Multiple Ion-Dependent and Substrate-Dependent Na^+/K^+-ATPase Conformational States. Transient and Steady-State Kinetic Studies†

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ABSTRACT: The hydrolysis of β-(2-furyl)acryloyl phosphate (FAP), catalyzed by the Na^+/K^+-ATPase, is faster than the catalyzed hydrolysis of ATP. This is due to catalyzed hydrolysis of the pseudosubstrate by K^+-dependent states of the enzyme, thus bypassing the Na^+-dependent enzyme states that are required and are rate limiting in ATP hydrolysis. Unlike ATP, FAP is a positive effector of the E_2 state. A study of FAP hydrolysis permits a detailed analysis of later steps in the overall ion translocation–ATP hydrolysis pathway. During the steady state of FAP hydrolysis in the presence of K^+, substantial phosphoryl-enzyme is formed, as is indicated by the covalent incorporation of ^{32}P from [^{32}P]FAP. A comparison of the phosphoryl-enzyme yield with the rate of overall hydrolysis reveals that at 25 °C the phosphoryl-enzyme formed is all kinetically competent. Both the yield of phosphoryl-enzyme and the rate of overall hydrolysis of FAP are [K^+] dependent. The transition $E_1 \rightleftharpoons E_2$ is also [K^+] dependent, but the rate of transition is differently affected by [K^+] than are the above-mentioned two processes. Two distinct roles for K^+ are indicated, as an effector of the E_1–E_2 equilibrium and as a "catalyst" in the hydrolysis of the E_2–P. In contrast to the results at 25 °C, a virtually stoichiometric yield of phosphoryl-enzyme occurs at 0 °C in the presence of Na^+ and the absence of K^+. At lower concentrations of K^+ and in the presence of Na^+, the hydrolysis of FAP at 0 °C proceeds substantially through the E_1–E_2 pathway characteristic of ATP hydrolysis. The selectivity of FAP for the E_2–K^+-dependent pathway is due to the thermal inactivation of E_1 at 25 °C in the absence of ATP or ATP analogues, even at high concentrations of Na^+. These results emphasize the existence of multiple functional "E_1" and "E_2" states in the overall ATPase–ion translocation pathway.

Sodium–potassium-activated adenosinetriphosphatase (Na^+/K^+-ATPase)[1] exhibits hydrolytic activity toward organophosphoryl derivatives such as *p*-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate, in the presence of Mg^{2+} and K^+. The absence of a Na^+ requirement for this reaction has led to the belief that this "phosphatase" activity reflects the terminal hydrolytic steps of the ATPase pathway (Cantley, 1981). Thus, certain conformational transitions essential for the mechanism of ATP hydrolysis and for ion translocation are bypassed. The rate of catalyzed hydrolysis of *p*-nitrophenyl phosphate (from which most mechanistic details of pseudosubstrate hydrolysis have been derived) is slow relative to that for ATP, so that the relevance of details of the mechanism to the overall ATP hydrolytic mechanism is unclear. More recently, another synthetic phosphoryl derivative, β-(2-furyl)acryloyl phosphate (FAP), has been shown to be a rapidly hydrolyzable substrate for the ATPase (Gache et al., 1976, 1977). FAP is hydrolyzed 3–4-fold faster than ATP at sodium and potassium concentrations optimal for ATP hydrolysis (Gache et al., 1977; Odom et al., 1981). This fact argues strongly that steps in the FAPase mechanism are relevant to the ATPase pathway; it is unreasonable that a highly efficient mechanism for the hydrolysis of FAP would have evolved, different from all of the stages of the ATPase sequence. Rather, it is likely that the ATPase pathway contains additional steps and/or that rate control is exerted at different stages of the pathway for the two substrates. Therefore, the kinetics of FAP hydrolysis should highlight different steps in the catalytic mechanism than do the kinetics of ATP hydrolysis.

The pathway for FAP hydrolysis, considered herein, is consistent with part of the conventionally accepted Albers–Post pathway for ATP hydrolysis. This pathway involves two principal enzyme conformations (E_1 and E_2). Our experiments involving the kinetics of FAPase suggest that at 25 °C E_2 is the only catalytically competent conformation, as is apparently the case in *p*-nitrophenyl phosphate hydrolysis (Post et al., 1972; Chipman & Lev, 1983).

With pseudosubstrates such as *p*-nitrophenyl phosphate it has not been possible to identify phosphoryl-enzyme as an intermediate in the overall catalyzed hydrolysis. In previous experiments reported from this laboratory (Odom et al., 1981) utilizing stopped-flow absorption spectrophotometric techniques, we were unable to demonstrate the transient formation of phosphoryl-enzyme from FAP. These experiments demonstrate that in the presence of low concentrations of potassium, the formation of the product (furylacrylate), is rate limiting. It was concluded that, in the absence of potassium, the ion-independent hydrolytic reaction rate exceeds the rate of phosphorylation of the enzyme by FAP. The variety of such experiments is restricted by the time and optical density res-

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[1] Abbreviations: Na^+/K^+-ATPase, sodium–potassium-activated adenosinetriphosphatase (EC 3.6.1.3); FAP, β-(2-furyl)acryloyl phosphate; DNPP, 2,4-dinitrophenyl phosphate; PNPP, *p*-nitrophenyl phosphate; Im-HCl, imidazole hydrochloride; EDTA, ethylenedinitrilotetraacetic acid; P_i, inorganic phosphate; $NaDodSO_4$, sodium dodecyl sulfate; PK, pyruvate kinase; LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide; AMP-PNP, adenyl-5′-yl imidodiphosphate.

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olution of the stopped-flow instrument. The quenched flow techniques for the measurement of rapid reactions permits a wider range of experimentation in this regard. The products of reaction can be separated one from another and analyzed independently at leisure. Such techniques have been applied to the formation of radioactively labeled [^{32}P]phosphoryl-enzyme in the Ca^{2+}-dependent ATPase from sarcoplasmic reticulum by G. Inesi and his collaborators [e.g., see Kurzmack et al. (1981)]. Utilizing radioactive [^{32}P]FAP in molar excess over enzyme, we have been able to investigate the formation of phosphoryl-enzyme during the steady state of FAP hydrolysis. In this paper we show that the pathway for ATPase-catalyzed FAP hydrolysis involves the transient formation of appreciable concentrations of phosphoenzyme (E_2–P). Hence, the kinetics of the FAPase reveals details of the E_2 part of the overall ATPase pathway that are not readily obtainable from kinetic studies of ATP hydrolysis.

Materials and Methods

Chemicals. Adenosine triphosphate (ATP Tris and disodium salts), *N*-ethylmaleimide (NEM), oligomycin A, *p*-nitrophenyl phosphate (PNPP dicyclohexylammonium salt), phosphoenolpyruvate (PEP trisodium salt), bovine muscle lactate dehydrogenase (LDH), rabbit muscle pyruvate kinase (PK), NAD^+, tetrasodium ethylenedinitrilotetraacetate (EDTA), and tris(hydroxymethyl)aminomethane (Tris) were the highest grade available from Sigma Biochemical Co. β-(2-Furyl)acrylic acid (Aldrich Chemical Co.) was twice recrystallized from toluene prior to use. Sodium dodecyl sulfate ($NaDodSO_4$) was purchased from BDH Chemical Ltd. Lubrol WX (Sepulco, Inc.) was twice recrystallized from diethyl ether. Imidazole (Im) was twice recrystallized from benzene prior to use. All other reagents were the highest grade available. Double glass distilled water was used throughout.

β-(2-Furyl)acryloyl phosphate (FAP) was synthesized as previously described (Odom et al., 1981), except that FAP was eluted at 4 °C from Dowex 1-X2 with either 0.80 or 1.0 M imidazole hydrochloride (pH 7.00 at 25 °C). At neutral pH and −70 °C, no measurable hydrolysis of this FAP solution was detected over several months. [^{32}P]FAP was prepared by equilibration of FAP and inorganic [^{32}P]phosphate (Kurzmack et al., 1981). Reaction products were separated on Dowex 1-X2 as described above. A typical yield from 25 μmol/FAP and 2.2–2.3 mCi of ^{32}P was 18–19 μmol at 50–60 Ci/mol (vs. 55–58 Ci/mol theoretical yield).

Enzyme. Na^+,K^+-ATPase was purified from the frozen electric organ of *Torpedo californica* by using the eel electroplax method of Dixon & Hokin (1978). The Lubrol concentration used for solubilization was 2.2% (w/v) at a ratio of 1 mL/4 g of tissue. On the basis of the Lowry protein determination, the specific activity of the purified material toward ATP was 6.5 μmol of P_i released (mg of protein)$^{-1}$ min^{-1} at 25 °C. The activity toward FAP was 18.2 μmol of P_i mg^{-1} min^{-1}. The ratio of oubain-sensitive ATPase to oubain-sensitive FAPase activity remains constant through the purification. On $NaDodSO_4$ denaturing gels, about 90% of the purified protein ran in the two bands corresponding to the ATPase subunits. Vanadate titrations (discussed later) gave approximately 2 nmol of sites/mg of protein, indicating that the preparation may be as little as 30–35% active ATPase.

The final ammonium sulfate fractionation of the purification produces a nearly opaque suspension of *Torpedo electroplax* enzyme. Since transmitting solutions are required for stopped-flow spectrophotometry, this step in the purification was omitted in preparing enzyme for this purpose, resulting in slightly cloudy solutions with 50–60% of the specific activity of the final product. Excess lubrol was removed from this preparation by chromatography on Sephadex G-25-300.

Assays. All kinetic assays were performed in 50 mM Im-HCl, 50 mM $MgCl_2$, and 20 mM KCl, pH 7.00 and 25 °C, unless otherwise noted, and were initiated by addition of enzyme. All experiments at 0 °C were carried out in 0.10 M Im-HCl prepared to pH 7.00 at 25 °C. The pH at 0 °C was 7.59 (Datta & Gryzbowski, 1966).

FAPase activity was measured as described by Odom et al. (1981) using $\Delta\epsilon$ = 670 $M^{-1}\cdot cm^{-1}$ at 356 nm. The FAP spectrum is shifted by Mg^{2+} ligation (Odom et al., 1981). At the various temperatures and consequently different pH and pK values for FAP, different $\Delta\epsilon$ values were obtained. The $\Delta\epsilon$ value at 356 nm is also affected by solvent. Consequently, we determined $\Delta\epsilon$ for each solvent environment and temperature. Absorbance was monitored on either a Varian 635 or Cary 14 or 16 spectrophotometer using water-jacketed cell holders.

PNPPase activity was assayed exactly as described by Odom et al. (1981). The $\Delta\epsilon_{max}$ for PNPP decreased 53% in 30% Me_2SO.

The pyruvate kinase–lactate dehydrogenase system (Bücher & Pfleiderer, 1955) was used to assay ATP turnover. Final concentrations were 3 mM ATP, 0.15 mM NADH, 4 mM PEP, 3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 25 μg/mL PK and 18 μg/mL LDH, at 25 °C and pH 7.00. The coupled reaction mixture was preincubated until the absorbance at 350 nm was constant, indicating that residual ADP had been phosphorylated. The reaction was started by addition of the ATPase.

Protein concentration was measured by using the microbiuret method of Brewer et al. (1974), which is less sensitive to interference by the Lubrol wx used to solubilize the electroplax enzyme than is the Lowry assay. Protein samples in $NaDodSO_4$ solution were extracted with 2 mL of diethyl ether/1 mL of aqueous protein prior to measurement of the absorbance. With bovine serium albumin as the protein standard, 50% more electroplax protein is estimated by the biuret assay than by the Lowry assay.

The enzyme active site concentration was measured by titration with vanadate as described by Cantley et al. (1977) except that FAPase rather than ATPase activity was titrated. Vanadate inhibition was identical for the two activities under all conditions tested. In the standard FAPase assay K_{diss} for vanadate was found to 9.0×10^{-9} M. Reactions were initiated by addition of FAP to mixtures of enzyme incubated 30–40 min in vanadate at 25 °C. Vanadate concentrations were chosen to cover the range from 20% to 80% inhibition of FAPase activity. Rates were determined from the first 0.5–2 min of reaction. From 14 such titrations, the turnover number of *Torpedo electroplax* enzyme for FAP under standard conditions is 133.6 ± 9.0 s^{-1}.

Rapid Kinetic Measurements. Rapid kinetic measurements were carried out on a Durrum D-110 single-beam stop-flow spectrophotometer. The instrument has a "dead" time of 5 ms. At 350 nm the absorbance sensitivity is 0.0005 OD. The instrument software permits on-line access to a Varian 1620 computer. Data were fit by programs developed at the University of Oregon.

Steady-State Phosphorylation Measurements. Reactions were carried out in 1.5-mL disposable centrifuge tubes incubated at 0 °C. Electroplax ATPase was extensively dialyzed vs. 5 mM Tris-HCl in 0.25 M sucrose to remove Na^+ and K^+. The reaction mixtures containing 0.8–1.0 nmol of enzyme were incubated 10–20 min at 0 °C. Reactions were initiated by

Table I: Kinetic Parameters for Potassium Stimulation of FAP Turnover at Saturating and Subsaturating FAP and Various Concentrations of Sodium, ATP, and AMP-PNP[a]

concentration conditions			kinetic parameters					
			activity at 1.5 mM FAP			activity at 0.2 mM FAP		
[Na^+] (mM)	[ATP] (mM)	[AMP-PNP] (mM)	v_T[b]	$K_{0.5}$ (mM)	n_H	v_T[b]	$K_{0.5}$ (mM)	n_H
0	0	0	100	4.1	1.7	53.4	4.63	1.84
5	0	0	106	5.0	1.64	54	7.1	1.9
20	0	0				55	5.4	1.7
100	0	0	99	4.2	1.7	39	3.7	0.84
100	0.05	0	92	3.1	1.77	36.8	1.71	1.6
100	0.20	0	81	2.88	1.64	27.5	2.0	1.26
0	0.05	0	103	11.9	1.37	53	20	1.5
0	0.20	0	108	26	1.40	42	29	1.9
0	0	5	89	13.0	1.38			
100	0	5	120	80	1.1			

[a] Conditions are 50 mM Mg^{2+}, pH 7.0 and 25 °C. Maximum velocity in the absence of effectors at 1.5 mM FAP is 150 s^{-1} (corresponding to V_T = 100) on the basis of vanadate sites. Data were analyzed by a binding expression with two terms of the form of eq 2, to account for the overlapping effects of K^+ stimulation and high-salt inhibition. Parameters are the following: V_T, maximum K^+-stimulated rate; $K_{0.5}$, [K^+] to give half-maximum stimulation; n_H, the Hill coefficient for K^+. [b] V_T contains a 1–2% contribution from an ion-independent, catalyzed rate of hydrolysis.

addition of less than 60 μL of [^{32}P]FAP solution at 0 °C. The total volume was 0.60–0.65 mL. Reactions were quenched after various time intervals (3–8 s) by the addition of an equal volume of aqueous trichloroacetic acid (10%). To calibrate nonphosphorylated protein blanks, [^{32}P]FAP was added after the enzyme solution was quenched by acid. Background radiation was below 10% of the lowest specific labeling observed.

To measure ^{32}P incorporation, the precipitated protein was collected by centrifugation. Soluble radioactivity was partially removed by resuspending the protein pellet in 5% trichloroacetic acid. The protein was again collected by centrifugation. The washed pellet still contained strongly absorbed [^{32}P]FAP. To remove it, the pellet was dissolved in detergent (2% $NaDodSO_4$, 0.10 M sodium acetate, pH 6.00, and 1.0 mM 2-mercaptoethanol). The dissolved protein was separated from small molecule components on a 3.0-mL Sephadex G-25-80 column by using the centrifuge chromatography technique of Tuszynski et al. (1980). Protein recovery was 80–95% in a volume identical with the sample volume. Adsorption of FAP to Sephadex causes remarkably efficient separation, reducing [FAP] by 5 orders of magnitude in one step. After chromatography, 0.20 mL of the recovered solution (0.60 mL total) was removed for estimation of protein by the microbiuret assay. The necessity to rely on this assay, which was performed on small quantities of protein in $NaDodSO_4$ solution, caused substantial imprecision in the phosphorylation yield measurements, and may also be largely responsible for some systematic variation in yield noted from experiment to experiment. The remaining volume was dissolved in 5 mL of aqueous scintillant and counted in a Packard TriCarb 3375 liquid scintillation counter.

Rapid Kinetics Quenched-Flow Experiments. Rapid mixing and quenching were carried out in a Durrum D-133 multimixing apparatus. The function timings and flow velocities were taken from traces of the instrument's drive plunger velocity, recorded on a Tektronix 5103N oscilloscope. The instrument dead time is approximately 23 and 33 ms at 25 and 0 °C, respectively. Temperature was controlled to ±0.5 °C with an ethanol/water bath. The quenching solution was 8% trichloroacetic acid. Equal volumes of reaction and quenching solution were utilized. About 1-mL samples were collected into 1.5-mL centrifuge tubes for determination of the phosphorylation as described.

Computing Procedure. The simultaneous fitting of the model given by Scheme I to both data sets in Figure 7 used programs developed by D. C. Teller (personal communication). All other data were fitted to functions described in the text by using the program MLAB (Knott & Reece, 1972) with a DEC PDP 10 computer.

Results

Conversion of Unliganded Enzyme to the K^+-FAPase. The slow rate of transition $E_1 \rightarrow E_2(K^+)$ (Karlish et al., 1978) has been used to test the hypothesis that $E_2(K^+)$ is the catalytically competent form of the ATPase for FAP hydrolysis at 25 °C. When hydrolysis of FAP, catalyzed by enzyme incubated in the absence of K^+ and Na^+, is monitored after rapid mixing of enzyme with K^+ plus FAP, a lag in the rate of disappearance of FAP is observed. Control experiments in which K^+ was included in the enzyme incubation mixture gave linear traces from the earliest time points (8–10 ms), confirming that K^+ induction of a second conformation is a prerequisite to FAP hydrolysis. The lag phase is well described by a first-order approach to steady state.

In experiments in which the approach to steady state was measured as a function of [K^+] (Figure 1), observed rate constants vary linearly with [K^+] to at least 10 mM. There is no evidence for saturation of the K^+ effect even at a [K^+] that gives 80% of maximum turnover. It proved impossible to measure rates at higher or lower [K^+] due to noisiness of the reaction traces. The lag amplitude was limited by low rates of hydrolysis (low [K^+]) and high rates of isomerization (high [K+]).

Inhibition of FAP Hydrolysis. The effect of ATP on potassium activation of FAP turnover at 25 °C was studied. In these experiments FAP turnover was titrated with K^+ at fixed concentrations of ATP and FAP. The Mg^{2+} concentration was high (50 mM) so that all ATP was present as MgATP. Bound FAP is nearly fully ligated at 50 mM Mg^{2+} (Odom et al., 1981). The concentrations of ATP were chosen to give 50% inhibition of turnover at the two concentrations of FAP used, when assayed at the standard FAPase condition of 20 mM K^+. The major effect of ATP (Table I) is to increase $K_{0.5(K^+)}$ to a higher potassium concentration. Small effects of ATP on the other parameters of Table I are detectable. Inhibition by ATP is completely reversed by increased [K^+], provided the FAP concentration is near saturation. This is indicative of a linked ligand effect on equilibria between enzyme states, rather than direct competition with FAP for binding to a single state.

Note that the addition of Na^+ can totally overcome the ATP inhibition of FAPase, provided the FAP concentration is near saturation. Addition of ATP plus Na^+ does not change $K_{0.5(K^+)}$, even at an ATP concentration that increases $K_{0.5(K^+)}$ by 6-fold

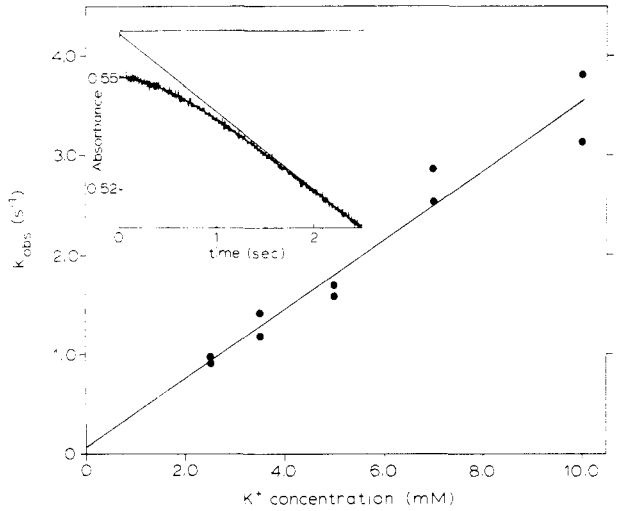


FIGURE 1: Effect of K^+ concentration on the rate of conversion of E_1 to active FAPase at 25 °C and pH 7.00. Each solution prior to mixing contained 0.23 M sucrose. Other conditions are standard as described under Materials and Methods. The initial concentration of FAP immediately after mixing was 0.23 mM. The solid line is the least-squares line with slope 34.8 s^{-1} M^{-1} and intercept 0.062 s^{-1}. (Inset) One of the reaction traces at 5.0 mM K^+. Absorbance is at 354 nm. The solid line through the trace is the best fit to the function $A_t = A_0 + A_{lag}e^{-kt} - \Delta At$. The straight line is the asymptotic steady-state limit given by the first and last terms only.

in the absence of sodium. The sensitivity of FAPase to [Na^+] and [ATP] is similar to that observed with other pseudosubstrates (Yoshida et al., 1969; Koyal et al., 1971; Gache et al., 1976) provided that the FAP concentration is subsaturating. Sodium alone is an activator of FAPase at low [K^+]; addition of ATP has little or no effect on this activation.

Beaugé & Glynn (1980) have observed that AMP-PNP, a nonphosphorylating analogue of ATP, is also an effector of the $E_1 \rightleftharpoons E_2(K^+)$ equilibrium. Na^+ is presumed to overcome the ATP inhibition of FAPase by initiating the catalytic pathway (see Discussion). If phosphorylation is the mechanism for overcoming inhibition (by generating E_2), very different results should be obtained for Na^+ plus the nonhydrolyzable analogue AMP-PNP. The results listed in Table I illustrate this prediction. Consistent with its role as a "static" effector of the E_1 state, AMP-PNP is inhibitory toward FAPase, both in the presence and in the absence of Na^+. Moreover, the ligation of the stable analogue leads to an increase in the concentration of K^+ required for effective FAPase. Note that, in the absence of Na^+, ATP and AMP-PNP have highly similar inhibitory properties.

In accord with the report of Albers & Koval (1972) we observe reversible inhibition of ATPase at concentrations up to at least 30% Me_2SO, with 50% inhibition occurring at 18% Me_2SO. FAP is similarly inhibited by Me_2SO. The inhibition of acetyl phosphatase by Me_2SO becomes substantial at much lower Me_2SO concentrations (Albers & Koval, 1972). In contrast, PNPPase is slightly activated by Me_2SO, reaching a maximum at about 18%. Higher concentrations inhibit PNPPase.

Oligomycin is thought to inhibit the E_1–P to E_2–P transition (Fahn et al., 1966; Fahn et al., 1968). Oligomycin inhibits the ATPase activity of the *Torpedo electroplax* enzyme ($K_I \approx 30$ μg/mL). We have found that oligomycin has no effect on FAPase activity both in the absence and in the presence of Na^+, nor is there any effect of oligomycin on PNPPase activity.

In other species, alkylation by NEM is reported to inhibit the E_1–P to E_2–P transition (Cantley, 1981). We attempted to utilize this modification procedure in order to observe E_1-dependent reactions with FAP. However, *Torpedo electroplax* ATPase is more resistant than other species to alkylation by NEM. The slow alkylation reaction leads to the same enzyme inactivation whether assayed by ATPase, FAPase, or PNPPase.

Table II: Yield of Phosphoenzyme during Steady-State Hydrolysis of FAP at 0 °C and Various Concentrations of Na^+ and K^+[a]

[K^+] (mM)	[Na^+] (mM)	fractional phosphorylation
0.0	0.0	0.09
5.0	0.0	0.56
20.0	0.0	0.56
0.0	5.0	0.35
0.0	25.0	0.90
0.0	100	0.93
5.0	100	0.50
20.0	100	0.43
0.0	100	0.97[b]
20.0	0.0	0.58[b]

[a] Conditions: 0.1 M Im-HCl, pH 7.59, 0.1 M sucrose, 25 mM $MgCl_2$, and 2.5 mM FAP. [b] Reaction time was 8 ± 0.5 s for these samples. For all others, reaction time was 3 ± 0.5 s.

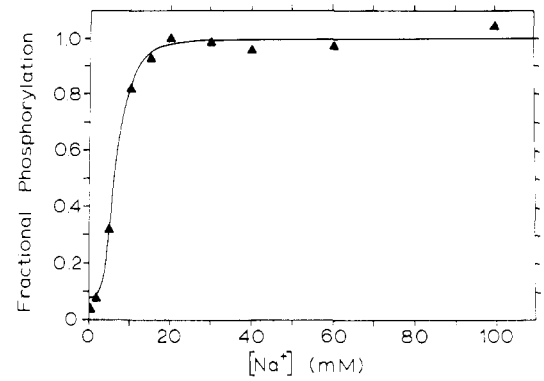


FIGURE 2: Sodium dependence of the yield of phosphoenzyme at 0 °C. Reaction conditions were 100 mM Im-HCl, pH 7.59, 25 mM $MgCl_2$, 2.5 mM [^{32}P]FAP, and 1.2 μM in enzyme sites. Reactions were initiated by addition of [^{32}P]FAP and quenched after 3 ± 0.5 s. The solid line represents the fit to the data of eq 1 with $\phi_p°$ set equal to 0.08. The maximum phosphorylation yield is 1.00 ± 0.01.

Phosphoryl-Enzyme Formation under Transient and Steady-State Conditions. Substantial acid-stable phosphoenzyme (expressed as a fraction of vanadate sites) is observed in the steady-state reaction of [^{32}P]FAP with Na^+,K^+-ATPase at 0 (Table II) and at 25 °C (see Figure 7). A short incubation time was chosen for these steady-state experiments to prevent a substantial decrease in the [FAP]. Evidence of the type presented in the last two rows of Table II argues that this short incubation time is sufficient to reach the steady state. Abundant evidence exists to prove that the number of vanadate sites is equal to the maximum number of ATP-phosphorylatable sites [e.g., see Perrone et al. (1975), Jorgensen (1977), Cantley et al. (1978), Peters et al. (1981), and Moczydlowski & Fortes (1981a)]. In all experiments, the total phosphorylation yield (rather than the vanadate-sensitive phosphorylation yield) is reported. Control experiments with a small excess of vanadate over ATPase indicate that all phosphorylation is vanadate sensitive.

A 2–3-fold higher yield of phosphoryl-enzyme is obtained from FAP than from ATP at 25 °C (Mårdh & Lindahl, 1977) under conditions optimal for hydrolysis of each substrate. This higher yield is consistent with the observed 2.8:1 ratio of FAPase to ATPase activity at 25 °C under optimal hydrolytic conditions, indicating that all of the phosphoryl-enzyme is catalytically competent.

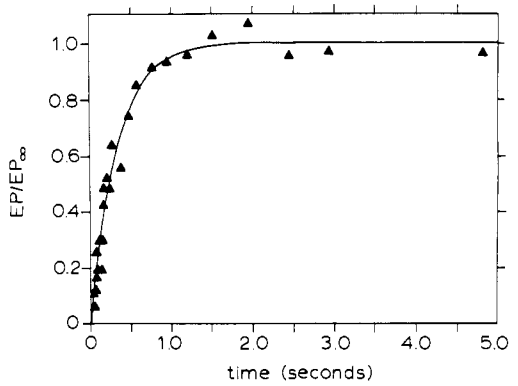


FIGURE 3: Transient formation of acid-stable phosphorylated enzyme at 0 °C in the presence of Na^+, using [^{32}P]FAP. Each syringe (A and B) contained 100 mM Im-HCl, pH 7.59, 50 mM $MgCl_2$, 100 mM NaCl, and 0.2 M sucrose. Syringe A contained 2.1 μM enzyme sites. Syringe B contained 1.9 mM [^{32}P]FAP. The solid line represents the fit to the data of the function $y/y\infty = \exp(-kt)$, for $k = 3.04\ s^{-1}$.

The dependence of phosphorylation yield on sodium concentration has been investigated at 0 °C in the absence of K^+ as is shown in Figure 2. Note the stoichiometric formation of phosphoryl-enzyme at sufficiently high sodium concentration. The dependence of the phosphorylation yield on Na^+ concentration is highly cooperative. These and other ion-dependent phosphorylation yield data were analyzed according to the phenomenological Hill equation where ϕ_p(max) and $\phi_p°$ are the maximum and ion-independent yields, respectively, and n_H is the Hill coefficient

$$\phi_p(\text{obsd}) = \phi_p° + \frac{\phi_p(\text{max})S^{n_H}}{K_{0.5}^{n_H} + S^{n_H}} \quad (1)$$

From the data of Figure 2, n_H is calculated to be 3.3 ± 0.3, a number similar to the number of Na^+ transported per high-energy phosphate hydrolyzed (three Na^+ per ATP).

The transient rate of sodium-dependent phosphorylation by FAP was measured at 0 °C by using rapid-mixing and quench techniques (Figure 3). The phosphoryl-enzyme yield is stoichiometric under these conditions. The rate constant (3.0 s^{-1}) is 6-fold less than the turnover rate for FAP hydrolysis in the presence of optimal K^+ at 0 °C.

The potassium-stimulated phosphorylation rate has been determined in order to investigate whether the phosphoryl-enzyme is a competent intermediate in the overall hydrolytic pathway. The rapid transient velocities of phosphorylation can only be resolved at subsaturating concentrations of FAP and at either low temperature or subsaturating [K^+]. The phosphorylation rate is accelerated by K^+. Typical results at 0 °C are shown in Figure 4. Even at the low K^+ concentration utilized, the phosphoryl-enzyme yield is maximal (see Figure 5). The calculated first-order rate constant for phosphoryl-enzyme formation (22 ± 3 s^{-1}) is the same as the turnover number under these conditions. The observed phosphorylation yield at 0 °C, 0.2 mM FAP, is just sufficient to account for the flux of FAP turnover (3.9 s^{-1}). At 25 °C and 2 mM K^+, and under conditions otherwise identical with those utilized at 0 °C, we obtain a first-order specific rate of phosphoryl-enzyme formation of 27 ± 6 s^{-1}.

The potassium concentration dependence of phosphorylation yield at 0 °C in the absence of Na^+ is as shown in Figure 5. A maximum yield of about 60% is reached at several millimolar potassium. At or above 50 mM K^+, a slight decrease in phosphorylation yield is always evident. The dependence of phosphorylation yield on K^+ concentration appears to be cooperative, but no attempt was made to evaluate a Hill

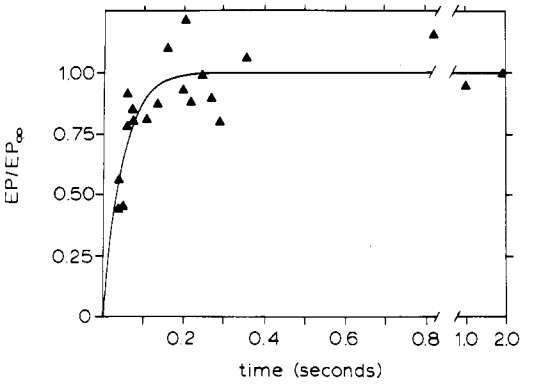


FIGURE 4: Transient formation of phosphorylated enzyme at 0 °C in the presence of K^+, using [^{32}P]FAP. Each syringe (A and B) contained 50 mM Im-HCl pH 7.59, 50 mM $MgCl_2$, 2.0 mM KCl, and 0.20 M sucrose. Syringe A contained 2.4 μM enzyme sites. Syringe B contained 0.40 mM [^{32}P]FAP. The solid line through the points represents the fit to the data of the function $y/y_\infty = \exp(-kt)$ for $k = 21.9\ s^{-1}$. The maximum phosphorylation yield was 0.154.

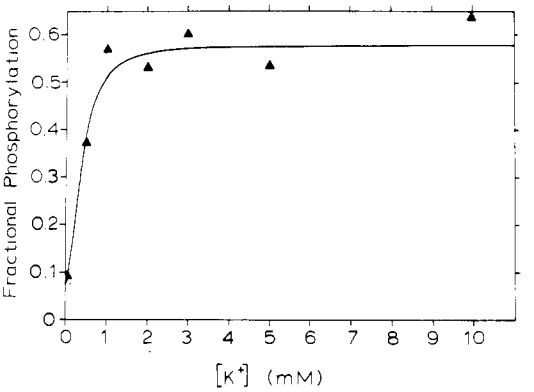


FIGURE 5: Potassium dependence of the level of phosphorylated enzyme during steady-state turnover of FAP. Final reaction conditions were 0 °C, 100 mM Im-HCl, pH 7.59, 25 mM $MgCl_2$, KCl as indicated, and 2.58 mM [^{32}P]FAP. Each reaction mixture (0.60 mL) contained 1.15 nmol of enzyme sites. Reactions were initiated by addition of [^{32}P]FAP and quenched after 3 ± 0.5 s. The solid line is the best fit to eq 1, assuming a Hill coefficient of 2, and assumes a yield of phosphorylated enzyme of 0.09 in the absence of cations (Table II). This gives $K_{0.5}$ = 0.38 mM and maximum phosphorylation yield equal to 0.58.

coefficient from the data. On the basis of n_H = 2.0, the half-saturation point ($K_{0.5}$) is calculated to be 0.4 ± 0.1 mM. This parameter is 3–4-fold smaller than the half-saturation point for K^+ stimulation of FAP turnover under the same conditions ($K_{0.5}$ = 1.45 ± 0.13 mM).

Since turnover of the [^{32}P]FAP can produce as much as 0.1–0.2 mM [^{32}P]P_i during these incubations, the possibility exists that some or all of the phosphorylation observed is due to inorganic phosphate, which is known to phosphorylate under similar conditions (Post et al., 1975). However, the effect of 1.0 mM unlabeled P_i on the yield of radioactive protein derived from [^{32}P]FAP is negligible. P_i is also a poor inhibitor of potassium-stimulated turnover of FAP ($K_I \simeq$ 25 mM at 25 °C).

In contrast, the sodium-dependent ^{32}P phosphorylation yield from [^{32}P]FAP is sensitive to [P_i]. The presence of 4 mM in labeled P_i reduces the ^{32}P phosphorylation yield to 60% at 0.6 mM FAP, similar to the results reported by Mårdh & Post (1977), who showed that phosphorylation by P_i interferes with Na^+-dependent phosphorylation by ATP. Higher concentrations of [^{32}P]FAP overcome this inhibition or dilution of radioactive phosphoenzyme by inorganic phosphate. It is likely that the long incubation of enzyme in Na^+ plus P_i, prior to the addition of [^{32}P]FAP, leads to the formation of some

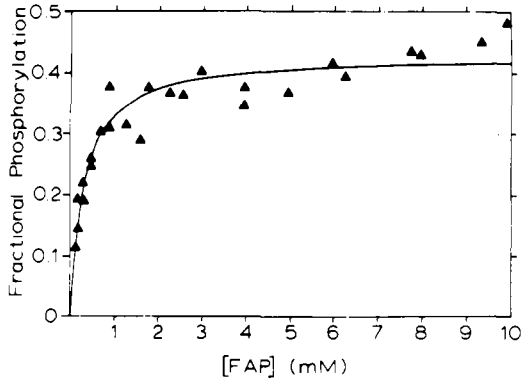


FIGURE 6: FAP concentration dependence of the yield of phosphorylated enzyme during steady-state hydrolysis at 0 °C. The reaction conditions were 0.10 M Im-HCl, pH 7.59, 25 mM $MgCl_2$, 2.0 mM KCl, and [^{32}P]FAP as indicated. Data from the three experiments at different enzyme concentrations are shown. The reaction time was 3 ± 0.5 s. The solid curve through the points represents the fit to the data (see text) and gives a value for the half-saturation point of 0.31 ± 0.04 mM. The K_m measured under these conditions is 0.24 ± 0.06 mM.

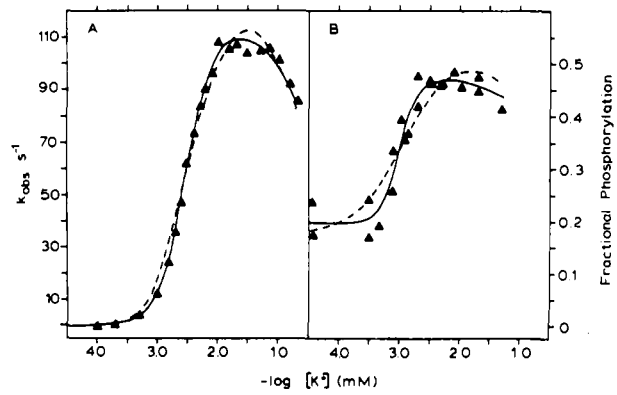


FIGURE 7: Potassium dependence of steady-state turnover of FAP and the yield of phosphorylated enzyme at 25 °C. Reaction conditions were 0.10 M Im-HCl, pH 7.00, 25 mM $MgCl_2$, and 2.5 mM FAP. (A) Turnover numbers (k_{obsd}) based on number of vanadate sites. The basal rate recorded in the absence of added K^+ (2.1 s^{-1}) has been subtracted from the points. The solid line represents the fit to the phenomenological two-term Hill expression (see footnote *a* to Table I). The parameters are given in the text. (B) Steady-state phosphorylation level based on the number of vanadate sites. Data from two experiments at slightly different enzyme concentrations are shown. The solid curve through the points represents the fit to the data of the expression described above. The parameters are given in the text. The dashed lines in both parts represent the simultaneous fit of eq 5a and 5b with $n = 3$ to the data in parts A and B, respectively. The parameters are listed in Table IV.

unlabeled E_1–P. In the absence of K^+, hydrolysis of E_1–P is slow. The presence of variable small amounts of P_i may account for the substantial variation in our measurements of the phosphorylation yield in the presence of Na^+ alone.

Since the sodium-dependent phosphorylation yield from ATP is also sensitive to the presence of the product ADP [e.g., see Mårdh (1975a,b) and Kuriki & Racker (1976)], the effect of the product FA^- on the sodium-dependent yield of phosphoryl-enzyme from FAP has been investigated. FA^- has no effect on the virtually stoichiometric phosphorylation yield at concentrations of up to 6 mM.

The dependence of steady-state phosphorylation yield on FAP concentration at 0 °C and 2 mM K^+ is shown in Figure 6. The K_m value for FAP obtained from Figure 5 is virtually identical with that obtained from the dependence of the rate of FAP hydrolysis on FAP concentration. We note that, under optimal phosphorylation conditions, the yield in Figure 5 is only 70% of that shown earlier (Figure 5). A substantial part of this discrepancy in yield is probably due to the imprecision in protein concentration determination in detergent solution, as noted under Materials and Methods. The consistency in the effects of ligand on the relative phosphorylation yield (see Discussion) leads us to a greater reliance on these results then would be apparent from the fluctuations in optimal phosphorylation yield from one set of experiments to another.

Unlike the transient phosphorylation rate studies, the steady-state phosphorylation yield and FAP turnover were better studied at 25 °C. Results at this temperature can be compared with existent parameters for ATP. Moreover, at 25 °C, the potassium-dependent hydrolytic pathway for FAP is particularly simple, as will be discussed.

Effect of K^+ on the Phosphoryl-Enzyme Yield and on the Rate of FAP Hydrolysis at 25 °C. The effect of K^+ concentration on both the phosphoryl-enzyme yield and the turnover rate at 25 °C at saturating FAP concentration is illustrated in Figure 7. The solid lines in Figure 7 are the calculated curves for the best values of the Hill equation parameters (eq 1). Figure 7A deals with the rate of FAP hydrolysis. Note that the dependence of reaction rate on K^+ concentration is substantially cooperative; the data can be fit to a Hill coefficient of 1.9 ± 0.1. For these results we calculate a V_{max} of 117 ± 2 s^{-1}. Note that, at very high K^+ concentration, there is some discernible inhibition of the FAPase activity.

The dependence of phosphoryl-enzyme yield on K^+ concentration is shown in Figure 7B. The Hill coefficient for the [K^+] dependence of the phosphoryl-enzyme yield is 2.9 ± 0.8. This value appears to differ from that for the [K^+] dependence of FAP hydrolysis, although the imprecision of the latter estimate makes the distinction uncertain. There is a more certain distinction between the $K_{0.5}$ for [K^+]-dependent phosphoryl-enzyme yield (1.0 ± 0.1 mM) vs. that for FAP hydrolysis (3.0 ± 0.1 mM). In the absence of K^+, the extent of phosphorylation is still substantial. In contrast, the ion-independent turnover is only about 2% of the V_{max} under optimal potassium concentrations.

Relationship between the Effect of Na^+ plus K^+ on the Phosphoryl-Enzyme Yield and the Rate of FAP Hydrolysis. Potassium stimulation of the FAPase activity at 25 °C was measured at several concentrations of sodium, at both 0.2 and 1.5 mM FAP. Note that K_M for FAP is 0.22 mM. At 1.5 mM FAP there is virtually no effect of Na^+ on the K^+-stimulated rate of hydrolysis (Table I). The K^+ dependence of the rate of FAP hydrolysis is also unaffected by [FAP] in the absence of Na^+. There is positive cooperativity in the dependence of reaction rate on [K^+]. Although there is inhibition of FAPase activity at very high [K^+] (as with other substrates; Gache et al., 1976; Pitts & Askari, 1971), the K^+ stimulation of FAPase activity in the presence or absence of Na^+ can be fit by a single-term Hill equation.

In contrast, at 0 °C, Na^+ effects the K^+-stimulated hydrolysis rate, even near saturating FAP. The phosphoryl-enzyme yield was studied at 0 °C and in the presence of potassium, both in the presence and in the absence of Na^+. These ion-dependent yields are compared with the rates of FAP hydrolysis (Figure 8).

The potassium concentration dependence of FAP turnover in the absence of Na^+ at 0 °C is not highly cooperative (Table III). The half-saturation value for the K^+ dependence of turnover is severalfold greater than for the K^+ dependence of phosphorylation yield. In contrast, K^+ activation of turnover in the presence of Na^+ is highly cooperative though it is not well described by a single-term Hill function.

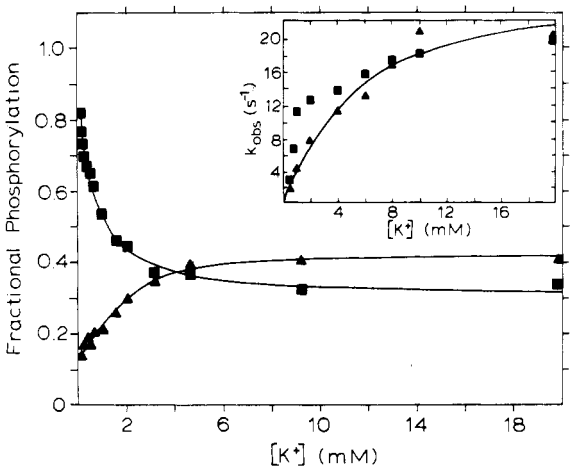


FIGURE 8: Effect of Na^+ on [K^+]-activated phosphorylation and turnover of FAP at 0 °C. Reaction conditions were 100 mM Im-HCl, pH 7.59, 50 mM $MgCl_2$, and 2.6 mM FAP, in the absence of Na^+ (▲) or in the presence of 100 mM Na^+ (■). The phosphoenzyme yield as a function of [K^+] is shown in the large figure. The reaction time was 5 ± 0.5 s. The inset illustrates the [K^+] dependence of FAP turnover. The solid lines represent the best fits of the experimental data to the binding expressions described in the text. Parameters are listed in Table III.

Table III: Potassium Stimulation Parameters for Fractional Phosphorylation and Steady-State Turnover in the Presence and Absence of Na^+ at 0 °C[a]

	experimental measurements		
	fractional phosphorylation, presence of Na^+[c]	fractional phosphorylation, absence of Na^+[d]	steady-state turnover, absence of Na^+[e]
ϕ_p° [k_0 (s^{-1})][b]	0.775	0.162	(0.44)
ϕ_p^{max} [k_{max} (s^{-1})][b]	−0.457	0.261	(24.2)
$K_{0.5(K^+)}$ (mM)	0.88	1.86	4.4
n_H	1.38	1.85	1.24
rms deviation to fitted curve	0.023	0.013	1.37 s^{-1}

[a] The data from Figure 8 were fit by nonlinear least-squares methods to eq 1 in the text. [b] For the steady-state turnover data in the absence of sodium, the parameters ϕ_p° and ϕ_p^{max} are replaced by k_0, the basal, cation-independent turnover rate, and k_{max}, the maximum potassium stimulated turnover rate. Data for turnover in the presence of Na^+ are not well described by eq 1 (see text). [c] Solid squares in Figure 8. [d] Solid triangles in Figure 8. [e] Solid triangles in inset to Figure 8. The parameter k_0 is set equal to 1.8% of k_{max}.

At low [K^+], Na^+ can increase both the turnover rate and the phosphoryl-enzyme yield severalfold (Figure 8). At high [K^+], Na^+ exerts little or no effect on either rate or yield.

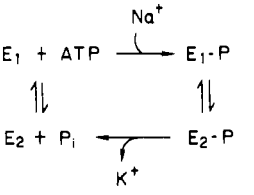
DISCUSSION

The rate of hydrolysis of FAP catalyzed by Na^+/K^+-ATPase is remarkably rapid. Pseudosubstrates designed to give an interpretable signal of reaction progress usually exhibit drastically reduced rates of catalyzed reaction. The lack of a Na^+ requirement for the overall hydrolysis of FAP, contrary to the results obtained with ATP, argue that a portion of the ATP pathway of hydrolysis has been bypassed. With other pseudosubstrates, this lack of a Na^+ requirement has been interpreted as a demonstration that these reactants enter the ATPase pathway in the K^+-dependent "phosphatase" phase of the reaction cycle. Indeed, the results presented herein argue strongly that this is the case with FAP at 25 °C. Since the rate of FAP hydrolysis exceeds that for ATP hydrolysis under optimal condtions, it follows that in the case of ATP steps preceding the phosphatase reaction must be rate limiting. Hence, the studies with FAP afford the opportunity to examine steps in the reaction cycle beyond the sodium-dependent phosphorylation of the enzyme by ATP.

Chirality experiments with Ca^{2+}-translocating ATPase and with phosphatases suggest that a single phosphoryl-enzyme intermediate is formed during the entire reaction cycle (Buchwald et al., 1982; Knowles, 1982). Since phosphoryl-enzyme is formed from ATP in the sodium-dependent phase of reaction, there was some question as to what the nature of the catalyzed hydrolysis of acyl phosphate (FAP) might entail in regard to the formation of a covalent reaction intermediate. The results we have presented demonstrate clearly that at 25 °C, where the K^+-dependent phosphatase pathway is exclusive, substantial phosphoryl-enzyme is formed from FAP; hence, both states of the enzyme, E_1 and E_2 of the Albers–Post mechanism, can react with the suitable phosphorylating reagent to generate the corresponding phospho(β-aspartyl)-enzyme.

The kinetics and the stoichiometry of formation of phosphoryl-enzyme from FAP at 25 °C indicate that this K^+-dependent phosphoryl-enzyme is a competent intermediate in the reaction pathway. The characteristic properties of the E_2–P encountered during ATP hydrolysis and the properties of phosphoenzyme described herein during FAP turnover are similar, suggesting that the key species are the same. A value of approximately 1 mM has been found for the dissociation constant for K^+, as calculated from its effect on the dephosphorylation rate, both in transient (Mårdh, 1975a) and in steady-state experiments (Robinson, 1975; Chipman & Lev, 1983) involving ATP. Our experiments with FAP yield a value of 0.5–1.0 mM for the apparent dissociation constant of K^+: the large uncertainty in this estimate arises from the uncertainty in the number of K^+ sites for K^+-dependent activation (see below). From the maximal phosphorylation yield at saturating [K^+] (0.46; Figure 7) and the turnover number (117 s^{-1}) we calculate the rate constant (k_2) for dephosphorylation to be 250 s^{-1} at 25 °C. Values in the range 230–300 s^{-1} have been measured for the transient breakdown of E_2–P(K^+) generated from ATP (Mårdh & Zetterqvist, 1974; Mårdh & Lindahl, 1977).

The conventionally accepted Albers–Post mechanism (Post et al., 1969; Siegel & Albers, 1967) for the Na^+,K^+-ATPase catalyzed hydrolysis of ATP is outlined in eq 2.



(2)

Post et al. (1972) explained the K^+ induction of phosphatase activity in terms of preferential affinity for one of the nonphosphorylated enzyme conformational states (E_2) for K^+. The remarkably slow $E_1 \rightarrow E_2(K^+)$ transition (Karlish et al., 1978) provides a test of the hypothesis that $E_2(K^+)$ is the catalyst for FAP hydrolysis. In the absence of K^+ the major enzyme form is E_1, both in the presence and in absence of Na^+ (Hegyvary & Post, 1971; Jorgensen, 1975; Jorgensen, 1977). The conformational transition, $E_1 \rightarrow E_2$, should limit the rate of FAP hydrolysis when enzyme (in the absence of K^+) is mixed with FAP and K^+, as is experimentally illustrated in Figure 1. The most attractive mechanism for the transition (Karlish et al., 1978) is given by eq 3. If the $E_1 \rightleftarrows E_2$

$$E_1 + K^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1{\cdot}K^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2{\cdot}K^+ \qquad (3)$$

equilibrium favors E_1, and K^+ ligation is fast relative to conformational changes, then for $[K^+] \ll K_1$, the dissociation constant for $E_1(K^+)$, the observed rate constant is given by eq 4. The K^+ concentration necessary to displace half the

$$k_{obsd} = k_2([K^+]/K_1) + k_{-2} \quad (4)$$

enzyme to E_2 is given by eq 5, where $K_2 = k_2/k_{-2}$. Thus, the

$$K^+ = K_1/(K_2 - 1) \quad (5)$$

rate is dependent upon binding of K^+ to low-affinity E_1 sites, but the poise of the equilibrium is drawn to the right by a large value of K_2.

Implicit in the application of this analysis to the turnover experiment is the assumption that enzyme conformations are either active or inactive with respect to catalysis of FAP hydrolysis. If true, the reaction rate at zero time would reflect the poise of the E_1, E_2 equilibrium in the absence of K^+. After correction for the basal ion-independent rate of enzyme-catalyzed hydrolysis, this ratio is found to be 0.06, similar to earlier reports of a very low (but finite) population of E_2 in the absence of K^+. The ratio of the (extrapolated) velocity at zero time after mixing to the later steady-state velocity is independent of the K^+ concentration although the individual rates are K^+ dependent. The independence of the velocity ratio on K^+ indicates that the same K^+-dependent process limits E_2-catalyzed hydrolysis at both early and late times. Only the ratio of E_2 to E_1 changes after the mixing of enzyme with K^+.

The presence of Na^+ influences the FAP reaction velocity only at low [FAP]. The lack of inhibition by Na^+ at high [FAP] demonstrates that FAP is itself a positive effector of the E_2 state. But even at high [FAP] the rate of hydrolysis is dependent on the concentration of K^+. In addition, the maximum phosphorylation yield is attained at lower potassium concentration than is the maximum rate of turnover. Hence, there must be a second role for K^+ (in addition to induction of E_2) in the hydrolytic reaction mechanism. Potassium activation parameters are the same at near saturating and subsaturating FAP concentrations (Table I), a result consistent with the hypothesis that the second role for K^+ lies in the activation of the hydrolytic rate of E_2–P, a role that requires sequential K^+-dependent forms of E_2–P. Similar conclusions have been reached by Chipman & Lev (1983).

It is not unprecedented to postulate that cation ligation induces additional states to the conventionally accepted E_1 and E_2 states, since ligation by Na^+ is known to be required for phosphorylation of E_1 by ATP (Mårdh & Post, 1977). An alternative hypothesis is that there exist discrete catalytic sites: a "kinase" site and a "phosphatase" site; each activity must be induced to an active conformation upon K^+ ligation at the appropriate site. Data presented herein and the results of Chipman & Lev (1983) demonstrate only a single set of K^+ binding sites. The model with two sets of K^+ sites requires multiple sites in each set, because of the cooperativity of K^+ activation of both turnover and phosphorylation yield. There is no compelling reason to invoke this more complicated model. Any detailed mechanism for catalysis and transport will have to take account of these additional K^+-induced E–P conformational states.

ATP in the presence of sodium can activate the potassium-dependent *p*-nitrophenylphosphatase activity (Nagai & Yoshida, 1966; Robinson, 1969; Post et al., 1972). The presence of Na^+ reverses the inhibition of FAPase by ATP (Table I). In the phosphatase activation, the mechanism is believed to involve the steady-state production of substantial $E_2(K^+)$ via the faster ATPase cycle [e.g., see Robinson & Flashner (1979)], since the transition $E_2 \rightarrow E_1$ is relatively slow at some concentrations of Na^+ and K^+ (Mårdh & Lindahl, 1977; Smith et al., 1980; Moczydlowski & Fortes, 1981b). Activation by Na^+ and ATP is clearly a consequence of the ATPase reaction pathway since the ATP analogue AMP-PNP is a more potent inhibitor in the presence of Na^+ than in its absence. If the only role of K^+ in the stimulation of FAP hydrolysis were to induce the E_2 state, then K^+ stimulation would be altered by the addition of Na^+ plus ATP, contrary to what is observed (Table I).

Table IV: Model-Dependent Parameters for K^+ Stimulation of Fractional Phosphorylation and Steady-State FAP Turnover at 25 °C[a]

parameter	value	parameter	value
n	3	Q_1 (mM)	1.20 ± 0.26
k_1 (s^{-1})	290 ± 48	Q_1 (mM)	150 ± 73
k_2 (s^{-1})	250 ± 28	Q_2 (mM)	0.68 ± 0.15

[a] The data in Figure 7 showing potassium dependence of steady-state FAP turnover and the level of phosphorylation were simultaneously fitted to the model given by eq 5. The errors in the parameters are rms estimates. These values predict the dashed curves in Figure 7.

Oligomycin, which blocks the E_1–P to E_2–P transition, does not affect FAPase activity at 25 °C, consistent with the exclusive phosphatase pathway at this temperature. In contrast, at 25 °C oligomycin inhibits ATPase by 50% under the same ionic conditions. Me_2SO blocks K^+-induced hydrolysis of phosphoenzyme (Albers et al., 1972). Since Me_2SO inhibits FAP turnover, it must affect the distribution among the various E_2–P states. Identical inhibition of FAPase and ATPase by Me_2SO is indicative of a common phosphorylated intermediate, whose hydrolysis is K^+ dependent. It is interesting that turnover of acetyl phosphate, where phosphoryl-enzyme is an intermediate (Bond et al., 1971), is also inhibited by Me_2SO.

The multiple sites involved in potassium activation of E_2–P hydrolysis might correspond with the sites involved in the transport of two K^+. However, curve fitting of the K^+-dependent rate, and phosphorylation yield, data does not permit a precise estimate of the Hill coefficient, and consequently of the number of binding sites for K^+; the desired value of n_H lies between 2 and 4 as will be discussed; see (Table IV). There are some differences in the K^+ affinity for E_2 vs. E_2–P. These differences are not very large. The large changes in K^+ affinity must therefore lie in the transition $E_2 \rightarrow E_1$.

A variety of results suggest that the binding of a *single* K^+ is required for effecting the transformation $E_1 \rightarrow E_2(K^+)$. This conclusion is suggested by the finding that the formation of active FAPase shows a hyperbolic rather than cooperative dependence on K^+ concentration (Figure 1) and by the studies of Karlish et al. (1978), Karlish (1979), and Beaugé & Glynn (1980), who found similar noncooperative K^+ dependence for the transition ($E_1 \rightarrow E_2$).

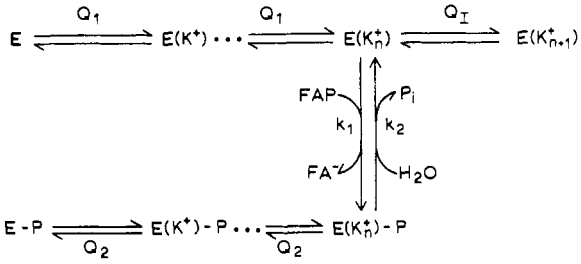
An analysis of the K^+-dependent phosphorylation yield and turnover rate experiment at 25 °C with FAP supports a maximum of only five parameters and leads to the model of Scheme I. The equations for turnover number (k_{obsd}) and fractional phosphorylation (ϕ_p) are given in eq 5a and 5b, respectively. Both the steady-state velocity of FAP hydrolysis

$$k_{obsd} = k_1k_2(K^+)^n/[k_1Q_2{}^n(1 + K^+/Q_2)^n + k_2Q_1{}^n(1 + K^+/Q_1)^n(1 + K^+/Q_1)] \quad (5a)$$

$$\phi_p = k_1Q_2{}^n(1 + K^+/Q_2)^n/[k_1Q_2{}^n(1 + K^+/Q_2)^n + k_2Q_1{}^n(1 + K^+/Q_1)^n(1 + K^+/Q_1)] \quad (5b)$$

and the steady-state phosphorylation yield (ϕ_p) can be quantitatively described by the model and derived parameters of Scheme I, where the number of K^+ sites that activate catalysis

Scheme I



is equal to 2–4. Since the best correspondence of model to the experimental data is obtained on the assumption that $n = 3$, the calculated parameters for $n = 3$ are listed in Table IV.

When Q_1, Q_2, and Q_I are combined according to Scheme I, the calculated $K_{0.5}$ is in good agreement with the observed value. This confirms our argument that linked ligand effects on both phosphorylated and nonphosphorylated enzyme states are appropriate to a correct description of the catalytic pathway.

FAP is rapidly hydrolyzed relative to ATP because phosphorylation of E_2 by FAP provides a reaction pathway which avoids the rate-limiting conformational transitions in the ATPase pathway. At 0 °C, but not at 25 °C, in the presence of Na^+ and absence of K^+ there is stoichiometric phosphorylation of the ATPase by FAP. This suggests that, at 0 °C, FAP is also a substrate for the Na^+/K^+-coupled ATPase pathway. Alternatively, E_1–P might also arise by an Na^+-induced conformational transition from E_2–P. The relative rates of sodium- and potassium-dependent phosphorylations, the expected poise of the $E_1 \rightleftarrows E_2$ equilibrium, and the rate of the $E_1 \rightarrow E_2$ transition argue against an E_2–P pathway for E_1–P formation under the ionic conditions that generate detectable E_1–P.

Sodium-dependent phosphorylation by ATP is rapid relative to K^+-induced ATP turnover so that appreciable phosphoryl-enzyme is observed in the steady state (Mårdh, 1975b; Mårdh & Lindahl, 1977). The difference between FAP and ATP as substrates for the ATPase pathway lies in the rate of formation of Na^+-dependent E_1–P. At concentrations of K^+ near saturation for FAP hydrolysis, any substantial induction of E_1 states by Na^+ must inhibit the turnover of FAP and the steady-state phosphorylation yield. At low $[K^+]$, where phosphorylation of E_2 is slow, appreciable Na^+-dependent phosphorylation of E_1 should activate turnover of FAP. At high FAP concentration and saturating K^+, no inhibition or activation of the K^+-stimulated FAPase by Na^+ is observable at either 0 or 25 °C. With respect to the difference in Na^+ effects at 0 vs. 25 °C, the important observations are that at 0 °C and low $[K^+]$, Na^+ increases both the rate of turnover and phosphorylation yield *severalfold* (the latter being stoichiometric in the absence of K^+), while at 25 °C and otherwise identical conditions, Na^+ has *no* measurable effect on the turnover rate and there is no evidence for transient formation of even a 5% yield of phosphoryl-enzyme (Odom et al., 1981). At 0 °C, in the presence of Na^+, at low $[K^+]$, there is more phosphoryl-enzyme formed from FAP than is needed to maintain the observed turnover from E_2–P. This excess must be E_1–P. The calculated residual phosphoryl-enzyme concentration (E_1–P) becomes negligible above 0.5 mM K^+, as the K^+-stimulated rate of phosphorylation of E_2 becomes appreciable (consistent with the loss of Na^+-dependent activation of turnover).

In regard to the qualitative differences in $[Na^+]$-dependent rates of FAP turnover at 0 vs. 25 °C, a simple calculation proves informative. From the extrapolated turnover numbers at saturating $[K^+]$ (33 s^{-1} at 0 °C; 150 s^{-1} at 25 °C) we calculate an activation energy of 9.8 kcal/mol for hydrolysis via the E_2 pathway exclusively, a value typical for other enzyme-catalyzed reactions. The coupling of the Na^+-dependent phosphorylation pathway and the K^+-dependent phosphoenzyme hydrolysis, which is detectable at 0 °C, does not occur appreciably at 25 °C, presumably due to thermal inactivation of E_1 at 25 °C. At 0 °C the $E_1(Na^+)$ phosphorylation rate is sufficiently fast that the two pathways will couple at moderate $[K^+]$. The extent of inactivation of the $E_1(Na^+)$ pathway for FAP hydrolysis at 25 °C is difficult to assess quantitatively from FAPase kinetics, because of the appreciable background cation-independent turnover rate at low $[K^+]$. At 25 °C in the absence of K^+ and at optimal Na^+ and half-saturating FAP, we find no evidence for a burst of phosphorylation by FAP within the time regime of the first FAP turnover (Odom et al., 1981). Therefore, at 25 °C, the Na^+-activated phosphorylation rate must be less than the cation-independent turnover rate (2 s^{-1}). Thus, the rate constant for phosphorylation of $E_1(Na^+)$ is less at 25 °C than at 0 °C, a further indication that a cooperative thermal destabilization of E_1, but not of E_2, occurs at 25 °C in the absence of ATP, the positive effector of E_1 structural stability.

It is interesting that there is no evidence for uncoupling of ATP hydrolysis at 25 °C under similar conditions. The overall Na^+/K^+-dependent optimal hydrolysis rate for ATP is only a few fold slower than the potassium-stimulated FAPase rate. Hence, the $E_1(Na^+)$ phosphorylation rate is far more rapid for ATP than for FAP. Since there is no evidence for phosphorylation of $E_2(K^+)$ by ATP, this process must be far slower with ATP than with FAP.

A wealth of experiments demonstrates that vectorial transport of ions via the Na^+,K^+-ATPase occurs concomitant with the $E_1 \rightarrow E_2$ conformational transition [for review, see Kyte (1981) and Robinson & Flashner (1979)]. ATP is special in its preferential ligation to E_1. Phosphorylation of the enzyme either by ATP or FAP leads to preferential stabilization of the E_2 state. FAP ligates preferentially to the E_2 state prior to phosphorylation, hence bypassing the translocation-dependent conformational transition upon phosphorylation. However, FAP hydrolysis can be coupled to this translocation-dependent conformational transition at 0 °C.

The ability to couple or uncouple the Na^+-dependent phosphorylation pathway to the K^+-dependent hydrolytic pathway by manipulation of temperature is a unique feature in the utilization of FAP as substrate. Studies of the transient details of the FAPase mechanism may provide new information about the coupling mechanism and its relation to ion translocation. The stereochemical and energetic differences that govern distinctions between ATP and FAP as substrate should be informative of the detailed mechanisms of energy utilization, as well as vectorial transport.

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