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COUPLING OF ATP PYROPHOSPHOROLYSIS TO TRANSPORT OF CA^{2+} IN MITOCHONDRIA

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SUMMARY: In the coupling of ATP pyrophosphorolysis to Ca^{2+} transport in beef heart mitochondria, for each molecule of ATP cleaved, one proton is released and one Ca^{2+} is transported into the interior space. With the use of tritium labelled ATP, it could be shown that ATP is pyrophosphorylyzed into a species equivalent to Pi that moves inward, and a species equivalent to ADP that is extruded into the aqueous space on the exterior of the mitochondrion. The species equivalent to Pi has been identified as a negatively charged form of Pi (PO^-) and the species equivalent to ADP as a positively charged form (ADP^+). The inward flow of PO^- is coupled to the inward flow of Ca^{2+} in 1:1 stoichiometry--a token that Ca^{2+} must enter in the form of $Ca^{2+}A^-$, where A^- is a monovalent anion. During ATP pyrophosphorolysis protons are released on the I side and taken up on the M side--one proton for each molecule of ATP cleaved. The alkalinization of the matrix space leads to the deposition of $Ca_3(PO_4)_2$ and to the extrusion of the two species released by this deposition (Pi, K^+). Two thirds of the PO^- is trapped as $Ca_3(PO_4)_2$ in the matrix space and one third is extruded into the external space. The extrusion of K^+ provides a mechanism by which protons can be continuously brought into the matrix space to sustain a high rate of coupled pyrophosphorolysis of ATP. The coupling pattern for Ca^{2+} transport driven by ATP pyrophosphorolysis is identical with the corresponding pattern for Ca^{2+} transport driven by electron transfer. This identity is suggestive that coupling mediated by the Fo-F_1 system and coupling mediated by electron transfer complexes are mechanistically indistinguishable.

INTRODUCTION: That ATP pyrophosphorolysis in mitochondria can drive active transport of cations or transhydrogenation in the same fashion as electron transfer has long been known (1-3). The site of ATP pyrophosphorolysis is the Fo-F_1 complex (4); the sites of electron transfer are Complexes I-IV. The coupling of electron transfer to transport of Ca^{2+} has been shown to be executed by each of the coupling electron transfer complexes (I, III and IV) (2, 5, 6). From the studies of Brierley et al. (2) showing that Ca^{2+} transport driven by ATP pyrophosphorolysis summates with Ca^{2+} transport driven by electron flow, it is a reasonable presumption that the Fo-F_1 complex is also competent to couple the pyrophosphorolysis of ATP to the transport of Ca^{2+}.

Pyrophosphorolysis of ATP and electron transfer are profoundly different processes. It was obvious to us that much could be learned from specifying

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what these two processes had in common in order to be interchangeable. The present communication is addressed to that objective. Some studies on the stoichiometry of ATP pyrophosphorolysis in mitochondria have already been reported (7, 8) but the scope of these studies was too limited to provide answers to the questions we have raised.

EXPERIMENTAL METHODS: Analytical procedures for studying ATP-energized transport of Ca^{2+}

Ca^{2+} uptake with $^{45}Ca^{2+}$ as label was measured by the rapid filtration method described by Brierley et al. (7) in which Gelman GN-6 membrane filters with glass fiber prefilters achieve filtration of the assay mixture in seconds with complete retention of the particles on the filter. Pi was determined by the procedure of Martin and Doty (9) as modified by Lindberg and Ernster (10). Mg^{2+} was determined by atomic absorption spectra in a perchloric acid extract of the particles that had been sedimented through 0.66 M sucrose. Nucleotide uptake was measured with tritium-labelled ATP using the filtration method referred to above.

Sources of error in the determination of the ratio of Ca^{2+} transported to ATP phosphorolyzed (CA^{2+}/ATP ratio)

Two sources of error are possible: the membrane loading of Ca^{2+} in which Ca^{2+} transport is not linked to $Ca_3(PO_4)_2$ deposition in the matrix space (11) and the transport of Ca^{2+} driven by electron transfer referable to endogenous electron transfer substrates in the mitochondrial preparation. The first hazard can be eliminated by measuring the ratio only after the membrane loading capacity has been fully utilized. The reaction is carried out in two stages: the initial period extended long enough to exhaust the membrane-loading capacity of the mitochondrial preparation that is about 100 nmol/mg protein (12) and then the actual period of measurement for which the initial period is the blank to be exhausted. The second hazard can be eliminated by using as a blank the same experimental system in presence of oligomycin. If there were any contributions made by electron transfer to the total of Ca^{2+} transported, subtraction of the oligomycin-inhibited blank should eliminate this contribution.

A possible source of error in the determination of the H^+/ATP ratio

In addition to the release of a proton referable to the pyrophosphorolysis of ATP, there is the added contribution of the ionization of ADP and Pi formed in the pyrophosphorolysis. At pH 6.2 this latter contribution is negligible but at pH 7 it is considerable. Thayer and Hinkle (7) correctly pointed out the desirability of studying the pyrophosphorolysis at pH 6.2 to avoid this complication.

Particle preparations

Heavy beef heart mitochondria were prepared by the method of Hatefi and Lester (13) and electron transfer particles by the method of Linnane and Ziegler (14).

RESULTS: Table 1 shows the results of a typical experiment designed to define the stoichiometries involved in the coupling of ATP pyrophosphorolysis to transport of Ca^{2+} with the precautions already specified in the Experimental Methods section. The H^+/Pi ratio was found to be close to 1, Pi released being taken as a measure of ATP pyrophosphorylyzed. The ratio of Ca^{2+} transported to ATP pyrophosphorylyzed was also found to be close to 1. The possibility that cyclical cation transport was a significant coupling option under

Table 1. Stoichiometry of the ATP-driven transport of Ca^{2+} by beef heart mitochondria

	nmol/mg prot/3 min
Net Ca^{2+} uptake	736*
Net Mg^{2+} uptake	10
Net Me^{2+} uptake	746
Net Pi release	850**
Me^{2+}/ATP = 0.88 (Theory = 1.0)	

*Corrected for Ca^{2+} uptake in presence of oligomycin.

**Corrected for Pi in the medium before addition of $^{45}CaCl_2$.

The assay mixture (10 ml) was 0.25 M in sucrose, 3.3 mM in imidazole (pH 7.2), 10 mM in $MgCl_2$ and 3 mM in Tris ATP. It contained, in addition, 2 mg/ml of heavy beef heart mitochondria. The mixture was incubated 2 min at 37° before addition of 2.3 mM $^{45}CaCl_2$. The reaction period was 3 min. The initial and final concentrations of Ca^{2+} and Pi in the filtrate were then determined as described in the Experimental Methods section.

the conditions imposed can be excluded on the basis of this value for the ratio. Both Ca^{2+} and Mg^{2+} are transported during ATP pyrophosphorolysis carried out in presence of both Ca^{2+} and Mg^{2+}. We considered it appropriate to add this small amount of Mg^{2+} transported to the total of divalent metal transported.

The data of Table 2 show that ADP formed by pyrophosphorolysis of ATP remains exclusively in the aqueous space external to the inner membrane, whereas Pi is distributed between the matrix space (two thirds) and the external space (one third). Since the deposition of $Ca_3(PO_4)_2$ does not require

Table 2. Distribution of ADP and Pi between the particle and the reaction medium during ATP driven transport of Ca^{2+}

	% Total
Tritium label of ATP in particle	0
Tritium label of ATP in supernate	100
Pi in particle	67
Pi in supernate	33

If all the Pi in excess of the requirements for $Ca_3(PO_4)_2$ formation were to leave the particle, the ratio of Pi in the particle to Pi in the supernate would be 2:1.

See legend of Table 1 for details of the assay mixture.

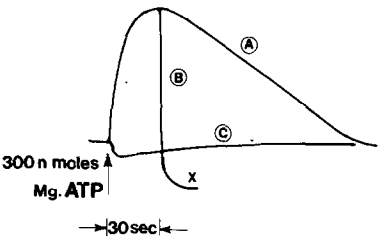

Figure 1. Design of experiments for evaluating ΔH^+ during ATP pyrophosphorolysis in electron transfer particles

The assay medium (6 ml) was 0.2 M in sucrose, 50 mM in KCl, 2.5 mM in K-MES (pH 6.1) and 5 mM in $MgCl_2$. The reaction mixture was maintained at 30°. After addition of the electron transfer particle suspension (24 mg), the pH was adjusted to 6.15 with alkali; and the mixture was incubated for 3 min before initiation of the pyrophosphorolysis reaction by addition of 300 nmol of an equimolar mixture of $MgCl_2$ and Tris ATP (brought to pH 6.15 by addition of alkali).

A - no addition other than magnesium ATP.

B - after 30 sec, 200 nmol standard HCl was added to calibrate the pH change and a sample was withdrawn (at X) for determination of the final level of Pi in the reaction mixture.

C - oligomycin (1 µg/mg prot) was added during the preincubation period and before the addition of magnesium ATP.

The analytical procedure for Pi analysis (10) was modified to increase sensitivity first by reducing the volume of the isobutanol phase from 5 ml to 2 ml and second by reducing the acidic ethanol phase from 8 ml to 5 ml. Under these conditions 1 nmol of Pi induced an absorbance change of 0.001.

Ca^{2+} and Pi in equimolar amounts, Pi in excess of the amount required for precipitation of $Ca_3(PO_4)_2$ would be released into the outer space by virtue of the high internal gradient. It is, therefore, reasonable to postulate that initially all the Pi in the form of the charged species formed by pyrophosphorolysis moves into the matrix space, and the excess of Pi not participating in $Ca_3(PO_4)_2$ deposition is then extruded into the outer space. The ratio of Pi in the matrix space to Pi in the external space (2:1) is theoretical for the distribution expected on the basis of the predicted efflux of excess Pi from the matrix space.

To measure the protonic changes on the M side of the Fo-F_1 complex, electron transfer particles were used. Schuster and Olson (15) have shown that these particles couple the transport of bound Mg^{2+} to electron transfer or pyrophosphorolysis of ATP. The transport is cyclical but the energized efflux of Mg^{2+} is so much faster than the nonenergized influx that in a short duration experiment no correction for cycling need be applied. The design of a series of experiments carried out to evaluate the H^+/ATP ratio in the electron transfer particles is shown in Fig. 1. The results of each such experi-

Table 3. H^+/Pi ratios during ATP-driven transport of Ca^{2+} in electron transfer particles at high and low concentrations of KCl ± valinomycin and ± oligomycin

Concentration of KCl (mM)	Addition of valinomycin	ΔPi (nmol)	ΔH^+ (nmol)	H^+/Pi mol/mol
50	+	180	160	0.89
5	0	180	178	0.99
5	+	144	162	1.12
5	0	132	156	1.18
50	0	0	0	
5	0	0	0	

For the design of these experiments and the experimental conditions, see the legend of Fig. 1. The concentration of valinomycin used was 0.1 µg/mg prot.

ment is shown in Table 3. During pyrophosphorolysis of ATP by the electron transfer particles (M side exterior) protons are taken up in an amount approaching one molecule/molecule of ATP pyrophosphorylyzed. The Δ proton curve flattens out in about 20 sec at 38° before reversing direction. When uncoupler is added at the phase of no change, the proton concentration in the external phase rapidly drops to exactly the value at the start of the ATP pulse. What this uncoupler effect suggests is that the protons released into the internal space during ATP pyrophosphorolysis are exactly equal to the protons taken up in the external space. We can thus say that the H^+/ATP ratio is -1 in the external space and +1 in the internal space. The amount of pyrophosphorolysis was estimated by the release of Pi. At the point of inflection of the proton uptake curve, the Pi released corresponded to the formation of less than one molecule of Pi per molecule of ATP cleaved. Thus in a short duration experiment we can exclude cleavage of more than one pyrophosphate bond per molecule of ATP added to the electron transfer particles. There is indeed adenylokinase activity in these particles; but fortunately this activity is relatively low compared to the rapid pyrophosphorylytic activity.

Some control experiments for H^+ uptake during ATP pyrophosphorolysis mediated by electron transfer particles are shown in Table 3. Oligomycin suppresses release of Pi; high or low concentrations of KCl in presence or in absence of valinomycin have no effect on the value of the H^+/ATP ratio or on the amount of Pi released--an indication that monovalent cations are not being transported by these particles.

The data presented in Table 2 have established that the charged species corresponding to Pi is the driving ion and the charged species corresponding to ADP is extruded into the external space. By analogy with electron-proton separation we could invoke PO^--ADP^+ charge separation--PO^- being the counterpart to the electron and ADP^+ being the counterpart to the proton. The protonic changes are consistent with this postulate. The extrusion of ADP^+ would lead to proton release on the I side as observed, and the transmembrane flux of PO^- would necessarily lead to proton uptake on the M side as observed.

In pyrophosphorolysis mediated by electron transfer particles, ATP has to move into the particle in exchange for ADP formed interiorly. This exchange (16) does not apply to pyrophosphorolysis in mitochondria. Does this exchange reaction complicate the protonic change pattern for electron transfer particles? The fact that addition of uncoupler completely abolishes the proton gradient established during pyrophosphorolysis in these particles establishes that the exchange reaction does not contribute significantly to the proton balance under the conditions imposed for studying the electron transfer particles.

DISCUSSION: ATP pyrophosphorolysis and electron transfer share three features: enzymic bond rupture; charge separation of two oppositely charged species; and charge substitution, the negatively charged species moving into the membrane with a cation and the positively charged species being extruded into the external aqueous space with an anion. Bond rupture involves cleavage of a P-O-P bond in pyrophosphorolysis and dehydrogenation in electron transfer; PO^- and ADP^+ are separated in pyrophosphorolysis, and e^- and H^+ are separated in electron transfer; PO^- moves into the membrane like the electron coupled to the movement of a cation, and ADP^+ like H^+ is extruded into the external aqueous phase together with an anion. To complete the analogy, a transport chain for PO^- analogous to the electron transfer chain would have to be invoked. The susceptibility of ATP pyrophosphorolysis to the inhibitory action of DCCD, oligomycin and tripropyltin is suggestive that the subunits of Fo containing the groups that are immobilized by these three reagents are intrinsic to the PO^- chain (4, 17, 18).

In coupled ATP pyrophosphorolysis, the passive role of the proton in energy coupling stands out sharply. No proton is directly released in the cleavage of the P-O-P bond. The extrusion of ADP^+ leads to the secondary release of a proton that is paired to the extrusion of an anion. The coupling species generated by pyrophosphorolysis is PO^-, not ADP^+.

The deposition of $Ca_3(PO_4)_2$ during coupled transport of Ca^{2+} has always posed a dilemma. The pH at which this deposition takes place is so alkaline

(> pH 9) that there would appear to be no possibility for the matrix space to provide a source of protons required for the termination of coupling. In the formation of $Ca_3(PO_4)_2$ from a mixture of $K_3(PO_4)$ and $CaCl_2$, at least 6 K^+ are released per molecule of $Ca_3(PO_4)_2$ formed. The efflux of K^+ goes parallel with the influx of a proton (21). Thus proton uptake in the matrix space required for energized coupling is compensated by proton influx resulting from K^+/H^+ exchange. This balancing apparently can take place at a fairly alkaline pH.

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