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Transient and Steady-State Kinetic Studies of Sodium–Potassium Adenosine Triphosphatase Using β-(2-Furyl)acryloyl Phosphate as Chromophoric Substrate Assay†

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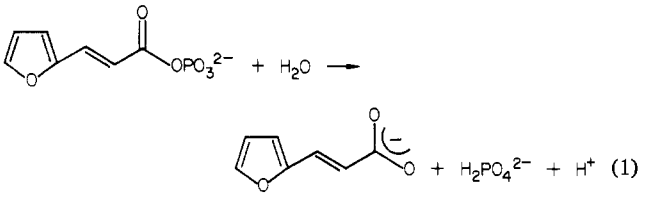
ABSTRACT: A convenient and highly specific continuous spectrophotometric assay for sodium–potassium adenosine triphosphatase activity utilizing the rapidly hydrolyzed and high-affinity chromophoric substrate β-(2-furyl)acryloyl phosphate (FAP) is described. The Na/K-ATPase-catalyzed hydrolysis of FAP is faster than that for ATP under all ionic conditions. The rate is neither inhibited nor activated by Na^+; it is dependent on $[K^+]$ and on $[Mg^{2+}]$. The hydrolysis of FAP to furylacrylate is accompanied by a large shift in the UV absorbance maximum. The spectrum of FAP, but not furylacrylate, is sensitive to noncovalent ligation with Mg^{2+}, a happenstance which permits the identification of Mg^{2+}FAP, and consequently allows for a probe of the role of Mg^{2+} in the catalysis. Mg^{2+} binding to the active site is essential for catalysis. MgFAP is more tightly bound to the site than is FAP^{2-}, but the complex is not obligatory for catalysis. The formation of a phosphoryl-enzyme intermediate is not evident in the reaction of FAP with the enzyme. Transient kinetic experiments, utilizing an excess of MgFAP, demonstrate a unique steady-state rate-limiting production of furylacrylate. These results indicate that the pathway demonstrated with ATP is not appropriate to the FAPase mechanism. The results suggest that acyl phosphates are good "phosphatase" substrates either because they are analogues of the phosphatase-specific phosphoryl-enzyme or because they react exclusively with the isomerized "E_2" form of the enzyme.

Ouabain-sensitive Na/K-ATPase[1] activity is conventionally measured by either quenched assays of the extent of inorganic phosphate formation or continuous assay via an ADP-dependent coupled enzymic process. The enzyme-catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP) has been used as a direct and continuous spectrophotometric assay for the K^+- and Mg^{2+}-dependent activity of Na/K-ATPase (Glynn & Karlish, 1975). The catalyzed hydrolysis of pseudosubstrates such as PNPP (the "phosphatase" activity) does not require sodium. Not surprisingly, the rate of catalyzed hydrolysis of PNPP is much slower than that of ATP. Recent studies with crab nerve Na/K-ATPase have shown that two other synthetic organic phosphates, 2,4-dinitrophenyl phosphate (DNPP) and β-(2-furyl)acryloyl phosphate (FAP), are enzymically hydrolyzed at a faster rate than ATP (Gache et al., 1976, 1977). The hydrolysis of these latter pseudosubstrates is unaffected by sodium concentrations up to that optimal for ATPase activity. At sodium and potassium concentrations optimal for ATP hydrolysis, FAP is hydrolyzed 3–4 times faster than ATP and twice as fast as DNPP. The enzymic hydrolysis of these chromophoric organic phosphates, like ATP hydrolysis, is inhibited by the cardiac glycosides ouabain and strophanthidin (Gache et al., 1977). The large difference in absorption spectrum between the product (furylacrylate anion) and the substrate (eq 1) facilitates the



(1)

analysis of the extent of hydrolysis (Malhotra & Bernhard, 1968). The kinetics of enzyme-catalyzed hydrolysis of FAP ("FAPase") in the transient and the steady state has been investigated in order to determine suitability of FAP for mechanistic studies as well as for routine quantitative Na/K-ATPase assays.

The results we present define such a routine quantitative assay. These results show that two less obvious factors merit

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[1] Abbreviations used: Na/K-ATPase, sodium–potassium-activated adenosine triphosphatase, EC 3.6.1.3; Ca^{2+}-ATPase, calcium-activated adenosine triphosphatase, EC 3.6.1.3; FAP, β-(2-furyl)acryloyl phosphate; PNPP, *p*-nitrophenyl phosphate; DNPP, 2,4-dinitrophenyl phosphate; Cl_3CCO_2H, trichloroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenedinitrilotetraacetic acid; P_i, inorganic phosphate; DMF, dimethylformamide.

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consideration before quantitation of the kinetic data. (1) The UV spectrum of FAP is shifted upon formation of the $Mg^{2+}FAP^{2-}$ complex so as to cause substantial changes in the Δ(absorbance) value for the conversion of FAP to furylacrylate. Failure to correct for this factor would lead to serious errors in stoichiometry, where the rate or amount of bond cleavage is being compared to the rate or amount of ion translocation, or in the transient state, where the rate of high-energy bond cleavage is compared to the rate of enzyme phosphorylation. (2) Sulfate ions from excess Na_2SO_4 in routine preparations of Na_2FAP solutions inhibit FAPase activity.

It should be noted that Kurzmack et al. (1981) and Rossi et al. (1979) have found the ion-translocating Ca^{2+}-ATPase from sarcoplasmic reticulum to have FAPase activity. The steady-state assay conditions we describe are appropriate for this latter enzyme if Ca^{2+} is added to the assay mixture. In contrast to the common steady-state FAPase and ATPase activities and the common transient-state ATPase mechanism exhibited by the two enzymes, the transient behavior toward FAP is notably different for the two enzymes. Transient kinetic studies of the FAPase activity of the Ca^{2+}-ATPase and their mechanistic implications are described in the accompanying paper.

Materials and Methods

Chemicals. β-(2-Furyl)acrylic acid was purchased from Aldrich Chemical Co. The sodium salt of *p*-nitrophenyl phosphate, the Tris salt of ATP, Dowex 50-W hydrogen form, isobutyl chloroformate, and strophanthidin were all purchased from Sigma Chemical Co. and used without further purification. Imidazole (Sigma) was recrystallized twice from benzene prior to use. Ion-exchange resin AG-1-X2 and Coomassie blue protein assay reagents were purchased from Bio-Rad Laboratories. Sodium cholate (pH 7.0) was prepared from cholic acid (Aldrich) and Lubrol WX (Supelco, Inc.) was used as received. All other reagents were of analytical grade. Solutions were prepared with twice-glass distilled water.

Preparation of Imidazolium PNPP. Dowex 50-W hydrogen form, washed with ethanol and then boiling distilled water, was packed to form a column 8 mm × 7.5 cm. The column was washed with 0.10 M imidazole until the pH was about 10, and a solution of the sodium salt of PNPP (100 mg in 0.35 mL of distilled water) was loaded onto the column. Distilled water was used to elute imidazolium PNPP. Fractions containing PNPP were detected spectrophotometrically (Gache et al., 1977). The pooled fractions (2.6 mL of 85 mM PNPP) were frozen and stored at −20 °C. The pH was adjusted to 7.0 with 0.1 M HCl before use in assays.

Preparation of Imidazolium FAP. The barium salt of FAP was synthesized according to the procedure of Malhotra & Bernhard (1968), slightly modified as follows. Triethylamine (27.7 mL; 0.2 mol), β-(2-furyl)acrylic acid (28 g; 0.2 mol), and ice-cold tetrahydrofuran (200 mL) were stirred in a reaction vessel in an ice bath. Isobutyl chloroformate (19.4 mL; 0.2 mol) was slowly added to the mixture with stirring. Sometimes the reaction mixture became too viscous, and consequently more ice-cold tetrahydrofuran was added. After 15 min the reaction mixture was filtered and the filtrate was concentrated to about 50 mL under reduced pressure with a rotary evaporator at about 15 °C. Pyridine (30 mL), concentrated (85%) phosphoric acid (20.4 mL; 0.3 mol), and dimethylformamide (DMF, 100 mL) were mixed. Immediately, the mixture was stirred into the concentrated furylacryloyl mixed anhydride filtrate maintained at 0 °C in an ice bath. Up to 100 mL of additional DMF was added as necessary to keep the mixture homogeneous. After about 20 min, 200–400 mL of ice-cold distilled water was added and the solution was rapidly extracted twice with diethyl ether. The clear DMF–H_2O bottom layer was stirred at 0 °C and brought up to pH 7.0 with NaOH, carefully avoiding warming. The barium salt of FAP was precipitated by the slow addition of 1.0 M $BaCl_2$ while the pH was maintained at 7. The precipitate was filtered, washed with ethanol until free of pyridine, and dried under vacuum; 75 g of solid containing some $Ba_3(PO_4)_2$ was obtained. The dried barium salt remained stable indefinitely when stored at −20 °C in the dark.

For studies on the ion dependence of the rate of enzyme-catalyzed hydrolysis of FAP, it was necessary to have FAP solutions that were free of sodium, potassium, phosphate, and sulfate ions. Since sulfate, which was present in the original procedure to free the dianion from the barium complex (Malhotra & Bernhard, 1968), was found to inhibit the enzymic hydrolysis of FAP, it was necessary to modify the procedure so as to avoid the use of sulfate. Finely powdered barium FAP (0.8 g) was stirred with 2 L of 1 mM imidazolium chloride (pH 6.2) for 1 h at room temperature. The solution was then spun at 7400*g* for 10 min to remove undissolved barium FAP and barium phosphate. The supernatant was loaded onto a short, broad, rapidly flowing column (33 mL/min) formed from about 7 mL of Bio-Rad AG-1-X2 resin equilibrated with 1 mM imidazolium chloride, pH 6.2, at 4 °C. The resin with its adsorbed FAP was then immediately removed and packed into a small column in a 10-mL disposable syringe. Barium was removed by washing the column with 1 mM imidazolium chloride at room temperature until the eluate no longer formed a precipitate with Na_2SO_4. The column was then washed with 0.2 M imidazolium chloride, pH 6.2, until the eluate was free of phosphate, as indicated by lack of precipitation with $BaCl_2$. FAP was eluted with 0.5 M imidazolium chloride, pH 5.5, and its elution was monitored spectrophotometrically at 306 nm. Furylacrylate [λ_{max} 292 nm ($\epsilon = 2.4 \times 10^4$ M^{-1} cm^{-1})] (Bernhard et al., 1965) was eluted from the column on the trailing edge of the FAP peak so only those fractions with a λ_{max} of 307 nm were pooled. The pooled fractions yielded 7–9 mL of 24 mM imidazolium FAP. The eluate could be stored for at least 2 months at −20 °C without noticeable hydrolysis. Slow hydrolysis occurred over a period of about 4 weeks at pH 7.0 and −20 °C. At room temperature FAP in solutions at pH 7.0 is stable for at least 1 day.

Malhotra has prepared FAP at low ionic strength by reprecipitating the purified FAP with $BaCl_2$ and redissolving the precipitate in a small aqueous volume in the presence of excess Dowex AG-50-W-X2 cation exchanger sufficient to bind all of the barium.

The preparation of [^{32}P]FAP is given in the accompanying paper (Kurzmack et al., 1981).

Enzyme. Na/K-ATPase, purified from the electric organ of *Electrophorus electricus* by using a modification of the method of Perrone et al. (1975), was kindly supplied by Dr. Lowell Hokin. The purified enzyme was stored at a concentration of 0.67 mg/mL in 250 mM sucrose–1 mM Tris–EDTA, pH 7.00, at −70 °C. The activity remained stable over a period of at least several months. Na/K-ATPase from the electric organ of *Torpedo californica* was purified by the method of Dixon & Hokin (1978) except that the final Lubrol concentration used for solubilization was 2.6%. Sheep kidney medulla were homogenized and microsomes were prepared using Kyte's procedure (Kyte, 1971). The microsomes were frozen and stored at −20 °C without further purification. The

Na/K-ATPase activity of the microsomes was stable over a period of 2 years.

Assays. Analysis of the purified eel enzyme by atomic absorption showed that the total amount of endogenous sodium added to the assay from the enzyme preparation was on the order of 10^{-6} M. The assay concentration of strophanthidin (2×10^{-4} M) gave maximum inhibition without need for preincubation. Activities are reported as the strophanthidin-sensitive activity unless otherwise noted. All assays for enzyme activity are at 25 °C, pH 7.0, in an assay volume of 1.0 mL. The assay was initiated by addition of enzyme. Spectrophotometric changes were monitored on a Cary 14 spectrophotometer, although much less elaborate instruments are equally appropriate. Spectrophotometrically determined rates were calculated from the linear change in optical density during the first 2–3 min of the reaction.

Although the maximum difference in extinction between FAP and furylacrylate occurs at about 330 nm, the hydrolysis of FAP was monitored at between 320 and 360 nm, generally at 356 nm ($\Delta\epsilon = 6.7 \times 10^2$ M^{-1} cm^{-1} in the presence of 50 mM Mg^{2+}), to obviate the high absorbance of FAP at lower wavelengths. Standard assay concentrations were 3 mM FAP, 50 mM $MgCl_2$, 20 mM KCl, and 100 mM imidazole–imidazolium chloride. Since the spectrum of FAP is Mg^{2+} sensitive, the molar extinction at any wavelength in the presence of Mg^{2+} was determined by the appropriate addition of Mg^{2+} to FAP solutions of known absorbance. The spontaneous hydrolysis of FAP under these conditions was negligible.

Enzyme activity with ATP ("ATPase") was measured by using the Fiske–Subbarow method (Fiske & Subbarow, 1925) with assay concentrations of 3 mM ATP, 30 mM histidine, 3 mM $MgCl_2$, and various concentrations of NaCl and KCl. PNPP activity was assayed spectrophotometrically by monitoring the appearance of nitrophenylate at 410 nm ($\epsilon = 8.0 \times 10^3$ M^{-1} cm^{-1}, pH 7.0). Concentrations were 5 mM PNPP, 20 mM $MgCl_2$, and 100 mM imidazole–imidazolium chloride. Na^+ and K^+ concentrations were variable.

Activities per site were determined by active-site titration of the enzyme with vanadate as described by Cantley et al. (1977) except that FAPase rather than ATPase activity was titrated.

Protein concentrations were determined by using the Lowry assay on Cl_3CCO_2H-precipitated protein as described by Bailey (Bailey, 1967), or by using the Bio-Rad protein assay based on the procedure developed by Bradford (Bradford, 1975).

Rapid Kinetic Measurements. The rapid kinetic experiments were all carried out with a single-beam stopped-flow spectrophotometer constructed at the University of Oregon and described previously (Seydoux & Bernhard, 1975). The instrument, equipped with a 0.8 cm pathlength combined mixing and flow cell, has a dead time of about 0.5–1.0 ms. In the wavelength range of these experiments (330–360 nm) it has a sensitivity of ±0.0001 OD. The instrument software permits on-line access to a Varian 1620 computer.

Results

Dependence of FAP Spectrum and "FAPase" on Magnesium. In the presence of sufficient Mg^{2+}, the absorption spectrum of FAP is shifted by 2–3 nm to higher wavelengths (Figure 1). Figure 1, line A, shows the difference spectrum of FAP in the presence and absence of 50 mM $MgCl_2$. For comparison, the difference spectrum for furyl-acrylate under the same conditions is shown in Figure 1, line B. In the presence of 50 mM Mg^{2+} (assay conditions) the absorption difference between FAP and furylacrylate was measured

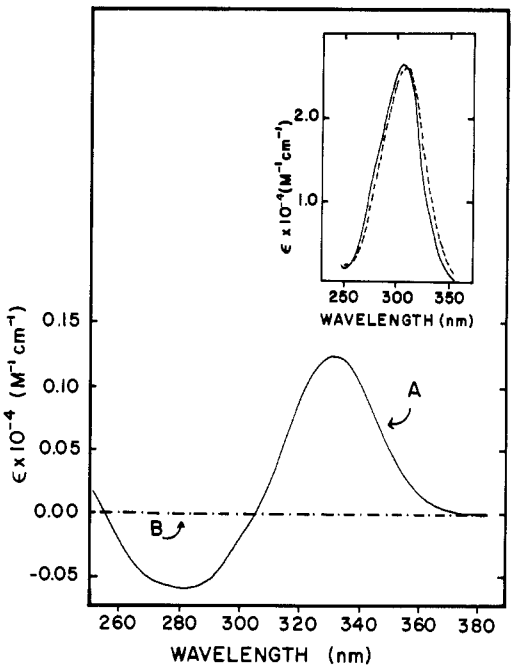


FIGURE 1: Difference spectra of FAP (A) and of furylacrylate (B) in the presence vs. the absence of 50 mM Mg^{2+}. All spectra were measured at pH 7.0, 25 °C, in the presence of 100 mM imidazole–imidazolium, 100 mM NaCl, and 20 mM KCl. Matched split cuvettes were used with FAP (or furylacrylate) in one side of the cuvette and Mg^{2+} in the other with all other components in equal concentration in both sides. The solutions in the sample cuvette were mixed and the difference spectrum was recorded. Furylacrylate was obtained by heating FAP in a sealed test tube 20 min at 70 °C. Insert: Spectrum of FAP in the absence (—) and presence (- - -) of 50 mM Mg^{2+}.

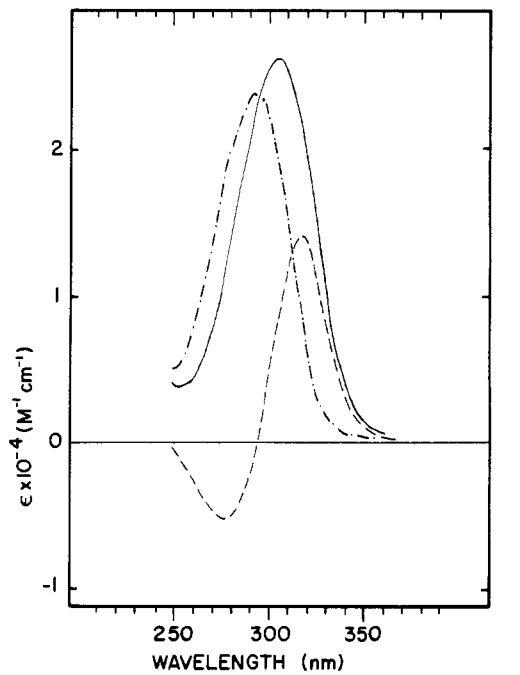


FIGURE 2: Absolute and difference spectra of FAP vs. furylacrylate in the presence of Mg^{2+}. Buffer conditions as in Figure 1, but with the addition of 50 mM $MgCl_2$. (—) Absolute spectrum of FAP; (·-·-) absolute spectrum of furylacrylate; (- - -) difference spectrum of FAP vs. furylacrylate. Sample and reference cuvettes both contained enzyme and FAP. Furylacrylate was generated by mixing the two sides of the reference cuvette and allowing complete enzymic hydrolysis of FAP.

(Figure 2). The absorption maximum shifts from 309 nm (MgFAP) to 292 nm (furylacrylate). The maximum difference is at ~330 nm.

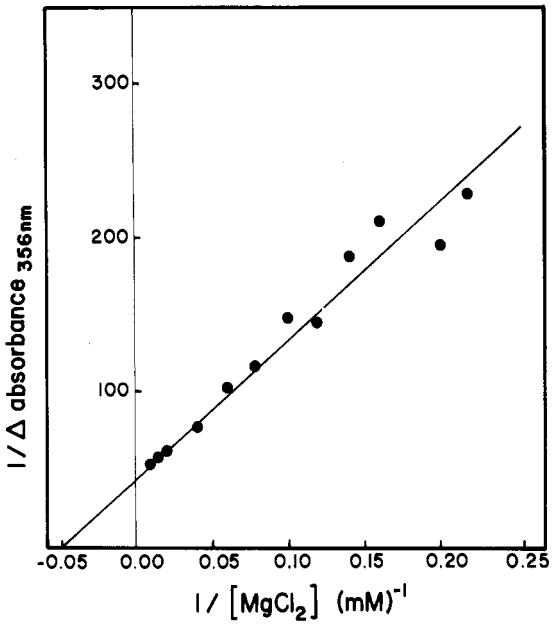


FIGURE 3: Interaction of FAP with magnesium. FAP (5 μL) was added to 3.00-mL samples containing imidazolium chloride, pH 7.0, and various concentrations of $MgCl_2$. The final concentration of FAP was 37 μM. Δ (absorbance) is the difference in the absorbance of FAP at 356 nm in the presence vs. the absence of Mg^{2+}.

Table I: Comparisons of Substrate Hydrolysis under Different Ionic Conditions[a]

ionic conditions	sub-strate	activity (μmol of P_i (mg of protein)$^{-1}$ min^{-1})
100 mM Na^+ + 20 mM K^+	ATP	7.0
	PNPP	0.3
	FAP	21.5
no Na^+ + 20 mM K^+	ATP	<0.1
	PNPP	0.57
	FAP	23

[a] All of the assays of the purified eel Na/K-ATPase are done at pH 7.0, 25 °C. ATP assay conditions: 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine; PNPP assay conditions: 5 mM PNPP, 20 mM $MgCl_2$, 100 mM imidazole-imidazolium chloride; FAP assay conditions: 3 mM FAP, 50 mM $MgCl_2$, 100 mM imidazole-imidazolium chloride.

The magnesium-dependent spectral shift was utilized to determine the dissociation constant for the Mg^{2+}–FAP complex. Since the concentration of Mg^{2+} was in large excess over the concentration of FAP, a linear double-reciprocal plot of (absorbance change)$^{-1}$ vs. $[Mg^{2+}]^{-1}$ is predicted for a model of a 1:1 reversible association of FAP^{2-} and Mg^{2+}. This is confirmed by the results of Figure 3. A dissociation constant of 21 mM was determined by utilizing the analytical method of Eisenthal & Cornish-Bowden (1974) and Porter & Trager (1977).

The effect of $[Mg^{2+}]$ on the rate of steady-state hydrolysis of FAP both at saturating (3 mM) and subsaturating (50 μM) concentrations of substrate is shown in Figure 4. Figure 4A shows that the effect of Mg^{2+} at high (saturating) concentrations of FAP is different from its effect on FAP complex formation. The velocity reaches an optimum value at concentrations of Mg^{2+} insufficient to form substantial MgFAP in solution.

Comparison of the Hydrolysis of ATP, PNPP, and FAP by Purified Eel Na/K-ATPase. The strophanthidin-sensitive activity of the purified eel Na/K-ATPase was measured by utilizing various substrates under conditions nearly optimal

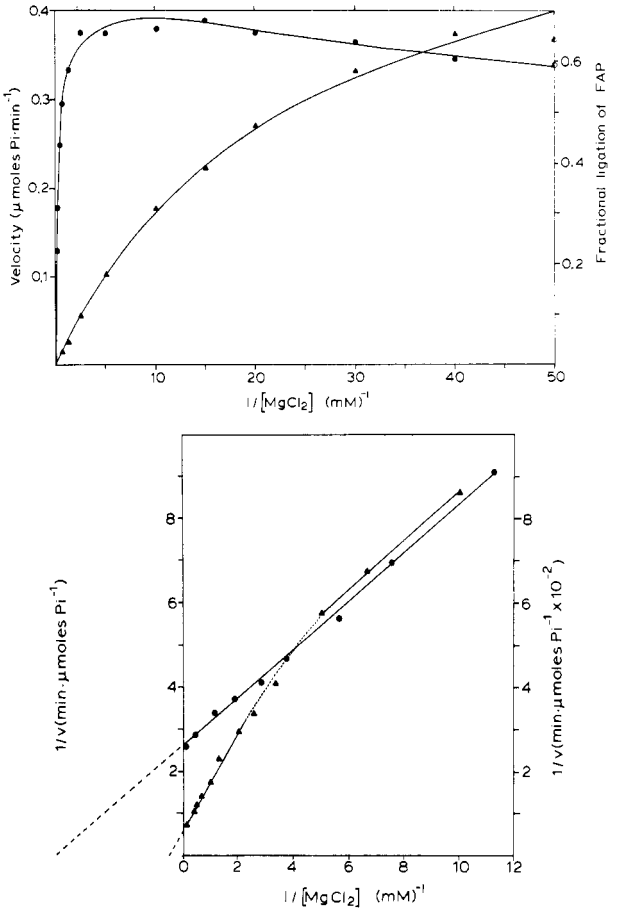


FIGURE 4: Effect of Mg^{2+} concentration on the steady-state rate of FAP hydrolysis. (A, upper) Assay conditions: 100 mM imidazolium chloride, pH 7.0, 100 mM KCl, 3 mM FAP, 0.12 mg of Torpedo electric organ enzymes; reaction volume 1 mL; temperature 25 °C. (●) Enzyme activity; (▲) fractional ligation of FAP with Mg^{2+} calculated from absorbances at t = zero (see Figure 3). Curve is theoretical for K_d of 21 mM. (B, lower) Assay conditions as in (A) except that (▲) FAP is 2.73 mM, (●) FAP is 50 μM, protein is 18 μg. Reaction monitored at 320 nm. Free Mg^{2+} calculated by using K_d for MgFAP complex of 21 mM.

Table II: Potassium Dependence of FAPase Activity[a]

$[K^+]$ (mM)	activity (μmol of P_i mg^{-1} min^{-1})
0.00	0.55
0.02	0.95
0.10	1.1
0.20	1.5
1.00	5.4
2.00	8.1
4.00	8.3
20.00	8.3

[a] All assays of the purified eel Na/K ATPase are done at pH 7.0, 25 °C, with assay conditions: 3 mM FAP, 50 mM $MgCl_2$, 100 mM NaCl, and 100 mM imidazole-imidazolium chloride.

for ATPase activity (100 mM NaCl, 20 mM KCl) and under nearly optimal phosphatase conditions (no NaCl, 20 mM KCl) at 25 °C, pH 7.0 (Table I). With the substrates tested, the activity was 92% (with PNPP) to 99% (with FAP and ATP) inhibitable by strophanthidin. Under both ATPase and phosphatase conditions the activity with FAP was about 3 times greater than the maximum activity with ATP. In the absence of sodium, activity with ATP is greatly reduced whereas FAP activity is essentially unchanged. The data in Table II show that the FAPase activity (like ATPase activity) is very sensitive to changes in potassium concentration up to

Table III: Strophanthidin Inhibition of Na/K-ATPase Activity

enzyme source	$[Na^+]$ (mM)	$[K^+]$ (mM)	substrate	strophanthidin-sensitive act. as fraction of total act.[a]
sheep kidney microsomes	100	20	ATP	0.55
	100	20	FAP	0.93
	0	20	ATP	*b*
	0	20	FAP	0.93
eel, purified	100	20	ATP	0.99
	100	20	FAP	0.99
	0	20	ATP	0.99
	0	20	FAP	0.99

[a] Concentration of strophanthidin was 2×10^{-4} M, 50 mM $MgCl_2$, pH 7.0, 25 °C. [b] Not detected.

about 10 mM K^+. The relative activities of the different substrates, found with eel Na/K-ATPase, are comparable to those obtained by Gache et al. (1977) using crab nerve Na/K ATPase. Likewise K_M for FAP was found to be similar with the two enzymes ($K_M \sim 0.5$ mM).

FAP hydrolysis is 25% inhibited in the presence of 15 mM sulfate under both ATPase and "phosphatase" conditions whereas ATPase activity is not measurably inhibited under these same conditions.

The results in Table I for FAP were obtained by using about 5 μg of the enzyme in 1 mL. Rates on the order of ~10 μM (furylacrylate) per minute could easily be measured at 356 nm. Rates as low as 0.1 μM/min could be measured by monitoring at shorter wavelengths by using a sensitive spectrophotometer with a precision of 0.001 OD. The rate of FAPase activity is 3.5 times that of the ATPase activity under optimal conditions for the Torpedo electroplax enzyme, giving a turnover number of 200 s^{-1} at 25 °C based on the molarity of vanadate binding sites. Therefore enzyme in the range of 10–100 pmol can be assayed conveniently. The sensitivity of the catalyzed-rate measurement is limited by the nonenzymic hydrolysis of FAP, which has a rate constant on the order of 10^{-4} min^{-1} under the conditions used for the assay.

Comparison of the Hydrolysis of ATP and FAP by Sheep Kidney Microsomes. Sheep kidney microsomes prepared according to the method of Kyte (1971) were assayed with both ATP and FAP (Table III). The hydrolysis of FAP was 93% inhibited by 2×10^{-4} strophanthidin, while only about 55% of the ATPase activity was so inhibitable. The strophanthidin-sensitive FAPase activity is about 3 times the strophanthidin-sensitive ATPase activity, a ratio which was also found for the purified eel enzyme. There appear to be strophanthidin-insensitive ATPases present in crude microsomal preparations for which FAP is a poor substrate.

Transient Reactions with FAP. The hydrolysis of ATP by the Na/K-ATPase can be shown to involve the steady-state formation of a significant fraction (0.2–0.4) of phosphoryl-enzyme per active site under near-optimal conditions of pH and specific ion concentrations (Glynn & Karlish, 1976; Mårdh & Lindahl, 1977). Since the turnover number for FAP hydrolysis is about 4 times that for ATP, one would expect a rapid, nearly stoichiometric pre-steady state burst of furylacrylate production *if* FAP forms a phosphoenzyme analogous to that formed from ATP. We therefore designed transient kinetic experiments to test for such a burst under conditions where we could have detected a burst on the order of 10% of enzyme-active sites. We chose wavelengths (350–356 nm) such that the initial total optical density due to 0.2 mM FAP would be less than 1, but a burst equivalent to the enzyme concentration used (10 μM) would lead to ~0.02 OD change. Nevertheless, we observed *no* significant burst of furylacrylate production, and the initial change in OD was as expected from steady-state turnover rates, whether the reaction was carried out in the absence or presence (to 0.1 mM) of K^+. A slow hydrolysis occurs in the absence of any added K^+, but we are uncertain as to whether this "basal" rate is due to K^+ contamination (Table II). We estimate from the precision of the measurements (Seydoux & Bernhard, 1975; Schwendimann et al., 1976) that any burst of furylacrylate production could not have been more than 10% of enzyme-active sites.

An attempt was made to detect stoichiometric furylacrylate production from excess FAP by strophanthidin-inhibited enzyme. Once again, no such reaction was detected within experimental limits (<5% of enzyme sites).

We have carried out preliminary experiments with labeled FAP in an attempt to detect phosphorylation of the enzyme. The enzyme from Torpedo was reacted with 4 mM [^{32}P]FAP (Kurzmack et al., 1981) and the reaction quenched after 3 s with Cl_3CCO_2H (Hokin et al., 1973). In the *presence* of 20 mM KCl and no Na^+ (conditions under which the turnover rate is maximal), the level of phosphorylation was not significantly greater than zero phosphoryl groups bound per vanadate binding site. In the presence of 100 mM NaCl and no added K^+ (conditions under which the turnover rate is 5% of maximum) about 0.3–0.4 equiv of phosphoryl groups was bound to the enzyme per vanadate binding site.

Discussion

FAPase Activity Measurement. The difference in the absorption spectrum of the reactant (FAP) vs. the product (furylacrylate) is useful for the continuous assay of Na/K-ATPase. The spectra of furylacryloyl derivatives are highly sensitive to noncovalent interactions with the environment (Bernhard et al., 1965). Thus it is possible to detect the interaction of specific ions such as Mg^{2+} with FAP. The detectable interaction can be exploited for the examination of the often discussed role of Mg^{2+} in enzyme-catalyzed nucleotide reactions.

The studies reported here show that at 50 mM Mg^{2+} (2.5 times the K_d) the FAP absorption spectrum is shifted to the red by 2 nm. Since there is no corresponding [Mg^{2+}]-dependent change in the furylacrylate spectrum, there is an increase in the absorption difference between substrate and product in the presence of 50 mM Mg^{2+}. This enhances the assay sensitivity at higher Mg^{2+} concentrations. Since a small shift in the absorption wavelength maximum results in a large change in this *difference* extinction at higher wavelengths, the [Mg^{2+}] perturbation of the FAP spectrum cannot be neglected when considering the stoichiometric relationships among bonds hydrolyzed, sites phosphorylated, and ions translocated.

The data of Figure 4 are consistent with a model in which E·Mg is the only catalytically competent species. The same apparent affinity for Mg^{2+} (K_d = 0.22 mM) is exhibited by the enzyme at high and at low extents of substrate saturation, at low concentrations of Mg^{2+}. At substantially higher concentrations of Mg^{2+}, a second [Mg^{2+}]-dependent effect is observable, but only at low extents of substrate saturation (Figure 4B). This is manifest in the nonlinear double-reciprocal plot. These data are quantitatively consistent with an enhanced affinity of the enzyme for MgFAP over FAP. A linear extrapolation of the rate data at higher [Mg^{2+}] and low [FAP] yields an estimated value of 1.7 mM for the K_d for Mg^{2+} in the Mg–enzyme-bound MgFAP complex. This value is an order of magnitude tighter than that for the aqueous MgFAP complex. Note, however, that the MgFAP complex is not required for catalysis.

At the concentrations utilized for solubilization of the enzyme, the detergents Lubrol WX and cholate did not interfere with the FAPase assay. The Fiske–Subbarow assay for inorganic phosphate (Banerjee et al., 1970) is seriously affected by the presence of these detergents. Higher concentrations of either detergent cause a loss of both ATPase and FAPase activity. The catalyzed hydrolysis of FAP by eel Na/K-ATPase is less susceptible to inactivation by detergents[2] than is the hydrolysis of ATP. Therefore, for development of purification methods involving detergents, the ATPase activity should be monitored. For routine purification by an established method, FAP provides a rapid means of monitoring ATPase activity.

In sheep kidney microsomes the fraction of FAPase activity which is not inhibited by strophanthidin is small (~7%) whereas strophanthidin-insensitive ATPase activity is substantial (~45%). This apparent selectivity of FAP by the Na/K-ATPase makes FAP a useful substrate for following the activity of this enzyme in the presence of other ATPases.

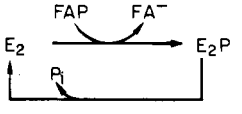
The use of FAP in assay of Na/K-ATPase has several advantages: (1) Enzymic activity is even higher than with ATP. (2) The use of FAP is easily adapted to the rapid assay of multiple samples. (3) FAP hydrolysis at saturating [K^+], in contrast to PNPP hydrolysis, is insensitive to [Na^+], and thus the presence of Na^+ does not interfere with the assay. (4) The sensitive spectrophotometric changes permit the measurement of transient enzyme–substrate reactions. Moreover, the synthesis of FAP is simple, and its long-term storage properties (both as the barium salt and in frozen solution as imidazolium FAP) make it readily available for use as needed.

Mechanistic Considerations. Despite controversy concerning the details of cationic sites and translocation mechanisms, it is generally agreed that the overall catalytic pathway for ATP hydrolysis in Na/K-ATPase follows the minimal mechanism of eq 2.

$$\begin{array}{ccc} \text{ATP} + E_1 & \rightleftharpoons & E_1P + \text{ADP} \\ \uparrow\downarrow & & \uparrow\downarrow \\ P_i + E_2 & \rightleftharpoons & E_2P \end{array} \qquad (2)$$

The breakdown of the total phosphoryl-enzyme must be at least partly rate determining, since a substantial fraction of phosphoryl-enzyme can be detected under conditions of high ATPase activity. Analysis of the transient kinetics of phosphoryl-enzyme formation from ATP (Mårdh & Lindahl, 1977) suggests that the rate-limiting step for this breakdown is in fact the conversion of E_1P to E_2P.

At lower K^+ concentrations, hydrolysis of E_2P becomes rate limiting. However, since with FAP we do not observe a significant amount of phosphoenzyme in the presence of K^+ and do not observe a burst of furylacryacrylate formation, the breakdown of phosphoryl-enzyme is clearly *not* rate limiting for the FAPase activity. Since the FAPase activity is higher than the ATPase activity (at any cation concentrations), this implies that the initial phosphoenzyme formed cannot be common to the two substrates. The most obvious alternative is that of eq 3. If the rate of hydrolysis of E_2P (~300 s^{-1}) estimated from experiments with ATP (Mårdh & Lindahl, 1977) were relevant to the FAPase reaction, the expected burst for the mechanism of eq 3 would have been near or below the precision limits of our experiments, but more significant phosphorylation in the presence of K^+ should still have been observed. An alternative mechanism is that FAP can be hydrolyzed directly by E_2 via a "phosphatase" mechanism. The proposal of a special mechanism, and hence a special site, for the very rapid hydrolysis of a pseudosubstrate is unattractive in principle. However, the phosphoryl-enzyme has been shown to be an acylphosphate (β-aspartoyl phosphate), and it may be that the FAP–enzyme complex is analogous to the E_2P structure which undergoes hydrolysis.[3]



(3)

It is important to note that with the Ca^{2+}-ATPase in SR vesicles, FAP clearly leads to substantial amounts of a kinetically competent phosphoenzyme and supports ion translocation (Kurzmack et al., 1981). It appears that in this case ATP and FAP form a similar phosphoenzyme. However, FAP hydrolysis, but not ATP hydrolysis, can be uncoupled from Ca^{2+} transport by dimethyl sulfoxide (Inesi et al., 1980). For both the Na/K- and Ca^{2+}-ATPases, phosphorylation from ATP and "phosphatase" activities are affected differently by detergents and organic solvents. It would appear that while the catalytic pathway for reaction of these two enzymes with ATP is similar, their reactions with FAP are different and variable, and reflect the details of the recognition sites for the nucleotide substrates (and, perhaps, for water). Such specific and conformation-dependent sites are, of course, a key element of the proposed coupling mechanisms for ion translation in these enzymes.

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[2] J. R. Brotherus, personal communication.

[3] One might also suggest that reaction with FAP leads to E_1P, but that the properties of E_1P formed from ATP are affected by nucleotides so that its breakdown is retarded via the slow $E_1P \rightarrow E_2P$ pathway, thus facilitating a mechanism of rapid hydrolysis without the accumulation of substantial phosphoenzyme. For reasons too complex for discussion here, this seems an unlikely hypothesis.

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Transient-State Kinetic Studies on the Mechanism of Furylacryloylphosphatase-Coupled Calcium Ion Transport with Sarcoplasmic Reticulum Adenosine Triphosphatase†

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ABSTRACT: Furylacryloyl phosphate (FAP) is an experimentally advantageous substrate for sarcoplasmic reticulum ATPase inasmuch as it sustains fairly high rates of transport (V_{max} $\simeq$ 15 nmol of calcium mg^{-1} s^{-1} vs. 90 nmol mg^{-1} s^{-1} with ATP) and displays chromogenic properties which can be utilized to monitor enzymic activity. This activity depends on Ca^{2+} concentrations similar to those observed for ATP, whereas higher Mg^{2+} concentrations ($\sim$10 mM) are required for optimal utilization of FAP compared to ATP. The substrate concentration dependence of the hydrolytic (phosphatase) activity and of the transport activity shows simple Michaelis-Menten behavior, in contrast to the bisphasic dependence obtained with ATP. The steady-state coupling ratio of FAP hydrolysis to Ca^{2+} transport is nearly 2.0 Ca^{2+} transported per phosphate generated at low Mg^{2+} concentrations but is reduced at higher Mg^{2+} concentrations (which are nevertheless required for optimal hydrolytic activity). Analogous to nucleotides, FAP hydrolysis by SR ATPase is via an intermediate phosphorylation of the enzyme. The mechanism of FAP degradation was monitored by stopped-flow and rapid-quench techniques utilizing absorption changes in the conversion of FAP to furylacrylate and by following the fate of ^{32}P radioactivity in [^{32}P]FAP. $^{45}Ca^{2+}$ transport was measured by rapid-quench methods in parallel experiments. It was found that upon addition of furylacryloyl phosphate, rapid phosphoryl transfer to the enzyme occurs. Maximal levels of phosphoenzyme are reached with a $t_{1/2} \simeq 60$ ms. A corresponding burst of furylacrylate production is detected with half-time $\simeq$ 100 ms. In addition, the initial enzyme phosphorylation is accompanied by translocation of 2 equiv of calcium per phosphoryl-enzyme bond formed. After these transient events, calcium transport and substrate hydrolysis proceed at a slower steady-state rate, demonstrating that a step subsequent to phosphorylation is rate limiting for recycling of the pump.

Both the Ca^{2+} transport and the hydrolytic activities of sarcoplasmic reticulum (SR) vesicles can be sustained by a variety of substrates which include ATP (Ebashi & Lipman, 1962; Hasselbach & Makinose, 1961, 1963), ITP (Makinose & The, 1965), acetyl phosphate (de Meis, 1969; Friedman & Makinose, 1970; Pucell & Martonosi, 1971), and *p*-nitrophenyl phosphate (Inesi, 1971; Nakamura & Tonomura, 1978). In addition to these substrates, furylacryloyl phosphate (FAP) was recently reported to be utilized by SR vesicles for both hydrolytic activity and Ca^{2+} transport (Rossi et al., 1979; Kurzmack et al., 1979).

FAP is an advantageous substrate because of its chromogenic properties which permit direct monitoring of furylacrylate production either upon hydrolytic cleavage of the substrate (Malhotra & Bernhard, 1968; Odom et al., 1981) or upon phosphoryl transfer to the enzyme. Taking advantage of these properties and of the availability of ^{32}P-labeled FAP, we have proceeded with a combination of steady-state and rapid kinetics methods to a detailed study of the mechanism of FAP utilization. Since we show that this substrate substitutes for ATP with high efficiency in the active vectorial transport of Ca^{2+}, we are confident that FAP-derived mechanistic conclusions are useful to the understanding of ATPase-coupled Ca^{2+} transport.

Materials and Methods

Materials. ATP, ITP, acetyl phosphate, and *p*-nitrophenyl phosphate were obtained from highest purity available commercial sources (Sigma, St. Louis, MO). NAD^+ was purchased from Boehringer Biochemicals.

Preparation of [^{32}P]*FAP.* β-(2-Furyl)acryloyl [^{32}P]phosphate was prepared via the equilibration of cold (unlabeled) FAP with inorganic [^{32}P]phosphate according to the catalytic pathway previously described (Malhotra & Bernhard, 1968, 1973).

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