Role of Phospholipid in the Intermediate Steps of the Sodium-plus-Potassium Ion-Dependent Adenosine Triphosphatase Reaction

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The phosphorylation and dephosphorylation steps of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase (adenosine triphosphatase) (EC 3.6.1.3) reaction have been compared in 'normal', lipid-depleted and 'restored' membrane ATPase preparations. Partial lipid depletion was achieved by a single extraction with Lubrol W, and 'restoration' by adding pure phosphatidylserine. γ - ^{32}P -labelled ATP was used for phosphorylation. The main findings were as follows. (1) Partial lipid depletion decreased but did not prevent Na^+ -dependent phosphorylation, although it virtually abolished both Na^+ -dependent and ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities. (2) 'Restoration' with phosphatidylserine produced an increment in phosphorylation that was the same in the presence and absence of added Na^+ . (3) K^+ decreased the extent of Na^+ -dependent phosphorylation of the depleted enzyme without producing a corresponding release of P_i . (4) K^+ rapidly decreased the extent of phosphorylation of the 'restored' enzyme to near-background value, with a concomitant release of P_i . (5) Na^+ -dependent ATP hydrolysis was not restored. (6) The turnover of the 'restored' enzyme seemed to be higher than that of the 'normal' enzyme. The reaction sequence is discussed in relation to these results and the fact that the depleted enzyme retained about 50% of K^+ -dependent phosphatase activity.

The finding that the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase* system (EC 3.6.1.3) is dependent on phospholipid [see Roelofsen & van Deenen (1973) for a summary and references] raises the question of which intermediate steps of the complete reaction sequence require the lipid. In addition, if lipid is not required for all of the partial reactions, the ability to inactivate and reactivate the enzyme by removal and restoration of phospholipid provides another parameter that can be varied and used to investigate the reaction sequence. So far rather little has been reported on this topic, in spite of the vast amount of work on the lipid requirement of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase and associated K^+ -dependent phosphatase activity, probably because of difficulties in reproducibly reactivating lipid-depleted enzymes. This factor is particularly important when extraction with detergent and subsequent salt fractionation procedures have been used to remove lipid, because only part of the original protein is retained in the final preparation and comparison with the original is questionable unless restoration of activity with exogenous phospholipid can be demonstrated. Hence it is not surprising that previous work has been confined to ATPases that have been digested with phospholipases, with the stress more on effects of lipid depletion than on the reactivated enzyme. Despite some apparently conflicting findings, the general

conclusions from those studies were similar: that phospholipid is important both for phosphorylation and dephosphorylation of the enzyme (Taniguchi & Tonomura, 1971; Goldman & Albers, 1973; Stahl, 1973).

The Lubrol extraction method for removing lipid from the ATPase (Palatini *et al.*, 1972; Wheeler *et al.*, 1975) not only produces depleted enzymes which can be reactivated very consistently and reproducibly by added phospholipid, but also provides a way of differentially inhibiting the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase and K^+ -dependent phosphatase activities (Wheeler & Walker, 1975). Thus a typical preparation after Lubrol extraction has a specific ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity amounting to only 5% of the original, whereas its specific K^+ -dependent phosphatase activity is still around 50% (Wheeler & Walker, 1975). Since the K^+ -dependent phosphatase activity is commonly thought to represent the last step of the complete ATPase reaction (see Whittam & Wheeler, 1970), these Lubrol-treated preparations appear to be very suitable for studying the partial reactions of the ATPase system. We have, therefore, compared the phosphorylation and dephosphorylation steps in the original, the lipid-depleted and the reactivated ATPase preparations, with the use of γ - ^{32}P -labelled ATP for phosphorylation and pure phosphatidylserine for reactivation.

A preliminary report of some of these findings has already been published (Wheeler, 1974).

* Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethylamine)tetra-acetate.

Experimental

Tissue preparations

Microsomal fractions were isolated from homogenates of rabbit kidney and treated with NaI as described previously (Wheeler *et al.*, 1975). These 'normal' ATPase preparations were partly depleted of lipid by a single extraction with Lubrol W, by the procedure of Palatini *et al.* (1972) as modified by Wheeler *et al.* (1975). The lipid-depleted preparations were stored at -20°C in the 'A-20' buffer solution described by Towle & Copenhagen (1970).

Radioactive ATP

γ - ^{32}P -labelled ATP was either obtained from The Radiochemical Centre, Amersham, Bucks., U.K., as the sodium salt, or made by the method of Glynn & Chappell (1964) in the form of the Tris salt.

Measurement of enzyme activity and phosphorylation

Samples of enzyme were incubated with γ - ^{32}P -labelled ATP in 2 ml of the appropriate medium contained in a water-jacketed vessel and continuously stirred with a small magnetic stirrer. The temperature was maintained at 38° or 3°C , according to the experiment. Incubation media contained 50 mM-Tris-HCl (pH 7.5), 0.3 mM-MgSO₄, 0.5 mg of bovine serum albumin/ml and 0.1 mM-NaH₂PO₄, with further additions of NaCl, KCl, EDTA, EGTA or phosphatidylserine, as indicated in the legends to the Figures and Table. Reactions were started by addition of the [^{32}P]ATP (final concentration 20–30 μM) and stopped, usually after 10 s, by rapid injection of 3 ml of an ice-cold solution containing 10% (w/v) trichloroacetic acid, 1 mM-ATP and 0.2 mM-NaH₂PO₄. Precipitated protein was sedimented by centrifugation for 15 min at about 2000g and 0 – 4°C , and the clear supernatant removed with a Pasteur pipette. The amount of tracer P_i in the supernatant was determined by mixing 2 ml samples with 0.5 ml of ammonium molybdate solution (4%, w/v, in 2.5 M-H₂SO₄), extracting the phosphomolybdate complex into 2.5 ml of 2-methylpropan-1-ol-benzene (1:1, v/v) and then diluting samples of the extract with ethanol for measurement of radioactivity. These measurements thus provided an assay of ATPase activity. Phosphorylation of the enzyme was determined as follows. The protein pellets were dispersed in the residual drops of trichloroacetic acid solution with the aid of a tightly fitting glass stirring rod, and washed by resuspension and centrifugation in 3 ml of the ice-cold solution of acid, ATP and P_i. This washing process was repeated to give a total of three or four washes, after which the pellets were dissolved in 1 M-NaOH. Samples of the NaOH solutions were used for radioactivity measurements and protein assays.

Radioactivity measurements

The Čerenkov-radiation method was used, all samples being counted in a Packard Tri-Carb liquid-scintillation counter, usually with a minimum of 10000 counts collected per sample. The counting efficiency was about 30%. Some quenching was

Fig. 1. Effect of incubation time on degree of activation by phospholipid

Lipid-depleted ATPase preparations (final concentration 0.03–0.08 mg of protein/ml) were incubated in solutions containing 100 mM-NaCl, 50 mM-Tris-HCl (pH 7.5), 0.3 mM-MgSO₄, 0.1 mM-NaH₂PO₄, 0.5 mg of bovine serum albumin/ml, with (Δ, ▲) or without (○, ●) 15 mM-KCl, and with (▲, ●) or without (Δ, ○) 0.38–0.59 mM-phosphatidylserine, for the times indicated at 38°C . After these preliminary incubations, [^{32}P]ATP (final concentration 20–40 μM) was added and the reaction allowed to proceed for 10 s. ATP hydrolysis (a) and enzyme phosphorylation (b) were measured as described in the Experimental section. Mean values from three similar but not identical experiments are given.

caused by the yellow colour of the phosphomolybdate complex in the organic solvents and that was checked and corrected for by the internal-standard method. Total radioactivity added as $[\text{P}^{32}]\text{ATP}$ to each vessel ranged from 10^5 to 10^6 c.p.m.

All other materials and methods used were exactly as described by Wheeler *et al.* (1975).

Results

Conditions for reactivation of lipid-depleted enzymes

There are several important differences in the conditions used to measure ATPase activity by the 'usual' and radioactive methods that could give rise to apparent discrepancies in results obtained by the two procedures. First, the protein concentration used for the radioactive method is usually about 10 times that 'normally' used (around 0.1 mg/ml compared with 0.01 mg/ml for our enzymes). Secondly, the concentration of ATP used for the radioactive work is usually about 20–30 μM , compared with about 2–3 mM for the conventional method. And thirdly, incubations for measuring phosphorylation are usually of the order 10–20 s, compared with 10–20 min

for the non-radioactive method. We found that both the short incubation times and the high concentration of protein required for phosphorylation measurements affected the reactivation of the lipid-depleted ATPase by added phospholipid.

The effect of time on the reactivation process is shown in Fig. 1(a). ATPase activity measured in a 10 s incubation under optimal conditions (that is with phosphatidylserine, Na^+ and K^+ present together) was doubled by a preliminary incubation of the enzyme with the phospholipid for 5–20 min before addition of the $[\text{P}^{32}]\text{ATP}$. In contrast, such preliminary incubation had no effect on the low ATPase activity measured in the absence of K^+ or phosphatidylserine. Fig. 1(b) shows the corresponding rates of phosphorylation of the ATPase. No clear effect of the preliminary incubation on the extent of phosphorylation was apparent to correlate with the increased rate of ATP hydrolysis, although in two out of three experiments we detected a small decrease in the rate of phosphorylation occurring in the presence of phosphatidylserine with K^+ .

In all other experiments reported here all the constituents of the incubation mixtures except the ATP were incubated together at 38°C for 10–20 min before starting the reaction with $[\text{P}^{32}]\text{ATP}$.

A comparison of the concentrations of phosphatidylserine required for maximal reactivation of a typical lipid-depleted ATPase when assayed by the two different methods is shown in Fig. 2. The concentration needed was about four times higher in the radioactive method, the concentration of ATPase being eight times higher.

Time-course of hydrolysis and phosphorylation

Typical time-courses of ATP hydrolysis and ATPase phosphorylation are illustrated in Fig. 3. Both the rates of hydrolysis and the amount of phosphorylation were reasonably constant during the 20 s incubation period under the conditions noted, and 10 s was chosen as a suitable and convenient incubation time for subsequent experiments.

Effect of enzyme concentration

ATP hydrolysis and phosphorylation of the ATPase were measured with enzyme concentrations ranging from 4 to 150 μg of protein/ml, in the presence and absence of 0.59 mM-phosphatidylserine (Fig. 4). Both the rate of ATP hydrolysis and the extent of phosphorylation increased linearly with increasing enzyme concentration up to about 50 μg of protein/ml, with small deviations from linearity at higher concentrations. Thus the general pattern of response to added phospholipid and K^+ was the same over the complete range of enzyme concentrations tested.

Fig. 2. Effect of concentration of phospholipid on ATPase activity under different incubation conditions

Lipid-depleted ATPase preparations were incubated with the concentrations of phosphatidylserine indicated under the following conditions. $\square$, $\blacksquare$, 120 mM- NaCl , 20 mM- KCl , 30 mM- Tris-HCl (pH 7.5), 3 mM- ATP , 3 mM- MgSO_4 , 0.5 mg of bovine serum albumin/ml, 0.5 mM- EGTA , 0.01 mg of enzyme protein/ml, with ($\square$) or without ($\blacksquare$) 0.1 mM-ouabain; 20 min incubation at 37–38°C. Δ , $\blacktriangle$, 100 mM- NaCl , 50 mM- Tris-HCl (pH 7.5), 30 μM - $[\text{P}^{32}]\text{ATP}$, 0.3 mM- MgSO_4 , 0.1 mM- NaH_2PO_4 , 0.5 mg of bovine serum albumin/ml, 0.08 mg of enzyme protein/ml, with ($\blacktriangle$) or without (Δ) 15 mM- KCl ; 10 s incubation at 37–38°C after 20 min preliminary incubation without the ATP. Mean values from two experiments are given.

Fig. 3. Time-course of ATP hydrolysis and enzyme phosphorylation

[³²P]ATP hydrolysis (a) and enzyme phosphorylation (b) were measured as described in the Experimental section, with lipid-depleted ATPase (0.13 mg of protein/ml), and 30 μM-[³²P]ATP. Symbols have the same meaning as in Fig. 1, with phosphatidylserine at 0.32 mM when present. The preliminary incubation was for 20 min and the incubation for 20 s, both at 37°C.

Fig. 4. Effect of enzyme concentration on ATP hydrolysis and enzyme phosphorylation

The conditions were as described in Fig. 3, except that the enzyme concentration was varied as indicated, the [³²P]ATP concentration was 20 μM, and the solutions contained 1 mM-EGTA. Symbols have the same meaning as in Figs. 1 and 3, with phosphatidylserine at 0.59 mM when present.

Table 1. *Effect of chelators on hydrolysis and labelling*

Lipid-depleted ATPase was incubated with [³²P]ATP in the presence and absence of phosphatidylserine and chelating reagents, and the rate of hydrolysis of the ATP and the extent of phosphorylation of the protein were measured as described in the Experimental section. All incubation media contained 100mM-NaCl, 15mM-KCl, 50mM-Tris-HCl (pH7.5), 0.3mM-MgSO₄, 0.1mM-NaH₂PO₄, 0.5mg of bovine serum albumin/ml and (A) 15μM-[³²P]ATP with 0.1mg of enzyme protein/ml, or (B) 20μM-[³²P]ATP with 0.08mg of enzyme protein/ml. The concentration of phosphatidylserine, when added, was 0.3mM in (A) and 0.6mM in (B).

Experiment	Chelator (mM)	ATP hydrolysis (%)		Phosphorylation (pmol of P _i /mg of protein)	
		Control	+Phosphatidylserine	Control	phosphatidylserine
(A)	—	12	50	48	17
	EDTA (0.2)	14	58	51	19
(B)	—	3	19	39	22
	EGTA (0.5)	3	21	33	21
	EGTA (1.0)	4	23	38	21
	EGTA (2.0)	4	24	33	19

Effect of chelators

Although the Lubrol-extracted enzymes were stored in a solution containing 1mM-EDTA, the effect of adding such chelating reagents to the incubation media used in the radioactive experiments was examined because of the possibility that the reactivation by phosphatidylserine resulted from its chelating properties rather than from a specific lipid effect. The results given in Table 1 exclude that possibility because neither EDTA nor EGTA mimicked the effect of phosphatidylserine, and neither prevented the phospholipid from reactivating. In fact, both reagents slightly enhanced the effect of phosphatidylserine on the ATPase activity, but did not affect the extent of phosphorylation significantly.

Effects of lipid depletion and restoration on intermediate reactions

(a) *General pattern.* The results already given in Figs. 1, 3 and 4, together with those in Table 1, present a consistent picture of the effects of lipid depletion and restoration on the phosphorylation and activity of the ATPase. The depleted enzyme was still phosphorylated when incubated with [³²P]ATP in the presence of Mg²⁺ and Na⁺; and the extent of phosphorylation decreased significantly when K⁺ was also added (Figs. 1(b), 3(b) and 4(b)). However, in contrast with the usual finding with 'normal' ATPase, this decrease in the extent of phosphorylation caused by addition of K⁺ was not accompanied by a corresponding increase in the rate of ATP hydrolysis (Figs. 1a, 3a and 4a). Addition of phosphatidylserine in the presence of Mg²⁺ and Na⁺ produced an increase in the amount of phosphorylation but, again, the rate of ATP hydrolysis was unaffected. When both phosphatidylserine and K⁺ were added to the system in the presence of

Fig. 5. *Effect of Na⁺ concentration on enzyme phosphorylation and ATP hydrolysis*

Lipid-depleted ATPase was dialysed overnight at 4°C against 125 vol. of 50mM-Tris-HCl (pH7.5) and 1mM-EGTA (pH7.5) to decrease its Na⁺ content. Samples (final concentration 0.07mg of protein/ml) were incubated with 20μM-[³²P]ATP, 1mM-EGTA, 0.3mM-MgSO₄, 50mM-Tris-HCl (pH7.5), 0.5mg of bovine serum albumin/ml, and the concentrations of NaCl indicated, for 10s at 37°C, after a preliminary incubation without the ATP for 20min. ATP hydrolysis (a) and enzyme phosphorylation (b) were measured as described in the Experimental section. ○, Control; ●, With 0.59mM-phosphatidylserine.

Mg²⁺ and Na⁺, however, the enzyme responded in a 'normal' way; the phosphorylation decreased to a very low value and the rate of ATP hydrolysis increased severalfold (Figs. 1, 3 and 4; Table 1).

The simplest interpretation of these results is that the phospholipid enhanced the steady-state amount of phosphorylation and was essential for the K⁺-dependent dephosphorylation step.

(b) *Effect of Na⁺ concentration.* Since the phosphorylation reaction that seems to be part of the complete ATPase reaction sequence is Na⁺-dependent (see Whittam & Wheeler, 1970) it was important to check the effect of Na⁺ concentration on the phosphorylation of these lipid-depleted enzymes. Fig. 5(a) showed that Na⁺ had no effect on the rate of ATP hydrolysis catalysed by the depleted preparations, regardless of whether or not phosphatidylserine was also added, whereas Fig. 5(b) shows that it had a marked effect on the extent of phosphorylation. However, both the magnitude of the Na⁺-dependent component of phosphorylation, and its variation with Na⁺ concentration, seemed to be the same in the presence and absence of added phosphatidylserine. Hence the increment in phosphorylation caused by the phospholipid was not similarly Na⁺-dependent.

(c) *Effects of K⁺ concentration.* The response of the lipid-depleted ATPase to various concentrations of K⁺, in the presence and absence of phosphatidyl-

serine, is shown in Fig. 6. Stimulation of ATP hydrolysis by K⁺ clearly depended on the presence of the phospholipid (Fig. 6a) whereas the lowering of the extent of phosphorylation by K⁺ did not (Fig. 6b). On the other hand, both the way in which, and the extent to which, the phosphorylation varied with K⁺ concentration were different in the presence and absence of phosphatidylserine. The data are not sufficiently precise to warrant detailed kinetic analysis, but the concentrations of K⁺ producing half-maximal effects provide some measure of those differences. In the absence of the phospholipid, half-maximal decrease in phosphorylation required about 1.8 mM-K⁺, whereas in the presence of phosphatidylserine only about 0.9 mM-K⁺ produced half-maximal decrease in phosphorylation and increase in ATPase activity.

(d) *Requirement of phospholipid for K⁺-dependent dephosphorylation.* Further evidence supporting the view that the phospholipid is essential for the K⁺-dependent dephosphorylation step is given in Fig. 7. The lipid-depleted enzyme was phosphorylated by brief incubation with [³²P]ATP in the absence of K⁺, with and without phosphatidylserine, and then an excess of unlabelled ATP was added, with and without K⁺. The excess of unlabelled ATP prevented any further detectable incorporation of [³²P] into the ATPase and thus allowed the rate of dephosphorylation to be monitored. In fact, accurate measurement

Fig. 6. Effect of K⁺ concentration on enzyme phosphorylation and ATP hydrolysis

[³²P]ATP hydrolysis (a) and enzyme phosphorylation (b) were measured as described in Fig. 1, with 0.07 mg of enzyme protein/ml, 33 μM-ATP, 0.2 mM-EDTA and the concentrations of KCl indicated. Preliminary incubation was for 20 min and incubation for 10 s, both at 37°C. Symbols have the same meaning as in Fig. 1, with phosphatidylserine at 0.36 mM when present.

Fig. 7. Effect of K⁺ on dephosphorylation of lipid-depleted ATPase, with and without phospholipid

Lipid-depleted ATPase (final concentration 0.14 mg of protein/ml) was incubated for 10 min at 37°C in a solution containing 100 mM-NaCl, 0.05 mM-EDTA, 0.3 mM-MgSO₄, 50 mM-Tris-HCl (pH 7.5), 0.5 mg of bovine serum albumin/ml, with (●, ▲) or without (○, △) 0.65 mM-phosphatidylserine, in a final volume of 2 ml. The temperature was then lowered to 3°C and [³²P]ATP added, giving a final concentration of 30 μM. After 15 s 0.1 ml of a solution containing 20 mM-ATP (○, ●) or 20 mM-ATP+200 mM-KCl (△, ▲) was added. The reaction was stopped after the times indicated and enzyme phosphorylation measured as described in the text.

of the rate of dephosphorylation would require the use of special rapid-reaction techniques, but the results obtained by our methods were good enough to show that K⁺ greatly accelerated the breakdown only of the intermediate formed in the presence of the phospholipid.

(e) *Comparison with 'normal' ATPase preparations.* The responses to Na⁺ and K⁺ of 'normal', lipid-depleted and 'restored' ATPase during incubation with [³²P]ATP are compared in Fig. 8. Note that, because of the way in which it is obtained, the depleted enzyme retains only a fraction of the protein from the original 'normal' preparation, so that the validity of direct comparison of absolute values for those two systems is questionable. Nevertheless, comparison of their phosphorylation relative to their ATPase activities is quite reasonable, and all comparisons of the properties of the depleted and 'restored' preparations are valid because they differ only in the addition of phosphatidylserine to

the latter. Several important points are revealed by these comparisons. (1) Partial lipid depletion did not prevent Na⁺-dependent phosphorylation, although it did abolish both Na⁺-dependent and (Na⁺+K⁺)-dependent ATPase activities. (2) The extent of Na⁺-dependent phosphorylation was lower in the depleted enzyme than in the 'normal' one. (3) Addition of K⁺ to the depleted enzyme caused a fall in the amount of Na⁺-dependent phosphorylation, even though no increase in ATP hydrolysis accompanied that effect. (4) Addition of phosphatidylserine to the depleted enzyme restored (Na⁺+K⁺)-dependent ATPase activity, and the original pattern of phosphorylation in the presence of Na⁺ plus K⁺, but did not restore Na⁺-dependent ATP hydrolysis. (5) In the absence of K⁺, the extent of phosphorylation of the 'restored' enzyme was higher than that in the depleted one, both with and without Na⁺. (6) The absolute (Na⁺+K⁺)-dependent ATPase activity of the restored preparations was higher than that of the original 'normal' enzyme, although the absolute amount of Na⁺-dependent phosphorylation was lower.

Effect of multiple extractions with lubrol

When the ATPase was subjected to three successive extractions with lubrol, instead of just a single extraction, all signs of enzyme activity were lost and no restoration of activity by added phospholipid was achieved (Wheeler *et al.*, 1975). Two such preparations were tested for phosphorylation with [³²P]ATP as described above: no incorporation of ³²P was detected under any condition tested.

Discussion

There are two aspects to the work described here: the attempt to find out where in the (Na⁺+K⁺)-dependent ATPase reaction sequence phospholipid is required, and the use of that phospholipid requirement to gain more information about the reaction sequence. Only the former aspect has been deliberately examined so far; but some of the results obtained show that the approach is potentially useful for the latter aspect.

As far as the location of the action of phospholipid is concerned, the results are unequivocal in showing that although the Lubrol-extracted ATPase underwent Na⁺-dependent phosphorylation by ATP, K⁺-dependent dephosphorylation leading to the release of P_i occurred only when phospholipid was added. Hence it is clear that the exogenous phospholipid was essential for the second part of the reaction sequence, subsequent to phosphorylation. However, the requirements for the initial phosphorylation steps are not absolutely clear because, in the absence of K⁺, added phospholipid also produced an increment in the extent of phosphorylation that

Fig. 8. Comparison of ATP hydrolysis and enzyme phosphorylation with 'normal', lipid-depleted and 'restored' ATPases

'Normal' and lipid-depleted ATPases were prepared as described in the Experimental section, and dialysed to remove Na⁺ as described in Fig. 5. A sample of the depleted enzyme was 'restored' by adding phosphatidylserine (final concentration 0.59 mM). After a preliminary incubation for 20 min at 37°C without ATP, samples of the enzyme preparations were incubated for 10 s at 37°C with 20 μM-[³²P]ATP, 0.3 mM-MgSO₄, 1 mM-EGTA, 0.5 mg of bovine serum albumin/ml, 50 mM-Tris-HCl (pH 7.5), with or without 100 mM-NaCl and 15 mM-KCl, as indicated. ATP hydrolysis and enzyme phosphorylation were measured as described in the Experimental section. The final concentration of enzyme protein was 0.2 mg/ml for the 'normal' preparation and 0.08 mg/ml for the depleted and 'restored' preparations. Mean values from two experiments are given.

might or might not have been an important part of the reaction sequence. On one hand, the extra phosphorylation seemed to be different from that measured in the absence of added lipid because it was either independent of Na⁺, or dependent on very low concentrations of Na⁺, its magnitude being approximately the same in the nominally Na⁺-free conditions as in 100 mM-NaCl. (The concentration of Na⁺ in the nominally Na⁺-free solutions could not have been more than 1 or 2 mM, and probably was less.) On the other hand, addition of K⁺ caused rapid decay of both kinds of phosphoryl enzyme, which argues against the possibility that the increment in labelling in the presence of added phospholipid resulted from some non-specific binding or occlusion of the label. The fact that ATPase preparations which had been subjected to repeated

extractions with lubrol could not be phosphorylated at all suggests that the residual lipid present after a single extraction was necessary for the Na⁺-dependent phosphorylation, unless such drastic treatment had caused either complete denaturation or complete loss of the enzyme protein. Clarification of these points will entail some modifications of the methods; but it is clear that any phospholipid requirement for the first part of the ATPase reaction, involving Na⁺-dependent phosphorylation, must be in some way different from that for the second part, involving K⁺-dependent dephosphorylation. This conclusion points to the potential usefulness of this approach for further study of the reaction sequence.

Wheeler & Walker (1975) showed that after a single extraction of the ATPase with Lubrol the K⁺-dependent phosphatase activity of the depleted

enzyme was, on average, still about 50% of the initial value, so that the requirement for exogenous phospholipid to produce K⁺-dependent release of P_i from the enzyme phosphorylated with ATP imposes quite narrow restrictions on the location of the lipid action. If the phosphatase activity is a manifestation of the K⁺-dependent dephosphorylation step in the complete ATPase reaction, then the added phospholipid must act somewhere between phosphorylation and dephosphorylation. Alternatively, the phosphatase activity must be regarded as being either separate from the ATPase activity, which seems unlikely in view of all the evidence available (see Whittam & Wheeler, 1970), or something different from a partial reaction of the ATPase. Since there is currently some dispute about the exact number and forms of phosphorylated intermediates in the overall reaction (Fukushima & Tonomura, 1973), the situation is perhaps best summarized thus:

(I) ATP binding and (Mg ²⁺ +Na ⁺)-dependent phosphorylation	}
(II) Transformation of phosphorylated forms or coupling to K ⁺ -dependent phosphatase	
(III) K ⁺ -dependent phosphatase	

parations, the results and conclusions of Winter (1972) about the effects of digitonin on the ATPase reaction could be re-interpreted exactly by the above scheme.

Further information about the reaction sequence is revealed by the observation that K⁺ lowered the steady-state amount of phosphorylation of the lipid-depleted ATPase without producing a corresponding release of P_i. This result not only provides new support for the claims that K⁺ does not just activate dephosphorylation (Siegel & Goodwin, 1972; Banerjee & Wong, 1972; Post *et al.*, 1972; Fukushima & Tonomura, 1973) but also appears to afford a relatively simple way of distinguishing between the different effects of K⁺.

Finally, it appears that these experiments may help to resolve another problem of the ATPase system, the 'Na⁺-dependent' ATPase activity. As shown in Fig. 8, 'normal' ATPase preparations exhibit

Either not dependent on lipid or dependent on tightly bound lipid

Phospholipid essential

{ Either not dependent on lipid or dependent on tightly bound lipid

(For the time being we are assuming that the K⁺-dependent phosphatase activity does represent a partial reaction of the ATPase.)

This scheme is not entirely different from those proposed by the investigators who used phospholipases to deplete ATPases of phospholipids, but it suggests that the phospholipid might be directly involved in a more limited fashion. Thus both Stahl (1973) and Goldman & Albers (1973) concluded that phospholipids are important for all stages of the ATPase reaction, and Taniguchi & Tonomura (1971) decided that they 'participate both in EP formation and decomposition, and ... are especially essential for the decomposition step'. The results discussed here suggest, instead, that some intramolecular transformation, or intermolecular coupling process is absolutely dependent on phospholipid that is relatively easily removed; whereas the initial and final stages of the reaction either do not require phospholipid, or else depend only on a limited and relatively tightly bound fraction. Unless reactivation by exogenous lipids can be demonstrated after complete, or almost complete, loss of the phosphorylation and phosphatase activities, the last two alternatives cannot readily be distinguished. If we assume that exposure to digitonin would remove some phospholipid from ATPase pre-

'Na⁺-dependent' ATPase activity under the conditions used for these experiments, this kind of activity being about equal to the (Na⁺+K⁺)-dependent hydrolysis. In contrast, neither the lipid-depleted nor the 'restored' enzymes showed any 'Na⁺-dependent' activity, although the 'restored' preparations possessed high (Na⁺+K⁺)-dependent activity. A possible explanation is that these observations provide support for the suggestion of Goldfarb & Rodnight (1970) that the apparent Na⁺-dependent hydrolysis of ATP is really (Na⁺+K⁺)-dependent ATPase activity that is activated by residual protein-bound K⁺. The lipid-depletion treatment used here involved not only exposure of the enzyme to a detergent (Lubrol W), but also successive washing in 0.2, 0.6 and 1.0M-NaCl. In retrospect, such a procedure seems ideal for replacing residual K⁺ with Na⁺, and would probably be more efficient at removing K⁺ than the EDTA solutions used by Goldfarb & Rodnight (1970). It should be possible to apply a suitably modified form of that washing procedure to 'normal' ATPase preparations without depleting them of lipid, and so to test this explanation.

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