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PHOSPHORYLATION AND DEPHOSPHORYLATION REACTIONS OF BOVINE BRAIN (Na^+–K^+)-STIMULATED ATP PHOSPHOHYDROLASE STUDIED BY A RAPID-MIXING TECHNIQUE

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SUMMARY

1. In order to allow a study of K^+-dependent dephosphorylation of (Na^+–K^+)-stimulated ATP phosphohydrolase (EC 3.6.1.3, (Na^+–K^+)-stimulated ATPase), a rapid-mixing apparatus with two consecutive mixing chambers and four syringes was developed. The shortest incubation time was about 1.5 ms, excluding the quenching time which was about 1 ms.

2. The pseudo first-order rate constant of the Na^+-dependent phosphorylation was estimated to be about 11 000 min^{-1} at 21 °C in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 100 μM [γ-^{32}P]ATP. This rate seemed to have reached a maximum at this concentration of [γ-^{32}P]ATP. The same seemed to hold for the amount of ^{32}P-labelled enzyme. Since this concentration of [γ-^{32}P]ATP only gives half maximal rate of the (Na^+–K^+)-stimulated hydrolysis of ATP the present data suggest a complex role of ATP, which is consistent with reports by others.

3. When studying the disappearance of protein-bound acyl-[^{32}P]phosphate upon the addition of 10 mM KCl, an apparent first-order rate constant of at least 14 000 min^{-1} was obtained at 21 °C. Within the time resolution of the rapid-mixing apparatus this rate constant seemed to be the same irrespective of which concentration of ATP was used in the range of 5–100 μM.

4. Since the steady-state concentration of the phosphorylatable enzyme form was unknown, the steady-state velocity of the phosphorylation could not be estimated. The concentration of ^{32}P-labelled enzyme at steady-state was about 0.2 nmole·mg^{-1} of protein. If it is assumed that all of this phosphoenzyme is dephosphorylated in one step, characterized by a first-order rate constant of at least 14 000 min^{-1}, the calculated rate of dephosphorylation at steady-state is at least 2800 nmoles·mg^{-1}·min^{-1}. However, the steady-state rate of (Na^+–K^+)-stimulated hydrolysis of ATP at 21 °C was only about 280 nmoles·mg^{-1}·min^{-1} in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl at 100 μM ATP. The consistency of these results with the current hypothesis on two different forms of the phosphorylated intermediate is discussed.

INTRODUCTION

Active Na^+ and K^+ transport across cell membranes is associated to a mem-

brane-bound ATPase system [1, 2] ($(Na^+–K^+)$-stimulated ATPase, EC 3.6.1.3). To understand the mechanism of this transport it is important to know the sequence of the partial reactions of the $(Na^+–K^+)$-stimulated ATPase. Part of this sequence is considered to be comprised of a Na^+-dependent phosphorylation by ATP and a K^+-dependent dephosphorylation [1, 2]. However, full details of the sequence are not yet known, and the same is true for the velocities of the partial reactions.

In a previous report from this laboratory the phosphorylation of a microsomal preparation of $(Na^+–K^+)$-stimulated ATPase from bovine brain by [γ-^{32}P]ATP was studied by a rapid-mixing technique [3]. ^{32}P-labelling of several components was obtained. However, it was shown that only the formation of [^{32}P]phosphate with stability properties consistent with acyl-[^{32}P]phosphate was dependent on Na^+ and rapid enough to be an intermediate reaction of the $(Na^+–K^+)$-stimulated ATPase reaction.

The results of rapid-mixing experiments at low concentrations of ATP reported by Kanazawa et al. [4] support the view that the $(Na^+–K^+)$-stimulated ATPase is intermediately phosphorylated. However, at low concentrations of ATP the enzyme is inhibited by K^+ [5–7]. It may, therefore, be questioned whether their results are applicable to the $(Na^+–K^+)$-stimulated hydrolysis of ATP at high ATP concentrations.

In the present work, the Na^+-dependent phosphorylation and the K^+-dependent dephosphorylation reactions were studied at concentrations of ATP sufficiently high to give K^+ stimulation of the overall hydrolysis. In order to make this possible, the rapid-mixing apparatus previously used [3] was modified so as to have two consecutive mixing chambers and more than two syringes.

EXPERIMENTAL PROCEDURE

Materials

Millipore filters, Type SSWP (3 μm), 25 mm in diameter, were used. ATP (disodium salt and tris salt) were purchased from Sigma. [γ-^{32}P]ATP was prepared by the method of Engström [8]. The specific radioactivity ranged from 0.2 to 0.3 μCi/nmole in the rapid-mixing experiments. All other chemicals were commercially available and of reagent grade. Doubly glass-distilled water was used throughout the work.

Analytical methods

Radioactivity was measured by means of a Geiger counter, or by measuring the Čerenkov radiation as previously described [3]. [^{32}P]orthophosphate was determined by a micromodification of the method of Martin and Doty [9], using silicotungstic acid to precipitate the protein. The final volume of each phase was 2.5 ml. The radioactivity was measured on aliquots of the organic phase.

Protein-bound acyl-[^{32}P]phosphate was assayed as described previously [3]. Protein was assayed by the method of Lowry et al. [10], with human serum albumin as a reference.

Enzyme preparation

Bovine brain cortex was used as the enzyme source and the microsomal frac-

tion was purified as described by Skou and Hilberg [11]. The preparation could be stored at −25 °C for up to 1 year without loss of activity.

The (Na^+-K^+)-stimulated ATPase activity was assayed as the (Na^+-K^+)-stimulated and ouabain-inhibited liberation of [^{32}P]orthophosphate from [γ-^{32}P]-ATP [3] or by coupling the appearance of ADP to that of pyruvate with the aid of phosphoenolpyruvate and pyruvate kinase [12].

Modified rapid-mixing apparatus

The general features of the rapid-mixing apparatus previously described [13] were adopted in a construction with two consecutive mixing chambers and an extra syringe (Fig. 1). The plungers of the syringes were pushed by a piston driven by com-

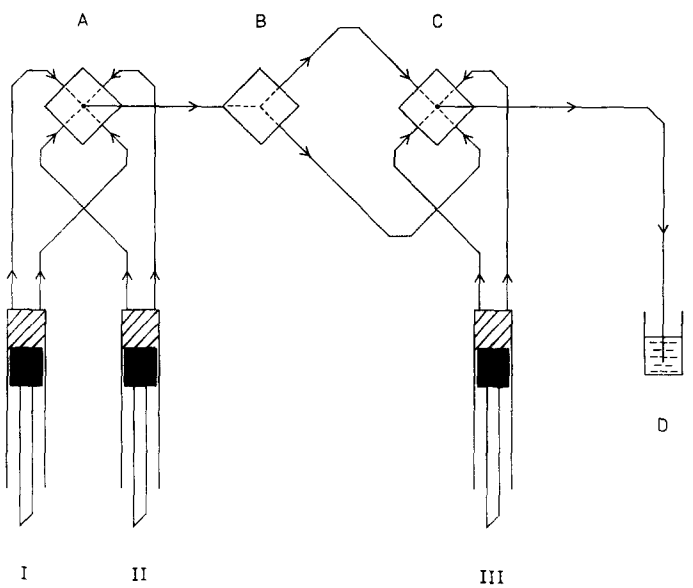

Fig. 1. Diagram showing the principles of the dephosphorylation experiments. A, first jet-mixer; B, Y-connection; C, second jet-mixer; D, vial containing 10% cold $HClO_4$ for quenching. Syringes I, II and III containing the solutions indicated in the text. At phosphorylation experiments, only Syringes I and II were used and the outlet tubing from A dipped into the vial with $HClO_4$. For further information, see Experimental Procedure.

pressed air. This arrangement allowed delivery of 1 ml from each syringe within 200 ms. The shortest possible incubation time in the apparatus was about 1.5 ms, including mixing but excluding quenching time.

The plungers were pushed at a constant rate. This was checked by means of a linear potentiometer, the slide of which was secured to the same moving block as were the plungers. The potentiometer was coupled to a DC source of 10 V and the potential of the slide was displayed by a Tectronix Model 564 storage oscilloscope. The sweep was triggered when two metal pins, arranged end-to-end, were separated as the plungers began to move, thus breaking the triggering circuit.

When two mixing chambers were used, the outlet tubing of the first mixing

chamber was coupled to a Y-connection (bore 0.5 mm), which split the flow into two streams, which were led to inlets 1 and 3, respectively, of the second mixing chamber. Inlets 2 and 4 received the third solution. Usually the second mixing was performed 60–70 ms after the first. The reaction time was a function of the volume of the outlet tubing, and of the rate of the flow, the latter being adjustable by altering the pressure on the piston.

The syringes were filled by reversing the flow of the compressed air acting on the piston of the driving device. In order to obtain gentle filling, the reversed air stream was passed through a constriction before reaching the piston.

The management of the apparatus was as described previously [3, 13]. In a typical experiment, Syringe I contained 1 ml of [γ-^{32}P]ATP and Syringe II 1 ml of the enzyme solution (0.5 mg of protein/ml). At the dephosphorylation experiments a third syringe, Syringe III, was also used and contained 1 ml of 30 mM KCl. All solutions contained the same concentrations of buffer and additional ions, i.e. 30 mM Tris-HCl buffer (pH 7.4), 3 mM $MgCl_2$ and 120 mM NaCl. In some phosphorylation experiments Syringes I and II also contained 10 mM KCl, as indicated. The experiments were carried out at 21.5 $\pm$ 0.5 °C.

In a control experiment the (Na^+–K^+)-stimulated ATPase activity was measured before and after the enzyme was forced through the rapid-mixing apparatus. Only about 5% of the activity was lost in the passage of the enzyme through the two consecutive jet mixers.

Quenching of the phosphorylation and dephosphorylation reactions

Quenching was obtained by expelling the reaction mixture into 5 ml of cold 10% (w/w) $HClO_4$. By extrapolation of the pseudo first-order plots of the phosphorylation experiments to the zero concentration of phosphorylated enzyme (Fig. 2), the quenching time was estimated. The value of the quenching time varies with the value of $[E\text{–}P]_\infty$ that is obtained, i.e. the maximal possible amount of phosphorylated enzyme that can be achieved. From 10 phosphorylation experiments at 3 mM $MgCl_2$, 120 mM NaCl and 100 μM [γ-^{32}P]ATP and after a reaction time of about 80 ms a value of $[E\text{–}P]_\infty$ of 0.499 $\pm$ 0.025 (2 S.D.) nmole·mg^{-1} of protein was obtained. The lower and upper limits represented quenching times of 0.7 and 1.3 ms, respectively. Since the shortest incubation time, including the time for mixing, was about 1.5 ms, the shortest total reaction time of the phosphorylation did not exceed 3 ms.

The efficiency of the quenching in the dephosphorylation experiments was estimated by the addition of K^+ to the quenching solution of a phosphorylation experiment performed as described above. The concentration of K^+ added to the quenching solution corresponded to the final concentration obtained in the quenching solution in dephosphorylation experiments (i.e. about 3.8 mM K^+). The decrease of the phosphorylation level was less than 5%. This shows that essentially all of the dephosphorylation with K^+ occurred before the reaction mixture reached the quenching solution.

Isolation of the ^{32}P-labelled material

Within 1 min after quenching the precipitate was collected on a Millipore filter and rinsed with 70 ml of cold, 5% (w/w) $HClO_4$, containing 10 mM sodium orthophosphate. Each collecting-rinsing procedure was completed in 5 min. The filter with

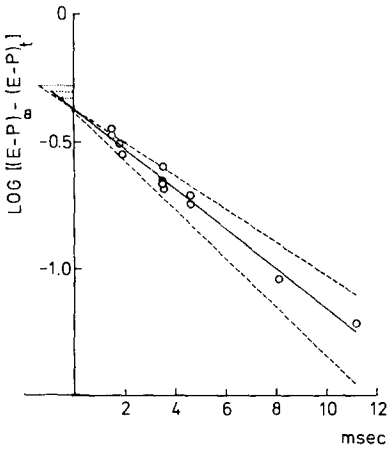

Fig. 2. Pseudo first-order plots of the rapid phosphorylation of microsomal ATPase. Rapid-mixing experiments were performed as described under Experimental Procedure. Phosphorylation was performed in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 100 μM [γ-^{32}P]ATP at pH 7.4 and 21 °C. $[E\text{–}P]_\infty$ represents the maximal amount of protein-bound acyl-[^{32}P]phosphate that could be obtained. $[E\text{–}P]_t$ represents the amount of acyl-[^{32}P]phosphate obtained at different times. $[E\text{–}P]_\infty$ was calculated from 10 phosphorylation experiments. A pseudo first-order plot corresponding to the mean value of $[E\text{–}P]_\infty = 0.499$ nmole · mg^{-1} of protein is shown. The curve is extrapolated to log ($[E\text{–}P]_\infty$ - $[E\text{–}P]_t$) at $[E\text{–}P]_t = 0$ to give the quenching time. Also shown are the plots corresponding to $[E\text{–}P]_\infty$ + 2 S.D. and $[E\text{–}P]_\infty$ − 2 S.D. to show the dependence of the quenching time on the value of $[E\text{–}P]_\infty$.

the rinsed precipitate was immediately transferred to a glass scintillation vial containing 4 ml of 0.1 M acetic acid at 21 °C, to which had been added 1 % sodium dodecylsulphate (pH 3.0). The filter was treated for 60 s with an MSE 150 W Ultrasonic Disintegrator, at setting Medium 5 with the 3/8 inch probe dipped 3 mm into the solution. The radioactive material dissolved in the acetic acid–sodium dodecylsulphate solution was analysed for protein-bound acyl-[^{32}P]phosphate and for total [^{32}P]-phosphate [3]. The remaining radioactivity in the filters was measured routinely by dissolving the filters in 1 ml of acetone in scintillation vials and measuring the Čerenkov radiation.

In the filtration procedure all protein was retained on the filter used (pore size 3 μm). Filters with a pore size of 0.45 μm gave the same results as did the 3-μm filters. No protein was detected in the filtrate by the method of Lowry et al., and neither could any ^{32}P-labelled protein be found in the filtrate by chromatography on Sephadex G-50 as described previously [3].

RESULTS

Assay of the phosphorylated enzyme

In order to allow a large number of rapid-mixing experiments within a limited period of time it was necessary to replace the time-consuming (2–3 h) Sephadex chromatography used in the previous studies [3] by the rapid (less than 10 min) filtration method described under Experimental Procedure. When pilot experiments were performed, the total ^{32}P-labelling of the precipitates was higher than was expected from the previous results (cf. [3]). This was particularly evident at higher con-

centrations of [γ-^{32}P]ATP. The precipitates, therefore, had to be selectively analysed for protein-bound acyl-[^{32}P]phosphate.

Protein-bound acyl-[^{32}P]phosphate was found to comprise about 70% of the total radioactivity of the washed precipitate obtained after incubation with 5 μM [γ-^{32}P]ATP, and about 55% with 25 μM and 100 μM [γ-^{32}P]ATP. When 10 mM KCl was present in the phosphorylation experiments even less (about 25%) of the total radioactivity displayed a stability property similar to that of acyl-[^{32}P]phosphate. The stability of the remaining radioactive material in acid and base was similar to that of [γ-^{32}P]ATP. The identity of this material with ATP was verified by high-voltage electrophoresis in 50 mM citric acid buffer (pH 3.5). That ATP remains bound to a preparation of (Na^+–K^+)-stimulated ATPase after treatment with acid is known also from experiments by others [14]. The importance of analysing the ^{32}P-labelled precipitate selectively for protein-bound acyl-[^{32}P]phosphate is thus apparent. It may be pointed out that it has not been investigated, in the present work, to what extent the [^{32}P]ATP binding detected represents a property of the native enzyme.

Dependence of the steady-state level of phosphorylation on the concentration of [γ-^{32}P]-ATP

In order to measure the maximal amount of $[E\text{–}P]_\infty$ that could be achieved under different conditions, the dependence of the steady-state level of phosphorylation on the concentration of [γ-^{32}P]ATP was investigated. At 3 mM $MgCl_2$, 120 mM NaCl and 5 μM [γ-^{32}P]ATP a steady-state level of acyl-[^{32}P]phosphate of about 0.3 nmole·mg^{-1} of protein was obtained (Fig. 3). However, at 100 μM [γ-^{32}P]ATP, the acyl-

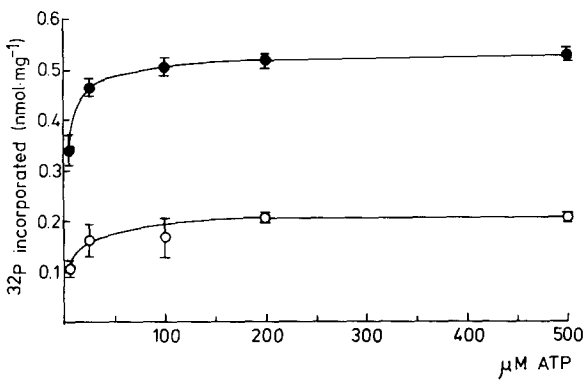

Fig. 3. Dependence of the steady-state level of phosphorylation on the concentration of [γ-^{32}P]ATP. Phosphorylation experiments were performed in the presence of 3 mM $MgCl_2$ and 120 mM NaCl (●—●), or 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl (○—○). The figures represent the amount of protein-bound acyl-[^{32}P]phosphate at steady state after an incubation time of about 80 ms. For further details, see Experimental Procedure.

[^{32}P]phosphate increased to about 0.5 nmole·mg^{-1} of protein. No increase in the amount of acyl-[^{32}P]phosphate was achieved at higher concentrations of [γ-^{32}P]ATP, i.e. 200 or 500 μM.

When 10 mM KCl was present in the incubation medium, the maximal amount of acyl-[^{32}P]phosphate obtained was about 40% of that when KCl was omitted, irre-

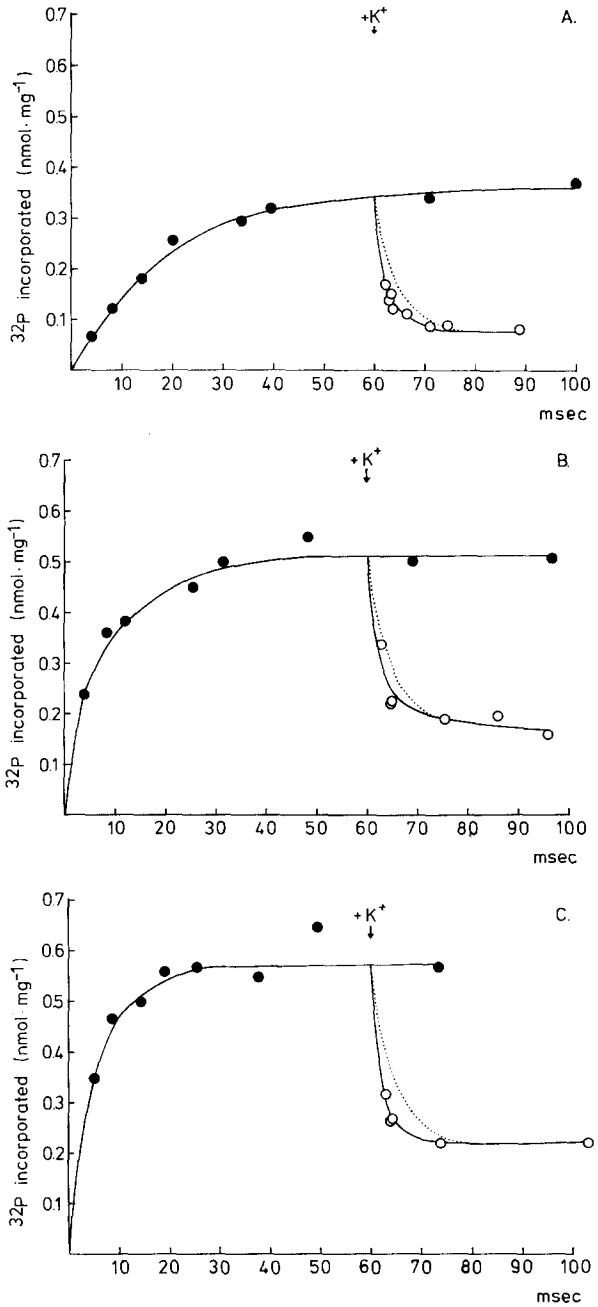

Fig. 4. Time dependence of phosphorylation and dephosphorylation. Rapid-mixing experiments were conducted as described under Experimental Procedure. Phosphorylation was performed in the presence of 3 mM $MgCl_2$, 120 mM NaCl and (A) 5 μM [γ-^{32}P]ATP, (B) 25 μM [γ-^{32}P]ATP and (C) 100 μM [γ-^{32}P]ATP. Dephosphorylation was performed by adding KCl to a final concentration of 10 mM after 60–70 ms of phosphorylation. Filled circles represent phosphorylation and open circles represent the dephosphorylation experiments. The dotted line represents a theoretical first-order dephosphorylation with a half-life time of 3 ms.

spective of the concentration of [γ-^{32}P]ATP. It is concluded that the maximal phosphorylation is already obtained at about 100 μM ATP irrespective of whether K^+ is present or not.

Time dependence of phosphorylation and dephosphorylation

In the presence of 3 mM $MgCl_2$ and 120 mM NaCl, the half-life time of the Na^+-stimulated phosphorylation was about 15 ms at 5 μM [γ-^{32}P]ATP (Fig. 4A). This corresponds to a rate constant of 2760 min^{-1}, which is calculated from a pseudo first-order plot similar to that shown in Fig. 2. At this calculation the concentration of [γ-^{32}]ATP is considered to be constant. The rate constant obtained at 5 μM [γ-^{32}P]ATP is in accordance with previous findings [3]. With 25 μM [γ-^{32}P]ATP, the half-life time was about 5 ms, corresponding to a pseudo first-order rate constant of about 8400 min^{-1}. At higher concentrations of [γ-^{32}P]ATP, i.e. 38, 50 (data not shown) and 100 μM (Figs 2 and 4C), the half-life time was only slightly decreased. Depending on the deviation (2 S.D. = 5%, n = 10) of $[E\text{–}P]_\infty$, the pseudo first-order rate constant of the phosphorylation at 3 mM $MgCl_2$, 120 mM NaCl and 100 μM [γ-^{32}P]ATP ranged from about 9000 min^{-1} to 13 000 min^{-1}.

When KCl was added, the final concentration being 10 mM, the protein-bound acyl-[^{32}P]phosphate was split at a rate at least as rapid as the rate of formation (Fig. 4). The apparent half-life of this K^+-dependent dephosphorylation did not exceed 3 ms, irrespective of the concentration of [γ-^{32}P]ATP used. This corresponds to an apparent first-order rate constant of not less than 14 000 min^{-1}.

Rate of hydrolysis of ATP

In order to compare the rates of the partial reactions with the rate of the overall reaction, the hydrolysis of ATP at 21 °C and different concentrations of ATP and different ionic environments were studied (Table I). In the presence of $MgCl_2$ some ATPase activity was always obtained, even in the absence of monovalent cations. The

TABLE I

THE EFFECT OF IONS, OUABAIN AND CONCENTRATION OF [^{32}P]ATP ON THE HYDROLYSIS OF [^{32}P]ATP BY THE MICROSOMAL ATPase

The ATPase activity was assayed as described under Experimental Procedure. The metal ions and ouabain were added as indicated in the table and at concentrations as indicated in the text. In those experiments which included ouabain, the enzyme was preincubated at pH 7.4 for 1 h at 21 °C before addition of [^{32}P]ATP.

Ions and ouabain	Hydrolysis of [^{32}P]ATP (nmole·mg^{-1}·min^{-1})			
	[^{32}P]ATP concn 5 μM	25 μM	100 μM	1 mM
Mg^{2+}	15	27	60	78
Mg^{2+}, ouabain	14	28	58	81
Mg^{2+}, Na^+	41	64	90	132
Mg^{2+}, Na^+, ouabain	16	29	58	85
Mg^{2+}, Na^+, K^+	45	140	342	698
Mg^{2+}, Na^+, K^+, ouabain	28	29	68	97

rate of hydrolysis was increased by the addition of 120 mM NaCl. A further increase was obtained when 10 mM KCl was also present. The (Na^+–K^+)-stimulated hydrolysis of ATP with 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl at 5, 25, 100 and 1000 μM ATP amounted to 30, 113, 282 and 620 nmoles·mg^{-1}·min^{-1}, respectively. The K^+-stimulated part of this hydrolysis was highest at higher concentrations of ATP, while at 5 μM ATP the addition of K^+ gave only a slight increase of the hydrolysis. This finding is in accordance with previous results [5–7]. In the presence of 0.1 mM ouabain the (Na^+–K^+)-stimulated ATPase activity was decreased approximately to that obtained with only $MgCl_2$ present in the incubation medium.

DISCUSSION

In this study both the rate of the Na^+-stimulated phosphorylation and the rate of the K^+-stimulated dephosphorylation were compared with the (Na^+–K^+)-stimulated hydrolysis of ATP at different concentrations of ATP. Rate constants of the initial phosphorylation were calculated from pseudo-first order plots as described in Fig. 2. From ADP/ATP exchange experiments by others [15, 16], it is evident that the rate of a backward reaction between ADP and the phosphoenzyme is very small with the conditions used in the present study. The method of estimation of the pseudo first-order rate constant thus seems to be justified.

The results of phosphorylation experiments at 5 μM [γ-^{32}P]ATP confirm the previous data [3]. At 25 μM [γ-^{32}P]ATP, 3 mM $MgCl_2$ and 120 mM NaCl, half-maximal phosphorylation was reached in about 5 ms. This corresponds to a pseudo first-order rate constant of about 8400 min^{-1}. However, at increased concentrations of [γ-^{32}P]ATP there was not a proportional increase of the pseudo first-order rate constant as would have been expected if the reaction is second order with respect to ATP and enzyme. The calculated pseudo first-order rate constant was about 11 000 min^{-1} at 100 μM [γ-^{32}P]ATP. Higher concentrations seemed to give no appreciable increase of the rate constant. One possible explanation of these data would be the existence of an enzyme–ATP complex, preceding the formation of the phosphoenzyme, as can be inferred from reaction models presented by others [14, 17, 18]. At high concentrations of ATP, the formation of this complex may be very rapid. It is, therefore, suggested that, at high concentrations of ATP, the rate-limiting step of the Na^+-stimulated phosphorylation, as studied in the present work, is represented by the reaction E–ATP $\rightarrow$ E–P + ADP. In such a case, the first-order rate constant of this step would have been approached by the apparent first-order constant obtained in the present work at 100 μM and higher concentrations of [γ-^{32}P]ATP.

On measuring the K_m for ATP in the (Na^+–K^+)-stimulated ATPase reaction, Kanazawa et al. [4] found a value of 181 μM. Similar results have been reported by others [6, 19], and can also be deduced from Table I of the present study. Thus the concentration of ATP at which maximal velocity of the phosphorylation reaction is approached, i.e. 100 μM ATP, is less than half-saturating with respect to the (Na^+–K^+)-stimulated hydrolysis of ATP. The present results, therefore, support previous suggestions [1, 4, 20] that ATP, in addition to its role as a substrate, has a function of controlling a rate-limiting step of the reaction sequence. However, our present data do not permit the identification of such a step.

Table II summarizes the present results of the partial reactions of (Na^+–K^+)-

TABLE II

PARTIAL REACTIONS OF (Na^+–K^+)-STIMULATED ATPase AND (Na^+–K^+)-STIMULATED HYDROLYSIS OF ATP AT 21 °C, 3 mM $MgCl_2$, 120 mM NaCl, 10 mM KCl AND 100 μM ATP

For details see Discussion.

	k (min^{-1})	v ($nmoles \cdot mg^{-1} \cdot min^{-1}$)
Phosphorylation (Na^+)	11 000	?
Dephosphorylation (K^+)	14 000	2800
ATP hydrolysis (Na^+, K^+)		280

stimulated ATPase and the (Na^+–K^+)-stimulated hydrolysis of ATP. The results are discussed below.

At 100 μM ATP the rate of (Na^+–K^+)-stimulated hydrolysis of ATP was about 280 $nmoles \cdot mg^{-1} \cdot min^{-1}$ in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl. Since the steady-state concentration of the enzyme form preceding the phosphorylation step was unknown the rate of phosphorylation at steady-state could not be estimated. However, the rate constant of the phosphorylation reaction was about 11 000 min^{-1}. This rate constant would allow the phosphorylation reaction to be an intermediate reaction of the (Na^+–K^+)-stimulated hydrolysis of ATP if, at steady state, as little as 0.025 $nmole \cdot mg^{-1}$ of protein was represented by the enzyme form preceding the phosphorylation step.

Fig. 4 shows that there is a very rapid decrease of the ^{32}P-label of the ATPase preparation on the addition of K^+. Since the efficiency of the quenching, in the presence of K^+, is apparent from control experiments described above, it is concluded that the results are consistent with very rapid, K^+-stimulated dephosphorylation.

The initial parts of the dephosphorylation curves represent incubation times near the limit of the time resolution of the rapid-mixing apparatus. It was, therefore, not possible to make precise calculations of the rate constant of the K^+-stimulated dephosphorylation. However, it was possible to define a lower limit of the constant by the following procedure.

As is evident from Fig. 4, the K^+-stimulated dephosphorylation was more rapid than a theoretical, first-order process with a half-life of 3 ms. However, the incubation times of the experimental points of Fig. 4 do not include the quenching time. This has been estimated to be about 1 ms (Fig. 2) in the absence of K^+ and is apparently not changed in its presence. Even if a time of 2 ms is added to the incubation times of the dephosphorylation experiments of Fig. 4, the experimental curve will still be drawn to the left of the theoretical curve. It, therefore, seems justifiable to state that the half-life of the K^+-dependent dephosphorylation does not exceed 3 ms. The apparent first-order rate constant of the dephosphorylation would, therefore, be at least 14 000 min^{-1}. This requires a steady-state concentration of the K^+-sensitive phosphoenzyme of not more than 0.02 $nmole \cdot mg^{-1}$ to account for the steady-state rate of the (Na^+–K^+)-stimulated hydrolysis of ATP at 3 mM $MgCl_2$, 120 mM NaCl, 10 mM KCl and 100 μM ATP.

This is quite an interesting result. The measured steady-state concentration of the phosphoenzyme under the same conditions is 10 times higher, i.e. 0.2 $nmole \cdot mg^{-1}$.

This suggests, though it does not prove, the existence of at least two kinetically different forms of the phosphoenzyme, the more abundant form being insensitive to K^+. Such a possibility is in accord with the current hypothesis of two phosphoenzyme forms, E_1–P and E_2–P, based on inhibition studies of the (Na^+–K^+)-stimulated ATPase by *N*-ethylmaleimide or by oligomycin and studies of the ADP/ATP exchange reaction [15, 21]. Rapid-mixing techniques of the kind described here may, therefore, offer a means of studying more directly the possible existence of multiple phosphoenzyme forms than has been possible hitherto.

One prerequisite for the rapid, K^+-stimulated decrease of phosphoenzyme to represent the K^+-stimulated step of the (Na^+–K^+)-stimulated hydrolysis of ATP is that a K^+-stimulated release of [^{32}P]orthophosphate occurs concomitantly at the same rate. Preliminary investigations of such an "initial burst" of [^{32}P]orthophosphate have shown that this type of measurement requires additional modifications of the rapid-mixing apparatus.

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