Gallant, J. A. (1979) *Annu. Rev. Genet. 13*, 393–415.
Gallant, J., & Lazzarini, R. A. (1976) in *Protein Synthesis: A Series of Advances* (McCartney, E. H., Ed.) Vol. 2, pp 309–359, Marcel Dekker, New York.
Gilbert, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 193–205, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Hamming, J., Gruber, M., & Ab, G. (1979) *Nucleic Acids Res. 7*, 1019–1033.
Hamming, J., Ab, G., & Gruber, M. (1980) *Nucleic Acids Res. 8*, 3947–3963.
Johnson, R. A., & Walseth, T. F. (1979) in *Advances in Cyclic Nucleotide Research* (Brooks, G., Greengard, P., & Robison, G. A., Eds.) pp 135–167, Raven Press, New York.
Kennerly, M. E., Morgan, E. A., Post, L., Lindahl, L., & Nomura, M. (1977) *J. Bacteriol. 132*, 932–949.
Kingston, R. E., & Chamberlin, M. J. (1981) *Cell (Cambridge, Mass.) 27*, 523–531.
Kingston, R. E., Gutell, R. R., Taylor, A. R., & Chamberlin, M. J. (1981a) *J. Mol. Biol. 146*, 433–449.
Kingston, R. E., Nierman, W. C., & Chamberlin, M. J. (1981b) *J. Biol. Chem. 256*, 2787–2797.
Kiss, A., Sain, B., & Venetianer, P. (1977) *FEBS Lett. 79*, 77–79.
Maaloe, O., & Kjeldgaard, N. O. (1966) *Control of Macromolecular Synthesis*, pp 94–96, W. A. Benjamin, New York.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787–3794.
Nierlich, D. P. (1978) *Annu. Rev. Microbiol. 32*, 393–432.
Oostra, B. A., van Ooyen, A. J. J., & Gruber, M. (1977) *Mol. Gen. Genet. 152*, 1–6.
Platt, T. (1981) *Cell (Cambridge, Mass.) 24*, 10–23.
Pribnow, D. (1975) *J. Mol. Biol. 99*, 419–443.
Sarmientos, P., Sylvester, J. E., Contente, E., & Cashel, M. (1983) *Cell (Cambridge, Mass.) 32*, 1337–1346.
Shen, W.-F., Squires, C., & Squires, C. L. (1982) *Nucleic Acids Res. 10*, 3303–3313.
Stent, G., & Brenner, S. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 2005–2014.
Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77–90.
Talmadge, K., & Gilbert, W. (1980) *Gene 12*, 235–241.
Travers, A. (1976a) *Mol. Gen. Genet. 147*, 225–232.
Travers, A. (1976b) *Nature (London) 263*, 641–646.
Travers, A., & Baralle, F. E. (1976) *Alfred Benzon Symp. 9*, 241–251.
Travers, A., Buckland, R., Goman, M., LeGrice, S. S. G., & Scaife, J. G. (1978) *Nature (London) 273*, 354–358.
Travers, A. A. (1980a) *J. Bacteriol. 141*, 973–976.
Travers, A. A. (1980b) *J. Mol. Biol. 141*, 91–97.
van Ooyen, A. J. J., Gruber, M., & Jorgenson, P. (1976) *Cell (Cambridge, Mass.) 8*, 123–128.
Vogelstein, B., & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 615–619.
Yanofsky, C. (1981) *Nature (London) 289*, 751–758.
Young, R. A., & Steitz, J. A. (1979) *Cell (Cambridge, Mass.) 17*, 225–234.

Kinetics of Calcium Uptake by Isolated Sarcoplasmic Reticulum Vesicles Using Flash Photolysis of Caged Adenosine 5′-Triphosphate†

Dorothy H. Pierce,* Antonio Scarpa, Michael R. Topp, and J. Kent Blasie

ABSTRACT: The kinetics of ATP-induced Ca^{2+} uptake by vesicular dispersions of sarcoplasmic reticulum were determined with a time resolution of about 10 ms, depending on the temperature. Ca^{2+} uptake was initiated by the addition of ATP through the flash photolysis of P^3-1-(2-nitrophenyl)-ethyl adenosine 5′-triphosphate utilizing a frequency-doubled ruby laser and measured with two different detector systems that followed the absorbance changes of the metallochromic indicator arsenazo III sensitive to changes in the extravesicular [Ca^{2+}]. The temperature range investigated was −2 to 26 °C. The Ca^{2+} ionophore A23187 was used to distinguish those features of the Ca^{2+} uptake kinetics associated with the formation of a transmembrane Ca^{2+} gradient. The acid-stable phosphorylated enzyme intermediate, E∼P, was determined independently with a quenched-flow technique. Ca^{2+} uptake is characterized by at least two phases, a fast initial phase and a slow phase. The fast phase exhibits pseudo-first-order kinetics with a specific rate constant of 64 ± 10 s^{-1} at 23–26 °C, an activation energy of 16 ± 1 kcal mol^{-1}, and a ΔS^* of ∼5 cal deg^{-1} mol^{-1}, is insensitive to the presence of a Ca^{2+} ionophore, and occurs simultaneously with the formation of the phosphorylated enzyme, E∼P, with a stoichiometry of ∼2 mol of Ca^{2+}/mol of phosphorylated enzyme intermediate. The slow phase also exhibits pseudo-first-order kinetics with a specific rate constant of 0.60 ± 0.09 s^{-1} at 25–26 °C, an activation energy of 22 ± 1 kcal mol^{-1}, and a ΔS^* of ∼16 cal deg^{-1} mol^{-1}, is inhibited by the presence of a Ca^{2+} ionophore, and has a stoichiometry of ∼2 mol of Ca^{2+}/mol of ATP hydrolyzed.

The sarcoplasmic reticulum (SR) membrane has an important role in the control of the cytoplasmic [Ca^{2+}] in muscle contraction and relaxation. In relaxation, calcium is removed from the cytoplasm across the SR membrane into the sarcotubular system (Ebashi et al., 1969; Hasselbach & Waas, 1982). The ATP-induced Ca^{2+} uptake by SR occurs against the Ca^{2+} concentration gradient, through a process mediated by the Ca^{2+}-ATPase in a cyclic, reversible mechanism of many steps (deMeis & Vianna, 1979; Chaloub & deMeis, 1980; Takakuwa & Kanazawa, 1981).

ATP-induced Ca^{2+} uptake by SR vesicles seems to consist of more than one phase. In quenched-flow experiments using radioactive Ca^{2+}, Inesi and co-workers obtained Ca^{2+} uptake

† From the Departments of Chemistry (D.H.P., M.R.T., and J.K.B.), and of Biochemistry and Biophysics (A.S. and J.K.B.), University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received April 12, 1983.* This work was supported by National Institutes of Health Grants HL-18708 and AA 05662 to J.K.B. and A.S.

0006-2960/83/0422-5254$01.50/0

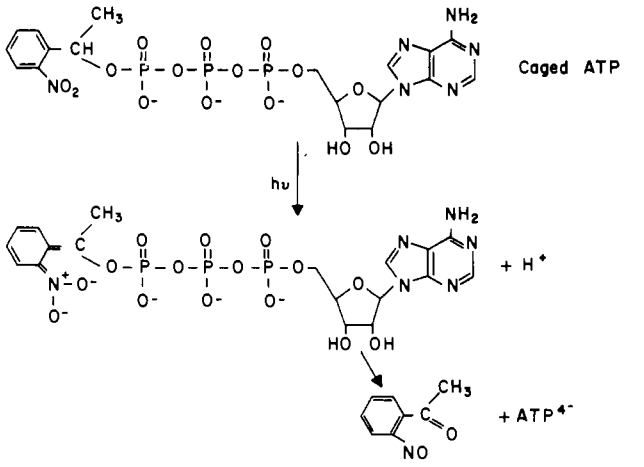

FIGURE 1: Chemical structure of the caged ATP molecule and its reaction in the photolytic release of ATP.

kinetics at 25 °C which extrapolated to a nonzero intercept at time $t = 0$ (Kurzmack et al., 1977; Verjovski-Almeida et al., 1978). Ikemoto et al. (1981) obtained results with low Ca^{2+} concentrations at 2–4 °C consistent with a fast phase preceding a slow phase of Ca^{2+} uptake. Dupont (1980) suggested on the basis of Millipore filtration quenching experiments at 0 °C that the fast phase may be Ca^{2+} "occlusion".

In this work, the kinetics of ATP-induced Ca^{2+} uptake by SR vesicles in dispersion were investigated with a time resolution of about 10 ms by combining the technique of laser photolysis of caged ATP[1] with the use of the metallochromic indicator arsenazo III to monitor extravesicular $[Ca^{2+}]$. Through the use of both the ionophore A23187, which equilibrates transmembrane Ca^{2+} gradients, and a range of temperatures from −2 to 26 °C, it was possible to distinguish and characterize two phases of Ca^{2+} uptake. A fast "occlusion" phase occurs simultaneously with phosphorylation of the enzyme, and a slower phase is associated with the transport of Ca^{2+} across the membrane profile.

Such investigations into the details of the kinetics of Ca^{2+} uptake by sarcoplasmic reticulum vesicles can provide important insights into the mechanism of the Ca^{2+} transport process itself and essential information for the interpretation of time-resolved structural studies of the sarcoplasmic reticulum membrane following stimulation of the Ca^{2+} transport process.

Materials and Methods

Vesicular dispersions of sarcoplasmic reticulum were isolated from the white muscle of the hind legs of rabbits (Herbette et al., 1977). The protein content of the dispersions was determined according to the Lowry method (Lowry et al., 1951).

Caged ATP was synthesized from ADP morpholidate and 1-(2-nitrophenyl)ethyl phosphate as described by Kaplan et al. (1978). 1-(2-Nitrophenyl)ethyl phosphate was prepared from the parent alcohol and *o*-phenylene phosphorochloridate by the method used for the synthesis of barium 2-cyanoethyl phosphate (Khwaja et al., 1970). Caged ADP was synthesized in the same way as caged ATP, with AMP morpholidate replacing ADP morpholidate. The chemical structure of caged ATP and the mechanism of the photolytic release are shown in Figure 1 (McCray et al., 1980). According to McCray et al., the release time of ATP is dependent on the dark reaction, which has a rate constant of 220 s^{-1} at pH 7.0 and 22 °C. The amount of ATP released is proportional to the photolysis energy. The caged compounds were in 100 mM TES,[1] pH 7.0.

The reaction mixture (82 μL) contained 40 mM Tris–maleate, 8 mM $MgCl_2$, 120 mM KCl, 100 μM arsenazo III, 200 μM glutathione, 2 mg of SR protein/mL, 50 μM $CaCl_2$ in addition to the $[Ca^{2+}]$ provided by the presence of SR protein, and 2.3 mM caged ATP, pH 7.0. Mg^{2+}, Ca^{2+}, and ATP concentrations were always in excess of the ATPase concentration. In addition, a large concentration of Mg^{2+} effectively prevented Ca^{2+} binding to ATP. In the ionophore experiments, the reaction mixture had two modifications: only 1 mg of SR protein/mL was present, and either 2 μL of ethanol or a solution of ethanol–ionophore A23187 was added. Solutions of ethanol–ionophore were comprised of either 0.75 or 1 mg of ionophore per mL of ethanol. Suitable ionophore concentrations were determined by ionophore titration. A lower concentration of SR was used in ionophore experiments as compared with experiments without ionophore, so as to minimize the amount of ethanol necessary for suitable ionophore concentrations and thus avoid any change in the Ca^{2+}-ATPase function that can result with the presence of >2% ethanol. The purpose of the ionophore experiments was to investigate the relationship between the formation of a transmembrane Ca^{2+} gradient and the phases of Ca^{2+} uptake.

A 100-μL quartz cell of 5-mm path length, 2-mm width contained the SR dispersions. The cell was placed in a closed, temperature-controlled chamber in such a way that the photolysis light was perpendicular to the path length.

A frequency-doubled ruby laser (Korad, Santa Monica, CA) was used in the photolysis of the caged compounds. The ruby laser was passively Q-switched with vanadyl phthalocyanine in nitrobenzene, OD ≃ 0.16 at 694 nm, and the pulse was frequency-doubled by a KDP[1] crystal. $CuSO_4$ solution blocked the 694-nm primary beam. A neon laser (Metrologic, Bellmawr, NJ) aided in the alignment of the ruby laser and KDP crystal. The 347-nm pulse energy ranged from 20 to 30 mJ. The pulse energy was measured with a Gen-Tec energy meter connected to a 564 Tektronix storage oscilloscope triggered by the laser. A quartz slide was placed in the frequency-doubled beam at 45° to reflect some of the pulse to an RCA 935 photodiode (PD), also connected to the oscilloscope. The signal from the photodiode was calibrated with respect to the energy readings from the Gen-Tec meter. Thus, during experiments with the sample chamber in the 347-nm beam, the laser energy was checked and maintained above 20 mJ for each experiment using the photodiode calibration.

Ca^{2+} uptake by SR was measured by monitoring changes in the absorbance spectrum of arsenazo III, which under specific conditions is a function of the free Ca^{2+} concentration and has a Ca^{2+} on and off time of ~2–5 ms (Scarpa, 1979). The absorbance changes of arsenazo III were measured with two different detector systems. One involved a time-sharing multiple wavelength spectrophotometer (Chance et al., 1975) with which the time-shared wavelength difference, 660–690 nm, was continuously monitored. The light from a Quartzline lamp passed through a rotating filter wheel (with 660- and

[1] Abbreviations: caged ATP, P^3-1-(2-nitrophenyl)ethyl adenosine 5′-triphosphate; E_a, activation energy; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; E~P, acid-stable phosphorylated enzyme (Ca^{2+}-ATPase); ΔG^*, free energy of activated complex; ΔH^*, enthalpy of activated complex; k_1, specific rate constant for first-order reaction; KDP, potassium dihydrogen phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCA, perchloric acid; butyl-PBD, 2-(4-biphenylyl)-5-(4-*tert*-butylphenyl)-1,3,4-oxadiazole; PD, photodiode; PMT, photomultiplier tube; ΔS^*, entropy of activated complex; SR, sarcoplasmic reticulum; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

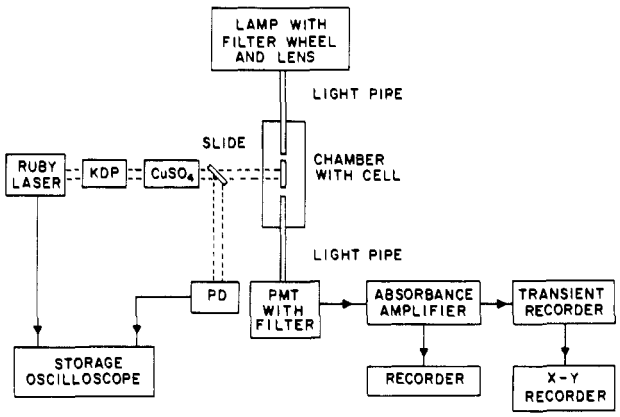

FIGURE 2: "Higher time" resolution spectrophotometer system with frequency-doubled ruby laser and accessories.

690-nm interference filters) driven by air at ~200 Hz, a collimating lens, and a light pipe into the chamber to the sample. The transmitted light was then collected through another light pipe by an EMI 9798B photomultiplier tube (PMT). The PMT voltage outputs for the two wavelengths were subtracted and averaged by the demodulator (~20-ms response time). The time dependence of the absorbance difference was followed with a storage oscilloscope and a chart recorder.

Since the demodulator system's time resolution and intrinsic noise level were somewhat limiting, a single wavelength measurement system of higher time resolution capability (10^{-5} s) and lower intrinsic noise level was also used. The measuring light (660 ± 1 nm) was detected with a PMT whose voltage output was sent to an absorbance amplifier (Johnson Foundation, University of Pennsylvania). The signal was simultaneously fed to a chart recorder and to a transient recorder (1200 data points per time interval ranging from 10 ms to 8 s) whose memory was displayed on an X–Y plotter. Figure 2 is a diagram of the higher time resolution equipment.

A high buffer concentration was necessary to hold the pH constant upon photolysis of the caged compound, which results in proton release. Glutathione was used to protect the Ca^{2+}-ATPase from any possible reaction with the ketone photolysis product of the caged nucleotide (Kaplan et al., 1978; Goldman et al., 1982). Light and the photolysis products due to flash photolysis of caged ATP have no effect on Ca^{2+} uptake, as evidenced by a comparison with results obtained upon direct ATP addition itself (Pierce, 1982).

The amount of photolytic release of ATP from caged ATP was determined through a comparison of Ca^{2+} uptake by SR initiated with flash photolysis of caged ATP and with direct addition of various known concentrations of ATP itself under otherwise identical conditions. This calibration of the photolytic conversion of caged ATP to ATP is relevant on the time scale of the dark reaction of caged ATP, namely, ~1–10 ms (McCray et al., 1980). The amount of release of ADP from caged ADP following photolysis was determined by the luciferin/luciferase assay (Strehler, 1968) once the ADP was converted to ATP by using a buffer solution at pH 8.0 containing Mg^{2+}, phosphoenolpyruvate, and pyruvic kinase. Release of ATP and of ADP from the respective caged nucleotide as a function of light intensity was found to be identical within experimental error.

Ca^{2+} calibrations were established for the arsenazo III in the SR dispersions over the temperature range investigated in the kinetic study using a 1-cm path-length, 2-mL cell. The 5-mm path-length, 100-μL cell could not be used directly for these calibrations due to inaccuracies inherent in the addition of very small volumes to small volumes. Beer's law was then applied to simply calibrate the arsenazo III in the SR dispersions in the 100-μL cell. The calcium calibrations are relevant on the time scale of the calcium on and off reaction of arsenazo III, namely, ~2–5 ms (Scarpa, 1979).

The Ca^{2+} calibration and photolytic conversion of caged ATP to ATP were tested for consistency. Because the concentrations of Ca^{2+} and ATP, not greatly in excess of the Ca^{2+}-ATPase concentration, were limiting as evidenced by negligible Ca^{2+} leakage following exhaustion of ATP, the Ca^{2+} transported to ATP hydrolyzed should have the well-established 2:1 mole ratio (MacLennan & Holland, 1975; Yamamoto et al., 1979; Inesi, 1979). Therefore, the Ca^{2+} calibration was checked by applying the 2:1 relationship to the Ca^{2+} uptake obtained upon direct addition of known amounts of ATP. Once the Ca^{2+} calibration was confirmed, it was applied along with the 2:1 ratio to the Ca^{2+} uptake obtained through flash photolysis of caged ATP in order to check the already determined amount of ATP release in flash photolysis of caged ATP.

In control experiments caged ADP replaced caged ATP, or the nucleotide or SR was omitted. The controls collectively checked the effects of energy dissipation from the laser flash and of nucleotide addition to the medium through the photolytic release reaction. Photolysis of caged ADP tested the combined effects of nucleotide addition, photolysis products, and energy dissipation from the light flash, because the released ADP is not a substrate for Ca^{2+} uptake by SR vesicles. The effect of the light flash on the dye was investigated through photolysis of the sample in the absence of caged nucleotide. The effects of the photolytic release reaction and of the light flash were determined by photolysis of caged ADP in the absence of SR. According to McCray et al. (1980), the caged nucleotide does not bind to the ATPase, and the 347-nm light does not cause protein damage.

In order to investigate the formation kinetics of the acid-stable phosphorylated ATPase at two temperatures, 0.5 and 26 °C, a quenched-flow technique (Gutfreund, 1969) was used. Syringes A and B each contained 20 mM MOPS,[1] 80 mM KCl, 5 mM $MgCl_2$, and 50 μM $CaCl_2$, pH 7.0. In addition, syringe A had 0.8 mg of SR protein/mL, and syringe B had 100 μM [γ-^{32}P]ATP (2.7–4.0 Ci/mol of ATP). The contents of the two syringes were rapidly mixed and then quenched with a 2.5% PCA,[1] 1 mM ATP, and 0.25 mM P_i solution while being stirred. The quenched reactions were kept on ice for 15 min and poured onto glass fiber disks previously soaked in the quenching solution. The disks were washed with deionized water 3 times, dried, and immersed in toluene–butyl-PBD[1] scintillation fluid for ^{32}P counting. For the zero-time data point, the contents of syringe A were added to the quenching solution with stirring, and then the contents of syringe B were added. Standards were obtained by taking 10, 25, and 50 μL of solution from syringe B. Blanks consisted of no solution. A correction was made for the base line by using the results for zero time. The conditions in the quenched-flow experiments were different from those for the Ca^{2+} uptake experiments, due to the nature of the different methods. A lower SR concentration was necessary with the quenched-flow technique to avoid the problem of settling of the vesicles. The aim of the quenched-flow experiments was to determine the maximum amount of phosphorylated enzyme, E~P, formed at the extreme temperatures, 0.5 and 26 °C.

Results

Upon laser photolysis with a pulse of about 20–25 mJ, the conversion of caged ATP to ATP or of caged ADP to ADP

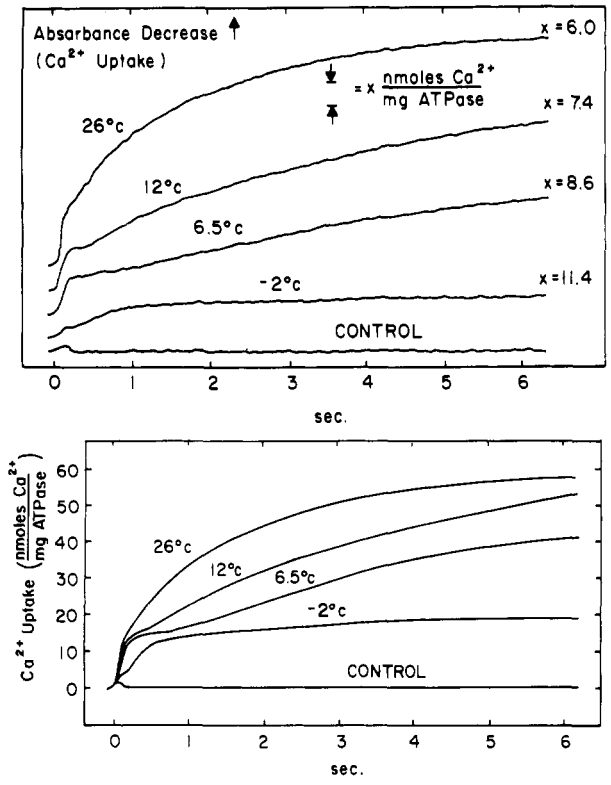

FIGURE 3: Typical experimental curves for the slow phase of Ca^{2+} uptake by SR vesicular dispersions at four different temperatures. Caged ADP, no nucleotide, or no SR served as controls. The curves were processed by the absorbance amplifier system and recorded on the chart recorder. Note: This figure is presented in two ways: (top panel) original curves with original Ca^{2+} calibrations indicated; (bottom panel) smooth curves with the Ca^{2+} calibrations applied.

was close to 2% of the total caged nucleotide. Multiple flashes each produced virtually identical nucleotide release and Ca^{2+} uptake kinetics. Within the measurement limitation of 0.1 °C, there was no change in the temperature of the reaction mixture upon a laser flash.

Ca^{2+} uptake kinetics on a time scale of seconds were obtained from experiments in which arsenazo III absorbance was followed with either the demodulator system (time resolution about 20 ms) or the absorbance amplifier, for a temperature range of −2 to 26 °C. Examples are shown in Figure 3. In the 6-s time interval shown, a slow phase of Ca^{2+} uptake is observed at the high and intermediate temperatures. These results illustrate the slowing of the reaction rate with a decrease in temperature. A fast phase of Ca^{2+} uptake is evident at −2 °C and at intermediate temperatures. Included are the controls of caged ADP, of no nucleotide, and of no SR, which show no absorbance change.

The total amount of Ca^{2+} uptake in the slow phase was 40 ± 4 nmol of Ca^{2+}/mg of protein, or 60 ± 6 nmol of Ca^{2+}/mg of ATPase, based on ~67% of the protein as ATPase (McFarland & Inesi, 1971). When compared with the moles of ATP present per milligram of ATPase, ~35, the total Ca^{2+} uptake expressed as moles of Ca^{2+} per moles of ATP hydrolyzed was about 2:1.

The slow phase of Ca^{2+} uptake exhibits pseudo-first-order behavior. The experimental conditions are such that the concentrations of the reactants are greater than the enzyme concentration. The specific rate constant (or apparent first-order rate constant) and half-life of the reaction were calculated for each temperature, as were the initial rate (expressed with respect to enzyme concentration) and enzyme activity. The term "enzyme activity" is used here to represent the initial

Table I: Kinetic Parameters of the Slow Phase of Ca^{2+} Uptake by SR Vesicular Dispersions

T (°C)	specific rate constant (s^{-1})	initial rate[a] [nmol of Ca^{2+} (mg of ATPase)$^{-1}$ s^{-1}]	half-life (s)	enzyme activity[b] [2 mol of Ca^{2+} (mol of E~P)$^{-1}$ s^{-1}]
25.0-26.0	0.60	42.0	1.1	3.5
12.0-13.5	0.11	8.8	6.3	0.73
6.8-8.0	0.053	3.3	13.0	0.28
−2.0-0.5	0.020	0.77	35.0	0.064

[a] The initial rate is expressed with respect to enzyme concentration. [b] The enzyme activity is based on the maximum of ~6 nmol of E~P/mg of ATPase. Each temperature is represented by eight to ten measurements, with a standard deviation of ±15%. Different detector systems and different SR preparations are involved.

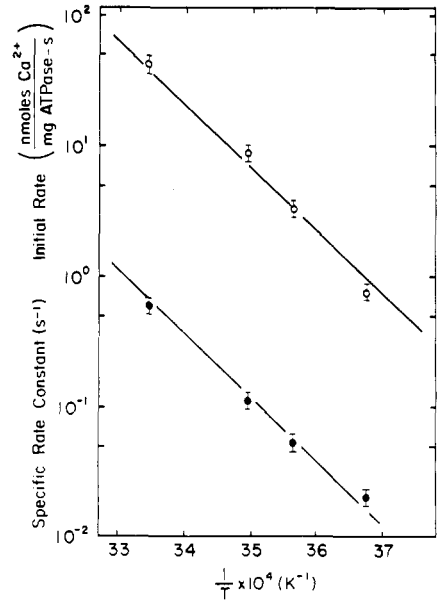

FIGURE 4: Arrhenius plot of the specific rate constants (●) and the initial rates (○) of the slow phase of Ca^{2+} uptake by SR vesicular dispersions. Each temperature is represented by eight to ten measurements with a standard deviation of ±15%. Different detector systems and SR preparations are involved.

rate or maximum rate of Ca^{2+} uptake in a given reaction step, expressed as 2 mol of Ca^{2+} (mol of E~P)$^{-1}$ s^{-1}. Table I lists the values of the specific rate constant, initial rate, half-life, and enzyme activity for data obtained with both the demodulator and absorbance amplifier systems. Each temperature is represented by eight to ten measurements with a standard deviation of ±15%, even using different SR preparations. Arrhenius plots of the specific rate constants[1] and initial rates are shown in Figure 4. The slope yields the value of the activation energy, E_a, according to

$$(\mathrm{d} \log k_1)/[\mathrm{d}(1/T)] = -[E_a/(2.303R)]$$

E_a for the slow step is 22 ± 1 kcal mol^{-1}.

Experiments with the absorbance amplifier and transient recorder time resolve the fast phase of Ca^{2+} uptake as shown in Figure 5 on a time scale of milliseconds for the temperature range of −2 to 26 °C. The dark reaction of caged ATP photolysis limits the resolution to about 10 ms, depending on the temperature (McCray et al., 1980). The slowing of the reaction rate with a decrease in temperature is apparent. At high temperatures, the slow phase of Ca^{2+} uptake is also ev-

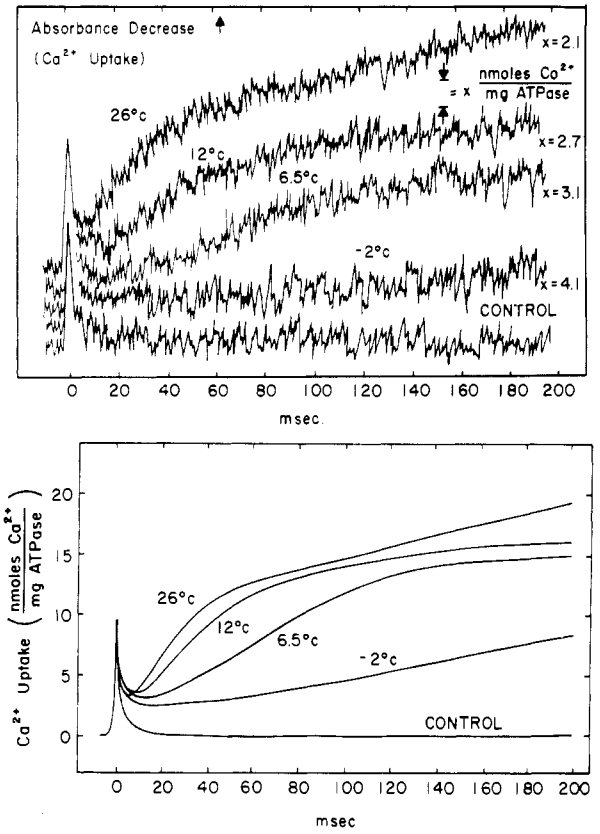

FIGURE 5: Typical experimental curves for the fast phase of Ca^{2+} uptake by SR vesicular dispersions at four different temperatures. Caged ADP, no nucleotide, or no SR served as controls. The curves were processed by the absorbance amplifier system and displayed on the X–Y plotter. Note: This figure is presented in two ways: (top panel) the original curves with original Ca^{2+} calibrations indicated; (bottom panel) smooth curves with the Ca^{2+} calibrations applied. At zero time, there is a spike due to the laser flash.

Table II: Kinetic Parameters of the Fast Phase of Ca^{2+} Uptake by SR Vesicular Dispersions

T (°C)	specific rate constant (s^{-1})	initial rate[a] [nmol of Ca^{2+} (mg of ATPase)$^{-1}$ s^{-1}]	half-life (ms)	enzyme activity[b] [2 mol of Ca^{2+} (mol of E~P)$^{-1}$ s^{-1}]
23.0–26.0	64.0	420	11	35
11.5–12.0	14.0	140	49	12
6.5–6.8	11.0	93	63	7.8
−2.0	3.1	74	220	6.2

[a] The initial rate is expressed with respect to enzyme concentration. [b] The enzyme activity is based on the maximum of ~6 nmol of E~P/mg of ATPase. Each temperature is represented by five to seven measurements, with a standard deviation of ±15%. Different detector systems and different SR preparations are involved.

ident. The controls of caged ADP, of no nucleotide, and of no SR are included and show no change in absorbance. There is a spike at zero time due to the laser flash.

The time course of the fast phase of Ca^{2+} uptake was obtained by substraction of the slow phase extrapolated back to zero time. The resulting fast phase likewise exhibits pseudo-first-order behavior. The experimental conditions are such that the concentrations of the reactants are greater than the enzyme concentration. The specific rate constant (or apparent first-order rate constant), half-life, initial rate, and enzyme activity were calculated for each temperature. Table II lists

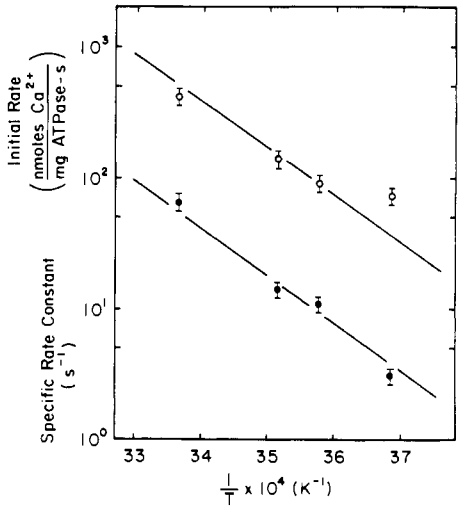

FIGURE 6: Arrhenius plot of the specific rate constants (●) and the initial rates (○) of the fast phase of Ca^{2+} uptake by SR vesicular dispersions. Each temperature is represented by five to seven measurements, with a standard deviation of ±15%. Data were obtained with use of the absorbance amplifier system. Different SR preparations are involved.

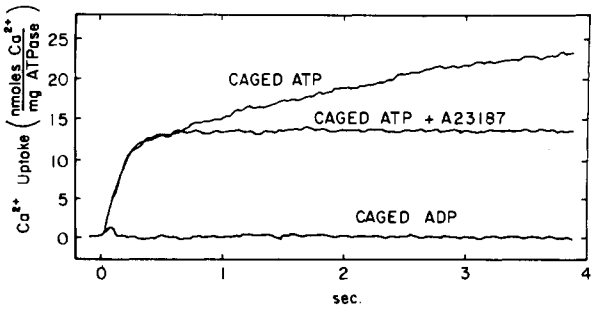

FIGURE 7: Typical experimental curves representing the effect of ionophore A23187 on Ca^{2+} uptake by SR vesicular dispersions at 10.5 °C. The curve labeled caged ATP is representative of Ca^{2+} uptake by SR vesicular dispersions with or without ~2% ethanol, initiated by laser photolysis of caged ATP. The curve labeled caged ATP + A23187 is representative of Ca^{2+} uptake by SR vesicular dispersions with ~2% ethanol–ionophore solution (0.75 or 1.0 mg of ionophore/mL of ethanol) initiated by laser photolysis of caged ATP. The control curve labeled caged ADP is representative of laser photolysis of caged ADP in SR vesicular dispersions with and without either ~2% ethanol or ~2% ethanol–ionophore solution (0.75 or 1.0 mg of ionophore/mL of ethanol). The curves were processed by the absorbance amplifier system and recorded on the chart recorder.

these values. Each temperature is represented by five to seven measurements with a standard deviation of +15%, even when different SR preparations are used. Arrhenius plots of the specific rate constants and initial rates are shown in Figure 6. The activation energy for the fast phase is 16 ± 1 kcal mol^{-1}.

The effect of ionophore A23187 on Ca^{2+} uptake by SR at 10.5 °C is shown in Figure 7. Similar results were obtained at 5.3, 11.6, and 17.8 °C. It is evident that the use of 2% ethanol has no detectable effect on the Ca^{2+} uptake. The ionophore at two different concentrations inhibits the slow phase without affecting the fast phase. Control experiments using caged ADP and omitting nucleotide are identical in both the presence and absence of ethanol or an ethanol solution of ionophore. The total amount of Ca^{2+} uptake in the fast phase is 6.7 ± 0.7 nmol of Ca^{2+}/mg of protein, or 10 ± 1 nmol of Ca^{2+}/mg of ATPase.

Figure 8 shows the results of the quenched-flow experiments carried out at 0.5 and 26 °C. The maximum amount of acid-stable phosphorylated enzyme, E~P, formation is 4 ±

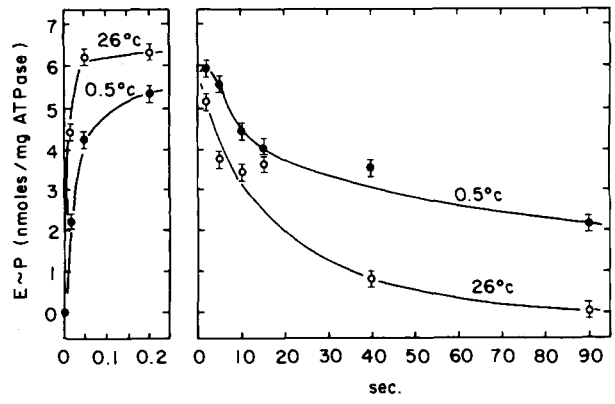

FIGURE 8: Amount of acid-stable phosphorylated enzyme present in SR vesicular dispersions at various times after the addition of ATP for two temperatures. Each point represents two to three measurements with a standard deviation of ±0.2.

0.13 nmol of E~P/mg of protein, or 6 ± 0.2 nmol of E~P/mg of ATPase.

According to the absolute reaction rate theory, thermodynamic properties of the activated complex may be calculated if the specific rate constant at a given temperature and the activation energy are known. The assumption is made that the reactants and activated complex are in equilibrium. Since dispersions can be considered as liquids, $\Delta H^* \simeq E_a$, where ΔH^* is the enthalpy or heat absorbed by the activated complex. The free energy of activation controls the rate of a given reaction at a certain temperature (Glasstone, 1954):

$$\Delta G^* = -RT \ln [(k_1 h)/(\tau k T)]$$

where k_1 is the specific rate constant, h is Planck's constant, k is Boltzmann's constant, and τ is the transmission coefficient assumed to be unity (Kreevoy, 1963). ΔG^* is thus ~15 kcal mol^{-1} for the Ca^{2+} uptake fast phase and ~18 kcal mol^{-1} for the slow phase. However, we must stress (see Discussion) that the release of ATP upon photolysis may be partially rate determining in the fast phase.

Determination of the entropy of the activated complex is possible through its relationship to the enthalpy and free energy:

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T$$

The ΔS^* is ~5 cal deg^{-1} mol^{-1} for the fast phase of Ca^{2+} uptake; the ΔS^* is ~16 cal deg^{-1} mol^{-1} for the slow phase.

Discussion

Laser photolysis of this caged ATP and the use of a metallochromic indicator in the study of Ca^{2+} uptake by SR vesicles in dispersion afford a time resolution of about 10 ms, depending on the temperature (McCray et al., 1980). This higher time resolution has made it possible for the first time to distinguish and characterize a fast phase of Ca^{2+} uptake from a slow phase over the broad temperature range of −2 to 26 °C.

Fast Phase of Calcium Uptake. The fast phase of Ca^{2+} uptake has a specific rate constant of 64 ± 10 s^{-1} at 23–26 °C (with the given experimental conditions) and an activation energy of 16 ± 1 kcal mol^{-1}. The total amount of Ca^{2+} uptake is 10 ± 1 nmol of Ca^{2+}/mg of ATPase, or 6.7 ± 0.7 nmol of Ca^{2+}/mg of protein.

The fast phase of Ca^{2+} uptake and the formation of the phosphorylated enzyme, E~P, occur in approximately the same time interval, indicating an interrelationship of the two processes. Ikemoto et al. (1981) and Sumida & Tonomura (1974) have performed acid quench experiments in which the appearance of a fast Ca^{2+} uptake phase and E~P formation seemed to coincide. Their experimental conditions were quite different, however, from the present work. Ikemoto et al. used low Ca^{2+} concentrations; Sumida and Tonomura omitted Mg^{2+}.

The maximum amount of acid-stable phosphorylated enzyme, E~P, formation is 6.0 ± 0.2 nmol of E~P/mg of ATPase, or 4.0 ± of 0.13 nmol of E~P/mg of protein, as determined by the quenched-flow experiment. Hence, the mole to mole ratio of fast Ca^{2+} uptake to E~P formation is 1.7 ± 0.2, or approximately 2:1.[2] Experiments with lower time resolution but similar concentration relationships support this finding. Inesi and co-workers (Kurzmack et al., 1977; Verjovski-Almeida et al., 1978), using a rapid mixing EGTA[1] quench technique with $^{45}Ca^{2+}$, obtained a 2:1 ratio at 25 °C by employing the Ca^{2+} uptake intercept value at time zero of 6–8 nmol of Ca^{2+}/mg of protein and the amount of E~P formed of 3.5–4.0 nmol of E~P/mg of protein. Dupont (1980) performed Millipore filtration EGTA quench experiments with radioactive Ca^{2+} at 0 °C and likewise found the ratio to be 2:1, with values of 5.5 nmol of Ca^{2+}/mg of protein and 3 nmol of E~P/mg of protein. At 10 °C, Yamada et al. (1970) also obtained the 2:1 ratio by using a Millipore filtration technique.

Because an ionophore forms lipophilic cation complexes (Scarpa et al., 1973; Simon et al., 1977), it can cause a rapid equilibration of an ionic gradient. When ionophore A23187 is present before addition of ATP, it prevents the establishment of a Ca^{2+} gradient across the SR membrane. The fast phase of ATP-dependent Ca^{2+} uptake, as evidenced by the fact that the dye in the extravesicular aqueous phase senses a rapid loss of Ca^{2+}, is unaffected by the presence of ionophore A23187 added to the reaction medium before photolytic release of ATP. Either (1) the Ca^{2+} remains accessible on the SR membrane vesicle exterior and there is an ATP-induced increase in the affinity of two binding sites for Ca^{2+} or (2) the Ca^{2+} is inaccessible and is relocated inside the membrane itself, away from either the inner or the outer surface. These two possibilities cannot be distinguished by the experiments in the present work. Dupont (1980) concluded from Millipore filtration EGTA quench experiments with $^{45}Ca^{2+}$ at 0 °C that the Ca^{2+} associated with the fast phase was not accessible from either side of the membrane, and called the Ca^{2+} "occluded".

Slow Phase of Calcium Uptake. The slow phase of Ca^{2+} uptake has a specific rate constant of 0.60 ± 0.90 s^{-1} at 25–26 °C (with the given experimental conditions) and an activation energy of 22 ± 1 kcal mol^{-1}. Because the fast phase of Ca^{2+} uptake could be kinetically differentiated from the slow phase due to the higher time resolution of the present experiments, it was possible to accurately determine for the first time their separate and characteristic activation energies. Inesi et al. (1973) measured the temperature dependence of Ca^{2+} transport by using $^{45}Ca^{2+}$ and Millipore filtration in the presence of oxalate, which, by forming a complex with the intravesicular Ca^{2+} transported, permits linear rates of Ca^{2+} transport for longer time periods. There was a transition in

[2] Due to the different conditions in the Ca^{2+} uptake and quenched-flow experiments necessitated by the different methods, the maximum E~P formation during the fast phase of Ca^{2+} uptake would be ≤6.0 ± 0.2 nmol/mg of ATPase (or ≤4.0 ± 0.13 nmol/mg of protein), and the resulting ratio of Ca^{2+} uptake to E~P formation would be ≥1.7 ± 0.2. In preliminary Ca^{2+} uptake experiments with 0.5 or 0.25 the amount of SR used in the Ca^{2+} uptake experiments described here, the amount of Ca^{2+} uptake in the fast phase was proportional to the amount of SR present. Therefore, it would seem reasonable to conclude that the ratio of fast Ca^{2+} uptake to maximum E~P formation is ~2:1.

the Arrhenius plot around 20 °C, and the values for the activation energy were 28 kcal mol^{-1} between 5 and 20 °C and 17 kcal mol^{-1} between 20 and 40 °C. Initial rates instead of specific rate constants were employed in the calculation of the activation energy. The values of E_a obtained by Inesi et al. may be more difficult to interpret in terms of Ca^{2+} transport because (1) the kinetics of the process could be affected by the presence of oxalate and (2) the fast Ca^{2+} uptake phase could not be differentiated from the slow phase.

The total amount of Ca^{2+} uptake in the slow phase is 60 ± 6 nmol of Ca^{2+}/mg of ATPase, or 40 ± 4 nmol of Ca^{2+}/mg of protein with flash photolysis of caged ATP when ~35 nmol of ATP/mg of ATPase or ~23 nmol of ATP/mg of protein is released. Therefore, the mole to mole ratio of Ca^{2+} uptake in the slow phase to ATP hydrolyzed is ~2:1, as would be expected under conditions with low ATP to ATPase ratios (about 3) and resulting small transmembrane Ca^{2+} gradients. The latter is supported by the results that multiple flashes generally produce similar Ca^{2+} uptake kinetics. This 2:1 ratio confirms the already established 2:1 ratio (MacLennan & Holland, 1975; Yamamoto et al., 1979; Inesi, 1979) obtained either under limiting conditions or during initial Ca^{2+} uptake.

When ionophore A23187 is present before the photolytic addition of ATP, the slow phase of Ca^{2+} uptake does not occur. Therefore, since in this case the ionophore prevents the establishment of a transmembrane Ca^{2+} gradient, the slow phase must be associated with the formation of this gradient, resulting from the translocation of Ca^{2+} across the membrane profile. With experiments of direct ATP addition, Scarpa and co-workers (Scarpa & Inesi, 1972; Scarpa et al., 1973) have added an ionophore after Ca^{2+} was accumulated by SR vesicles and observed the subsequent release of the Ca^{2+} at 24 °C.

Flash Photolysis of Caged ATP To Initiate Calcium Uptake. The advantage of using this caged ATP photolyzed with a frequency-doubled ruby laser is the rapid release of ATP molecules, dependent on the caged compound's dark reaction, which has a rate constant of 220 s^{-1} at pH 7.0 and 22 °C (McCray et al., 1980). As a result, the Ca^{2+} transport processes are effectively synchronized among the ensemble of ATPase molecules. It was thus possible to time resolve the fast phase of Ca^{2+} uptake by SR because the specific rate is considerably slower than that of (1) the release of ATP from caged ATP, (2) the diffusion of the released ATP to the neighboring ATPase, and (3) the response of the dye arsenzo III, whose on and off reaction with Ca^{2+} is on the order of a millisecond (Scarpa, 1979). However, since the specific rates of the fast phase of Ca^{2+} uptake and of ATP release differ only by a factor of 3–4, the kinetics of the fast phase may be partially limited by the dark reaction of caged ATP photolysis.

Quenched-flow techniques can have a time resolution similar to that of flash photolysis of this caged ATP. A disadvantage, however, to quenched-flow techniques is that the results are less precise, since scattered points are obtained (Ikemoto et al., 1981) instead of continuous kinetic curves, as in the present work. Recently, Froehlich and Heller (J. P. Froehlich and P. H. Heller, personal communication), using a quenched-flow procedure and $^{45}Ca^{2+}$ at 21 °C with time resolution in the millisecond regime, also obtained biphasic kinetics for Ca^{2+} uptake. They determined a specific rate constant of 15 s^{-1} at 21 °C for the initial, fast phase of Ca^{2+} uptake, which is reasonable compared with our value of 64 s^{-1} at 23–26 °C, considering the different reactant concentrations in the two experiments.

The technique of flash photolysis of caged ATP requires the use of suitable controls, such as the replacement of caged ATP with caged ADP, or the omission of caged nucleotide or SR. No change in absorbance was detected in any of the control experiments, indicating that there is no effect due to energy dissipation from the laser flash, addition of nucleotide, or the nonsubstrate photolysis reaction products on our measurements of Ca^{2+} uptake kinetics.

The determination of the kinetics of Ca^{2+} uptake with this high time resolution, and the differentiation and identification of at least two phases of Ca^{2+} uptake, should provide useful information for an understanding of the mechanism of the Ca^{2+} transport process as well as for the interpretation of parallel dynamic structural studies (Blasie et al., 1982, 1983). Dynamic structural experiments utilizing flash photolysis of caged ATP to initiate the Ca^{2+} transport process can be performed either (1) with time resolution or (2) with low-temperature trapping of an intermediate within the observation time window.

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Registry No. Calcium, 7440-70-2; ATPase, 9000-83-3.

References

Blasie, J. K., Herbette, L., Pierce, D. H., Pascolini, D., Scarpa, A., & Fleischer, S. (1982) *Ann. N.Y. Acad. Sci. 402*, 478–484.

Blasie, J. K., Herbette, L., Pierce, D. H., Pascolini, D., Skita, V., Scarpa, A., & Fleischer, S. (1983) in *Sarcoplasmic Reticulum: Structure and Function* (Fleischer, S., & Tonomura, T., Eds.) Academic Press, New York (in press).

Chaloub, R. M., & deMeis, L. (1980) *J. Biol. Chem. 255*, 6168–6172.

Chance, B., Legallais, V., Sorge, J., & Graham, N. (1975) *Anal. Biochem. 66*, 498–514.

deMeis, L., & Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 280–282.

Dupont, Y. (1980) *Eur. J. Biochem. 109*, 231–238.

Ebashi, S., Endo, M., & Ohtsuki, I. (1969) *Q. Rev. Biophys. 2*, 351–384.

Glasstone, S. (1954) in *Textbook of Physical Chemistry*, p 1104, Van Nostrand, New York.

Goldman, Y. E., Gutfreund, H., Hibberd, M. G., McCray, J. A., & Trentham, D. R. (1982) *Biophys. J. 37*, 125a.

Gutfreund, H. (1969) *Methods Enzymol. 56*, 229–249.

Hasselbach, W., & Waas, W. (1982) *Ann. N.Y. Acad. Sci. 402*, 459–469.

Herbette, L., Marquardt, J., Scarpa, A., & Blasie, J. K. (1977) *Biophys. J. 20*, 245–272.

Ikemoto, N., Garcia, A. M., Kurobe, Y., & Scott, T. L. (1981) *J. Biol. Chem. 256*, 8593–8601.

Inesi, G. (1979) in *Membrane Transport in Biology* (Tosteson, D. C., Ed.) Vol. II, pp 353–393, Springer-Verlag, New York.

Inesi, G., Millman, M., & Eleter, S. (1973) *J. Mol. Biol. 81*, 483–504.

Kaplan, J. H., Forbush, B., III, & Hoffman, J. F. (1978) *Biochemistry 17*, 1929–1935.

Khwaja, T. A., Reese, C. B., & Stewart, J. C. M. (1970) *J. Chem. Soc. C*, 2092–2100.

Kreevoy, M. M. (1963) in *Technique of Organic Chemistry* (Friess, S. L., Lewis, E. S., & Weissberger, A., Eds.) Vol. VIII, p 137, Wiley, New York.

Kurzmack, M., Verjovski-Almeida, S., & Inesi, G. (1977) *Biochem. Biophys. Res. Commun. 78*, 772–776.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 263–275.
MacLennan, D. H., & Holland, P. C. (1975) *Annu. Rev. Biophys. Bioeng. 4*, 377–404.
McCray, J. A., Herbette, L., Kihara, T., & Trentham, D. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7237–7241.
McFarland, B. H., & Inesi, G. (1971) *Arch. Biochem. Biophys. 145*, 456–464.
Pierce, D. H. (1982) Ph.D. Dissertation, University of Pennsylvania, Philadelphia, PA.
Scarpa, A. (1979) *Methods Enzymol. 56*, 301–338.
Scarpa, A., & Inesi, G. (1972) *FEBS Lett. 22*, 273–276.
Scarpa, A., Baldassare, J., & Inesi, G. (1973) *J. Gen. Physiol. 60*, 735–749.
Simon, W., Morf, W. E., & Ammon, D. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E. Kretsinger, R. H., MacLennan, D. H., & Siegel, F. L., Eds.) pp 50–62, Elsevier, New York.
Strehler, B. L. (1968) *Methods Biochem. Anal. 16*, 99–181.
Sumida, M., & Tonomura, Y. (1974) *J. Biochem. (Tokyo) 75*, 283–297.
Takakuwa, Y., & Kanazawa, T. (1981) *J. Biol. Chem. 256*, 2691–2700.
Verjovski-Almeida, S., Kurzmack, M., & Inesi, G. (1978) *Biochemistry 17*, 5006–5013.
Yamada, S., Yamamoto, T., & Tonomura, Y. (1970) *J. Biochem. (Tokyo) 67*, 789–794.
Yamamoto, T., Takisawa, H., & Tonomura, Y. (1979) *Curr. Top. Bioenerg. 9*, 179–236.

Distance Measurement between the Active Site and Cysteine-177 of the Alkali One Light Chain of Subfragment 1 from Rabbit Skeletal Muscle†

Diana J. Moss‡ and David R. Trentham*

ABSTRACT: Förster energy-transfer techniques have been applied to labeled myosin subfragment 1 from rabbit skeletal muscle to determine an intramolecular distance and whether this distance changes during magnesium-dependent ATPase activity. The alkali one light chain was labeled at Cys-177 with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) and then exchanged into subfragment 1. High specificity of labeling was indicated by high-performance liquid chromatography analysis of a tryptic digest of the labeled light chain. 2'(3')-*O*-(2,4,6-Trinitrophenyl)adenosine 5'-diphosphate (TNP-ADP) was bound to the labeled protein at the ATPase active site. The efficiency of energy transfer between the probes was 0.09 when measured by both steady-state and time-resolved fluorescence. Anisotropy measurements of the bound AEDANS indicated considerable freedom of motion of the probe. The probable distance between the probes was 57 Å. This distance was unchanged during triphosphatase activity. Two further sites of TNP-ADP interaction with subfragment 1 were found. The effect of these interactions on the energy-transfer measurements was reduced to a minimum by careful choice of reaction conditions.

The myosin and actomyosin ATPase[1] mechanisms have been intensively studied to characterize the intermediates and measure their rates of interconversion (Trentham et al., 1976; Taylor, 1979). This has led to a detailed description of the hydrolysis of ATP (Webb & Trentham, 1983).

$$\mathrm{M} + \mathrm{ATP} \overset{1}{\rightleftharpoons} \mathrm{M{\cdot}ATP} \overset{2}{\rightleftharpoons} \mathrm{M^*{\cdot}ATP} + \mathrm{H_2O} \overset{3}{\rightleftharpoons} \mathrm{M^{**}{\cdot}ADP{\cdot}P_i} \overset{4}{\rightleftharpoons} \mathrm{M^*{\cdot}ADP{\cdot}P_i} \overset{5}{\rightleftharpoons} \mathrm{M^*{\cdot}ADP} + \mathrm{P_i} \overset{6}{\rightleftharpoons} \mathrm{M{\cdot}ADP} \overset{7}{\rightleftharpoons} \mathrm{M} + \mathrm{ADP} \quad (1)$$

Equation 1 shows a seven-step mechanism for the myosin ATPase in which the asterisks are used to distinguish intermediates. In eq 1 conformation changes (steps 2, 4, and 6), which have so far been characterized predominantly by kinetic methods, are associated with substrate binding and the release of each product. The nature of the conformation changes implied in the myosin and actomyosin ATPase mechanisms is not understood. It is not clear, for example, whether the conformation changes have a direct role in contraction, possibly through the movement of large segments of protein, or whether their role is more indirect and is limited to small perturbations of structure in the vicinity of the ATPase active site.

Many techniques have been used to probe the structural features of actin and myosin. Of these, utilization of Förster energy transfer is especially promising as a means to determine the relative location of points of interest and to examine the size and extent of the inter- and intramolecular protein motion (Stryer, 1978). Over the past few years several measurements

† From the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104. *Received March 15, 1983.* Supported by grants from the National Institutes of Health (AM 23030), the Muscular Dystrophy Association of America, and Whitehall Foundation. This work has been described in part in Moss & Trentham (1980).

‡ Present address: MRC Cell Biophysics Unit, London WC2B 5RL, U.K.

[1] Abbreviations: ATPase, adenosine-5'-triphosphatase; TNP-ADP, 2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine 5'-diphosphate (other TNP containing compounds are similarly abbreviated); 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; A1 and A2 light chains, the alkali one and alkali two light chains of myosin (Weeds & Pope, 1977); S1A1 and S1A2, subfragment 1 containing A1 and A2 light chains, respectively; A1-AEDANS light chain, A1 light chain labeled at Cys-177 with 1,5-IAEDANS (other 1,5-IAEDANS-labeled species are similarly abbreviated); DEAE, diethylaminoethyl; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography.

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