THE RECONSTITUTION OF THE MITOCHONDRIAL ENERGY-LINKED TRANSHYDROGENASE

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SUMMARY: Complex I (NADH-ubiquinone reductase) catalyzes pyridine nucleotide transhydrogenase at rates several fold higher than those found in submitochondrial particles from bovine heart. An ATP-dependent reduction of $NADP^+$ by NADH was demonstrated after combination of Complex I with phospholipids, hydrophobic proteins derived from bovine heart mitochondria, and mitochondrial ATPase (F_1)[1]. The reaction was inhibited by oligomycin, uncoupling agents and low concentrations of Triton X-100.

Hatefi and Hanstein (1) have shown that pyridine nucleotide transhydrogenase activity is found in "Complex I" from bovine heart mitochondria and that this activity resembles the non-energy-linked transhydrogenase activity of submitochondrial particles (2, 3), with respect to its response to pH and to inhibition by palmitoyl-SCoA. It has been suggested that the energy-linked (4) and non-energy-linked transhydrogenase reactions may be catalyzed by the same enzyme system in mitochondria (e.g. 5, 6) and in this communication we describe the restoration of ATP-dependent pyridine nucleotide transhydrogenase activity of Complex I.

METHODS: Soybean phospholipids (7), submitochondrial particles from bovine heart (8), Complex I (9), and F_1 (10) were prepared as described in the references. Hydrophobic proteins were prepared by extraction of submitochondrial particles with cholate and subsequent ammonium sulphate fractionation (11). Reconstitution of energy-linked transhydrogenase consisted of Complex I, hydrophobic proteins and phospholipids, and was performed as

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[1]Abbreviations: F_1, coupling factor 1 (ATPase); FCCP p-trifluoromethoycarbonylcyanide phenylhydrazone.

previously described (7). Binding of F_1 to the vesicles was achieved by incubation for 10 minutes at room temperature. Energy-linked transhydrogenase was measured (12) with the inclusion of an ATP regenerating system consisting of phosphoenolpyruvate and pyruvate kinase. Protein was estimated by the biuret method (13). Phospholipid concentrations were determined by phosphate analysis (14).

RESULTS: Figure 1 shows the activity of the reconstituted energy-linked transhydrogenase. Addition of $NADP^+$ caused a slow increase at 340 nm, attributable to non-energy-linked transhydrogenation. Addition of ATP resulted in a considerable stimulation of this rate, similar to that observed with submitochondrial particles.

As indicated in Table I, energy-linked transhydrogenase activity was greatly reduced by omission of F_1. The residual activity is probably a consequence of incomplete removal of F_1 from the "hydrophobic proteins" (7). Crude hydrophobic protein preparations are also contaminated with NADH

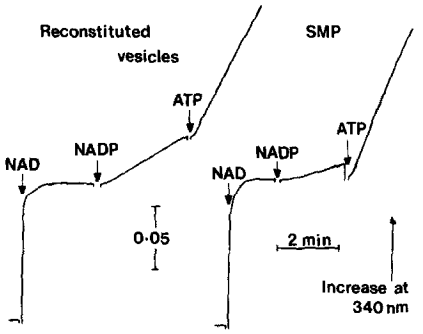


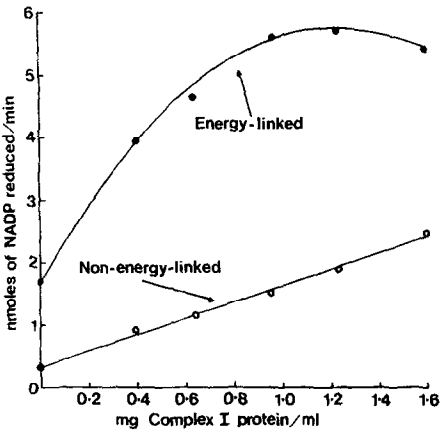
Fig. 1. Comparison of the energy-linked transhydrogenase activity of the reconstitution system (reconstituted) and of submitochondrial particles (SMP). Reconstitution was performed with 2 mg of hydrophobic proteins, 16 µmoles of phosphatidylethanolamine, 4 µmoles of phosphatidylcholine and 0.64 mg of Complex I in 1 ml. 0.2 ml (0.5 mg of protein) aliquots of the reconstituted system were supplemented with 20 µg of F_1 and then mixed with 250 µmoles of sucrose, 50 µmoles of Tris-acetate, pH 7.0, 10 µmoles of $MgSO_4$, 57 µmoles of ethanol, 10 µg of rotenone, 1.5 mg of defatted bovine serum albumin, 80 µg of alcohol dehydrogenase, 4 µmoles of phosphoenolpyruvate and 50 µg of pyruvate kinase in a final volume of 1 ml. Additions as indicated were 20 nmoles of NAD^+, 0.2 µmole of $NADP^+$ and 2 µmoles of ATP. 0.5 mg of submitochondrial particles were similarly treated and assayed. The demonstration of the activity (12) was done at temperature 30° in a 1 cm cuvette by measurement of the change of absorbance at 340 nm.

TABLE I. Effects of the Components on the Reconstitution of Energy-Linked Transhydrogenase. Reconstitution and assay were conducted as described in Fig. 1, except that 0.96 mg of Complex I per ml was used. The rates shown are corrected for the non-energy-linked rate.

System	Energy-linked transhydrogenase activity
	nmoles/min
Complete	5.0
- F_1	1.5
- Complex I	1.0
+ Oligomycin (5 μg/ml)	0.13
+ FCCP (0.25 μg/ml)	0.20
+ Triton X-100 (0.1%)	0.90

dehydrogenase (7). The latter shows transhydrogenase activity (15), since a slow energy-linked transhydrogenase reaction could be demonstrated in the system composed of hydrophobic proteins and phospholipids alone. This rate, however, was only about 20% of the rate obtained in the presence of Complex I (Table I). The reconstituted ATP-dependent transhydrogenase activity was inhibited by oligomycin, FCCP and low concentrations of Triton X-100, sufficient to cause lysis of the vesicles. The non-energy-linked rate was unaffected by these reagents.

In Fig. 2 the effect on the reconstitution of varying the concentration of Complex I is demonstrated. The non-energy-linked transhydrogenase activity increased linearly with increasing Complex I concentration, but the energy-linked rate reached saturation at about 1 mg of Complex I protein per ml. This behavior follows from the previously demonstrated maximum incorporation of Complex I into the reconstitution system (7). The non-energy-linked rate would, however, be independent of the degree of incorporation.



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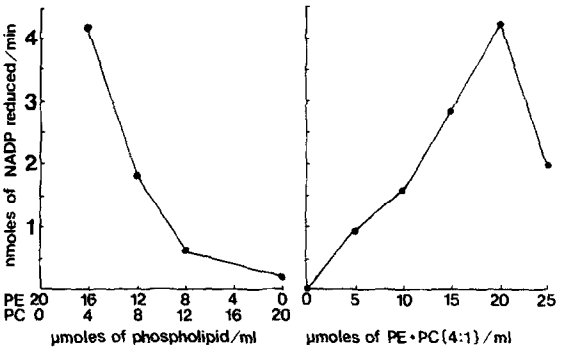

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Fig. 2. Energy-linked and non-energy-linked transhydrogenase as a function of Complex I concentration. Reconstitution and assay were as described in Fig. 1, except that the Complex I concentration was varied as indicated. The curve labeled "non-energy-linked" is the rate obtained on adding $NADP^+$. The curve labeled "energy-linked" is the rate obtained on adding ATP, after subtraction of the non-energy-linked rate.

Fig. 3. The effect of phospholipid composition and concentration on the reconstitution of the energy-linked transydrogenase. Reconstitution and assay were as described in Fig. 1, except that the phospholipid was varied as indicated, and the Complex I concentration was 0.96 mg of protein/ml. PE, phosphatidylethanolamine; PC, phosphatidylcholine.

At the lowest concentration of Complex I employed in the experiment of Fig. 2, the specific activities of non-energy-linked and energy-linked transhydrogenase were 2.0 and 8.2 nmoles/min/mg of protein, respectively. Under the same conditions of assay, the rates obtained with submitochondrial particles were 2.6 and 17.0 nmoles/min/mg of protein, respectively. In the case of the reconstituted transhydrogenase, both rates were increased by increasing the Complex I concentration but the ratio of energy-linked to non-energy-linked activity declined. This ratio of energy-linked to non-energy-linked activity was maximally 4.1 in reconstituted system which was 63% of the corresponding ratio (i.e. 6.5) of submitochondrial particles.

The effect of varying the phospholipid concentration and composition in reconstitution is shown in Fig. 3. A molar ratio of phosphatidyl-

ethanolamine to phosphatidylcholine of 4:1 was the most effective. It was not possible to test phosphatidylethanolamine alone since precipitation occurred in the assay after addition of Mg^{++}. Increasing the proportion of phosphatidylcholine caused a marked decrease in the rate of energy-linked transhydrogenase.

Figure 3 also shows the reconstituted activity as a function of the phospholipid concentration with the molar ratio of phosphatidylethanolamine to phosphatidylcholine at 4:1. The optimum concentration was found to be 20 μmoles of phospholipid per ml. Both the optimal concentration and composition are the same as those found for the reconstitution of oxidative phosphorylation at Site I (7).

DISCUSSION: The reconstituted ATP-driven transhydrogenase described here is comparable to that catalyzed by submitochondrial particles in its specific activity, its sensitivity to uncoupling agents and oligomycin, and in its requirement for F_1. These facts illustrate again the general applicability of the methods used to reconstitute other mitochondrial energy-linked reactions (11, 16, 7). To date, energy-driven electron transport has not previously been observed in reconstituted systems. The demonstration in such systems of ATP-driven reversed electron transport through the Site I region, for example, may be complicated by the high energy requirement and interference from forward electron transport through uncoupled respiratory complexes. Neither of these difficulties is encountered with the transhydrogenase reaction where the uncoupled reaction proceeds in the same direction as the coupled reaction, and the former is selectively suppressed by the presence of Mg^{++} and ATP in the assay (17).

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