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THE SUBUNIT STRUCTURE OF BOVINE HEART MITOCHONDRIAL TRANSHYDROGENASE

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Summary

Reaction of purified bovine heart transhydrogenase with bifunctional cross-linking reagents dimethyl adipimidate, dimethyl pimelimidate, dimethyl suberimidate, and dithiobis(succinimidyl propionate) results in the appearance of a dimer band on sodium dodecyl sulfate polyacrylamide gels with no higher oligomers formed. Treatment of the enzyme with 6 M urea led to inactivation and prevented cross-linking by dimethyl suberimidate. Transhydrogenase reconstituted into phosphatidylcholine proteoliposomes also yielded a dimer band on cross-linking. These data indicate that soluble and functionally reconstituted transhydrogenase possesses a dimeric structure.

Transhydrogenase has recently been purified to homogeneity from bovine heart submitochondrial particles [1,2]. The molecular weight of the enzyme is about 120 000 as determined by SDS-polyacrylamide gel electrophoresis [1]. On reconstitution into proteoliposomes, transhydrogenation between NADPH and NAD^+ is coupled to the inward translocation of protons [3] according to the equation:

$$H^+_{out} + NADPH + NAD^+ \rightleftharpoons H^+_{in} + NADP^+ + NADH$$

These studies confirmed and extended the prior observations of Moyle and

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Mitchell [4,5] that showed transhydrogenase-dependent proton fluxes across the mitochondrial inner membrane.

Singer [6] has suggested that all membrane transport systems may consist of dimeric proteins. Studies on transport systems including ($Na^+ + K^+$)-ATPase [7] and ADP-ATP carrier protein [8], support this hypothesis. In this paper the subunit structure of purified solubilized and reconstituted transhydrogenase has been investigated using a variety of bifunctional cross-linking reagents.

Transhydrogenase was prepared as previously described [1], except that Lubrol WX was exchanged for 0.05% sodium cholate upon final concentration and assayed at 25°C [9]. Reconstitution of transhydrogenase into phosphatidylcholine liposomes was as described [10]. All analyses were performed on SDS-polyacrylamide gel electrophoresis gels using the buffer system of Weber and Osborn [11]. Cross-linked products were separated on either 5% acrylamide or 3% acrylamide, 0.5% agarose gels run at 8 mA/gel for 7.5 h at 25°C. Gels were stained for protein with Coomassie Blue R250, diffusion destained in 25% isopropanol — 10% acetic acid (v/v) and scanned at 560 nm. The molecular weights of cross-linked products were estimated from plots of log molecular weight against electrophoretic mobility [11]. Rabbit muscle glycogen phosphorylase *a* (0.3 mg/ml) was cross-linked with 10.9 mM dimethyl suberimidate in 0.2 M triethanolamine-HCl, pH 8.5, for 60 min at room temperature to generate a mixture of monomers (molecular weight 92 500) dimers, trimers and tetramers which were used as standards.

Homogeneous solubilized transhydrogenase was cross-linked with dimethyl suberimidate, then electrophoresed on gels containing 5% acrylamide. As shown in Fig. 1, cross-linking diminishes the staining intensity of the monomer band and results in the formation of three closely migrating dimer bands having apparent molecular weights of 210 000, 220 000 and 230 000. These

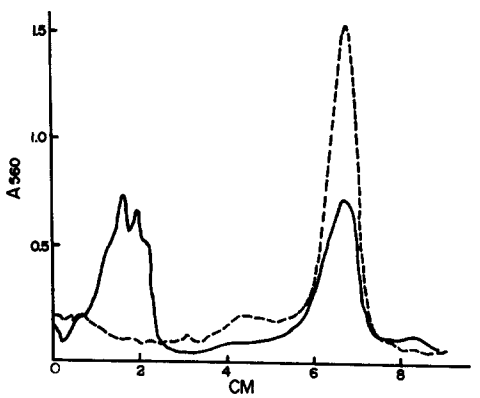

Fig. 1. Cross-linking of purified solubilized transhydrogenase. Transhydrogenase (0.44 ml, 0.1 mg/ml) containing 1% lysophosphatidylcholine was dialyzed for 1.5 h at 4°C against 100 ml of 0.2 M triethanolamine-HCl, pH 8.5, containing 1 mM dithiothreitol and 0.05% sodium cholate (w/v). The dialyzed enzyme was then reacted at 25°C with 10.9 mM dimethyl suberimidate. At zero time (- - - - -) and 40 min (———) samples (15 μg protein) were withdrawn for SDS-polyacrylamide gel electrophoresis on 5% acrylamide gels.

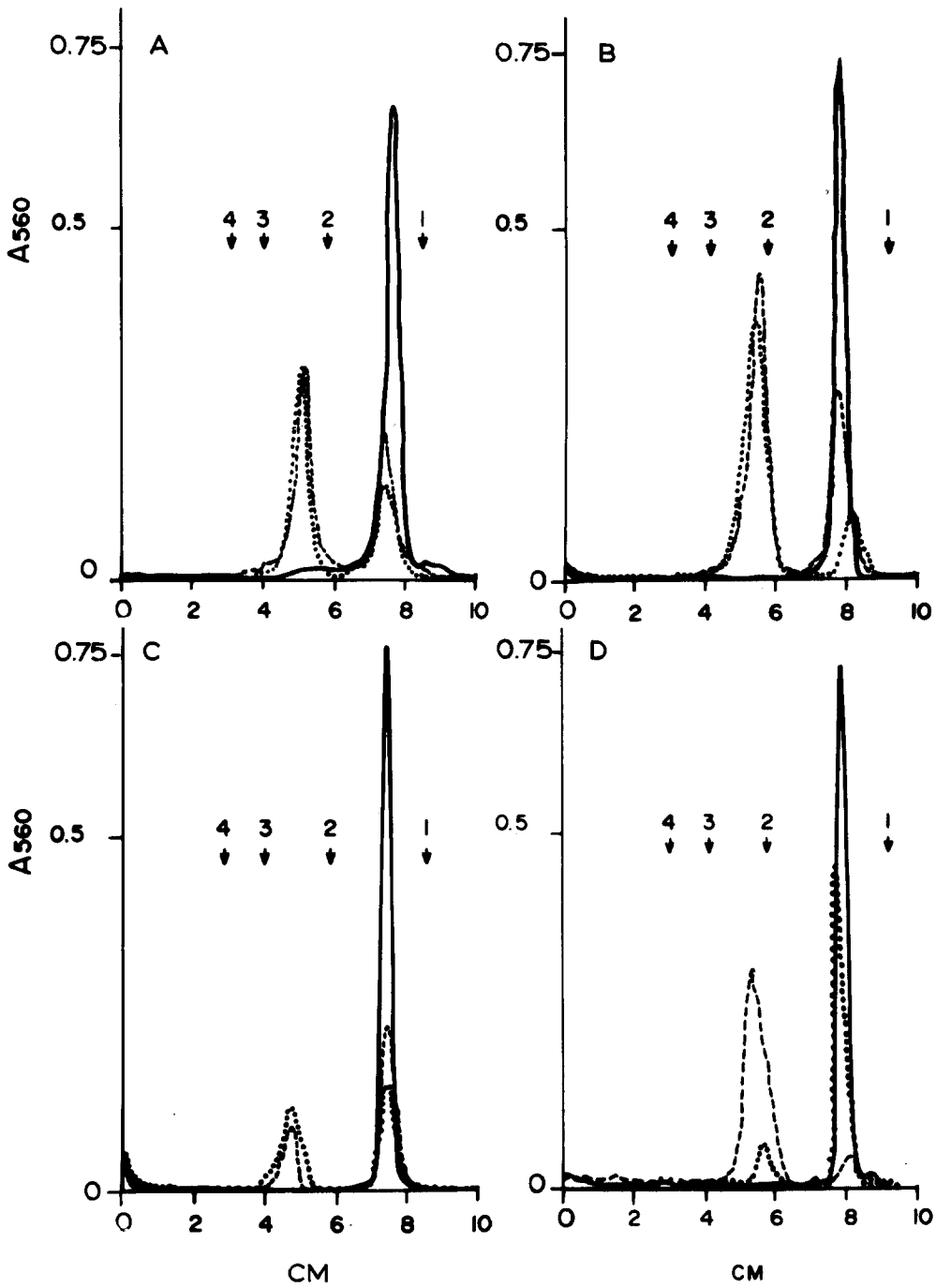

Fig. 2. Electrophoretic patterns of transhydrogenase after cross-linking with various diimidates. Transhydrogenase (0.4 ml) was dialyzed for 1.5 h at 4°C against 100 ml of 0.2 M triethanolamine-HCl, pH 8.5, containing 1 mM dithiothreitol and 0.05% sodium cholate (w/v). The dialyzed enzyme was then reacted at room temperature with (A) dimethyl adipimidate (12.2 mM) 0.14 mg protein/ml, (B) dimethyl pimelimidate (11.4 mM) 0.13 mg protein/ml and (C) dimethyl suberimidate (10.9 mM) 0.21 mg protein/ml. At zero time (———), 30 min (- - - - - -), and 60 min (· · · · · ·) samples (10 μg protein) were with-

dimeric species probably represent 'cross-link isomers' [12] in which cross-linking occurs at different sites on the constituent subunits. This, as observed previously [12,13], would be expected to produce dimers of differing structure and hence altered mobility.

To investigate the formation of higher cross-linked transhydrogenase oligomers, gels containing 3% acrylamide and stabilized with 0.5% agarose were employed. Fig. 2 shows that transhydrogenase cross-linked by a series of bifunctional reagents, dimethyl adipimidate, dimethyl pimelimidate, dimethyl suberimidate and dithiobis(succinimidyl propionate) yielded products in the molecular weight range of 220 000—250 000, with no higher oligomers. Cross-link isomers were not resolved in these gels. The lack of higher oligomer formation at the low protein concentration studied argues against a collisional mechanism [14] for dimer formation, since it is apparent that the potential for such intra-dimer cross-links exists, i.e. more than one lysine residue on each monomer is available for reaction. With dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate (Fig. 2A—C) cross-linking was essentially complete after 60 min. No further conversion of the enzyme to the stable dimer was seen at longer reaction times. In the case of dithiobis(succinimidyl propionate) (Fig. 2D) almost complete conversion of the monomer band to a dimer band occurred within 30 min. Treatment of the cross-linked product with β-mercaptoethanol, to cleave the cross-linker disulfide bond, regenerated nearly completely the monomer band with a corresponding decrease in the dimer band. Transhydrogenase functionally reconstituted into phosphatidylcholine liposomes [10] was modified with dimethyl suberimidate. The results obtained were identical to those presented in Fig. 2C. Modification with all the cross-linking reagents resulted in a decrease in staining intensity of the protein bands. This was particularly evident with dimethyl suberimidate. Reaction of the enzyme with the monofunctional reagent, methyl acetimidate also reduced the staining intensity of the monomer. It is concluded that this modification of the transhydrogenase interferes with the efficacy of Coomassie Blue staining.

These data indicate that active transhydrogenase exists as a dimer in solution. To provide additional support for this conclusion, the enzyme was cross-linked with dimethyl suberimidate under denaturing conditions. Dialysis against 2 M urea before reaction with dimethyl suberimidate neither inactivated nor prevented cross-linking (Fig. 3B), whereas treatment with 6 M urea (Fig. 3C) completely inactivated the enzyme and nearly completely prevented cross-linking. Although these data suggest that in 6 M urea transhydrogenase dimers

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drawn for SDS-polyacrylamide gel electrophoresis on 3% acrylamide, 0.5 agarose gels. Cross-linking with dithiobis(succinimidyl propionate) (D) was performed by dialyzing transhydrogenase (0.4 ml, 0.27 mg/ml) for 1.5 h at 4°C against 50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, containing 0.05% sodium cholate (w/v), followed by addition of dithiobis(succinimidyl propionate) in dimethyl sulfoxide to a final concentration of 1 mM. At zero time (———) and 30 min (- - - - -) samples were withdrawn for SDS-polyacrylamide gel electrophoresis (10 μg protein). The samples were reacted with 15 mM iodoacetamide for 15 min at room temperature in the dark prior to boiling for 1 min in electrophoresis buffer. Another sample was withdrawn at 30 min (· · · · · ·) and boiled for 3 min in electrophoresis buffer containing 0.42 M β-mercaptoethanol to cleave the cross-linker disulfide bond. The numbers (1—4) correspond to the positions of the monomer (1), dimer (2), trimer (3), and tetramer (4) bands of rabbit muscle glycogen phosphorylase *a*.

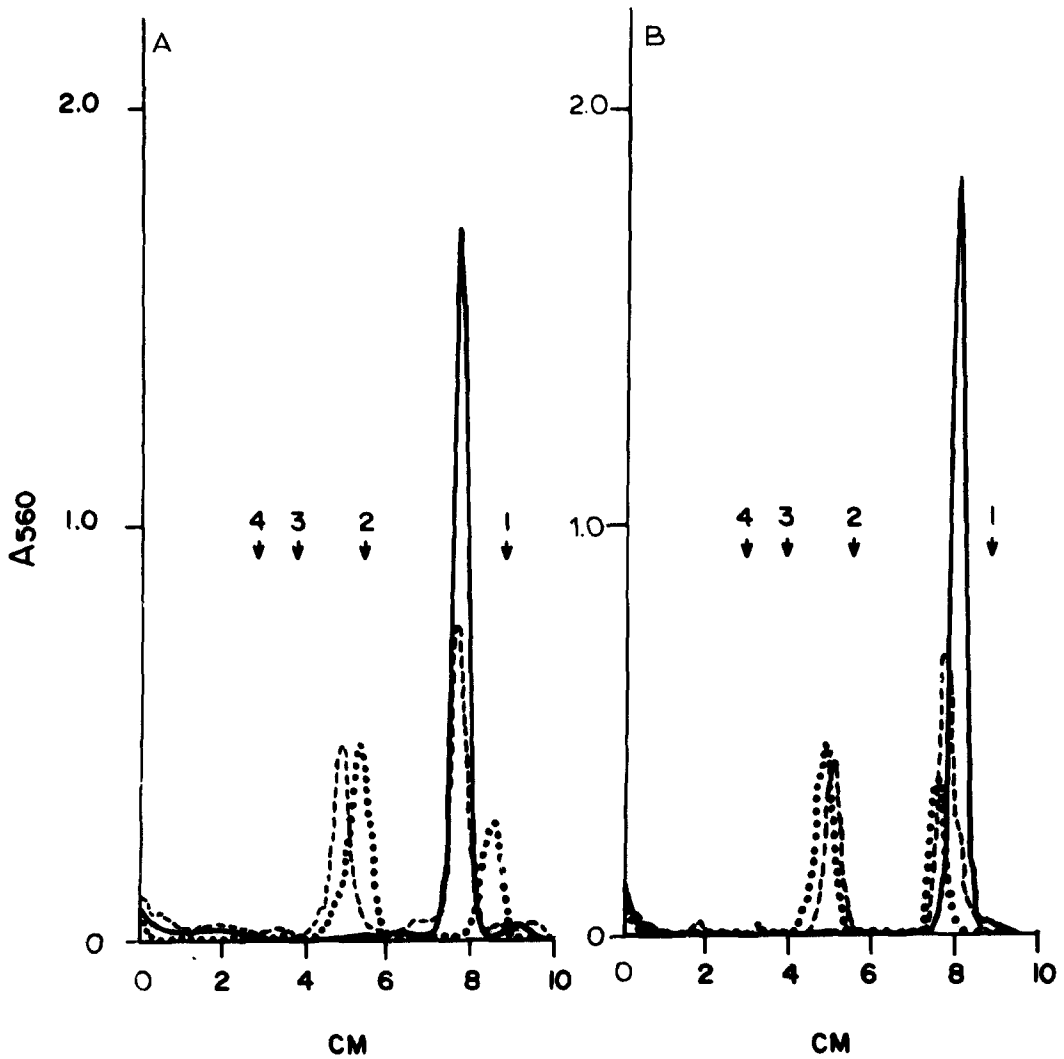
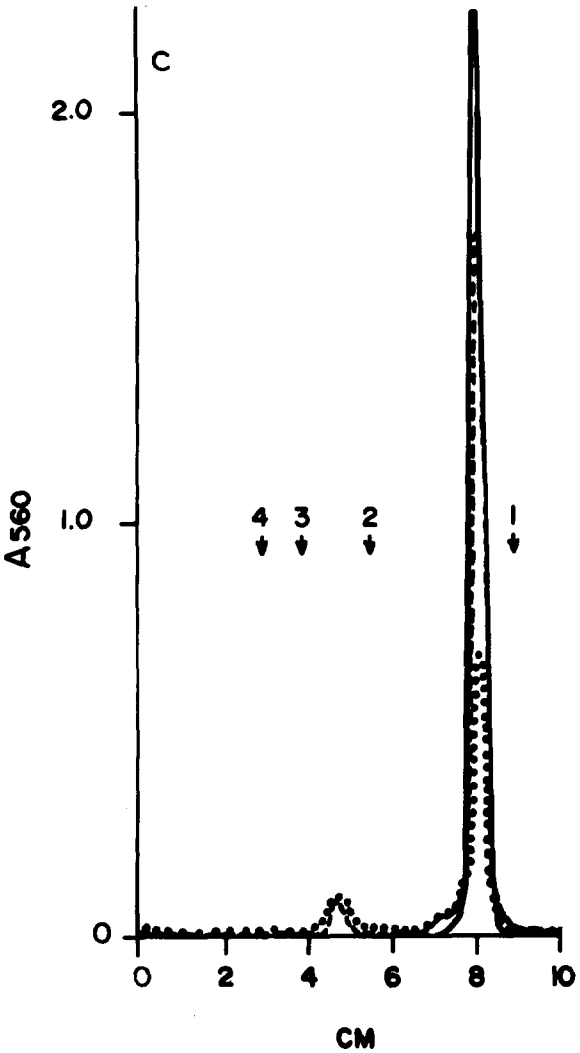


Fig. 3. Cross-linking of purified solubilized transhydrogenase with dimethyl suberimidate in the absence and presence of urea. Transhydrogenase (0.4 ml, 0.23 mg/ml) was dialyzed for 1.5 h at 4°C against 100 ml of 0.2 M triethanolamine-HCl, pH 8.5, containing 1 mM dithiothreitol, 0.05% sodium cholate (w/v) and (A) no urea, (B) 2 M urea, and (C) 6 M urea. The dialyzed enzyme was then reacted with 10.9 mM dimethyl suberimidate at room temperature and at zero time (———), 30 min (- - - - - -) and 60 min (· · · · · ·) samples (15 μg protein) were withdrawn for enzymatic assay and SDS-polyacrylamide gel electrophoresis on 3% acrylamide, 0.5 agarose gels. The numbers designate the positions of cross-linked glycogen phosphorylase *a* as described in Fig. 1.

are dissociated, the possibility that the conformation of the dimeric enzyme is altered to an extent that the cross-linked amino acids are not properly spaced cannot be eliminated.

While purified and reconstituted transhydrogenase appears to be composed of a dimer of identical subunits, the subunit structure of the enzyme in the mitochondrial membrane remains to be determined. These studies are currently underway.



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