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On the reversibility of the energy-linked transhydrogenase

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SUMMARY

In submitochondrial particles derived from beef-heart mitochondria, the reduction of NAD^+ by NADPH can furnish sufficient energy to phosphorylate ADP to ATP. Additional evidence for the reversibility of the energy-linked transhydrogenase was obtained by showing that the reduction of NAD^+ by NADPH leads to enhancement of the 8-anilinonaphthalene sulphonic acid fluorescence, and to a red shift in the spectrum of reduced cytochrome *b*.

Ever since the discovery by Danielson and Ernster[1,2] of an energy-linked transhydrogenation in submitochondrial particles, the question of the reversibility of this reaction has intrigued many investigators. It is now firmly established that one high-energy bond is expended in the transfer of two reducing equivalents from NADH to $NADP^+$ (refs. 2–5).

Recently Skulachev and coworkers[6–8] showed that the addition of NADPH and NAD^+ to suspensions of submitochondrial particles or chromatophores led to energization of the membranes, as measured by the uptake of lipid-soluble anions. This important result demonstrated for the first time that the energy-linked transhydrogenase reaction is reversible.

Prompted by their findings, we have examined whether the reversibility of the energy-linked transhydrogenase reaction could be demonstrated by other criteria as well. It was found that reversal of the transhydrogenase leads to induction of the energy-dependent red shift of cytochrome *b*[9,10], and to enhancement of 8-anilinonaphthalene sulphonic acid (ANS) fluorescence[11]. Furthermore, it was found that the reduction of NAD^+ by NADPH can furnish sufficient energy to synthesize ATP from ADP and phosphate.

Abbreviation: ANS, 8-anilinonaphthalene sulphonic acid.

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In Fig. 1 the effects of addition of the components of the transhydrogenase reaction on the ANS fluorescence and on the cytochrome *b* red shift are shown. Increased fluorescence or increased absorbance reflects energization of the submitochondrial particles. This was only observed when the concentrations of the nicotinamide nucleotides were such that the net reaction of the transhydrogenase was in the direction of reduction of NAD^+. It can be seen that equilibration resulted in a disappearance of the response of the probes. The de-energization was strongly accelerated by addition of uncoupler or of $NADP^+$. In agreement with Skulachev and coworkers[6,7] it was found that when the concentrations of the nicotinamide nucleotides were such that the net reaction of the transhydrogenase was in the direction of reduction of $NADP^+$ there was no response. Similar results were obtained with ammonia particles[12] recoupled by oligomycin (not shown). The extent of the responses recorded in Fig. 1 was diminished by 50% by the addition of 30 mM KCl, by 80% when 0.2 μg valinomycin was added together with KCl, and by nearly 100% when KCl, 0.2 μg valinomycin and 0.2 μg nigericin were added.

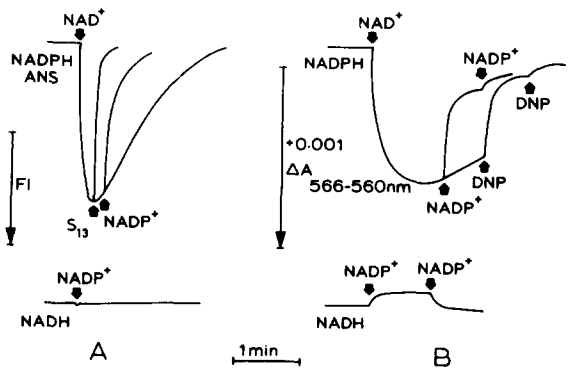

Fig. 1. The response of ANS fluorescence and cytochrome *b* absorbance upon addition of NADPH and NAD^+. A. Response of ANS fluorescence. Mg–ATP particles[14] (1.0 mg protein) were suspended in a medium (final volume, 2 ml) containing 250 mM sucrose, 20 mM Tris acetate (pH 7.5), 5 mM $MgCl_2$, 5 mM KCN, 5 μg rotenone and 10 μM ANS at 25°. After adjustment of the fluorescence reading upon addition of 0.15 mM NAD(P)H, the reaction was started by adding 0.15 mM $NAD(P)^+$. Further additions were 0.15 mM $NADP^+$ and 0.25 μM of the uncoupler S_{13}. Excitation wavelength 313 + 366 nm, emission at 470–3000 nm. Fluorescence was measured in an Eppendorf fluorimeter. The units in the figure are arbitrary. B. Response of cytochrome *b* absorbance. Mg–ATP particles (1.1 mg protein) were suspended in a medium (final volume, 0.9 ml) containing 250 mM sucrose, 20 mM Tris acetate (pH 7.5), 5 mM $MgCl_2$, 10 mM succinate, 10 mM ascorbate, 50 μM *N,N,N',N'*-tetramethyl-*p*-phenylene diamine, 6 mM Na_2S and 5.4 μg rotenone at 25°. After addition of 0.5 mM NAD(P)H the reaction was started by adding 0.5 mM $NAD(P)^+$. Further additions were 0.5 mM $NADP^+$ and 0.2 mM 2,4-dinitrophenol (DNP). Absorbance changes were recorded in a dual-wavelength spectrophotometer at 566–560 nm.

The results of measurements of ATP synthesis coupled to the transhydrogenase reaction are reported in Table I. In these experiments regenerating systems for NADPH and NAD^+ were used. Omission of the regenerating enzymes resulted in a markedly decreased ^{32}P incorporation. When glucose-6-phosphate dehydrogenase was omitted and NAD was kept completely reduced by having 150 mM ethanol, 15 I.U. alcohol dehydrogenase and 2 mM semicarbazide present, ATP formation was decreased from 22 to 10.8 nmoles (Expt. 1). This latter value was approximately equal to that found in the control without

alcohol dehydrogenase, and gives the maximal contribution of exchange reactions *plus* NADH oxidation to the ^{32}P incorporation in the system. In parallel experiments the rate of NAD reduction in the absence of alcohol dehydrogenase was measured. In the absence of other additions this varied between 70 and 90 nmoles/min per mg protein.

TABLE I

SYNTHESIS OF ATP COUPLED TO THE TRANSHYDROGENASE REACTION

A reaction mixture (final vol., 1 ml) containing 200 mM sucrose, 20 mM Tris acetate (pH 7.5), 30 mM glucose, 5 mM $MgCl_2$, 2 mM ADP, 15 mM glucose 6-phosphate, 15 mM acetaldehyde, 10 mM phosphate buffer (pH 7.5; 3 μC/ml ^{32}P), 4 mM Na_2S, 0.5 mM $NADP^+$, 5 units hexokinase, 5 units glucose-6-phosphate dehydrogenase and 15 units alcohol dehydrogenase was equilibrated for 5 min at 30°. After this equilibration, 0.5 mg Mg–ATP particles (pretreated at 0° for 5 min with 2 μg rotenone and 0.2 μg antimycin per mg protein) was added. The reaction was started 1 min later by adding 0.5 mM NAD^+. After 10 min, the reaction was stopped with 1 ml 10% trichloroacetic acid. Inorganic phosphate was extracted from the deproteinized reaction mixture according to Nielsen and Lehninger[15]. Radioactivity in organic phosphate was counted in a Nuclear Chicago gas-flow counter. All enzymes used were obtained from Boehringer and were dialysed for two periods of 3 h each against an excess of 10 mM Tris acetate buffer (pH 7.5) prior to use.

Expt.	*Omissions*	*Additions*	*ATP formation (nmoles/min per mg protein)*
1	None	None	22
	Alcohol dehydrogenase	None	11
	Glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, $NADP^+$	NADPH	7
	Glucose-6-phosphate dehydrogenase, acetaldehyde	Ethanol, semicarbazide	10.8
	None	Oligomycin (10 μg)	1.2
	None	S_{13} (0.2 μM)	1.0
2	None	None	13.8
	Alcohol dehydrogenase	None	5.9
3	None	None	22.4
	Alcohol dehydrogenase	None	13.5
4	None	None	10.8
	Alcohol dehydrogenase	None	5.3
	None	$(NH_4)_2SO_4$ (30 mM)	6.4
	None	$(NH_4)_2SO_4$ (100 mM)	3.5

The ATP synthesis was more than 90% inhibited by oligomycin and by the uncoupler S_{13}. The velocity of ^{32}P incorporation was constant for at least 10 min in all cases. Table I also shows that $(NH_4)_2SO_4$ inhibited the formation of ATP. However, the rate of NADH formation (alcohol dehydrogenase absent) was also inhibited. It is of interest that the reaction in the opposite direction, the energy-dependent reduction of $NADP^+$ by NADH, is *stimulated* by NH_4Cl (ref. 13).

In summary, it can be said that all parameters show that the energy-linked

transhydrogenase reaction is in reversible equilibrium with the energy-conserving system of submitochondrial particles.

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