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Flow–force relationships during energy transfer between mitochondrial proton pumps

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The effect of inhibitors of proton pumps, of uncouplers and of permeant ions on the relationship between input force, $\Delta\tilde{\mu}_{H^+}$, and output flows of the ATPase, redox and transhydrogenase H^+-pumps in submitochondrial particles was investigated. It is concluded that: (1) The decrease of output flow of the transhydrogenase proton pump, defined as the rate of reduction of $NADP^+$ by NADH, is linearily correlated with the decrease of input force, $\Delta\tilde{\mu}_{H^+}$, in an extended range of $\Delta\tilde{\mu}_{H^+}$, independently of whether the H^+-generating pump is the ATPase or a redox pump, or whether $\Delta\tilde{\mu}_{H^+}$ is depressed by inhibitors of the H^+-generating pump such as oligomycin or malonate, or by uncouplers. (2) The output flows of the ATPase and of the site I redox H^+-pumps exhibit a steep dependence on $\Delta\tilde{\mu}_{H^+}$. The flow–force relationships differ depending on whether the depression of $\Delta\tilde{\mu}_{H^+}$ is induced by inhibitors of the H^+-generating pump, by uncouplers or by lipophilic anions. (3) With the ATPase as H^+-consuming pump, at equivalent $\Delta\tilde{\mu}_{H^+}$ values, the output flow is more markedly inhibited by malonate than by uncouplers; the latter, however, are more inhibitory than lipophilic anions such as ClO_4^-. With redox site I as proton-consuming pump, at equivalent $\Delta\tilde{\mu}_{H^+}$ values, the output flow is more markedly inhibited by oligomycin than by uncouplers; again, uncouplers are more inhibitory than ClO_4^-. (4) The results provide further support for a delocalized interaction of transhydrogenase with other H^+-pumps.

Introduction

The establishment of $\Delta\tilde{\mu}_{H^+}$ as the sole and competent intermediate for free-energy transfer between H^+-generating and H^+-consuming pumps [1] requires a detailed kinetic and thermodynamic analysis. One approach is that of analyzing the relationship between input force and output flow of the H^+-consuming pump under conditions where the input force is varied by the addition of different uncoupling agents and pump inhibitors.

If $\Delta\tilde{\mu}_{H^+}$ is assumed to be the sole and competent intermediate in free-energy transfer between H^+-generating and H^+-consuming pumps, the pattern of flow–force relationships should be independent of how $\Delta\tilde{\mu}_{H^+}$ is varied. Several studies on flow–force relationships have failed to demonstrate such an independence [2–9]. Recently, however, a number of objections have been raised against these results [4,10,11]. Thus, it has been claimed [4,10,11] that the assays employed for determining $\Delta\tilde{\mu}_{H^+}$ suffer from several experimental errors which render the conclusions based on the $\Delta\tilde{\mu}_{H^+}$measurements uncertain.

In a study on the transhydrogenase reaction, Ernster et al. [12] showed that oligomycin, uncouplers and the ATPase F_1-inhibitor protein have different effects on the rates of ATP-driven succinate-linked NAD^+ reduction and of ATP-driven transhydrogenation, and

Abbreviations: $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical proton gradient; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential gradient; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Ap5A, P^1P^5-di(adenosine-5'-)pentaphosphate; J_{NADH}, rate of NAD^+ reduction by reverse electron flow at redox site I; J_{NADPH}, rate of NADPH formation by transhydrogenation; J_{ATP}: Rate of phosphorylation.

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concluded that the mechanism of energy coupling in these two reactions are different, i.e., localized in the latter case (for a review on transhydrogenase see ref. 13). Eytan et al. [14] compared ATP hydrolysis and reduction of $NADP^+$ by NADH in reconstituted liposomes containing both purified mitochondrial ATPase and transhydrogenase. At limiting ATP concentrations, 3 mol of NADPH were formed per ATP hydrolyzed. On the basis of an assumed ATPase stoichiometry of 3 H^+ translocated per ATP hydrolyzed, it was concluded that one H^+ is translocated per NADPH formed. In the vesicles, the ATPase may be replaced by bacteriorhodopsin as primary H^+-pump. The data were taken to support a role of the bulk $\Delta\tilde{\mu}_{H^+}$ in the interaction between the two pumps. This conclusion was also supported by a recent study using so-called double inhibitor and inhibitor/uncoupler titrations, with submitochondrial particles or reconstituted transhydrogenase-ATPase vesicles as the source of transhydrogenase [15]. Thus, the available evidence strongly suggests that the mitochondrial transhydrogenase interacts in a delocalized manner with the bulk $\Delta\tilde{\mu}_{H^+}$ regardless of the nature of the primary H^+-pump. A similar conclusion was reached by Jackson and coworkers utilizing *Rhodobacter capsulatus* chromatophores [16,17].

As revealed by ATP-driven succinate-linked reduction of NAD^+, coupling at the first site appears to be more complicated, and has been suggested to be localized [18,19,29]. However, the latter conclusion has been challenged by the observation that effects of inhibitor-uncouplers on ATP-driven succinate-linked reduction of NAD^+ in rat liver mitochondria are compatible with a chemiosmotic mechanism [20]. The reason for the different behaviour of intact mitochondria and submitochondrial particles in some inhibitor-uncoupler titrations still remains unclear.

Data on the flow–force relationships characterizing various energy-transduction processes and on the manner by which these relationships are altered by inhibitors are necessary in order to draw correct conclusions from double-inhibitor and inhibitor-uncoupler titrations. The present investigation was therefore carried out in order to test whether the previously obtained results and interpretations would be compatible with a more refined analysis based on the relationship between the rates of the energy-linked reactions involved and the magnitude of $\Delta\tilde{\mu}_{H^+}$.

Materials and Methods

Determination of output flows: J_{NADH}, J_{ATP}, J_{NADPH}

Submitochondrial particles were incubated for a 5 min period in 2 ml of the selected medium (see legends to the figures) in thermostatted cuvettes. Generation of $\Delta\tilde{\mu}_{H^+}$ by ATP hydrolysis was carried out with 2 mM ATP in the presence of non-limiting amount of creatine kinase and 10 mM phosphocreatine, whereas generation of $\Delta\tilde{\mu}_{H^+}$ by succinate oxidation was achieved by adding 10 mM succinate to the medium. After incubation, depending on the reaction to be measured, the energy-driven reaction was started by the addition of NAD^+, $NADP^+$ or ADP.

ATP-driven succinate-linked reduction of NAD^+ (J_{NADH}) was followed by monitoring NADH formation spectrophotometrically at 340 nm, in the presence of 0.5 mM NAD^+, 10 mM succinate, 0.5 μg/ml antimycin A, 2 mM ATP and the ATP-regenerating system described above. During the assay of reduction of $NADP^+$ by NADH by transhydrogenase (J_{NADPH}), the concentration of NADH was kept constant by regenerating NADH with 0.2 M ethanol and non-limiting amounts of alcohol dehydrogenase in the presence of 5 mM hydrazine [21].

In the assay for ATP synthesis (J_{ATP}) the medium contained 5 mM glucose, 0.5 mM $NADP^+$, and non-limiting amounts of glucose-6-phosphate dehydrogenase and of hexokinase. ATP synthesis was followed by monitoring the formation of NADPH spectrophotometrically at 340 nm. The medium also contained 50 μM Ap5A to inhibit the adenylate kinase reaction. J_{ATP} was corrected for residual adenylate kinase activity by subtracting the rate of ATP formation (4–5 nmol/min per mg) measured in a parallel incubation supplemented with 1 μg/mg protein oligomycin, 1 μM antimycin and 1 μM FCCP.

Control experiments were routinely carried out in order to check that the above-mentioned regenerating systems were not rate-limiting; doubling either the particle concentration or the enzyme concentration did not change the measured rates of the energy-driven reactions. Measurement of $\Delta\psi$ in the presence of 1 μM Oxonol VI did not interfere with the rates of the transhydrogenase-catalyzed reduction of $NADP^+$ by NADH, or with aerobic ATP synthesis. However, Oxonol VI had an inhibitory effect (20–30%) on the rate of ATP-driven succinate-linked NAD^+ reduction. In order to take this effect into account, NADH formation was followed in the presence of the same amount of Oxonol VI as used in the assay.

Determination of $\Delta\tilde{\mu}_{H^+}$

All measurements were carried out in the presence of 10 mM NH_4^+ (as $(NH_4)_2HPO_4$), the presence of which resulted in an undetectable ΔpH as monitored by 9-aminoacridine fluorescence [22], in the presence as well as in the absence of ClO_4^- (as $KClO_4$) as permeant anion. The lower limit of ΔpH detection was 0.5 units. $\Delta\tilde{\mu}_{H^+}$ determinations were thus carried out by measuring only $\Delta\psi$, which was assayed by monitoring the energy-linked Oxonol VI response (see below). The use of this technique allowed us to record $\Delta\psi$ and energy-driven reactions in parallel samples under the

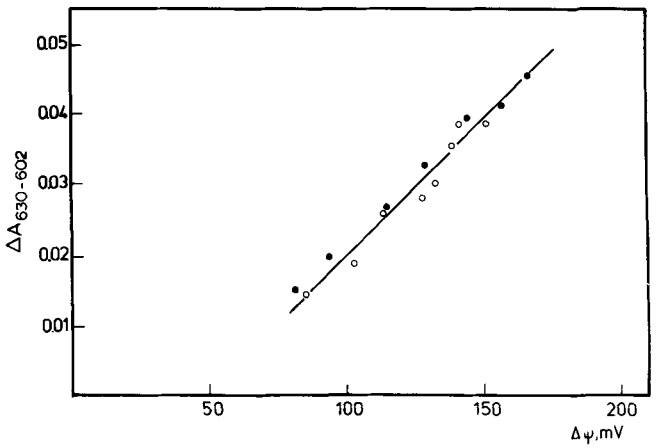

Fig. 1. Calibration of Oxonol VI response by ClO_4^- uptake. Submitochondrial particles were incubated in a medium containing 0.18 M sucrose, 30 mM Tris-Mops (pH 7.3), 0.5 mM EGTA-Tris, 5 mM magnesium Acetate, 5 mM $(NH_4)_2HPO_4$, 16 mM $KClO_4$ and 4 μM rotenone. The temperature was 25°C and the concentrations of protein were 0.14 mg/ml and 2 mg/ml in the determinations of Oxonol VI response and $\Delta\psi$, repectively. Additions were: 10 mM succinate-Tris, 0–10 mM malonate. The measurements were carried out in the absence (○) or in the presence (●) of 0.15 μg/ml oligomycin. $A_{630-602}$ refers to the absorbance by Oxonol VI and $\Delta\psi$ to the electrode-based determination of the membrane potential.

same experimental conditions, including protein concentrations and incubation times.

Oxonol VI is a well-established indicator of membrane potential [23–25] when monitored at 630–602 nm [26]. Calibration of the Oxonol VI response was achieved electrometrically in parallel samples in suspensions of succinate-oxidizing submitochondrial particles, in which the extent of energization was varied by the addition of malonate (Fig. 1). The electrometric determination of $\Delta\psi$ was carried out on the basis of the distribution of a permeant anion, ClO_4^- [27], measured in an open thermostatted and stirred suspension of submitochondrial particles (see legend to Fig. 1 for medium composition), at different $\Delta\psi$ values using a ClO_4^--sensitive electrode. Anaerobiosis was avoided by continuously oxygenating the suspension. ClO_4^- was added stepwise to the deenergized submitochondrial particle suspension, after which the submitochondrial particles were energized with succinate in the presence of varying concentrations of malonate. Deenergized conditions gave a correction factor for probe binding; no further corrections for probe binding [27] and activity coefficients [28] were applied. The internal volume was taken as 1 μl/mg protein. In order to measure the Oxonol VI response, submitochondrial particles were incubated in the selected medium in the presence of 1 μM Oxonol VI, and the increase in Oxonol VI absorbance was monitored. After 5 min of incubation the H^+-consuming pump was started by the addition of the appropriate nucleotide, leading to a new lower value due to a decreased membrane potential. The actual change in membrane potential was then calculated.

Assays

The changes in NADH and NADPH absorbance were measured at 340 nm in a Perkin-Elmer Lambda-5 spectrophotometer, using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Oxonol VI absorbance was monitored at 630–602 nm in an Aminco DW-2 dual wavelength spectrophotometer fitted with a magnetic stirrer. The ClO_4^- sensitive electrode was built by sticking a PVC membrane containing Aliquat 336, an anion exchanger, to the body of a Radiometer F2112 selectrode. Output was fed to a Radiometer pHM26 mVoltmeter and to a Linseis L512 chart recorder. 9-Aminoacridine fluorescence measurements were carried out by using a Perkin-Elmer 650-40 fluorimeter at 400 and 460 nm as excitation and emission wavelength, respectively. All measurements were carried out under thermostatted conditions.

Preparations

Bovine heart submitochondrial particles were prepared according to Petronilli et al. [29] in a medium containing 0.25 M sucrose, 10 mM Tris-Mops, 5 mM ATP, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM succinate and 2 mM dithiothreitol.

Chemicals

Oxonol VI was a gift from Dr. W.G. Hanstein (Department of Biochemistry, Bochum University, F.R.G.). Enzymes and nucleotides were purchased from Sigma.

Results

Fig. 2 shows the effects of oligomycin on J_{NADH}, the rate of ATP-driven, succinate-linked NAD^+ reduction at site I, on J_{NADPH}, the rate of the transhydrogenase reaction, and on the levels of $\Delta\psi$ ($\Delta\tilde{\mu}_{H^+}$). Low concentrations of oligomycin induced a slight increase in $\Delta\psi$, parallelled by an enhancement of the rate of the transhydrogenase reaction. This effect is in agreement with previous observations that in the presence of low amounts of oligomycin the leakiness of the membrane for H^+ was lowered by the reaction of oligomycin with the ATPase complex lacking F_1 or other subunits [12,15]. In agreement with the observations by Ernster et al. [12,30], J_{NADH} was markedly depressed by the addition of even low concentrations of oligomycin (Fig. 2). Furthermore, oligomycin also had a markedly higher inhibitory effect on J_{NADH} than on J_{NADPH} and on the level of $\Delta\psi$ ($\Delta\tilde{\mu}_{H^+}$). J_{NADPH} correlated well with the level of $\Delta\psi$ ($\Delta\tilde{\mu}_{H^+}$), while J_{NADH} did not.

The relationship between J_{NADPH} and $\Delta\psi$ in the presence of increasing concentrations of oligomycin

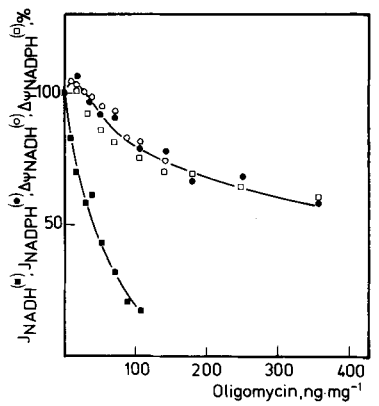

Fig. 2. Effects of oligomycin on the rates of ATP-driven NAD^+ reduction at site I (J_{NADH}) and transhydrogenation (J_{NADPH}) and on $\Delta\psi$. Submitochondrial particles (0.14 mg/ml) were incubated for 5 min in a medium composed of 0.18 M sucrose, 30 mM Tris-Mops (pH 7.3), 5 mM magnesium acetate, 5 mM $(NH_4)_2HPO_4$, 0.5 mM EGTA, 2 mM ATP, 10 mM phosphocreatine, excess of creatine kinase, 0.5 μg/ml antimycin A, and the amounts of oligomycin indicated. The temperature was 25°C. Additions: for NAD^+ reduction the medium also contained 10 mM succinate-Tris and 1 μM Oxonol VI, and the reaction was started by the addition of 0.5 mM NAD^+; for $NADP^+$ reduction the medium also contained 50 μM NADH, 0.2 M ethanol, excess alcohol dehydrogenase, 5 mM hydrazine and 4 μM rotenone, and the reaction was started by the addition of 0.5 mM $NADP^+$. Assays of $\Delta\psi$ were carried out in a parallel sample in the presence of 1 μM Oxonol VI. Symbols denote: ■, J_{NADH}; ●, J_{NADPH}; ○, $\Delta\psi$ during NAD^+ reduction; □, $\Delta\psi$ during $NADP^+$ reduction. In the absence of oligomycin, J_{NADH} and $\Delta\psi$ were 60 nmol/min per mg and 156 mV, respectively, during NAD^+ reduction. During $NADP^+$ reduction the corresponding values were 72 nmol/min per mg and 144 mV.

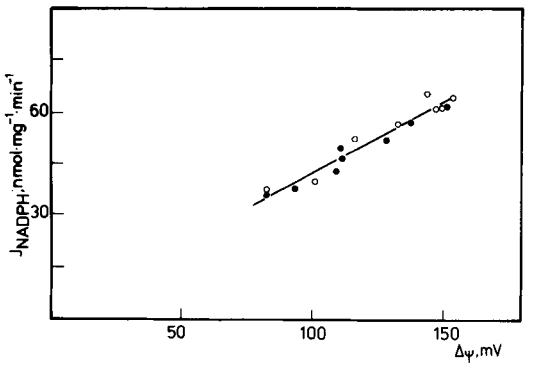

Fig. 3. Relationship between the rate of ATP-driven $NADP^+$ reduction (J_{NADPH}) and $\Delta\psi$ in the presence of varying concentrations of oligomycin or FCCP. The measurements were carried out as described in Fig. 2. Additions were: ○, 0–259 ng oligomycin/mg protein; ●, 0–200 pmol FCCP/mg protein.

and FCCP is shown in Fig. 3. In accordance with the data in Fig. 2, J_{NADPH} increased linearly with $\Delta\psi$ over a wide range. Interestingly, the same linear relationship was found with both oligomycin and FCCP. Thus, with the transhydrogenase as the H^+-consuming pump, the flow–force relationship was independent of the agent depressing $\Delta\tilde{\mu}_{H^+}$.

Determination of the effects of oligomycin and FCCP on ATP-driven J_{NADH} (site I reversal) and $\Delta\psi$ yielded flow–force relationships with considerably more pronounced slopes than in the case of J_{NADPH} (Fig. 4A). Inhibition of J_{NADH} by FCCP was accompanied by a somewhat larger depression of $\Delta\psi$ than in the case of oligomycin, so that the slope of the plot was less steep. It should be stressed that a difference in the slope of the flow–force relationships obtained in the presence of oligomycin or FCCP was found independently of whether a small $\Delta\tilde{\mu}_{H^+}$ increase was induced by pretreatment with low oligomycin concentrations (not shown). In all cases the depression of $\Delta\psi$ at equivalent extents of inhibition of J_{NADH} was larger in the presence of uncouplers. Occasionally, low concentrations of oligomycin induced an increase in $\Delta\psi$, which, unexpectedly, was accompanied by a depression rather than an increase of J_{NADH} (Fig. 4B).

A permeable anion, i.e., ClO_4^- was then used to depress $\Delta\tilde{\mu}_{H^+}$. Fig. 5 shows the relationship between J_{NADH} and $\Delta\psi$ ($\Delta\tilde{\mu}_{H^+}$) in the presence of FCCP or ClO_4^-. As can be seen in Fig. 5 the slope of the plot was much less steep in the case of ClO_4^- as compared to FCCP. Thus, with ClO_4^- as $\Delta\tilde{\mu}_{H^+}$-depressing agent, much larger changes of $\Delta\tilde{\mu}_{H^+}$ were required to achieve an inhibition of J_{NADH} equivalent to that observed with FCCP. However, because of the limited sensitivity

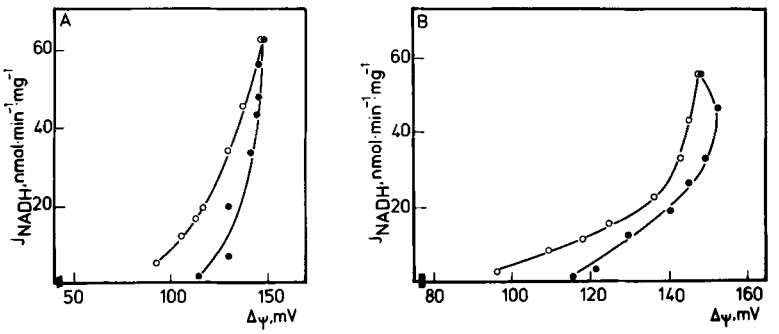

Fig. 4. Relationship between the rate of ATP-driven NAD^+ reduction at site I (J_{NADH}) and $\Delta\psi$ as obtained in titrations with oligomycin or FCCP. The measurements were carried out as described in the experimental section and in the legend of Fig. 2. Additions: (A): ●, 0–125 ng oligomycin/mg protein; ○, 0–144 pmol FCCP/mg protein; (B): ●, 0–95 ng oligomycin/mg protein; ○, 0–150 pmol FCCP/mg protein.

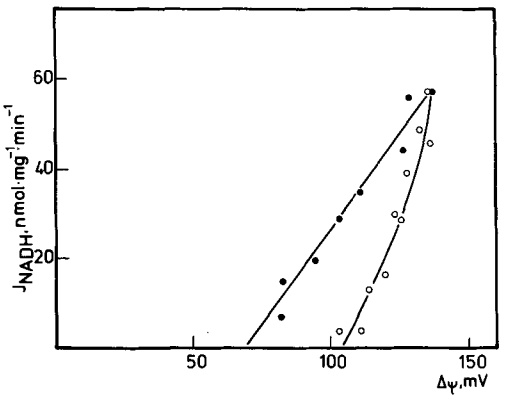

Fig. 5. Titrations of J_{NADH} and $\Delta\psi$ with FCCP or ClO_4^-. The measurements were carried out as described in the legend of Fig. 2. Additions: ○, 0–214 pmol FCCP/mg protein; ●, 0–1.6 mM $KClO_4$. ΔpH was found to be negligible (< 0.5 units) in parallel experiments with 9-aminoacridine.

of the ΔpH probe, an undetectable increase of ΔpH (lower than 0.5 units) might occur in the presence of ClO_4^-, which would lead to a less steep slope of the plot.

Inhibition of succinate oxidation by malonate indicated that this agent had an effect on J_{NADPH}, J_{ATP} and $\Delta\psi$ ($\Delta\tilde{\mu}_{H^+}$) (Fig. 6) which was comparable to that of oligomycin (cf. Fig. 2). Interestingly, at the same malonate concentration, the level of $\Delta\psi$ during trans-

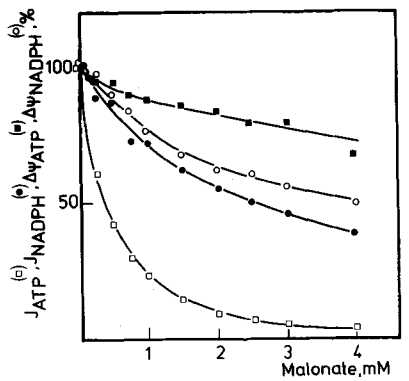

Fig. 6. Malonate titrations of the rates of succinate-driven ATP synthesis (J_{ATP}) and transhydrogenation (J_{NADPH}), and of $\Delta\psi$. Submitochondrial particles (0.14 mg/ml) were incubated for 5 min in a medium containing 0.18 M sucrose, 30 mM Tris-Mops (pH 7.3), 5 mM magnesium acetate, 5 mM $(NH_4)_2HPO_4$, 0.5 mM EGTA, 10 mM succinate-Tris, 4 μM rotenone and the indicated concentrations of malonate. The temperature was 25°C. In the case of ATP synthesis additions were: 50 μM Ap5A, excess glucose-6-phosphate dehydrogenase and hexokinase, 5 mM glucose, 0.5 mM NADP, and 5 mM P_i-Tris; the reaction was started by the addition of 0.2 mM ADP. In the case of $NADP^+$ reduction additions were: 50 μM NADH, 1.5 μg/ml oligomycin, 0.2 M ethanol, excess of alcohol dehydrogenase and 5 mM hydrazine; the reaction was started by the addition of 0.5 mM $NADP^+$. determinations were carried out in parallel samples in the presence of 1 μM Oxonol VI. Symbols denote: □, J_{ATP}; ●, J_{NADPH}; ■, $\Delta\psi$ during ATP synthesis; ○, $\Delta\psi$ during $NADP^+$ reduction.

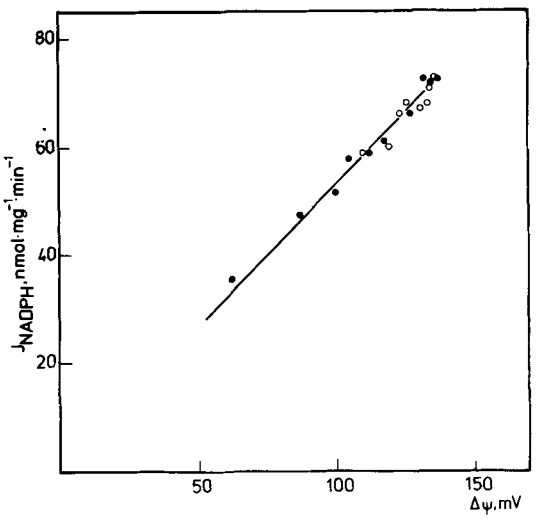

Fig. 7. Relationship between succinate-driven J_{NADPH} and $\Delta\psi$ in titrations with malonate or FCCP. The measurements were carried out as described in the legend of Fig. 6. Additions were: ●, 0–5 mM malonate; ○, 0–214 pmol FCCP/mg protein.

hydrogenation was lower than that found during ATP synthesis.

The effect of malonate and FCCP on J_{NADPH} and $\Delta\psi$ is shown in Fig. 7. In accordance with the data of Fig. 6 and similar to the flow–force relationship of Fig. 3, J_{NADPH} decreased linearily with $\Delta\psi$ in the range investigated, regardless of whether malonate or FCCP was used to depress $\Delta\tilde{\mu}_{H^+}$.

Malonate and FCCP also caused a marked depression of J_{ATP} (Fig. 8). Although the difference was small, the slope of the plot was steeper with malonate than with FCCP (see however, Sorgato et al., Ref. 10). Fig. 9 shows the relationship between J_{ATP} and $\Delta\psi$ in the presence of FCCP and ClO_4^-. Similar to the results in Fig. 5, a more extensive decrease of $\Delta\tilde{\mu}_{H^+}$ was necessary with ClO_4^- to achieve extents of inhibition of

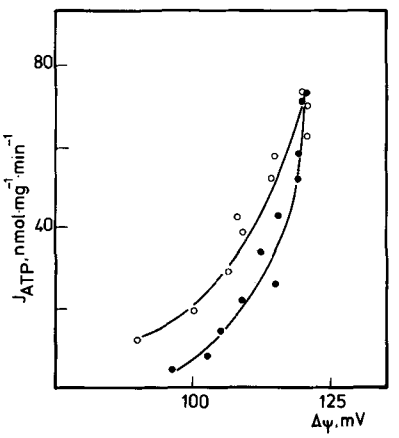

Fig. 8. Relationship between succinate-driven J_{ATP} and $\Delta\psi$ in titrations with malonate or FCCP. The measurements were carried out as described in the legend of Fig. 6. Additions: ●, 0–2.5 mM malonate; ○, 0–214 pmol FCCP/mg protein.

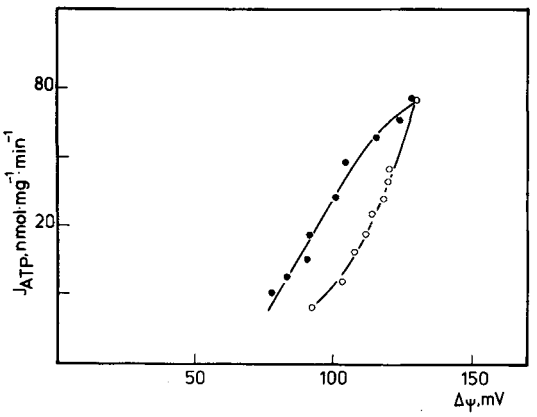

Fig. 9. Relationship between succinate-driven J_{ATP} and $\Delta\psi$ in titrations with FCCP and ClO_4^-. The measurements were carried out as described in the legend of Fig. 6. Additions: ○, 0–214 pmol FCCP/mg protein; ●, 0–2 mM ClO_4^-.

J_{ATP} equivalent to those observed with FCCP. This rendered the relationship J_{ATP} vs. $\Delta\tilde{\mu}_{H^+}$ less steep with ClO_4^- than with FCCP.

Discussion

It is now generally accepted that inhibition of redox pumps with respiratory inhibitors leads to a marked decrease in ATP synthesis, with comparatively small changes in $\Delta\tilde{\mu}_{H^+}$ [5,9]. This behaviour, which reflects a very steep dependence of the rate of ATP synthesis on $\Delta\tilde{\mu}_{H^+}$, has been analysed by the use of a non-linear non-equilibrium thermodynamic treatment [31–33].

The experiments of the present study indicate that the effect of oligomycin on the ATP-driven succinate-linked reduction of NAD^+ is similar to that of malonate on succinate-linked ATP synthesis. Addition of inhibitors of the H^+-generating pump results in a marked inhibition of the rate of the H^+-consuming pump in the presence of little or no depression of $\Delta\tilde{\mu}_{H^+}$. This leads to a very steep flow–force relationship between J_{NADH} or J_{ATP} and $\Delta\tilde{\mu}_{H^+}$. In contrast, in the case of the transhydrogenase, inhibition of ATPase with oligomycin and inhibition of succinate oxidation with malonate result in a parallel decrease in J_{NADPH} and $\Delta\tilde{\mu}_{H^+}$ An additional difference between transhydrogenase on one hand, and ATP synthesis and the site I redox pump (as H^+-consuming pumps) on the other, is that the linear relationship between J_{NADPH} and $\Delta\tilde{\mu}_{H^+}$ is identical with malonate and FCCP.

The linearity and proportionality characterizing the relationship between J_{NADPH} and $\Delta\psi$ ($\Delta\tilde{\mu}_{H^+}$) (Figs. 3, 7) contrast sharply with the form of the dependence on the same driving force of the rate of ATP synthesis and of NAD^+ reduction at Site I (Figs. 4, 8, 9). The difference may be related to the different ΔG° values for these reactions: about zero [29] for transhydrogenation, and more than 19 kJ/mol for ATP-driven succinate-linked NAD^+ reduction and ATP synthesis [35–37]. A ΔG° value close to zero implies that if ΔG is held close to ΔG°, small fluctuations in either $\Delta\tilde{\mu}_{H^+}$ or the nicotinamide nucleotide redox potential around the equilibrium point (i.e., zero) will lead to a transition from NADPH formation to NADPH oxidation, or vice versa. Therefore, the plot of J_{NADPH} vs. $\Delta\mu_{H^+}$ is expected to go through the origin. In the case of the other pumps, if $\Delta G = \Delta G^\circ$, the flow will be zero at $\Delta\tilde{\mu}_{H^+} = \Delta G^\circ/n$ (where n is the stoichiometry of the H^+-consuming pump). In other words, a threshold value of $\Delta\tilde{\mu}_{H^+}$ is expected to be evident in the flow–force relationships of these pumps. Whether the difference in ΔG° between the two classes of H^+ pumps may be sufficient to explain all the observed differences remains in any case to be clarified.

A number of reports have appeared in the last 10 years indicating that the relationship between output flow (for example rate of ATP synthesis) and $\Delta\tilde{\mu}_{H^+}$ depends on how $\Delta\tilde{\mu}_{H^+}$ is varied [3,4]. In particular, it has been stressed that agents causing a decrease in $\Delta\tilde{\mu}_{H^+}$ by activating various ion transport systems, such as protonophores or valinomycin plus K^+, yield flow–force relationships which are less steep than those observed in the presence of agents which act directly at the level of the H^+-generating pump [5], e.g., malonate or antimycin A (cf. Ref. 38). In general, the present results agree with these observations, i.e., (i) there is a pronounced difference in the slope of the flow–force relationship for different combinations of pumps, for example with the ATPase (inhibitor: oligomycin) or redox site II (inhibitor: malonate) as H^+-generating and redox site I as H^+-consuming pumps; (ii) with both combinations of H^+-generating and H^+-consuming pumps the difference in slope is slight between titrations with FCCP or with oligomycin, or with FCCP vs. malonate, but it becomes larger when the effect of the inhibitors of the H^+-generating pump is compared with that of lipophilic anions.

A major argument against the validity of the flow–force relationships has often been claimed to be a less-than-reliable determination of $\Delta\tilde{\mu}_{H^+}$ [4,10,11]. However, the present investigation clearly shows that the flow–force relationships for transhydrogenase are linear and almost proportional, and hold independently of whether the H^+-generating pump is the ATPase or succinate-driven respiration, and of whether $\Delta\tilde{\mu}_{H^+}$ is depressed by inhibitors of the H^+-generating pump or by uncouplers. The fact that the flow–force relationships of the other pumps show strikingly different patterns strongly suggests that these patterns, as well as those of the transhydrogenase, are consequences of the inherent pump properties rather than a result of erroneous $\Delta\tilde{\mu}_{H^+}$ assays.

The present results may be compared with those reported by Jackson and co-workers using *Rhodobacter capsulatus* chromatophores [16,17]. The transhydro-

genase of that organism also obeys a nearly linear and proportional relationship between J_{NADPH} and $\Delta\tilde{\mu}_{H^+}$, which does not depend on the means used to modulate these parameters. Cotton and Jackson [17] report a unique relationship between the rates of transhydrogenation and ATP synthesis in these chromatophores. This is taken to imply that the rate of phosphorylation also depends in a unique way on the $\Delta\tilde{\mu}_{H^+}$ (the relationship was not determined directly). They also observe that the rate of the transhydrogenase reaction is more resistant than ATP synthesis to addition of uncouplers or respiratory inhibitors. This is explained as being due to a differential regulation by $\Delta\tilde{\mu}_{H^+}$ of ATP synthesis and transhydrogenation. In our view this differential sensitivity is not dissimilar to the less steep dependence of J_{NADPH} on a $\Delta\tilde{\mu}_{H^+}$ observed in the present work. In previous work Melandri and collaborators had favoured a localized coupling model in chromatophores [7,8].

In summary, we have to consider three features of the flow–force relationships when either the transhydrogenase is coupled to the redox or the ATPase proton pump or the redox and the ATPase pumps are coupled together. The three features are: (i) linearity; (ii) proportionality; and (iii) differential effects of various agents or inhibitors. In the case of the transhydrogenase of submitochondrial particles it appears that the flow–force relationship has the characteristics of linearity and proportionality and in addition no differential effects between the various agents or inhibitors are observed. In the case of the ATPase and the redox pumps, on the other hand, all three features are different in that there is no linearity and no proportionality, and the effects of the various inhibitors are not identical. As discussed previously, while non-linearity and non-proportionality may be attributed to the thermodynamic and kinetic properties of the redox and ATPase proton pumps, as compared to the transhydrogenase proton pump, this explanation is not sufficient for the differential effects of the various inhibitors. For these latter effects we are left with the alternative of either some specific interaction of the inhibitor with either of the pumps participating in the coupling process or with the concept of localized interaction between the pumps. We have no experimental evidence to choose between the two alternatives. However, the choice between one of these two alternatives has important implications as for the nature of the proton circuit connecting the transhydrogenase to the other proton pumps. In fact, the view that no localized proton circuits are operating in the case of the transhydrogenase proton pump, i.e., that this pump is completely delocalized with respect to the redox and ATPase proton pumps, provides indirect support for the parallel concept of the existence of localized proton circuits between the redox and ATPase proton pumps.

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