SELECTIVE SOLUBILIZATION OF NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE FROM THE MITOCHONDRIAL INNER MEMBRANE

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Summary: Nicotinamide nucleotide transhydrogenase was solubilized from beef heart submitochondrial particles employing Triton X-100 or lysolecithin. Lysolecithin was considerably more efficient and selective and released over 80 % of the transhydrogenase acdtivity from the membrane together with succinate dehydrogenase. Solubilization of NADH dehydrogenase and cytochrome oxidase was more efficiently accomplished with Triton than with lysolecithin. Both detergents released ATPase to various extents. Transhydrogenase remaining bound to particles after treatment with lysolecithin still catalyzed energy-linked transhydrogenation.

Mitochondrial nicotinamide nucleotide transhydrogenase catalyzing the reaction NADH + $NADP^+ \rightleftarrows NAD^+$ + NADPH constitutes one of the few respiratory chain linked enzymes that so far has resisted solubilization and characterization in its isolated form. After the discovery by Danielson and Ernster (1) that submitochondrial particles catalyze both nonenergy-linked and energy-linked transhydrogenation, several authors (2-4) have reported partial purification of the enzyme. However, the methods employed result in preparations that appear to be contaminated by several, if not all, of the components of submitochondrial particles (5). The difficulties in obtaining reproducible results with various preparations were pointed out by Kaplan (6) in a recent review. In the course of kinetic investigations of transhydrogenase and its mode of coupling to the respiratory chain carried out in this laboratory (7-10), the need for an isolated form of the transhydrogenase became apparent. Therefore, as a first step towards isolation of the enzyme a systematic study of the various possible means for solubilization of the transhydrogenase from submitochondrial particles was undertaken. The present paper describes the effect of various detergents, including lysolecithin, on the transhydrogenase and its solubilization from submitochondrial particles.

MATERIALS AND METHODS

EDTA-submitochondrial particles were prepared as described by Lee and Ernster (11). After two washes with 0.25 M sucrose the particles were suspended and washed twice with bi-distilled water. The final pellet was suspended in water (at a concentration of 20 mg prot. per ml) and stored at -15°C. Particle protein was determined by the biuret method or by the Lowry method. When protein was determined in supernatants containing high concentrations of detergents, the same concentration of detergent was added to the standard samples. NADH dehydrogenase was assayed as NADH-ferricyanide reductase as described by Minakami et al. (13). Succinate dehydrogenase was assayed as succinate-ferricyanide reductase in the presence of 3 mM ferricyanide as described by King (14). Cytochrome oxidase activity was estimated spectrophotometrically using reduced cyt. c according to Sottocasa et al. (15) and the activity of ATPase was determined as described by Pullman et al. (16). Phospholipid was extracted with chloroform-methanol (17) and P_i was determined according to Bartlett (18). Supernatants were concentrated using an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass., USA) equipped with a PM10 filter. Triton was removed by Bio-Beads SM-2 (Bio-Rad, Richmond, Calif., USA) as described by Holloway (12). Nonenergy-linked and energy-linked transhydrogenation was measured as described earlier (7,10). Chromatographically pure lysolecithin (from egg) was obtained from Koch-Light Laboratories Ltd (Colnbrook, Bucks, England). Digitonin was purchased from E. Merck (Darmstadt, Germany). Other biochemicals were purchased from Sigma Chem. Co. (St. Louis, USA) or Boehringer Mannheim GmbH (Mannheim, Germany).

RESULTS

It may be seen (Fig. 1A) that within the concentration range tested, the ionic detergents deoxycholate and cholate did not remove any transhydrogenase

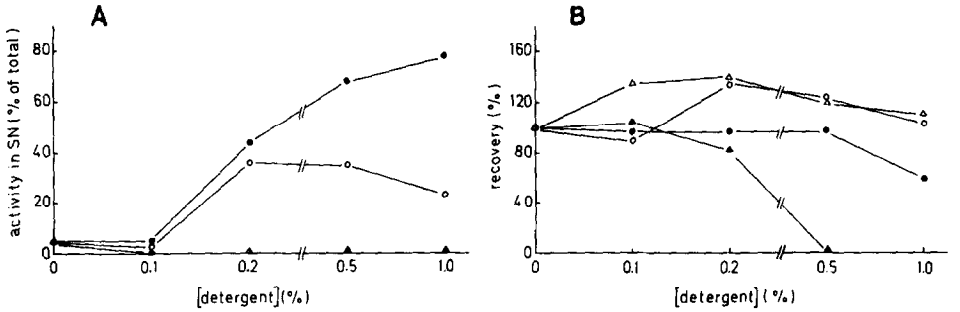

Fig. 1. Effect of detergents on the release of transhydrogenase activity from submitochondrial particles (A) and on recovery of total activity (B). Detergents used were: ●, Triton X-100; ○, digitonin; Δ, cholate; ▲, deoxycholate. Incubation mixture contained 0.1 M tris-acetate pH 7.4, 3 mM EDTA, 1 mM dithioerythritol (DTE), and 10 mg EDTA particles. Detergents were added to the indicated concentrations. Final volume was 10 ml and temperature was kept at 0-5°C. After 30 min incubation the mixture was centrifuged at 100,000 x g for 40 min. The supernatant (SN) was poored off carefully and the pellet was suspended in 1 ml tris-acetate-EDTA-DTE medium. Transhydrogenase activity was assayed as reduction of NAD^+ by NADPH using lactate dehydrogenase plus pyruvate as regenerating system.

activity from the particles. At concentrations above 0.1 % and 1 %, respectively, both deoxycholate (Fig. 1B) and cholate (not shown) inactivate. In contrast, , at concentrations below 1 %, the nonionic detergents digitonin and Triton X-100 removed a substantial part of total transhydrogenase activity from the particles (Fig. 1A) without a simultaneous decrease in recovery (Fig. 1B); Triton concentrations of 1 % or higher were inhibitory.

Triton supernatants obtained in the experiments of Fig. 1 may be frozen or concentrated up to ten times without any loss of activity. Freezing of digitonin supernatants led to a complete loss of activity. An additional advantage with Triton over digitonin is that Triton may be removed efficiently and specifically by adsorption to hydrophobic polymers without altering the transhydrogenase activity (cf. Methods).

Using Triton as the detergent of choice, various parameters that affect the release of protein from the EDTA particles, e.g. concentration of Triton and of protein, were more carefully investigated. Release of phospholipids and of other enzymatic activities from the particles, i.e. NADH dehydrogenase, succinate dehydrogenase, cytochrome oxidase and ATPase, were followed in parallel to that of transhydrogenase. To ensure that components recovered in the supernatant after centrifugation may be called soluble, the incubation mixtures were centrifuged for 4 hours at 200,000 x g (average g value).

As shown in Fig. 2A, with increasing concentrations of Triton, solubilization of transhydrogenase started at about 0.1 %, reached a plateau between 0.2 and 0.4 % and declined above 0.5 % presumably due to inactivation. Succinate dehydrogenase and NADH dehydrogenase appeared to be solubilized at lower Triton concentrations. Transhydrogenase, succinate dehydrogenase and NADH dehydrogenase reached a maximum corresponding to 50, 55 and 25 % solubilization, respectively. In contrast to transhydrogenase, the two latter enzymes were not inactivated by higher concentrations of Triton. ATPase and cytochrome oxidase were released to a small extent only, and the detergent caused a marked decrease in recovery of the former activity (not shown). Re-

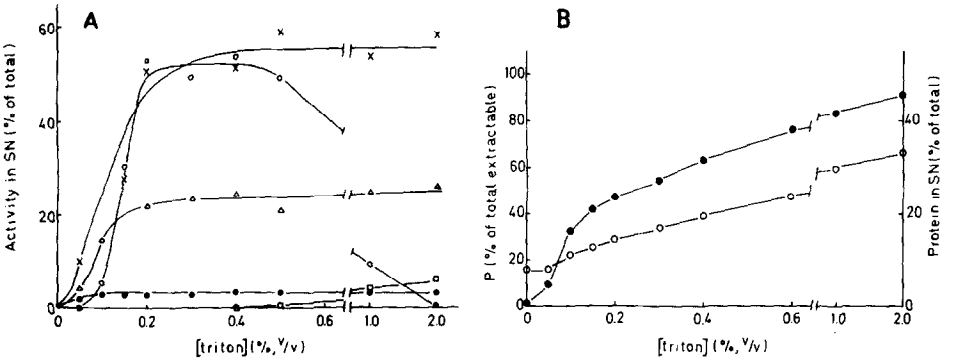

Fig. 2. Effect of Triton X-100 on solubilization of various activities (A) and protein and phospholipids (B) from submitochondrial particles. Assays were (A): o, transhydrogenase; Δ, NADH dehydrogenase; x, succinate dehydrogenase; ●, ATPase; □, cytochrome oxidase. (B): o, protein, ●, phospholipids. Incubation mixture was the same as that described in Fig. 1 except that 14 mg EDTA particles was added.

lease of phospholipids started at about 0.1 % and increased continuously to over 90 % at 2 % Triton (Fig. 2B) whereas only about 30 % of total protein was solubilized at this concentration of Triton (Fig. 2B). Accordingly, the specific activity of transhydrogenase recovered in the supernatant was increased about 4 fold as compared to submitochondrial particles. A decrease of the concentration of protein at fixed concentration of Triton (0.3 %) led to an increased degree of solubilization without inactivation of any of the activities tested (not shown). Maximal solubilization of transhydrogenase was found to be close to 90 %.

Phospholipids, in particular lysophosphatides, e.g., lysolecithin, have been reported by Komai et al. (19) to be effective solubilizing agents for membrane-bound proteins. As may be seen in Fig. 3A solubilization of transhydrogenase and succinate dehydrogenase by lysolecithin was considerably more selective as compared to that by Triton; higher concentrations of lysolecithin (about 1 %) were not inhibitory. Preliminary data indicate that the lysolecithin extract was devoid of all cytochromes except cytochrome c. A maximal solubilization of transhydrogenase of about 80 % was achieved at 0.08 % lysolecithin. Moreover, a decrease of the concentration of protein at fixed

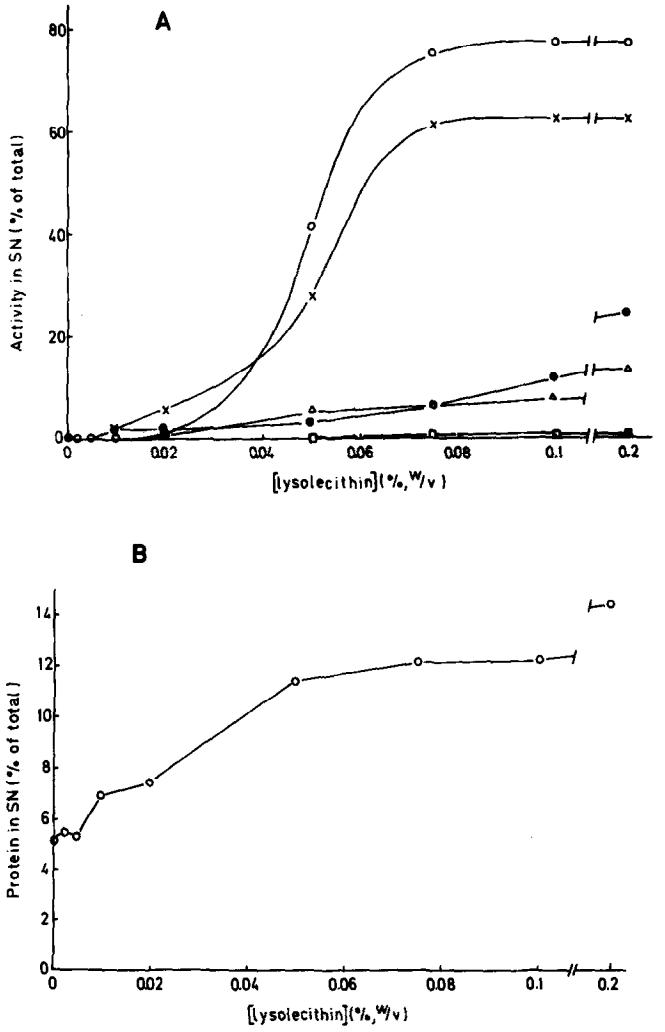

Fig. 3. Effect of lysolecithin on solubilization of various activities (A) and protein (B) from submitochondrial particles.
Assays were (A): o, transhydrogenase; Δ, NADH dehydrogenase; x, succinate dehydrogenase; ●, ATPase; □, cytochrome oxidase. Incubation mixture was the same as that described in Fig. 2 except that Triton was exchanged for lysolecithin.

concentration of lysolecithin (0.1 %) did not alter significantly the extent of solubilization of the enzymes tested (not shown). Recovery of protein in the supernatant amounted to only about 12 % (Fig. 3B) giving an increase in specific activity of 7 fold as compared to submitochondrial particles; release of phospholipids was not measured due to interference with added lysolecithin.

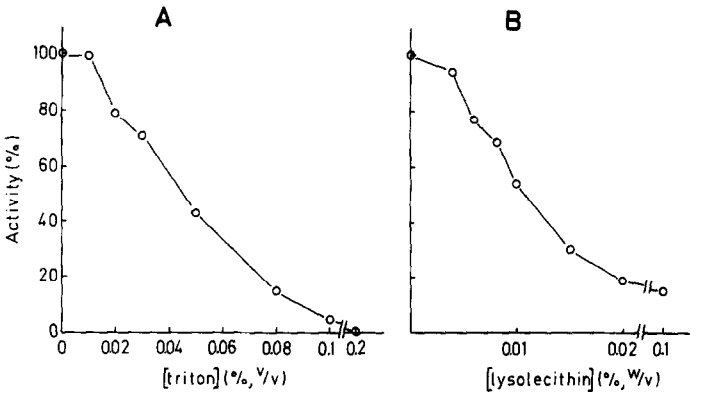

Fig. 4. Effect of Triton X-100 (A) and lysolecithin (B) on energy-linked transhydrogenase.
Composition of incubation mixture was the same as that described in Figs. 2 and 3 except that the final volume was 1 ml. After 30 min at 0°C aliquots of 0.2 ml were removed and assayed for energy-linked transhydrogenase using the alcohol dehydrogenase system (cf. Methods).

Figs. 4A and 4B show the effect of Triton and lysolecithin on the energy-linked transhydrogenase reaction (corrected for the nonenergy-linked reaction (cf. Methods)) using ascorbate plus phenazine methosulphate as energy source. Both Triton and lysolecithin were inhibitory at concentrations below those that detach the enzyme from the membrane. A striking difference was, however, that at high concentration of lysolecithin the activity remaining associated with the submitochondrial vesicles according to Fig. 3A appeared to be coupled to energy; this was not the case with Triton.

DISCUSSION

The present data show that Triton X-100 and lysolecithin readily detach transhydrogenase from submitochondrial particles. In the former case succinate dehydrogenase, ATPase and NADH dehydrogenase were solubilized to various extents together with transhydrogenase whereas with lysolecithin a more selective solubilization of transhydrogenase and succinate dehydrogenase occurred. The 29,000 MW protein extracted with lysolecithin from submitochondrial particles by Capaldi et al. (20) may therefore be identical to a subunit of either of the two latter enzymes (cf. ref. 21). Selective solubilization of

ATPase by cardiolipin has been reported earlier by Toson et al. (22). Lee et al. (23) showed later that this solubilization involves a specific binding of cardiolipin to the membrane. Specific solubilization of transhydrogenase and succinate dehydrogenase by lysolecithin may reflect a similar phenomenon. It remains to be established, however, if the specificity exerted is dependent on general physical properties of the detergent rather than structural properties. A complete investigation of various phospholipids with respect to their solubilization properties is presently being carried out.

Energy-coupling in lysolecithin-treated submitochondrial particles reported by Komai et al. (19) and Hunter et al. (24) is supported by the occurrence of energy-linked transhydrogenation in these particles. However, the problem whether this coupling occurs with a preparation that contains closed vesicles or nonvesicular complexes (cf. ref. 19,24) appears as yet unsolved

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