Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1977) *J. Chem. Phys. 71*, 4546-4553.
Kallis, G. B., & Holmgren, A. (1980) *J. Biol. Chem. 255*, 10261-10265.
Katti, S., LeMaster, D. M., & Eklund, H. (1990) *J. Mol. Biol.* (in press).
Kumar, A., Wagner, G., Ernst, R. R., & Wüthrich, K. (1981) *J. Am. Chem. Soc. 103*, 3654-3658.
Lee, M. S., Gippert, G., Soman, K. Y., Case, D. A., & Wright, P. E. (1989) *Science 245*, 635-637.
LeMaster, D. M., & Richards, F. M. (1988) *Biochemistry 27*, 142-150.
Marion, D. I., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967-974.
Mark, D. F., & Richardson, C. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 780-784.
Neuhaus, D., Wagner, G., Vasák, M., Kägi, J. H. R., & Wüthrich, K. (1985) *Eur. J. Biochem. 151*, 257-273.
Pace, C. N., Grimsley, G. R., Thomson, J. A., & Barnett, B. J. (1988) *J. Biol. Chem. 263*, 11820-11825.
Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479-485.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167-339.
Stryer, L., Holmgren, A., & Reichard, P. (1967) *Biochemistry 6*, 1016-1020.
Wagner, G., Braun, W., Havel, T. F., Schaumann, T., Go, N., & Wüthrich, K. (1987) *J. Mol. Biol. 196*, 611-641.
Weiner, S. J., Kollman, P. A., Nguyen, D. T., & Case, D. A. (1986) *J. Computat. Chem. 7*, 230-252.
Williamson, M. P., Havel, T. F., & Wüthrich, K. (1985) *J. Mol. Biol. 182*, 295-315.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Wüthrich, K., Billeter, M., & Braun, W. (1983) *J. Mol. Biol. 169*, 949-961.

Mitochondrial Energy-Linked Nicotinamide Nucleotide Transhydrogenase: Effect of Substrates on the Sensitivity of the Enzyme to Trypsin and Identification of Tryptic Cleavage Sites†

Mutsuo Yamaguchi, Sadao Wakabayashi,‡ and Youssef Hatefi*

Division of Biochemistry, Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, California 92037

Received November 27, 1989; Revised Manuscript Received January 8, 1990

ABSTRACT: The mitochondrial nicotinamide nucleotide transhydrogenase catalyzes hydride ion transfer between NAD(H) and NADP(H) in a reaction that is coupled to proton translocation across the inner mitochondrial membrane. The enzyme (1043 residues) is composed of an N-terminal hydrophilic segment (~400 residues long) which binds NAD(H), a C-terminal hydrophilic segment (~200 residues long) which binds NADP(H), and a central hydrophobic segment (~400 residues long) which appears to form about 14 membrane-intercalating clusters of ~20 residues each. Substrate modulation of transhydrogenase conformation appears to be intimately associated with its mechanism of proton translocation. Using trypsin as a probe of enzyme conformation change, we have shown that NADPH (and to a much lesser extent NADP) binding alters transhydrogenase conformation, resulting in increased susceptibility of several bonds to tryptic hydrolysis. NADH and NAD had little or no effect, and the NADPH concentration for half-maximal enhancement of trypsin sensitivity of transhydrogenase activity (35 μM) was close to the K_m of the enzyme for NADPH. The NADPH-promoted trypsin cleavage sites were located 200-400 residues distant from the NADP(H) binding domain near the C-terminus. For example, NADPH binding greatly increased the trypsin sensitivity of the K_{410}-T_{411} bond, which is separated from the NADP(H) binding domain by the 400-residue-long membrane-intercalating segment. It also enhanced the tryptic cleavage of the R_{602}-L_{603} bond, which is located within the central hydrophobic segment. These results, which suggest a protein conformation change as a result of NADPH binding, have been discussed in relation to the mechanism of proton translocation by the transhydrogenase.

The mitochondrial nicotinamide nucleotide transhydrogenase catalyzes the 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 proton translocation across the inner membrane with a H^+/H^- stoichiometry close to unity (see eq 1; H_c^+ and H_m^+ are protons on the cytosolic and the matrix side of the inner membrane, respectively) (Fisher & Earle, 1982).

$$NADH + NADP + H_c^+ \rightleftarrows NAD + NADPH + H_m^+ \quad (1)$$

The bovine enzyme has been sequenced from cDNA clones (Yamaguchi et al., 1988a), and the sequence of its signal peptide (43 residues) has been determined from the sequence of the mRNA (Yamaguchi et al., 1988b). The mature transhydrogenase has 1043 amino acids and a molecular

† Supported by U.S. Public Health Service Grant GM 24887.
* To whom correspondence should be addressed.
‡ Present address: Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan.

0006-2960/90/0429-4136$02.50/0

weight of 109 228 (Yamaguchi et al., 1988a) and appears to exist as a homodimer in the mitochondrial inner membrane (Anderson & Fisher, 1981). A segment of the protein at the N-terminus corresponding to about 45 kDa is hydrophilic and contains the NAD(H) binding domain. Also a portion of the molecule at the C-terminus corresponding to about 25 kDa is hydrophilic and carries the NADP(H) binding domain. These segments protrude from the inner mitochondrial membrane into the mitochondrial matrix where they form the enzyme catalytic site. The central part of the enzyme (~40 kDa) appears to be composed of about 14 hydrophobic clusters of about 20 amino acid residues each. This segment is considered, therefore, to traverse the inner mitochondrial membrane many times.

The transhydrogenase is readily inhibited by a large number of protein-modifying reagents, including trypsin. The exceptional trypsin sensitivity of the enzyme was first demonstrated by Ernster and co-workers in preparations of submitochondrial particles [Juntti et al., 1970, see also Djavadi-Ohaniance and Hatefi (1975)]. Subsequently, Blazyk et al. (1976) demonstrated that the trypsin sensitivity of the transhydrogenase activity of rat liver submitochondrial particles was increased 3–4-fold in the presence of NADPH [see also Anderson and Fisher (1978)]. The stimulatory effect of NADPH is not confined to trypsin sensitivity of the enzyme. It has also been reported in connection with inhibition of the transhydrogenase by thiol modifiers (Earle et al., 1978; Modrak et al., 1988; Yamaguchi & Hatefi, 1989), dicyclohexylcarbodiimide (Phelps & Hatefi, 1984), and ethoxyformic anhydride and dansyl chloride (Yamaguchi & Hatefi, 1985). Our recent studies with *N*-ethylmaleimide as the inhibitor have shown that NADPH accelerates and NADP retards the inhibition rate, and that the pK_a of the target residue (Cys-893) changes from 9.1 in the absence of added substrates to 8.7 in the presence of NADPH and to 9.5 in the presence of NADP (Yamaguchi & Hatefi, 1989).

This paper shows the effects of substrates on inactivation of purified bovine heart transhydrogenase by trypsin, identifies the trypsin cleavage sites, and demonstrates that NADPH binding results in a global enzyme conformation change. The implications of the results with regard to the mechanism of action of the transhydrogenase are discussed.

Materials and Methods

Materials. NAD, NADH, NADP, and NADPH were obtained from Calbiochem. Soybean trypsin inhibitor, L-α-lysophosphatidylcholine, Brij 58, and 3-acetylpyridine adenine dinucleotide were obtained from Sigma. *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington. Ponceau S was obtained from Aldrich and poly(vinylidene difluoride) (PVDF)[1] membrane from Millipore. NAD–agarose (type I) was prepared by the method of Mosbach et al. (1972). Bovine mitochondrial transhydrogenase was prepared as described by Phelps and Hatefi (1984). The specific activity of the enzyme used was 35–40 μmol of AcPyAD reduced by NADPH min^{-1} (mg of protein)$^{-1}$ at 37 °C.

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 μg of L-α-lysophosphatidylcholine, and 0.33 mM 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 by using a value of 6.38 mM^{-1} cm^{-1} for the absorbance difference of AcPyADH and NADPH at the above wavelength pair (Phelps & Hatefi, 1981). Protein concentration was determined by the method of Peterson (1977), using bovine serum albumin as the standard.

Trypsin Digestion of Transhydrogenase. Transhydrogenase (1.6–2.9 mg/mL) was digested with TPCK-treated trypsin (trypsin:transhydrogenase ratio = 1:400) at 23 °C in 50 mM Tris–acetate, pH 7.5, containing 0.001% potassium cholate with or without substrate. At the indicated time intervals, aliquots of the reaction mixture were removed and mixed with a 3-fold excess of soybean trypsin inhibitor to stop further digestion. Samples of this solution were then used for assay of the remaining enzyme activity and analysis of the digestion pattern of the polypeptide by SDS–polyacrylamide gel electrophoresis.

Gel Electrophoresis and Electrotransfer. Protein samples were incubated for 1 h at room temperature in 63 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue and subjected to electrophoresis on 12.5% SDS–polyacrylamiode slab gels (Laemmli, 1970). Gels were stained with Coomassie blue and destained, and the bands were quantitated by scanning and measuring the area under each peak.

Electrotransfer of peptides from polyacrylamide gel to the PVDF membrane for N-terminal sequencing was conducted by a modification of the procedure of Matsudaira (1987). Purified enzyme (0.8 mg in 0.4 mL) was treated with trypsin for 120 min in the absence or presence of 0.4 mM NADPH, and the reaction was stopped by the addition of trypsin inhibitor. Samples containing 60 μg of protein were subjected to slab gel electrophoresis in multiple lanes and transferred to PVDF membranes for 2 h at 30 V in 20 mM Tris–acetate, pH 8.3, containing 1 mM EDTA and 0.2 mM dithiothreitol. PVDF membranes were stained with a mixture of Ponceau S (0.09%) and Coomassie blue (0.01%) and destained, and protein bands were excised for amino acid sequencing.

N-Terminal Sequence Analysis of Peptides. Sequence analysis of peptides was performed with an Applied Biosystems (Foster City, CA) Model 470 sequenator equipped with on-line phenylthiohydantoin analysis using the program 03RPTH and reverse-phase HPLC on a Brownlee C_{18} column. Five or six N-terminal residues of each peptide were sequenced to identify the tryptic cleavage sites. The amount of each peptide sequenced was 10–40 pmol.

Gel Filtration of the Trypsin Digest of Transhydrogenase on Sephadex G-200. Transhydrogenase (0.92 mg in 0.5 mL) was treated with trypsin (trypsin:transhydrogenase ratio = 1:400) at 23 °C for 60 min in the presence of 50 mM Tris–acetate, pH 7.5, containing 0.4 mM NADPH and 0.001% potassium cholate. The tryptic digest was loaded on a column (0.94 × 46 cm) of Sephadex G-200 equilibrated and eluted with the same buffer, and 0.4-mL fractions were collected. To each fraction was added 0.6 mL of water, and its absorbance at 230 nm and enzyme activity (using 50-μL aliquots) were measured.

Affinity Chromatography of the 43-kDa Fragment on NAD–Agarose. The N-terminal 43-kDa fragment was isolated by Sephadex G-200 gel filtration as described above, and 210 μg of this fragment in 0.5 mL was loaded on a NAD–agarose column (1.1 × 8 cm) equilibrated with 10 mM sodium

[1] Abbreviations: SDS, sodium dodecyl sulfate; PVDF, poly(vinylidene difluoride); DCCD, *N,N'*-dicyclohexylcarbodiimide; FSBA, [(*p*-(fluorosulfonyl)benzoyl]-5'-adenosine; NEM, *N*-ethylmaleimide; CD, circular dichroism; AcPyAD, 3-acetylpyridine adenine dinucleotide.

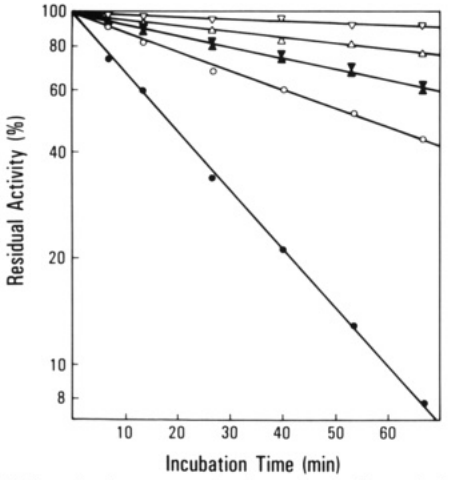

FIGURE 1: Effect of substrates on the time course of trypsin inactivation of the transhydrogenase. The enzyme (2.0 mg/mL) was treated with trypsin (trypsin: transhydrogenase ratio = 1:400) at 23 °C in a buffer containing 50 mM Tris–acetate, pH 7.5, and 0.001% potassium cholate in the absence (▲) or presence of 0.4 mM each of NADPH (●), NADP (○), NADH (△), and NAD (▼). The symbol (▽) shows the control in the absence of trypsin. At the intervals shown, aliquots were withdrawn from each incubation mixture and assayed for activity.

phosphate, pH 7.0, containing 0.01% Brij 58 and 1.0 mM dithiothreitol (column buffer). The column was washed with 20 mL of column buffer and eluted sequentially with 10 mL of the same buffer containing 20 mM NaCl, 6 mL of column buffer, 5 mL of column buffer containing 0.5 mM NADH, and 10 mL of column buffer. Fractions of 1.4 mL each were collected and their protein concentrations determined.

Results

Effect of Substrates on Tryptic Inactivation and Fragmentation of the Transhydrogenase. Shown in Figure 1 are data on the inactivation time course by trypsin of purified bovine heart nicotinamide nucleotide transhydrogenase in the absence and presence of substrates. It is seen that enzyme inactivation by trypsin followed pseudo-first-order kinetics and was very slow at pH 7.5 and a trypsin:transhydrogenase ratio of 1:400 (50% inactivation after 95 min at 23 °C). Addition of NAD had no effect on the inactivation rate, NADH retarded the rate by 50%, NADP nearly doubled it, and NADPH increased it by 6-fold. The NADPH effect was concentration-dependent, with half-maximal effect occurring at 35 μM NADPH (data not shown). This value is close to the K_m of the enzyme for NADPH (20 μM) at pH 7.5 and suggests that the NADPH-induced sensitivity to trypsin results from binding of the nucleotide at the enzyme active site.

The effect of substrates on fragmentation of the transhydrogenase by trypsin is visualized on the SDS–polyacrylamide gel slab shown in Figure 2. Lane 1 contains molecular weight standards, and lanes 2 and 3 show the purified transhydrogenase in the absence and presence of trypsin + soybean trypsin inhibitor, respectively. The faint bands seen in lanes 2 and 3 are degradation products of the transhydrogenase itself, which are produced during detergent exchange on hydroxyapatite columns, concentration on Centricon-30, and overnight storage of the purified enzyme. Lane 4 demonstrates the pattern of degradation by trypsin after 67-min digestion under the same conditions as in Figure 1, and lanes 5, 6, 7, and 8 show, respectively, the effects of 0.4 mM each of NADPH, NADP, NADH, and NAD when added to the digestion mixture. It is clear that the results are in full agreement with those of Figure 1; i.e., NADPH greatly enhanced the digestion of the enzyme by trypsin and NADP was also

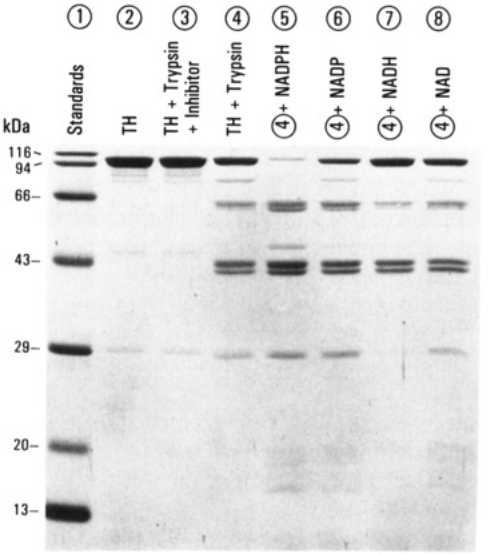

FIGURE 2: SDS–polyacrylamide gel electrophoresis of transhydrogenase treated with trypsin in the absence or the presence of substrates. Conditions were the same as in Figure 1. After 67 min of incubation, the digestions were stopped by addition of excess soybean trypsin inhibitor to each tube. Then the peptides were denatured and subjected to SDS–polyacrylamide gel electrophoresis. Lane 1, molecular weight standards; lane 2, untreated transhydrogenase; lane 3, transhydrogenase incubated with trypsin plus excess trypsin inhibitor; lanes 4–8, transhydrogenase treated with trypsin in the absence or presence of 0.4 mM each, respectively, of NADPH, NADP, NADH, and NAD. The amount of protein loaded on each gel was 4.3 μg.

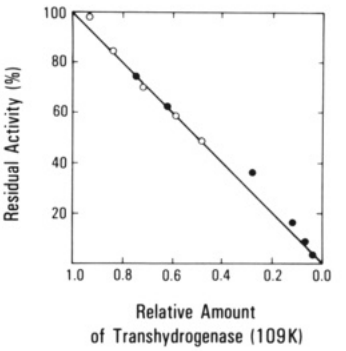

FIGURE 3: Relationship between the remaining enzyme activity of transhydrogenase treated with trypsin for various lengths of time and the amount of undigested transhydrogenase protein. Conditions were the same as in Figure 6. Open and closed circles indicate, respectively, the absence and the presence of 0.4 mM NADPH in the digestion mixture. The amount of undigested transhydrogenase after each period of incubation was estimated as described in Figure 6.

somewhat stimulatory, while NADH seems to have had a slight negative effect and NAD little or no effect. In addition to differences in the intensity of the transhydrogenase band itself, Figure 2 also shows differences in the pattern of polypeptides produced, which will be discussed below. Figure 3 shows that the loss of transhydrogenase activity in the absence or the presence of NADPH was well correlated with the diminished intensity of the transhydrogenase band on SDS gels, as determined from densitometric traces of stained gels scanned at 560 nm. These results indicate that the smaller molecular weight bands seen in Figure 2 are devoid of transhydrogenase activity and are not in agreement with the data of Weis et al. (1987) which seemed to suggest that the loss of the transhydrogenase band due to proteolysis precedes the loss of transhydrogenase activity.

Identification of Tryptic Cleavage Sites. As seen in Figure 2, the major polypeptides produced from the transhydrogenase as a result of tryptic digestion have approximate relative

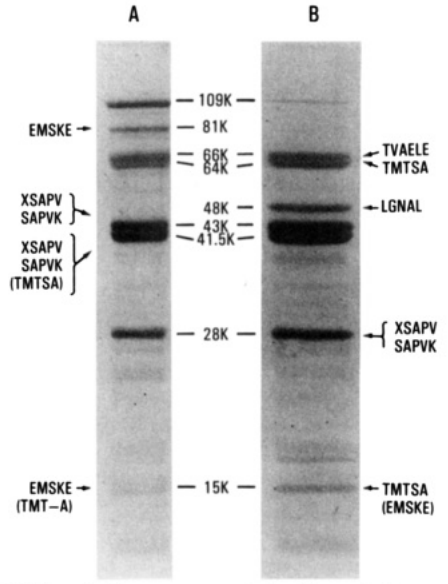

FIGURE 4: N-Terminal sequence analysis of transhydrogenase tryptic peptides electrotransferred onto PVDF membranes. Digestions were carried out as in Figure 1 in the absence (A) and the presence (B) of 0.4 mM NADPH. SDS–polyacrylamide gel electrophoresis of the tryptic digests and electrotransfer to PVDF membranes were carried out in triplicate as described under Materials and Methods. The respective peptide bands were excised from the membrane and combined for loading on a gas-phase sequenator and determination of five to six N-terminal residues. The N-terminus of the transhydrogenase preparations is frayed, as described in the text. Therefore, tryptic peptide bands containing the transhydrogenase N-terminus were composed of two polypeptides, one containing and the other lacking Cys at the N-terminus (see text). Peptides with their N-terminal sequences shown in parentheses were present as minor contaminants.

molecular weights (M_r) of 81K, 66K, 64K, 48K, 43K, 41.5K, 28K, and 15K (where K denotes $\times 10^3$). These polypeptides were electrotransferred from SDS gels similar to lanes 4 and 5 of Figure 2 onto PVDF membranes (lanes A and B of Figure 4, respectively), then excised, and subjected to N-terminal sequence analysis for five to six cycles. The sequence data are also shown in Figure 4 for each polypeptide. However, before these results are described, it is important to recall one point about the N-terminus of the transhydrogenase. Sequencing of the transhydrogenase messenger RNA indicated that the N-terminus of the mature enzyme is Cys (Yamaguchi et al., 1988b). When the transhydrogenase was carboxymethylated and then subjected to manual Edman degradation, both (carboxymethyl)cysteine and serine were identified as the N-terminal residues (Yamaguchi et al., 1988b). Other attempts at identification of the N-terminal residue of the unmodified enzyme suggested that about 40% of the chains were one residue shorter than the rest (Yamaguchi et al., 1988a). Thus, preparations of the transhydrogenase appeared to be composed of two polypeptides, one starting at the N-terminus with CSAPVK..., and the other with SAPVK..., and unless the enzyme was carboxymethylated, the N-terminal Cys was destroyed during Edman degradation, resulting in the first cycle in an unidentifiable residue (X) from the longer chain and in Ser from the shorter chain. This problem is reflected in the analyses of the polypeptides of Figure 4, where the bands representing the N-terminal segment of the transhydrogenase contained two polypeptides, one with the N-terminal sequence of SAPVK and the other with XSAPV.

The data of Figure 4 are summarized in Figure 5A where vertical arrows marked TRP show the trypsin cleavage sites. Other arrows show the residues modified by FSBA and DCCD in the NAD(H) binding domain near the N-terminus, by NEM at positions 626 and 893, and by FSBA in the NADP(H) binding domain near the C-terminus. Going down the chain in Figure 5A, it is seen that the first trypsin cleavage site is in the NAD(H) binding domain between the sites of modification by FSBA and DCCD (Wakabayashi & Hatefi, 1987a,b). This cleavage is consistent with the presence of the 28K and the 81K polypeptides in lane A of Figure 4. As seen in lane 7 of Figure 2, very little 28K and 81K polypeptides were formed in the presence of NADH. The presence of NAD in the digestion mixture appears to have slightly reduced the levels of these polypeptides also (Figure 2, lane 8). These results agree with our assignment of the NAD(H) binding site of the enzyme (Wakabayashi & Hatefi, 1987a) and suggest that NAD(H) binding interferes with tryptic cleavage of the K_{247}–E_{248} bond. Compared to these results, lane 5 of Figure 2 (see also lane B of Figure 4) shows essentially no 81K band, but a considerable amount of the 28K fragment. The reason for this is that, in the presence of NADPH, the enzyme is rapidly cleaved at K_{410}–T_{411} (see below). As a result, little or no 81K fragment is formed. However, cleavage of the K_{410}–T_{411} bond results in a 43K N-terminal fragment, which is digested further at K_{247}–E_{248} to yield a 28K N-terminal polypeptide.

Next in Figure 5A is the cleavage site at K_{410}–T_{411}, which is consistent with the formation of the two polypeptides with relative molecular weights of 43K and 66K (Figure 4). There is also a cleavage site at K_{428}–T_{429}, which appears to have produced the 64K fragment. The latter bond is essentially at the end of the N-terminal hydrophilic region of the transhydrogenase molecule. The fourth tryptic cleavage site identified is at R_{602}–L_{603}, which produced the 48K C-terminal polypeptide. The 48K polypeptide and the 15K fragment having N-terminal residues TMTSA appear to have resulted from further proteolysis of the 64K polypeptide discussed above. The R_{602}–L_{603} bond is within the central hydrophobic stretch of the transhydrogenase molecule. However, the hydropathy plot of the transhydrogenase indicates a very short hydrophilic segment in this region between two hydrophobic clusters (arrow in Figure 5B). Thus, the tryptic digestion data are consistent with the possibility that a very short segment of the molecule between the sixth and the seventh hydrophobic clusters protrudes into the aqueous phase and is accessible to proteolytic attack. As seen in Figure 4, the 15K band appears to contain two polypeptides, one with the N-terminal residues TMTSA, which was discussed above, and another with the N-terminal residues EMSKE. The latter polypeptide would be expected from cleavage of the K_{247}–E_{248} bond in the N-terminal 43K fragment.

Time Course of the Appearance of Tryptic Fragments and Effect of NADPH. Study of the time course of the appearance of the tryptic fragments of the transhydrogenase in the absence and the presence of NADPH has indicated the following. As seen in Figure 6, the fragment that appears first, and its relative concentration rapidly increases, especially in the presence of NADPH (Figure 6, panel B), is the 43K polypeptide, and the next fragment produced most is the 66K piece. These and other results not shown have suggested that the most sensitive bond to trypsin, especially in the presence of NADPH, is the K_{410}–T_{411} bond. Other data shown in Figure 6 (see also Figures 2 and 4) suggest that the 41.5K and the 64K fragments are produced by further digestion, respectively, from the 43K and the 66K polypeptides. Then the 64K piece is cleaved at the R_{602}–L_{603} bond to yield the 15K and 48K polypeptides. This sequence of events is summarized in Figure 7, where the NADPH-promoted steps are identified with heavy

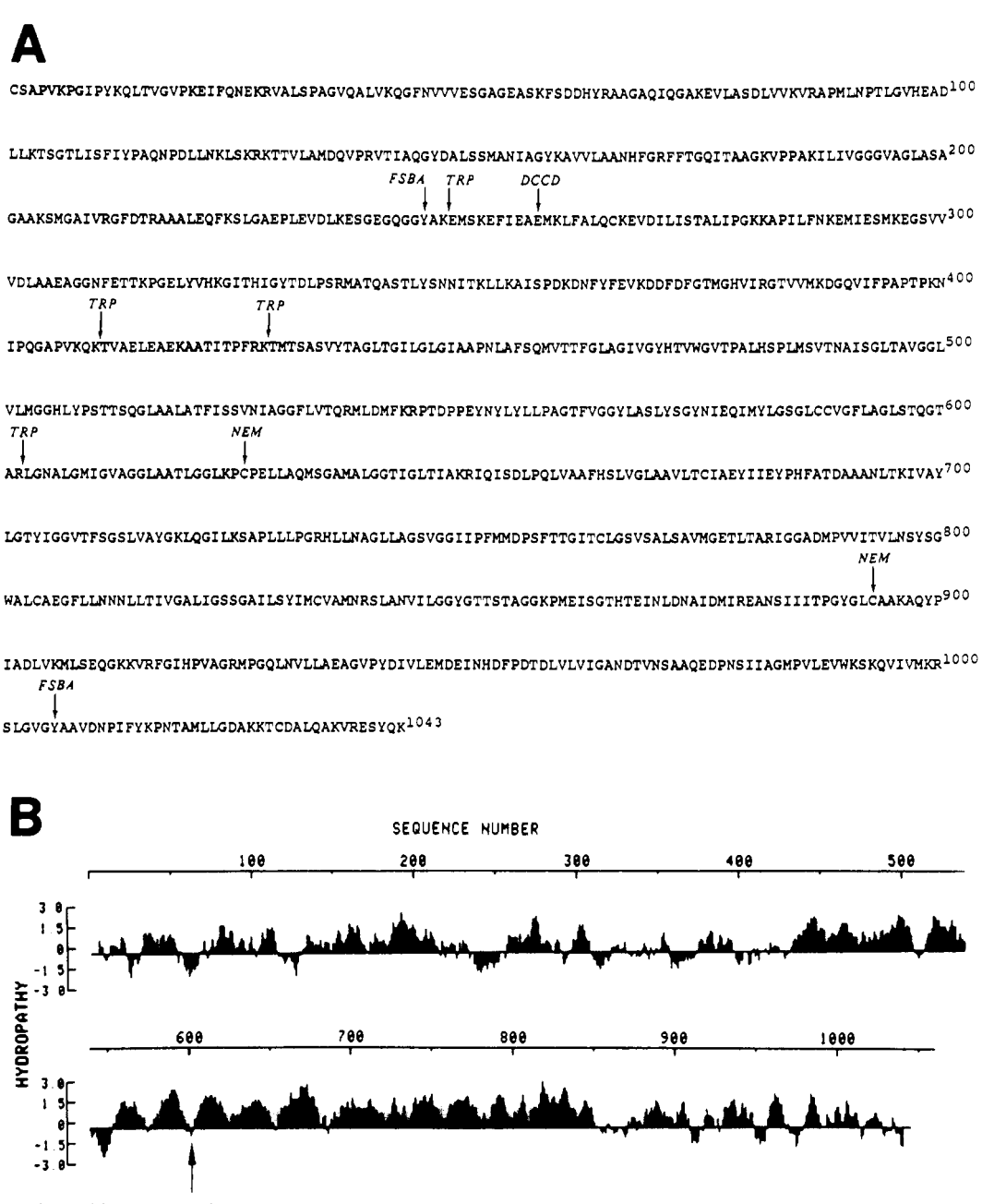

FIGURE 5: (A) Amino acid sequence of the mature bovine transhydrogenase showing four identified tryptic cleavage sites (TRP) and residues modified by [(*p*-fluorosulfonyl)benzoyl]-5′-adenosine (FSBA) and *N,N′*-dicyclohexylcarbodiimide (DCCD) at the NAD(H) binding domain near the N-terminus, the tyrosyl residue modified by FSBA at the NADP(H) binding domain near the C-terminus, and the cysteine residues modified by *N*-ethylmaleimide (NEM). (B) Hydropathy profile of the transhydrogenase showing the position of the trypsin-sensitive bond R_{602}–L_{603} (arrow) between two hydrophobic clusters.

arrows. This figure also shows how the N-terminal 28K fragment could be obtained without concomitant production of the 81K polypeptide (see Figure 4 and above). As seen in Figures 2 and 4, the presence of NADP(H) resulted in the appearance of the 48K fragment, and this polypeptide was not seen in the absence of NADP(H) in the digestion mixtures. It was, therefore, of interest to know whether the cleavage of the R_{602}–L_{603} bond is a consequence of the NADP(H)-promoted conformational change of the enzyme, thus making this bond accessible to trypsin attack, or whether the appearance of the 48K fragment in digestion mixtures containing NADP(H) is due simply to accelerated breakdown of the transhydrogenase by trypsin. Thus, two digestion mixtures were prepared, one containing NADPH and a trypsin:transhydrogenase ratio of 1:400 and the other lacking NADPH but containing a 6-fold higher concentration of trypsin. Under these conditions, the rate of disappearance of transhydrogenase activity was nearly the same in the two mixtures. After 40 min of incubation at 23 °C, samples were withdrawn from the two mixtures, denatured, and subjected to SDS gel electrophoresis. The results (Figure 8) clearly show that, even under comparable rates of digestion of the transhydrogenase by trypsin, only the mixture containing NADPH contained the 48K fragment. It is important to note, however, that both mixtures contained a 15K polypeptide with the N-terminal residues TMTSA and another with the N-terminal residues EMSKE (better visualized in Figure 4), except that the relative concentrations of the two 15K polypeptides were different in lanes 2 and 3 of Figure 8 (see also Figure 4). In the absence of NADPH, the 15K(EMSKE) was produced more, while in the presence of NADPH the 15K(TMTSA) was the dominant species (Figure 4). These results suggest the following. In the absence of NADPH, the 64K polypeptide is fragmented at R_{602}–L_{603} to produce the 15K(TMTSA) and the 48K po-

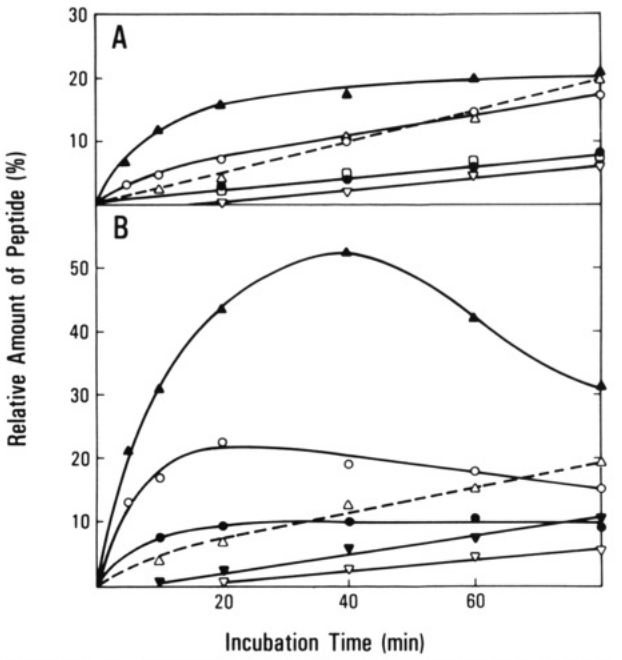

FIGURE 6: Sequence of appearance and accumulation of transhydrogenase tryptic peptides as a function of duration of incubation in the absence (A) and the presence (B) of NADPH. The enzyme (160 μg of protein) in 100 μL of 50 mM Tris–acetate, pH 7.5, containing 0.001% potassium cholate was treated with trypsin as in Figure 1 in the absence (A) or presence (B) of 0.4 mM NADPH. At the time intervals shown, 3-μL aliquots were removed, treated with excess trypsin inhibitor, denatured, and subjected to SDS–polyacrylamide gel electrophoresis. At the same time intervals, 1-μL aliquots were removed and assayed for residual transhydrogenase activity. The gels, after staining and destaining, were scanned by a densitometer at 560 nm, and the relative amount of each band was estimated as described under Materials and Methods. The data points in the figure show the estimated amount of each polypeptide expressed as the percent of the initial amount of the transhydrogenase. (□) 81K; (○) 66K; (●) 64K; (▼) 48K; (▲) 43K; (△) 41.5K; (▽) 28K.

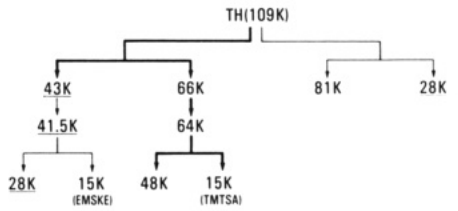

FIGURE 7: Summary of the results of Figures 2, 4, and 6, showing the sequence of tryptic fragmentation of the transhydrogenase. Heavy lines show the steps accelerated in the presence of NADPH. In the presence of this nucleotide, breakdown of the enzyme into an 81K and a 28K fragment does not seem to occur, probably because of the rapid cleavage of the molecule into a 66K and a 43K polypeptide. TH, the intact transhydrogenase molecule. Fragments containing the N-terminus of the transhydrogenase are underlined. For other details, see text and Figure 5.

lypeptides, but the latter is further degraded into small fragments not detectable on SDS gels. In the presence of NADPH, cleavage of R_{602}–L_{603} is accelerated. However, the resultant 48K fragment, which contains the NADP(H) binding site, is stabilized against further hydrolysis in the presence of NADPH.

Characteristics of the N-Terminal 43K Fragment. As stated above, tryptic digestion of the transhydrogenase in the absence or presence of substrates produces an N-terminal 43K polypeptide, which is partially hydrolyzed further near its C-terminus to a 41.5K fragment. These polypeptides are relatively stable to further degradation, suggesting retention of tertiary structure (see the relative intensities of these bands in Figures 2, 4, and 8). It was, therefore, of interest to see

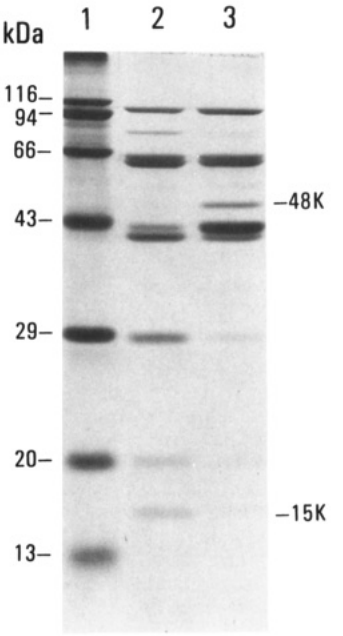

FIGURE 8: Comparison of tryptic peptides formed in the absence and presence of NADPH at the same rate of transhydrogenase inactivation. The enzyme (117 μg of protein) was treated at 23 °C with 1.8 μg of trypsin (lane 2) or with 0.3 μg of trypsin in the presence of 0.4 mM NADPH (lane 3) in 40 μL of 50 mM Tris–acetate, pH 7.5, containing 0.001% potassium cholate. After 40 min of incubation, digestion was stopped by addition of excess trypsin inhibitor, and the digests were subjected to SDS gel electrophoresis. Lane 1, standard proteins. The amount of protein loaded on lanes 2 and 3 was 7.2 μg each.

whether these fragments were capable of NAD(H) binding, especially for the following reasons. The bovine transhydrogenase has 50% sequence identity with the *Escherichia coli* enzyme, which is composed of two subunits, α with M_r = 54K and β with M_r = 48.7K (Clarke et al., 1986). In the NAD(H) and the NADP(H) binding domains, as identified in the bovine enzyme (Wakabayashi & Hatefi, 1987a), the sequence identity with the *E. coli* transhydrogenase is ≥65% (Yamaguchi et al., 1988a). The α subunit of the *E. coli* enzyme is made up of a hydrophilic N-terminal part of about 410 residues plus a hydrophobic C-terminal end of about 80 residues. Thus, the 43K fragment of the bovine enzyme would be expected to be similar to the *E. coli* α subunit lacking its short C-terminal hydrophobic segment.

Accordingly, the bovine enzyme in the presence of 0.4 mM NADPH was subjected to tryptic digestion for 60 min, and the digest was placed on a column of Sephadex G-200. The column was eluted as described under Materials and Methods, and the eluted fractions were monitored at 230 nm. As seen in Figure 9, three major peaks were collected. The first peak (A) contained transhydrogenase activity due to undigested enzyme plus tryptic fragments of M_r 66K, 64K, and 48K (see Figure 9 inset, lane A). Peak C was mainly due to the absorbance of the added NADPH at 230 nm, while peak B contained the 43K and the 41.5K polypeptides (see Figure 9 inset, lane B). Calibration of the column with protein standards (Figure 9, arrows) suggested that the peak B material had a molecular mass close to 90 kDa, i.e., twice the size of the N-terminal tryptic peptides eluted at that position. This suggested that both monomers of the dimeric enzyme had been digested by trypsin at similar positions, thus producing a dimer composed of two N-terminal 43K (and/or 41.5K) polypeptides. Results of cross-linking of the material of peak B with dimethyl pimelimidate followed by SDS gel electrophoresis agreed with the above possibility. The gel showed the presence of bands

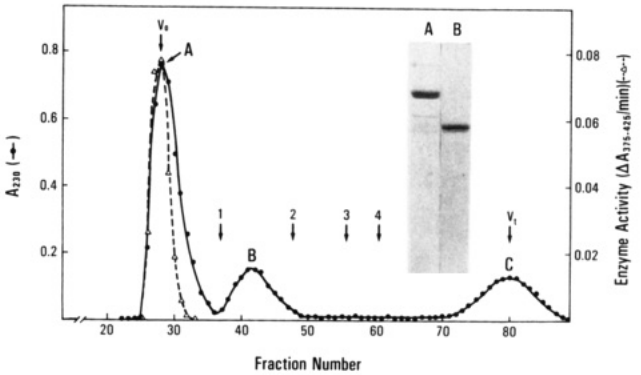

FIGURE 9: Gel filtration of the tryptic digest of the transhydrogenase on Sephadex G-200. Conditions for tryptic digestion and gel filtration were the same as described under Materials and Methods. (●) Absorbance at 230 nm; (△) transhydrogenase activity. V_0 and V_t show the elution positions of blue dextran and ferricyanide, respectively. Numbered arrows show the elution positions of (1) yeast alcohol dehydrogenase (150 kDa), (2) bovine serum albumin (66 kDa), (3) carbonic anhydrase (29 kDa), and (4) soybean trypsin inhibitor (20 kDa). Inset: SDS–polyacrylamide gel electrophoresis of fractions A and B eluted from Sephadex G-200. Forty microliters of each fraction was mixed with 10 μL of SDS denaturation buffer (313 mM Tris-HCl, pH 6.8, 10% SDS, 25% β-mercaptoethanol, 50% glycerol, and 0.01% bromphenol blue), incubated for 1 h at room temperature, and subjected to SDS gel electrophoresis. The gel was stained with Coomassie blue and destained. For other details, see text.

at about 43K and 90K plus higher molecular weight aggregates (data not shown). Like the native enzyme, the peak B material of Figure 9 was capable of binding to NAD–agarose (data not shown). Washing of the column with buffer or 20 mM NaCl did not elute it, but it could be eluted with 0.5 mM NADH, again like the native enzyme (Phelps & Hatefi, 1984).

Discussion

As stated in the introduction, inhibitory modification of the transhydrogenase by several reagents of different target specificities is accelerated in the presence of NADPH, thus suggesting that NADPH binding alters the structure of the enzyme to a more open conformation. Substrate modulation of transhydrogenase conformation is important because of its mechanistic implications, which will be discussed below. However, study of this phenomenon has been difficult, because (a) modifiers of sulfhydryl, amino, and carboxyl groups (even phenolic, guanido, and imidazole moieties) which might have been used as fluorescent probes inhibited the enzyme and (b) the circular dichroism spectrum of the transhydrogenase was not affected by substrates (unpublished results). On the other hand, despite its devastating result, trypsin could be employed to demonstrate that NADPH binding has a profound effect on the conformation of the transhydrogenase. The findings may be summarized as follows:

(1) It has been shown that the high trypsin sensitivity of the transhydrogenase is due to susceptibility of the K_{410}–T_{411} bond to cleavage and that the NADPH acceleration of enzyme inactivation by trypsin is mainly because of the increased rate of cleavage of this bond in the presence of the nucleotide. Hydrolysis of the K_{410}–T_{411} bond produced a 43K fragment, containing the N-terminus and the NAD(H) binding domain of the transhydrogenase, plus a 66K fragment, representing the remainder of the enzyme molecule and carrying the NADP(H) binding domain (see Figures 5 and 7). The 43K fragment appeared slowly to undergo further cleavage near its C-terminus to produce a 41.5K polypeptide. Otherwise, these N-terminal fragments seemed to be relatively resistant to further proteolysis. The 43/41.5K polypeptides appeared to be dimeric and were capable of affinity binding to NAD–agarose, suggesting retention of tertiary/quaternary structure after cleavage from the remainder of the transhydrogenase molecule.

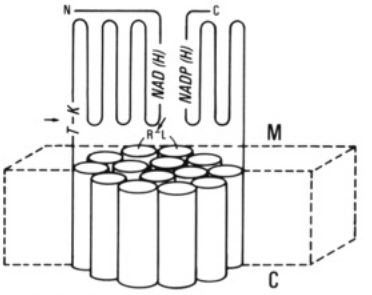

FIGURE 10: Hypothetical model of transhydrogenase showing the N- and C-terminal extramembranous segments, the central hydrophobic segment containing 14 hydrophobic clusters of amino acids (depicted here as membrane-intercalating α-helical cylinders), and the K_{410}–T_{411} and R_{602}–L_{603} bonds (arrows). This is a stylized drawing; there has been no attempt to represent various segments to relative scale. Dashed box, inner mitochondrial membrane; M, matrix side; C, cytosolic side. For other details, see text.

(2) The K_{410}–T_{411} bond, whose tryptic hydrolysis is accelerated in the presence of NADPH, is separated from the NADP(H) binding domain of the transhydrogenase by a long (~400 residues) stretch of hydrophobic, membrane-intercalating amino acids. Therefore, it is possible that the effect of NADPH binding is conveyed to the K_{410}–T_{411} bond through this hydrophobic segment. Not in agreement with this possibility is the preliminary finding that the CD spectrum of the enzyme, which showed a strong Cotton effect presumably due to the multiple membrane-spanning helical structures expected to be present in the center of the molecule, was not altered upon NADPH addition to the enzyme (data not shown). Another possibility is that the accessibility of the K_{410}–T_{411} bond to trypsin is increased upon NADPH binding by the enzyme. This could be envisioned by assuming that the C-terminal, extramembraneous segment of the transhydrogenase bearing the NADP(H) binding site overlaps the N-terminal, extramembranous segment and that depending on the absence or the presence of bound NADPH the C-terminal segment interferes more or less with access of trypsin to the K_{410}–T_{411} bond (see Figure 10).

(3) The results summarized in Figure 7 indicated that the presence of NADPH in the digestion mixture accelerated the tryptic cleavage of other transhydrogenase bonds in addition to K_{410}–T_{411}. This includes conversion of the 66K to the 64K fragment, and further cleavage of these fragments at R_{602}–L_{603} to produce a C-terminal 48K polypeptide. These results suggest that the presence of NADPH increases the trypsin sensitivity of those polypeptides containing the NADP(H) binding domain until a 48K C-terminal fragment is produced. Then, the presence of NADPH stabilizes this fragment against further digestion. In other words, it appears that NADPH binding promotes tryptic cleavage of the transhydrogenase only at locations distinct from the NADP(H) binding domain. In addition, these data together with the results discussed in paragraph (1) above suggest that the NAD(H) and the NADP(H) binding domains of the transhydrogenase are capable of binding their respective nucleotides independently. This conclusion agrees with the random mechanism of the transhydrogenase (Blazyk & Fisher, 1975; Hanson, 1979; Rydström, 1981) as well as with the fact that in the *E. coli* enzyme the corresponding domains are on two separate subunits (Clarke et al., 1986).

The NADPH-induced increase in trypsin sensitivity of several peptide bonds located 200–400 residues away from the

NADP(H) binding domain of the transhydrogenase strongly suggests that NADPH binding is associated with a significant conformation change of the enzyme. As indicated above, these results are important in view of their mechanistic implications. Consider, for example, the reversal of eq 1 in which hydride ion transfer from NADPH to NAD is coupled to proton translocation from the matrix (m) to the cytosolic (c) side of the inner mitochondrial membrane. This process results in creation of a proton electrochemical potential, which can be used to drive an endergonic reaction, such as ATP synthesis (Van de Stadt et al., 1971). The important point, however, is that there is very little difference (~5 mV only) in the reduction potentials of NADPH/NADP and NADH/NAD (Olson & Anfinsen, 1953; Kaplan et al., 1953). Therefore, the only source of energy for m → c proton translocation during reverse transhydrogenation is the difference in the concentrations of reactants (NADPH + NAD) and products (NADP + NADH). Moreover, since the scalar transhydrogenase reaction does not involve release or uptake of protons, and hydride ion transfer between NAD(H) and NADP(H) is direct, one would have to conclude that in reverse transhydrogenation substrate binding energies are transduced via protein conformation change to a membrane electrochemical potential. In other words, as a result of substrate-induced conformation change, the enzyme takes up protons on the matrix side and releases protons on the cytosolic side. This possibility is supported by our recent results showing that the *N*-ethylmaleimide-sensitive Cys-893 of the transhydrogenase changes its pK_a by 0.8 pH unit depending on whether the enzyme binds NADP or NADPH (Yamaguchi & Hatefi, 1989).

Acknowledgments

We thank C. Munoz for the preparation of mitochondria and A. Haghighi for expert technical assistance.

References

Anderson, W. M., & Fisher, R. R. (1978) *Arch. Biochem. Biophys. 187*, 180–190.

Anderson, W. M., & Fisher, R. R. (1981) *Biochim. Biophys. Acta 635*, 194–199.

Blazyk, J. F., & Fisher, R. R. (1975) *FEBS Lett. 50*, 227–232.

Blazyk, J. F., Lam, D., & Fisher, R. R. (1976) *Biochemistry 15*, 2843–2848.

Clarke, D. M., Loo, T. W., Gillam, S., & Bragg, P. D. (1986) *Eur. J. Biochem. 158*, 647–653.

Djavadi-Ohaniance, L., & Hatefi, Y. (1975) *J. Biol. Chem. 250*, 9397–9403.

Earle, S. R., O'Neal, S. G., & Fisher, R. R. (1978) *Biochemistry 17*, 4683–4690.

Fisher, R. R., & Earle, S. R. (1982) in *The Pyridine Nucleotide Coenzymes* (Everse, J., Anderson, B., & You, K.-S., Eds.) pp 279–324, Academic Press, New York.

Hanson, R. L. (1979) *J. Biol. Chem. 254*, 888–893.

Juntti, K., Torndall, U. B., & Ernster, L. (1970) in *Electron Transport and Energy Conservation* (Tager, J. M., Papa, S., Quagliariello, E., & Slater, E. C., Eds.) pp 257–271, Adriatica Editrice, Bari.

Kaplan, N. O., Colowick, S. P., & Neufeld, E. F. (1953) *J. Biol. Chem. 205*, 1–15.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

Modrak, D. E., Wu, L. N. Y., Alberta, J. A., & Fisher, R. R. (1988) *Biochemistry 27*, 7665–7671.

Mosbach, K., Guilford, H., Ohlsson, R., & Scott, M. (1972) *Biochem. J. 127*, 625–631.

Olson, J. A., & Anfinsen, C. B. (1953) *J. Biol. Chem. 202*, 841–856.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Phelps, D. C., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 8217–8221.

Phelps, D. C., & Hatefi, Y. (1984) *Biochemistry 23*, 4475–4480.

Rydström, J. (1981) in *Mitochondria and Microsomes* (Lee, C. P., Schatz, G., & Dallner, G., Eds.) pp 317–335, Addison-Wesley, Reading, MA.

Van de Stadt, R. J., Niewenhuis, F. J. R. M., & Van Dam, K. (1971) *Biochim. Biophys. Acta 234*, 173–176.

Wakabayashi, S., & Hatefi, Y. (1987a) *Biochem. Int. 15*, 915–924.

Wakabayashi, S., & Hatefi, Y. (1987b) *Biochem. Int. 15*, 667–675.

Weis, J. K., Wu, L. N. Y., & Fisher, R. R. (1987) *Arch. Biochem. Biophys. 257*, 424–429.

Yamaguchi, M., & Hatefi, Y. (1985) *Arch. Biochem. Biophys. 243*, 20–27.

Yamaguchi, M., & Hatefi, Y. (1989) *Biochemistry 28*, 6050–6056.

Yamaguchi, M., Hatefi, Y., Trach, K., & Hoch, J. A. (1988a) *J. Biol. Chem. 263*, 2761–2767.

Yamaguchi, M., Hatefi, Y., Trach, K., & Hoch, J. A. (1988b) *Biochem. Biophys. Res. Commun. 157*, 24–29.