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BOVINE BRAIN Na^+, K^+-STIMULATED ATP PHOSPHOHYDROLASE STUDIED BY A RAPID-MIXING TECHNIQUE. DETECTION OF A TRANSIENT [^{32}P]PHOSPHOENZYME FORMED IN THE PRESENCE OF POTASSIUM IONS

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Summary

1. Conditions for binding of [γ-^{32}P]ATP to bovine brain Na^+,K^+-stimulated ATPase were investigated by the indirect technique of measuring the initial rate of ^{32}P-labelling of the active site of the enzyme.

2. At 100 μM [γ-^{32}P]ATP in the presence of 3 mM $MgCl_2$, approximately the same very high rate of formation of [^{32}P]phosphoenzyme was obtained irrespective of whether [γ-^{32}P]ATP was added to the enzyme simultaneously with, or 70 ms in advance of the addition of NaCl. A comparatively slow rate of phosphorylation was obtained at 5 μM [γ-^{32}P]ATP without preincubation. However, on preincubation of the enzyme with 5 μM [γ-^{32}P]ATP a rate of formation of [^{32}P]phosphoenzyme almost as rapid as at 100 μM [γ-^{32}P]ATP was observed.

3. A transient [^{32}P]phosphoenzyme was discovered. It appeared in the presence of K^+, under conditions which allowed extensive binding of [γ-^{32}P]-ATP. The amount of [γ-^{32}P]ATP that could be bound to the enzyme seemed to equal the amount of [^{32}P]phosphorylatable sites.

4. The formation of the transient [^{32}P]phosphoenzyme was inhibited by ADP. The transient [^{32}P]phosphoenzyme was concluded mainly to represent the K^+-insensitive and ADP-sensitive E_1-^{32}P.

5. When KCl was present in the enzyme solution before the addition of NaCl only a comparatively slow rate of phosphorylation was observed. On preincubation of the enzyme with [γ-^{32}P]ATP an increase in the rate of formation of [^{32}P]phosphoenzyme was obtained, but there was no transient [^{32}P]-phosphoenzyme. The transient [^{32}P]phosphoenzyme was, however, detected

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when the enzyme solution contained NaCl in addition to KCl and the phosphorylation was started by the addition of [γ-^{32}P]ATP.

Introduction

A Na^+,K^+-stimulated ATPase system (ATP phosphohydrolase EC 3.6.1.3) seems to be connected with the transport of Na^+ and K^+ against an electrochemical gradient in the animal cell [1—5]. It is well recognized that in order to reveal details of this connection, knowledge of the partial reactions of Na^+, K^+-stimulated ATPase is of the greatest importance [1—5].

The rapid-mixing technique has proved to be a valuable tool for obtaining information on velocities of the partial reactions [6—10]. An Na^+-stimulated formation of [^{32}P]phosphoenzyme from [γ-^{32}P]ATP was found to be sufficiently rapid for the ^{32}P-labelled enzyme to be an intermediate of the overall hydrolysis of ATP [8,9]. In addition, biphasic dephosphorylation, one initial very rapid K^+-stimulated phase and one later slow K^+-insensitive phase, was observed [10]. The rapid K^+-stimulated phase corresponded to a simultaneous and equally rapid burst of $^{32}P_i$ from the [^{32}P]phosphoenzyme. The results provided independent evidence of the existence of two kinetically different forms of the [^{32}P]phosphoenzyme.

In an earlier study it was found that the rate of the Na^+-stimulated phosphorylation of the enzyme reached its maximum at about 100 μM [γ-^{32}P]ATP [9]. This was apparently inconsistent with the requirements of the overall reaction, for which about 1 mM ATP in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl was needed for maximal rate. In agreement with models presented by other authors this result was explained by the existence of an E-AT^{32}P complex, preceding the formation of [^{32}P]phosphoenzyme.

In the present study the conditions for binding of [γ-^{32}P]ATP and the existence of E-AT^{32}P were investigated by means of a rapid-mixing technique. In these experiments a transient formation of [^{32}P]phosphoenzyme, in the presence of KCl, was discovered. The formation of this [^{32}P]phosphoenzyme was inhibited by ADP. It is suggested that the transient phosphorylation constitutes independent kinetic evidence of a K^+-insensitive and ADP-sensitive form of [^{32}P]phosphoenzyme, i.e. E_1-^{32}P.

Experimental procedure

Materials

Millipore filters, type SSWP (pore size 3 μm), 25 mm in diameter, were used. The disodium salt and tris salt of ATP were obtained from Sigma. [^{32}P]-orthophosphate was a product of The Radiochemical Centre, Amersham (U.K.). [γ-^{32}P]ATP was prepared by a modification of the method of Engström [11] which has been described previously [10].

Analytical Methods

Radioactivity was determined by measuring the Čerenkov radiation as described in another study [10].

[^{32}P]orthophosphate and protein-bound acyl-[^{32}P]phosphate were assayed as previously described [9].

Protein was assayed by the method of Lowry et al. [12], with human serum albumin as reference.

Enzyme preparation

Na^+,K^+-stimulated ATPase was prepared from bovine brain cortex as described by Skou and Hilberg [13]. The enzyme activity was assayed as Na^+,K^+-stimulated and ouabain-sensitive ATPase [9]. The same batch of enzyme as used in another study [10] was also used throughout the present investigation. The Na^+,K^+-stimulated ATPase activity at 21 °C in the presence of 3 mM $MgCl_2$, 120 mM NaCl, 10 mM KCl and 1 mM ATP was about 408 nmol · mg^{-1} · min^{-1}. The maximal amount of [^{32}P]phosphoenzyme obtained at 21 °C and at 3 mM $MgCl_2$, 120 mM NaCl and 100 μM [γ-^{32}P]ATP was 0.35 nmol · mg^{-1} of protein.

Rapid-mixing experiments

The rapid-mixing apparatus has been described previously [9]. Three syringes of the apparatus were used. The volume of each syringe was 0.6 ml in

TABLE I

CONTENTS OF THE SYRINGES OF THE RAPID-MIXING APPARATUS IN THE EXPERIMENTS OF FIGS 1—5

The number of each experiment is indicated in the appropriate figure. All three syringes contained 3 mM $MgCl_2$ in 30 mM Tris-HCl buffer (pH 7.4). Syringe II contained the enzyme solution at a protein concentration of 0.6 mg/ml.

Expt.	Syringe: I			II		III			
	Concentration of reagents (mM)								
	NaCl	KCl	[γ-^{32}P]ATP	NaCl	KCl	NaCl	KCl	[γ-^{32}P]ATP	ATP
1	—	—	—	—	—	360	—	0.300	—
2	—	—	0.200	—	—	360	—	0.100	—
3	—	—	—	—	—	360	—	0.015	—
4	—	—	0.010	—	—	360	—	0.005	—
5	—	—	—	—	—	360	30	0.300	—
6	—	—	0.200	—	—	360	30	0.100	—
7	—	—	—	—	—	360	30	0.015	—
8	—	—	0.010	—	—	360	30	0.005	—
9	—	—	0.010	—	—	360	—	—	3.0
10	—	—	0.010	—	—	360	30	—	3.0
11*	—	—	0.200	—	—	360	30	0.100	—
12	—	10	—	—	10	360	10	0.015	—
13	—	10	0.010	—	10	360	10	0.005	—
14	—	10	—	—	10	360	10	0.300	—
15	—	10	0.200	—	10	360	10	0.100	—
16**	120	10	0.200	120	10				

* Syringe III also contained 15 mM ADP.

** Only two syringes were used. After mixing in the first jet mixer the solutions were directly expelled into the quenching solution.

all experiments. In addition, the syringes always contained 3 mM $MgCl_2$ and 30 mM Tris-HCl buffer (pH 7.4). Monovalent ions, enzyme, [γ-^{32}P]ATP, unlabelled ATP and ADP were added as indicated in Table I. The experiments were performed at 21—22° C. In a separate experiment only two syringes were used, containing the [γ-^{32}P] ATP solution and the enzyme solution, respectively. Both syringes also contained 3 mM $MgCl_2$ 120 mM NaCl and 10 mM KCl in 30 mM Tris-HCl buffer (Table I).

The background ^{32}P-labelling was less than 10% of maximal amount of [^{32}P]phosphoenzyme. This background seemed to correspond to unspecifically adsorbed $^{32}P_i$ to the enzyme-protein and to the filter [10].

Since the enzyme was forced through the rapid-mixing apparatus at different compositions of the incubation medium, the activity of the enzyme was controlled before and after passage through the apparatus at different combinations of $MgCl_2$, NaCl, KCl and ATP. The final concentrations of the additions were 3 mM, 120 mM, 10 mM and 5 μM, respectively. When the enzyme was forced at maximum velocity through two jet mixers and one Y-connection, a maximal loss of only about 10% of the original Na^+,K^+-stimulated ATPase activity was observed.

Results and Discussion

In order to make it easier to understand the design of the experiments and to follow the discussion a simplified scheme of the partial reactions of Na^+,K^+-stimulated ATPase [2—5] is presented.

$$ATP + E_1 \rightleftharpoons E_1 - ATP$$

$$E_1 - ATP \underset{\rightleftharpoons}{\overset{Mg^{2+},\ Na^+}{}} E_1\text{-}P + ADP$$

$$E_1\text{-}P \rightleftharpoons E_2\text{-}P$$

$$E_2\text{-}P \overset{K^+}{\rightleftharpoons} E_2 + P_i$$

$$E_2 \rightleftharpoons E_1$$

The two different forms of phosphoenzyme presented in this reaction scheme seem to differ only in their kinetic characteristics [10]. The chemical analysis of [^{32}P]phosphoenzyme (protein-bound acyl[^{32}P]phosphate) used in the present investigation cannot distinguish between these two forms of [^{32}P]-phosphoenzyme [9]. The sum of these forms will therefore be measured.

The finding of a maximal rate of formation of phosphoenzyme at as low a concentration of ATP as about 100 μM, which was less than half saturating with respect to the Na^+,K^+-stimulated ATPase activity, supported the view that an E-ATP complex precedes the phosphoenzyme [9]. It was suggested that the formation of E-ATP is very rapid at high concentrations of ATP compared to the step from E-ATP to phosphoenzyme. However, at low concentrations of ATP the rate of formation of E-ATP would probably have determined the rate of the formation of phosphoenzyme.

In order to further investigate this hypothesis the enzyme was preincu-

bated with [γ-^{32}P]ATP under different ionic conditions and at a low (5 μM) and a high (100 μM) concentration of [γ-^{32}P]ATP. At 100 μM [γ-^{32}P]ATP approximately the same rate of formation of [^{32}P]phosphoenzyme was obtained in the present investigation as was found previously [9], irrespective of whether the [γ-^{32}P]ATP was added to the enzyme at the same time as or 70 ms prior to the addition of NaCl (Expts 1 and 2, Fig. 1A).

In agreement with previous results [8,9], a comparatively slow rate of phosphorylation was obtained at 5 μM [γ-^{32}P]ATP (Expt 3, Fig. 1B). The slow rate of phosphorylation may be explained by a slow rate of formation of E-AT^{32}P preceding the phosphorylation step. By preincubation of the enzyme with 5 μM [γ-^{32}P]ATP for about 70 ms, a rate of formation of [^{32}P]phosphoenzyme almost as rapidly as at 100 μM [γ-^{32}P]ATP was achieved (Expt 4, Fig. 1B). Thus, the preincubation at 5 μM [γ-^{32}P]ATP seemed to allow an accumulation of E-[^{32}P]ATP sufficiently high to give approximately the same initial rate of phosphorylation as at 100 μM [γ-^{32}P]ATP.

In order to investigate in what way K^+ interfered with this rapid formation of [^{32}P]phosphoenzyme, phosphorylation was started by adding NaCl plus KCl to the final concentrations of 120 mM and 10 mM, respectively. At 100 μM [γ-^{32}P]ATP formation of a transient [^{32}P]phosphoenzyme was observed (Expt 5, Fig. 2A). By preincubation of the enzyme with 100 μM [γ-^{32}P]ATP the amount of the transient [^{32}P]phosphoenzyme was slightly increased (Expt 6, Fig. 2A). At 5 μM [γ-^{32}P]ATP the transient increase of [^{32}P]phosphoenzyme was significant only when the start of phosphorylation was preceded by preincubation of the enzyme with [γ-^{32}P]ATP (Expts 7 and 8, Fig. 2B). It

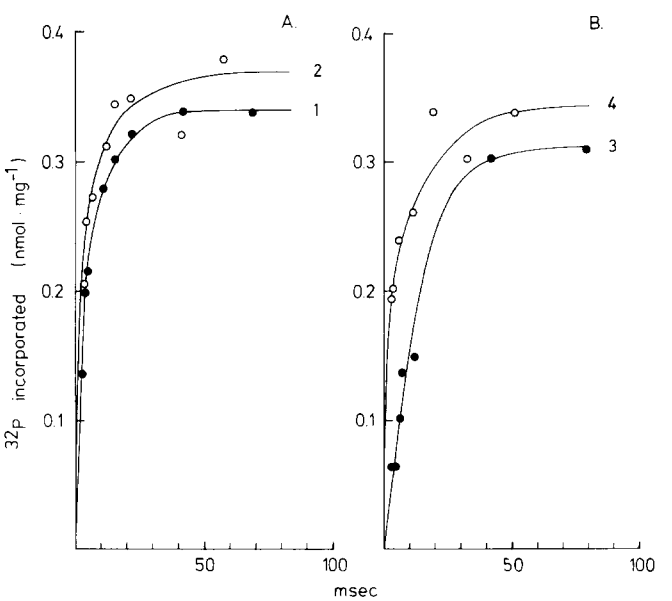

Fig. 1. Phosphorylation of Na^+, K^+-stimulated ATPase in the absence of KCl. All solutions contained 3 mM $MgCl_2$ and 30 mM Tris-HCl buffer (pH 7.4). Phosphorylation was started by the addition of NaCl plus [γ-^{32}P]ATP (●———●) or by NaCl added 70 ms after the [γ-^{32}P]ATP (○———○), final concentration of NaCl was 120 mM. (A) 100 μM [γ-^{32}P]ATP, (B) 5 μM [γ-^{32}P]ATP. The numbers on the curves refer to the corresponding experiments as described in Table I.

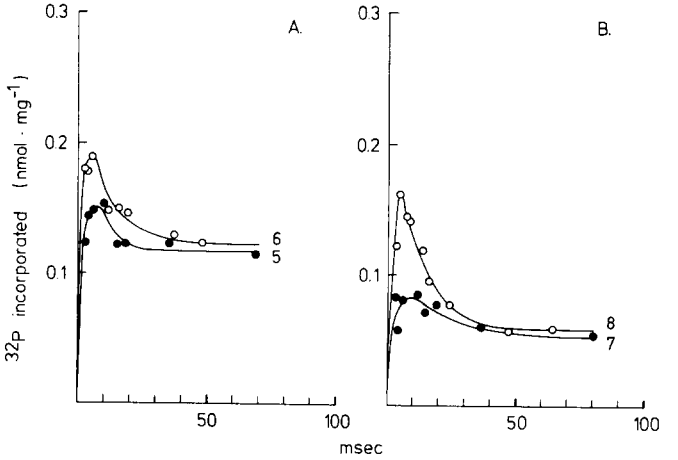

Fig. 2. Phosphorylation of Na^+,K^+-stimulated ATPase in the presence of KCl. All solutions contained 3 mM $MgCl_2$ and 30 mM Tris-HCl buffer (pH 7.4). Phosphorylation was started by the addition of NaCl, [γ-^{32}P]ATP plus KCl (●——●) or by NaCl plus KCl 70 ms after the addition of [γ-^{32}P]ATP (○——○). Final concentrations of the cations added were 120 mM Na^+ and 10 mM K^+. (A) 100 μM [γ-^{32}P]ATP, (B) 5 μM [γ-^{32}P]ATP. The numbers on the curves refer to the corresponding experiments as described in Table I.

is thus concluded that the formation of the transient [^{32}P]phosphoenzyme requires considerable binding of [γ-^{32}P]ATP to the enzyme before the [^{32}P]-phosphorylation is started. This would give a very high initial rate of formation of [^{32}P]phosphoenzyme.

The apparent first-order rate constant of the K^+-stimulated dephosphorylation (at least 14 000 min^{-1}) was shown to be at least slightly higher than that of the Na^+-stimulated phosphorylation (11 000 min^{-1}) [9]. If phosphorylation is performed in the presence of 10 mM KCl an accumulation of a K^+-sensitive [^{32}P]phosphoenzyme will be essentially prevented. The phosphoenzyme formed in the presence of 10 mM KCl will therefore probably consist of a K^+-insensitive form, E_1-P (compare Figs 2,3 and 5). The transient [^{32}P]-phosphoenzyme is suggested to consist of the K^+-insensitive form, E_1-^{32}P, which is dephosphorylated via a K^+-sensitive form, E_2-^{32}P. The rate-limiting step of the dephosphorylation of the transient [^{32}P]phosphoenzyme would thus be the reaction E_1-^{32}P → E_2-^{32}P. This is supported by the fact that the rate of decrease of the transient [^{32}P]phosphoenzyme (see also Expts 10 and 16 of Figs 3 and 5) was similar to the rate of the slow phase of dephosphorylation, which was obtained by addition of excess cold ATP or (1,2-cyclohexylenedinitrilo)tetraacetic acid (CDTA) to a [^{32}P]phosphoenzyme formed in the presence of KCl [10]. This slow phase of dephosphorylation was concluded to consist of dephosphorylation of E_1-^{32}P via E_2-^{32}P.

Interpretation of the transient [^{32}P]phosphoenzyme as suggested above requires the existence of an E-[^{32}P]ATP complex which has already been accumulated, or rapidly accumulates when Na^+ is added. These requirements were therefore further investigated. The enzyme was preincubated with 5 μM [γ-^{32}P]ATP in the presence of 3 mM $MgCl_2$ for about 70 ms before the addition of NaCl to start the formation of [^{32}P] phosphoenzyme. By addition

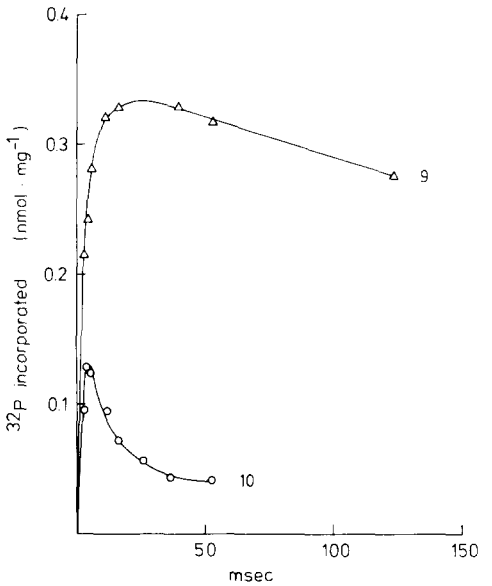

Fig. 3. Phosphorylation of Na^+,K^+-stimulated ATPase in the presence of a high concentration of unlabelled ATP, performed after preincubation with [γ-^{32}P]ATP. The Na^+,K^+-stimulated ATPase was preincubated for 70 ms at 5 μM [γ-^{32}P]ATP and 3 mM $MgCl_2$. Phosphorylation was started by the addition of NaCl plus unlabelled ATP (△——△) or NaCl and unlabelled ATP plus KCl (○——○). The final concentrations of the reagents added were 120 mM NaCl, 1 mM unlabelled ATP and 10 mM KCl. The numbers on the curves refer to the corresponding experiments as described in Table I.

of NaCl plus excess unlabelled ATP to the preincubated enzyme, an almost maximal amount of [^{32}P]phosphoenzyme was obtained (Expt 9, Fig. 3). The results support the view that binding of [γ-^{32}P]ATP to the active site is a prerequisite for [^{32}P]phosphorylation. By extrapolation to zero time, an amount of about 0.35 nmol · mg^{-1} of the E-[^{32}P]ATP formed before the addition of NaCl and unlabelled ATP could be obtained. This amount of E-[^{32}P]ATP was the same as the amount of phosphorylatable sites. Although this is an indirect technique to measure ATP-binding to the enzyme, the results were in accordance with those of Hegyvary and Post [14] which were obtained by a dialysis rate technique. However, the finding of a number of ATP-binding sites equal to the number of phosphorylatable sites were in contrast to the 1 : 2 ratio reported by Jørgensen [15]. Jørgensen used a centrifugation procedure for his ATP-binding experiments which may not be rapid enough to avoid hydrolysis of the bound ATP. Even if the binding and centrifugation were performed at 0° C and in the presence of a high concentration of EDTA, a small hydrolysis of E-ATP would have given an underestimation of the ATP-binding capacity. Apparently, no correction was made for any hydrolysis of ATP. It is further concluded from the present results that the rate of exchange between bound [γ-^{32}P]ATP and free, unlabelled ATP is slow. The rate of the backward reaction E-[^{32}P]ATP → E + [^{32}P]ATP thus seems to be slow compared with the rate of formation of [^{32}P]phosphoenzyme. The slow, constant rate of decrease of the [^{32}P]phosphoenzyme observed after the initial rapid [^{32}P]phosphorylation was approximately the same as the rate of the overall hydrolysis in the absence of KCl.

By addition of NaCl and unlabelled ATP plus KCl to an enzyme which had been preincubated for 70 ms with 5 μM [γ-^{32}P]ATP, a transient [^{32}P]-phosphoenzyme was observed (Expt 10, Fig. 3). The increase and decrease of the transient [^{32}P]phosphoenzyme was similar to those in the experiments presented in Fig. 2, but since further ^{32}P-labelling was prevented the amount of [^{32}P]phosphoenzyme decreased below the steady-state levels obtained in the experiments of Fig. 2. It is known from previous investigations that K^+ decreases the amount of E-ATP [14,16]. However, the present results indicate that the backward reaction E-[^{32}P]ATP $\rightarrow$ E + [^{32}P]ATP is slow compared with the rate of formation of [^{32}P]phosphoenzyme even when 10 mM KCl is present in the incubation medium (compare Expt 9, Fig. 3).

In order to investigate the effect of ADP on the formation of the transient [^{32}P]phosphoenzyme, the Na^+,K^+-ATPase was first incubated with 100 μM [γ-^{32}P]ATP in the presence of 3 mM $MgCl_2$ for about 70 ms before the addition of ADP (Expt 11, Fig. 4). The preincubation made it possible to form the E-[^{32}P]ATP complex before the addition of ADP. This seemed to be important since ADP otherwise would interfere with the binding of [γ-^{32}P]ATP to the enzyme and thus inhibit the formation of the E-[^{32}P]ATP preceding the phosphorylation step. After the preincubation, NaCl and KCl were added simultaneously with ADP. Final concentrations of the additions were 120 mM, 10 mM and 5 mM, respectively. The [^{32}P]phosphorylation was inhibited by ADP and there was no transient increase of [^{32}P]phosphoenzyme. These results support the previous suggestion in this report that the transient [^{32}P]-phosphoenzyme represents the K^+-insensitive E_1-^{32}P, which also has been shown to be sensitive to ADP [2—5,10].

Since both the binding of ATP to the enzyme [14,16] and the [^{32}P]-phosphorylation by [γ-^{32}P]ATP [17] are antagonized by K^+, it seemed of special interest to further investigate the effect by K^+ on the appearance of the transient [^{32}P]phosphoenzyme. The enzyme was mixed with KCl, as well as with $MgCl_2$, and the phosphorylation was started by the addition of NaCl. Radioactive ATP was added, either together with NaCl or 70 ms prior to the addition of NaCl, i.e. preincubation. At 5 μM [γ-^{32}P]ATP, only a very low yield of [^{32}P]phosphoenzyme was obtained irrespective of whether the enzyme was preincubated with [γ-^{32}P]ATP or not (Expts 12 and 13, Fig. 5A). However, with preincubation (Expt 13) the rate of phosphorylation was increased. This increase in the rate of formation of [^{32}P]phosphoenzyme on preincubation was also observed at 100 μM [γ-^{32}P]ATP (Expts 14 and 15, Fig. 5B) and suggests that [γ-^{32}P]ATP, during preincubation increases the level of an E-[^{32}P]ATP complex. No transient [^{32}P]phosphoenzyme was formed, which implies that the level of E-[^{32}P]ATP preceding the phosphorylation step was too low to give the necessary rate of formation of [^{32}P]phosphoenzyme. This may be explained by a conversion of a major part of the enzyme to a K^+-stabilized form (E_2). If the change from this form to a rapidly phosphorylatable E-[^{32}P]ATP complex (E_1-[^{32}P]ATP) is slow, the addition of NaCl would give a rapid [^{32}P]phosphorylation only of the E_1-[^{32}P]ATP complex already formed.

When both 120 mM and 10 mM KCl were present in the enzyme solution, addition of 100 μM [γ-^{32}P]ATP resulted in the formation of the transient

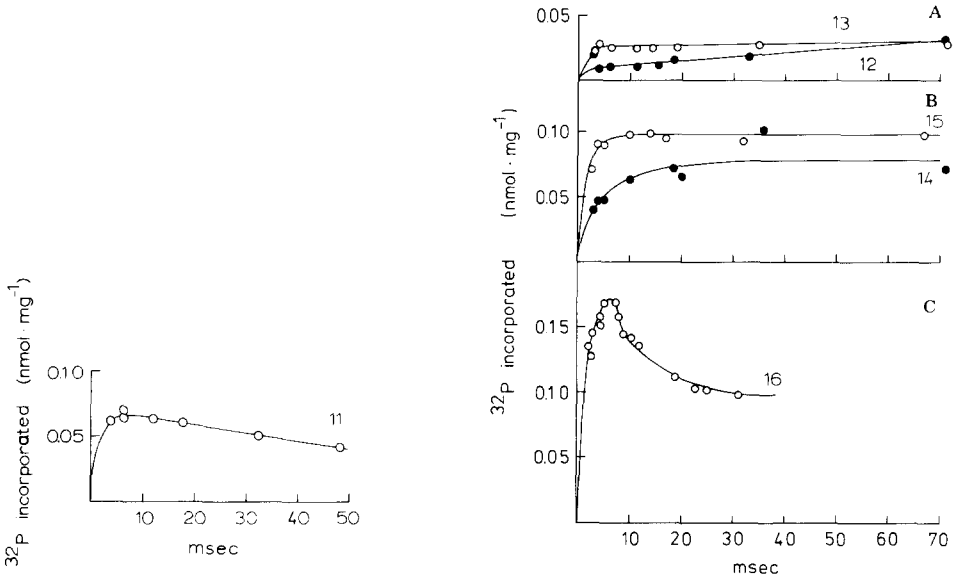

Fig. 4. Effect of ADP on the formation of the transient [^{32}P]phosphoenzyme. The enzyme was preincubated at 3 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP in 30 mM Tris-HCl buffer (pH 7.4). After about 70 ms NaCl, KCl and ADP were added to the final concentrations of 120 mM, 10 mM and 5 mM, respectively. The number on the curve refers to the corresponding experiment as described in Table I.

Fig. 5. Phosphorylation of Na^+,K^+-stimulated ATPase after pretreatment with KCl. All solutions contained 3 mM $MgCl_2$ plus 10 mM KCl in 30 mM Tris-HCl buffer (pH 7.4). Phosphorylation was started by the addition of NaCl plus [γ-^{32}P]ATP (●———●) or by addition of NaCl 70 ms after the addition of ([γ-^{32}P]ATP (○———○). The final concentration of NaCl was 120 mM. (A) 5 μM [γ-^{32}P]ATP, (B) 100 μM [γ-^{32}P]ATP. (C) In addition to 3 mM $MgCl_2$ and 10 mM KCl, 120 mM NaCl was also included in the enzyme solution as well as in the [γ-^{32}P]ATP solution (○———○). Phosphorylation was started by mixing these solutions in the first jet mixer. After mixing, the solutions were expelled directly into the quenching solution. The numbers of the curves refer to the corresponding experiments as described in Table I.

[^{32}P]phosphoenzyme (Expt 16, Fig. 5C). If the accumulation of E_1-[^{32}P]ATP to a high level is a prerequisite for the formation of the transient [^{32}P]phosphoenzyme, it is also concluded that Na^+ overcomes the inhibitory effect of K^+ by stabilizing an enzyme form that easily combines with ATP.

Acknowledgements

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References

1 Skou, J.C. (1965) Physiol. Rev. 45, 596—617
2 Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727—756
3 Whittam, R. and Wheeler, K.P. (1970) Annu. Rev. Physiol. 32, 21—60
4 Skou, J.C. (1971) in Current Topics in Bioenergetics (Sanadi, D.R., ed.), Vol. 4, pp. 357—398, Academic Press, New York
5 Hokin, L.E. and Dahl, J.L. (1972) in Metabolic Pathways (Hokin, L.E., ed.), Vol. 6, pp. 269—315, Academic Press, New York

6 Kanazawa, T., Saito, M. and Tonomura, Y. (1967) J. Biochem. 61, 555—566
7 Kanazawa, T., Saito, M. and Tonomura, Y. (1970) J. Biochem. 67, 693—711
8 Mårdh, S. and Zetterqvist, Ö. (1972) Biochim. Biophys. Acta 255, 231—238
9 Mårdh, S. and Zetterqvist, Ö. (1974) Biochim. Biophys. Acta 350, 473—483
10 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 448—463
11 Engström, L. (1962) Ark. Kemi. 19, 129—140
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Skou, J.C. and Hilberg, C. (1969) Biochim. Biophys. Acta 185, 198—219
14 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234—5240
15 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53—67
16 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
17 Post, R.L., Kume, S. and Rogers, F.N. (1973) in Mechanisms in Bioenergetics (Azzone, G.F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds), pp. 203—218, Academic Press, New York