Gache, C., Rossi, B., & Lazdunski, M. (1977) *Biochemistry 16*, 2957-2965.
Glynn, I. M., & Karlish, S. J. D. (1975) *Annu. Rev. Physiol. 37*, 13-55.
Hokin, L. E., et al. (1973) *J. Biol. Chem. 248*, 2593.
Inesi, G., Kurzmack, M., Nakamoto, R., de Meis, L., & Bernhard, S. A. (1980) *J. Biol. Chem. 255*, 6040.
Kurzmack, M., Inesi, G., Tal, N., & Bernhard, S. A. (1981) *Biochemistry* (following paper in this issue).
Kyte, J. (1971) *Adv. Cellular Metabolic Regulation.*
Malhotra, O. P., & Bernhard, S. A. (1968) *J. Biol. Chem. 243*, 1243-1252.
Mårdh, S., & Lindahl, S. (1977) *J. Biol. Chem. 252*, 8058-8061.
Perrone, J. R., Hackney, J. F., Dixon, J. F., & Hokin, L. E. (1975) *J. Biol. Chem. 250*, 4178-4184.
Porter, W. R., & Trager, W. F. (1977) *Biochem. J. 161*, 293-302.
Rossi, B., Loome, F., Gache, C., & Lazdunski, M. (1979) *J. Biol. Chem. 254*, 302.
Schwendimann, B., Ingbar, D., & Bernhard, S. A. (1976) *J. Mol. Biol. 108*, 123-138.
Seydoux, F., & Bernhard, S. A. (1975) *Biophys. Chem. 1*, 161-174.

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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0006-2960/81/0420-0486$01.00/0

Five milliliters of the equilibrating mixture originally contained 4.5 μmol of FAP, 0.125 μmol of halibut GPDH (18 mg), 2.5 μmol of NAD^+, and 5 μmol of inorganic phosphate (pH 7.0, containing a total of 1.0 mCi of $^{32}P_i$). This solution was maintained at pH 7.0 and 5 °C in an ethylenediamine–HCl buffer (7.4 mM) and the enzyme was stabilized by the addition of EDTA (3.7 μmol) and KCl (to a total concentration of 0.074 M). The reaction vessel was covered with aluminum and shielded, and the reaction was allowed to proceed overnight (18 h). Preliminary analyses indicated that this was nearly sufficient time to approach isotopic equilibrium in FAP. Still longer incubation times lead to substantial hydrolysis of the acyl-enzyme, an irreversible process. The high concentration of NAD^+ utilized is required both for effective catalysis (since the acyl-enzyme has low affinity for the NAD^+ cocatalyst (Malhotra & Bernhard, 1973) and in order to inhibit hydrolysis of the acyl-enzyme. The ratio of hydrolysis to phosphorolysis is lower at 5 °C than at room temperature. Halibut GPDH, prepared essentially according to the procedure of Seydoux et al. (1973) for sturgeon GPDH, was kindly furnished by biochemistry laboratory students at the University of Oregon. The yield of enzyme from this muscle source (~10 g/kg of muscle) and its very high specific activity (360–400 units/mg by the Ferdinand (1964) assay) make it an excellent source for "reagent"-scale acyl-enzyme preparations.

After 18 h, the radioactive mixture (5.0 mL) was placed at 5 °C on a 20-mL column of Dowex 1-X2 which had been pretreated as follows: The column was first washed with ~200 mL of a solution which was 1 M in KCl and 0.01 M in ethylenediamine–HCl (adjusted to pH 6.2). The column was then washed with 200 mL of a solution which was 0.1 M in KCl and contained 0.01 M ethylenediamine–HCl buffer and 0.001 M EDTA, pH 6.2. The reaction mixture was then placed on the column and eluted with 160 mL of this latter solution. This elution resulted in the removal of all of the radioactivity present in the form of inorganic phosphate. Following the elution of inorganic phosphate, a linear gradient of the above 0.1 M KCl, pH 6.2, buffer vs. a 1.0 M KCl–ethylenediamine buffer, pH 6.4, was prepared by utilizing 45 mL of each component. During the course of this gradient elution, all of the FAP was eluted as one sharp band at about 0.6 M KCl. The molar activity of the isolated [^{32}P]FAP was 50% of that of the original inorganic phosphate (theoretical yield at equilibrium 67%).

Methods. Ca^{2+} uptake by SR vesicles was started by the addition of substrate to a reaction mixture as specified in the legends to the figures. In steady-state kinetic experiments, it was interrupted by Millipore filtration (Martonosi & Feretos, 1964). Rapid kinetic experiments were carried out on a Durrum D-133 multimixing apparatus. The reaction mixture was quenched with EGTA or La^{3+} previous to Millipore filtration (Chiesi & Inesi, 1979). The amount of calcium taken up by the vesicles or the concentration of calcium remaining in the medium was determined by measuring radioactive ^{45}Ca tracer in the filters or in the filtrate.

FA production was followed in an Aminco dual-wavelength spectrophometer, taking advantage of the spectral shift due to the conversion of FAP to FA^- (Malhotra & Bernhard, 1968; Odom et al., 1981). The high extinction coefficient of FAP predictated a choice of wavelengths on the FAP spectral shoulder (340 vs. 365 nm or 350 vs. 370 nm).

Rapid kinetic experiments were carried out on a Durrum D-137 dual-wavelength stopped-flow photometer, using 340- and 365-nm interference filters (Ditric Optics, Marlboro, MA).

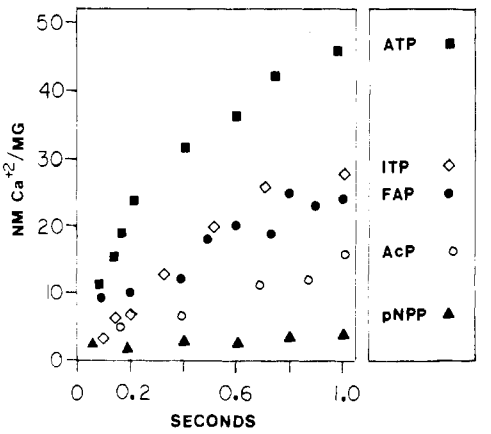

FIGURE 1: Transient ^{45}Ca uptake by SR vesicles (measured by rapid quenching) with several substrates. All substrate concentrations were 1 mM except *p*-NPP which was 10 mM. Reaction mixtures contained 80 mM KCl, 20 mM Mops (pH 6.9), 10 mM $MgCl_2$, 30–40 μM $^{45}CaCl_2$, and 0.25-0.35 mg of SR protein/mL.

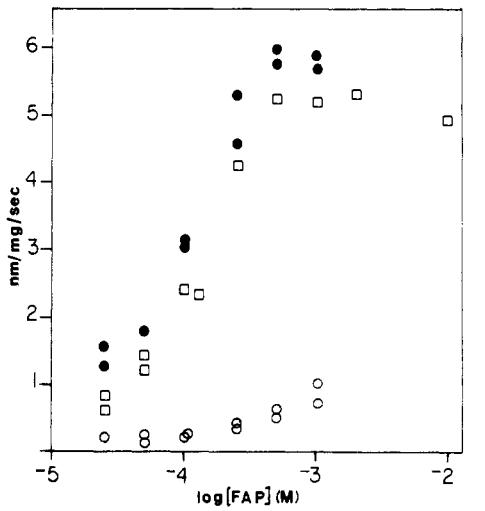

FIGURE 2: FAP concentration dependence of the steady-state rate of FA production (●), Ca uptake (□), and FA production in the absence of Ca (○). Enzymic rates are normalized per mg of SR protein.

Phosphoenzyme formation and P_i release following addition of [^{32}P]FAP to SR was determined as previously described for [γ-^{32}P]ATP (Chaloub et al., 1979). Rapid mixing and quenching was obtained in a Durrum D-133 multimixing apparatus.

Results

Steady-State Kinetics. SR vesicles utilize a variety of high-energy phosphate ester substrates for coupling the phosphorolytic reaction to active Ca^{2+} transport (Makinose & The, 1965; de Meis, 1969; Friedman & Makinose, 1970; Pucell & Martonosi, 1971; Rossi et al., 1979; Kurzmack et al., 1979). Comparative measurements of Ca^{2+} uptake by SR vesicles following addition of ATP, ITP, FAP, AcP, and PNPP at nearly saturating concentrations are shown in Figure 1. It is clear that even though ATP is the substrate which is utilized most rapidly, FAP can also sustain significant transport activity. Like ATP (Hasselbach & Makinose, 1961, 1963), FAP utilization for Ca^{2+} transport can be prolonged by the addition of oxalate.

The dependence of steady-state rate of transport and of hydrolysis on the FAP concentration (in the presence of oxalate) is shown in Figure 2. It should be noted that such a dependence is monophasic, indicating a single k_m of approx-

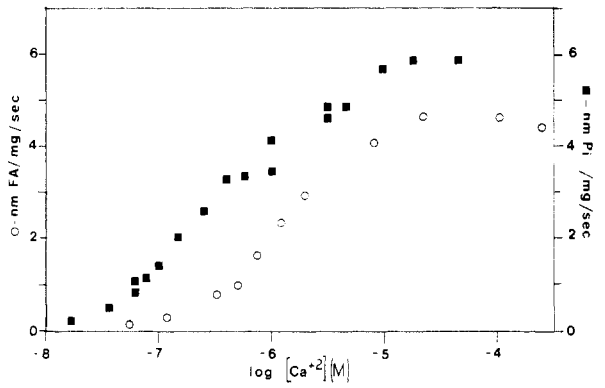

FIGURE 3: Ca^{2+} concentration dependence of the steady-state rate of FAPase and ATPase activity. The reaction mixture contained 20 mM Mops (pH 6.9), 80 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM potassium oxalate, 0.1 mM FAP or ATP, and 0.092 mg of SR/mL. In every experiment $[Ca^{2+}]_{total} \gg E_0$. A dissociation constant of 5.0×10^{-7} M (Schwartzenbach, 1957) was assumed for the Ca–EGTA complex.

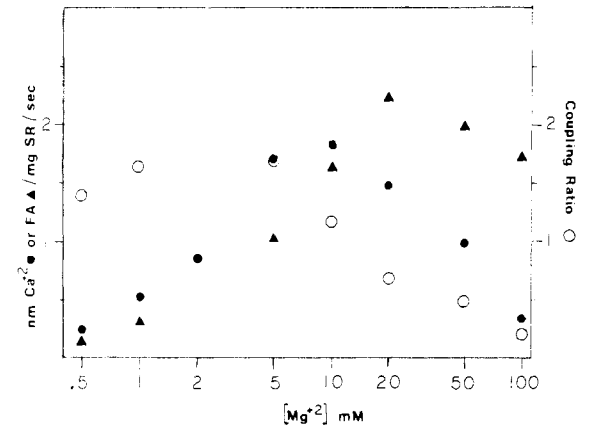

FIGURE 4: Mg^{2+} concentration dependence of the steady-state rate of FA production (▲) and Ca transport (●). FAP concentration was 100 μM. Other conditions are as in Figure 4. The ratio of $^{45}Ca^{2+}$ transport per E~P hydrolyzed is also indicated (○).

imately 2×10^{-4} M. On the contrary, it is well-known (Inesi et al., 1967; Yamamoto & Tonomura, 1967) that the dependence of transport and hydrolytic activity on ATP concentration follows a complex biphasic curve, with activation at high ATP concentrations. Just such an interesting difference between ATP and pseudosubstrates was recently pointed out by Rossi et al. (1979).

The dependence of the velocity of FAP hydrolysis on Ca^{2+} concentration is illustrated in Figure 3. Enzyme activation is obtained between 10^{-7} and 10^{-5} M Ca^{2+}. This concentration dependence is only slightly higher than that observed with ATP. In the absence of Ca^{2+} ($[Ca^{2+}] < 10^{-8}$), only very low rates of hydrolysis are obtained at high FAP concentrations, analogous to the basal ATPase activity (Hasselbach & Makinose, 1961, 1963).

It is shown in Figure 4 that FAP, like ATP, utilization by SR requires Mg^{2+}. The same $[Mg^{2+}]$ dependence is observable in the hydrolysis of FAP catalyzed by Na/K-dependent ATPase (Gache et al., 1977; Odum et al., 1981). With this latter enzyme it has been shown that FAP affinity but not reactivity, is Mg^{2+} concentration dependent. Mg^{2+} is, however, a constituent of the catalytic site. Optimal activity with SR ATPase is obtained with 10 mM Mg^{2+}. Such an Mg^{2+} concentration is higher than that required for optimal utilization of ATP or for FAP hydrolysis with Na/K-ATPase (Odom et al., 1981), presumably due to a lower affinity constant for formation of E·FAP·Mg complex. Calcium transport is coupled to ATP

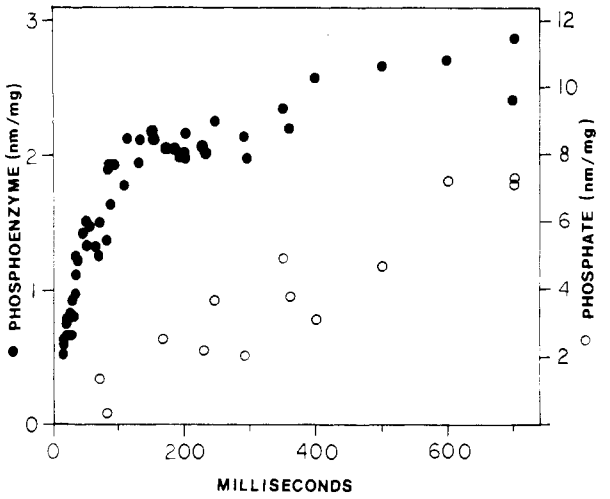

FIGURE 5: Transient formation of an acid-stable phosphoenzyme (●) and $^{32}P_i$ production (○) using [^{32}P]FAP. The reaction mixture contained 20 mM Mops (pH 6.9), 80 mM KCl, 10 mM $MgCl_2$, 0.1 mM Ca, 0.28 mg of SR/mL, and 0.1 mM [^{32}P]FAP.

hydrolysis under optimal conditions with a coupling ratio of $2Ca^{2+}/E{\sim}P$. The coupling ratio between calcium transport and FAP hydrolysis under conditions optimal for ATP-coupled transport is variable; it is usually less than two Ca^{2+} transported per FAP hydrolyzed (as can be calculated from the data of Figure 4). The most favorable ratios (Ca/FAP = 1.7) are obtained at low $[Mg^{2+}]$. Recently the uncoupling effect of organic solvents on the Ca^{2+} translocation–FAP hydrolysis has been reported (Inesi et al., 1980).

Rapid Kinetics: The ATPase Reaction. It is well-known that utilization of ATP as a substrate for the Ca^{2+}-dependent SR ATPase involves the rapid formation of a phosphorylated enzyme intermediate via the γ-phosphoryl of ATP (Yamamoto & Tonomura, 1967; Makinose, 1969; Martonosi, 1969; Inesi et al., 1970). By the use of [^{32}P]FAP we were able to demonstrate that a phosphorylated enzyme intermediate is also formed when FAP is the substrate. The reaction is Ca^{2+} dependent, and negligible enzyme phosphorylation occurs in the absence of Ca^{2+} ($[Ca^{2+}] < 10^{-8}$ M).

Satisfactory resolution of the enzyme–FAP phosphorylation kinetics is obtained by rapid-quench methods (Figure 5). In these experiments, the slower rate of P_i production can be shown to be concommitant with the formation of phosphoryl-enzyme; P_i formation proceeds linearly after steady-state levels of phosphoenzyme are attained.

The chromogenic properties of FAP as a substrate permit utilization of stopped-flow and optical methods to monitor furylacrylate (FA) production directly. This type of measurement is illustrated in Figure 6, in which oscilloscope traces of light absorption changes detected by double-wavelength differential photometry are shown. The absorbance change is due to formation of FA and, therefore, to either enzyme phosphorylation by or hydrolysis of FAP. It should be noted that an initial "burst" production of 3–4 nmol of FA/mg of protein occurs within the first 300 ms (see Figure 6B). FA release then continues at a slower rate (compare Figure 5 with Figure 6B,C) of approximately 8 nmol mg^{-1} s^{-1}. This rate is, within experimental uncertainties, the same as the rate of P_i production.

Rapid Kinetics: Ca^{2+} Translocation. Previously (Kurzmack et al., 1977), it was shown that enzyme phosphorylation by ATP is rapidly *followed by* translocation of the activating calcium ion from the outer side of SR vesicles. This translocation, which involves two calcium ions per phosphoryl transfer, is a kinetically distinct phenomenon (corresponding

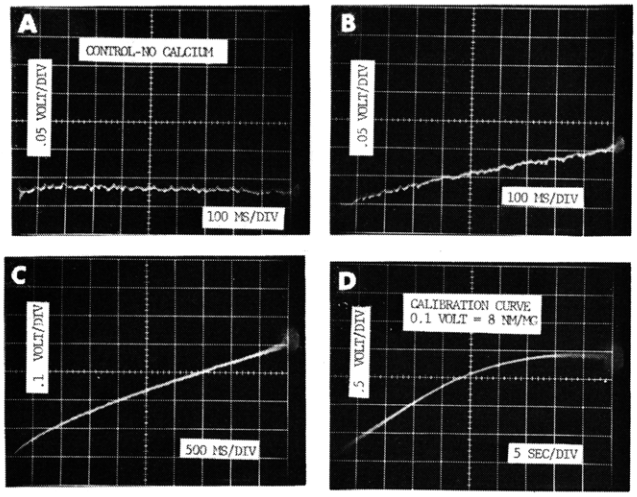

FIGURE 6: Transient production of FA at 25 °C, pH 6.9 (Dionex Durrum D-137 dual-wavelength stopped-flow spectrophotometer). The reaction mixture for (A) contained 20 mM Mops, 80 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM FAP, and 0.63 mg of SR/mL. For (B–D) 1 mM $CaCl_2$ was added to the reaction mixture to give a free Ca^{2+} concentration of about 20 μM; hence $[Ca^{2+}] \gg E_0$. Calibration of the scale was obtained by allowing all the FAP to react (D).

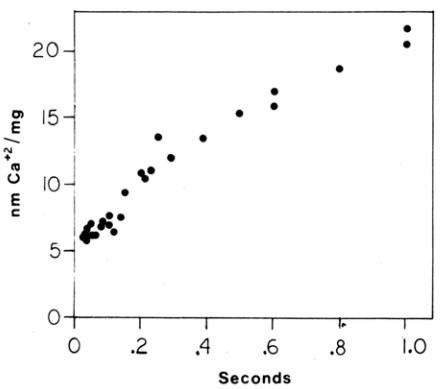

FIGURE 7: Transient calcium transport at 25 °C (multimixer with a lanthanum quench). The reaction mixture contained 20 mM Mops (pH 6.9), 80 mM KCl, 10 mM Mg, 30 μM $^{45}CaCl_2$, 100 μM FAP, and 0.3 mg of SR/mL. The reaction was quenched with 10 mM lanthanum chloride, 20 mM Mops, and 80 mM KCl.

temporally to the first cycle of the pump). P_i production and further calcium transport then continued at slower steady-state rates.

These transient-state phenomena can be demonstrated in rapid kinetic experiments by quenching the reaction with EGTA or La^{3+} and measuring fluxes of calcium tracer in serial samples. As is shown in Figure 7, when FAP is added as a substrate, an initial burst of calcium uptake (7–8 nmol/mg of protein) is observed as with ATP. The burst corresponds to two Ca^{2+} ions translocated per equivalent of enzyme which is phosphorylated within the first 300 ms of reaction (Figures 5 and 7).

It should be pointed out that when enzyme, preincubated with Ca^{2+}, is mixed with substrate, and either EGTA or La^{3+} is added 20 ms thereafter, the first enzyme turnover occurs even in the presence of these quenchers (Chiesi & Inesi, 1979). This is due to the rapid phosphorylation of the enzyme as compared to the slow dissociation of activating calcium from the enzyme (Sumida et al., 1978). For this reason, calcium translocation is already observed in a burst reaction (Figure 7) before dephosphorylation of the phosphoenzyme (compare Figures 5 and 7).

After the first enzyme turnover, binding of activating calcium to the recycling enzyme can be prevented by EGTA or La^{3+}. Further substrate utilization is thus completely inhibited; quenching with EGTA or La^{3+} is practically instantaneous, as demonstrated by isotope chase experiments (Chiesi & Inesi, 1979). After the initial "burst" translocation, further calcium transport occurs at a rate of 12–14 nmol mg^{-1} of protein s^{-1}, concomitantly with P_i production (Figure 7).

Discussion

Our experiments show that FAP, a pseudosubstrate with a high rate of coupled transport and hydrolytic activity, is utilized through a mechanism analogous to that of ATP. It is useful and convenient for studies of intermediate formation and for elucidating the rate-limiting steps, particularly in the form [^{32}P]FAP.

The catalytic cycle appears to be similar with FAP and ATP. Both require Mg^{2+} as an activator. The requirement for higher Mg^{2+} concentrations for optimal FAPase (Figure 4) may be in part related to the more facile dissociation of the Mg^{2+}·FAP complex, as compared to Mg^{2+}·ATP (Odom et al., 1981).

The Ca^{2+} concentration dependences of ATPase and FAPase activities are similar. The slightly higher Ca^{2+} concentrations required for FAPase activation (Figure 3) are likely related to the higher free Mg^{2+} concentrations used with this substrate: Mg^{2+} is weakly competitive for the Ca^{2+}-activating enzymic sites (Inesi et al., 1967).

An interesting aspect of these studies is the coupling ratio between Ca^{2+} transport and FAP hydrolysis in the steady state. We find this ratio to be variable under concentration conditions for which the ratio remains optimal with ATP. It is lower in the presence of high Mg^{2+} concentrations. It should be noted, however, that coupling ratios as high as 1.7–1.8 Ca^{2+} translocated per molecule of FAP hydrolyzed are obtained at lower (up to ~0.5 mM) Mg^{2+} concentrations. Therefore, it seems likely that the transport mechanism involves translocation of two calcium ions per enzyme cycle with both FAP and ATP as substrates under optimal coupling conditions.

The pathway of FAP utilization, as with ATP, includes the Ca^{2+}-dependent formation of a phosphorylated enzyme intermediate. The rates of enzyme phosphorylation with FAP are considerably slower than for ATP ($K \simeq 100\ s^{-1}$ at ATP saturation) (Froehlich & Taylor, 1975; Verjovski-Almeida et al., 1978). Nevertheless, the FAP-mediated phosphorylation rates are still sufficiently rapid to allow the buildup of substantial phosphoenzyme. In this regard, note that optimal phosphoenzyme yield is nearly the same with both ATP and FAP as substrate, and hence that this optimal yield must represent a virtual stoichiometric limitation rather than a balance between opposing kinetic processes. This result implies, therefore, that rate-limiting degradation of the phosphoenzyme, to a catalytic form competent for another round of catalysis and transport, is in some way dependent on protein interactions with the particular substrate (ATP or FAP) and or product (ADP or FA) structure. It should be pointed out that other pseudosubstrates such as *p*-nitrophenyl phosphate have very slow rates of phosphorylation (Inesi, 1971), rendering inhibition of the hydrolytic cleavage of the phosphoenzyme necessary in order to demonstrate appreciable levels of phosphoenzyme (Nakamura & Tonomura, 1978).

Enzyme phosphorylation is accompanied by the rapid formation of furylcrylate anion (FA) which can be monitored spectrophotometrically in stopped-flow experiments (Figure 6) (Gache et al., 1976, 1977; Odom et al., 1981). The ability to monitor the appearance of the leaving group concomitant

with phosphoryl transfer to the enzyme and efficient Ca^{2+} translocation is a feature of experiments with FAP. After the rapid formation of the enzyme intermediate and of FA, a slower rate of FA prodction which parallels the P_i production (Figures 5 and 6) is observed. The specific rate for this slower process is 4 s^{-1}. Since the rate of hydrolysis of the phosphoenzyme from inorganic phosphate is 17 s^{-1} under these conditions (Chaloub et al., 1979), rate control must be exerted prior to hydrolytic cleavage of the phosphoenzyme formed from FAP.

An initial burst of calcium uptake is observed in rapid-quench experiments with both ATP (Kurzmack et al., 1977) and FAP (Figure 7). These results indicate that calcium, bound to specific activating sites accessible to the bulk solvent, is displaced from the outer surface to a location which is not exposed to quenching reagents such as EGTA. Therefore, the phosphorylation reaction and the subsequent calcium translocation must be completed within a time shorter than that required for Ca^{2+} dissociation from the high-affinity solvent-accessible sites. This rate limit (17 s^{-1}) has been observed by Sumida et al. (1978) and by Rauch et al. (1978).

Extrapolation of the transient Ca^{2+} uptake data of Figure 7 to early times yields a burst amplitude of two Ca^{2+} ions per equiv of phosphoenzyme with FAP as with ATP. During the subsequent steady state this coupling ratio of 2:1 does not necessarily hold but can be less than 2 depending on environmental conditions, e.g., Mg^{2+} concentration (see Figure 4).

Our direct observations on the making and breaking of covalent bonds and on ion translocation demonstrate the minimal number of partial reaction steps illustrated below for utilization of FAP. The scheme is analogous to schemes already proposed on a qualitatively less direct basis for ATP-coupled transport.

$$E + 2Ca_{out} \rightleftarrows E{\cdot}2Ca$$

$$E{\cdot}2Ca + FAP \rightleftarrows FAP{\cdot}E{\cdot}2Ca$$

$$FAP{\cdot}E{\cdot}2Ca \rightleftarrows FAE{\sim}P{\cdot}2Ca$$

$$FA{\cdot}E{\sim}P{\cdot}2Ca \rightleftarrows E{-}P{\cdot}2Ca + FA$$

$$E{-}P{\cdot}Ca_2 \rightleftarrows E{-}P + 2Ca^{2+}_{in}$$

$$E{-}P \rightleftarrows E + P_i$$

In regard to the mechanism of enzyme catalysis, it should be noted that the phosphorylated intermediate of SR ATPase is an acyl (β-aspartoyl) phosphate arising from the transfer of the terminal phosphoryl of ATP to the enzyme (Degani & Boyer, 1973; Bastide et al., 1973). When the enzyme is presented with a substrate such as FAP which is itself an acyl phosphate, such a substrate is not hydrolyzed directly. Rather, FAP breakdown follows the pathway which includes the phosphorylated enzyme intermediate. This indicates that in SR ATPase, the phosphorylation and hydrolytic steps are coupled in an obligatory sequence which involves in addition the obligatory sequestering and translocation of Ca^{2+}.

The results presented herein appear notably different from those described in the preceding article on Na/K-FAPase. With SR ATPase, the pathways of Ca^{2+} transport and of phosphate bond cleavage are essentially the same for ATP and FAP. The enhanced FAPase activity and the lack of evidence for phosphoenzyme formation in the Na/K-FAPase reaction suggest an uncoupling of the FAPase and the cation (Na^+) transport in the detergent-solubilized enzyme. The possibility remains open that vesicular structural constraints rather than inherent enzyme protein structural and functional differences are responsible for the disparate results with the two enzymes. In this regard the results with SR ATPase vesicles which preserve the coupling, albeit more tenuously, even with the pseudosubstrates is presumably of greater physiological relevance. The similar ATPase mechanisms proposed for the two enzymes suggests that nucleotide interactions are important for the tight coupling of enzyme phosphorylation to ion translocation.

Acknowledgments

We thank Drs. Om Malhotra and Odile Viratelle, who supplied valuable advice and assistance during the preparation of this paper.

References

Bastide, F., Meissner, G., Fleischer, S., & Post, R. L. (1973) *J. Biol. Chem. 248*, 8385.

Chaloub, R., Guimaraes-Motta, H., Verjovski-Almeida, S., DeMeis, L., & Inesi, G. & (1979) *J. Biol. Chem. 254*, 9464.

Chiesi, M., & Inesi, G. (1979) *J. Biol. Chem. 254*, 10370.

Degani, C., & Boyer, P. (1973) *J. Biol. Chem. 248*, 8222.

De Meis, L. (1969) *Biochim. Biophys. Acta 172*, 343.

Ebashi, S., & Lipman, F. (1962) *J. Cell Biol. 14*, 389.

Ferdinand, W. (1964) *Biochem. J. 92*, 578.

Friedman, Z., & Makinose, M. (1970) *FEBS Lett. 11*, 69.

Froehlich, J., & Taylor, E. (1975) *J. Biol. Chem. 250*, 2013.

Gache, C., Rossi, B., & Lazdunski, M. (1976) *Eur. J. Biochem. 65*, 293.

Gache, C., Rossi, B., & Lazdunski, M. (1977) *Biochemistry 16*, 2957.

Hasselbach, W., & Makinose, M. (1961) *Biochem. Z. 333*, 518.

Hasselbach, W., & Makinose, M. (1963) *Biochem. Z. 339*, 94.

Inesi, G. (1971) *Science (Washington, D.C.) 171*, 901.

Inesi, G., Goodman, J. J., & Watanabe, S. (1967) *J. Biol. Chem. 242*, 4637.

Inesi, G., Maring, E., Murphy, A., & McFarland, B. (1970) *Arch. Biochem. Biophys. 138*, 285.

Inesi, G., Kurzmack, M., Nakamoto, R., DeMeis, L., & Bernhard, S. (1980) *J. Biol. Chem. 255*, 6040.

Kurzmack, M., Verjovski-Almeida, S., & Inesi, G. (1977) *Biochem. Biophys. Res. Commun. 78*, 772.

Kurzmack, M., Inesi, G., & Bernhard, S. (1979) *Biophys. J. 25*, 109.

Makinose, M. (1969) *Eur. J. Biochem. 10*, 74.

Makinose, M., & The, R. (1965) *Biochem. Z. 343*, 383.

Malhotra, O., & Bernhard, S. (1968) *J. Biol. Chem. 243*, 1243.

Malhotra, O., & Bernhard, S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2077.

Martonosi, A. (1969) *J. Biol. Chem. 244*, 613.

Martonosi, A., & Feretos, R. (1964) *J. Biol. Chem. 239*, 648.

Nakamura, Y., & Tonomura, Y. (1978) *J. Biochem. (Tokyo) 83*, 571.

Odom, T., Chipman, D., Betts, G., & Bernhard, S. (1981) *Biochemistry* (preceding paper in this issue).

Pucell, A., & Martonosi, A. (1971) *J. Biol. Chem. 246*, 3389.

Rauch, B., Chak, D., & Hasselbach, W. (1978) *FEBS Lett. 93*, 65.

Rossi, B., Leone, F., Gache, C., & Lazdunski, M. (1979) *J. Biol. Chem. 254*, 2302.

Schwartzenbach, G., Senn, H., & Anderegg, G. (1957) *Helv. Chim. Acta 40*, 1186.

Seydoux, F., Bernhard, S., Pfenninger, O., Payne, M., & Malhotra, O. (1973) *Biochemistry 12*, 4290.
Sumida, M., Wang, T., Mandel, F., Froehlich, J., & Schwartz, A. (1978) *J. Biol. Chem. 253*, 8772.
Verjovski-Almeida, S., Kurzmack, M., & Inesi, G., (1978) *Biochemistry 17*, 5006.
Yamamoto, T., & Tonomura, Y. (1967) *J. Biochem. (Tokyo) 62*, 558.

Affinity Labeling of Rabbit Muscle Pyruvate Kinase by a New Fluorescent Nucleotide Alkylating Agent 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

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ABSTRACT: This paper describes the synthesis and characterization of a new fluorescent nucleotide analogue, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine, which is capable of reacting covalently with nucleophilic groups in proteins. This nucleotide analogue, with a fluorescence emission maximum at 412 nm, functions as an active site directed irreversible inhibitor of rabbit muscle pyruvate kinase. The inactivation follows pseudo-first-order kinetics at pH 8.0. A plot of the rate constant for inactivation vs. the analogue concentration yields hyperbolic kinetics, indicative of reversible binding of the analogue prior to covalent reaction. Significant protection is afforded by phosphoenolpyruvate, while MgATP, MgADP, and Mg alone decrease the rate of inactivation but not to the same extent as does phosphoenolpyruvate. The metal-free nucleotides ADP or ATP as well as pyruvate have no effect on the rate of inactivation. The incorporation of 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine into pyruvate kinase was measured from the fluorescence of modified enzyme. A total of 2 mol of reagent is incorporated/mol of enzyme subunit when the enzyme is 100% inactivated. However, if the modified enzyme is treated with dithiothreitol or β-mercaptoethylamine, only 1 mol of reagent is found incorporated/enzyme subunit. Concomitant with this decrease in incorporation upon treatment of the modified enzyme with thiols is a reactivation that is proportional to the loss of incorporated reagent. Thus, there are two sites of modification per enzyme subunit: reaction of 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine at one site leads to the formation of inactive enzyme which can be reactivated by the addition of dithiothreitol or β-mercaptoethylamine, whereas reaction at the second site has no effect on enzyme activity and under the conditions used is not reversible by dithiothreitol. The reagent incorporated cannot be attributed to reaction at tyrosine or lysine residues. The reversibility by thiols of modification at the site responsible for inactivation suggests that the amino acid residue modified may be a cysteine. These studies indicate that 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine may provide a means of introducing a covalently bound fluorescent probe at nucleotide sites in other proteins.

Affinity labeling using nucleotide analogues with reactive functional groups has proved to be an effective approach to exploring the nucleotide binding sites of proteins (Colman et al., 1977; Hampton et al., 1977; Gulyaev et al., 1976). Among the most generally applicable of the nucleotide alkylating agents is 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, which labels specifically nucleotide binding sites of glutamate dehydrogenase (Pal et al., 1975), rabbit muscle and yeast pyruvate kinases (Wyatt & Colman, 1977; Likos et al., 1980), phosphofructokinase (Mansour & Colman, 1978; Pettigrew & Frieden, 1978; Weng et al., 1980), mitochondrial F_1 ATPase[1] (Esch & Allison, 1978; DiPietro et al., 1979), chloroplast ATPase (DeBenedetti & Jagendorf, 1979), cAMP-dependent protein kinase (Zoller & Taylor, 1979; Hixson & Krebs, 1979), malate dehydrogenase (Roy & Colman, 1979), glutamine synthetase (Foster & Kingdon, 1980), and an ADP receptor protein of platelets (Bennett et al., 1978; Mills et al., 1980). Fluorescent analogues of natural biochemical compounds as well as fluorescent labeling agents have been valuable in probing the environment of binding sites in proteins and in elucidating distances between defined site markers on proteins by energy transfer (Brand & Witholt, 1967; Horton & Koshland, 1967; Stryer, 1978). A fluorescent nucleotide alkylating agent should thus provide an effective tool for introducing into a defined nucleotide binding site a covalent fluorescent probe which can then be used to examine the properties of that site and its interactions with other sites of the protein. In order to interpret this type of information, it is necessary first to characterize the reaction of the analogue with a particular protein. This paper describes the synthesis and structure determination of the new fluorescent nucleotide alkylating agent, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. Rabbit muscle pyruvate kinase has been shown to utilize 1,N^6-ethenoadenosine diphosphate as a substitute for ADP in its catalytic reaction (Secrist et al., 1972; Barrio et

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received July 16, 1980.* This work was supported by a grant from the American Cancer Society (BC-138) and by a U.S. Public Health Service Postdoctoral Fellowship (1F32CA06556) (J.J.L.).

[1] Abbreviations used: 5′-*p*-FSO_2BzεAdo, 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine; CBS-Lys, N^{ϵ}-(4-carboxybenzenesulfonyl)lysine; CBS-Tyr, *O*-(4-carboxybenzenesulfonyl)tyrosine; 5′-*p*-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; 3′-*p*-FSO_2BzAdo, 3′-[*p*-(fluorosulfonyl)benzoyl]adenosine; ATPase, adenosine 5′-triphosphatase; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide, NADH, reduced NAD; Tris, tris(hydroxymethyl)aminomethane; AMP, adenosine 5′-phosphate.

0006-2960/81/0420-0491$01.00/0