain is not homologous to any other known amino acid sequence and is clearly located in a soluble region, relatively far from the sequence that has been predicted to constitute the last transmembrane α helix [3,7], approximately residues β241–262. Among residues β351–420 are four fully conserved acidic residues, i.e., βE361, βE374, βD383 and βD392, and two semiconserved acidic residues, β401 and β413. The only additional conserved acidic residue in the β subunit, βD213, has previously been shown not to be essential [3].

The above-mentioned conserved acidic residues βE361, βE374, βD383 and βD392, as well as βE371 which is not conserved but located in the same domain, were replaced by site-directed mutagenesis, and the effects of the mutations on various transhydrogenase activities were tested. *E. coli* containing approximately 70-fold overexpressed transhydrogenase genes was used as source of enzyme. Mutagenesis and transformation of *E. coli* K12 strain TG1 with plasmid pDC21 or pSA2 carrying wild-type or mutated *pnt* genes, and isolation of cytosolic membrane vesicles were carried out as described [3,8–10]. Catalytic and proton pumping activities were assayed as reduction of 3-acetylpyridine-NAD^+ ($AcPyAD^+$) by NADPH at pH 7.0 [3,8,9] and reduction of $AcPyAD^+$ by NADH in the presence of NADPH at pH 6.0 [10], and quenching of ACMA at pH 7.4 [10], respectively. Proper membrane insertion and folding was checked by SDS-PAGE [3]. The variability in the activity determinations was less than 10%.

Transhydrogenase mutant DNA was sequenced by the dideoxy chain termination method [11]. Alignment of all known transhydrogenase gene sequences were carried out as described [12]. These sequences were the genes for the *E. coli* (GenBank accession No. X04195), bovine mitochondrial (J08392), *Eimeria tenella* (L07954), *Rhodospirillum rubrum* (U01158), *Entamoeba histolytica* (V13421), mouse (Z49204) and human (Z50101) transhydrogenases.

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                ß351                                                    ß410
E. coli     LPGHMNVLLA EAKVPYDIVL EMDEINDDFA DTDTVLVIGA NDTVNPAAQD DPKSPIAGMP
Bovine      MPGQLNVLLA EAGVPYDIVL EMDEINHDFP DTDLVLVIGA NDTVNSAAQE DPNSIIAGMP
Mouse       MPGQLNVLLA EAGVPYDIVL EMDEINSDFP DTDLVLVIGA NDTVNSAAQE DPNSIIAGMP
Human       MPGQLNVLLA EAGVPYDIVL EMDEINHDFP DTDLVLVIGA NDTVNSAAQE DPNSIIAGMP
R. rubrum   MPPHMNVLLA EANVPYDEVF ELEEINSSFQ TADVAFVIGA NDVTNPAAKT DPSSPIYGMP
E. histolytica LPGHMNVLLA EANVPYKIVF AMEE.AEDLE NVDVAIVVGA NDTVNPIAET DPTSPLAGMP
E. tenella  LPGHMNVLLA EADVPYKIVK EMSEVNPEMS SYDVVLVVGA NDTVNPAAL. EPGSKISGMP
                **  ***** ** ***  *     *:        * : *:** **::*  *   :*     ***

                ß411                                                    ß462
E. coli     VLEVWKAQNV IVFKRSMNTG YAGVQNPLFF KENTHMLFGD AKASVDAILL AL•
Bovine      VLEVWKSKQV IVMKRSLGVG YAAVDNPIFY KPNTAMLLGD AKKTCDALQA KVRESYQK•
Mouse       VLEVWKSKQV IVMKRSLGVG YAAVDNPIFY KPNTAMLLGD AKKTCDALQA KVRESYQK•
Human       VLEVWKSKQV IVMKRSLGVG YAAVDNPIFY KPNTAMLLGD AKKTCDALQA KVRESYQK•
R. rubrum   ILDVEKAGTV LFIKRSMASG YAGVENELFF RNNTMMLFGD AKKMTEQIVQ AMN•
E. histolytica IIDIYKAKVC VVNKRSLNQG IAAVDNPLFF YSNTRMFLSD .KKGFEELNE EIK
E. tenella  VIEAWKARRV FVLKRSMAAG YASIENPLFH LENTRMLFGN AKNTTSAVFA RVN
                :::: *        ***   *  *   * :*    **  *     *
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Fig. 2. Conserved and semiconserved acidic residues in the β352–420 domain of transhydrogenases. Multiple alignment of the C-terminal part of the β-subunit of *E. coli* transhydrogenase (β351–462) with the corresponding sequences of bovine (925–1043), mouse (925–1043), human (925–1043), *R. rubrum* (B352–B464), *E. histolytica* (416–527) and *E. tenella* (348–459) transhydrogenases. Stop codons are indicated with a dot. The alignment is underscored with stars for identity and colons for high similarity. Conserved and semiconserved negatively charged residues are bold.

Table 1
Effects of site-specific mutation on catalytic and proton pumping activities catalyzed by *E. coli* transhydrogenase

Mutant	AcPyAD$^+$/ NADPH activity (%)	Proton pumping activity (%)	NADH/AcPyAD$^+$ (+NADPH) activity (%)
Wild type	100	100	100
TG1	3	3	4
βE361Q	20	40	113
βE371Q	71	78	34
βE374L	63	108	33
βD383L	49	79	n.d.
βD383R	92	123	n.d.
βD392A	1	0	44
βD392K	3	0	2
βD392N	2	0	13
βD392Q	3	0	2
βD392T	2	0	2

Control activities (100%) for reduction of AcPyAD$^+$ by NADPH and for reduction of AcPyAD$^+$ by NADH in the presence of NADPH catalyzed by wild-type cytoplasmic membrane vesicles were 3.3 μmol/min per mg protein and 8.3 μmol/min per mg protein, respectively. Proton pumping was measured as initial decrease in ACMA fluorescence/min per mg protein expressed as % of control.
n.d., not determined.

Of these, the *E. coli* [13,14], bovine [7], *Eimeria tenella* [15], *Rhodospirillum rubrum* [16,17] and *Entamoeba histolytica* [18] sequences have been published previously. The *Eimeria acervulina* transhydrogenase (L08392) [19] has only been sequenced with regard to the domain corresponding to the α subunit of the *E. coli* enzyme and is therefore not included in this study. The mouse and human transhydrogenase genes were cloned recently by our group (Lagberg, E. and Rydström, J., unpublished data).

Activities for reduction of AcPyAD$^+$ by NADPH, reduction of AcPyAD$^+$ by NADH in the presence of NADPH, and proton pumping, all catalyzed by cytosplasmic membrane, were determined for the mutant transhydrogenases. As shown in Table 1, replacement of all residues except βD392 resulted in active mutant transhydrogenases. Indeed, the activities of the βD383R and βE361Q mutants were even significantly higher than control with regard to proton pumping and reduction of AcPyAD$^+$ by NADH in the presence of NADPH. In general, all active mutant transhydrogenases, i.e., βE361Q, βE371Q, βE374L, βD383L and βD383R, catalyzed proton pumping at a rate which apparently was proportionally higher than that for the primary reaction, reduction of AcPyAD$^+$ by NADPH, which is generally explained by the non-linearity of the proton-pumping assay. The activities of the βAsp392 mutants βD392A, βD392K, βD392N, βD392Q and βD392T were approximately 0–3% of the wild-type control activity with respect to reduction of AcPyAD$^+$ by NADPH and proton pumping, whereas the background activities of the genomic TG1 transhydrogenase were approximately 3%. Consequently, all βAsp392 mutants were inactive. However, the activity for reduction of AcPyAD$^+$ by NADH in the presence of NADPH was still substantial for the βD392A and βD392N mutants, i.e., 44% and 13%, respectively, but only 2% for the βD392K, βD392Q and βD392T mutants. It was therefore concluded that the βD392 mutant transhydrogenases were essentially inactive with regard to reduction of AcPyAD$^+$ by NADPH and proton pumping, but that a partial reaction was retained to a considerable extent in the βD392A and βD392N mutants.

Expression levels and folding of mutated transhydrogenases were tested by SDS-PAGE (cf. Ref. [3]). All mutants in Table 1 were equally expressed, inserted in the membrane and folded as compared to wild type except for the βD392K mutant which appeared to be normally expressed but subsequently partially degraded (not shown).

Like other proton pumps transhydrogenase presumably translocates protons through the protein by some type of proton wire. βHis91 located in the predicted transmembrane helix 9 has previously been concluded to form part of a proton wire and to accept protons from the periplasmic space [1,3,4,10]. Obviously, the opposite end of the assumed wire may be located in the cytoplasmic part of either the α subunit or the β subunit. One possibility is that the β subunit, which undergoes dramatic conformational changes upon NADP(H) binding and proton translocation [1,2], indeed harbors the cytosolic part of the proton wire, and that the main function of the α subunit is to provide reducing equivalents for NADP$^+$ bound to the β subunit.

The present results indicate that βAsp392 is essential for reduction of AcPyAD$^+$ by NADPH and proton pumping and that it may form part of a proton wire. The finding that βGlu361, βGlu371, βGlu374 and βAsp383 were not essential for activity or proton pumping does not exclude that these residues participate in proton pumping, but rather that they are not essential, i.e., if they are participating in proton pumping their function may be shared with or replaced by other residues. This possibility and the roles of the semiconserved residues βAsp401 and βAsp 413 are being investigated. In addition to βHis91 and βAsp392, other conserved basic residues and/or serine, tyrosine and threonine residues in the β subunit are being considered as components of a transhydrogenase proton wire. The only other essential residues identified earlier are αGly172 [12] and βGly314 [20], which both are essential for the structure of the βαβ fold of the NAD(H) and NADP(H)-binding sites, respectively.

Reduction of AcPyAD$^+$ by NADH at low pH in the presence of NADPH has been suggested to reflect a cyclic reaction in which NADH reduces AcPyAD$^+$ via bound NADP(H) [1,10,21–23]. This reaction has been shown not to pump protons [10]. A similar reaction is catalyzed by the bovine enzyme [24–26]. Bound NADP(H) participating

in this cyclic reaction may be cellular NADP(H) [23] or NADP(H) added to the isolated enzyme [9,21–26]. Routinely, this cyclic reaction is always catalyzed by transhydrogenases which are active in the reduction of $AcPyAD^+$ by NADPH and proton pumping. However, the reverse is not always the case, i.e., if the transhydrogenase is modified in a manner which does not allow NADP(H) to dissociate sufficiently rapid, the cyclic reaction may be active but not, or much less so, the reduction of $AcPyAD^+$ by NADPH or proton pumping. The present finding that βD392A and to some extent βD392N, but not βD392K, βD392Q or βD392T, catalyzed the cyclic reaction, strongly suggests that the mutation did not cause a global change/inactivation of the transhydrogenase, but rather a subtle local change that influenced the binding/dissociation of NADP(H) from its binding site. Since βAsp392 is close to the predicted binding site [2], it is possible that this residue constitutes part of the NADP(H) site.

In conclusion, in a domain containing 6 acidic conserved/semiconserved residues, βAsp392 was found to be essential for catalysis and proton pumping. It is proposed that the protonation/deprotonation state of βAsp392, as a component of the transhydrogenase proton wire and possibly the NADP(H) site, indirectly regulates the association/dissociation of NADP(H) through a conformational change.

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