Biochimica et Biophysica Acta, 597 (1980) 100—111

BBA 78659

STUDIES ON ($Na^+ + K^+$)-ACTIVATED ATPase

XLIV. SINGLE PHOSPHATE INCORPORATION DURING DUAL PHOSPHORYLATION BY INORGANIC PHOSPHATE AND ADENOSINE TRIPHOSPHATE

F.M.A.H. SCHUURMANS STEKHOVEN, H.G.P. SWARTS, J.J.H.H.M. DE PONT and S.L. BONTING

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received May 9th, 1979)

Key words: ($Na^+ + K^+$)-ATPase; Phosphate incorporation; Inorganic phosphate; ATP

Summary

($Na^+ + K^+$)-ATPase can be phosphorylated by its substrate ATP as well as by its product inorganic phosphate. The maximal capacity for phosphorylation by either of these two substances is one mol phosphate per mol enzyme. In order to investigate whether the enzyme molecule possesses only one phosphorylation site common to ATP and P_i, or two phosphorylation sites, one for ATP and one for P_i, dual phosphorylation of the enzyme has been carried out. Under conditions, which are maximally favourable for each type of phosphorylation, successive phosphorylation by P_i and ATP leads to a maximal incorporation of only one mol phosphate per mol enzyme. The phosphorylation capacity for ATP decreases by the same amount as the P_i-phosphorylation level increases, without an effect on the apparent affinity for ATP.

The results can be explained by assuming either a single common phosphorylation site for P_i and ATP, or a conformational change of the enzyme following phosphorylation by P_i, which excludes phosphorylation by ATP.

Introduction

The ($Na^+ + K^+$)-stimulated ATPase system, which is responsible for active transport of Na^+ and K^+ across the plasma membrane, is phosphorylated by ATP in the presence of Na^+ and Mg^{2+} [1,2]. The resulting phosphoenzyme is sensitive to K^+-stimulated hydrolysis, and the rate of the dephosphorylation reaction exceeds that of the overall ($Na^+ + K^+$)-ATPase reaction [3,4]. The ATP-phosphorylation capacity is also proportional to the activity of the ($Na^+ +$

K^+)-ATPase preparation [1,5,6]. Hence, this phosphoenzyme can be considered to be an intermediate in the forward reaction sequence.

As early as 1966, it was recognized that the ATP-phosphorylated enzyme would undergo a conformational change for transport of Na^+ [7], later designated as the $E_1 \sim P \rightleftharpoons E_2$-P transition [8]. It is now fairly well documented, e.g. via inhibition by oligomycin, that this transition is involved in the transport of sodium ions via uncoupled Na^+ extrusion [9] and via exchange of intracellular versus extracellular Na^+ by erythrocytes [10].

The enzyme can also be phosphorylated by inorganic phosphate (P_i) in the presence of Mg^{2+}, but this phosphorylation is inhibited by Na^+, in contrast to that by ATP [11]. The reaction appears to be involved in the outward transport of K^+ as it occurs in the exchange of intracellular against extracellular K^+ [12]. The inward K^+ transport would involve a subsequent $E_2 \cdot K \rightarrow E_1 \cdot K$ conformational transition, caused by ATP binding without phosphorylation [13,14].

In previous studies it has been found that the ATP- and P_i-phosphorylated intermediates are chemically indistinguishable [15—17], that both are located in a 100 000-dalton subunit of the enzyme and that the maximal capacities for phosphorylation by either ATP or P_i are equal [18] and do not exceed one mol per mol of enzyme [19,20]. However, in addition to a high-affinity ATP binding and phosphorylation site (K_m = 0.2—0.5 μM, Refs. 1,21) involved in outward Na^+ transport [22], the enzyme also contains a low-affinity ATP binding site (K_m = 0.1—0.5 mM, Refs. 22,23) involved in inward K^+ transport [12]. The high-affinity ATP site would be involved in hydrolysis of nucleotides, whereas the low-affinity ATP site would be involved in hydrolysis of phosphatase substrates like *p*-nitrophenyl phosphate [23]. Inorganic phosphate competitively inhibits *p*-nitrophenyl phosphatase activity [24] and exchanges with enzyme-bound phosphate derived from *p*-nitrophenyl phosphate [25].

These data could suggest that while ATP would phosphorylate the enzyme via its high-affinity binding site, P_i might phosphorylate the site with low affinity for ATP. Under appropriate conditions it might then be possible to obtain a double phosphorylated enzyme via consecutive phosphorylation by P_i and ATP. This possibility has been investigated in the present study.

Materials and Methods

Preparation of (Na^+ + K^+)-ATPase

(Na^+ + K^+)-ATPase has been prepared and purified from rabbit kidney outer medulla microsomes according to Jørgensen [26] with slight modification: 340 mg microsomes were incubated for 1 h at 20° C in a medium containing 0.58 mg/ml sodium dodecyl sulfate (SDS) in 25 mM imidazole-HCl (pH 7.5), 3 mM ATP and 2 mM EDTA (1.4 mg protein/ml). The suspension was then centrifuged at 200 000 × *g* for 30 min at 5° C. The pellet was homogenized in 50 ml of the same medium lacking SDS. This homogenate was centrifuged on a linear gradient between 20 and 45% sucrose. Removal of ATP, subsequent washing and storage of this purified preparation have been described elsewhere [27]. The specific (Na^+ + K^+)-ATPase activity of these preparations, determined

by the non-radioactive assay method [27], ranged from 1.1 to 2.3 mmol hydrolyzed ATP/mg protein per h.

Phosphorylation with ATP

Phosphorylation was carried out with 20 μM [γ-^{32}P]ATP (The Radiochemical Centre, Amersham, U.K.; specific radioactivity was adjusted with non-radioactive ATP to 20—50 Ci/mol) in a buffer medium (0.1 ml) containing 50 mM NaCl, 5 mM $MgCl_2$, 50 mM imidazole-HCl (pH 7.0) and 0.25 mg/ml ($Na^+ + K^+$)-ATPase protein. The reaction was started by rapid mixing of equal volumes of medium containing the enzyme with medium containing the ATP at 0°C and was stopped after 3 s by addition of 2 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 μm pore width Selectron-filter (Sleicher and Schüll, Dassel, F.R.G.), which was then washed three times with stopping solution. Incorporated ^{32}P was determined by liquid scintillation counting. For blank values, the stopping solution was mixed with the [γ-^{32}P]ATP prior to addition of the enzyme.

Phosphorylation with inorganic phosphate

Phosphorylation with $^{32}P_i$ or $^{33}P_i$ (1 mM) was carried out at 0° C for various periods in a medium containing 5 mM $MgCl_2$ and 50 mM imidazole-HCl (pH 7.0) at 0.25—0.5 mg protein/ml. Carrier free $^{32}P_i$ or $^{33}P_i$ (NEN Chemicals GmbH, Dreieichenhain, F.R.G.) was freed for this purpose from radioactive contamination as previously described [27] and mixed with non-radioactive imidazole phosphate (pH 7.0) to give a specific radioactivity of 20—50 Ci/mol. The reaction was started and stopped as described for phosphorylation by [γ-^{32}P]ATP. The denatured phosphoprotein was collected, washed and the incorporated P_i determined.

Dual phosphorylation with P_i and ATP

Initial phosphorylation with $^{33}P_i$ was carried out as described in the preceding paragraph in a volume of 0.1 ml. For subsequent phosphorylation by ATP 50 μl was added of a solution, containing 60 μM [γ-^{32}P]ATP, 150 mM NaCl, 5 mM $MgCl_2$, 50 mM imidazole-HCl (pH 7.0) and 1 mM $^{33}P_i$ of the same specific radioactivity as used for the initial phosphorylation by P_i. The presence of $^{33}P_i$ during phosphorylation by ATP, which keeps concentration and specific radioactivity of $^{33}P_i$ constant throughout the experiment, is needed to prevent dissociation of the ^{33}P-phosphorylated product. It did not further increase the ^{32}P-phosphorylation level of the enzyme.

The reaction was stopped 3—15 s after addition of ATP by adding 2 ml 5% trichloroacetic acid containing 100 mM phosphoric acid. After filtration and washing of the phosphoprotein, incorporated ^{32}P and ^{33}P was determined by means of a liquid scintillation counter, which was properly programmed for double label analysis. The radioactivity of each isotope was calculated by means of the external standard ratio method.

Results

Optimal conditions for phosphorylation by P_i, ATP, and P_i + ATP

Effect of pH. Fig. 1 shows that the optimal pH for phosphorylation by P_i

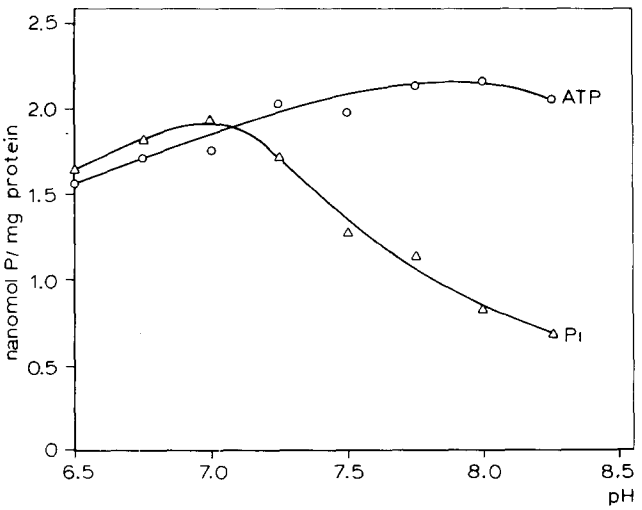

Fig. 1. Phosphorylation by P_i and ATP in dependence on pH. Phosphorylation of $(Na^+ + K^+)$-ATPase (0.25 mg protein/ml) by ATP (22.7 μM) in the presence of 100 mM NaCl and 4 mM $MgCl_2$ was allowed to proceed for 3 s, phosphorylation by P_i in the presence of 5 mM $MgCl_2$ for 4 min.

(pH 7.0) is one unit below that for phosphorylation by ATP (pH 8.0). This difference suggests the existence of different optimal conformations for each phosphorylation. The best compromise for dual phosphorylation would lie between pH 7.0 and 7.25. At pH 7.0 phosphorylations by P_i and ATP are 100% and 88% of the maximum, respectively, while at pH 7.25 they are 90% and 95%, respectively. Below pH 7.0 both phosphorylations decline, whereas above pH 7.25 the phosphorylation level for P_i declines more than the phosphorylation level for ATP rises. Hence a pH of 7.0 has been chosen for dual phosphorylation.

Reaction time. Phosphorylation by ATP is a rapid process, which under our experimental conditions is complete within 1 s, even at 0°C, whereas the ATP phosphorylation level remains stable at that temperature for at least 15 s (Ref. 28, pp. 23,24). Therefore, routinely an ATP phosphorylation time of 3 s has been chosen. Phosphorylation by P_i in the absence of ouabain at 0°C, on the other hand, is a slow process, which at this temperature takes 15—30 min for completion (Fig. 2). Consequently, when it was necessary to reach maximal phosphorylation levels by this substrate, 30 min was allowed for equilibration.

Influence of Na^+. Phosphorylation by ATP requires Na^+ [1], whereas phosphorylation by P_i is inhibited by this cation [11]. Fig. 3 shows the effect of Na^+ on the two types of phosphorylation. While phosphorylation by ATP (3 s at 0°C) becomes maximal in the presence of 50 mM Na^+ ($K_{0.5}$ = 5 mM), phosphorylation by P_i (4 min at 0°C) is 90%-inhibited at this Na^+ concentration (I_{50} = 5 mM). As will be shown below, inhibition by Na^+ of phosphorylation by P_i at 0°C is a relatively slow process, and its onset at 50 mM Na^+ becomes apparent only after 30—40 s. This delay is long as compared to the 3-s period, sufficient for completion of phosphorylation by ATP, but short as compared to the 30 min required for maximal phosphorylation by P_i. Hence, in dual phosphorylation experiments we have chosen to phosphorylate

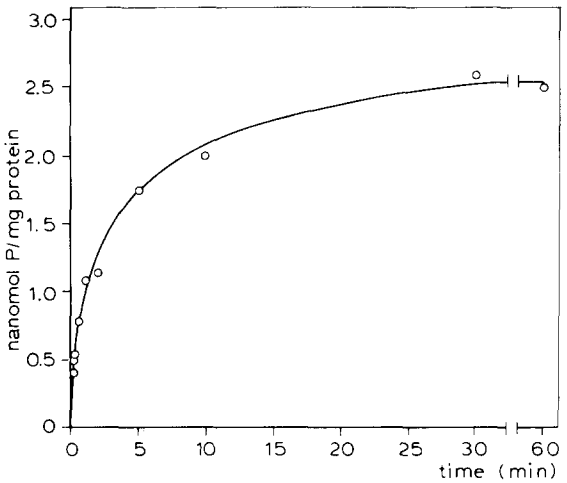

Fig. 2. Time course of phosphorylation by P_i. $(Na^+ + K^+)$-ATPase (0.31 mg protein/ml) was phosphorylated by P_i at 0°C for 3 s—60 min.

first with $^{33}P_i$ and to allow sufficient time (30 min) for maximal phosphorylation (in the absence of Na^+) before subjecting the preparation to a 3—15 s period of phosphorylation by ATP in the presence of 50 mM Na^+. This Na^+ concentration should not inhibit P_i phosphorylation within this short time interval.

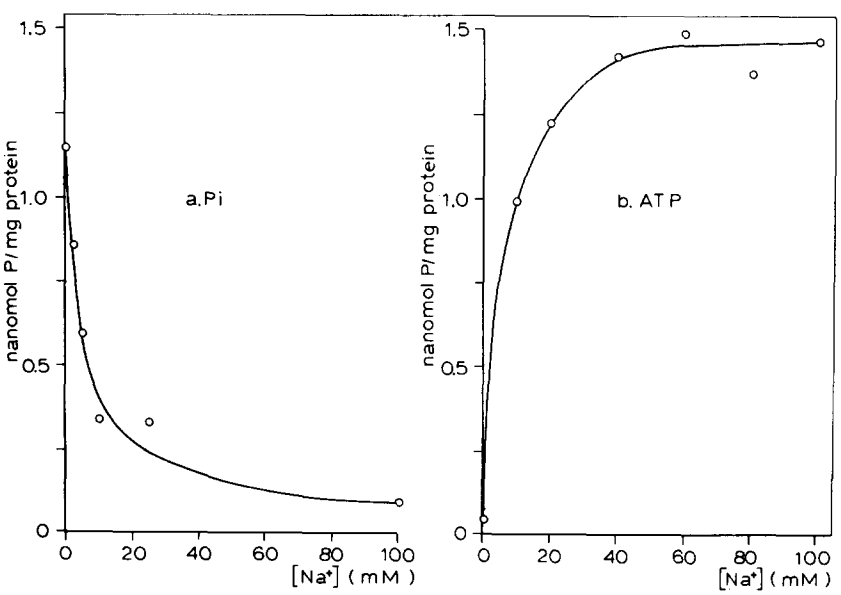

Fig. 3. Na^+ dependence of phosphorylation by P_i (a) and ATP (b). (a): $(Na^+ + K^+)$-ATPase, preequilibrated with $MgCl_2$ was mixed with NaCl + P_i. Final concentrations in the mixture were 0.52 mg protein/ml, 5 mM Mg^{2+}, 1 mM P_i and Na^+ in the indicated concentrations. The reaction at 0°C was stopped after 4 min. (b): $(Na^+ + K^+)$-ATPase, preequilibrated with NaCl plus $MgCl_2$ was mixed with ATP. Final concentrations were 0.25 mg protein/ml, 4 mM Mg^{2+}, 19 μM ATP and Na^+ in the indicated concentrations. The reaction at 0°C was stopped after 3 s.

Influence of Mg^{2+}. The K_m for Mg^{2+} for phosphorylation by P_i is 0.8 mM at 0°C. Phosphorylation by ATP is maximal at or below 0.2 mM Mg^{2+}. We have, therefore, chosen a concentration of 5 mM for our assays, which gives nearly (90%) maximal phosphorylation by P_i and maximal phosphorylation by ATP.

Substrate concentrations. For phosphorylation by P_i at 0°C, 1 mM is saturating (K_m = 70 μM) and for phosphorylation by ATP, 10 μM (K_m = 0.05—0.25 μM). For dual phosphorylation, we have routinely used 1 mM P_i and 20 μM ATP sequentially.

Effect of temperature. The maximal phosphorylation level at 0°C for P_i usually amounts to 50—70% of the level obtained with ATP in the absence of P_i (Fig. 6 and Table I). After treatment with ouabain prior to phosphorylation by P_i, equal phosphorylation capacities for P_i and ATP (without ouabain) are found at 0°C [18], which is probably due to stabilization of the phosphorylated intermediate by ouabain. However, ouabain cannot be applied in dual phosphorylation experiments, since it inhibits binding of ATP [29] and hence phosphorylation by this substrate [1].

The difference in phosphorylation capacities for ATP and P_i, apparent at 0°C, can be abolished by raising the temperature to 35°C, which increases both the rate and the final level of P_i phosphorylation (Fig. 4). The ATP phosphorylation levels in the absence of P_i are the same at both temperatures [28]. Unfortunately, at higher temperature the rate of inhibition by Na^+ on phosphorylation by P_i is also increased. The delay in inhibition by Na^+ is reduced from 30—40 s at 0°C, to 10 s at 5°C, and to 3 s at 10°C (Fig. 5), and even to less at higher temperatures. The relatively long delay of inhibition by

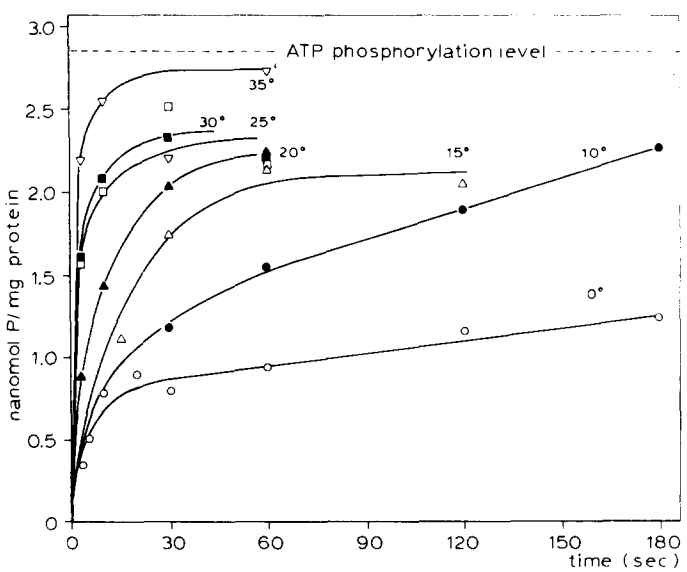

Fig. 4. Temperature dependence of P_i-phosphorylation. ($Na^+ + K^+$)-ATPase (0.29 mg protein/ml) was phosphorylated with P_i for the indicated times and at the indicated temperatures. The dashed line indicates the 3 s ATP phosphorylation level at 0°C.

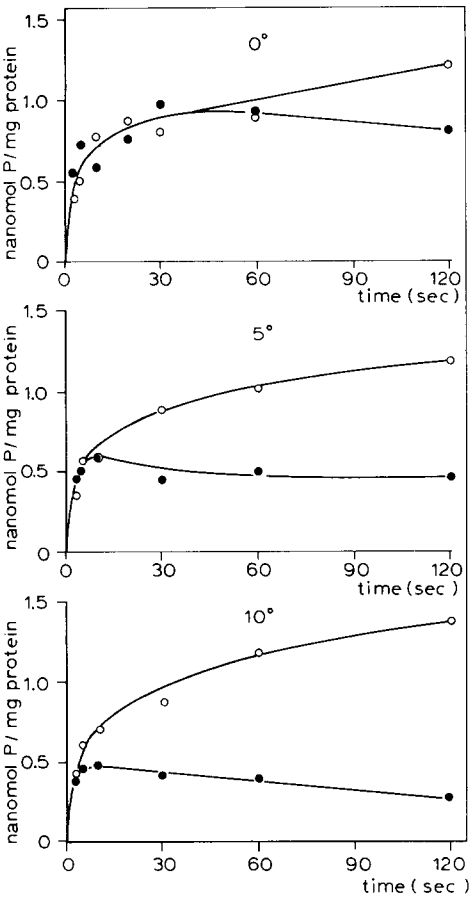

Fig. 5. Time course of the effect of Na^+ on phosphorylation by P_i. Shown is phosphorylation at 0, 5 and 10°C of (Na^+ + K^+)-ATPase (0.27 mg protein/ml in top figure, 0.25 mg/ml in middle and bottom figure), preequilibrated with Mg^{2+} and added at zero time to P_i plus Na^+ (●) or to P_i without Na^+ (○). The Na^+ concentration was 50 mM for the experiment at 0°C and 25 mM for the experiments at 5 and 10°C.

Na^+ on phosphorylation by P_i at 0°C is favourable for achieving phosphorylation levels unaffected by inhibition by the Na^+ present during ATP phosphorylation. For this reason we have chosen 0°C as the temperature for sequential phosphorylation by P_i and ATP.

Dual phosphorylation by P_i and ATP

In the experiment, depicted in Fig. 6, phosphorylation by P_i is allowed to proceed for 30 min. Then Na^+ or Na^+ + ATP is added and the P_i and ATP-phosphorylation levels are traced for a subsequent 15 s and compared with the ATP-phosphorylation level in the absence of P_i. Fig. 6 shows that Na^+ or Na^+ + ATP do not significantly affect the P_i-phosphorylation level within the time course shown. This is predictable for Na^+ from Fig. 5 (top), where inhibition became apparent only after 30—40 s. On the other hand, the ATP-phosphorylation level is 50% lower than that obtained with ATP without prior phosphorylation with P_i. The sum of the phosphorylation levels for P_i

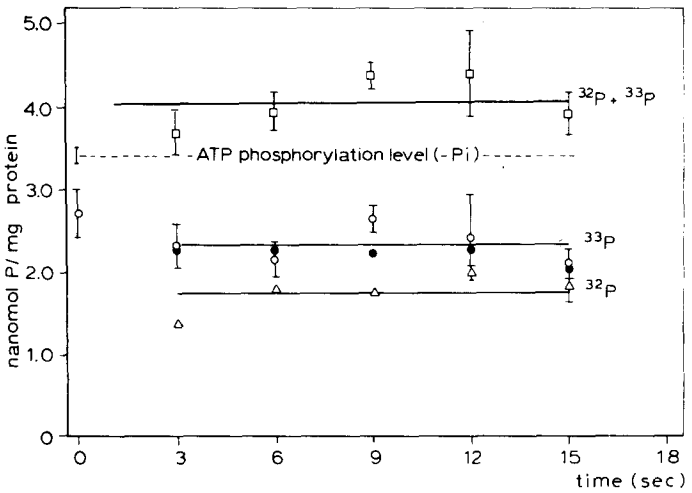

Fig. 6. Dual phosphorylation assay with $^{33}P_i$ and [γ-^{32}P]ATP. Following phosphorylation of ($Na^+ + K^+$)-ATPase (0.25 mg/ml) with P_i for 30 min, the assay medium was mixed at zero time with $Na^+ + {}^{33}P_i$ + [γ-^{32}P]ATP or $Na^+ + {}^{33}P_i$ without ATP. The phosphorylation level for P_i in the presence (○) or absence (●) of ATP, as well as the ATP-phosphorylation level in the presence of P_i (△) was traced for another 15 s. The dashed line indicates the ATP-phosphorylation level in the absence of P_i; (□) indicates the sum of (○) + (△), i.e. the sum of the P_i- and ATP-phosphorylation levels. All points are averages for triplicate determinations with standard errors of the mean. The standard errors for P_i phosphorylation (●) in the absence of ATP have been omitted, but are commensurate to those for P_i phosphorylation following addition of ATP (○).

and ATP in each other's presence amounts to 4 nmol/mg protein, or 1 mol phosphate/250 000 dalton ($Na^+ + K^+$)-ATPase. This sum is not significantly different from the ATP-phosphorylation level in the absence of P_i (Fig. 6).

This point has been further checked by decreasing the P_i-phosphorylation level through shortening the time of reaction with P_i (Table I). The sum of phosphorylation by P_i and ATP remains constant and equal to the phosphorylation level for ATP without P_i (time, 0 min in Table I). In other words, the ATP-phosphorylation level is reduced to the same extent as to which P_i

TABLE I

DUAL PHOSPHORYLATION OF ($Na^+ + K^+$)-ATPase BY $^{33}P_i$ AND [γ-^{32}P]ATP

($Na^+ + K^+$)-ATPase, at a concentration varying from 0.025—0.25 mg/ml in different experiments, was first phosphorylated for the indicated times with 1 mM $^{33}P_i$ and subsequently for 3 s with 0.25—100 μM [γ-^{32}P]ATP. The ATP-phosphorylation levels are maxima, determined from Lineweaver-Burk plots; the maximum at zero time was determined in the absence of P_i. The experimental data represent averages of 2—3 determinations with S.E.

Time (min)	(1) P_i phosphorylation (nmol P/mg protein)	(2) Max. ATP phosphorylation (nmol P/mg protein)	(1 + 2) Total phosphorylation (nmol P/mg protein)	n
0	—	3.08 ± 0.09	3.08 ± 0.09	3
0.5	0.75 ± 0.10	2.34 ± 0.14	3.09 ± 0.17	2
2	0.99 ± 0.04	2.28 ± 0.18	3.27 ± 0.18	3
5	1.16 ± 0.02	1.97 ± 0.06	3.13 ± 0.06	3
30	1.59 ± 0.17	1.35 ± 0.05	2.94 ± 0.18	3

has phosphorylated the enzyme. The maximal phosphorylation capacity is in several experiments less than 4 nmol/mg protein, i.e., 1 mol phosphate per mol enzyme of 250 000 dalton. This does not necessarily reflect a higher molecular weight than 250 000, nor a low protein purity of the enzyme preparation as judged by SDS gel electrophoresis, but rather a variable, partial inactivation during purification. This is also shown by the variability of the specific (Na^+ + K^+)-ATPase activity (see Materials and Methods).

We have also checked whether the reduction in the ATP phosphorylation level, due to P_i phosphorylation, is caused by a decreased affinity for ATP or is due to exclusion of the phosphorylation site without change in K_m. To this end, the enzyme has been phosphorylated with 1 mM P_i for 0.5—30 min and subsequently for 3 s with ATP in variable concentration. The dependence of the ATP-phosphorylation levels on the ATP concentration is presented in a Scatchard plot (Fig. 7). It shows a series of almost parallel lines with slopes representing the reciprocal of the K_m and with intercepts on the abscissa giving the maximal ATP-phosphorylation levels. The maximal ATP-phosphorylation levels decrease, whereas the K_m values remain virtually constant, suggesting total exclusion of the enzyme molecules that have already been phosphorylated by P_i from subsequent phosphorylation by ATP. A non-competitive inhibition of the Na^+-dependent ATPase reaction by P_i, which is antagonized by increasing Na^+ concentration, has been observed by Robinson [24]. This inhibition is probably also due to phosphorylation by P_i with proportional decrease of the ATP phosphorylation, since we find that Na^+ counteracts the phosphorylation by P_i (Fig. 3).

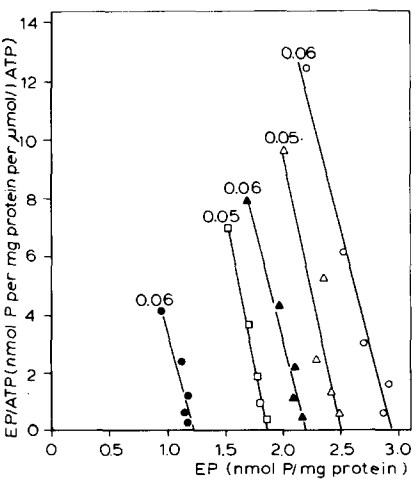

Fig. 7. Effect of prior phosphorylation by P_i on capacity and affinity of phosphorylation by ATP. The enzyme (25 μg protein/ml) was first phosphorylated with 1 mM P_i for 0.5 min (△), 2 min (▲), 5 min (□) and 30 min (●). The fifth line (○) represents a preparation not treated with P_i. The preparations were then phosphorylated with [γ-^{32}P]ATP (0.25—5 μM) for 3 s. ATP-phosphorylated enzyme levels (EP) are shown as a Scatchard plot. The ATP concentrations have been corrected for utilization (0.6—30%) through phosphorylation. The lines through the experimental points are calculated according to the least squares method. The value above each line indicates the K_m value (μM) for ATP phosphorylation, determined from the slope.

Discussion

The sum of sequential phosphorylation by P_i and ATP does not exceed the maximal phosphorylation level by ATP alone, and does not exceed the capacity of one mol phosphate incorporated per mol of the enzyme. The 1 : 1 replacement of ATP-phosphorylated intermediate by P_i-phosphorylated intermediate is not due to a diminished affinity for ATP, since the K_m value for ATP remains unchanged during phosphorylation by P_i. This strongly suggests that there exists only one identical phosphorylation site for ATP and P_i per two 100 000 dalton subunits present in the enzyme molecule [19,20]. This conclusion is supported by the evidence for physicochemical identity of the phosphorylation centres [15—17] and by the non-competitive product inhibition by P_i on ATP in the Na^+-dependent ATPase reaction [24]. Thus the possibility, mentioned in the Introduction, that P_i would phosphorylate a low-affinity ATP binding site, distinct from a high-affinity binding and phosphorylation site for ATP, is at first glance not supported by our findings. One would then expect up to two mol phosphate incorporated during dual phosphorylation.

There is, however, another possible explanation for our finding of a maximal phosphate incorporation of one mol per mol enzyme during dual, sequential phosphorylation by P_i and ATP. Recent experiments on the binding of the non-phosphorylating ATP analogue AMP-adenylyl imidodiphosphate have shown that P_i-phosphorylation also causes an equimolar decrease of the binding of this nucleotide (Schuurmans Stekhoven et al., to be published). This leads us to the conclusion that phosphorylation by P_i changes the enzyme from a conformation with high affinity for ATP to one with low affinity for ATP. In the latter conformation phosphate must probably first dissociate from the enzyme before ATP can bind.

Evidence that a conformational change is involved in the process, derives from the finding that P_i-phosphorylation is driven by a change in entropy [18]. There are some additional arguments for assuming different conformations of the enzyme, which are optimal for phosphorylation by ATP or P_i. The two phosphorylations have different pH optima (Fig. 1), they are conversely affected by Na^+ ($K_{0.5}$ for Na^+ in the ATP-dependent process is 5 mM, equal to the I_{50} for Na^+ in the P_i-dependent process, Fig. 3), and the optimal Mg^{2+} concentrations differ. Hence, the conformation required for phosphorylation by ATP and that obtained upon phosphorylation by P_i are to all probability mutually exclusive. This also limits the sum of their phosphorylations to one mol per mol of enzyme, even in the presence of two different, but chemically identical phosphorylation sites. The location of these two sites might, for instance, be spatially separated on the two 100 000 dalton subunits present in the enzyme molecule, one on each subunit.

Post et al. [11] have also described a decreased ATP phosphorylation upon prior or subsequent phosphorylation with P_i. This would be due to transformation of the ATP-phosphorylated enzyme into the P_i-phosphorylated enzyme via the dephosphoenzyme. We have found no evidence for this process during the 15 s allowed in Fig. 6 for ATP phosphorylation, i.e., the ^{33}P-level does not increase, presumably because P_i phosphorylation already has reached its maximum. Since Post et al. used a crude membrane preparation as enzyme

source, the phosphorylation capacities per molecule of enzyme cannot be calculated from their results. Moreover, the relative contribution by either ATP or P_i was not assessed by double label experiments. Nevertheless, they found that Na^+ as well as ATP decreases inhibition by P_i when added in this sequence, the more so, the higher the concentration of Na^+ or ATP. In addition, when ATP and P_i are added simultaneously [30] not only the ATP phosphorylation level is reduced as compared to that in the absence of P_i, but also the P_i-phosphorylation level as compared to that in the absence of ATP. The sum of the two reduced phosphorylation levels reaches a slightly higher value than the steady-state ATP-phosphorylation level in the absence of P_i. We also observe this in the experiment shown in Fig. 6, where the P_i-phosphorylation level is not reduced by ATP. So the reverse of our statement may also be true, i.e., that conformations for P_i phosphorylation and following phosphorylation by ATP (with Na^+ and/or ATP bound to it) are also mutually exclusive.

In summary, two explanations may apply to the finding that the sum of phosphorylation by P_i and ATP is equal to the level of phosphorylation by ATP alone, i.e. maximally 1 mol per mol of the enzyme:

(1) There is only one phosphorylation site per enzyme molecule, which is the same for P_i and ATP;

(2) There is one phosphorylation site for P_i and another one for ATP, which are spatially separated, but following phosphorylation by P_i a conformation of the enzyme is obtained, which cannot be phosphorylated by ATP and vice versa.

The second explanation does not exclude the first one, i.e. there may be only one phosphorylation site, but the conformations following phosphorylation by P_i or ATP could still oppose the conformations normally required for phosphorylation by ATP or P_i, respectively.

Acknowledgements

This investigation was supported in part by the Netherlands Organization for the Advancement of Basic Research (ZWO), through the Netherlands Biophysics Foundation.

References

1 Post, R.L., Sen, A.K. and Rosenthal, A.S. (1965) J. Biol. Chem. 240, 1437—1445
2 Fahn, S., Koval, G.J. and Albers, R.W. (1968) J. Biol. Chem. 243, 1993—2002
3 Mårdh, S. and Zetterqvist, Ö. (1974) Biochim. Biophys. Acta 350, 473—483
4 Jørgensen, P.L. (1975) Quart. Rev. Biophys. 7, 239—274
5 Nakao, T., Nakao, M., Nagai, F., Kawai, K., Fujihira, Y., Hara, Y. and Fujita, M. (1973) J. Biochem. 73, 781—791
6 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53—67
7 Fahn, S., Koval, G.J. and Albers, R.W. (1966) J. Biol. Chem. 241, 1882—1889
8 Siegel, G.J. and Albers, R.W. (1967) J. Biol. Chem. 242, 4972—4979
9 Karlish, S.J.D. and Glynn, I.M. (1974) Ann. N.Y. Acad. Sci. 242, 461—470
10 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. 192, 217—235
11 Post, R.L., Toda, G. and Rogers, F.N. (1975) J. Biol. Chem. 250, 691—701
12 Simons, T.J.B. (1974) J. Physiol. 237, 123—155
13 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530—6540

14 Simons, T.J.B. (1975) J. Physiol. 244, 731—739
15 Post, R.L., Kume, S., Tobin, T., Orcutt, B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306s—326s
16 Siegel, G.J., Koval, G.J. and Albers, R.W. (1969) J. Biol. Chem. 244, 3264—3269
17 Bonting, S.L., Schuurmans Stekhoven, F.M.A.H., Swarts, H.P.G. and De Pont, J.J.H.H.M. (1979) in Sodium, Potassium-ATPase: Structure and Kinetics (Skou, J.C. and Nørby, J.G., eds.), pp. 317—330, Academic Press, New York
18 Schuurmans Stekhoven, F.M.A.H., van Heeswijk, M.P.E., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 422, 210—224
19 Perrone, J.R., Hackney, J.F., Dixon, J.F. and Hokin, L.E. (1975) J. Biol. Chem. 250, 4178—4184
20 Jørgensen, P.L. and Petersen, J. (1977) Biochim. Biophys. Acta 466, 97—108
21 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234—5240
22 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. 256, 465—496
23 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006—1019
24 Robinson, J.D. (1978) Biochim. Biophys. Acta 509, 419—428
25 Inturrisi, C.E. and Titus, E. (1970) Mol. Pharmacol. 6, 99—107
26 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
27 Schoot, B.M., Schoots, A.F.M., De Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1977) Biochim. Biophys. Acta 483, 181—192
28 Schoot, B.M. (1978) Ph.D. Thesis, University of Nijmegen, The Netherlands
29 Hansen, O., Jensen, J. and Nørby, J.G. (1971) Nature New Biol. 234, 122—124
30 Post, R.L. (1977) in Biochemistry of Membrane Transport (Semenza, G. and Carafoli, E., eds.), pp. 352—362, Springer-Verlag, Berlin