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ESTIMATION OF H^+-TRANSLOCATION STOICHEIOMETRY OF MITOCHONDRIAL ATPase BY COMPARISON OF PROTON-MOTIVE FORCES WITH CLAMPED PHOSPHORYLATION POTENTIALS IN SUBMITOCHONDRIAL PARTICLES

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The proton-motive forces generated in submitochondrial particles by both hydrolysis of ATP and oxidation of succinate have been measured by flow dialysis and compared with the ambient phosphorylation potentials. It is concluded that three H^+ are translocated for each ATP molecule hydrolysed or synthesised. By utilising rat liver mitochondria respiring with β-hydroxybutyrate as a new system for regeneration of ATP from ADP and P_i, phosphorylation potentials were clamped at a range of values by using mixtures of particles and mitochondria in various ratios. As the rate of ATP hydrolysis by the particles was lowered, the proton-motive force decreased only slightly except at the very lowest rates, these results paralleling earlier studies on the relation between rate of respiration-driven proton translocation and proton-motive force.

There is uncertainty about the stoicheiometry of proton translocation [H^+/ATP] that is catalysed by the mitochondrial ATPase. Correlation of the quantity of proton translocation with the amount of ATP hydrolysed has given estimates for H^+/ATP of either 2 [1–3] or 3 [4]. An alternative method is to compare the magnitudes of the proton-motive forces, Δp, and phosphorylation potentials, ΔG_p, that are generated by respiring mitochondria or submitochondrial particles. Extensive studies of this type have also given estimates for H^+/ATP of 2 [5–9] or 3 [10,11]. The latter type of measurement will tend to give an underestimate for H^+/ATP because complete equilibration between Δp and ΔG_p may not be reached [12]. Therefore, complementary measurements of ΔG_p and Δp are required under conditions in which ATP hydrolysis generates Δp. A value for H^+/ATP so obtained will tend to be an overestimate because H^+ back-flow across the mitochondrial membrane is expected to lower Δp below the value at which it would be in equilibrium with ΔG_p. Thus, values of H^+/ATP obtained from experiments in which respiration or ATP hydrolysis is generating Δp will provide lower or upper limits for the value of H^+/ATP.

Submitochondrial particles have been chosen for studies on the value of H^+/ATP because the comparison of ΔG_p with Δp sustained by the particles is free of complications that arise with intact mitochondria; in the latter system the external ΔG_p is believed to be related to Δp not only by H^+/ATP but also by an extra term that expresses

Abbreviations: Δp, proton-motive force $= \Delta\tilde{\mu}_{H^+}/F = \Delta\psi + 2.303RT\,\Delta pH/F$, where F is the Faraday constant; ΔG_p, phosphorylation potential $= \Delta G^{o\prime} + RT\ \ln[ATP]/[ADP][P_i]$; $\Delta\psi$, membrane potential (in mV); ΔpH, pH gradient across the membrane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Ap_5A, P^1,P^5-di(adenosine-5'-)pentaphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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the net movement of protons into the matrix associated with the combined process of phosphate transport and ATP-ADP exchange [12]. The flow-dialysis method has been used as a non-perturbing assay of the uptake into respiring submitochondrial particles of the indicators of membrane potentials, $\Delta\psi$, and pH gradient, ΔpH. A disadvantage of this method is that the relatively large amounts of particles required, and the typical length of the experiments (between 11 and 14 min in the present work), can result in large changes in the concentration of the substrate that is responsible for generating Δp. This is particular problem for the ATP hydrolysis reaction because both the term in $\Delta G^{o\prime}$ and the term containing the log of the reactant and product concentration ratio make comparable contributions to ΔG_p. Hence, during a flow-dialysis experiment in which ATP is initially added, ΔG_p will change continuously and considerably, thus making comparison with Δp difficult. The usual regenerating systems for ATP, e.g., pyruvate kinase, alleviate this problem only partially as P_i concentration can still change considerably during an experiment. To overcome this problem we have used mitochondria oxidising β-hydroxybutyrate as a novel method to regenerate ATP from ADP and P_i. This substrate was chosen because it is not utilised by submitochondrial particles unless NAD^+ is also added.

Bovine heart MgATP submitochondrial particles that are relatively deficient in ATPase-inhibitor protein were prepared according to the procedure for 'Type-II' particles given in Ref. 13, and finally resuspended in 200 mM sucrose, 5 mM phosphate-Tris (pH 7.3), 5 mM $Mg(CH_3COO)_2$. Liver mitochondria from rats that had been starved overnight were isolated by standard centrifugation procedures in 220 mM mannitol, 70 mM sucrose, 3 mM Hepes (pH 7.4) essentially as described in Ref. 14 and were finally suspended in 200 mM sucrose, 1 mM EGTA (pH 7.4). Flow-dialysis measurements at 25 ± 1°C of the uptake of 6 μM $S^{14}CN$, 15–20 μM $^{14}CH_3NH_2$ or 20 μM $[^{14}C](CH_3)_2N$ (all from the Radiochemical Centre, Amersham, Bucks., U.K.) and determinations of ΔG_p were done as described previously [10,11]. Protein was determined by the biuret method [15]. DL-3-Hydroxybutyrate and ADP were obtained from Boehringer, Ap_5A from Sigma.

Before using mitochondria plus β-hydroxybutyrate as the regenreating system for supplying ATP to submitochondrial particles in flow-dialysis experiments, the following controls were done: (i) Submitochondrial particles were found not to respire in the presence of β-hydroxybutyrate, except when NAD^+ was also added, nor was any uptake of $S^{14}CN^-$ by the particles detected in the presence of β-hydroxybutyrate. (ii) Prolonged incubation (up to 11 min) of mitochondria with submitochondrial particles did not result in any detectable $S^{14}CN^-$ uptake by the particles in the presence of β-hydroxybutyrate. Hence, there was no leakage of NAD^+ from the mitochondria. (iii) Mitochondria oxidising β-hydroxybutyrate did not bind or accumulate $S^{14}CN^-$. In addition, it was found that mitochondria did not alter $S^{14}CN^-$ accumulation into the particles respiring with succinate nor did they interfere with the dialysis of $S^{14}CN^-$ from the flow-dialysis cell. Mitochondria were found to bind $^{14}CH_3NH_2$ (cf. Ref. 12). However, as under the reaction conditions used in the present work (see also ref. 11) no detectable ΔpH (using either CH_3NH_2 or $(CH_3)_3N$ as indicator of ΔpH) was generated in particles by either respiration or ATP hydrolysis in the absence of mitochondria, measurements of ΔpH were not required in experiments when mitochondria were present to regenerate ATP. The results of all these control experiments showed that the ATP-dependent Δp in submitochondrial particles could be determined without interference from the respiring mitochondria.

In a mixture of mitochondria and submitochondrial particles supplied with β-hydroxybutyrate, ADP and P_i, ΔG_p was found, from periodically measuring the adenine nucleotide and P_i concentrations, to remain constant throughout an experiment for measurement of $\Delta\psi$. The steady-state value of ΔG_p depended on the ratio of the concentration of particles to the concentration of mitochondria; the higher this ratio the lower the value of ΔG_p. Fig. 1 shows the results of a number of experiments in which Δp maintained in particles by ATP hydrolysis was measured over a range of clamped ΔG_p values that were determined by the choice of the particle to mitochondria ratio. The values of ΔG_p (Fig. 1) refer to the bulk aqueous phase of the suspension and therefore in con-

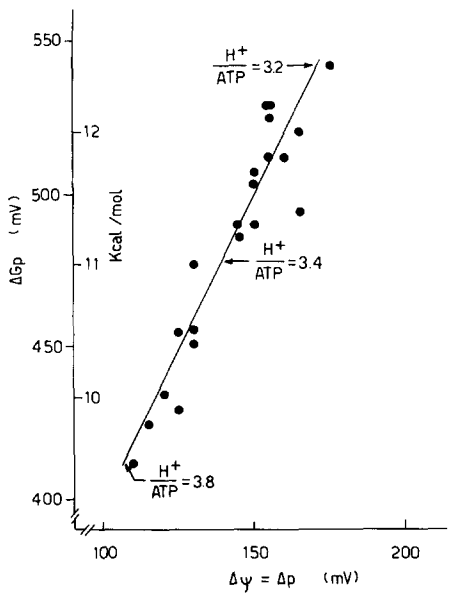

Fig. 1. $\Delta\psi$ generated in submitochondrial particles at different clamped values of ΔG_p. To the stirred upper chamber of the flow-dialysis cell were added 200 mM sucrose, 5 mM phosphate-Tris (pH 7.3), 5 mM $Mg(CH_3COO)_2$, 0.36 mM Ap_5A, 4 mM ADP, 50 mM DL-β-hydroxybutyrate and between 0.5 and 18 mg protein of mitochondria. 4 min later, 3.5–6 mg protein of submitochondrial particles that had been preincubated with Ap_5A were added. The final volume was 1 ml and water-saturated O_2 was blown over the suspension throughout. Temperature was 25 ± 1°C. After allowing 5 min for a steady-state value of ΔG_p to be attained, $S^{14}CN^-$ was added and the collection of the diffusate from the flow-dialysis cell was started. Oligomycin (1.5 μg per mg of total protein), was added after a further 6 min to inhibit both ATP synthesis and hydrolysis and to cause efflux of $S^{14}CN^-$, thus permitting measurement of $\Delta\psi$ [10]. Identical results were obtained when either 15 μM FCCP or Triton X-100 to a final concentration of 0.3–0.4% (v/v) was added in place of oligomycin. For determination of ΔG_p identical reaction mixtures, except that $S^{14}CN^-$ was omitted, were made and samples were taken and quenched with acid for measurements of adenine nucleotide and P_i [11] from two separate reaction mixtures between the ninth and eleventh minutes of incubation in the presence of particles.

tact with the ATPases of the particles. It was shown in separate experiments that the mitochondrial matrix concentrations of adenine nucleotide and P_i were negligible in comparison with the total concentrations of these molecules.

The data can be fitted by regression analysis to the linear plot shown in Fig. 1 with a correlation coefficient of 0.88. Estimates for H^+/ATP obtained from comparisons of Δp with ΔG_p became progressively lower as ΔG_p increased (Fig. 1). This behaviour is suggested to arise because when ΔG_p, and thus ATP/ADP, is relatively high, the ATPase enzyme will hydrolyse ATP faster than at lower values of ΔG_p, when inhibition of activity by ADP will be more significant. At the lower values of ΔG_p the value of Δp can be expected to deviate further below its theoretical value, owing to the relatively increasing importance of the rate of H^+ leakage from the lumen of the particles in determining the steady-state value of Δp. Thus, the value of H^+/ATP obtained from comparison of Δp with ΔG_p is expected to approach most closely its true value at high values of ΔG_p, when the rate of proton pumping is also at its highest. Consequently, the data in Fig. 1 are taken to show that the maximum value for H^+/ATP is no greater than 3.2.

Under identical conditions to those used for the experiments shown in Fig. 1, except that mitochondria and β-hydroxybutyrate were omitted but 30 mM sodium succinate was added, respiring submitochondrial particles were found to generate a Δp of 168.5 ± 6.5 mV and a ΔG_p of 11 ± 0.3 kcal/mol (average of eight experiments). Comparison of these two values gives an estimate of 2.8 as a minimum value for H^+/ATP. Taken together with the estimate of a maximum value of 3.2 obtained in the experiments with ATP as substrate, this finding constitutes good evidence that H^+/ATP is 3.

The slope of the plot shown in Fig. 1 is 2.01. This could be taken as an alternative upper limit for the value of H^+/ATP, as an estimate obtained in this way should be independent of systematic errors. Furthermore, the plot shown in Fig. 1 does not extrapolate through the origin but extrapolates to $\Delta p = -98$ mV at $\Delta G_p = 0$. Consideration therefore needs to be given to the question of whether Δp has been systematically underestimated by approx. 98 mV, and to the associated question of whether the plot shown in Fig. 1 can be justifiably subjected to a linear extrapolation to the origin.

Underestimation of Δp by as much as 98 mV would mean that the concentration of SCN^- within the particles must in turn have been underestimated by more than 30-fold [10]. An error of this magnitude is far beyond the error limits of the

flow-dialysis method for measuring SCN^- uptake, and therefore could only arise from a grossly incorrect determination of the internal volume of the particles. However, our estimate of the latter, 0.75 μl per mg protein, is comparable to other estimates under similar conditions to those used in the present work [11,16,17]. Additionally, we have shown that oxidation by the particles of ascorbate (30 mM) plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (100 μM), or of 50 mM succinate, in the presence of added cytochrome *c* (100 μM), did not drive detectable uptake into the particles of the triphenylmethylphosphonium cation that is accumulated by respiring mitochondria [18]. This result demonstrated that particles with the same orientation as mitochondria did not contribute significantly to the total internal volume enclosed by the particles, unless cytochrome oxidase in any particles with the mitochondrial orientation were incapable of generating a membrane potential. A third possible source of underestimation of Δp could be that a large ΔpH has been overlooked, but, as discussed earlier, and elsewhere [10,11], the experimental evidence strongly indicates that ΔpH is very small. It is concluded that any underestimation of Δp must be considerably less than 98 mV. Although under some conditions, different from those used in the present work, we have reported higher values for Δp than described in the present paper [11], there are conditions under which Δp is probably overestimated owing to binding of SCN^- and $CH_3NH_3^+$ to the particles (Hinkle, P.C., personal communication).

It is improbable that the plot shown could be expected to extrapolate to the origin, because at lower values of ΔG_p inhibition of ATPase activity by ADP will be so severe that Δp will be increasingly out of equilibrium with ΔG_p. At $\Delta p = 0$, ΔG_p is predicted to be much greater than 0. Therefore, although over the range that was experimentally accessible the data fit a straight line (Fig. 1) with a good correlation coefficient, it is considered that estimates for the maximum value of H^+/ATP should be taken from the individual data points rather than from the slope of a linear plot of the data. It should also be pointed out that, irrespective of any argument whether the plot (Fig. 1) should extrapolate to the origin, a value of 2 for H^+/ATP requires that the respiration-dependent Δp of 168 mV generated by particles would have to have been underestimated by approx. 75 mV so that the underestimate for H^+/ATP obtained under these conditions would be below 2. Taking all these considerations into account, together with the evidence that Δp has not been grossly underestimated, it is concluded that $H^+/ATP = 3$ for mitochondrial ATPase, which is in agreement with the estimated stoicheiometry for the chloroplast ATPase [12], and some but not all [12] the estimates for the mitochondrial enzyme.

The type of experiment reported here also permits some conclusions about the relationship between the rate of ATP-driven H^+ translocation and the steady-state value of Δp. At all ratios of particles to mitochondria used in the present work, the capacity for ATP hydrolysis by the particles was more than sufficient to allow the mitochondria to attain their maximum rate of ATP synthesis and therefore to respire in State 3 [19]. This was routinely established with a Clark-type oxygen electrode. In the steady state the total rate of ATP hydrolysis by the particles therefore equalled the known rate of ATP synthesis by the mitochondria, and thus the specific rate of ATP hydrolysis (i.e., nmol/min per mg particle protein) could always be calculated. Combination of this information with the measurement of Δp showed a non-linear relationship between Δp and the rate of ATP hydrolysis or H^+ pumping (Fig. 2), paralleling previous findings when H^+ translocation was driven by electron transfer [5,18,20].

The data in Fig. 2 are presented on the basis that the only reaction for producing ATP is mitochondrial oxidative phosphorylation. A second reaction that can produce ATP is adenylate kinase activity, but the rate of ATP synthesis catalysed by this enzyme was expected to be very low because the inhibitor Ap_5A was present. This was confirmed by showing that no significant changes in the steady-state concentration of AMP were detected during experiments with a range of ratios of particles to mitochondria. However, the very lowest calculated rates of ATP hydrolysis (Fig. 2) may be underestimated because under these conditions the very low rate of production of AMP, which would have accompanied a significant contribution to the rate of ATP synthesis from adenylate kinase, would have been difficult

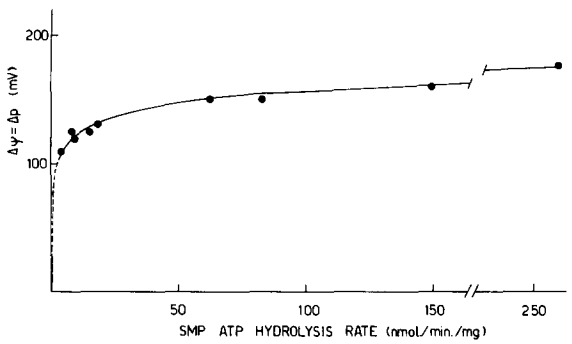

Fig. 2. Relationship between rate of ATP hydrolysis and $\Delta\psi$ in submitochondrial (SMP) particles. Experimental conditions as in Fig. 1. The rates of ATP hydrolysis were inferred as described in the text.

to detect. Nevertheless, irrespective of the source of ATP, the rate of ATP hydrolysis must have varied considerably over the experiments shown in Fig. 2. This is because at the highest rate of ATP hydrolysis shown in Fig. 2 the concentrations of ATP and ADP were 3.9 and 0.3 mM, respectively, whereas at the lowest rate they were 0.7 and 2.5 mM, with the consequence that strong product inhibition by ADP [21] ensured considerable changes in the rate of ATP hydrolysis. The conclusion about a non-linear relationship between rate of H^+ translocation and magnitude of Δp is therefore not altered, even if the estimates of the lowest rates of ATP hydrolysis were to be increased to allow for a possible contribution from adenylate kinase to the steady-state rate of ATP synthesis.

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