PROTON TRANSLOCATION QUOTIENT FOR THE ADENOSINE TRIPHOSPHATASE OF RAT LIVER MITOCHONDRIA

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1. Introduction

The hydrolysis of ATP to ADP and P_i by intact rat liver mitochondria gave an overall $\rightarrow H^+/P$ quotient near 2.0 [1, 2]. As discussed previously, however, the $\rightarrow H^+/P$ quotient for (intracristal) hydrolysis of ATP added to suspensions of intact mitochondria does not necessarily represent the $\rightarrow H^+/P$ quotient characteristic of the ATPase, because any proton translocation associated with the porter-catalysed entry of ATP and exit of ADP and P_i during the ATP hydrolysis would also be included [2–4]. Cristae membrane vesicles produced by sonication of mitochondria are mostly inside out [5–7], and the ATPase is directly accessible to ATP added to the medium in which the sonic vesicle preparations are suspended, as shown by their insensitivity to atractyloside [8]. We have therefore used sonic vesicle preparations of rat liver mitochondria to estimate the $\rightarrow H^+/P$ quotient characteristic of the ATPase in experiments otherwise similar to those with intact mitochondria.

We show here that the $\rightarrow H^+/P$ quotient characteristic of the ATPase of sonic vesicles of rat liver mitochondria is near 2.0; we confirm that an $\rightarrow H^+/P$ quotient near 2.0 is also obtained for the overall translocation plus ATPase reaction catalysed by intact mitochondria; and we conclude that there is no net proton translocation associated with the porter-catalysed entry of ATP and exit of ADP and P_i under the conditions of our experiments with intact mitochondria.

Abbreviations:

FCCP : carbonylcyanide *p*-trifluoromethoxyphenylhydrazone;
pH_0 : $-\log_{10}$(chemical activity of H^+ ions in the outer aqueous phase);
ΔH_0^+ : quantity of protons added to (or entering) the outer aqueous phase;
$\rightarrow H^+$: quantity of protons translocated;
$\rightarrow H^+/P$: proton translocation quotient giving number of protons translocated per ATP molecule hydrolysed.

2. Materials and methods

Rat liver mitochondria were isolated as described previously [9]; and the time-course of proton translocation by intact mitochondria and by sonic vesicle preparations was estimated from pH_0 recordings in the presence of 10 μg of valinomycin per g of mitochondrial protein or 60 μg of valinomycin per g of vesicle protein, using corresponding experiments in the presence of 1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to give base lines, as before [2, 10]. Sonic vesicles were prepared from mitochondrial suspensions (30 mg of protein/ml) at 4° in 250 mM sucrose containing 5 mM $MgCl_2$ and 20 mM sucrose containing 5 mM $MgCl_2$ and 20 mM glycylglycine-KOH buffer at pH 7.5, using an MSE 60 W sonicator operated at full power for 60 sec. The vesicles were isolated by differential centrifugation at 4°; they were washed twice on the centrifuge in 150 mM KCl, and were finally suspended in 250 mM sucrose at a concentration corresponding to 80 mg of protein/ml. The rate of ATP hydrolysis by the sonic vesicles was estimated by the pH method [11], essentially as before [12]. The rate of proton translocation was given by the slope of the observed time-course of proton translocation (i.e. the apparent rate of proton translocation) plus the rate of decay of the proton displacement. At any given time, the rate of decay of the proton displacement was

given by the product of the proton displacement and the velocity constant for the decay of this displacement. As pulses of respiration (and the accompanying proton translocation) terminate more abruptly than pulses of ATP hydrolysis, the velocity constant, which is equal to (ln 2)/(time for half decay), was obtained from recordings of the decay of the proton displacement after respiratory pulses [7, 9, 10], using 1 mM NADH as substrate and injecting small amounts of oxygen under conditions otherwise similar to those used in the ATP hydrolysis experiments. Aurovertin was donated by Prof. R.B. Beechey (Shell Research, Sittingbourne, Kent). Atractyloside was a gift from Prof. J.B. Chappell (Bristol University).

3. Results and conclusions

3.1. *Intact mitochondria*

Fig. 1 shows the number of acid equivalents produced in the external medium per mole of ATP injected into suspensions of intact rat liver mitochondria ($\Delta H_0^+/ATP$) under various conditions. The rapid initial alkalinisation that occurs in presence of FCCP is attributable to an inward translocation of negative charge, as discussed previously [2–4]. In the absence of aurovertin, ATP hydrolysis was relatively sluggish (+FCCP), and there was a correspondingly slow outward proton translocation (+valinomycin), which, using a rather complicated procedure [2], extrapolates to an $\rightarrow H^+/P$ quotient near 2.0. In the presence of aurovertin, which greatly increases the affinity of the ATPase for ATP relative to ADP [12], ATP hydrolysis proceeded rapidly to completion (+FCCP), and there was a correspondingly rapid outward proton translocation (+ valinomycin), extrapolating, in this typical experiment, to a $\Delta H_0^+/ATP$ value near 2.8. As the net acidification due to the hydrolysis of ATP near pH 7.0 corresponds to 0.8 H^+ ions per ATP molecule, the extrapolated overall $\rightarrow H^+/P$ quotient is near 2.0.

The results of 16 experiments corresponding to that of fig. 1 in presence of aurovertin gave a mean $\rightarrow H^+/P$ quotient of 2.00 ± 0.18, thus confirming our earlier findings.

3.2. *Sonic vesicles*

The pH_0 recordings showing the time-course of ATP hydrolysis and proton translocation in suspensions of sonic vesicles were essentially similar to those published previously [7]. They differed from those obtained with suspensions of intact mitochondria in that proton translocation was inward instead of outward, and no fast initial pH_0 change, attributable to a shift of charge across the membrane, was observed in the presence of FCCP.

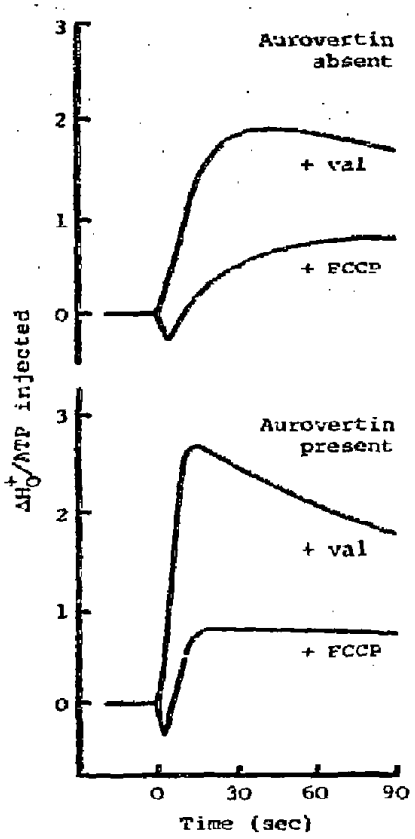

Fig. 1. Time-course of changes of pH_0 during hydrolysis of ATP by intact rat liver mitochondria, expressed as $\Delta H_0^+/ATP$ injected. Mitochondria (6 mg of protein/ml) were suspended in 3.3 ml of anaerobic medium containing 150 mM KCl, 25 mM sucrose, 3.3 mM glycylglycine-KOH buffer and 1 mM EDTA at pH_0 7.0–7.1 and at 25°. In the experiments shown in the lower part of the figure, 1 mg of aurovertin/g of mitochondrial protein was present. Where indicated, 10 μg of valinomycin/g of protein (val) or 1 μM FCCP were also present. At zero time (after a 15-min preincubation period), 50 nmoles of ATP (5 μl of an anaerobic 10 mM ATP solution adjusted to pH 7.05) was injected into the mitochondrial suspensions.

In order to obtain optimum conditions for measuring $\rightarrow H^+/P$ quotients in suspensions of sonic vesicles, it was necessary to work at acid pH, where the time for half decay of the proton displacement was consider-

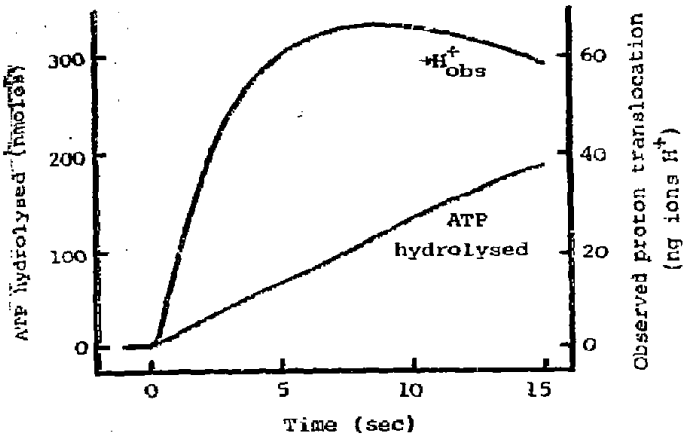

Fig. 2. Time-course of hydrolysis of ATP and observed inward translocation of protons, $\rightarrow H^+_{obs}$, (derived from recordings of pH_0 obtained in the presence of valinomycin or FCCP as explained in the Materials and methods section), by sonic vesicle preparations from rat liver mitochondria. The sonic vesicles (5 mg of protein/ml) were suspended in 3.3 ml of anaerobic medium containing 150 mM KCl, 15 mM sucrose, 3.3 mM glycylglycine, 5 mM $MgCl_2$, 20 mM K_2SO_4, 0.2 mg of carbonic anhydrase (Sigma), 40 µl of 3 mM atractyloside and 0.22 mg of oligomycin/g of vesicle protein, at pH_0 6.2–6.3 and at 25°. At zero time (after a 15-min preincubation period), 500 nmoles of Mg-ATP (10 µl of an anaerobic solution containing equimolar ATP and $MgCl_2$ adjusted to pH 6.25, was injected into the vesicle suspensions.

ably longer than at pH 7. We used a suspension medium near pH 6.25 that routinely contained 5 mM $MgCl_2$; and 20 mM sulphate was included because it was previously found to activate the ATPase, and to delay the onset of inhibition by ADP [13, 14]. Atractyloside was added to inhibit possible ATP hydrolysis by membrane vesicles that were not inside out.

Preliminary experiments, in which the vesicle suspensions were titrated with oligomycin, showed that the observed extent of proton translocation during ATP hydrolysis was optimised by a quantity of oligomycin (0.22 mg/g of vesicle protein) that increased the half time of decay of the proton displacement near pH 6.25 from 2.5 sec to 4.5 sec. This quantity of oligomycin was about the same as that used by Lee and Ernster (0.30 mg/g of vesicle protein) [15] to optimise oxidative phosphorylation in sonic vesicle preparations of beef heart mitochondria.

Fig. 2 shows the time-course of hydrolysis of ATP and the observed inward translocation of protons ($\rightarrow H^+_{obs}$) in a suspension of sonic vesicles of rat liver

Table 1
$\rightarrow H^+/P$ quotient for the ATPase of rat liver mitochondria.

Rate of ATP hydrolysis (nmoles/sec)	Observed rate of proton translocation (ng ions/sec)	Rate of decay of proton displacement (ng ions/sec)	$\rightarrow H^+/P$
13.0	17.5	5.0	1.74
13.0	18.3	5.1	1.80
12.4	16.5	4.8	1.72
11.8	17.0	4.1	1.79
11.6	14.5	4.0	1.60

The values in the table were obtained from experiments similar to those illustrated in fig. 2, and the rate of decay of the proton displacement was estimated as described in the Materials and methods section. The measurements were made at 2 sec after injecting the ATP.

mitochondria in the presence of the optimum concentration of oligomycin. The rate of ATP hydrolysis remained almost constant during the first 10 sec, and the observed rate of proton translocation did not begin to fall rapidly until more than 2 sec after the addition of the ATP. We have therefore used the observed rates of ATP hydrolysis and proton translocation at 2 sec after the addition of the ATP to calculate the value of the $\rightarrow H^+/P$ quotient. The values of $\rightarrow H^+/P$ quotients from five experiments corresponding to those of fig. 2 are shown in table 1, together with the data used to obtain them.

The mean $\rightarrow H^+/P$ quotient obtained in this work is 1.73 ± 0.10. As our sonic vesicle preparations may contain some ATPase situated in membrane material that is not topologically closed or that has a relatively high proton conductance, it is to be expected that the $\rightarrow H^+/P$ quotient may be under-estimated, in spite of the allowance made for the dissipation of the proton displacement on the basis of the decay rate of this displacement observed in the vesicle suspension as a whole. We conclude that the $\rightarrow H^+/P$ quotient of the proton-translocating ATPase is near 2.0.

4. Discussion

In our earlier work on the proton-translocating

ATPase of rat liver mitochondria, we assumed that the oligomycin-insensitive ATPase activity was not attributable to the proton-translocating ATPase [2]; and the fact that some proton translocation could be observed in suspensions of intact mitochondria in the presence of high concentrations of oligomycin was therefore taken as evidence for net proton translocation associated with the entry of ATP and exit of ADP and P_i through the specific porter systems [3]. Klingenberg and collaborators [4] reached a closely related conclusion. When we observed that oligomycin does not completely inhibit the proton-translocating ATPase of rat liver mitochondria, but only decreases V_{max} to a lower, but significant, value [14], it became evident that our earlier conclusion [3] concerning the net proton-translocating property of the ATP, ADP and P_i porter systems was open to doubt. The present finding that the $\rightarrow H^+/P$ quotient of the proton-translocating ATPase of the sonic vesicle preparation is not significantly different from the $\rightarrow H^+/P$ quotient characteristic of the hydrolysis of externally added ATP by intact mitochondria shows that the entry of ATP and the exit of ADP and P_i through the porter systems of the cristae membrane is not associated with net proton translocation under the conditions of our experiments.

According to the chemiosmotic hypothesis, the P/O quotient is given by the $\rightarrow H^+/O$ quotient characteristic of respiratory chain activity divided by the $\rightarrow H^+/P$ quotient characteristic of (reversible) ATPase activity [16]. Thus, our observation that the $\rightarrow H^+/P$ quotient is not significantly less in sonic vesicle preparations than in whole mitochondrial suspensions (and that there is thus no proton-translocation contribution by the system catalysing ATP/ADP antiport and P_i uniport) is consistent with the fact that limiting P/O quotients are not greater in sonic vesicle preparations than in suspensions of intact mitochondria [15].

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