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ATP-driven transhydrogenase provides an example of delocalized chemiosmotic coupling in reconstituted vesicles and in submitochondrial particles

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The mechanism of coupling between mitochondrial ATPase (EC 3.6.1.3) and nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) was studied in reconstituted liposomes containing both purified enzymes and compared with their behavior in submitochondrial particles. In order to investigate the mode of coupling between the transhydrogenase and the ATPase by the double-inhibitor and inhibitor-uncoupler methods, suitable inhibitors of transhydrogenase and ATPase were selected. Phenylarsine oxide and A3′-*O*-(3-(*N*-(4-azido-2-nitrophenyl)amino)propionyl)-NAD^+ were used as transhydrogenase inhibitors, whereas of the various ATPase inhibitors tested aurovertin was found to be the most convenient. The inhibition of the ATP-driven transhydrogenase activity was proportional to the inhibition of both the ATPase and the transhydrogenase. Inhibitor-uncoupler titrations showed an increased sensitivity of the coupled reaction towards carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) — an uncoupler that preferentially uncouples localized interactions, according to Herweijer et al. (Biochim. Biophys. Acta 849 (1986) 276–287) — when the primary pump was partially inhibited. However, when the secondary pump was partially inhibited the sensitivity towards FCCP remained unchanged. Similar results were obtained with submitochondrial particles. These results are in contrast to those obtained previously with the ATP-driven reverse electron flow. In addition, the amount of uncoupler required for uncoupling of the ATP-driven transhydrogenase was found to be similar to that required for the stimulation of the ATPase activity, both in reconstituted vesicles and in submitochondrial particles. Uncoupling of reversed electron flow to NAD^+ required much less uncoupler. On the basis of these results, it is proposed that, in agreement with the chemiosmotic model, the interaction between ATPase and transhydrogenase in reconstituted vesicles as well as in submitochondrial particles occurs through the $\Delta\tilde{\mu}_{H^+}$. In contrast, the energy transfer between ATPase and NADH-ubiquinone oxidoreductase appears to occur via a more direct interaction, according to the above-mentioned results by Herweijer et al.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NAP_3NAD^+, A3′-*O*-(3-(*N*-(4-azido-2-nitrophenyl)amino)propionyl)-NAD^+; $AcPyAD^+$, oxidized 3-acetylpyridine adenine nucleotide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Introduction

A major unsolved question in the field of energy transduction concerns the mechanism of interaction between energy-transducing complexes. The generally adopted chemiosmotic hypothesis states that a delocalized proton gradient across the

membrane constitutes the intermediate in energy transduction [1].

While it is generally agreed that the energy-transducing complexes can function as proton pumps [2], the chemiosmotic concept has been criticised on the basis of results obtained from thermodynamic and kinetic tests [3]. In the former case, it is reasoned that in a sequence of steps each of the intermediates should be of a lower energy content than its predecessor. Through measurements of the forces involved information on the stoichiometry of the coupling reactions can also be derived [4].

Kinetic tests of the chemiosmotic hypothesis primarily involve measurements of the rate of the proton movement in relation to the electron transfer and phosphorylation reactions. More recently, the so-called 'double-inhibitor titrations' have been applied again to the question of whether the interaction between energy-transducing complexes is of a localized or a delocalized nature [3,5–11]. Attempts have been made to investigate this matter by comparing the properties of reconstituted systems, consisting of complexes derived from unrelated organisms, with those of intact energy-transducing membranes [7]. Thus, it could be shown via inhibitor-uncoupler titrations that the behavior of reconstituted vesicles that contained ATPase from yeast and bacteriorhodopsin from *Halobacterium halobium* corresponded to that expected for a delocalized energy transduction. In contrast, energy-linked reversal of electron flow in submitochondrial particles indicated a localized behavior in some inhibitor-uncoupler titrations [10]. Double-inhibitor titrations seemed not to be discriminatory between localized and delocalized energy transduction.

Nicotinamide nucleotide transhydrogenase has recently been purified, reconstituted in vesicles and shown to be a proton pump. In the reconstituted system the proton/NADPH ratio of the transhydrogenase reaction has been shown to be 1 (for reviews, see Refs. 12 and 13), whereas in intact rat liver mitochondria the ratio has been claimed to be 2 [14,15]. The coupling between transhydrogenase and other proton pumps in the mitochondrial inner membrane, e.g., the ATPase, has been proposed to be indirect and delocalized [12,13,16] or localized [17–19]. Recently, the ATP-driven transhydrogenase reaction has been reconstituted by the incorporation of purified transhydrogenase and bovine heart mitochondrial ATPase in the same vesicle [20,21]. In the present paper we have extended the double-inhibitor and inhibitor-uncoupler titrations to the ATP-driven transhydrogenase catalyzed by reconstituted transhydrogenase-ATPase vesicles or submitochondrial particles. It is shown that, in contrast to the energy-linked reverse electron transfer in the respiratory chain, the ATP-driven transhydrogenase in both of these systems behaves according to the predictions for a delocalized system.

Materials and Methods

Preparations

Submitochondrial particles (MgMnATP) used in this study were prepared from beef heart mitochondria essentially according to the method of Hansen and Smith [22], with the modification that the Tris-HCl buffer was replaced by 10 mM Hepes/KOH (pH 7.5). Bovine heart transhydrogenase was prepared essentially as described by Persson et al. [23], except that the concentration of Triton X-100 used in the FPLC step was 0.08–0.1%, and that the media used for wash and elution of the enzyme from the calcium phosphate gel were 2 mM sodium phosphate containing 0.5% potassium cholate, and 200 mM sodium phosphate containing 0.5% potassium cholate, respectively.

Oligomycin-sensitive ATPase from beef heart was prepared according to the method of Stigall et al. [24].

Reconstitution of vesicles

Reconstitution of transhydrogenase and co-reconstitution of transhydrogenase and ATPase by a cholate dialysis procedure was carried out essentially as described [21], using a phospholipid mixture composed of 42.5% phosphatidylcholine, 42.5% phosphatidylethanolamine, 10% lysophosphatidylcholine and 5% phosphatidylserine (all w/w). The phospholipids were dried under nitrogen, redissolved in diethyl ether, dried and suspended to a final concentration of 20 mg/ml in a reconstitution buffer composed of 4 mM Hepes/KOH (pH 7.5), 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. For reconstitution by the cholate dialysis procedure [25], transhydrogenase in 0.5% potassium cholate/200 mM sodium phosphate (pH 7.5), ATPase and liposomes were mixed, dissolved by the addition of potassium cholate to a final concentration of 1% and dialyzed overnight at 0 °C against 1 l of reconstitution buffer. When transhydrogenase was reconstituted alone FCCP routinely caused a 30-fold stimulation of the activity. Transhydrogenase-ATPase vesicles catalyzed at least a 10–20-fold ATP-dependent stimulation of the reduction of thio-$NADP^+$ by NADH. The extent of stimulation of transhydrogenase and ATPase in the co-reconstituted system by uncouplers was about 4 and 1.5-fold, respectively. The corresponding values for the activities of submitochondrial particles were no change and 3-fold, respectively.

Assays

Transhydrogenase activity of the purified, soluble enzyme preparations was normally assayed as reduction of $AcPyAD^+$ by NADPH at 375–420 nm in a medium containing 80 mM Hepes/KOH (pH 7.5), 0.025% Triton X-100 and 0.4 mg/ml lysophosphatidylcholine (egg yolk) at 25 °C. The reaction was started by he addition of 200 μM $AcPyAD^+$ and 200 μM NADPH. Unless otherwise indicated, the transhydrogenase activity of the reconstituted systems was assayed as reduction of thio-$NADP^+$ by NADH at 395–460 nm, at 25 °C in 2.5 ml medium containing 80 mM Hepes/KOH (pH 7.5), 8 mM $MgCl_2$, 5 mM ATP, 0.5 mM phospho*enol*pyruvate and 4 units of pyruvate kinase. In the absence of ATP and uncoupler the transhydrogenase activity was less than 10% of that obtained in the presence of ATP. The ATP-driven transhydrogenase activity was not corrected for the activity obtained in theabsence of ATP (cf. Ref. 13). The reaction was started by the addition of 200 μM thio-$NADP^+$ and 200 μM NADH.

ATP hydrolysis in the reconstituted system was assayed as oxidation of NADH at 340 nm, at 25 °C in 80 mM Hepes/KOH (pH 7.5), 8 mM $MgCl_2$, 0.5 mM phospho*enol*pyruvate, 4 units of pyruvate kinase, 3 units of lactate dehydrogenase and 200 μM NADH. The reaction was started by the addition of 5 mM ATP. Fully uncoupled activities were assayed in the presence of 1.6 μM FCCP.

Transhydrogenase and ATPase activities of submitochondrial particles were estimated as reduction of thio-$NADP^+$ by NADH and oxidation of NADH, respectively, under the same conditions as those used in the reconstituted systems, except that 1 μM antimycin, 2 mM potassium cyanide and 2 μM rotenone was added.

ATP-driven reduction of NAD^+ by succinate catalyzed by submitochondrial particles was as-

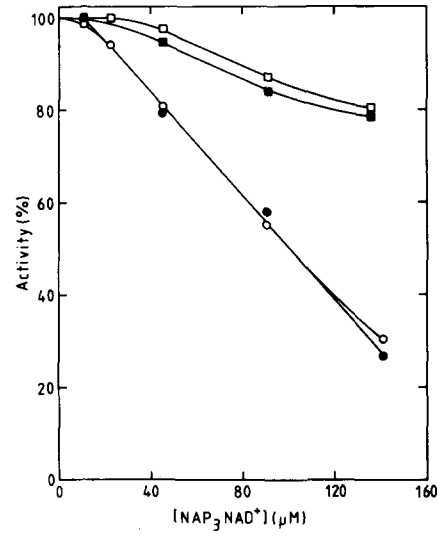

Fig. 1. Titration with NAP_3NAD^+ of ATP-driven, uncoupled transhydrogenase and ATPase activities catalyzed by reconstituted transhydrogenase-ATPase vesicles, in the absence and in the presence of uncoupler. Penefsky-type column-treated vesicles containing 85.6 μg transhydrogenase/ml and 1.11 mg ATPase/ml were preincubated on ice for 15 min with 51.3 μM ATP prior to the NAP_3NAD^+ addition. Photolabeling was achieved by illuminating the transhydrogenase-ATPase vesicles at 366 nm on ice in the presence of various concentrations of NAP_3NAD^+. After incubation for 60 min unreacted probe was removed by centrifuging the samples in 1 ml Penefsky-type columns. Transhydrogenase and ATPase activities were monitored as reduction of thio-$NADP^+$ by NADH and oxidation of NADH, respectively. 100% activity of the ATP-driven transhydrogenase reaction (○) corresponds to 610 nmol/min per mg transhydrogenase and for the FCCP uncoupled reaction (●) to 215 nmol/min per mg transhydrogenase. 100% activity of ATPase in the coupled system (□) amounted to 0.83 μmol/min per mg ATPase and in the FCCP uncoupled reaction (■) to 2.04 μmol/min per mg ATPase. Relative activities are plotted.

sayed at 340 nm, at 25 °C in a medium containing 80 mM Hepes/KOH (pH 7.5), 8 mM $MgCl_2$, 1 mM NAD^+, 10 mM succinate, 1 μM antimycin and 2 mM potassium cyanide. The reaction was started by the addition of 5 mM ATP.

Photoaffinity labeling

Labeling of aliquots of reconstituted transhydrogenase-ATPase vesicles and submitochondrial particles with $NADP_3NAD^+$ was performed on ice during illumination with near-ultraviolet light at a wavelength of 366 nm. The light source was a CAMAG TL-900/U ultraviolet lamp. Sephadex G-50 coarse columns [26] equilibrated with 10 mM Tris-HCl (pH 7.5) were used for buffer exchange of the reconstituted vesicles prior to photolabeling and after photolabeling to remove unreacted probe.

Chemicals

Phospholipids were obtained from Lipid Products (Nutley, U.K.). $NADP_3NAD^+$ [27] and 8-azido-ATP [28] were prepared and analyzed by Mr. A.F. Hartog (Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam). Aurovertin D [29] was isolated from *Calcarisporium arbusculum* by Dr. R.M. Bertina (Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam). Sephadex G-50 coarse was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and obtained from Sigma.

Results

Inhibitor titrations

Fig. 1 shows the normalized inhibitory effect of the photoactivated NAD^+ analogue NAP_3NAD^+ on various activities catalyzed by reconstituted transhydrogenase-ATPase vesicles. The inhibition of the uncoupled transhydrogenase activity by covalently bound NAP_3NAD^+ was essentially linear with the concentration of inhibitor present during the illumination period, up to about 140 μM, indicating a relatively high k_D value for the analogue-enzyme complex. In the presence of 140 μM analogue an inhibition by covalent binding of 70% was obtained. A more extensive inhibition, up to 100%, was obtained when the illumination procedure was repeated with newly added ligand, but such high levels of inhibition were not required in our experiments. Also some covalent attachment of the photolabel to inhibitory sites on the ATPase occurred, probably at ATP-binding sites, but this binding was less extensive, causing 20% inhibition of the ATPase when the transhydrogenase was inhibited by more than 70%. At low levels of inhibition of the transhydrogenase the ATPase was not significantly inhibited. It can be seen in Fig. 1 that the ATP-driven transhydrogenase activity was inhibited by covalently bound ligand to the same extent as the uncoupled transhydrogenase activity at low as well as at high concentrations of the inhibitor. The protective effect of NAD^+ on the inhibition of the transhydrogenase by NAD_3NAD^+ after illumination was due to occupation of the NAD(H)-binding site by the analogue (Hu, P.S., Persson, B. and Rydström, J., unpublished observations). The residual activity of the uncoupled enzyme, therefore, is a measure of the amount of active enzyme present. The finding that the ATP-driven transhydrogenase showed the same sensitivity towards NAP_3NAD^+ as the uncoupled transhydrogenase indicates that the transhydrogenase was fully rate-limiting for the overall process, i.e., the flux control coefficient of transhydrogenase in the coupled system equalled 1 [30].

Although it is not an irreversible inhibitor, the F_1-inhibitor aurovertin was tested because of its well-known specificity for F_1 [31]. Treatment of reconstituted transhydrogenase-ATPase vesicles with aurovertin led to an approximately equal loss of ATPase activity independently of whether the vesicles were coupled or uncoupled (Fig. 2A). The normalized figure also shows the inhibitory effect of aurovertin on the ATP-driven transhydrogenase activity of the vesicles. Apparently, the ATP-driven transhydrogenase was less sensitive to the inhibitor than the ATPase itself (Fig. 2A). However, as shown in Fig. 2B, aurovertin stimulated the uncoupled transhydrogenase reconstituted alone in vesicles maximally about 50% at a concentration of 4 μM, but had little or no effect on the purified soluble enzyme. Also, partially coupled transhydrogenase was stimulated to the same extent (not shown). Subtraction of the latter stimulation from the activity of the ATP-driven transhy-

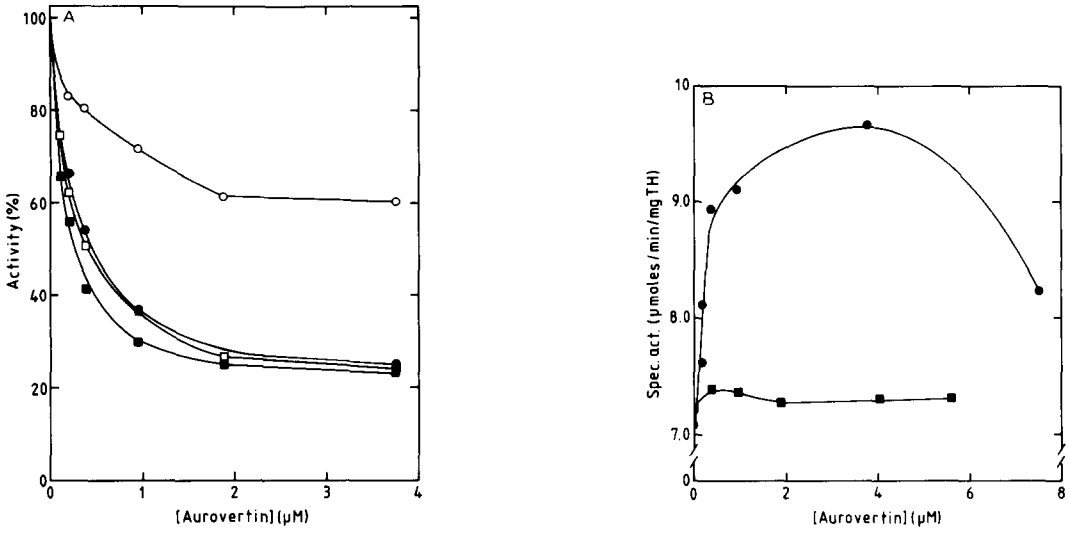

Fig. 2. Titration with aurovertin of ATPase and ATP-driven transhydrogenase activities catalyzed by reconstituted transhydrogenase-ATPase vesicles in the absence and in the presence of uncoupler (A), and soluble transhydrogenase and transhydrogenase activities catalyzed by uncoupled reconstituted transhydrogenase vesicles (B). (A) Vesicles containing 1.88 μg transhydrogenase and 24.4 μg ATPase were preincubated for 3 min in 2.5 ml assay medium containing various concentrations of aurovertin. 100% activity of ATPase in the absence of FCCP (□) was 1.44 μmol/min per mg ATPase and in the presence of FCCP (■) 3.88 μmol/min per mg ATPase. 100% ATP-driven transhydrogenase activity (○) was 1.45 μmol/min per mg transhydrogenase. ● denotes the ATP-driven transhydrogenase activity when corrected for the aurovertin-mediated stimulation of membrane-bound transhydrogenase activity in Fig. 2B. (B) Vesicles containing 1.88 μg transhydrogenase were preincubated for 3 min in 2.5 ml assay medium containing various concentrations of aurovertin. Transhydrogenase (TH) activity was assayed as reduction of $AcPyAD^+$ by NADPH in 80 mM Hepes/KOH (pH 7.5) and 1.6 μM FCCP. Reaction was started by the addition of 200 μM $AcPyAD^+$ and 200 μM NADPH (●). Solubilized transhydrogenase was preincubated for 3 min at a concentration of 1.20 μg per 2.5 ml assay medium containing various concentrations of aurovertin and assayed for $AcPyAD^+$ reduction by NADPH (■) as described in Materials and Methods. In (A) relative activities are plotted and in (B) absolute activities are plotted.

drogenase in the presence of aurovertin yielded a normalized inactivation profile of the ATP-driven transhydrogenation which was almost identical to that found for the inhibition of coupled and uncoupled ATPase activities (Fig. 2A). The correction for the stimulation of the transhydrogenase by aurovertin is justified by the previous finding that the flux control coefficient of transhydrogenase equals 1 in the coupled system (cf. Fig. 1). The fact that the ATP-driven transhydrogenase activity was inhibited by aurovertin to the same extent as the ATPase itself means that also the flux control coefficient of the ATPase equals 1. The apparent failure of the summation theorem (the sum of all flux control coefficients equals 1) is briefly discussed in the Discussion.

Double-inhibitor titrations

This method employs the inhibition of the primary pump (ATPase) and the secondary pump (transhydrogenase) separately or simultaneously using the specific inhibitors aurovertin and phenylarsine oxide. Phenylarsine oxide has recently been demonstrated to be a potent inhibitor of transhydrogenase with little effect on the ATPase in the concentration range used [32]. Fig. 3 shows a double-inhibitor titration experiment where the ATPase activity of reconstituted transhydrogenase-ATPase vesicles was inhibited by aurovertin and the residual ATP-driven transhydrogenase activity further inhibited by phenylarsine oxide. The ATP-driven and the uncoupled transhydrogenase activities were titrated with phenylarsine oxide in the absence and in the presence of aurovertin. As may be seen in Fig. 3B, the normalized values derived from the experiment in Fig. 3A indicate that the relative change in activity by inhibition with phenylarsine oxide was essentially

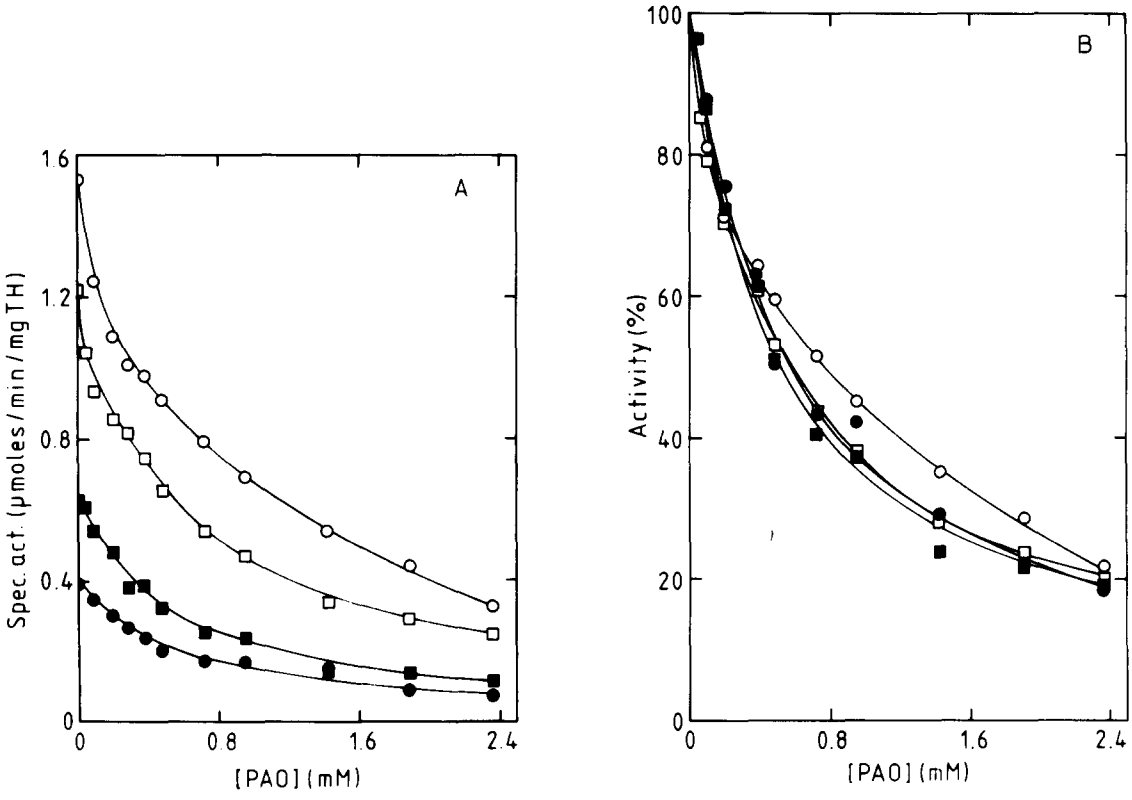

Fig. 3. Titration with phenylarsine oxide (PAO) of the ATP-driven and uncoupled transhydrogenase activities catalyzed by reconstituted transhydrogenase-ATPase vesicles with and without previous inhibition of the ATPase by aurovertin. Vesicles containing 2.35 μg transhydrogenase and 30.5 μg ATPase were preincubated for 3 min in 2.5 ml assay medium without and with 1.88 μM aurovertin after which the activities were assayed with different concentrations of phenylarsine oxide. Transhydrogenase activity was assayed as reduction of thio-$NADP^+$ by NADH. ○ and ● denote the ATP-driven and the uncoupled transhydrogenase activities in control vesicles, respectively; □ and ■, the ATP-driven and the uncoupled transhydrogenation in aurovertin-treated liposomes, respectively. In (A) absolute activities are plotted and in (B) relative activities are plotted.

independent of the presence of aurovertin. The aurovertin-mediated stimulation of membrane-bound uncoupled transhydrogenase activity seen in Fig. 2B was observed also in this experiment. Thus, independent of the relative activity of transhydrogenase, the inhibition of the ATP-driven transhydrogenase activity was always identical with the inhibition of the uncoupled transhydrogenase, i.e., the control strength of the ATP-driven transhydrogenase/ uncoupled transhydrogenase remains 1. This was valid also when the ATPase was substantially inhibited, resulting in a low ATP-driven transhydrogenase activity. The slight deviation of the curve for the inhibition of the ATP-driven transhydrogenase by phenylarsine oxide in the absence of aurovertin does not seem to be significant since it was absent when NAP_3NAD^+ was present (cf. Fig. 1) and when aurovertin was added to inhibit the ATPase (cf. Fig. 2A).

Inhibitor-uncoupler titrations

A second approach to study the interaction beween two proton pumps is the method of inhibitor-uncoupler titrations [33]. These experiments were carried out either by partially inhibiting the primary pump (ATPase) or by partially inhibiting the secondary pump (transhydrogenase), in both cases followed by titration with an uncoupler. Fig. 4 shows an inhibitor-uncoupler experiment where the ATP-driven transhydrogenase activity of reconstituted transhydrogenase-ATPase vesicles, inhibited to various extents by NAP_3NAD^+, was titrated with FCCP. The normalized inhibition of

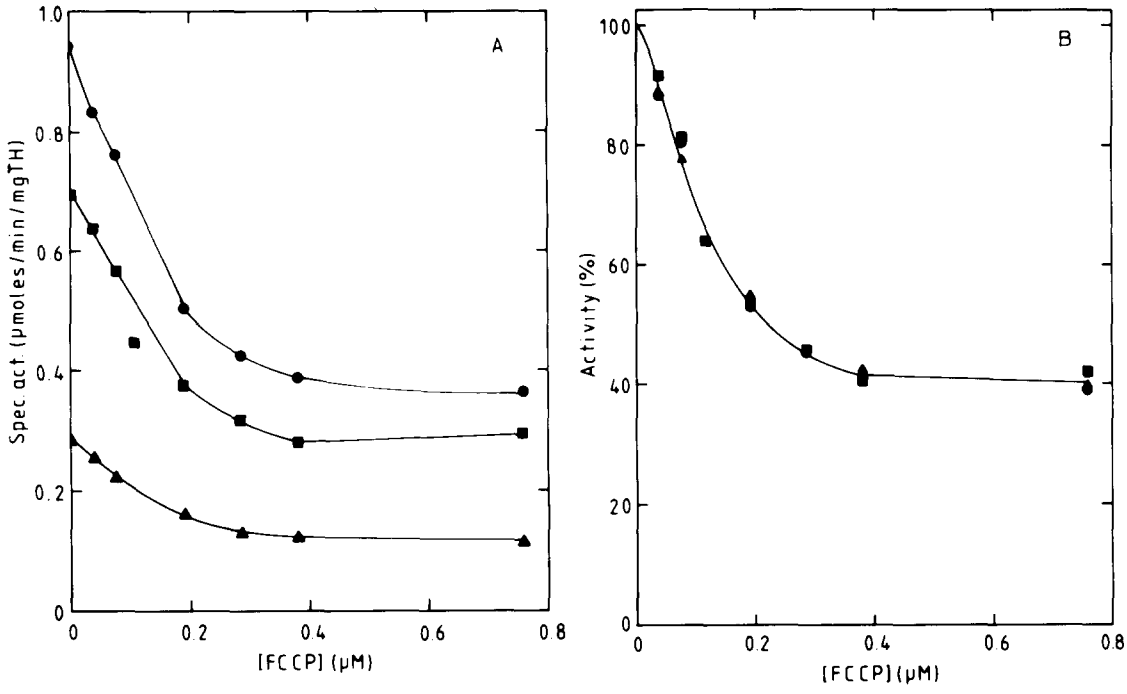

Fig. 4. Titration with FCCP of the ATP-driven transhydrogenase (TH) activity catalyzed by transhydrogenase-ATPase vesicles in which transhydrogenase has been inhibited to varying extents by NAP_3NAD^+. Penefsky-type column-treated vesicles containing 85.6 μg transhydrogenase and 1.11 mg ATPase/ml were preincubated on ice for 15 min in the presence of 51.3 mM ATP prior to the NAP_3NAD^+ addition. Vesicles were illuminated at 366 nm on ice in the absence (●) or in the presence (■) of 45.5 μM and 136 μM (▲) NAP_3NAD^+. After 60 min of incubation unreacted probe was removed by centrifuging the samples in 1-ml Penefsky-type columns. ATP-driven transhydrogenation was assayed as reduction of thio-$NADP^+$ by NADH and titrated with increasing concentrations of FCCP. In (A) absolute activities are plotted and in (B) relative activities are plotted.

the ATP-driven transhydrogenase by FCCP in Fig. 4B, based on the rates shown in Fig. 4A, was found to remain constant with various levels of inhibition of the transhydrogenase by covalently bound NAP_3NAD^+. The concentration of FCCP required under these conditions to give 50% uncoupling of the ATP-driven transhydrogenase reaction was about 0.11 μM. The activity in the presence of 0.75 μM FCCP (fully uncoupled activity) was equal to the activity of the soluble enzyme. In Fig. 2B is shown that the activity of transhydrogenase in uncoupled vesicles equalled the activity of the soluble enzyme also when the reverse reaction, i.e., reduction of $AcPyAD^+$ by NADPH, was measured.

Fig. 5 shows an inhibitor-uncoupler experiment similar to the one presented in Fig. 4, but in this case the transhydrogenase activity of the reconstituted transhydrogenase-ATPase vesicles was inhibited by phenylarsine oxide. Both in the absence and in the presence of phenylarsine oxide, the normalized uncoupling by FCCP in Fig. 5B, based on the rates shown in Fig. 5A, was found to remain essentially constant with various concentrations of FCCP, with a slight deviation at concentrations of FCCP higher than 0.3 μM. The concentration of FCCP required to give 50% uncoupling of the ATP-driven transhydrogenase reaction was found to be about 0.15 μM (Fig. 5A and B). Thus, these results confirm the conclusions of the experiment shown in Fig. 4.

Inhibition of the primary pump (ATPase) by aurovertin in an inhibitor-uncoupler experiment was then performed with the same type of reconstituted transhydrogenase-ATPase vesicles. ATP-driven transhydrogenase activities (Fig. 6) as well as ATPase activities (not shown) were titrated with FCCP in the absence and in the presence of aurovertin. The results in Fig. 6 show that the more the ATP-driven transhydrogenase was re-

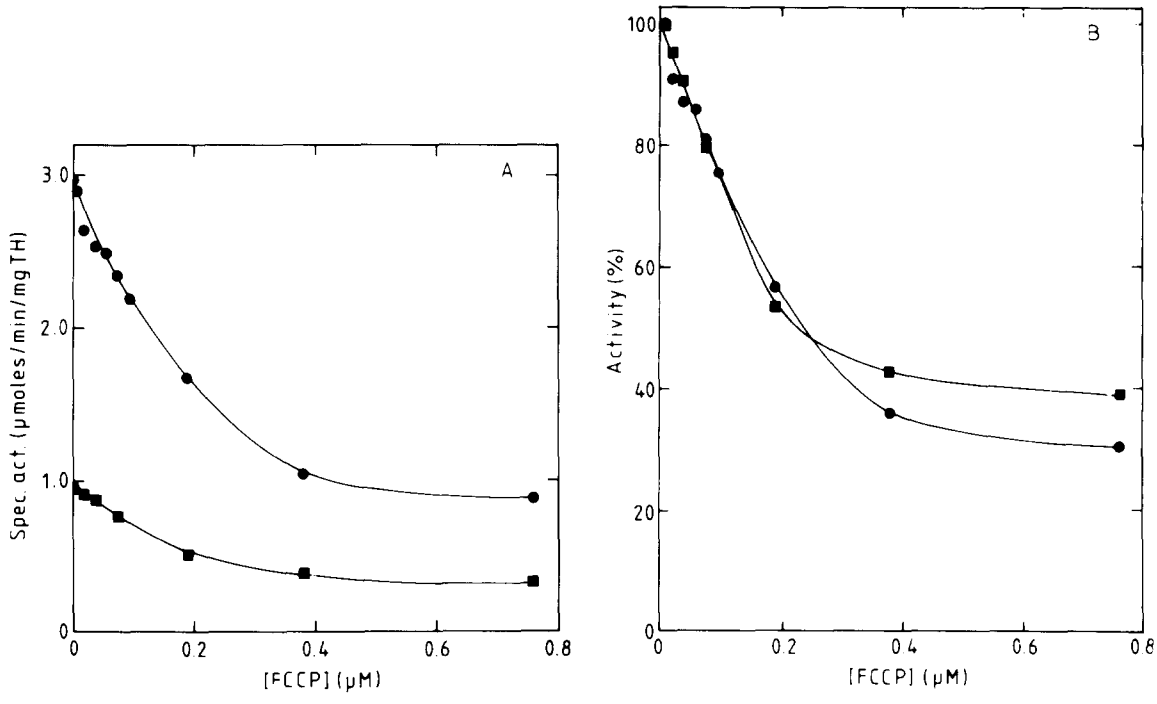

Fig. 5. Titration with FCCP of ATP-driven transhydrogenase (TH) activity catalyzed by transhydrogenase-ATPase vesicles in which transhydrogenase has been inhibited to varying extents by phenylarsine oxide. Vesicles containing 2.35 μg transhydrogenase and 30.5 μg ATPase were preincubated for 3 min at 25 °C in 2.5 ml assay medium in the absence (●) and in the presence (■) of 332 μM phenylarsine oxide. ATP-driven transhydrogenation, monitored as reduction of thio-NADP$^+$ by NADH, was titrated with increasing concentrations of FCCP. In (A) absolute activities are plotted and in (B) relative activities are plotted.

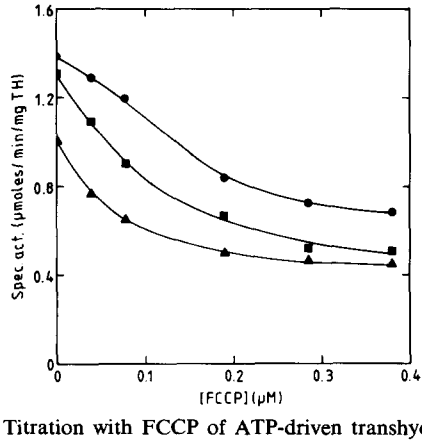

Fig. 6. Titration with FCCP of ATP-driven transhydrogenase (TH) activity catalyzed by transhydrogenase-ATPase vesicles in which ATPase has been inhibited to varying extents by aurovertin. Vesicles containing 1.88 μg transhydrogenase and 24.4 μg ATPase were preincubated for 3 min in 2.5 ml assay medium containing various concentrations of aurovertin. Control samples (●), samples treated with 0.38 μM (■) and 0.94 μM (▲) aurovertin were assayed for ATP-driven transhydrogenase activity when titrated with increasing concentration of FCCP. Transhydrogenation was monitored as reduction of thio-NADP$^+$ by NADH. Values denote absolute activities.

stricted by inhibition of the ATPase the less uncoupler is needed to achieve 50% uncoupling of the transhydrogenase reaction. It should be mentioned that, as reported above (cf. Fig. 2A), the ATPase activity was inhibited more extensively than the ATP-driven transhydrogenase activity, due to the stimulatory effect of aurovertin on the transhydrogenase itself. In this context it is relevant (cf. Discussion) to mention that the amount of uncoupler needed to inhibit the ATP-driven transhydrogenase activity by 50% also stimulated the ATPase activity 50% as compared to the fully uncoupled ATPase activity.

Since the experiments of Figs. 4 and 5 agree with the notion that in reconstituted vesicles only a delocalized chemiosmotic behavior can be observed [7], the experiment of Fig. 4, in which the transhydrogenase was partially inhibited by covalently bound NAP_3NAD^+, was repeated with submitochondrial particles instead of reconstituted vesicles. Fig. 7A shows the inhibitor-uncoupler

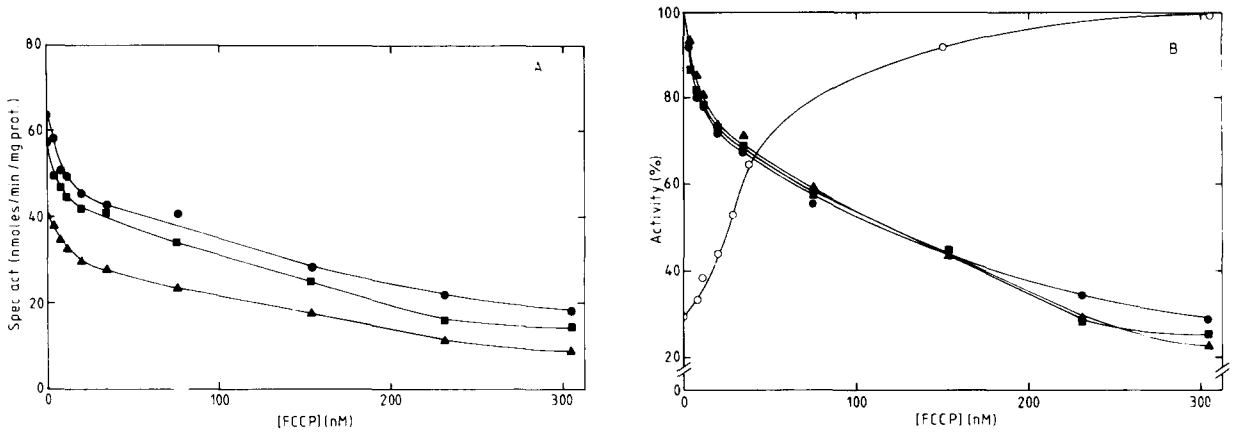

Fig. 7. Titration with FCCP of ATP-driven transhydrogenase and ATPase activities catalyzed by submitochondrial particles with and without inhibition of transhydrogenase by NAP_3NAD^+. Submitochondrial particles at a concentration of 14 mg/ml were preincubated on ice for 15 min in the presence of 51.3 mM ATP prior to the addition of NAP_3NAD^+. Particles in the absence (●) or in the presence of 83.3 μM (■) or 208 μM (▲) NAP_3NAD^+ were illuminated at 366 nm on ice. After 60 min of incubation the ATP-driven transhydrogenase activity was immediately assayed as reduction of thio-$NADP^+$ by NADH and titrated with increasing concentration of FCCP. In (B) is included the FCCP enhancement of ATPase activity (○) when assayed separately in the particle preparation used above in the absence of NAP_3NAD^+. In (A) absolute activities are plotted and in (B) relative activities are plotted.

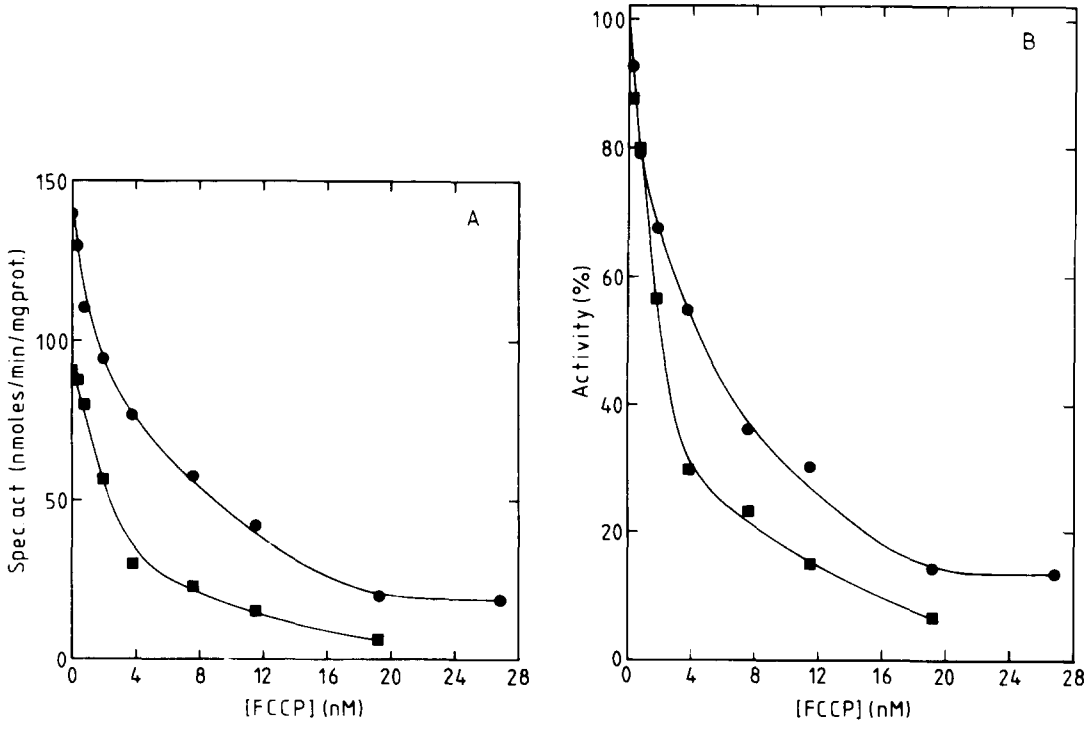

Fig. 8. Titration with FCCP of ATP-driven reduction of NAD^+ catalyzed by submitochondrial particles in the absence and presence of rotenone. Particles at a concentration of 14 mg/ml were preincubated on ice without (●) and with 25 pmol rotenone/mg (■) for 10 min prior to assay. In (A) absolute activities are plotted and in (B) relative activities are plotted.

titration carried out with submitochondrial particles where the ATP-driven transhydrogenase was partially inhibited by NAP_3NAD^+ prior to titration with uncoupler. The normalized values of the data in Fig. 7A, as well as the normalized values for the stimulation of the ATPase by FCCP, are presented in Fig. 7B. Half-maximal stimulation of the ATPase activity by FCCP was obtained at a slightly lower concentration as that found for half-maximal uncoupling of the ATP-driven transhydrogenase, i.e., about 50 nM. As in previous inhibitor-uncoupler titrations approximately the same uncoupler concentration was required to inhibit the ATP-driven transhydrogenase by 50% regardless of whether the transhydrogenase was inhibited by NAP_3NAD^+ or not (Fig. 7B), an observation indicating that transhydrogenase and ATPase in their native milieu are behaving in a delocalized chemiosmotic manner.

Since the results presented above clearly differ from those reported by Herweijer et al. [10], who measured the ATP-driven reduction of NAD^+ instead of the ATP-driven transhydrogenase, an inhibitor-uncoupler experiment was also carried out with the ATP-driven reduction of NAD^+ in the present investigation as a control. Fig. 8 shows such an experiment, in which submitochondrial particles (taken from the same batch as those used for the experiment of Fig. 7) were preincubated in the absence and in the presence of rotenone, after which the ATP-driven reduction of NAD^+ was titrated with FCCP. From the normalized values in Fig. 8B it may be seen that, when NAD^+ reduction was 40% inhibited by rotenone, approx. 50% of the FCCP concentration was required to give a 50% uncoupling of the inhibited ATP-driven reduction of NAD^+, as compared to the FCCP concentration required for 50% uncoupling of the uninhibited reaction. These results suggest a localized coupling for the ATP-driven reduction of NAD^+, in agreement with the data reported by Herweijer et al. [10].

Discussion

Both the photoaffinity probe NAP_3NAD^+ and the dithiol reagent phenylarsine oxide [32] have been shown to react quite specifically with the transhydrogenase in a system in which also ATPase is present. Other photoaffinity probes like 8-azido-ATP reacted about equally well with both enzymes (not shown). Inhibition of transhydrogenase, catalyzed by reconstituted transhydrogenase-ATPase vesicles, by either phenylarsine oxide or NAP_3NAD^+ resulted in an equal inhibition of the ATP-driven transhydrogenase and the uncoupled transhydrogenase activity. In the former reaction the hydrolysis of ATP (catalyzed by ATP synthase) was used to drive the reduction of thio-$NADP^+$ by NADH (catalysed by transhydrogenase). In the absence of ATP (and uncoupler) the rate of reduction of thio-$NADP^+$ was less than 10% of that obtained in its presence.

The finding that the ATP-driven transhydrogenase activity was inhibited to the same extent as the uncoupled transhydrogenase activity under conditions that the transhydrogenase was inhibited by a stoichiometric, irreversible inhibitor, indicates that the transhydrogenase is the 'rate-limiting factor' in the presence of ATP, the activity of the coupled system being proportional to the amount of active transhydrogenase. On the other hand, the inhibition of ATP hydrolysis by aurovertin also resulted in a proportional inhibition of the coupled system. Although aurovertin is not an irreversible inhibitor of ATP synthase, a certain concentration of this agent inhibited uncoupled ATP hydrolysis to the same extent as the coupled ATPase, indicating that the inhibitory effect did not vary with the extent of coupling of the system. The F_0-inhibitor oligomycin not only inhibited ATP hydrolysis but also decreased the transhydrogenase activity in the absence of ATP and uncoupler (not shown). This result indicated that in the presence of oligomycin the leakiness of the membrane for protons was lowered by the reaction of oligomycin with the ATPase complex lacking F_1 or other subunits. For that reason we preferred aurovertin to oligomycin. A complicating factor in the experiments with aurovertin was the unexpected stimulatory effect of aurovertin on the transhydrogenase. This effect, which was only seen with membrane-bound transhydrogenase, was very reproducible and may be ascribed to a structural effect on the membrane since soluble transhydrogenase was not affected and the fluorescence of aurovertin was not enhanced when added to transhydrogenase vesicles. The sensitivity of the

transhydrogenase to changes in membrane structure might be an interesting subject for further studies.

Since we have established that the ATP-driven transhydrogenase activity is proportional to the activity of the transhydrogenase itself (see above), the effect of aurovertin on the coupled reaction could be corrected for any effect on the transhydrogenase. When this correction was made the inhibition of the coupled system by aurovertin appeared to be equal to the inhibition of the ATP hydrolysis. Previously, Ernster and coworkers [19] obtained similar results using the F_1-inhibitor protein to inhibit ATP hydrolysis. Titrations with inhibitors of one of the participating pumps, therefore, show that in the coupled reaction both the ATPase activity and the transhydrogenase activity are completely 'rate-limiting', i.e., the flux control coefficient of both enzymes is equal to 1 [34,35]. As a test for this conclusion one of the two enzymes was first inhibited, in our case the ATPase, to change the relative capacity of the two participating proton pumps. By using aurovertin we not only inhibited ATP hydrolysis but activated the transhydrogenase as well, and even under these conditions inhibition of the transhydrogenase resulted in a proportional inhibition of the whole system. According to the arguments given by several authors [5,6,10,36], this could mean that the interaction between the two proton pumps is localized, the two systems behaving like a one-enzyme system. On the other hand, Van der Bend et al. [7] showed that in their co-reconstituted vesicles both ATPase and the light-driven proton pump bacteriorhodopsin were 'rate-limiting', while no localized interaction could be expected. When no localized interaction is present the sum of all flux control coefficients should be 1 [30], while in the present system this seems to be 2. This anomaly disappears when a third reaction is involved in the system with a flux control coefficient of -1. This third reaction could be the leak of protons through the membrane: increasing the leak (by uncouplers) results in a proportional decrease of the ATP-driven transhydrogenase activity (cf. Ref. 35). Recently, Chen [37] has shown, on the basis of a theoretical kinetic analysis, that indeed also in systems in which the interaction between the participating pumps is delocalized, double-inhibitor titration curves might be obtained that are usually considered to be indicative of a direct interaction between the participating components. Such a result is related to the kinetic properties of the individual systems. For these reasons the results of double-inhibitor titrations cannot be used as a sole argument against delocalized chemiosmosis and should be complemented with other data such as inhibitor-uncoupler titrations.

Previously, Van der Bend et al. [7] have argued that uncoupler titrations of systems in which the secondary pump is inhibited to various degrees should provide a means of discriminating between localized and delocalized interaction. Recently, on the basis of a simple chemiosmotic model in which it is assumed that the fluxes are linearly related to the forces, Herweijer [38] has simulated the effect of inhibition of the primary or secondary pump on the uncoupler titrations. The simulation shows that partial inhibtion of the primary pump results in an increased efficiency of the uncoupler, but that this is not the case with partial inhibition of the secondary pump. Pietrobon and Caplan [11] have made similar simulations and they concluded that also a partial inhibition of the secondary pump resulted (or could result) in an increased efficiency of an uncoupler. However, in these latter simulations the effect was minimal (relative to some experimental data) and obtained under unrealistic conditions [11]. Also, when a non-linear model of chemiosmotic energy coupling was assumed the possible increase of uncoupling efficiency was much smaller than the proportional increase experimentally found in experiments on reversed electron flow and only present at intermediate concentrations of uncouplers [39]. According to the proposals for a localized interaction, the efficiency of protonophoric uncouplers such as FCCP, S-13 or dinitrophenol should increase nearly proportional when either the primary or the secondary pump is partially inhibited [10]. Uncoupler titrations under conditions when the secondary pump is inhibited to a certain extent should therefore be discriminatory between localized and delocalized interactions. In the present investigation, the uncoupling by FCCP was found to be more efficient when ATPase was partially inhibited by aurovertin but not when transhydrogenase was partially inhibited. Thus, according to

the latter criterium and in agreement with the chemiosmotic model, the interaction between transhydrogenase and ATPase in the reconstituted transhydrogenase-ATPase vesicles is delocalized.

Since it is possible that the conditions for direct interaction between transhydrogenase and ATPase are lost upon isolation and reconstitution of the two enzymes, the relevant inhibitor-uncoupler titrations were repeated with submitochondrial particles, a preparation that represents a system closer to the physiological situation. In contrast to what has been reported for the interaction between NADH : Q oxidoreductase and ATPase [10], and confirmed by us (Fig. 8), the results obtained with transhydrogenase measurements in submitochondrial particles were similar to those obtained with reconstituted transhydrogenase-ATPase vesicles. In relation to the arguments presented by Kell [8] we have made an estimate of the turnover number of the ATP-driven transhydrogenase activity in submitochondrial particles and in reconstituted transhydrogenase-ATPase vesicles. In our submitochondrial particles the turnover number was 24 s^{-1} considering the dimer of transhydrogenase as the active unit. In the reconstituted transhydrogenase-ATPase vesicles this number equals 16 s^{-1}. The ATPase/transhydrogenase molar ratio is about 5 in submitochondrial particles, while in the reconstituted vesicles the ratio equals 6. We may thus conclude that the ATPase/transhydrogenase molar ratio and the turnover number of the ATP-driven transhydrogenase are of the same order in both systems. For the system described in Ref. 7 the turnover of ATP synthesis in the reconstituted vesicles was much lower than in the native system. The findings presented here strengthen previous arguments that inhibitor-uncoupler titrations of reverse electron flow are in disagreement with the chemiosmotic model and favor the concept of a localized interaction between NADH : Q oxidoreductase and ATPase [10].

In agreement with Ernster et al. [18] and the proposal of Herweijer et al. [10] for the mechanism of ucoupling by protonophoric uncouplers, we have also found that for uncoupling of reverse electron flow by FCCP much lower concentrations of the uncoupler are needed than for uncoupling of the ATP-driven transhydrogenase where uncoupling requires the dissipation of $\Delta\tilde{\mu}_{H^+}$. The latter concentrations were similar to the concentrations needed to uncouple (stimulate) ATP hydrolysis. The protonophoric uncouplers preferentially equilibrate protons available in the membrane during the process of energy transfer, and the concentrations needed for uncoupling are therefore proportional to the rate of the energy-transduction process when this transduction is localized. If the transduction is delocalized, then uncoupling requires dissipation of the bulk $\Delta\tilde{\mu}_{H^+}$ and the amount of uncoupler needed is proportional to the activity of the primary pump, whether a secondary pump is operative or not. If no secondary pump is operative, as in the case when only ATP hydrolysis is measured, then uncoupling requires dissipation of $\Delta\tilde{\mu}_{H^+}$ and with uncouplers like FCCP and S-13 a higher concentration is needed than for uncoupling localized energy transduction. With an uncoupler like gramicidin all uncoupling occurs via dissipation of $\Delta\tilde{\mu}_{H^+}$ since gramicidin cannot uncouple localized interactions directly. It should be kept in mind that also for localized energy transduction the existence of a $\Delta\tilde{\mu}_{H^+}$ is essential: upon dissipation of $\Delta\tilde{\mu}_{H^+}$ the rate of extrusion of protons into the bulk increases, thereby decreasing the level of localized energy transduction. When $\Delta\tilde{\mu}_{H^+}$ is zero, also localized energy transfer has ceased. Noteworthy is the reproducible phenomenon that at low concentrations of FCCP, i.e., below 2 nM, the efficiency of uncoupling of reversed electron flow was not increased after partial inhibition of the NADH : Q oxidoreductase. This phenomenon has not been seen in previous experiments where S-13 and dinitrophenol were used [10], nor in the experiments of Hitchens and Kell [6,40]. In both cases the authors had to assume that diffusion of the uncoupler is very fast and not rate-limiting for the overall process of localized uncoupling. Our data suggest that, under our conditions (the reversal activity is 50% of the activity measured in Ref. 10), the diffusion of FCCP at very low concentrations of the uncoupler is rate-limiting for the uncoupling process while at higher concentrations the protonophoric activity itself is the rate-limiting step.

In conclusion, we may state that the experimental data are in line with the contention that, contrary to the interaction between NADH : Q

oxidoreductase and ATPase in submitochondrial particles, the interaction between transhydrogenase and ATPase both in reconstituted transhydrogenase-ATPase vesicles and in submitochondrial particles occurs via the bulk $\Delta\tilde{\mu}_{H^+}$.

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