Biochimica et Biophysica Acta 938 (1988) 7–16
Elsevier

BBA 73838

Some total and partial reactions of Na^+/K^+-ATPase using ATP and acetyl phosphate as a substrate

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(Received 20 July 1987)

Key words: ATPase, Na^+/K^+-; Sodium/potassium pump; Acetyl phosphate; Sodium ion transport; Potassium ion transport; (Pig kidney)

Acetyl phosphate, as a substrate of $(Na^+ + K^+)$-ATPase, was further characterized by comparing its effects with those of ATP on some total and partial reactions carried out by the enzyme. In the absence of Mg^{2+} acetyl phosphate could not induce disocclusion (release) of Rb^+ from $E_2(Rb)$; nor did it affect the acceleration of Rb^+ release by non-limiting concentrations of ADP. In K^+-free solutions and at pH 7.4 sodium ions were essential for ATP hydrolysis by $(Na^+ + K^+)$-ATPase; when acetyl phosphate was the substrate a hydrolysis (inhibited by ouabain) was observed in the presence and absence of Na^+. In liposomes with $(Na^+ + K^+)$-ATPase incorporated and exposed to extravesicular (intracellular) Na^+, acetyl phosphate could sustain a ouabain-sensitive Rb^+ efflux; the levels of that flux were similar to those obtained with micromolar concentrations of ATP. When the liposomes were incubated in the absence of extravesicular Na^+ a ouabain-sensitive Rb^+ efflux could not be detected with either substrate. Native $(Na^+ + K^+)$-ATPase was phosphorylated at 0 °C in the presence of NaCl (50 mM for ATP and 10 mM for acetyl phosphate); after phosphorylation had been stopped by simultaneous addition of excess *trans*-1,2-diaminocyclohexane-*N,N,N′,N′* tetraacetic acid and 1 M NaCl net synthesis of ATP by addition of ADP was obtained with both phosphoenzymes. The present results show that acetyl phosphate can fuel the overall cycle of cation translocation by $(Na^+ + K^+)$-ATPase acting only at the catalytic substrate site; this takes place via the formation of phosphorylated intermediates which can lead to ATP synthesis in a way which is indistinguishable from that obtained with ATP.

Introduction

The active Na^+/K^+ coupled transport is catalyzed by a membrane-bound structure which uses ATP as the natural substrate [1–6]. As a whole, the overall biochemical and transport events are coupled. However, when partial reactions are considered, or under certain nonphysiological conditions, it is possible to find a translocation of ions which does not have a biochemical counterpart and biochemical reactions which do not lead to

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; E_1, $(Na^+ + K^+)$-ATPase free of ligands or in the form with high affinity for ATP; E_2, $(Na^+ + K^+)$-ATPase conformation with low affinity for ATP; E_1-P, phosphorylated $(Na^+ + K^+)$-ATPase with an ADP-sensitive dephosphorylation rate; E_2-P, phosphorylated $(Na^+ + K^+)$-ATPase with a K^+-sensitive dephosphorylation rate; $E_2(Rb)$, unphosphorylated $(Na^+ + K^+)$-ATPase with Rb^+ ions occluded in it.

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Na^+ or K^+ transport. Although several of the intermediary steps and enzyme forms are known, their complete assembly in the cycle of active transport is still under debate [1–6]. On the other hand, the $(Na^+ + K^+)$-ATPase can also catalyze the hydrolysis of non nucleotide phosphate esters; this phosphatase activity, whose reaction sequence is still more elusive, does not seem to be associated with cation translocation, although it is related to enzyme forms involved in the ATPase reaction [1,3,6]. Recently, it has been shown that at least one of the phosphatase substrates, acetyl phosphate, can also act as substrate for the ATPase and fuel Na^+ transport sensitive to ouabain in liposomes with $(Na^+ + K^+)$-ATPase incorporated [7,8]. Therefore, with its dual substrate properties, acetyl phosphate may be an important tool to investigate the sequence $(Na^+ + K^+)$-ATPase reactions as well as the relationship(s) between ATPase and phosphatase activities. The evidence collected so far indicates that acetyl phosphate can form a phosphorylated intermediate [9–11] and induce occlusion of Na^+ ions whose release is accelerated by ADP [8]. This latter effect indicates the formation of an $E_1P(Na)$ intermediate; however, as this was seen in enzyme almost completely inactivated with chymotrypsin it is not certain that the same event will take place in native enzyme. On the other hand, although a ouabain-sensitive acetyl phosphate-dependent Na^+ transport was observed [7,8], there are no data on the ability of this alternative substrate to promote the transport of K^+ in the other direction giving rise to a complete cycle of Na^+/K^+ coupled translocation. The experiments described in this paper were designed to answer these questions and to look into some ATPase total and partial reactions, comparing the effects of ATP with those of acetyl phosphate. A brief account of the results has been given elsewhere [12].

Methods

The experiments were carried out with pig kidney $(Na^+ + K^+)$-ATPase partially purified according to Jørgensen [13]. The specific activity varied from 13 to 20 units/mg and remained stable for months when stored at −85 °C in 25 mM imidazole (pH (20 °C) 7.5)/2 mM EDTA/10% sucrose. Before use the enzyme was washed twice (1 : 10, v/v) and resuspended in the same solution without sucrose.

ATPase activity was determined as in Ref. 14 following the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$. Labelling of $[\gamma\text{-}^{32}P]ATP$ was done according to Glynn and Chappell [15] as modified by de Meis [16]. The hydrolysis of acetyl phosphate was measured as in Ref. 17 by the hydroxylamine method of Stadtman [18]. In both cases the reported values are the difference between those observed in the absence and presence of 10^{-4} M ouabain.

Protein was usually determined by the method of Lowry et al. [19] except in Rb^+ occlusion experiments where we used Peterson's [20] modification of that method.

Enzyme phosphorylation and dephosphorylation from $[\gamma\text{-}^{32}P]ATP$ and acetyl $[^{32}P]$phosphate was carried out incubating the enzyme at 0 °C; the reaction was stopped at different times, and after interacting with different ligands, by the addition of 20% trichloroacetic acid/1 mM ATP (or 50 mM acetyl phosphate)/50 mM inorganic phosphate (all final concentrations). The mixture was allowed to stand for 15 min at 0 °C and the denatured protein was collected on a Whatman GF/F glass fiber filter; the filter was then washed with 30 ml of 5% trichloroacetic acid/10 mM inorganic phosphate and counted in a liquid scintillator counter using a toluene-based scintillator. The composition of solutions is described in the legend of the corresponding figure and tables. The acetyl $[^{32}P]$phosphate was synthesized as proposed by Stadtman [18] with $[^{32}P]P_i$ purified as in Ref. 21.

Release of occluded Rb^+ from unphosphorylated $(Na^+ + K^+)$-ATPase was followed as described by Glynn et al. [22] using two syringes, the contents of which were mixed immediately prior to the passage through a cation exchange resin column. Syringe A contained 0.1 mg enzyme protein suspended in 0.5 ml of a medium containing 100 μM $[^{86}Rb]RbCl$, 100 mM Tris/Tris-HCl, (pH 7.4 at 20 °C) and 1 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA). Syringe B contained an equal volume of a similar solution with no enzyme plus the addition of acetyl phosphate and/or ADP at twice the desired final concentration. At 0.3 s following mixing, the

enzyme suspension went through a Dowex 50-X 8-400 cation exchange resin; the contact time in the resin was 0.7 s. The protein content of the column effluents was assayed by Lowry et al. [19] as modified by Peterson [20].

The experiments on ATP- or acetyl phosphate-promoted Rb^+ transport were performed following Rb^+ efflux from liposomes with $(Na^+ + K^+)$-ATPase incorporated. The general technique is described in detail in Ref. 7. Loading of $^{86}RbCl \cdot KCl$ took place during enzyme incorporation by sonication; total efflux times ranged from 1 to 35 min at 20 °C. In all cases 2.5 mM phosphocreatine and 5 units of creatine phosphokinase were present in the extravesicular solution. The ATP regenerating system was included to offset ATP consumption at 0.002 mM ATP but was present as a control in all experimental conditions. The total composition of intra- and extravesicular solutions is given in the corresponding table and table legends.

Net synthesis of ATP was investigated at 0 °C exposing $(Na^+ + K^+)$-ATPase phosphorylated from 'unlabeled' ATP and acetyl phosphate to media containing [^{14}C]ADP. Phosphorylation took place in 50 mM (ATP) or 10 mM (acetyl phosphate) NaCl, 50 mM imidazole (pH (20 °C) 7.5), 1 mM $MgCl_2$ and 10 μM ATP or 5 mM acetyl phosphate. The exposure to 10 μM [^{14}C]ADP lasted 45 s and was started together with the addition of 1 M NaCl and 10 mM CDTA (final concentrations). The reaction was stopped with 0.5% (final concentration) sodium dodecyl sulfate. Nucleotide separation was carried out by a modification of the procedure described by Beaugé and Campos [23]. The modification consisted of a different loading of the columns; as the nucleotides were in media of high ionic strength (a little more than 1 M) the solutions were first diluted (1 : 10) with water and the columns were loaded with aliquots of 1 ml at the time. With this precaution there was no loss of radioactivity during loading (which was otherwise observed) and nucleotide separation was as effective as described before [23].

All solutions were made with de-ionized bi-distilled water. NaCl, KCl and RbCl were spectrometric grade; the other chemicals were reagent grade. ATP (vanadium-free), ADP, Tris, imidazole, phosphocreatine, creatine phosphokinase and ouabain were from Sigma Chemical Co., U.S.A. Compounds containing Na^+ were made Na^+ free by passing them through a cation exchange Amberlite column in a Tris form. [^{32}P]P_i was purchased from the Comisión Nacional de Energía Atómica of Argentina and [^{14}C]ADP was from New England Nuclear, U.S.A.

Counting was performed in a liquid scintillation counter with automatic quenching correction for times long enough to give a standard error of counting of about 1%.

To estimate ionized Mg^{2+} concentrations the following dissociation constants were used (mM): ATP-magnesium, 0.091; acetyl phosphate-magnesium, 10 [8,14].

Results

Interaction site of acetyl phosphate with $(Na^+ + K^+)$-ATPase enzyme during the ATPase cycle

The evidence accumulated so far suggests that acetyl phosphate can interact with the high-affinity catalytic site of the $(Na^+ + K^+)$-ATPase. Thus, acetyl phosphate competitively antagonizes phosphorylation by ATP at the same time that it itself is able to phosphorylate the enzyme [10,11] and to sustain a ouabain-sensitive Na^+ transport in liposomes with $(Na^+ + K^+)$-ATPase incorporated [7,8]. However, it must be noted that phosphorylation is just a partial and early reaction in the whole ATPase cycle; in addition, although the acetyl phosphate-promoted Na^+ transport seems to go through the $E_1P(Na)$-E_2PNa intermediates [8] it has not yet been proven that it proceeds following the complete reaction sequence of the coupled Na^+/K^+ translocation. On the other hand, the fact that in the presence of 'extracellular' K^+ the levels of Na^+ fluxes in liposomes with $(Na^+ + K^+)$-ATPase incorporated were similar with 5–10 mM acetyl phosphate and micromolar concentrations of ATP [7,8] suggests that if the $E_2(K)$-E_1 transition takes place with acetyl phosphate it is not accelerated by this compound. This is supported by experiments where acetyl phosphate did not prevent the formation of the E_2 (Rb) occluded conformation [8]. There are two ways to account for these results: (i) acetyl phosphate does not bind to the low affinity ATP site,

TABLE I

LACK OF ACETYL PHOSPHATE EFFECTS ON THE RELEASE OF Rb^+ FROM Rb^+-OCCLUDING UNPHOSPHORYLATED ($Na^+ + K^+$)-ATPase IN THE ABSENCE AND PRESENCE OF NON-SATURATING CONCENTRATIONS OF ADP AS DISOCCLUDING AGENT

The release of occluded Rb^+ from unphosphorylated ($Na^+ + K^+$)-ATPase was determined using two syringes, the contents of which were mixed before the passage through a Dowex 50 X8-400 cation exchange resin [22]. Syringe A contained 0.5 ml of enzyme (0.1 mg protein) equilibrated with 100 μM [^{86}Rb]RbCl, 100 mM Tris-HCl, pH (20°C) 7.4 and 1 mM CDTA; syringe B contained an equal volume of a similar solution without the enzyme plus the addition of the ligands indicated in the table at twice their final concentration. Temperature was 20°C. Each entry is the mean ± S.E. of triplicate determinations.

Ligand in final enzyme suspension	Rubidium remaining in effluent (nmol/mg)	
	total	total minus that at 2 mM ADP
—	2.96 ± 0.12	2.71 ± 0.16
2 mM ADP	0.25 ± 0.10	–
1 mM Acetyl phosphate	2.93 ± 0.15	2.68 ± 0.18
3 mM Acetyl phosphate	2.88 ± 0.15	2.63 ± 0.14
5 mM Acetyl phosphate	2.91 ± 0.11	2.66 ± 0.15
0.02 mM ADP	1.61 ± 0.11	1.36 ± 0.15
5 mM Acetyl phosphate plus 0.02 mM ADP	1.65 ± 0.10	1.40 ± 0.14

or (ii) it does bind but there is no detectable effect due to the binding. If the second alternative is true, one could expect that in the presence of acetyl phosphate the binding of ATP (or its analogues) to the 'regulatory site' would somehow be impaired. We approached this problem following the effects of acetyl phosphate on the release of occluded Rb^+ from unphosphorylated enzyme in the absence and presence of limiting ADP concentrations.

These experiments, one of which is illustrated in Table I, showed that acetyl phosphate does not accelerate the release of Rb^+ from unphosphorylated ($Na^+ + K^+$)-ATPase; at the same time, even at 5 mM concentration, it does not interfere with the release of Rb^+ induced by an unsaturating (20 μM) concentration of ADP. The most logical conclusion is that the inability of acetyl phosphate to influence the events related to the E_2-E_1 transition is due to the fact that this compound does not bind to the 'regulatory' ATP site.

Hydrolysis of ATP and acetyl phosphate in the presence and absence of Na^+

In its capacity as alternate ATPase substrate, acetyl phosphate leads to a ouabain-sensitive transport of Na^+, presumably through a biochemical route similar to that seen with ATP [7,8]. Within the errors of measurement, the Na^+ fluxes fueled by acetyl phosphate in liposomes with ($Na^+ + K^+$)-ATPase incorporated were not different from those obtained with micromolar concentrations of ATP. On the assumption that phosphorylation by neither phosphate is rate limiting, if the ATP/Na^+ and the (acetyl phosphate)/Na^+ ratios (mol of phosphate compound hydrolyzed per mol of Na^+ translocated) are the same, one would expect a rate of ouabain-sensitive acetyl phosphate hydrolysis via the ATPase cycle similar to that of ATP in K^+-free Na^+-containing media. Experimentally this could be verified, provided that the ouabain-sensitive hydrolysis of acetyl phosphate in K^+-free Na^+ goes only via the ATPase pathway.

The ouabain-sensitive rates of ATP and acetyl phosphate hydrolysis under different ionic conditions are compared in Table II. The results are very interesting in the sense that some go as predicted, whereas others are completely unexpected. Thus, in K^+-free 130 mM Na^+ the hydrolysis of ATP (Na^+-ATPase activity) is within the

TABLE II

EFFECTS OF MONOVALENT CATIONS COMPOSITION ON OUABAIN-SENSITIVE HYDROLYSIS OF ATP OR ACETYL PHOSPHATE BY PIG KIDNEY ($Na^+ + K^+$)-ATPase

The incubation solutions contained in addition 180 − ($Na^+ + K^+$) mM Tris-HCl, 3 mM $MgCl_2$, 0.1 mM EGTA and 3 mM ATP or 5 mM acetyl phosphate. The experiments were performed at 37°C and each entry is the mean ± the S.E. of triplicate determinations. The specific activity of the enzyme was 12.8 units/mg.

Monovalent cation (mM)	Ouabain-sensitive hydrolysis (μmol/mg per min)	
	ATP	acetyl phosphate
130 Na^+-20 K^+	12.8 ± 0.03	5.3 ± 0.10
130 Na^+- 0 K^+	0.385 ± 0.005	0.229 ± 0.010
0 Na^+- 10 K^+	0.007 ± 0.005	8.7 ± 0.14
0 Na^+- 0 K^+	0.010 ± 0.006	0.237 ± 0.015

TABLE III

EFFECT OF BUFFER COMPOSITION ON OUABAIN-SENSITIVE HYDROLYSIS OF ACETYL PHOSPHATE BY ($Na^+ + K^+$)-ATPase INCUBATED IN K^+-FREE SOLUTIONS INT HE PRESENCE AND ABSENCE OF Na^+

The incubation solutions had in addition the following composition: 5 mM acetyl phosphate, 3 mM $MgCl_2$, 0.1 mM EGTA; when present, the concentration of Tris-HCl was 180 − (NaCl) mM, that of choline chloride was 150 − (NaCl) mM and that of imidazole was 30 mM. The experiments were carried out at 37 °C and pH 7.4. Each entry is the mean ± the S.E. of triplicate determinations. The ($Na^+ + K^+$)-ATPase specific activity of this preparation was 12.5 units/mg.

NaCl (mM)	Ouabain-sensitive hydrolysis of acetyl phosphate (μmol/mg per min)		
	Tris/Tris-HCl (pH 7.4)	choline chloride/ imidazole (pH 7.4)	difference imidazole − Tris
0	0.244 ± 0.024	0.275 ± 0.026	0.031 ± 0.035
130	0.226 ± 0.010	0.239 ± 0.011	0.013 ± 0.015
Difference (0 Na^+ − 130 Na^+)	0.018 ± 0.026	0.036 ± 0.028	

expected range (3% of the maximal); the rates of acetyl phosphate hydrolysis are lower (close to 2% of the maximal ($Na^+ + K^+$)-ATPase activity) but, assuming they are the expression of an 'ATPase' reaction, they fall within the possible range given the values and precision of Na^+ fluxes measured in liposomes [8]. Also as expected, in the absence of Na^+ and with 10 mM KCl, conditions where only the phosphatase pathway should be available [1,3,6], there was no detectable hydrolysis of ATP, whereas the hydrolysis of acetyl phosphate was within the values seen before for phosphatase activity with this substrate [24]; interestingly enough, these high rates of acetyl phosphate hydrolysis were also seen in the concurrent presence of 130 mM Na^+ and 20 mM K^+, where the maximal rate of ATPase activity was detected. On the other hand, and in agreement with previous observations (see Ref. 25) in the absence of both Na^+ and K^+, and with normal ionic strength, there was no detectable ouabain-sensitive ATP hydrolysis; however, under these conditions a ouabain-sensitive hydrolysis of acetyl phosphate, not significantly different from that seen in K^+-free 130 mM Na^+, was observed. The ability of partially purified ($Na^+ + K^+$)-ATPase to hydrolyze acetyl phosphate in the absence of Na^+ and K^+ is also shown in Table III. In addition, this table shows that a similar rate of hydrolysis takes place in 180 mM Tris/Tris-HCl as well as in 30 mM imidazole/150 mM choline chloride solutions. It should be stressed that the Na^+ concentrations in solutions nominally free of that cation checked by atomic absorption spectrophotometry were not higher than 10 μM.

ATP and acetyl phosphate-promoted rubidium transport

If the ouabain-sensitive Mg^+-dependent Na^+ transport promoted by acetyl phosphate in liposomes with ($Na^+ + K^+$)-ATPase incorporated [8] follows the complete cycle of ATPase reaction and cation translocation it should be possible to detect, under the conditions of those experiments, an acetyl phosphate dependent K(Rb) transport taking place in a direction opposite to that of Na^+ ions. In addition, these K(Rb) fluxes should have a magnitude around that seen for the fluxes of Na^+. Furthermore, this approach could be used to analyze the behaviour of K^+ transport in the absence of cytoplasmic Na^+ comparing them with the ouabain-sensitive acetyl phosphate hydrolysis observed in ($Na^+ + K^+$)-free conditions (see above).

Table IV summarizes these experiments. In the presence of extravesicular (intracellular) Na^+, ATP and acetyl phosphate can promote an efflux of Rb^+ which is abolished in liposomes containing ouabain. The levels of fluxes with 0.002 mM ATP are the same as with 10 mM acetyl phosphate, and represent about 5% of those observed with 3 mM ATP. On the other hand, in the absence of extravesicular (intracellular) Na^+ both substrates fail to sustain any ouabain-sensitive K^+ transport.

TABLE IV

ATP AND ACETYL PHOSPHATE-STIMULATED K^+ (^{86}Rb-LABELLED) EFFLUX FROM LIPOSOMES WITH $(Na^+ + K^+)$-ATPase INCORPORATED

The extravesicular (intracellular) media contained in addition 100 mM Tris-HCl, pH (20 °C) 7.0, 3 mM $MgCl_2$, 2.5 mM phosphocreatine, 5 units of creatine phosphokinase and $5 \cdot 10^{-4}$ M ouabain. The intravesicular (extracellular) solution consisted of 200 mM KCl labeled with [^{86}Rb]RbCl, 25 mM imidazole, pH (20 °C) 7.0 and 1 mM EDTA. The assays were started by adding 0.1 ml of vesicle suspension to 0.3 ml of incubation solution. Efflux times lasted 1 min for 3 mM ATP and 5 min under all other conditions. Temperature was 20 °C. Each entry is the mean ± S.E. of quadruplicate determinations. The basal efflux in the absence of ATP or acetyl phosphate averaged 0.006 ± 0.004 nmol per 10 μl of initial vesicles per min ($n = 20$).

Expt. No.	Extravesicular			Intravesicular ouabain (mM)	K^+ (^{86}Rb-labelled) efflux (nmol/10 μl original vesicles per min)
	ATP (mM)	acetyl phosphate (mM)	NaCl (mM)		
1	3	–	100	–	0.943 ± 0.035
	0.002	–	100	–	0.045 ± 0.012
	–	10	100	–	0.033 ± 0.007
2	3	–	100	–	0.898 ± 0.028
	0.002	–	100	–	0.032 ± 0.009
	–	10	100	–	0.038 ± 0.008
3	3	–	100	–	1.040 ± 0.043
	–	10	100	–	0.048 ± 0.014
	–	10	–	–	0.020 ± 0.013
4	0.002	–	–	–	0.014 ± 0.009
	–	10	–	–	0.021 ± 0.010
5	0.002	–	–	–	−0.002 ± 0.010
	–	10	–	–	0.011 ± 0.008
6	–	10	–	–	−0.006 ± 0.009
7	0.002	–	–	–	0.008 ± 0.012
	–	10	–	–	0.025 ± 0.012
8	3	–	100	0.1	0.019 ± 0.014
	0.002	–	100	0.1	−0.006 ± 0.011
	–	10	100	0.1	0.007 ± 0.009
9	0.002	–	–	0.1	−0.006 ± 0.011
	–	10	–	0.1	0.007 ± 0.009

Reactivity to ligands of $(Na^+ + K^+)$-ATPase phosphorylated from ATP and acetyl phosphate

One of the strongest arguments in favor of the idea that $(Na^+ + K^+)$-ATPase phosphorylated from acetyl phosphate and ATPase phosphorylated from ATP (both in the presence of Mg^{2+} and Na^+) were different was the inability to detect either acetyl phosphate–acetate or acetyl phosphate–ADP phosphoryl group exchange [11,26]. On that basis it was suggested that, whereas with ATP the enzyme went through the E_1P-E_2P transition, with acetyl phosphate, phosphorylation gave rise directly and irreversibly to E_2P bypassing the E_1P form [9,11]. The sensitivity to ADP of the acetyl phosphate-dependent Na^+ occlusion in $(Na^+ + K^+)$-ATPase treated with chymotrypsin contradicts the view stated above; however, it could be argued that following chymotrypsin digestion, where the E_2P state is not reached [8,28] the enzyme is 'forced' into an $E_1P(Na)$ form which is different from that attained in its native state. The use of phosphoryl exchange reactions to detect an $E_1P(Na)$ in native $(Na^+ + K^+)$-ATPase phosphorylated by acetyl phosphate is not an easy

task: on the one hand, acetate ions have been shown not to interact with the ADP binding site [8], and on the other the extremely high affinity differences of the catalytic site for ATP (less than 1 μM [1–6]) and acetyl phosphate (about 2–3 mM) (Refs. 10, 11, and Campos, M., unpublished results) would result in competitive inhibition of the forward reaction (phosphorylation by acetyl phosphate) even with micromolar concentrations of synthesized ATP. For these reasons we decided to attack the problem by following the net synthesis of ATP during a single dephosphorylation cycle; the idea was to allow phosphorylation of native enzyme, which is expected at least with ATP to go mostly into E_2P form [1–6] and then force it back into $E_1P(Na)$ by increasing the concentration of Na^+ (see Ref. 29) in the presence and absence of ADP.

In a preliminary experiment we studied the feasibility of this approach by looking into the sensitivity to ADP of the phosphoenzymes formed with [γ-^{32}P]ATP and [^{32}P]acetyl phosphate. The procedure used to detect newly synthesized ATP was designed to take account of: (i) the need to produce as little ADP as possible during the preliminary phosphorylation with ATP (this turned out to be about 1 μM); (ii) the need to form enough phosphoenzyme initially, and (iii) a high sensitivity to ADP of both phosphoenzymes. Previous experiments had shown that at 0°C, 1 s phosphorylation with ATP and 30 s with acetyl phosphate yielded enough phosphoenzyme; that phosphoenzyme was sensitive to K^+ but insensitive to ADP (results not shown). An unexpected observation was that increasing NaCl concentration progressively inhibited the rate of E-P formation, and that this was much more pronounced when acetylphosphate was the phosphorylating substrate (not shown); for that reason the concentration of NaCl was 50 mM during phosphorylation from ATP and 10 mM during phosphorylation from acetyl phosphate. When this protocol was followed it was seen that stopping E-P formation with 10 mM CDTA plus 1 M NaCl (final concentrations) drastically changed the reactivity of the phosphoenzymes which now became highly sensitive to ADP; this was the case when phosphorylation was produced with ATP (Fig. 1A) or with acetyl phosphate (Fig. 1B). Roughly, the rates of dephosphorylation increased about 3-fold upon the addition of 10 μM ADP (final concentration).

The experiments on net ATP synthesis followed in general the protocol outlined in Fig. 1. Aliquots of 0.5 mg ($Na^+ + K^+$)-ATPase were incubated in 0.5 ml with unlabeled ATP or acetyl phosphate in the presence and absence of ionized magnesium (CDTA was added to the Mg^{2+}-free solutions). After the indicated times (1 s for ATP and 30 s for acetyl phosphate) 10 mM CDTA/1 M NaCl/10 μM [^{14}C]ADP (all final concentrations) were added and the reaction was stopped 45 s later with 0.5% SDS (see Methods). Chelation of Mg^{2+} during dephosphorylation was expected not only to stop further phosphorylation but to prevent the hydrolysis of the newly formed ATP as well as accumulation of ADP. The labeled ADP and ATP were separated as indicated in Methods. In Table

TABLE V

NET SYNTHESIS OF ATP BY ($Na^+ + K^+$)-ATPase PHOSPHORYLATED FROM ATP OR ACETYL PHOSPHATE AND DEPHOSPHORYLATED IN THE PRESENCE OF 10 μM ADP AND 1 M NaCl

Aliquots of 0.5 mg ($Na^+ + K^+$)-ATPase were incubated at 0°C in 0.5 ml of media with 50 or 10 mM NaCl, 30 mM imidazole, pH (20°C) 7.4, 0.1 mM EGTA and 10 μM ATP or 5 mM acetyl phosphate. One half of the ATP- and acetyl phosphate-containing tubes had also 1 mM $MgCl_2$; in the other half $MgCl_2$ was excluded and 5 mM CDTA was added instead. After 1 s (ATP) or 30 s (acetyl phosphate) a mixture of 10 μM [^{14}C]ADP/1 mM NaCl was added to all tubes and the reaction was stopped 45 s later with 0.5% sodium dodecyl sulfate (all final concentrations). Nucleotide separation was performed by mean of a step pH and concentration gradient in columns made with 1.4 ml Eppendorf tubes containing 0.4 ml of Dowex 1-X8-400 resin. The table summarizes the results of two experiments. Each entry is the difference of means (with and without $MgCl_2$) ± S.E. of triplicate determinations. The radioactivity found in the 250 mM HCl eluent solution in the absence of $MgCl_2$ was similar to that found in blank tubes without enzyme. For details see Methods.

Expt. No.	ATP or acetyl phosphate concn.	Mg^{2+}-dependent net ATP synthesis (nmol/mg protein per 45 s)
1	0.01 mM ATP	1.030 ± 0.055
	5 mM acetyl phosphate	0.860 ± 0.046
2	0.01 mM ATP	0.909 ± 0.048
	5 mM acetyl phosphate	0.760 ± 0.036

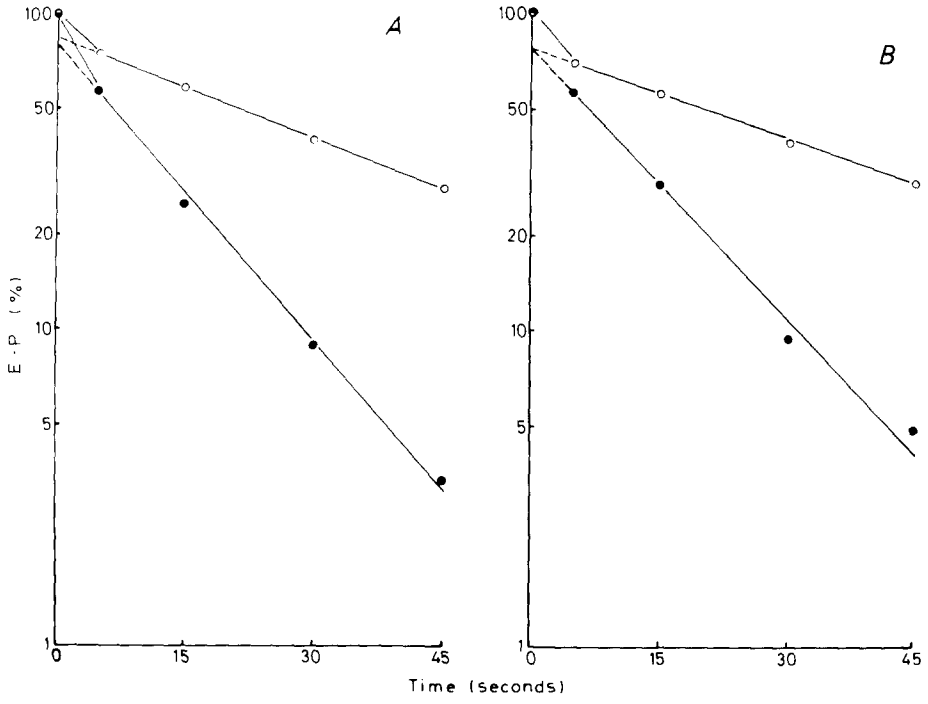

Fig. 1. Effects of ADP on the rate of dephosphorylation of $(Na^+ + K^+)$-ATPase phosphorylated from ATP (A) or acetyl phosphate (B). Aliquots of 0.05 mg enzyme protein were incubated at 0 °C in a total volume of 0.3 ml containing 50 or 10 mM NaCl, 30 mM imidazole pH (20 °C) 7.4, 1 mM $MgCl_2$, 0.1 mM EGTA and either 10 μM [γ-^{32}P]ATP or 5 mM acetyl [^{32}P]phosphate. After 1 s (ATP) or 30 s (acetyl phosphate) in one group of tubes the reaction was stopped with 20% trichloroacetic acid/1 mM ATP (or 50 mM acetyl phosphate)/50 mM inorganic phosphate (all final concentrations). The rest of the tubes were used to estimate the phosphoenzyme remaining after phosphorylation was interrupted (at 1 and 30 s as before) adding 10 mM CDTA/1 M NaCl with (●) and without (○) 10 μM ADP (final concentrations); at the times indicates by the experimental points, the reaction was stopped in the same way as for enzyme phosphorylation. The denatured membrane fragments spent 15 min in an ice-cold bath and were then retained in Whatman GF/F glass fiber filters; the filters were washed with 30 ml of 5% (w/v) trichloroacetic acid/10 mM inorganic phosphate and counted in a liquid scintillation counter using a toluene-based scintillator. Blank tubes consisted of acid-denatured enzyme or native enzyme without Mg^{2+} in the incubation solutions; the values of phosphorylation were identical in both groups representing less than 2% of the maximal [^{32}P]P_i incorporation. The levels of phosphoenzyme at zero time (100% phosphorylation) were 1.9 nmol/mg in A and 1.49 nmol/mg in B. The rate constants for dephosphorylation, estimated from the linear portions of the graphs, were: (A): 0.025 s^{-1} and 0.072 s^{-1} without and with ADP; (B): 0.022 s^{-1} and 0.064 s^{-1} without and with ADP, respectively. Each point in the graphs represents the mean of duplicate determinations.

V the results of two experiments are summarized as the differences in ATP synthesis between media with and without Mg^{2+} during the 'phosphorylation phase'. It can be clearly seen in the Table that with ATP as well as with acetyl phosphate there is a Mg^{2+}-dependent synthesis of ATP; that synthesis, which is a little smaller with acetyl phosphate, falls close to the expected values (see Discussion).

Discussion

The results presented in this paper provide new information regarding the interactions of acetyl phosphate with the $(Na^+ + K^+)$-ATPase when the former acts as substrate of the ATPase reaction. On the other hand, the evidence conclusively indicates that only the high affinity catalytic site is involved in that interaction, whereas the regulatory site is unable to accept acetyl phosphate, at least at the concentrations tested (Table I). Obviously then, some important structural differences must exist between these sites for, in addition to what was shown here, there is an indication that *p*-nitrophenyl phosphate, another phosphatase substate, cannot interact with the catalytic moiety [30], whereas it can replace ATP in accelerating

the release of Rb^+ from occluded $E_2(Rb)$ pushing the enzyme into the E_1 state [14]. This leads to another important conclusion which is that a nucleotide structure is not an absolute requirement to mimic either the catalytic or regulatory functions of ATP.

The fact that acetyl phosphate can promote a ouabain-sensitive Rb^+ transport (Table IV) supports the notion that, regardless of the structure of the phosphate donor molecule, once the enzyme has been phosphorylated in the presence of Na^+ and Mg^{2+}, the complete events of Na^+ and K^+ translocation are triggered; therefore, with the intracellular environment exposed to Mg^{2+} and Na^+ and the extracellular environment to K^+, acetyl phosphate can catalyze an active Na^+/K^+ coupled transport. Unfortunately, the errors involved in flux determinations (see also Ref. 8) do not allow an accurate estimate of the Na^+/K^+ coupling ratio but, within those errors, there is no indication that the ratio will be different from that with ATP.

The hydrolysis of acetyl phosphate sensitive to ouabain observed in $(Na^+ + K^+)$-free solutions was independent of the buffer (Tris or imidazole) and occurred at pH 7.4 and normal ionic strength (Tables II and III), conditions under which an ouabain-sensitive hydrolysis of ATP was undetected (Table II). On the other hand, a significant fraction of ouabain-inhibitable ATP hydrolysis was seen when the ionic strength [25,31] or the pH [32–35] were lowered; in the latter case, the effect was observed with imidazole but not with Tris [33,34]. The experiments on K(Rb) transport in liposomes could have elucidated the mechanism of this hydrolysis of acetyl phosphate (ATPase or phosphatase reaction) only if they had been positive; the failure of acetyl phosphate to sustain K^+ transport without Na^+ in the cytosol (Table IV) does not settle the point because the rest of the ionic composition (200 mM K^+ inside and some likely K^+ accumulation outside due to leak) is different from that of the experiments on hydrolysis. Therefore, the question remains an open one. On the other hand, in the presence of 130 mM Na^+/20 mM K^+, all or almost all of the high rate of acetyl phosphate hydrolysis must be an expression of phosphatase activity. The rationale of this assertion comes from the fact that if it were due to the ATPase reaction, considering the Na^+ fluxes observed in K^+-containing liposomes [8] it would require an (acetyl phosphate)/(Na^+) ratio far beyond the values seen for ATP.

Finally, the experiments on dephosphorylation and net synthesis of ATP indicate that the phosphoenzymes produced from ATP and acetyl phosphate in the presence of Na^+ and Mg^+ have similar properties. In both cases, the prevailing form in a steady state appears to be E_2P, since dephosphorylation is ADP insensitive but is accelerated by K^+ (result not shown). In addition, both phosphoenzymes can be taken into an ADP-sensitive state by high concentrations (1 M) of NaCl (Fig. 1); as ADP not only increases the rate of dephosphorylation but leads to net synthesis of ATP (Table V), the most logical explanation is that the conformation upon which ADP acts is $E_1P(Na)$ [29]. Actually, the amounts of ATP synthesized are close to the expected on the basis of the levels of phosphoenzymes attained (1.9 nmol/mg with ATP and 1.49 nmol/mg with acetyl phosphate), the total dephosphorylation in 45 s (about 96%) and the fraction of that dephosphorylation that was sensitive to ADP (0.65–0.66). This predicts 1.19 nmol/mg of ATP synthesized with the phosphoenzyme obtained from ATP and 0.94 nmol/mg with that from acetyl phosphate. The observed values averaged 0.97 nmol/mg and 0.81 nmol/mg, respectively. A phosphoenzyme susceptible of being converted into an ADP-sensitive form by 1 M NaCl concentration could also result from phosphorylation by contaminant inorganic phosphate [29]. However, there are two reasons that make this a rather unlikely event in the conditions of our experiments [36]: (i) phosphorylation was carried out in the presence of Na^+ and with no K^+ in the media, and (ii) the dephosphorylation rate of that phosphoenzyme was increased by K^+ (not shown). One last consideration calls for comments. The present results show that the phosphorylation step with acetyl phosphate is not irreversible, for the phosphoenzyme formed, like that formed from ATP, can be forced into an almost pure E_1P state. This does not necessarily prove that when going in the forward direction the phosphoenzyme formed with acetyl phosphate also follows the sequence E_1P-E_2P, but it makes that likely [8]; recent data on

fluorescence experiments [37,38] are also consistent with this view.

Acknowledgements

This work was supported by Grants from CONICET (0155) and CONICOR. M.C., G.B. and L.B. are established investigators of CONICET. We wish to thank M. Siravegna for her skillful technical assistance and Frigorífico Regional Colonia Tirolesa for supplying the pig kidneys.

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