Mitochondrial Energy-linked Nicotinamide Nucleotide Transhydrogenase

MEMBRANE TOPOGRAPHY OF THE BOVINE ENZYME*

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The mitochondrial energy-linked nicotinamide nucleotide transhydrogenase is a homodimer of monomer $M_r = 109,228$. Hydropathy analysis of its cDNA-derived amino acid sequence (1043 residues) has indicated that the molecule is composed of 3 domains: a 430-residue-long hydrophilic N-terminal domain which binds NAD(H), a 200-residue-long hydrophilic C-terminal domain which binds NADP(H), and a 400-residue-long hydrophobic central domain which appears to be made up mainly of about 14 hydrophobic clusters of ~20 residues each. In this study, antibodies were raised to the hydrophilic N- and C-terminal domains cleaved from the isolated transhydrogenase by proteolytic digestion, and to a synthetic, hydrophilic pentadecapeptide, which corresponded to position 540-554 within the central hydrophobic domain. Immunochemical experiments with mitoplasts (mitochondria denuded of outer membrane) and submitochondrial particles (inside-out inner membrane vesicles) as sources of antigens showed that essentially the entire N- and C-terminal domains would be expected to come together to form the enzyme's catalytic site. Treatment of mitoplasts with several proteolytic enzymes indicated that large protease-sensitive masses of the transhydrogenase are not exposed on the cytosolic side of the inner membrane, which agreed with the expectation that the central highly hydrophobic domain of the molecule should be largely membrane-intercalated. Trypsin, $\alpha$-chymotrypsin, and papain had little or no effect on the mitoplast-embedded transhydrogenase. Proteinase K, subtilisin (Nagarase), thermolysin, and pronase E each split the mitoplast-embedded enzyme into two fragments only, a fragment of ~70 kDa containing the N-terminal hydrophilic domain, and one of ~40 kDa bearing the C-terminal hydrophilic domain. The cleavage site of proteinase K was determined to be $A^{488}_G$-$A^{491}_C$, which is located in a small hydrophilic segment within the central hydrophobic domain. This protease-sensitive loop appears to be exposed on the cytosolic side of the inner membrane. The proteinase K-nicked enzyme containing two peptides of 71 and 39 kDa was isolated from mitoplasts and shown to have high transhydrogenase activity.

The mitochondrial energy-linked nicotinamide nucleotide transhydrogenase catalyzes the direct and stereospecific transfer of a hydride ion between the 4A position of NAD(H) and the 4B position of NADP(H) in a reaction that is coupled to transmembrane proton translocation with $H^+\text{H}^+$ stoichiometry close to unity (see Equation 1 where $H^2_\text{z}$ and $H^2_\text{a}$ are matrix and cytosolic protons, respectively) (1-3).

\[
\text{NADH} + \text{NADP} + H^2_\text{a} = \text{NAD} + \text{NADPH} + H^2_\text{z}
\]  

(1)

The enzyme from bovine heart mitochondria is a homodimer of monomer $M_r = 109,228$. It is nuclearly encoded, extramitochondrially synthesized, and transported into mitochondria (4, 5). The amino acid sequences of the mature protein (1043 residues) and its signal peptide (43 residues) have been deduced, respectively, from cDNA clones and the mRNA (6, 7). *Escherichia coli* contains a similar enzyme. It is composed of two subunits, $\alpha$ with $M_r = 54,000$ and $\beta$ with $M_r = 48,700$ (8). The amino acid sequences of these subunits have also been deduced from the nucleotide sequences of the genes (8). There is considerable sequence identity between the *E. coli* and the bovine enzymes, especially in the nucleotide binding domains (~65% identity) as determined for the latter enzyme (6).

The bovine transhydrogenase contains a 430-residue-long N-terminal domain, which is hydrophilic and binds NAD(H), and a 200-residue-long C-terminal domain, which is also hydrophilic and binds NADP(H) (6, 9). The central 400-residue-long segment of the protein is hydrophobic, and hydropathy analyses have indicated that it is composed mainly of 14 hydrophobic clusters of ~20 residues each (6). There are in this central segment only 25 charged residues, 6 Asp, 7 Glu, 6 Lys, and 6 Arg. Thus, one would expect a priori that the hydrophilic N- and C-terminal domains would be extramembranous, protruding into the mitochondrial matrix where they would together form the catalytic site of the enzyme, and the central hydrophobic stretch would intercalate mainly into the membrane and make up the proton channel of the molecule. As will be seen below, the results of our studies on the membrane topography of the bovine transhydrogenase are in full accord with these expectations.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-rabbit IgG-alkaline phosphatase conjugate, and anti-rabbit IgG-peroxidase conjugate were obtained from Calbiochem.

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Proteinase K (Type XI), papain, a-chymotrypsin (Type VII), subtilisin (Nagarase), pronase E, thermolyzin, leupeptin, 4-chloro-1-naphthol, p-nitrophenyl phosphate, and 3-acetylpyridine adenine dinucleotide were obtained from Sigma. N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin was from Worthington. Lubrol PX was obtained from Aldrich. Mitochondrial preparations were from Millipore. Protein A, \(^{125}\)I-labeled with Bolton and Hunter reagents (10), was from Amersham. Bovine mitochondrial transhydrogenase was prepared as reported previously (11).

**Assay of Transhydrogenase Activity—** Transhydrogenation from NADPH to AcPyAD was assayed at 37°C in a reaction mixture containing 100 mM sodium phosphate, pH 6.5, 4.5 \(\mu\)M of L-\(\alpha\)-lysophosphatidylcholine, and 0.3 mM concentration each of NADPH and AcPyAD. The reaction was started by the addition of enzyme, and the reduction of AcPyAD was followed at 375 minus 425 nm in an Aminco DW2a dual-wavelength spectrophotometer. Rates were calculated using a value of 6.38 \(\text{mM cm}^{-1}\) for the absorbance difference of AcPyAD and NADPH at the above wavelength pair. When the enzyme activity was measured on SMP or mitoplasts, L-\(\alpha\)-lysophosphatidylcholine was omitted from the reaction mixture, and 1 \(\mu\)M rotenone was added. In the case of mitoplasts, 0.1% Lubrol PX was also added.

**Preparation of SMP, Mitochondria, and Mitoplasts—** SMP were prepared according to Ref. 12. Intact bovine heart mitochondria for preparation of mitoplasts were prepared by the method of Hatefi et al. (13), and mitoplasts were prepared according to Krebs et al. (14). The residual transhydrogenase activity after the treatment of mitoplast membranes with n-octyl-\(\beta\)-D-glucopyranoside (matrix enzyme) in the mitoplast preparation were <10% and >95%, respectively, of the total activities that were present in the original sample of mitochondria.

**Purification of Transhydrogenase from Proteinase K-treated Mitoplasts—** Mitoplasts (280 mg in 28 ml of STA buffer) were digested by the addition of 5.4 mg of proteinase K for 80 min at 37°C; then 500 \(\mu\)l of 0.2 M PMSF in methanol were added to stop further digestion. The residual transhydrogenase activity was 85% as compared to the activity of control mitoplasts not treated with proteinase K. Preparation of SMP from proteinase K-treated mitoplasts and purification of the enzymatic preparation therefrom followed the peptides as described previously (11). From 280 mg of mitoplasts, 70 mg of SMP were obtained, and from the SMP 270 \(\mu\)g of transhydrogenase with a specific activity of 24.6 \(\mu\)mol of AcPyAD reduced (min-\(\mu\)g protein)\(^{-1}\).

**Isolation of the N-terminal 43-kDa and C-terminal 20-kDa Fragments—** Purified transhydrogenase was digested with trypsin in the presence of 0.4 mM NADPH as described previously (15). The trypptic digest was subjected to SDS-polyacrylamide gel electrophoresis, and the digests were separated into pellet and supernatant fractions using a Beckman Airfuge (100,000 rpm, 5 min) and analyzed as described above before use.

**Detection of Peptides by Immunoblotting—** Protein samples were denatured by the addition of 2 volumes of SDS denaturation buffer (94 mM Tris-HCl, pH 6.8, 15% glycerol, 7.5% \(\beta\)-mercaptoethanol, 3% SDS, and 5% urea) followed by immersion in boiling water for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (20). Protein samples were subjected to the same procedure as described above before use.

**Treatment of SMP and Mitoplasts with Various Proteases—** SMP, 3.8 mg in 0.8 ml of STa containing 0.4 mM NADPH, were treated with 50 \(\mu\)g of papain or 16 \(\mu\)g of trypsin at 37°C for 40 min, and the digestion was stopped by the addition of 8 \(\mu\)l of 0.2 M PMSF in methanol and 50 \(\mu\)l of leupeptin (1 mg/ml). Mitoplasts, 1 mg of protein in 100 \(\mu\)l of STA (0-25 mM sucrose containing 10 mM Tris acetate, pH 7.5), were treated with 10 \(\mu\)g of various proteases at 23°C for 40 min, the digests were separated into pellet and supernatant fractions as described above before use.

**Detection of Peptides by Immunoblotting—** Protein samples were denatured by the addition of 2 volumes of SDS denaturation buffer (94 mM Tris-HCl, pH 6.8, 15% glycerol, 7.5% \(\beta\)-mercaptoethanol, 3% SDS, and 5% urea) followed by immersion in boiling water for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (22). Peptides were transferred onto PVDF membranes using a Bio-Rad Mini Trans-Blot apparatus. The electrophoretic buffer solution contained 20 mM Tris acetate, pH 8.3, 1 mM EDTA, and 0.2 mM dithiothreitol. Electrotransfer was performed at 30 V for 1 h. After the transfer, membranes were washed several times with PBS and incubated for 1 h with anti-rabbit IgG-peroxidase conjugate diluted 5000-fold in the dilution buffer described above. The blots were washed with PBS three times and stained by incubation in 25 ml of PBS with 5 ml of ethanol containing 15 mg of 4-chloro-1-naphthol (110 \(\mu\)l) and 15 ml of 0.05 M NaCl. Protein A, \(^{125}\)I-labeled with Bolton and Hunter reagents (10), was from Amersham. Bovine mitochondrial transhydrogenase was prepared as reported previously (11). From 280 mg of mitoplasts, 70 mg of SMP were obtained, and from the SMP 270 \(\mu\)g of transhydrogenase with a specific activity of 24.6 \(\mu\)mol of AcPyAD reduced (min-\(\mu\)g protein). The peptide DMFKRPTDPPEYNYL, with inclusion of a cysteine residue at the C terminus, was synthesized by Dr. John Tomich (Medical Genetics Division, Children's Hospital, Los Angeles) essentially as reported (18) and was conjugated to the carrier protein, keyhole limpet hemocyanin (KLH), using the hetero-bifunctional reagent succinimidyl (4-N-(maleimidomethyl)cyclohexane-1-carboxylate as described by the manufacturer. The peptide (12 mg) was reduced with diithiothreitol and denatured in 4 M guanidine HCl. Just prior to use, the reducing agent was removed by gel filtration, and the peptide was mixed with 10 mg of the activated carrier. The mixture was allowed to react for 3 h at 25°C, then stored at 4°C until it was used to inoculate the animals. The KLH-peptide conjugate (~200 \(\mu\)g) in 0.75 ml of PBS was mixed with the same volume of incomplete Freund's adjuvant and 3 mg of M. butyricum and injected subcutaneously in several places along the back of the rabbit. Fourteen days later, the same solution lacking M. butyricum was similarly injected. Seven days later, 200 \(\mu\)g of KLH-peptide conjugate in 0.6 ml of PBS was mixed well with 0.4 ml of 10 mg/ml Al(OH)\(_3\), and injected intraperitoneally (10). Ten weeks later, boosting was carried out in the same way as the third injection, and, 14 days later blood was collected.

**Affinity Purification of Antibodies—** Purified transhydrogenase (~2 mg of protein) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane as described below (20). The membrane was stained with 0.5% Ponceau S in 1% acetic acid for 8 min and destained with water, and the transhydrogenase band was excised. This piece of membrane was used for purification of antibodies against the N-terminal 43-kDa and the C-terminal 20-kDa peptides as well as the synthetic peptide described above. Affinity purification of the antisera followed the method of Bisson and Schiavo (21). Preimmune sera were subjected to the same procedure as described above before use.

**Detection of Peptides by Immunoblotting—** Protein samples were denatured by the addition of 2 volumes of SDS denaturation buffer (94 mM Tris-HCl, pH 6.8, 15% glycerol, 7.5% \(\beta\)-mercaptoethanol, 3% SDS, and 5% urea) followed by immersion in boiling water for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (22). Peptides were transferred onto PVDF membranes using a Bio-Rad Mini Trans-Blot apparatus. The electrophoretic buffer solution contained 20 mM Tris acetate, pH 8.3, 1 mM EDTA, and 0.2 mM dithiothreitol. Electrotransfer was performed at 30 V for 1 h. After the transfer, membranes were washed several times with PBS and incubated for 1 h with anti-rabbit IgG-peroxidase conjugate diluted 5000-fold in the dilution buffer described above. They were then washed with PBS three times and stained by incubation in 25 ml of PBS with 5 ml of ethanol containing 15 mg of 4-chloro-1-naphthol (110 \(\mu\)l) and 15 ml of 0.05 M NaCl. Protein A, \(^{125}\)I-labeled with Bolton and Hunter reagents (10), was from Amersham. Bovine mitochondrial transhydrogenase was prepared as reported previously (11). From 280 mg of mitoplasts, 70 mg of SMP were obtained, and from the SMP 270 \(\mu\)g of transhydrogenase with a specific activity of 24.6 \(\mu\)mol of AcPyAD reduced (min-\(\mu\)g protein).
were kept overnight at 4°C and centrifuged for 5 min at 100,000 rpm in a Beckman Airfuge. The pellets were washed twice by suspension in STA and recentrifuged. Then, 50 μl of 100 Autogramma counter. before. The radioactivity in the pellets was counted in a Packard tubes were centrifuged and the pellets were washed three times as described above. The activity in the pellets was counted in a Packard Autogramma counter.

**Enzyme-linked Immunosorbent Assay (ELISA)—**MicroTest wells were coated with 100 μl of antigen in PBS or STA, and the plates were incubated for 1 h at 37°C. After the plates were washed with the same buffer, 200 μl of 2% bovine serum albumin in PBS or STA was added to each well, and the plates were further incubated as before. The wells were then washed several times with the same buffer, and each was treated with 100 μl of control IgG or purified IgG at the dilutions shown in the figures. The plates were incubated for 2 h at 37°C, followed by thorough washing of the wells with buffer. Anti-rabbit IgG conjugated to alkaline phosphatase was diluted 2500-fold in PBS or STA, and 100 μl were added to each well. The plates were incubated for 1 h at 37°C. After the wells were washed, 100 μl of substrate solution (1 mg/ml p-nitrophenyl phosphate dissolved in a solution containing 1 M diethanolamine, 0.5 mM MgCl₂, and 0.02% Na₂SO₃, pH 9.8) were added to each well, the plates were allowed to stand at room temperature for 90 min for color development, and the absorbance of each well was read at 405 nm in the Titertek Multiskan spectrophotometer.

**N-Terminal Sequencing of Peptides—**Peptides were separated by SDS-polyacrylamide gel electrophoresis (12% Laemmli gel, Ref. 22) and transferred to PVDF membranes. Peptides were located by staining with a mixed solution of Ponceau S (0.09%) and Coomassie Blue (0.01%) in 10% methanol, then excised from the membranes. They were loaded onto a gas phase sequencer, and the N-terminal amino acids (4-6 residues) of each peptide were determined as reported previously (15).

**Protein Assays—**Protein concentration was measured by the method of Peterson (23), with bovine serum albumin as standard. For mitochondria, mitoplasts, and submitochondrial particles, protein concentration was estimated by the biuret method (24) in the presence of 0.1% sodium deoxycholate.

**RESULTS**

The strategy used for study of the membrane topography of the transhydrogenase was 2-fold. (a) By controlled proteolytic digestion, the hydrophilic N- and C-terminal domains of the protein were cleaved and isolated from the purified enzyme. Also, an antigenic peptide (DMFKRPTDPPEYYNYL), which in the transhydrogenase is located at position 540-554 within the central hydrophobic domain of the molecule, was synthesized and linked to KLH. Antibodies were raised against these three fragments in the rabbit and tested in immunoabsorbents of whole mitochondria or SMP. Moreover, the reactivity of each antisem was blocked when it was pretreated with an excess of its respective antigen. When the anti-43-kDa and the anti-20kDa IgG were tested against intact mitochondria, mitoplasts, and SMP, using 125I-protein A binding to monitor antigen-antibody interaction,

Fragmentation of purified transhydrogenase by papain and isolation of the N- and the C-terminal hydrophilic domains. Conditions for papain digestion and gel filtration of the digest on Sephadex G-200 have been described under "Experimental Procedures." The absorbance at 230 nm of transhydrogenase activity measured using 50-μl aliquots of each fraction. Vₐ and Vₛ show the elution positions of blue dextran and potassium ferricyanide, respectively. Numbered arrows show the elution positions of yeast alcohol dehydrogenase (150 kDa) (1), bovine serum albumin (66 kDa) (2), ovalbumin (43 kDa) (3), and soybean trypsin inhibitor (20 kDa) (4). Inset, SDS-polyacrylamide gel electrophoresis of fractions A, B, and C eluted from Sephadex G-200. Forty microliters of each fraction were mixed with the SDS denaturation buffer, incubated for 4 min in a boiling water bath, and subjected to SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue and de-stained.

**FIG. 1. Fragmentation of purified transhydrogenase by papain and isolation of the N- and the C-terminal hydrophilic domains.**
the results with mitochondria and mitoplasts were essentially negative (Fig. 2), the slight positive response at high antibody concentration being probably due to low levels of mitochondrial fragments in the preparations. By comparison, however, the results with SMP were strongly positive (Fig. 2). These data indicated that epitopes from the N-terminal 43-kDa and the C-terminal 20-kDa domains of the transhydrogenase were exposed on the matrix, but not on the cytosolic, side of the inner membrane. Anti-43-kDa antiserum partially inhibited the transhydrogenase activity of SMP, but anti-20-kDa antiserum had no inhibitory effect at comparable levels (Fig. 3).

Results similar to those of Fig. 2 were obtained with the D540-L554 anti-peptide antibody. Using ELISA to monitor antigen-antibody interaction, it was shown that the antipeptide antiserum reacted strongly with the purified enzyme and SMP, but showed marginal reactivity toward mitoplasts (Fig. 4). Again, the low reactivity with mitoplasts could be due to the presence of low levels of fragmented mitoplasts, which is a likely occurrence during the manipulations involved in ELISA. These results indicated, therefore, that epitopes from the D540-L554 peptide are also exposed on the matrix side of the inner membrane.

Extramembranous Segments of the Transhydrogenase Molecular While it is highly likely that the hydrophilic D540-L554 pentadecapeptide is largely extramembranous and exposed to the medium on the matrix side of the inner mitochondrial membrane, a similar conclusion does not follow from the results of Fig. 2 for the N- and C-terminal hydrophilic domains. Fig. 2 only shows that epitopes from these segments which are reactive toward our antibodies are exposed on the matrix side. Indeed, hydrophathy analyses of these domains,

FIG. 2. Membrane sidedness of the N- and C-terminal hydrophilic domains of the transhydrogenase as determined by radioimmunolabeling with 125I-protein A. Solid lines, affinity-purified antibodies to the N-terminal 43-kDa (●) and the C-terminal 20-kDa (○) peptides; dashed lines, preimmune sera after "affinity purification." Procedural details are described under "Experimental Procedures." The abscissas in this figure and Fig. 4 show the number of times the IgG fractions were diluted before use.

FIG. 3. Effects of antibodies on the transhydrogenase activity of submitochondrial particles. SMP (38 μg) were incubated in PBS with antisera (●) raised to the N-terminal 43-kDa (A) and the C-terminal 20-kDa (B) peptides or with preimmune sera (△) in a total volume of 100 μl for 15 min at 23°C. Then enzyme activity was determined as described under "Experimental Procedures."

especially of the N-terminal 430 residues, show several hydrophobic stretches of amino acids, one or more of which could be intramembranous. Therefore, it was important to find out how much of the transhydrogenase protein mass is extramembranous on the matrix as well as on the cytosolic side of the inner membrane.

For this purpose, SMP and mitoplasts were treated with papain as described under "Experimental Procedures." Aliquots containing 25 μg of protein from untreated and papain-treated SMP were assayed for transhydrogenase activity (30% of control) and electrophoresed on 12% SDS-polyacrylamide gels. Pellet and supernatant fractions were obtained by centrifuging 400 μl of papain-treated SMP, and equivalent aliquots of these fractions were also electrophoresed. Then, proteins were transferred to PVDF membranes, and the membranes were incubated with antibodies to the N-terminal 43-kDa (panel A) and the C-terminal 20-kDa (panel B) peptides. Lane 1, untreated SMP; lane 2, papain-treated SMP; lane 3, pellet of papain-treated SMP; lane 4, supernatant of papain-treated SMP. Papain cleavage resulted near the N terminus in N-terminal 42- and 44-kDa (panel A, lanes 2–4) and C-terminal 66-kDa (panel B, lanes 2 and 3) peptides, and near the C terminus in N-terminal 82-kDa (panel A, lanes 2 and 3) and C-terminal 26-kDa (panel B, lanes 2–4) peptides.
pellet of papain-treated SMP, and supernatant of papain-treated SMP. Membranes in panel A were blotted with the N-terminal 43-kDa antibodies and those in panel B with the C-terminal 20-kDa antibodies. It is clear from the results that papain treatment of SMP had caused solubilization of two fragments with $M_r$ values of about 42,000 and 44,000, which blotted to the N-terminal 43-kDa antibodies (panel A, lane 4), and a smaller peptide with $M_r$ (apparent) of 26,000, which blotted to the C-terminal 20-kDa antibodies (panel B, lane 4). The latter peptide was excised from the PVDF membranes, and its N-terminal sequence was determined to be GGKPME, which showed that papain had hydrolyzed the same bond, i.e. A$_{485-488}^{GKPM}$, in SMP-bound transhydrogenase as it had done in the purified enzyme. These results indicated, therefore, that essentially the entire 430-residue-long N-terminal hydrophilic domain, and the 200-residue-long C-terminal hydrophilic domain of the transhydrogenase molecule are extramembranous and bathe in the matrix milieu of mitochondria. This conclusion was confirmed when SMP were treated with trypsin instead of papain. Two N-terminal segments were solubilized with $M_r$ values exactly the same as those reported previously for the N-terminal tryptic fragments isolated from the purified enzyme, i.e. about 43,000 and 41,000 (15). The most abundant C-terminal fragment found in the soluble fraction exhibited a $M_r$ of 14,000, which in agreement with the primary sequence of the enzyme indicated that the potential tryptic cleavage sites in the C-terminal domain are downstream of the papain cleavage site at A$_{485}^{GKPM}$.

Essentially similar experiments were carried out to investigate the segments of the transhydrogenase molecule exposed on the cytosolic side of the inner mitochondrial membrane, except that in this case mitoplasts (mitochondria denuded of outer membrane) were used instead of SMP. As seen in Fig. 6, treatment of mitoplasts with various proteolytic enzymes produced either no detectable transhydrogenase fragments or split the enzyme into two pieces, an N-terminal piece of $M_r$ ~71,000 plus a C-terminal piece of $M_r$ ~39,000. These results suggested that a large transhydrogenase segment is not exposed on the cytosolic side of the inner mitochondrial membrane, because one would have expected such an exposed segment to be susceptible to multiple cuts by the proteases used, thus resulting in N- and C-terminal fragments whose molecular weight would not add up to that of the intact protein. This reasoning is, of course, in excellent agreement with predictions based on the hydrophathy analysis of the transhydrogenase primary sequence. It has already been shown above that the hydrophilic N- and C-terminal domains of the enzyme protrude into the matrix. What remains is the central highly hydrophobic domain of the molecule, which one would not expect to be extramembranous to any great extent. The hydrophathy analysis of this central segment suggests that it is composed of about 14 hydrophobic clusters of ~20 residues each. If one assumes that these clusters span the membrane, then there would have to be links between the clusters that are exposed on the matrix and the cytosolic sides of the inner membrane. These hydrophilic links, as suggested by the hydrophathy data, are all small, and Fig. 6 suggests that those exposed on the cytosolic side are resistant to proteolytic attack, except for one link which is broken by proteinase K, subtilisin, thermolysin, and pronase E (a mixture of several proteases) and results in a 71-kDa N-terminal and a 39-kDa C-terminal fragment.

Further characterization of the above fragments was carried out, using proteinase K. First, it was shown that proteinase K treatment of mitoplasts to the extent that essentially all transhydrogenase molecules were split produced only the 71- and the 39-kDa bands seen in Fig. 6 (data not shown). Second, it was demonstrated that, after complete conversion of the intact 110-kDa transhydrogenase into the 71- and the 39-kDa fragments, the detergent-solubilized mitoplasts had retained 85-90% transhydrogenase activity (NADPH $\rightarrow$ AcPyAD) as compared to control mitoplasts not treated with proteinase K (data not shown). Third, it was found that the proteinase K-nicked enzyme could be isolated from mitoplasts, still retaining >70% activity. These results are shown in Fig. 7. Lane 1 is a Coomassie blue-stained SDS gel of the nicked transhydrogenase isolated from proteinase K-treated mitoplasts. It shows a thin band of the intact transhydrogenase (110 kDa) and two bands of about 71 and 39 kDa, which represent the nicked enzyme. Lanes 2 and 3 are immunoblots of the material of lane 1 blotted with the N-terminal 43-kDa antibodies in lane 2 and with the C-terminal 20-kDa antibodies in lane 3. In a similar experiment, mitoplasts were treated with proteinase K to the extent that the intact transhydrogenase band was about 90% cleaved. Then, the enzyme was purified from the proteinase K-treated and untreated mitoplasts and assayed for activity. The purified intact enzyme had a transhy-
Transhydrogenase activity of 32 nmol of AcPyAD reduced by NADPH per min per mg of protein; the purified nicked enzyme (containing ≤10% intact transhydrogenase) exhibited an activity of 24.6. Finally, the 39-kDa C-terminal fragment produced by proteinase K treatment of mitoplast-bound transhydrogenase was isolated, and its N-terminal sequence was determined to be AANLT, which indicated that the bond cleaved by proteinase K is A⁴⁶⁹⁰-A⁴⁶⁹¹. According to our hydrophyt analysis, these residues are located in a hydrophilic segment between hydrophobic clusters 9 and 10 of the transhydrogenase molecule (see “Discussion”). Thus, one may conclude that this small hydrophilic segment is extramembranous and protrudes from the cytosolic side of the inner membrane into the intermembrane space.

**DISCUSSION**

The hydrophyt profile of the bovine mitochondrial transhydrogenase based on the enzyme’s predicted amino acid sequence suggested that the transhydrogenase molecule is composed of three domains, a 430-residue-long N-terminal hydrophilic domain, a 400-residue-long central hydrophobic domain, and a 200-residue-long C-terminal hydrophilic domain (6). The N- and the C-terminal domains were shown, respectively, to bind NAD(H) and NADP(H) (9). These domains could be isolated in soluble form after appropriate and limited proteolytic digestion of the purified transhydrogenase, and the purified N-terminal hydrophilic domain (a dimer containing two peptides with _M_ values mainly of 43,000 and partly of 41,500) was shown to bind NAD with high affinity (15). These findings allowed certain predictions regarding the topography of the transhydrogenase molecule in the mitochondrial inner membrane: (i) that the N- and the C-terminal hydrophilic domains are extramembranous, (ii) that these extramembranous domains protrude into the mitochondrial matrix where together they form the catalytic site of the enzyme, and (iii) that the central hydrophobic domain is largely membrane-intercalated because of its low content of charged amino acids (25 residues only). These considerations also led to the further prediction that there is probably very little transhydrogenase mass protruding from the inner membrane on the cytosolic side, this despite the contrary conclusions of Weis et al. (25) which will be discussed below.

The above predictions were substantiated by the following experiments. The N- and the C-terminal hydrophilic domains (43 and 20 kDa, respectively) were cleaved by appropriate proteolytic digestion from the purified transhydrogenase, purified, identified by N-terminal sequencing of 5 to 6 residues, and injected into rabbits for production of polyclonal antibodies. A hydrophilic pentadecapeptide (D⁴⁴⁶-L⁵⁵⁴) located within the central hydrophobic domain, which was expected to be extramembranous and antigenic, was also synthesized and used for production of antipeptide antibodies. The antibodies raised were checked for reactivity against purified transhydrogenase, then used for topography studies against mitoplasts and SMP as sources of antigens. The results showed clearly that epitopes from the isolated N- and C-terminal domains as well as from the central hydrophilic peptide were exposed in SMP, but not in mitoplast preparations.

In order to determine how much of the N- and C-terminal hydrophilic domains were protruding from SMP into the medium, the particles were treated in one experiment with papain, and in another with trypsin, then the soluble and the particulate fractions were separated by centrifugation, and the sizes of the solubilized N- and C-terminal antigens were determined after SDS gel electrophoresis of the supernatants and immunoblotting with antibodies to the 43-kDa N-terminal and the 20-kDa C-terminal fragments. The results indicated that essentially the entire N- and C-terminal hydrophilic domains are extramembranous and cleavable by appropriate proteolytic digestion of SMP. These findings are summarized in Fig. 8, which shows the N- and the C-terminal hydrophilic domains in abbreviated form and the central hydrophobic domain as interpreted from a Kyte-Doolittle hydrophyt analysis with a setting of 9 residues (6, 26). The bonds hydrolyzed by trypsin in the N-terminal domain and by papain in the C-terminal domain are marked, and the antigenic pentadecapeptide (D⁴⁴⁶-L⁵⁵⁴) protruding into the matrix space is outlined with dashes.

To determine what parts of the transhydrogenase molecule were exposed on the cytosolic side of the inner membrane, mitoplasts were treated with seven different proteolytic enzymes. Two considerations governed the selection of these proteases. One was to have among them a wide substrate specificity, and another to be able to stop their activity before SDS gel electrophoresis of the mitoplasts. The results showed that trypsin, α-chymotrypsin, and papain had little or no effect on the transhydrogenase embedded in mitoplasts, while proteinase K, subtilisin (Nagarse), thermolysin, and pronase E (a mixture of several proteases) each cleaved the mitoplast-embedded transhydrogenase into a 71- and a 39-kDa fragment. Immunoblotting indicated that the 71-kDa fragment contained the N-terminal hydrophilic domain, while the 39-kDa fragment included the C-terminal hydrophilic domain. Since the _M_ values of the two fragments added up precisely to the _M_ of intact transhydrogenase (110,000), these results argued against the cytosolic exposure of large segments of the enzyme, which would be susceptible to digestion by one or another of the seven proteases employed. What appeared more likely was the cytosolic exposure of a single small peptide sensitive to four of the seven proteases mentioned. Only with such an arrangement could one obtain two fragments of approximately 70 and 40 kDa and no fragments of other sizes with the use of several different proteolytic enzymes.

In contrast to the above considerations, Weis et al. (25) have published the following results and conclusions. Using polyclonal antibodies raised against bovine heart transhydrogenase, Weis et al. (25) showed that when rat liver mitoplasts were treated with proteinase K, the antiserum recognized in the pellet an antigen of _M_ = 75,000 as the final proteolytic product of rat liver transhydrogenase (_M_ = 110,000). No antigens were found in the supernatant of mitoplasts treated with proteinase K. When rat liver SMP were treated with proteinase K, a transient antigen of _M_ = 52,000 was released into the supernatant. The authors concluded from these results that the transhydrogenase in rat liver mitochondria is composed of a 52-kDa domain that protrudes into the matrix, a 23-kDa domain (75 – 52 = 23) that intercalates into the membrane, and a 35-kDa domain (110 – 75 = 35) that protrudes from the cytosolic side of the inner membrane. What Weis et al. (25) did not investigate was the fate of the 35-kDa domain, and this introduces a problem with their conclusions. Assume that in mitoplasts proteinase K had fragmented the rat-liver transhydrogenase into two pieces of 75 and 35 kDa, both membrane-bound. If the antiserum used recognized the 75-kDa fragment, but not the 35-kDa piece, then the immunoblots of the mitoplasts would not show the presence of the 35-kDa piece in the membranes. As a result, it could be assumed that the 35-kDa segment had been cleaved by proteinase K from the mitoplasts and further digested into undetectable fragments. This problem does not complicate our conclusions, because our experiments do not leave any piece of the transhydrogenase unaccounted for.
In order to identify the protease-sensitive segment of the bovine transhydrogenase, which is exposed on the cytosolic side of the mitochondrial inner membrane, the following experiments were performed. Mitoplasts were treated with proteinase K until the transhydrogenase band as checked by immunoblotting had been nearly completely converted into the 71- and the 39-kDa fragments. Assay of the transhydrogenase activity of mitoplasts solubilized with Lubrol showed that, even when the 110-kDa band of intact transhydrogenase had disappeared, the particles still retained 85–90% of their original transhydrogenase activity. This prompted us to isolate the nicked enzyme. The attempt succeeded, and a highly active preparation containing >80% nicked transhydrogenase was isolated. Thereupon, the 39-kDa fragment was excised from SDS gels of the nicked enzyme and subjected to N-terminal sequencing. This sequence, AANLT, is seen in the hypothetical arrangement shown in Fig. 8, where the proteinase K cleavage site is marked. As stated above, Fig. 8 is simply an interpretation of the hydropathy profile of the central hydrophobic domain of the transhydrogenase. It is, therefore, interesting that the proteinase K cleavage site should occur in the largest extramembranous segment on the cytosolic side of this hypothetical picture. Also interesting is that one can rationalize the effects of subtilisin, thermolysin, and pronase E on this same extramembranous loop, in each case resulting in two transhydrogenase fragments with approximate \( M \), values of 70,000 and 40,000.

Fig. 8 also shows a trypsin cleavage site on the matrix side in the center of the picture, and an N-ethylmaleimide-modifiable cysteine on the cytosolic side. These sites were identified previously in the purified transhydrogenase (15, 27). Their sidedness, as shown in Fig. 8, is not proven, but is simply a consequence of our interpretation of the hydropathy profile. However, in order to alter this arrangement, without changing the positions of the D\(^{340}\)-L\(^{554}\) pentadecapeptide on the matrix side and the protease-sensitive loop on the cytosolic side, one would have to pull out of the membrane two of the hypothetical membrane-intercalating clusters (boxes in Fig. 8) that are located between these extramembranous loops. This is, of course, possible, except that \( a \) priori the extreme hydrophobicity of the intervening clusters is more suited to an arrangement close to that shown in Fig. 8.

The fact that the proteinase K-nicked transhydrogenase is active and can be purified presents two points of comparison with the \( E. coli \) transhydrogenase, which is composed of two subunits with \( M \), values of 54,000 and 48,700 (8). (a) It is possible that what holds the two subunits of the \( E. coli \) enzyme and the two fragments of the nicked bovine transhydrogenase together is interaction between the extramembranous N- and C-terminal hydrophilic domains. Indeed, the proximity of these domains is necessitated by the fact that hydride ion transfer between NAD(H) located on the N-terminal domain and NADP(H) bound to the C-terminal domain is direct (1–3). Thus, it is not surprising that the nicked bovine enzyme should retain its transhydrogenase activity, and, like the \( E. coli \) enzyme, be purifiable. (b) As mentioned earlier, the \( E. coli \) transhydrogenase is highly homologous to the bovine enzyme, except that it lacks a stretch of 32 residues where the bovine pentadecapeptide marked in Fig. 8 is located. As a result, the \( \alpha \) subunit of the \( E. coli \) enzyme contains a short stretch of hydrophobic residues at its C-terminal end, while its \( \beta \)-subunit starts with a long stretch of hydrophilic amino acids at its N-terminal end. By comparison, this situation is reversed in the proteinase K-nicked bovine transhydrogenase. Its 71-kDa fragment, bearing the NAD binding site, contains a long hydrophilic tail, while its 39-kDa piece, bearing the NADP binding site, carries a short hydrophilic stretch. Since proton translocation by the transhydrogenase is driven by the difference in the binding energies of substrates (NADPH + NAD) versus products (NADP + NADH) and is mediated via protein conformation change by the hydrophobic, membrane-intercalating amino acid residues of the protein, it would be of considerable mechanistic interest to see whether the proteinase K-nicked enzyme is capable of proton translocation.

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Fig. 8. Membrane topography of the bovine mitochondrial transhydrogenase. Rectangular boxes show hypothetical membrane-intercalating clusters of amino acids as interpreted from a Kyte-Doolittle hydropathy analysis of the amino acid sequence of the transhydrogenase. The abbreviated 430-residue-long N-terminal and the 200-residue-long C-terminal hydrophilic domains are shown protruding from the membrane on the matrix (\( M \)) side. Also largely exposed on the \( M \) side is the segment D\(^{340}\)-L\(^{554}\) which is outlined by a dashed line. The exposed protease-sensitive loop is shown on the cytosolic (\( C \)) side connecting the presumed membrane-intercalating clusters 9 and 10. The bonds cleaved by trypsin (\( TRP \)), papain, and proteinase K, and the cysteine residue modified by N-ethylmaleimide (\( NEM \)) are marked. For other details, see text.
Topography of Transhydrogenase 5735

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