Biosynthesis of Rat Liver Transhydrogenase *in Vivo and in Vitro*

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The biosynthesis of pyridine dinucleotide transhydrogenase, a homodimeric inner mitochondrial membrane redox-linked proton pump, has been studied in isolated rat hepatocytes. Newly synthesized transhydrogenase, having an apparent molecular weight identical to the enzyme of isolated liver mitochondria, was selectively immunoprecipitated from detergent extracts of isolated hepatocytes which were labeled with [35S]methionine. That the enzyme is a nuclear gene product is indicated since 1) synthesis was inhibited by cycloheximide, but not by chloramphenicol and 2) no synthesis could be demonstrated in hepatocyte ghosts which are competent only in mitochondrial translation. In addition to the mature form of the enzyme, a species about 2000 daltons larger was also immunoprecipitated from pulse-labeled cells. The half-life of the larger form during a subsequent chase at 37 °C was about 2 min, whereas the mature form was not degraded. The relationship between the two forms of the enzyme was established by *in vitro* studies. A protein approximately 2000 daltons larger than mature transhydrogenase was immunolabeled from a rabbit reticulocyte lysate system programmed with sucrose gradient fractionated rat liver mRNA. This protein was converted to a species having the same size as mature enzyme after incubation with either intact rat liver mitochondria or a soluble matrix fraction derived from mitoplasts.

These studies indicate that transhydrogenase is synthesized in the cytoplasm as a higher molecular weight precursor which is post-translationally processed to the mature protein by a soluble matrix protease during or after membrane insertion.

Although the mitochondrial genome is capable of coding for the synthesis of a limited number of proteins, approximately 90% of the protein of the organelle is coded by nuclear genes and translated on cytoplasmic ribosomes. The cytoplasmic gene products destined for import to the inner membrane and matrix are generally, though not always, higher molecular weight precursors that are cleaved post-translationally by a matrix-processing protease (1). Recent studies indicate that processing may occur subsequently to, rather than concomitantly with, energy-dependent membrane insertion or translocation (2, 3).

Much emphasis has been placed, particularly in lower eucaryotes, on the synthesis, processing, and assembly of the energy-transducing complexes of oxidative phosphorylation, i.e. cytochrome c oxidase, ubiquinol-cytochrome c reductase (b-c1 complex), and F0-F1-ATPase. A general feature of these hetero-oligomeric complexes is that certain of their subunits are cytoplasmically synthesized, whereas others are mitochondrial gene products (1). For example, in rat liver (4, 5) as in yeast (6, 7) and Neurospora crassa (8) the three largest subunits (I to III) of cytochrome c oxidase are synthesized in the mitochondrion and the smaller subunits (IV to VII) are of cytoplasmic origin. Two of the liver oxidase subunits, IV (9, 10) and V (11), have been reported to be synthesized as precursors being 1500 to 3000 daltons larger than the mature species. Little is known about the biosynthesis of b-c1 complex of F0-F1-ATPase in higher eukaryotes. Although it has been reported that one subunit of the b-c1 complex and two subunits of the F0 segment of F0-F1-ATPase are products of mitochondrial translation (5, 12, 13), no information is available concerning the nature or processing of the cytoplasmically synthesized subunits. Analogous studies on yeast (14, 15) and N. crassa (16) suggest that most of the cytoplasmic subunits of these complexes in higher eukaryotes are likely to be synthesized as extended precursor forms.

This communication describes our initial studies on the biosynthesis and mechanism of insertion of pyridine dinucleotide transhydrogenase into the inner membrane of rat liver mitochondria. The enzyme couples the reversible transfer of a hydride ion between matrix NADPH and NAD⁺ to vectorial proton translocation from the matrix to the cytosol (17, 18). Unlike the other mitochondrial energy-transducing complexes, bovine heart transhydrogenase of the native membrane possesses a simple subunit structure consisting of a dimer of apparently identical 110-kDa subunits (19). Here, we have employed monospecific antibody prepared against bovine heart transhydrogenase to identify and characterize the enzyme synthesized by isolated rat hepatocytes and by rabbit reticulocyte lysates programmed with partially purified liver mRNA.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

Identification of Rat Liver Transhydrogenase by Immunoblot Analysis—Mitochondrial transhydrogenase has been, to date, purified to homogeneity only from bovine heart (17, 20).

1 Portions of this paper (including "Experimental Procedures" and Figs. 1, 3, and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2457, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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Monospecific antibody has been prepared against purified transhydrogenase (19, 22). To investigate the biosynthesis of transhydrogenase in rat liver, it was initially necessary to establish that heart anti-transhydrogenase specifically recognizes the corresponding enzyme from rat hepatocytes. To accomplish this, immunoblot analysis of heart and rat liver submitochondrial particle proteins resolved by SDS\(^2\)-polyacrylamide gel electrophoresis was employed. Fig. 1 illustrates that heart submitochondrial particle transhydrogenase (lane 2) migrates identically with purified heart enzyme (lanes 1 and 5). Rat liver submitochondrial particles also gave a single immunoreactive species (lane 4) that migrated slightly more slowly into the gels than heart transhydrogenase. When membranes from both sources were electrophoresed together (lane 3), a broadened band appeared, indicative of a composite of two proteins having slightly different molecular weights. Under the electrophoresis conditions used, resolution of proteins in the 100-kDa range differing by as little as 500 daltons would not be observed. In other studies (not shown) the antibody was found to effectively inhibit the reduction of the 3-acetylpyridine analog of NAD\(^+\) by NADPH (24) catalyzed by rat liver submitochondrial particles, providing further evidence that the antibody specifically complexes with liver transhydrogenase.

**Site of Transhydrogenase Synthesis in Rat Hepatocytes**—The low rate of protein synthesis in isolated higher eukaryotic cells has hampered investigations on the cytoplasmic biosynthesis, import, and processing of low abundance inner mitochondrial membrane proteins in mammals, although a few have been reported (9–11, 31). The emphasis on that aspect of higher eukaryote mitochondrial biogenesis directed by the nuclear genome has been focused on the uptake of relatively high abundance soluble matrix and intramembrane space enzymes (31–39). Transhydrogenase represents less than 1% of the protein of the inner mitochondrial membrane (20) and, therefore, may be classified as a low abundance protein of the organelle. Although sequence analysis of mammalian mitochondrial DNA provides no evidence for reading frames that could code for the synthesis of a protein having a molecular weight in excess of about 67 kDa (40), Bhat et al. (41) have reported that isolated rat liver mitochondria are capable of synthesizing 19–24 polypeptide species ranging in size to approximately 110 kDa. It is noteworthy that the highest molecular weight putative mitochondrial translation product reported corresponds to that of transhydrogenase. To determine the site of transhydrogenase synthesis, isolated hepatocytes were continuously labeled for 2 h with \(^{35}\)S methionine under conditions where either cytoplasmic or mitochondrial protein synthesis was blocked. During the incubation at 37 °C total protein synthesis was inhibited by 80–90% in the presence of 0.7 mM cycloheximide and by 5–15% in the presence of 0.1 mM chloramphenicol. The enzyme was immunoprecipitated from detergent extracts of labeled cells by addition of heart anti-transhydrogenase followed by adsorption to Protein A-bearing *Staphylococcus aureus* cells. Newly synthesized transhydrogenase was visualized by fluorography following SDS-polyacrylamide gel electrophoresis of the immune complexes. As shown in Fig. 2, a single \(^{35}\)S-labeled protein was immunoprecipitated from hepatocytes incubated in the absence of protein synthesis inhibitors (lane 2) that co-migrated with purified heart \(^{35}\)Iotranshydrogenase marker added to and immunoprecipitated from an unlabeled hepatocyte extract (lane 1). Hepatocytes labeled in the presence of cycloheximide did not express transhydrogenase (lane 3). However, chloramphenicol did not suppress synthesis of the enzyme (lane 4). These results strongly suggest that transhydrogenase is a nuclear gene product. To eliminate any ambiguity relating to the intracellular site of transhydrogenase synthesis, it was of interest to demonstrate that the enzyme could not be immunoprecipitated from hepatocyte ghosts that are capable only of mitochondrial protein synthesis (26). To this end, hepatocyte ghosts were prepared by digitonin treatment of intact cells and labeled with \(^{35}\)S methionine according to the procedure of Kuzela et al. (26). As shown in Fig. 3 protein synthesis by the ghosts (lane 2) was inhibited totally by chloramphenicol (lane 3) but not inhibited by cycloheximide (lane 4). These data demonstrate that the ghost preparation is completely incapable of cytoplasmic protein synthesis. That transhydrogenase was not immunoprecipitated from labeled ghosts (lane 5) confirms that the enzyme is of cytoplasmic origin.

**The Presence of Transhydrogenase Precursor in Hepatocytes**—Although relatively long pulses of hepatocytes with \(^{35}\)S methionine resulted in the immunoprecipitation of a single labeled protein having an apparent molecular weight corresponding to the mature form (Fig. 2), shorter pulses revealed an additional radioactive band of higher apparent molecular weight (\(\Delta M_i = 2000\)). This is illustrated in Fig. 4, where the two bands are clearly visible after a 10-min pulse (lane 1). As expected for a precursor, the larger species disappeared during a subsequent chase in the presence of unlabeled methionine and cycloheximide, giving an apparent half-life of about 2 min (lanes 2–6). By contrast, the amount of

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\(^2\) The abbreviation used is: SDS, sodium dodecyl sulfate.
precipitate was visualized obtained after precursor in hepatocytes. Methionine and cycloheximide methionine for 10 min and then chased in the presence of cold methionine and cycloheximide as described under “Experimental Procedures.” At various times during the chase, aliquots were taken for detergent extraction and immunoprecipitation. Transhydrogenase was visualized as described in Fig. 3. Lane 1 contained the immunoprecipitate after the pulse. Lanes 2-6 contain immunoprecipitates obtained after 2, 4, 6, 8, and 10 min of chase, respectively.

mature form of the enzyme remained stable during the chase after a perceptible increase during the first 2 min. This apparent half-life is similar to those reported for the processes of mammalian matrix precursor proteins (cf. Ref. 42). The addition of purified heart transhydrogenase to the labeled cell extract totally prevented the immunoprecipitation of both forms of the enzyme.

Synthesis of Transhydrogenase Precursor by Cell-free Translation.—To verify that transhydrogenase is synthesized in the cytoplasm as a higher molecular weight precursor, rat liver mRNA was translated in a rabbit reticulocyte lysate system in the presence of [35S]methionine. Initial attempts to selectively immunoprecipitate the enzyme from lysates programmed with total mRNA were unsuccessful due to contamination with many protein species of both higher and lower molecular weights. Accordingly, it was found to be necessary to further fractionate the poly(A) RNA on a sucrose gradient to both enrich the transhydrogenase message and to obtain contaminant-free immunoprecipitates. The translation products obtained from each mRNA fraction. A trend of increasing molecular weights of the products encoded from fraction a to fraction f is apparent. The translation products obtained in Fig. 5B were subjected to immunoprecipitation to locate transhydrogenase mRNA (Fig. 5C). No immunoprecipitable labeled protein was detected in fractions a through c (only fraction c is shown, lane 2). Fraction d (lane 3) gave a highly contaminated precipitate, which may arise from the non-specific binding of many products to the Protein A adsorbent, with no indication for the presence of a species with a mobility similar to transhydrogenase or its putative precursor. Fraction e (lane 5) gave a single faint band, which was more intense in fraction f (lane 6). The immunoprecipitated protein of fractions e and f had a slightly lower mobility in SDS-polyacrylamide gels than heart [35S]iodotranshydrogenase marker (lanes 1, 4, and 7).

To better define the difference in apparent molecular weight between mature hepatocyte transhydrogenase and its putative in vitro synthesized precursor, both immunoprecipitated [35S]-labeled products were electrophoresed under conditions developed to optimize separation of proteins in the 100-kDa range. As shown in Fig. 6, bovine heart [125I]iodotranshydrogenase (lanes 1 and 3) and mature hepatocyte transhydrogenase (lane 2) migrated identically, whereas the in vitro synthesized product migrated slower (lane 4). The specificity of the transhydrogenase antibody is indicated by the complete prevention of in vitro product precipitation in the presence of added purified bovine heart transhydrogenase (lane 5). The difference in apparent molecular weights between the in vivo and in vitro products, as indicated by molecular weight markers including β-galactosidase (116,000) and phosphorylase b (97,400), was calculated to be approximately 2000.

Processing of Transhydrogenase Precursor by Intact Rat Liver Mitochondria and a Matrix Subfraction.—In order to establish the precursor relationship of the in vitro synthesized product to mature transhydrogenase the cell-free translation products were exposed to intact rat liver mitochondria. After incubation, the mitochondria were separated from the suspending medium by centrifugation, and transhydrogenase was then immunoprecipitated from both fractions. The band present in the supernatant fraction migrates identically with that of the translation mixture, whereas all of the radiolabeled product found in the mitochondrial fraction migrates faster and corresponds to the mature form of the enzyme. In other studies (data not shown), transhydrogenase taken up by mitochondria, but not the protein of the supernatant fraction, has been shown to be resistant to degradation by trypsin. This indicates, but does not prove, that the protein has been inserted into the inner membrane. Precursor processing protease activity has been typically found to reside in the matrix compartment of both higher and lower
Products were incubated with intact mitochondria and matrix fraction. In Panel A, in vitro translated products were incubated with intact rat liver mitochondria. The mitochondrial fraction (M) was separated from the supernatant fraction (S) by centrifugation, and then both fractions were immunoprecipitated, electrophoresed on 5-15% gradient gels as in Fig. 6, and fluorographed. pTH represents the position where transhydrogenase immunoprecipitated directly from the translation mixture migrated. In Panel B, in vitro translated products were incubated in the presence (lane MP) or absence (lane pTH) of a rat liver mitochondrial matrix fraction. Transhydrogenase was then immunoprecipitated, electrophoresed, and fluorographed. Details are described under “Experimental Procedures.”

eukaryotes (1). Fig. 7B illustrates that this is true also for transhydrogenase. After incubation of translation products with a matrix fraction derived from detergent lysis of mitochondria, the larger transhydrogenase form, under conditions employed, is partially converted into a smaller form that corresponds in apparent mass to mature enzyme.

These studies strongly indicate that the in vitro synthesized transhydrogenase represents a precursor form of the enzyme, identical to that found in short-term pulsed intact cells. However, it remains unknown if the in vitro processing of the precursor occurs at the N terminus of the protein, if processing faithfully produces mature enzyme, if processing results in the proper insertion of the protein in the inner membrane, and if processing is required to generate substrate-binding sites characteristic of an active enzyme. These aspects are currently being investigated.

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Supplemental Material

Biosynthesis of Rat Liver Transhydrogenase in Vivo and In Vitro

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Environmental Procedures

Materials. Beige heart mitochondrial transhydrogenase was purified to homogeneity as previously described (20). Radiolabeled marker enzymes for fluorography were prepared by reacting 104 cpm of purified transhydrogenase in 0.4 ml of a reaction buffer containing 50 mM sodium phosphate and 6.0% sodium chloride, pH 7.4, with 30 nCi of [35S] iodoacetamide in the presence of four bovine insulin for 10 min at 4°C. [35S] iodoacetamide was separated from free insulin by column chromatography (see methods) 0.5 M sucrose, 4 mM Hepes (pH 7.4). Beige heart and rat liver mitochondria were prepared and assayed as described (20, 21). Rat liver histones and matrix fractions were derived from mitochondria prepared by the procedure of Schumacker and Greenstein (21). Mitochondria used for in vitro processing were suspended in a medium containing 100 mM potassium, 140 mM sucrose, and 4 mM Hepes (pH 7.4). Beige heart and rat liver mitochondria were prepared by the procedure of Schumacker and Greenstein (21) and diluted to a concentration of 0.2 g/ml with a solution containing 6.0% sodium chloride, 50 mM Hepes (pH 7.4), 10 mM KCl, 1 mM MgCl2, 1 mM EDTA, 0.1 M Tris and 10 mM dithiothreitol. Histones were prepared as described (40). Polypeptides were separated by electrophoresis on an 11% polyacrylamide gel from which the gels were stained according to Laemmli (21). Total rat liver RNA was prepared by the method of Chirgwin et al. (22). Poly(A)RNA was prepared by oligo(dT) cellulose chromatography (28). Fractionation of poly(A)RNA was performed by layering 100-500 μg RNA in a discontinuous sucrose gradient comprising 1.0 M sucrose, 0.9, 1.3, 1.7, and 2.0 M sucrose containing 0.1 M EDTA, and 0.8 M glycerol, and 0.4 M EDTA, and 0.1 M MgCl2, and 0.1 M NaCl in 10 mM Tris, 10 mM EDTA, and 0.1 M sucrose. After centrifugation in a Beckman SW 41 rotor at 27,000 rpm for 26 h, 0.8 M fractions were collected from the top of the gradient and the 20% RNA was fractionated. Polypeptides were prepared as described in Fig. 5A and diluted with an equal volume of sterile water. After addition of 2% sodium acetate to a final concentration of 0.2 M, the RNA was precipitated overnight with two volumes of absolute ethanol at 0°C. The RNA was recovered by centrifugation at 10,000 rpm for 1 h in a Beckman JA 20 rotor. The pellets were dissolved in 10 ml of sterile 0.2 M NaCl and the RNA was again precipitated with two volumes of ethanol. After centrifugation, the RNA pellets were washed three times with 0.5 M sodium acetate, pH 6. The pellets were dissolved in 4 ml of water and the RNA was precipitated with two volumes of ethanol in the presence of 18% polyethylene glycol, pH 8. Finally, 20 μg RNA was added to centrifugation, dried in vacuo, dissolved in 10-20 μl of water and stored at -70°C.

For some other materials were: sodium [35S] iodoacetate, 35S methionine (1000 Ci/mmol) and rabbit reticulocyte lysate system programmed with rat liver mRNA. The translation mixture (20-100 μl containing 2.5 mM [35S]methionine was incubated at 37°C for 1 h. Incorporation of [35S]methionine into protein was determined by spotting 1 μl of the translation mixture onto 3MM Whatman filter discs, that were washed for 10 min in 10% trichloric acid, washed twice with water, ether, and acetone, dried and counted by liquid scintillation spectrometry. For immunoprecipitation, the translation mixtures were made 2% SDS, heated for 2 min in a boiling water bath, and diluted to 1 ml giving a final concentration containing 150 mM NaCl, 150 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.2% SDS, 0.1% Triton X-100, 10 μg each of leupeptin, antipain, pepstatin, pepstatin, 0.5 mM PMSF and 60 μl of anti-transhydrogenase antibodies. Samples were spotted onto nitrocellulose paper and incubated overnight at 4°C. Immunoprecipitates were washed and disassembled as described above, except that immunoprecipitates were collected after centrifugation at 5000 rpm for 1 min in a Beckman JA 40 rotor.

In vivo transduction. Protein synthesis was performed in a nucleoside-treated rabbit reticulocyte lysate system programmed with rat liver mRNA. The translation mixture (20-100 μl containing 2.5 mM [35S]methionine was incubated at 37°C for 1 h. Incorporation of [35S]methionine into protein was determined by spotting 1 μl of the translation mixture onto 3MM Whatman filter discs, that were washed for 10 min in 10% trichloric acid, washed twice with water, ether, and acetone, dried and counted by liquid scintillation spectrometry.
Eppendorf Microfuge Model 5414. The supernatant fraction was carefully removed and the pellet fraction was washed once with 100 μL of isolation medium (23M containing 25 μg chymostatin. The pellet fraction was solubilized in 100 μL of a solution containing 1.5% SDS, 1% Triton X-100, and 25 μg chymostatin. To the supernatant fraction, or that containing the matrix fraction, was added 15 μL 10% SDS, 10 μL Triton X-100, and 5 μL of 1 mg/mL chymostatin. Fractions were then placed in a boiling water bath for 3 min, clarified by centrifugation, diluted to 1 mL, and immunoprecipitated as described under "in vitro translations".

Fig. 1. Cross-reaction of bovine heart anti-transhydrogenase with the rat liver enzyme. Purified bovine heart transhydrogenase and subcellular particles from bovine heart and rat liver were resolved by 5-15% gradient SDS-polyacrylamide gel electrophoresis and the enzyme visualized by immunoblot analysis as described under "Experimental Procedures". Lanes 1 and 5 contained 0.85 μg purified transhydrogenase; lane 2, 7.5 μg heart subcellular particles; lane 3, 7.5 μg heart subcellular particles plus 15 μg liver subcellular particles; lane 4, 15 μg liver subcellular particles.

Fig. 3. Transhydrogenase is not synthesized by hepatocyte ghosts. Hepatocyte ghosts were labeled with [35S] methionine in the presence or absence of 1 mM chloramphenicol or chloramphenicol and detergent extracts prepared as described under "Experimental Procedures". The extracts were analyzed for total translation products and for those immunoprecipitated by anti-transhydrogenase. Proteins were resolved by 5-15% gradient SDS-polyacrylamide gel electrophoresis and visualized by fluorography. Lane 1 contains marker [125I] sodotranshydrogenase; lane 2, total translation products from ghosts labeled in the absence of inhibitors (20,000 dpm); lane 3, total translation products from ghosts labeled in the presence of chloramphenicol; lane 4, total translation products from ghosts labeled in the presence of cycloheximide; lane 5, immunoprecipitate from hepatocyte ghosts labeled in the presence of chloramphenicol; lane 6, represents a control experiment in which transhydrogenase was immunoprecipitated from labeled intact hepatocytes.

Fig. 5. Partial purification of rat liver transhydrogenase mRNA. Rat liver poly(A) mRNA was resolved by discontinuous sucrose gradient centrifugation. Various mRNA fractions were translated in a rabbit reticulocyte lysate system and the synthesis of transhydrogenase was analyzed by immunoprecipitation with anti-transhydrogenase followed by SDS-polyacrylamide gel electrophoresis (Panel B, 5% acrylamide, Panel C, 10% acrylamide) and fluorography as described under "Experimental Procedures". Panel A shows the A254 of fractions obtained from the sucrose gradient. The mRNA contained in pooled fractions, designated a through f, was recovered after repeated ethanol precipitation. Panel B, a fluorogram illustrating total synthesis products obtained when 4 μL of mRNA isolated from each pooled fraction was translated in a reticulocyte lysate system, shows 7 bands. Lane 1, represents translation products from non-labeled mRNA. Lane 2, represents translation products from mRNA isolated from fractions a through f. Lanes 3-7 contained [125I] sodotranshydrogenase. Lanes 2, 3, 5, and 6, immunoprecipitates of translation products from mRNA obtained from sucrose gradient fractions a, c, e, and f, respectively.