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Correlation between Active Form and Dimeric Structure of Mitochondrial Nicotinamide Nucleotide Transhydrogenase from Beef Heart

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The active form of purified mitochondrial nicotinamide nucleotide transhydrogenase from beef heart was investigated by crosslinking with dimethylsuberimidate and SDS-PAGE, with or without pretreatment with the inactivating detergent Triton X-100. In the absence of detergent, crosslinked isomers of the dimeric form of 208–235 kDa were obtained. Addition of detergent led to the simultaneous loss of the dimers and the bulk of the activity. Removal of the detergent led to a partial restoration of both activity and the dimeric forms. The results suggest that the active form is a dimer, and that the detergent-dependent conversion to the largely inactive monomer is reversible. It is proposed that the mechanism of inactivation of transhydrogenase by Triton X-100 involves a disruption of essential hydrophobic interactions between the membrane-spanning regions of the monomers.

KEY WORDS: Transhydrogenase; subunit structure, mitochondria, proton pump

INTRODUCTION

Mitochondrial nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) is a proton pump which catalyzes the reversible transfer of hydrogen between NAD and NADP linked to translocation of protons across the inner membrane. Beef heart transhydrogenase was recently cloned, its *c*DNA sequenced, and its exact molecular weight determined to be 109, 212 (Yamaguchi *et al.*, 1988). Based on hydropathy plots, a topology model for the transhydrogenase involving 14 transmembrane α-helices accounting for approximately 35% of the total protein was proposed (Yamaguchi and Hatefi, 1991a) (for reviews, see Rydström *et al.*, 1987 and Jackson, 1991).

Crosslinking (Anderson and Fisher, 1981; Wu and Fisher, 1983) and radiation inactivation (Persson *et al.*, 1987) of transhydrogenase have indicated that the active form is a homodimer. Based on crosslinking as well as radiation inactivation, the same conclusion has been reached in the case of the *E. coli* transhydrogenase, i.e., that the active enzyme is composed of two sets of the α and β subunits (α plus β correspond to the monomer of the bovine enzyme) and thus has the composition $\alpha_2\beta_2$ (Hou *et al.*, 1990). Exposure of the purified beef heart enzyme to 3% Triton X-100 results in inactivation and conversion to the monomer form (Persson *et al.*, 1987). Even though these findings agree with the general notion that active membrane proteins, especially proteins involved in transport, often are dimers (Klingenberg, 1981), they neither support nor refute the possibility that the transhydrogenase monomer may be active, or that the dimer/monomer transition is reversible. The present investigation has attempted to address these questions by a quantitation of the correlation between active form and quaternary structure.

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MATERIALS AND METHODS

Preparation of Transhydrogenase

Transhydrogenase from beef heart mitochondria was purified as described (Persson *et al.*, 1984; Eytan *et al.*, 1990). Transhydrogenase (500 μl of a preparation containing 0.41 mg/ml) in 200 mM sodium phosphate (pH 7.5) and 0.05% sodium cholate (neutralized) were dialyzed for 2 h against 200 ml of 0.2 M triethanolamine (pH 8.5) and 0.05% sodium cholate (neutralized). The sample was diluted four times with dialysis buffer to give 2 ml of 100 μg/ml.

Addition and Removal of Triton X-100

The dialyzed transhydrogenase sample was made 1% with respect to Triton X-100 by the addition of 100 μl of 20% Triton X-100 (v/v) in 0.2 M triethanolamine (pH 8.5). Removal of Triton X-100 was accomplished by a method described previously using Bio-Beads (Holloway, 1973), a neutral porous styrene divinyl benzene copolymer. Moist washed copolymer (0.6 g) was added to the solution, which was gently stirred at 4°C. Aliquots for the determination of Triton X-100 (10 μl), transhydrogenase activity (5 μl), protein (20 μl), and crosslinking (20 μl) were withdrawn before and after the addition of Triton X-100, and at different time intervals during 3 h.

Crosslinking of Transhydrogenase

The crosslinker DMS[4] was used, dissolved in 0.2 M triethanolamine (pH 8.5). To 20 μl aliquots (2 μg transhydrogenase protein) of the Triton X-100 mixture, 16 μl H_2O was added, followed by 4 μl of 109 mM DMS, giving a final concentration of 10.9 mM DMS. After crosslinking for 45 min at room temperature, the reaction was stopped by adding 16 μl of concentrated Laemmli (1970) sample buffer (composed of 0.2 M Tris-HCl, 30% glycerol, 6% SDS, and 10% sodium laurylsulfate), and 4 μl 0.1% bromphenol blue. Samples were boiled for 2 min and 4 μl of mercaptoethanol was added before the samples were applied on the SDS-PAGE.

Assays

Transhydrogenase activity was determined on an Aminco-Chance DW-2 recording spectrophotometer using a medium composed of 80 mM potassium phosphate (pH 6.3), 200 μM NADPH, 200 μM $AcPyAD^+$, 0.05% Triton X-100, and 0.4 mg/ml of lysophosphatidylcholine. Reduction of $AcPyAD^+$ by NADPH was followed at 375–420 nm. Volume was 1 ml and temperature 30°C. The concentration of Triton X-100 was estimated by diluting the 10 μl aliquots in 1 ml of water and measuring the absorbance at 274 nm, after correcting for protein absorbance (Holloway, 1973).

Protein concentration was estimated by a modified Lowry method (Peterson, 1977). Slab gel electrophoresis (SDS-PAGE) was carried out on a 1-mm SDS-polyacrylamide gel according to Laemmli (1970), with a 3% concentration gel and a 7.5% separation gel. The gel was run overnight with a constant current of 5 mA and stained according to a modified silver staining method (Goldman *et al.*, 1981), and dried on to a filter paper. The gel was then scanned using an LKB Ultroscan XL laser densitometer.

Chemicals

Dimethylsuberimidate dihydrochloride was purchased from Sigma Chem. Co. (St. Louis, Missouri). Bio-Beads SM-2 (20–50 mesh) were obtained from Bio-Rad Co. (Richmond, California). All other biochemicals were obtained from Sigma or Beohringer Mannheim (Mannheim, Germany).

RESULTS

Like many membrane proteins, transhydrogenase is highly sensitive to detergents that interfere with the hydrophobic surfaces which normally are exposed to the interior of the membrane. Figure 1 shows the rapid inactivation of purified beef heart transhydrogenase by the addition of 1% Triton X-100. In the presence of Triton X-100 the activity decreased from 17.3 μmol/min/mg to 4.3 μmol/min/mg, i.e., the maximal extent of inactivation under these conditions was about 75%. Following the addition of Bio-Beads to remove micellar Triton X-100, the concentration of the detergent decreased to about 0.06% in 3 h. Concomitantly with the removal of the detergent, the activity of transhydrogenase was partially restored, from 23% to about 54%, of the initial activity prior to the addition of the detergent.

During the incubation with Triton X-100 and the subsequent removal of the detergent, samples were withdrawn, crosslinked with suberimidate, and subjected to SDS-PAGE (cf. Materials and Methods). As

[4] Abbreviations: $AcPyAD^+$, oxidized 3-acetylpyridine-NAD^+; SDS-PAGE, polyacrylamide gel electrophoresis carried out in the presence of sodium dodecyl sulfate; DMS, dimethylsuberimidate.

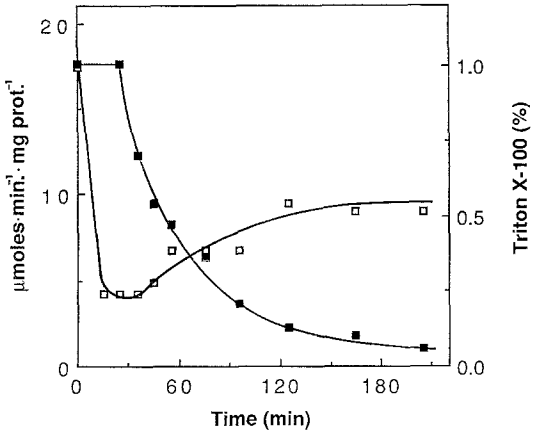

Fig. 1. Reversible inactivation of nicotinamide nucleotide transhydrogenase by Triton X-100. Triton X-100 (1%) was added at $t = 0$ min and removed by Bio-Beads at $t = 25$ min. Symbols denote: □, specific transhydrogenase activity; ■, concentration of Triton X-100 (%).

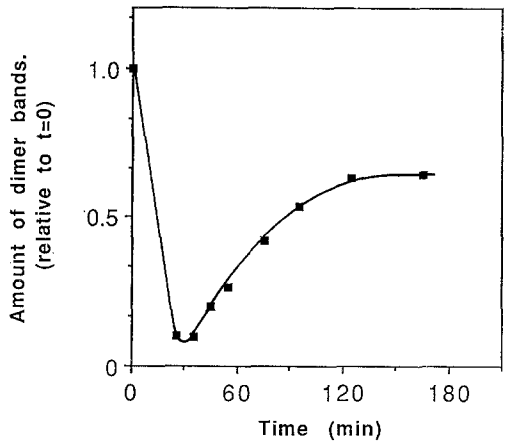

Fig. 3. Quantitation of dimeric transhydrogenase following the addition and the removal of Triton X-100. The 208-, 234-, and 242-kDa dimeric transhydrogenase bands were quantitated by densitometry as described in Materials and Methods.

seen in Fig. 2, lanes 2 and 3, the dominating band both in the absence and in the presence of Trition X-100 represents the transhydrogenase monomer of about 110 kDa. A couple of additional weak bands with slightly lower molecular weights may also be seen, which result from proteolytic degradation generated during the activation of the purified homogeneous enzyme by phospholipids (Peterson *et al.*, 1984). In the

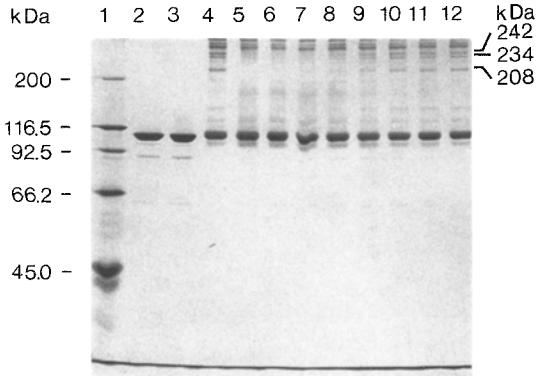

Fig. 2. Crosslinking of transhydrogenase by DMS. Transhydrogenase was crosslinked by DMS in the absence and in the presence of 1% Triton X-100. Samples were: lane 1, molecular weight markers; lane 2, transhydrogenase in the absence of Triton X-100; lane 3, transhydrogenase in the presence of Triton X-100; lane 4, crosslinked transhydrogenase in the absence of Triton X-100; lane 5, transhydrogenase crosslinked after exposure to 1% Triton X-100 for 25 min.; lanes 6–12, transhydrogenase exposed to 1% Triton X-100 for 25 min followed by the addition of Bio-Beads and crosslinked, after a total incubation time of 35, 45, 55, 75, 95, 125, and 165 min, respectively.

absence of Triton X-100 but in the presence of the crosslinking agent suberimidate, a certain fraction of the total transhydrogenase protein was recovered in the dimeric region as 3 bands. These may be referred to as crosslinked isomers (cf. Anderson and Fisher, 1981; Wu and Fisher, 1983). The molecular weights of the dimeric structures were 208, 234, and 242 kDa, respectively, which closely correspond to previously published reports (Wu and Fisher, 1983). In addition to these bands, other bands with lower or higher molecular weights appeared. However, these were not affected by the Triton X-100 treatment. As shown in Fig. 2 the intensity of the dimeric bands varied from sharp prior to the addition of detergent (lane 4), to very weak in the presence of the detergent (lane 5), and again to sharp bands after detergent removal (lanes 9–12). The proteolytic products apparently formed a band in all crosslinked samples, which migrated close to and ahead of the monomer transhydrogenase band. Alternatively, they may be linked to the high-molecular-weight crosslinked products.

A quantitation of the 208-, 234-, and 242-kDa bands by laser densitometry showed that the sum of these bands was decreasing after the addition of the detergent (Fig. 3). A minimum level of about 8% was obtained already after less than 30 min. Removal of the detergent then led to a time-dependent recovery of the bands, with an optimum of about 62% after 2 h. The time required for optimal regeneration of the dimers (Fig. 3) approximately coincided with that required for maximal recovery of the transhydrogenase activity (Fig. 2). However, although the

maximal extent of recovery of the dimeric bands was approximately the same as the recovery of activity, the minimum in dimers was lower than the corresponding minimum in activity.

DISCUSSION

The present results indicate for the first time that inactivation of the mitochondrial transhydrogenase by detergents, such as Triton X-100, involves a reversible dissociation to the monomer with a molecular weight of about 110 kDa. In the absence of detergent the appearance of dimeric bands with molecular weights of 208, 234, and 242 kDa suggests that, in agreement with previous findings, the active form of transhydrogenase is a dimer (Anderson and Fisher, 1981; Wu and Fisher, 1983; Persson *et al.*, 1987). Reappearance of the dimeric bands after removal of the bulk of the detergent was accompanied by a partial restoration of the catalytic transhydrogenase activity, providing further evidence that the active form of the enzyme indeed is a dimer. That only a partial reactivation was obtained may be explained by the fact that Triton X-100 could not be removed completely from the incubation mixture without time-consuming dialysis, which tends to inactivate the enzyme irreversibly (not shown). Addition of concentrations of Triton X-100 below 1% gave a lower degree of inhibition but also a higher final extent of reactivation upon removal of the detergent (not shown), whereas addition of 3% Triton has been shown previously to cause inactivation and an essentially irreversible monomerization (Persson *et al.*, 1987).

Since the method employed for estimating the active form involves crosslinking with DMS followed by SDS-PAGE analysis, there are some characteristic features of this method that need clarification. In order to avoid extensive intermolecular crosslinking between different transhydrogenase dimers or monomers, the concentration of the crosslinking agent has to be minimal. However, as a consequence, only a certain fraction of the total enzyme will be crosslinked. Crosslinking may also involve several reactive groups, which leads to the formation of crosslinked dimeric "isomers" (Anderson and Fisher, 1981; Wu and Fisher, 1983), i.e., in the present case at least three main dimeric bands with apparent molecular weights of 208, 234, and 242 kDa. Since all of these bands thus represent equivalent dimers, total active transhydrogenase was assumed to be related to the sum of these bands.

Detergents of the Triton X-100 type do not influence significantly soluble enzymes such as the transhydrogenase from *Pseudomonas aeruginosa* (J. Rydström, unpublished). Inactivation of the beef heart transhydrogenase by Triton X-100 may therefore be visualized to involve mainly the hydrophobic membrane part of the enzyme. An intriguing consequence of this argument would be that the hydrophobic membrane parts of the two monomers in the active dimer constitute essential points of interaction in forming the dimeric structure. Hydrophobic interactions between transmembrane α-helices rather than hydrophilic interactions between nonmembranous regions of the enzyme have recently been proposed to be responsible for maintaining the active structure of the beef heart transhydrogenase peptide bisected by proteinase K (Yamaguchi and Hatefi, 1991b).

Inactivation of the beef heart transhydrogenase by Triton X-100 raises the question whether the monomer is inactive or not. Higher concentrations of Triton have been shown to inactivate the enzyme and cause monomerization (Persson *et al.*, 1987), and the present investigation shows a clear correlation between bulk catalytic activity and dimeric forms. However, the present investigation also showed that approximately 23% of the native transhydrogenase activity remained apparently in the presence of only 8% of the dimeric form, indicating that the monomer was not completely inactive. However, problems such as a possible inhibitory effect of Triton on a hypothetically low-activity monomer have to be solved before a definite conclusion in this regard may be drawn.

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