Biochimica et Biophysica Acta, 681 (1982) 319–322
Elsevier Biomedical Press

BBA Report

BBA 40006

HYDROLYSIS OF ITP GENERATES A MEMBRANE POTENTIAL IN SUBMITOCHONDRIAL PARTICLES

M. CATIA SORGATO [a], FRANCESCA GALIAZZO [a], MARINA VALENTE [a], LUCIA CAVALLINI [a] and STUART J. FERGUSON [b]

[a] *Centro Studio Fisiologia Mitocondriale CNR and Istituto Chimica Biologica dell'Università di Padova, 35100 Padova (Italy) and* [b] *Department of Biochemistry, University of Birmingham, Birmingham B15 2TT (U.K.)*

(Received February 1st, 1982)

Key words: ITP hydrolysis; ATP hydrolysis; Proton translocation; Membrane potential; ATPase; Electron transport; (Bovine heart, Rat liver, Submitochondrial particle)

ITP hydrolysis catalysed by the ATPase of submitochondrial particles from both bovine heart and rat liver is shown to be linked to the generation of a membrane potential, and therefore also to proton translocation. The magnitude of the membrane potential is similar to that observed during ATP hydrolysis at equivalent concentrations of phosphate and nucleoside tri- and diphosphates. An explanation is suggested for why in other reports ITP was found to be a poor substrate for supporting energy-linked reactions that are driven by the membrane potential.

Elucidation of enzymatic reaction mechanisms is often facilitated by studies with non-physiological substrates. Substitution of ITP for ATP as substrate for the H^+-translocating mitochondrial ATPase has been proposed to be accompanied by a change in the major energy-releasing step from substrate binding (for ATP) to triphosphate hydrolysis (for ITP) [1]. It has also been proposed [1,2] that only energy released upon substrate binding can be coupled to H^+ translocation, and that therefore ITP, although hydrolysed at as much as 50% of the rate of ATP [2], is a very ineffective source of energy for driving reversed electron transfer from succinate to NAD in submitochondrial particles. Some instances of reversed electron transfer in the presence of ITP have been suggested to arise from transphosphorylation by nucleoside diphosphokinase of contaminating ADP to give ATP, which in turn was responsible for driving the reaction [2]. A mechanistic model in which ITP hydrolysis is essentially uncoupled from H^+ translocation has, however, been recognised [2] as difficult to reconcile with observations that both the transhydrogenase reaction [2,3] and the enhancement of 8-anilino-1-naphthalenesulphonate fluorescence [2,4] are seemingly readily driven by hydrolysis of ITP.

As comparison of the mechanisms of ATP and ITP hydrolysis may provide insight into the mechanism of coupling of nucleoside triphosphate hydrolysis to H^+ translocation, the question of whether or not ITP hydrolysis is linked to H^+ translocation needs to be resolved. A direct approach is to measure one or both (membrane potential, $\Delta\psi$, and pH gradient, ΔpH) of the components of the proton-motive force that is generated in submitochondrial particles by H^+ translocation. The present paper is concerned with mea-

Abbreviations: $Ap_5A = P^1,P^5$-di(adenosine-5′-pentaphosphate); $\Delta\psi$, membrane potential; ΔpH, pH gradient across the membrane; $\Delta G_p = \Delta G^{o\prime} + RT$ ln[ATP or ITP]/[ADP or IDP][P_i] = phosphorylation potential.

TABLE I

COMPARISON BETWEEN MEMBRANE POTENTIALS SUPPORTED BY ITP AND ATP HYDROLYSIS IN BOVINE HEART SUBMITOCHONDRIAL PARTICLES

$\Delta\psi$ was determined at 25 ± 1°C by measurements with the flow-dialysis method of the uptake of 10 μM $S^{14}CN^-$ (60 μCi/μmol) into the particles [5]. The experimentally determined internal volume of submitochondrial particles was 0.75 μl/mg protein. A reaction medium of 200 mM sucrose, 5 mM P_i/Tris (pH 7.3), 25 mM $Mg(CH_3COO)_2$, 20 mM glucose (except with submitochondrial particles of preparation 1), 0.35 mM Ap_5A together with 5 mg protein/ml of particles was used in the upper chamber of the flow-dialysis cell. The particles were washed three (preparation 2) or four (preparation 1) times by repeated centrifugation. 25 units of NH_4^+-free lyophylised hexokinase were added when indicated. ΔG_p was determined [16,17] in parallel to $\Delta\psi$ after 4–5 min incubation, which corresponded approximately to the time when $\Delta\psi$ was determined by addition of oligomycin (2.5 μg/mg protein) to inhibit nucleoside triphosphate hydrolysis and induce $S^{14}CN^-$ efflux from the particles.

Preparation	Nucleotide added (20 mM)	Further addition	$\Delta\psi$ (mV)	ΔG_p (kcal/mol) (mV)
1	ITP	–	110	10.20 (442)
1	ATP	–	115	10.20 (442)
2	ITP	–	110	10.30 (447)
2	ITP	Hexokinase	75	9.7 (421)
2	ATP	–	110	10.25 (445)
2	ATP	Hexokinase	65	8.8 (382)

surements of this type under conditions in which $\Delta\psi$ has been shown previously [5,6] to be the sole component of the proton-motive force.

Bovine heart submitochondrial particles that were relatively depleted of the ATPase inhibitor protein were prepared according to the method for type II particles given in Ref. 7. Rat liver particles were prepared by the method of Lemasters [8]. ATPase and ITPase activities were determined at 25°C by measurement of H^+ release under exactly the same conditions used for flow-dialysis measurements (Table I), or by coupling nucleoside diphosphate production via pyruvate kinase and lactate dehydrogenase to oxidation of NADH using the same reaction mixture as for flow dialysis but a lower concentration of particles (0.09 mg/ml).

Reversed electron transport from succinate to NAD was measured as described in ref. 2 but at 25°C and in the same incubation mixture as for $\Delta\psi$ determination. Contamination of ITP by ATP or ADP was assessed using high-pressure liquid chromatography essentially as described in Ref. 9. The ATP content of Ap_5A and submitochondrial particles after denaturation with acid was measured with a luciferine-luciferase assay using an LKB photometer and the LKB recommended procedure. Ap_5A and ITP were purchased from Sigma, and ATP from Boehringer. Protein was determined by the biuret method [10].

Table I shows that in the presence of added ATP or ITP almost identical values of $\Delta\psi$ were found for submitochondrial particles. In parallel to the $\Delta\psi$ determination the free energy of nucleoside triphosphate hydrolysis was also determined. It was similar for the two nucleosides (assuming equal values of $\Delta G^{\circ\prime}$), and so thermodynamically $\Delta\psi$ would be expected to be the same with both substrates.

Furthermore, at the time of $\Delta\psi$ measurement the rates of nucleoside triphosphate hydrolysis were equal (approx. 200 nmol/min per mg protein) and thus there was no kinetic limitation on the rate of H^+ translocation driven by ITP. The rate of ATP hydrolysis was initially markedly faster than that of ITP, but progressively decreased owing to accumulation of ADP which is a strong inhibitor [2], whilst the ITP hydrolysis rate was relatively constant because IDP is a very weak inhibitor [2]. ATP hydrolysis can generate higher values of $\Delta\psi$ when both the free energy and rate of hydrolysis are greater [11] than in the experiments shown in Table I. Similar results to those in Table I were found for rat liver submitochondrial particles, although $\Delta\psi$ was less stable in these particles (data not shown).

The evidence that hydrolysis of ITP, and not

contaminating amounts of ATP, was responsible for the observed membrane potentials is the following: (i) Neither ATP nor ADP was detected in the ITP. (ii) The total concentration of ATP present in a flow-dialysis experiment was in the range 10–18 μM. This included adenine nucleotide present in the Ap_5A that was added to inhibit adenylate kinase activity, and also any nucleotide that was tightly bound to the particles and therefore not necessarily available as a substrate for the nucleoside diphosphokinase reaction. (iii) The relative K_m values for ITP and ATP hydrolysis are 3.4 and 0.13 mM, respectively [12]. Therefore, even if nucleoside diphosphokinase could maintain an ATP concentration of about 15 μM, ATP hydrolysis in the presence of millimolar concentrations of ITP should be inhibited. This argument is not negated by the observation [13] that ITP concentrations up to 5 mM can stimulate the hydrolysis of low concentrations of ATP by purified mitochondrial ATPase. In a flow-dialysis experiment the ITP concentration fell from its initial value of 20 mM to approx. 11 mM ($\Delta G_p = 10.2$ kcal/mol (Table I)) at the time when oligomycin was added and $\Delta\psi$ determined. At these concentrations of ITP, ATP hydrolysis is expected to be inhibited by ITP [13], provided the observations with purified ATPase can be extrapolated to submitochondrial particles. (iv) The addition of hexokinase plus glucose lowers both $\Delta\psi$ and the concentration of either ITP or added ATP (Table I), while the level of contaminating ATP remained at 11 μM (as detected by luciferine-luciferase) when hexokinase was added. Therefore, if hydrolysis of contaminating ATP were responsible for generating $\Delta\psi$ in the presence of ITP, then no change in $\Delta\psi$ should have been seen on addition of hexokinase. (v) $\Delta\psi$ was undetectable when 20 μM ATP plus pyruvate kinase, 2 mM K_2SO_4 and phospho*enol*pyruvate for the regeneration of ATP, were added to particles. Thus, even in the absence of competition from ITP, the rate of hydrolysis of a micromolar concentration of ATP was insufficient to generate $\Delta\psi$. These observations taken together are strong evidence that contrary to previous proposals [1,2], ITP hydrolysis by mitochondrial ATPase is coupled to H^+ translocation. The extent of coupling for both ATP and ITP must be essentially equivalent as similar $\Delta\psi$ values are observed with the two substrates (Table I).

The finding of approximately equal membrane potentials with ATP and ITP (Table I) led us to investigate the rate of reversed electron transfer from succinate to NAD, reproducing as closely as possible the conditions under which $\Delta\psi$ was measured. Thus, the initial concentrations of ATP or ITP (approx. 12 mM), ADP or IDP (approx. 7 mM) and P_i (approx. 11 mM) were very similar to those present in the flow-dialysis experiments (Table I) at the time when $\Delta\psi$ was determined. The concentration of particles, 0.5 mg/ml, was lower so that reduction of NAD could be measured spectrophotometrically. Under these conditions, the initial rates of reversed electron transfer driven by ATP and ITP were, respectively, 20 and 10 nmol/min per mg protein. The difference between ATP and ITP is considerably smaller than has been observed in some previous work [2,12], but in the present work the comparison has been made under conditions where both ATPase and ITPase activities and ΔG_p for both reactions are almost equal. When reversed electron transfer is measured immediately after adding 5 mM ATP or ITP to an assay, rates of 145 and 27 nmol/min per mg protein have been observed. This is consistent with the higher rate of ATPase and value of $\Delta\psi$ found under such conditions in which ATPase activity is not inhibited by ADP and ΔG_p is higher [11].

A possible reason why there are variations in the reported capacity of ITPase activity to drive reversed electron transfer ([2,3,14] and present work) is that the proton-motive force has differed, owing to variations in the specific rates of ITP-driven H^+ translocation and the rates of H^+ leakage from the interior of the particles. Small changes in the size of the proton-motive force are likely to be accompanied by relatively large changes in the rate of reversed electron transfer. The sharp dependence of the rate of ATP synthesis on the proton-motive force in at least some systems [15] provides a precedent for this type of behaviour. The low rates of the ITP $\rightleftharpoons$ P_i exchange reaction relative to ATP $\rightleftharpoons$ P_i [2,14] can be explained on a similar basis, except that, in addition to the influence of proton-motive force on the rate of triphosphate resynthesis, the relative K_m values for the nucleoside diphosphate must also be taken into account. The transhydrogenase reaction has

long been known to be less sharply dependent on the energised state, or proton-motive force, then reversed electron transfer [3], and thus any decrease in the proton-motive force upon replacing ATP by ITP can be accompanied by relatively small changes in the rate of transhydrogenation.

The proposal that relative rates of reversed electron transfer might be related to the value of $\Delta\psi$ (or total proton-motive force) clearly needs to be tested by further experiments to decide whether observations with different nucleoside triphosphates [2], the relatively small inhibitory effects of ADP [2] and the effects of inhibitors of the ATPase [12] fit the predicted pattern. Two factors contribute to determining the magnitude of $\Delta\psi$ in these circumstances, the rate of nucleoside triphosphate hydrolysis and the ΔG_p value [11], and therefore it is not easy to predict the behaviour of $\Delta\psi$.

It is concluded that the behaviour of various nucleotides as substrates of the ATPase requires measurements of the proton-motive force or H^+ uptake, and that conclusions about their efficacy in driving H^+ translocation cannot be safely made from measurements of reversed electron transfer or other energy-linked reactions. Finally, it should be pointed out that the conclusion that ITP hydrolysis is coupled to H^+ translocation does not necessarily rule out the proposition [1,2] that the energetics of individual steps of ITP hydrolysis differ from those for ATP hydrolysis. Such a difference in mechanisms for ATP and ITP hydrolysis would suggest that the energy for driving H^+ translocation must be regarded as originating from the overall hydrolysis of a nucleotide rather than from an individual step in the reaction pathway.

The authors thank Mrs. M. Cuccia and Mr. P. Dalan for secretarial and technical help. M.C.S. and S.J.F. thank the North Atlantic Treaty Organisation for Grant No. 1771.

References

1 Harris, D.A., Dall-Larsen, T. and Klungsøyr, L. (1981) Biochim. Biophys. Acta 635, 412–428
2 Harris, D.A., Gomez-Fernandez, J.C., Klungsøyr, L. and Radda, G.K. (1978) Biochim. Biophys. Acta 504, 364–383
3 Ernster, L., Juntti, K. and Asami, K. (1973) J. Bioenerg. 4, 149–159
4 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1976) Biochim. Biophys. Acta 423, 174–188
5 Sorgato, M.C., Ferguson, S.J., Kell, D.B. and John, P. (1978) Biochem. J. 174, 237–256
6 Branca, D., Ferguson, S.J. and Sorgato, M.C. (1981) Eur. J. Biochem. 116, 341–346
7 Ferguson, S.J., Harris, D.A. and Radda, G.K. (1977) Biochem. J. 162, 351–357
8 Lemasters, J.J. (1980) FEBS Lett. 110, 96–100
9 Juengling, E. and Kammermeier, H. (1980) Anal. Biochem. 102, 358–361
10 Gornall, A.G., Bardawill, C.J. and David, M.A. (1949) J. Biol. Chem. 177, 751–766
11 Sorgato, M.C., Branca, D., Simon, S., Stefani, L. and Ferguson, S.J. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria, (Palmieri, F., Quagliariello, E., Siliprandi, N. and Slater, E.C., eds.), pp. 407–410, Elsevier/North-Holland, Amsterdam
12 Ernster, L., Asami, K., Juntti, K., Coleman, J. and Nordenbrand, K. (1976) in The Structure of Biological Membranes (Nobel Symp. 34) (Abrahamson, S., ed.), pp. 135–156, Plenum Press, New York
13 Schuster, S.M., Gertschen, R.J. and Lardy, H.A. (1976) J. Biol. Chem. 251, 6705–6710
14 Pedersen, P.L. (1976) J. Biol. Chem. 251, 934–940
15 Ferguson, S.J. and Sorgato, M.C. (1982) Annu. Rev. Biochem. 51, 185–217
16 Bergmeyer, H.U. (1970) Methods of Enzymatic Analysis Vol. 4, pp. 2101–2110, Verlag-Chemie, Weinheim
17 Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400