Energy-linked Transhydrogenase

EFFECTS OF VALINOMYCIN AND NIGERICIN ON THE ATP-DRIVEN TRANSHYDROGENASE REACTION CATALYZED BY RECONSTITUTED TRANSHYDROGENASE-ATPase VESICLES

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Reconstituted transhydrogenase-ATPase vesicles obtained with purified beef heart transhydrogenase and oligomycin-sensitive ATPase were investigated with respect to the mode of interaction between the two proton pumps, with special reference to the relative contributions of the membrane potential and proton gradient using valinomycin and nigericin in the presence of potassium. In the absence of ionophores and at low ATP concentrations, below 20 μM, the ATPase generated a proton motive force which was predominantly due to a membrane potential, whereas at saturating concentrations of ATP the proton gradient was the predominant component. The ATP-dependence of the rate of the ATP-driven transhydrogenase reaction showed apparent \( K_m \) values in the low and high ATP concentration range of about 3 and 56 μM, respectively, with a corresponding difference in \( V_{max} \) of about 3-fold. It is concluded that the reconstituted transhydrogenase can utilize both a membrane potential and a proton gradient, separately or combined, where the relative contributions of these components depend on the activity of the ATPase. In the reconstituted vesicles, the maximally active transhydrogenase is apparently driven by an electrochemical proton gradient where the membrane potential and the proton gradient contribute one-third and two-thirds, respectively. The rate-dependent relative generation of a proton motive force and pH gradient presumably reflects the proton pump characteristics of the ATPase and/or buffering/permeability characteristics of the vesicles rather than the properties of the transhydrogenase per se. These results are discussed in relation to current models for transhydrogenase-linked proton translocation.

Mitochondrial nicotinamide nucleotide transhydrogenase (EC 1.6.1.1.) is an integral protein localized in the inner membrane catalyzing the reversible transfer of hydride ion between NADH and NADP⁺. The enzyme has been purified and shown to be a dimer of 2 identical subunits of approximately 109 kDa each. It is a \( N,N' \)-dicyclohexylcarbodiimide-sensitive proton pump which, when reconstituted in liposomes, has been shown to catalyze a reduction of NAD⁺ by NADPH associated with an inward translocation of one proton/NADP⁺ formed. In submitochondrial particles, the proton motive force generated by a second proton pump, e.g. the ATPase or either of the electron transport-driven proton pumps of the respiratory chain, is utilized by the transhydrogenase through an outward translocation of protons. In submitochondrial particles the latter translocation stimulates the rate of reduction of NADP⁺ (or analogs) by NADH and shifts the nicotinamide nucleotide ratio \([\text{NADPH}] / [\text{NADP}^+]\) from the thermodynamic equilibrium of close to unity to approximately 500 (for reviews see Refs. 1–3). In this manner the mitochondrion and the cytoplasm may achieve an increased availability of NADPH (cf. 4). Transhydrogenase from beef heart was recently sequenced and found to have an exact molecular weight of 109,212 (5).

The demonstration that the reaction catalyzed by the purified and reconstituted transhydrogenase is electrogenic (6, 7) and involves proton translocation (8–10), led to the suggestion that the energy-linked transhydrogenase is operating by a chemiosmotic mechanism (2, 3, 11). However, mainly due to the lack of a suitable model system, the detailed characteristics of especially the ATP-driven reaction have not yet been investigated. Coreconstitution of purified mitochondrial transhydrogenase and oligomycin-sensitive ATPase has recently been shown to produce vesicles that catalyze a pronounced ATP-dependent stimulation of the rate of reduction of NADP⁺ by NADH as well as an increased nicotinamide nucleotide ratio (12). At low concentrations of ATP, hydrolysis of 1 mol of ATP was found to give 3 mol of NADPH, consistent with an inward translocation of three protons/ATP hydrolyzed and one proton/NADPH formed (12). Incorporation of bacteriorhodopsin in place of ATPase gave a light-driven transhydrogenase (13), indicating that the interaction between transhydrogenase and the primary proton pump occurs through a bulk proton motive force. That also transhydrogenase and ATPase in reconstituted transhydrogenase-ATPase vesicles as well as in submitochondrial particles interact through a delocalized chemiosmotic coupling was shown by inhibitor-uncoupler titrations (14).

Transhydrogenase-ATPase and transhydrogenase-bacteriorhodopsin vesicles thus offer a unique model system for further elucidating the detailed mechanism of interaction between transhydrogenase and a primary proton pump in the absence of other proteins. In the present investigation transhydrogenase-ATPase vesicles were used for clarifying the relative contributions of a membrane potential and a pH gradient in the ATP-driven transhydrogenase reaction at different rates of ATP hydrolysis.

**MATERIALS AND METHODS**

Preparation of Transhydrogenase and ATPase—Nicotinamide nucleotide transhydrogenase from beef heart was prepared essentially.
as described previously (10), except that the medium used for wash and elution of the enzyme bound to the calcium phosphate gel was 2 mM sodium phosphate containing 0.5% potassium cholate, and 200 mM sodium phosphate containing 0.5% potassium cholate, respectively. Preparations of mitochondrial oligomycin-sensitive ATPase (complex V) and a similar preparation (38–45F), both from beef heart submitochondrial particles, followed the procedure described by Stigall et al. (15).

Reconstitution of Transhydrogenase-ATPase Vesicles—Reconstitution was carried out essentially as described previously (12). A phospholipid mixture composed of 42.5% phosphatidylcholine, 42.5% phosphatidylethanolamine, 10% lysophosphatidylcholine, and 5% phosphatidylserine (all w/w) was dried under nitrogen, redissolved in dimethyl sulfoxide, and suspended to a final concentration of 20 mg/ml in a reconstitution buffer composed of 4 mM Hepes (pH 7.5), 50 mM potassium sulfate, 10% methanol, 1 mM EDTA, and 1 mM dithiothreitol. Liposomes were prepared by sonication of the phospholipid suspension to clarity in a bath-type sonicator (80W, Laboratory Supply Co., Inc., Hicksville, NY). Reconstitution of transhydrogenase by the cholate dialysis procedure (cf. 6, 7, 15) was carried out by mixing (in this order) transhydrogenase (in 0.5% potassium cholate, 200 mM sodium phosphate, pH 7.5), varying amounts of ATPase and liposomes, 20% potassium cholate to a final concentration of 1%, followed by dialysis overnight at 0°C against 1 liter of reconstitution buffer. The ATP-dependent activity of vesicles was measured at 4°C and were stable for at least 1 week.

**Assays**—Transhydrogenase activity was assayed essentially as described using the NADP⁺ analog tNADP⁺ (17). Absorbance changes were followed at the wavelengths 355–460 nm. Rates of reduction of tNADP⁺ were calculated using an extinction coefficient of 16.7 μM⁻¹ cm⁻¹ (17). The assay mixture contained 200 μM tNADP⁺, 200 μM NADH, and 2 mM MgCl₂ in 80 mM potassium phosphate, pH 7.5. These concentrations of tNADP⁺ and NADH were saturating under the conditions used. The ATP-dependent transhydrogenase reaction was assayed at room temperature (23°C) by the addition of varying concentrations of ATP and, when the concentration of ATP was 1 mM, with an ATP-regenerating system composed of 10 μg of dialyzed pyruvate kinase and 2 mM phosphoenolpyruvate. Final volume was 1 ml. The ATP-dependent activity, which was always 100% inhibited by 10 μg of oligomycin, was taken as the difference between the activity obtained after and prior to the addition of ATP, corrected for the slight activation (less than 10%) obtained by CCCP in the absence of ATP.

The spectrophotometric assays were carried out with an Aminco DW-2 dual wavelength spectrophotometer adjusted for a maximal signal/noise ratio at the wavelengths used. In order to minimize noise due to particles (bubbles, etc.) 5 s were allowed to pass after each addition/mixing before recording the trace; this procedure did not affect the initial rate measurement. The reaction was started by the addition of vesicles following the addition of ATP.

In order to assay the transhydrogenase/ATPase activity ratio of the vesicles, radioactive ATP (0.1 μCi/ml) was added to the transhydrogenase assay mixture, the absorbance changes were measured as described above, and 0.2-ml samples were withdrawn after 1 and 2 min for extraction and estimation of released inorganic phosphate (15). The fluorescence response of ACMA was monitored with an assay mixture containing 80 mM potassium phosphate, pH 7.5, 2 mM MgCl₂, 200 μM NADH, 200 μM tNADP⁺, 1 μM ACMA, 2 mM phosphoenolpyruvate, pyruvate kinase, and transhydrogenase-ATPase vesicles (30 μg/ml). The fluorescent changes were assayed in an Aminco-Bowman fluorimeter using excitation and emission wavelengths of 418 and 480 nm, respectively.

**Chemicals**—Chromatographically pure phosphatidylcholine (egg), phosphatidylethanolamine (egg), lysophosphatidylcholine (egg), and phosphatidylserine (bovine spinal cord) were purchased from Lipid Products (Nutfield, United Kingdom). ACMA was prepared as described (20). γ-32P-labeled ATP was purchased from Amersham, United Kingdom. Oxonol VI was a gift from Dr. W. Hanstein (University of Tubingen, Bochum, Federal Republic of Germany (F. R. G.). Cholic acid was purified and potassium cholate prepared as described (15). Other chemicals were purchased either from Sigma or Boehringer (Mannheim, F. R. G.).

**RESULTS**

As shown in Fig. 1, trace A, 1.25 mM ATP caused a 20-fold stimulation of the rate of reduction of tNADP⁺ by NADH catalyzed by reconstituted transhydrogenase-ATPase vesicles, which was partially inhibited by valinomycin. Substitution of valinomycin by a low concentration of oligomycin (0.12 μM) gave a similar extent of inhibition (trace B), whereas a high concentration of oligomycin (12 μM) brought about a complete inhibition (trace C). However, when the experiments in traces A–C were repeated with 10 μM ATP, the inhibition by valinomycin was close to complete (trace D). In contrast, the inhibitions by the low and high concentrations of oligomycin were essentially unchanged (traces E and F).

In order to further establish the correlation between concentration of ATP, ATP-driven transhydrogenase activity and inhibition by valinomycin and oligomycin, the ATP-driven transhydrogenase reaction was measured as a function of ATP concentration in the absence and in the presence of oligomycin or valinomycin (Fig. 2). For the sake of clarity the high ATP concentration range, 5–2500 μM (A), is shown separate from the entire ATP concentration range investigated, 1–2500 μM (B). In the absence of inhibitors, the ATP dependence of the transhydrogenase activity did not follow Michaelis-Menten kinetics but was apparently biphasic above

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**Fig. 1. Effect of valinomycin and oligomycin on ATP-driven transhydrogenase activity catalyzed by reconstituted transhydrogenase-ATPase vesicles.** ATP-driven transhydrogenase activity catalyzed by reconstituted transhydrogenase-ATPase vesicles was measured in the presence of high or low concentrations of ATP, oligomycin, and valinomycin. The reaction mixture contained 15 μl of vesicles. Additions were: A, 1250 μM ATP and valinomycin (0.45 μM); B, 1250 μM ATP and oligomycin (0.12 μM); C, 1250 μM ATP and oligomycin (12 μM); D, 10 μM ATP and valinomycin (0.45 μM); E, 10 μM ATP and oligomycin (0.12 μM); F, 10 μM ATP and oligomycin (12 μM).
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FIG. 2. Double reciprocal plot of ATP-driven transhydrogenase activity as a function of ATP concentration at 5-2500 µM (A) and 1-2500 µM (B), in the absence and in the presence of oligomycin and valinomycin. ATP-driven transhydrogenase activity catalyzed by reconstituted transhydrogenase-ATPase vesicles (ves.) was assayed in the presence of 1-2500 µM ATP. Additions were: (○—○), none; (●—●), valinomycin (val, 0.45 nM); (□—□), oligomycin (oligo, 0.12 µM). The extrapolated lines intercept at the $-1/K_m$ value on the abscissa for the low and high ATP range, respectively.

At approximately 30 µM ATP, and monophasic below this concentration (A and B). The two extreme phases obtained at high and low ATP concentrations corresponded to apparent $K_m$ values of about 3 and 56 µM, respectively (A and B). The difference in the corresponding $V_{max}$ values was about 3-fold. At low ATP concentrations, addition of valinomycin gave a pronounced inhibition, which decreased relatively with increasing ATP concentrations. Addition of oligomycin caused an inhibition that was essentially constant in the concentration range of ATP tested.

The concentration dependences of the inhibition of the ATP-driven transhydrogenase reaction by valinomycin as well as by nigericin and CCCP were then determined at two concentrations of ATP, 5 µM and 1.5 mM, representing the low and high ATP ranges, respectively (Fig. 3). The activity in the low ATP range was abolished by 125 nM valinomycin (A), whereas the activity in the high ATP range was unaffected by up to 125 nM valinomycin and was inhibited only 35% by 500 nM valinomycin (A). Nigericin inhibited both the low and high ATP activities with a more pronounced effect on the low ATP concentration activity (B). In this case, the difference in sensitivity between the two activities was smaller as compared to valinomycin (B). CCCP also had a more pronounced effect on the low ATP activity (C).

A possible interpretation of the results in Figs. 2 and 3 is that the ATP-driven transhydrogenase activities obtained with 5 µM and 1.5 mM ATP reflect different populations of transhydrogenase-ATPase vesicles with different properties, e.g. as revealed by different sensitivities to oligomycin and/or different behavior with regard to incorporation and function in vesicle membranes. This possibility of different ATPase populations appears unlikely in view of the experiments in Figs. 4 and 5, in which the two activities were assayed in the presence of increasing concentrations of oligomycin (Fig. 4) or vesicles with various ATPase/transhydrogenase ratios (Fig. 5). In both cases the relative activities obtained with 5 µM and 1.5 mM ATP were affected similarly.

The finding that the selective effects of valinomycin and other inhibitors on the ATP-driven transhydrogenase reaction apparently depend on the rate of the reaction, may suggest that the composition of the driving ATP-induced proton motive force, i.e. the membrane potential and the pH...
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FIG. 4. Effect of oligomycin on the ATP-driven transhydrogenase reaction. ATP-driven transhydrogenase activity catalyzed by reconstituted transhydrogenase-ATPase vesicles was assayed in the presence of 5 μM ATP (●) or 1.5 mM ATP (○). Control specific activities were 2 and 10 μmol/min/mg, respectively. Oligomycin was added at the concentrations indicated.

FIG. 5. ATP-driven transhydrogenase catalyzed by transhydrogenase-ATPase vesicles containing a fixed amount of transhydrogenase and varying amounts of ATPase. Transhydrogenase-ATPase vesicles were reconstituted as described under "Materials and Methods" except that the ATPase concentration was varied as indicated in the abscissa. ATP-driven transhydrogenase was assayed in the presence of either 5 μM ATP (●) or 1.5 mM ATP (○). Specific activities at the ATP concentrations 5 μM and 1.5 mM and an ATPase/transhydrogenase ratio of 50 were 2 and 10 μmol/min/mg, respectively.

As shown in Figs. 6 and 7, the initial response of the membrane potential probe Oxonol VI (19) in the absence of ionophores increased with the concentration of ATP and reached a maximum at about 100 μM. At high ATP concentrations the membrane potential assayed with Oxonol VI was partially dissipated within minutes (Fig. 6, traces B and C), presumably due to its conversion into a pH gradient (see below). Addition of nigericin had little or no effect below 10 μM ATP (Fig. 6, traces A and B; Fig. 7), but the effect increased markedly with increasing concentrations of ATP (Fig. 6, trace C). At a concentration of 2.5 mM ATP, the addition of nigericin tripled the Oxonol response (Fig. 7). As expected, addition of valinomycin (Fig. 6, trace C) abolished the response. Essentially the same traces were obtained with transhydrogenase-ATPase vesicles in the absence of transhydrogenase substrates or vesicles containing only ATPase (not shown).

FIG. 6. ATP-dependent change in Oxonol response by transhydrogenase-ATPase vesicles. Changes in Oxonol response were determined in the presence of various concentrations of ATP. Additions were: trace A, 1 μM ATP and nigericin (0.14 μM); trace B, 10 μM ATP and nigericin (0.14 μM); trace C, 1250 μM ATP, nigericin (0.14 μM), and valinomycin (0.46 μM).

FIG. 7. Effect of nigericin and ATP concentration on ATP-dependent change in Oxonol response by transhydrogenase-ATPase vesicles. The Oxonol response was determined in the absence (●) and in the presence (○) of nigericin (0.14 μM) as a function of the concentration of ATP. The order of additions and other conditions were the same as in Fig. 6. 100% absorbance change corresponds to 0.09 A.
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ATP

**FIG. 8.** ATP-dependent ACMA response by transhydrogenase-ATPase vesicles. Quenching of ACMA fluorescence was assayed after addition of various concentrations of ATP. Additions were: A, 10 μM ATP; B, 33 μM ATP; C, 100 μM ATP; D, 1250 μM ATP.

**FIG. 9.** Effect of valinomycin on ATP-dependent ACMA response by transhydrogenase-ATPase vesicles. Quenching of ACMA fluorescence was assayed after addition of valinomycin and different concentrations of ATP. Additions were: A, 10 μM ATP, valinomycin (0.45 μM), and CCCP (4.9 μM); B, valinomycin (0.45 μM), 10 μM ATP, and CCCP (4.9 μM).

induced by 2.5 mM ATP in a concentration-dependent manner (Fig. 10, traces A–C).

**DISCUSSION**

The successful reconstitution of purified beef heart transhydrogenase with ATPase (12) or bacteriorhodopsin (13) in vesicles that show an energy-linked transhydrogenase activity similar or superior to that of submitochondrial particles, has provided ideal model systems for investigating the functional properties of transhydrogenase as a proton pump. The present study has aimed at a clarification of the relative roles of the membrane potential and proton gradient in driving the ATP-driven energy-linked transhydrogenase reaction, with emphasis on qualitative rather than quantitative aspects. Qualitative aspects have been emphasized because it is extremely difficult to accurately determine the magnitude of ΔpH and membrane potential in small vesicles of the type used in the present investigation, the reason being that the free bulk inside the vesicles is almost nonexistent and that most interactions therefore occur next to the membrane surface. Because different relative sizes of ΔpH and membrane potential often are related to the rate of proton pumping in coupling systems (22), proton pumping in the transhydrogenase-ATPase vesicles was varied by limiting the concentration of ATP in the range 1–2500 μM. That this concentration range indeed influences the activity of the ATPase is shown by the fact that the K_m of the reconstituted ATPase for ATP is in the mM range, probably above rather than below 5 mM (12).

The results show that, at a low ATPase activity, the membrane potential component predominates, whereas at a high ATPase activity the proton gradient predominates as driving force. The membrane potential-driven transhydrogenase has a K_m with respect to ATP of about 3 μM, whereas the proton gradient-driven transhydrogenase has a K_m which is close to 20-fold higher, i.e. about 56 μM. However, the difference in V_max is only 3-fold. The inhibitory effects of the ionophores valinomycin, nigericin, and CCCP on the rate of the ATP-driven transhydrogenase reaction showed that, at a low rate, all three ionophores were effective, whereas at a high rate nigericin and CCCP were still fairly effective but valinomycin essentially ineffective. In the case of nigericin it is possible that the effect of high concentrations may be due to uncoupling (cf. Figs. 3 and 6), a phenomenon observed previously. _Taken together these results show for the first time that the mitochondrial transhydrogenase can be driven in a reconstituted system either by a membrane potential or a proton gradient, or both, generated by ATP hydrolysis.

An interesting problem is whether this dependence of the transhydrogenase on a membrane potential and/or a proton gradient actually is related to the transhydrogenase, the ATPase and/or the permeability characteristics of the vesicular membrane. The fact that the dye responses are essentially independent of the presence of transhydrogenase, suggests that the ATP-dependent relative magnitude of ΔpH and membrane potential is unrelated to the transhydrogenase itself. At low ATP the predominating membrane potential could be due to the buffering capacity of the phosphatidylethanolamine headgroups for example and/or the permeability properties of the membrane. In this case transhydrogenase may be driven directly by the membrane potential or by a

2 J. Rydstrom, unpublished observations.
ΔpH generated locally in the transhydrogenase by the membrane potential. At high ATP the transhydrogenase is driven almost exclusively by a ΔpH.

Mechanistically, the possibility that the interaction of transhydrogenase with a membrane potential occurs by a conversion to a proton gradient through a proton well, or by a membrane potential-dependent increase in charge translocation across the enzyme, has been considered and discussed by Jackson and co-workers (23). Based on the pH dependence of the effect of membrane potential on the rate of the transhydrogenase in chromatophores from the photosynthetic bacteria *Rhodobacter capsulatus*, these workers favored the latter model (23, cf. 24). Although the present investigation has not aimed specifically at this problem, our data are consistent with the conclusion of Jackson and co-workers (23). One of the prerequisites of this model is that the rate of the energy-linked transhydrogenase should be essentially independent of the pH of the medium. Indeed, when reconstituted with a relatively pH-insensitive proton pump, e.g. bacteriorhodopsin, the resulting light-driven transhydrogenase activity was also essentially pH independent over a wide pH range (13).

In conclusion, it has been shown that reconstituted transhydrogenase-ATPase vesicles at a relatively low ATPase activity catalyze a membrane potential-driven energy-linked transhydrogenase reaction, whereas the reaction driven by a high ATPase activity involves a proton gradient or, depending on the conditions, a proton gradient plus a membrane potential. A common mechanistic basis for these two extreme situations remains to be elucidated.

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REFERENCES