Reaction Mechanism of the Cardiac Sarcotubule Calcium(II) Dependent Adenosine Triphosphatase†

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ABSTRACT: Cardiac sarcoplasmic reticulum reacts with γ-$AT^{32}P$ to form a labeled phosphorylated intermediate, which is stable at low temperatures, at acidic pH, in the presence of calcium and in the absence of magnesium ions. Formation of the intermediate is highly dependent on calcium and is not affected by ouabain or azide. Low temperature (2°) increases the yield of the intermediate. In the presence of 5 mM calcium and at 2°, the maximum concentration of phosphoprotein was 1.2 nmol/mg of protein. In order to study the hydrolytic reaction and the reaction of the phosphorylated intermediate with ADP, the labeled sarcoplasmic reticulum was separated from ATP and hydrolysis products by ion exchange chromatography. The equilibrium constant for the reversible phosphorylation varied with the ionic environment. In the presence of 1 mM $CaCl_2$, the equilibrium constant was 5, while in the presence of 2 mM EDTA, the constant was 1. The rate constant for the hydrolysis of the intermediate was dependent on the magnesium concentration (10^{-5}–10^{-2} M) and the maximum value observed was 3.2 min^{-1}.

Subcellular fractions can be prepared from mammalian cardiac muscle which contain an ATP-dependent calcium transport system. These fractions are apparently enriched in materials similar to sarcotubular preparations obtained from skeletal muscle (Fuchs *et al.*, 1968). Although the mechansim of ATP hydrolysis in the sarcotubular fractions of skeletal muscle has been extensively studied (Makinose, 1969; Martonosi, 1969; Inesi *et al.*, 1970; Kanazawa *et al.*, 1971; Panet *et al.*, 1971), equivalent studies have not appeared with cardiac sarcotubular preparations. A few brief reports (Namm *et al.*, 1972; Fanburg and Matsushita, 1973; Pang and Briggs, 1973) indicate that cardiac sarcotubule fractions form a phosphoprotein intermediate from ATP during its hydrolysis. In the present study, three partial reactions of the ATPase were studied. They are the formation of the intermediate, the hydrolysis of the intermediate, and the backward reaction between ADP and the intermediate (Kanazawa *et al.*, 1971).

Materials and Methods

Preparation of the Sarcotubule-Enriched Fraction. Canine hearts removed from dogs anesthetized with sodium pentobarbitol were used as starting material. The ventricular muscle was chilled in ice and washed free of blood with cold saline, cut into small pieces, and homogenized in 4 volumes of 0.3 M sucrose–10 mM imidazole (pH 7.0) for 40 sec with a Sorvall Omnimixer. Following centrifugation at 17,300*g*-max for 20 min to remove myofibrils and mitochondria, the sarcoplasmic reticulum pellet was collected by centrifugation at 34,800*g*-max for 25 min. The pellet which was washed with 0.6 M KCl–10 mM imidazole (pH 7.0) (1.3 ml per gram of heart) to remove contaminant proteins was reclaimed by centrifugation at 144,000*g*-max for 30 min and resuspended in a small volume of 0.3 M sucrose–10 mM imidazole (pH 7.0). The protein concentration averaged 5 mg/ml.

Incubation of Cardiac Sarcoplasmic Reticulum with γ-$AT^{32}P$. Unless otherwise stated, incubation of sarcoplasmic reticulum (0.8–1.0 mg/ml) was carried out at 2° with γ-labeled $AT^{32}P$ (Amersham/Searle, specific activity, 500 Ci/mol). The reaction was started by the addition of 0.1 ml of 8×10^{-5} M ATP with 0.4 μCi γ-labeled $AT^{32}P$ to 3.9 ml of an incubation medium containing 51.5 mM KCl, 1.03 mg of sarcoplasmic reticulum/ml, and 10.3 mM imidazole (pH 7.0). When added, the concentrations of the following were: $CaCl_2$, 10^{-4}–10^{-2} M; $MgCl_2$, 10^{-4}–10^{-2} M; EGTA,[1] 0.1–2 mM; EDTA, 0.1 mM; ouabain, 0.1 mM; and sodium azide, 10 mM. The incubation mixture which was sampled (0.5 ml) at intervals between 12 sec and 1.8 min after the addition of γ-$AT^{32}P$ was pipetted into 1 ml of ice-cold 1.2 M perchloric acid containing 80 g/l. of silicotungstic acid. Since addition of cold 10^{-4} M ATP and 10^{-3} M inorganic phosphate with the perchloric acid was not found to influence the apparent level of phosphoprotein intermediate, these additions were abandoned. The denatured protein, after precipitation, was centrifuged at 10,000*g*-max for 10 min. The resultant pellet was used for measurement of the phosphoprotein intermediate and the supernatant for the measurement of inorganic ^{32}P.

A. Measurement of Phosphoprotein. For the measurement of the phosphoprotein intermediate, the sediment was washed successively with 4 ml of ice-cold 5% trichloroacetic acid and 4 ml of ice-cold 2% trichloroacetic acid. The amount of radioactivity in the supernatant, after the 2% trichloroacetic acid wash, was found to be insignificant. The final protein pellet was resuspended in 2 ml of 2% Na_2CO_3 in 0.1 N NaOH and heated in a boiling water bath for 15 min. Aliquots were assayed for protein by the method of Lowry *et al.* (1951) and for radioactivity by liquid scintillation.

B. Measurement of Radioactive Inorganic Phosphate. The radioactive inorganic phosphate in 1 ml of supernatant was extracted by the method of Wahler and Wollenberger (1958) and counted by liquid scintillation. Quench curves for the butyl acetate were prepared by addition of butyl acetate to $^{32}P_i$ standards.

C. Calculation of ATP Concentrations. The fraction of ATP

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[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*-tetraacetic acid.

remaining in the incubation medium was calculated as follows.

fraction of ATP remaining =

$$1 - \frac{\text{phosphoprotein (nmol/mg)} + \text{PO}_4\text{ (nmol/mg)}}{\text{initial ATP (nmol/ml)/SR (mg/ml)}}$$

Measurement of Magnesium in Sarcoplasmic Reticulum (SR). Sarcoplasmic reticulum was digested with 70% perchloric acid and assayed for magnesium in the presence of 0.9% La^{3+} and 0.09 N hydrochloric acid by atomic absorption spectroscopy.

Purification of Phosphorylated Sarcoplasmic Reticulum. The phosphoprotein intermediate was purified on an anion exchange resin at 2° as described by Panet *et al.* (1971). The sarcoplasmic reticulum–ATP reaction mixture (6 ml) was put on a column (1 × 3.5 cm) of Dowex AG 1-X8 chloride form, 100–200 mesh, preequilibrated with a solution containing 10 mM imidazole (pH 7.0)–1 mM $CaCl_2$. The first 0.5 ml of eluate was discarded and the subsequent cloudy phosphoprotein intermediate fraction was collected over the next 5 min. To determine the amount of contaminating nucleotides, the purified phosphoprotein intermediate fraction was centrifuged at 34,000*g*-max for 30 min and the optical density at 260 mμ (Vischer and Chargaff, 1948) was measured against a blank consisting of the supernatant from a sarcoplasmic reticulum fraction which had been eluted the same way except that ATP was omitted. No soluble nucleotide was measurable in the purified phosphoprotein intermediate fraction. About 90% of the phosphoprotein intermediate was recovered.

ATP Formation from ADP and Phosphoprotein. ADP (0.2 ml, 0.032–1.600 mM) in the presence and absence of 32 mM EDTA was added to 3.0 ml of purified phosphoprotein intermediate fraction (0.3–0.6 nmol of $^{32}P_i$/mg of protein and 0.5 mg of protein/ml) in 1.0 mM $CaCl_2$–10 mM imidazole (pH 7.0). For control experiments, 0.2 ml of EDTA (32 mM) or 0.2 ml of deionized water was added instead; 0.5 ml of sample was taken at intervals between 12 sec and 2.0 min after the addition of ADP and pipetted into 1 ml of ice-cold 1.2 M perchloric acid containing 80 g/l. of silicotungstic acid. The denatured protein, after precipitation, was centrifuged at 10,000*g*-max for 10 min. The resultant pellet was used for measurement of the phosphoprotein intermediate and the supernatant for the measurement of radioactive inorganic phosphate.

The equilibrium constant, K_{eq}, for the reaction E + ATP $\rightleftarrows$ EP + ADP can be expressed as

$$K_{eq} = [\text{ADP}][\text{EP}]/[\text{E}][\text{ATP}]$$

or

$$K_{eq} = \frac{[\text{ADP}_0 - (\text{EP}_0 - \text{EP}_t)](\text{EP}_t)}{(E_0 - \text{EP}_t)(\text{EP}_0 - \text{EP}_t)}$$

where E_0 is the total number of phosphorylated sites on the sarcoplasmic reticulum; EP_0, the phosphoprotein intermediate (EP) concentration at time zero; EP_t, the EP concentration at equilibrium; ADP_0, the ADP concentration at time zero.

Decomposition of Phosphoprotein. $MgCl_2$ (0.2 ml, 0.16–64 mM) dissolved in EGTA (32 mM) was added to 3.0 ml of purified phosphoprotein intermediate fraction (0.3–0.6 nmol of $^{32}P_i$/mg of protein and 0.5 mg of protein/ml) in 1.0 mM $CaCl_2$–10 mM imidazole (pH 7.0). For control experimens, 0.2 ml of deionized water or 0.2 ml of EGTA (32 mM) was added; 0.5-ml samples were taken at intervals between 12 sec and 1 min. The phosphoprotein intermediate level and the rate of radioactive phosphate liberation were measured.

The rate of decomposition of phosphoprotein intermediate (EP), v_t, can be expressed as

$$v_t = K_d\text{EP}_t$$

where K_d = the rate constant for the reaction EP → E + P_i and EP_t = the EP concentration at time *t*.

Results

Characterization of the ATPases in the Sarcoplasmic Reticulum Fraction. Although it has been demonstrated by Namm *et al.* (1972), Fanburg and Matsushita (1973), and Pang and Briggs (1973) that fractions enriched in cardiac sarcotubular material form a phosphoprotein intermediate during ATP hydrolysis, evidence that the EP is derived from the ATPase of the sarcosplasmic reticulum and not from other ATPases contaminating the fraction has only been presented by Pang and Briggs (1973), who showed that ouabain and azide had no influence on the phosphoprotein intermediate level when either calcium or magnesium was added to the incubation mixture. In order to interpret the effects of various conditions and circumstances on phosphoprotein intermediate levels, it is necessary to know the extent to which ATPases other than the sarcotubular enzyme are competing for ATP and forming P_i. Without this information, it is not possible to distinguish between P_i formation due to phosphoprotein intermediate turnover and P_i formation due to other enzymes. In order to evaluate this problem, various inhibitors were added to the incubation mixture: 10 mM azide was added to inhibit mitochondrial ATPase (Fanburg and Gergely, 1965), EGTA was added to inhibit calcium-stimulated ATPase activity, and ouabain was added to depress ($Na^+ + K^+$) ATPase activity (Post *et al.*, 1965). The conditions chosen for this study were optimal for Mg–ATPase activity and otherwise similar to those found (temperature and ATP concentration) to be favorable for the phosphoprotein intermediate studies. The results shown in Table I indicate that about 50% of the ATPase activity originated from calcium-dependent ATPase activity (as indicated by the extent of inhibition by EGTA) and about 50% from the mitochondrial ATPase. Addition of ouabain did not affect the ATPase activity, suggesting that under the conditions of these experiments, the ($Na^+ + K^+$) ATPase was not active.

Effect of Temperature on Phosphate Liberation and Phosphoprotein Level. Figure 1 shows the effect of temperature, 2°, 12°, and 26°, on the rate of phosphate liberation (Figure 1a) and the level of phosphoprotein intermediate (Figure 1b). The experiments shown in these figures were done at the same time on the same sarcoplasmic reticulum (see Methods). The terminal phosphate of ATP thus appears either as phosphoprotein or inorganic phosphate. In the presence of magnesium, the rate of phosphate liberation was so high that even at 2° the phosphate obtainable from ATP was almost completely liberated in 0.7 min (Figure 1a). At the same time, a very low level of phosphoprotein intermediate (0.4 nmol of $^{32}P_i$/mg was observed at 0.2 min at 2° (Figure 1b) and the level decreased rapidly thereafter. Since in the presence of

TABLE I: ATPase Activity of Cardiac Sarcoplasmic Reticulum.[a]

Conditions	ATPase Activity (nmol mg^{-1} min^{-1})
Control	7.2 ± 0.4
Azide (10 mM)	4.6 ± 0.3
EGTA (1 mM)	4.1 ± 0.2
Azide (10 mM) + EGTA (1 mM)	0.4 ± 0.0
Ouabain (0.1 mM)	7.5 ± 0.7

[a] Cardiac sarcoplasmic reticulum (0.50–0.75 mg/ml) was incubated in 5 mM $MgCl_2$–10 mM imidazole (pH 7.0)–50 mM KCl–2 μM γ-$AT^{32}P$ at 2° for 0.2 min. Values were expressed as mean ± SEM. Number of experiments was 3.

magnesium all the $AT^{32}P$ had been hydrolyzed to $^{32}P_i$ (Figure 1a) with negligible $E^{32}P$ measurable (Figure 1b) at 12 and 26°, little information was obtained about sarcotubular ATP hydrolysis under these conditions. Presumably phosphoprotein intermediate formation and hydrolysis were too fast to be observed. In the presence of calcium, however, the rate of phosphate liberation was lower and increased with temperature. At 2°, the amount of phosphate liberated appeared to plateau after 1.3 min (Figure 1a). A less clearly defined plateau was seen at 12 and 26°. At 2°, the plateau in the phosphoprotein intermediate level (Figure 1b) and the lack of further phosphate liberation between 1.2 and 1.8 min (Figure 1a) suggest that the rate of breakdown of phosphoprotein to phosphate is very low at this temperature. The early rise in the phosphate level at 2° cannot, therefore, be derived from the phosphoprotein intermediate and must be due to the presence of mitochondrial fragments. As the level of ATP drops, largely due to phosphoprotein intermediate formation, the rate at which this second enzyme will hydrolyze ATP will also drop. (At 2° and 0.7 min, the sum of EP + P_i is 1.9 nmoles/mg; thus the ATP level must be about 0.1 μM, which presumably is too low for the mitochondrial enzyme.) In the presence of calcium, the phosphoprotein intermediate level was observed to fall more rapidly at 26 than at 2°. (Since the sum of EP + P_i = 2.0 nmol/mg after 0.5 min at 26°, the fall in level of phosphoprotein intermediate represents phosphoprotein intermediate hydrolysis rather than phosphoprotein intermediate turnover.) The important conclusion reached through these temperature studies is that although the phosphoprotein intermediate is readily formed at 2°, it is only slowly broken down at this temperature in the presence of calcium. Thus 2° is an optimal temperature for the formation of the phosphoprotein intermediate.

Effect of Substrate Concentration on Phosphate Liberation and Phosphoprotein Level. Figure 2a shows that increasing the ATP concentration from 2 to 10 μM at 2° increases the rate of phosphate liberation when either magnesium or calcium is the added divalent cation. The phosphate liberation observed in the presence of added calcium must not be coming from the phosphoprotein intermediate, since the phosphoprotein intermediate does not hydrolyze rapidly at that temperature and must, therefore, be coming from another enzyme (Table I). Addition of EGTA with magnesium reduced the rate of phosphate liberation (Figure 2a), suggesting that some of the enzyme activity was dependent upon contaminating calcium. In Figure 2b, the level of the phosphoprotein intermediate formed in the presence of 10 μM ATP was equal to that at 2 μM when calcium was the added divalent cation, but was markedly different when magnesium was the added cation. This suggests that the rate of phosphoprotein intermediate formation is dependent on ATP concentration. At 10 μM ATP, the rate of formation is sufficiently greater than the rate of breakdown to produce measurable levels of the phosphoprotein intermediate. That the formation of the phosphoprotein intermediate depends, however, on the presence of some calcium is shown by the fact that when EGTA is added the level of the phosphoprotein intermediate becomes immeasurable even though ATP is 10 μM. It appears, therefore, that 2 μM ATP is sufficient for phosphoprotein intermediate formation if the rate of phosphoprotein intermediate hydrolysis is minimized by the lack of magnesium or low temperature.

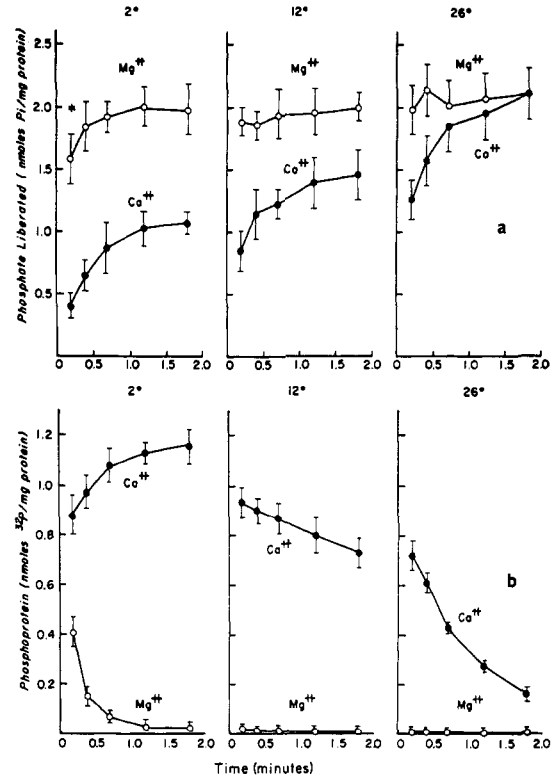

FIGURE 1: (a) Effect of temperature on the ATPase of cardiac sarcoplasmic reticulum. Sarcoplasmic reticulum (0.8–1.0 mg/ml) was incubated with 5 mM $CaCl_2$ or $MgCl_2$–2 μM ATP with 0.1 μCi/ml of γ-$AT^{32}P$ in the presence of 50 mM KCl and 10 mM imidazole (pH 7.0) at 2°. In this and all subsequent figures the values presented are expressed as mean ±SEM; *2 nmol/mg represents hydrolysis of all ATP. (b) Effect of temperature on the level of phosphoprotein in cardiac sarcoplasmic reticulum. The conditions were the same as in Figure 1A.

Effect of Divalent Metals on the Phosphoprotein Levels. Although the effects of magnesium and calcium on phosphoprotein intermediate levels have been shown in previous figures, the effects of changing the concentrations of these metals were not described. Figure 3 shows that magnesium concentrations ranging from 10^{-4} to 10^{-2} M have little effect on the level. It appears, therefore, that the rate of phosphoprotein intermediate breakdown is not much altered within this range of magnesium concentrations. Experiments designed to test this conclusion more directly will be described in a later section. Reducing the calcium level from 10^{-2} to 10^{-4} M does, however, influence phosphoprotein intermediate levels. Although the rate of rise in the phosphoprotein intermediate appears to be particularly influenced by the calcium levels,

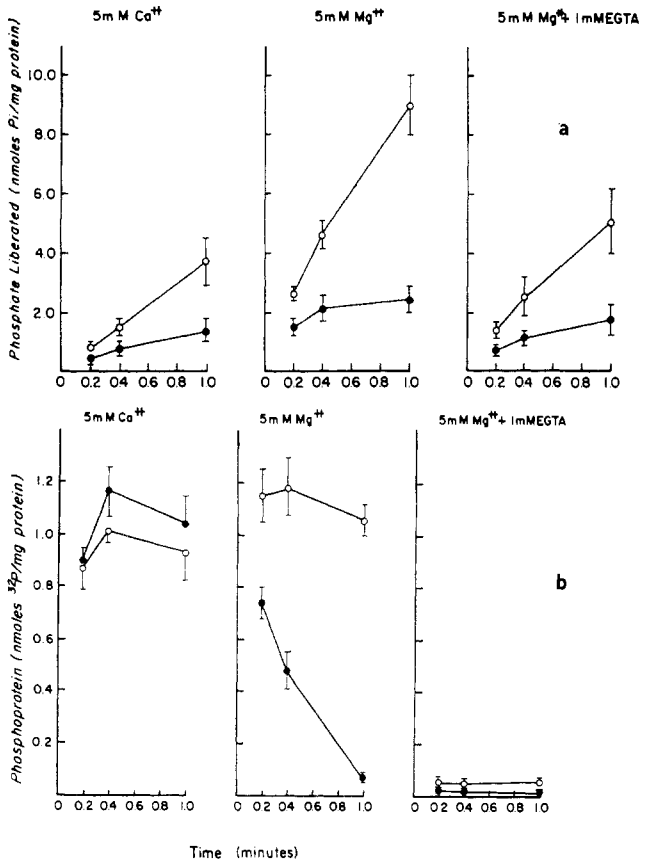

FIGURE 2: (a) Effect of ATP concentration on the ATPase activity of cardiac sarcoplasmic reticulum. The conditions were the same as in Figure 1; (●) 2 μM ATP. (○) 10 μM ATP. (b) Effect of ATP concentration on the level of phosphoprotein in cardiac sarcoplasmic reticulum. The conditions were the same as in Figure 2a.

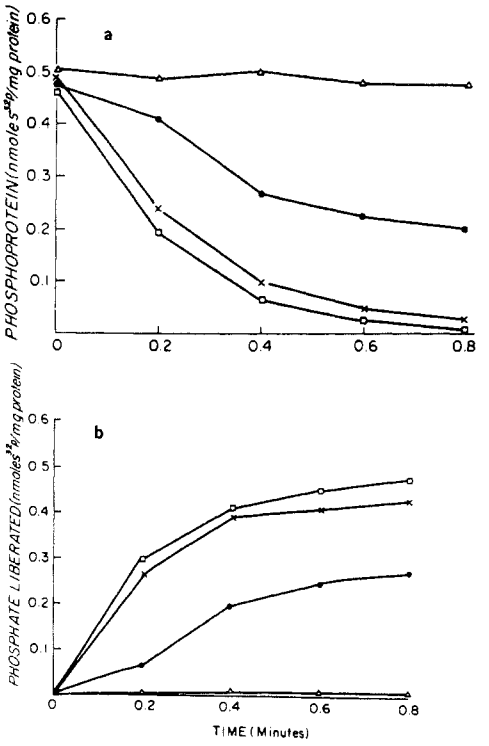

FIGURE 4: (a) Effect of magnesium and EGTA on the hydrolysis of phosphoprotein in cardiac sarcoplasmic reticulum. Purified phosphoprotein (0.5 mg/ml) was incubated in 1.0 mM $CaCl_2$–10 mM imidazole (pH 7.0). Magnesium and EGTA were added as described in Methods. This figure shows a typical experiment: (△) control, (●) 2 mM EGTA, (×) 2 mM EGTA + 0.2 mM Mg^{2+}, (□) 2 mM EGTA + 1.0 mM Mg^{2+}. (b) Effect of magnesium and EGTA on the liberation of phosphate from phosphoprotein in cardiac sarcoplasmic reticulum. Conditions were the same as in Figure 4a.

the maximum phosphoprotein intermediate level achieved did not increase between 10^{-4} and 10^{-3} M of calcium.

Effect of Magnesium on Phosphoprotein Hydrolysis. Figure 4a and b shows the typical effects of magnesium on the breakdown of the phosphoprotein intermediate to P_i. These experiments were carried out with the phosphoprotein intermediate isolated by anion exchange chromatography as described by Panet *et al.* (1971). The decrease in the phosphoprotein intermediate levels (Figure 4a) corresponded very closely to the rise in P_i level, Figure 4b. From Figure 4a it can be seen that the phosphoprotein intermediate is stable in the presence of 1 mM calcium (control). If calcium was chelated by the addition of EGTA (2 mM), measurable phosphoprotein intermediate hydrolysis was observed. This breakdown of the phosphoprotein intermediate may be related to the magnesium (23–40 nmol/mg of protein) which accompanies the sarcoplasmic reticulum. Addition of magnesium increased the rate of hydrolysis of the phosphoprotein intermediate in proportion to the level of added magnesium. Computation of the hydrolytic rate constant, K_d, from the data in Figure 4a and b indicates that K_d was independent of the phosphoprotein intermediate concentration. Figure 5 shows the dependence of K_d on total magnesium. (The magnesium present in the sarcoplasmic reticulum has been included.) The rate of the phosphoprotein intermediate breakdown appears to increase as a hyperbolic function on total magnesium concentration. Though not shown, the K_d value observed at 4.01 mM magnesium was 3.24 ± 0.15 min^{-1}. A plot of $1/K_d$ against the reciporcal of the magnesium concentration (insert of Figure 5) shows that the values could be fit by a straight line. The relation between K_d and the magnesium concentration is given by (Kanazawa *et al.*, 1971)

$$K_d = \frac{K_{d_{max}}}{1 + K_{Mg}/[Mg^{2+}]}$$

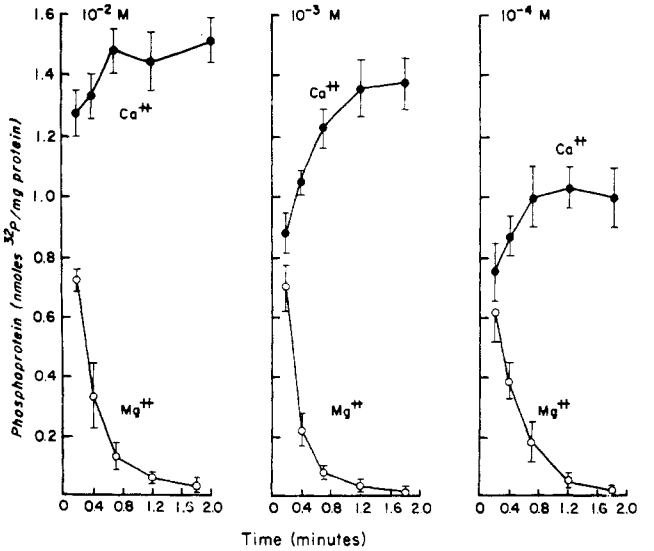

FIGURE 3: Effect of divalent ion concentrations on the level of phosphoprotein in cardiac sarcoplasmic reticulum. The conditions were the same as in Figure 1.

TABLE II: Apparent Equilibrium Constant for the Phosphorylation Reaction of Cardiac Sarcoplasmic Reticulum.[a]

Conditions	No. of Observations	ADP_0 (μM)	EP_0 (μM)	EP_t (μM)	K_{eq}
1 mM $CaCl_2$	2	100	0.26	0.01	7.38
	1	70	0.20	0.01	6.80
	3	50	0.22	0.01	4.39
	1	30	0.19	0.01	3.06
	3	20	0.21	0.02	3.93
	5	10	0.20	0.04	4.82
	5	5	0.17	0.06	5.44
	5	2	0.20	0.11	5.30
					5.09 ± 0.20
2 mM EGTA	3	0.25	0.31	0.21	0.92
	3	0.50	0.30	0.16	1.05
	3	0.75	0.30	0.12	0.88
	3	1.00	0.27	0.10	1.08
	3	2.00	0.28	0.07	1.24
					1.03 ± 0.05

[a] Experimental conditions are given in Methods. All values are expressed in μM. The maximum level of phosphoprotein (E_0) formed was 0.55 μM for these experiments.

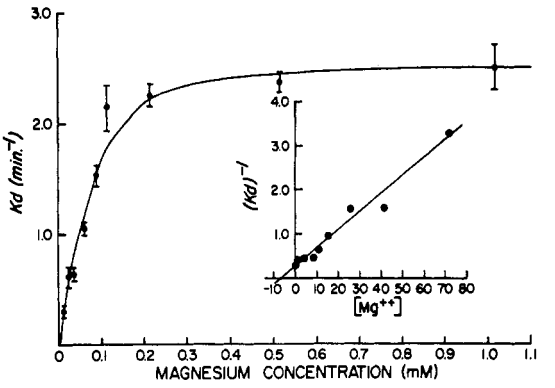

FIGURE 5: Effect of magnesium on rate constant of phosphoprotein hydrolysis (K_d) in cardiac sarcoplasmic reticulum. Conditions were the same as in Figure 4. K_d values were expressed as mean ±SEM.

where $K_{d_{max}}$ is the hydrolytic rate constant at infinite magnesium concentration and K_{Mg} is the dissociation constant of the phosphoprotein intermediate–magnesium ion complex. Using the plot shown in the insert of Figure 5, $K_{d_{max}}$ was found to be 3.72 min^{-1} and $K_{Mg} = 1.5 \times 10^{-4}$ M.

Re-Formation of ATP from ADP and the Phosphoprotein Intermediate. Since the phosphoprotein intermediate can phosphorylate ADP to form ATP, high ADP levels can, by forcing the reaction backwards, inhibit the ATPase activity of the sarcoplasmic reticulum. Figure 6a and b shows the change in the phosphoprotein intermediate levels that follows the addition of ADP to purified phosphoprotein intermediate. Although not shown, the appearance of inorganic phosphate was very low (0.02–0.04 nmol mg^{-1} min^{-1}); thus contaminating ATPases apparently are quite ineffective at these levels of ATP (0.3 μM) and magnesium (intrinsic levels = 23–40 nmol/mg). In the experiment described in Figure 6a, added calcium was 1 mM and the contaminating level of magnesium was between 12 and 20 μM. The response to the addition of ADP in this experiment, in contrast to that shown in Figure 6b in which the divalent metals were chelated with 2 mM EDTA, was the tendency for the phosphoprotein intermediate (EP) level to decline initially to a low level and then to increase during the ensuing incubation period. Since this phenomenon was not observed in the presence of EDTA, it appears to be somehow dependent on one of the metals chelated by EDTA. Data of the type shown in Figure 6a and b were used to calculate the apparent equilibrium constant, K_{eq}, of the reaction E + ATP $\rightleftarrows$ EP + ADP. In order to make this calculation, the total amount of E in the reaction was estimated from the level of phosphoprotein intermediate formed under optimal conditions (5 mM $CaCl_2$–2 μM $AT^{32}P$, 2°). The phosphoprotein intermediate level, 0.8 min after the addition of ADP, was used as the equilibrium level. Table II shows the apparent equilibrium constant measured in the presence of added calcium or added EDTA. The data show that addition of EDTA to the reaction mixture influences the equilibrium constant, decreasing it from a value of 5 to 1. This change may be due to the chelation of calcium or magnesium or both ions in the extravesicular medium, or may reflect an imbalance of these ion concentrations between the inside and the outside of the sarcoplasmic reticulum. (The sarcoplasmic reticulum is impermeable to EDTA under the conditions used in these experiments (Duggan and Martonosi, 1970).)

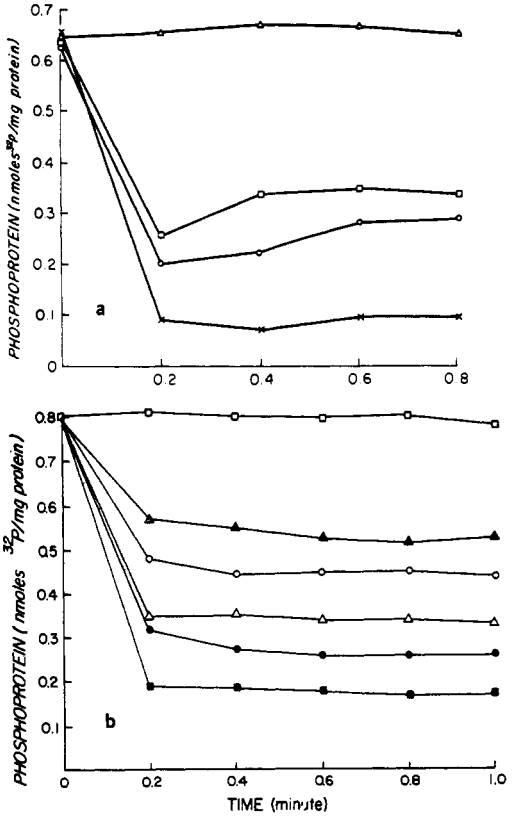

FIGURE 6: (a) Effect of ADP on the level of phosphoprotein in cardiac sarcoplasmic reticulum. ADP was added as described in Methods. This figure shows a typical experiment: (△) control, (□) 2 μM ADP, (○) 5 μM ADP, (×) 10 μM ADP. (b) Effect of ADP and EDTA on the level of phosphoprotein in cardiac sarcoplasmic reticulum. Conditions were as described in Figure 6a: (□) control (2 mM EDTA), (▲) 0.25 μM ADP, (○) 0.50 μM ADP, (△) 0.75 μM ADP, (●) 1.00 μM ADP, (■) 2.00 μM ADP.

Discussion

The results of the present study suggest that the ATPase of sarcotubular membranes from cardiac muscle form a phosphoprotein intermediate under conditions which are virtually identical with those described for skeletal muscle (Makinose, 1969; Martonosi, 1969; Inesi *et al.*, 1970; Kanazawa *et al.*, 1971; Panet *et al.*, 1971). The maximal phosphoprotein intermediate levels found in this study, 1.2 nmol/mg of protein, are substantially higher than the value of 0.05 nmol/mg reported by Fanburg and Matsushita (1973) and 0.2 nmol/mg reported by Namm *et al.* (1972). Since their incubation conditions were similar to the ones described in this paper, it is likely that they were working with less pure sarcotubule preparations. The value of 1.2 nmol/mg of protein found for cardiac muscle is, however, substantially less than the value of 3–5 nmol/mg observed in skeletal sarcoplasmic reticulum preparations (Martonosi, 1969; Panet *et al.*, 1971). It seems likely that the difference in phosphoprotein levels may again be due to differences in the purity of the sarcoplasmic reticulum fractions obtained from these two different types of muscle. We know that our cardiac sarcoplasmic reticulum fraction contains some ($Na^+ + K^+$) ATPase activity (unpublished result) and mitochondrial ATPase activity (Table I) and must, therefore, contain fragments of sarcolemmal and/or T-tubules and mitochondria. Baskin and Deamer (1969) and Katz *et al.* (1970) have also presented evidence that cardiac sarcoplasmic reticulum fractions are relatively impure. We are confident that the conditions used to study phosphoprotein intermediate formation excluded phosphoprotein intermediate formation by either mitochondrial (Cross *et al.*, 1970) or sarcolemmal (Post *et al.*, 1965) ATPases, since neither azide nor ouabain affected phosphoprotein intermediate levels in the presence of calcium or magnesium (Pang and Briggs, 1973). The level of phosphoprotein intermediate in cardiac sarcoplasmic reticulum as in skeletal sarcoplasmic reticulum (Makinose, 1969; Martonosi, 1969; Inesi *et al.*, 1970; Kanazawa *et al.*, 1971; Panet *et al.*, 1971) was promoted by calcium and depressed by magnesium (Figures 1–3). When calcium was at very low levels following addition of EGTA (Figure 2), phosphoprotein intermediate levels were undetectable.

We have found, as Panet *et al.* (1971) did for skeletal preparation, that a reasonably stable phosphoprotein intermediate preparation (Figures 4 and 6) could be separated from the reaction products by ion exchange chromatography. This made it possible to study directly the hydrolytic step in a cardiac sarcoplasmic reticulum preparation. The rate constant for hydrolysis, K_d, was found to be particularly sensitive to magnesium in the range of 2.5×10^{-5}–1×10^{-4} M (Figure 5). The hydrolytic rate constant for skeletal phosphoprotein intermediate has also been shown to be dependent on the magnesium concentrations (Kanazawa *et al.*, 1971). The maximum hydrolytic rate observed in cardiac sarcoplasmic reticulum was 3.2 min^{-1}. Considering that the maximum number of phosphorylating sites in cardiac sarcoplasmic reticulum is 1.2 nmol/mg (Figure 1b), the maximum ATPase activity at 2° would be 3.8 nmol mg^{-1} min^{-1} ($V_{max} = K_d E_0$). This value is quite similar to that measured in Table I (3.0 nmol mg^{-1} min^{-1}). Thus it appears that phosphoprotein intermediate hydrolysis is the rate-limiting step in the course of the ATPase reaction. It is not possible to compare directly the K_d (3.2 min^{-1}) in this study to the one (30 min^{-1}) described by Tonomura and coworkers (Kanazawa *et al.*, 1971) for skeletal muscle, because they made their observations at 15° and our experiments were performed at 2°. If one assumes a Q_{10} of 2–3 for the hydrolytic rate constant, it would appear that the skeletal rate constant is still 3–4 times greater than the cardiac rate constant. This low hydrolytic rate constant for cardiac sarcoplasmic reticulum may also be due to the relative impurity of the fraction. The differences in the rate constants and the phosphoprotein intermediate levels between skeletal and cardiac sarcoplasmic reticulum preparations support this possibility.

Using the purified phosphoprotein intermediate preparation, we have also been able to show that addition of ADP will reduce the phosphoprotein intermediate level (Figure 6). Since there is no significant release of inorganic phosphate associated with this decrease in the phosphoprotein intermediate levels, we have assumed that ATP is being formed by the addition of ADP to cardiac phosphoprotein, as it does when added to skeletal phosphoprotein intermediate (Kanazawa *et al.*, 1971; Panet *et al.*, 1971). The equilibrium constant for this reaction was found to depend upon the level of external divalent metal (Ca^{2+}, Mg^{2+}) ion concentrations (Table II). The equilibrium constant found by Panet *et al.* (1971) in skeletal sarcoplasmic reticulum in the absence of EDTA was 1 rather than 5 as found in this study with cardiac sarcoplasmic reticulum. It is possible that this discrepancy is due to differences in the internal levels of divalent metals between cardiac and skeletal sarcoplasmic reticulum. This decrease in the equilibrium constant is entirely compatible with Tonomura's hypothesis (Kanazawa *et al.*, 1971) that calcium in the external medium drives the reaction $E + ATP \rightleftarrows EP + ADP$ toward the right, while the calcium inside the sarcoplasmic reticulum shifts the equilibrium toward the left. Addition of EDTA would chelate only the calcium ions on the outside (Duggan and Martonosi, 1970) and thus would decrease the tendency for the reaction to proceed to the right. These results suggest that the equilibrium constant is somewhat ambiguous since the ionic environment inside the sarcoplasmic reticulum cannot be known and could not, therefore, be specified.

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Conversion of Aldehyde to Acid in the Bacterial Bioluminescent Reaction†

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ABSTRACT: Bacterial luciferase catalyzes the emission of light in the presence of reduced flavine mononucleotide, oxygen, and a long chain aliphatic aldehyde. The role of the aldehyde and whether it directly participates in the chemical reaction have not been clear. In the present study, the function of the aldehyde in the luminescent system has been investigated using [1-^{14}C]decanal in a coupled enzyme system. The substrates for the bioluminescent reaction (aldehyde and $FMNH_2$) were generated in the reaction mixture by horse liver alcohol dehydrogenase and FMN reductase. The reaction products were quantitatively analyzed and the distribution of radioactivity has shown that aldehyde is exclusively converted to acid by bacterial luciferase. A linear relation between light emission and acid production was obtained with a quantum yield of 0.10 quanta emitted per molecule of acid produced. Investigations on two different experimental systems in which FMN reductase is absent and $FMNH_2$ is injected resulted in an identical yield of light per molecule of acid produced. These results thus support the proposal that aldehyde oxidation to acid provides the necessary energy for light emission in the bacterial bioluminescent reaction.

Bacterial luciferase catalyzes the emission of light at 490 nm in the presence of $FMNH_2$, O_2 and a long chain aliphatic aldehyde (Cormier and Strehler, 1953; Strehler *et al.*, 1954). Although the aldehyde is essential for high quantum yields, the fate of this molecule in the bioluminescent reaction is unknown. Investigations by a number of workers have shown that the total amount of light emitted is proportional to the amount of added aldehyde (McElroy and Green, 1955; Cormier and Totter, 1957; Lee, 1972; Shimomura *et al.*, 1972). It has been proposed that the aldehyde is converted to the corresponding long chain acid since this reaction would provide sufficient energy for the emission of a quantum of light at 490 nm (McElroy and Green, 1955). Recent support for this proposal has come from the detection by mass spectroscopy of acid in the bioluminescent reaction mixture (Shimomura *et al.*, 1972). However, it is important to establish that acid production does not occur in the absence of luciferase. Indeed, the slow turnover of luciferase ($t_{1/2}$ = 2 sec with decanal) and low yield of products have been the major reasons for the delay in identification of the chemical products of the reaction.

In the present studies, we have investigated the fate of the aldehyde in the bioluminescent reaction mixture and in controls missing a component essential for light emission. Acid production was observed and quantitated both in the controls and in the light emitting system. A net difference in the acid production between the bioluminescent reaction mixture and the controls was directly related to the total amount of emitted light. This result is in agreement with a recent communication of McCapra and Hysert (1973). Furthermore, the present studies show that acid is the only product arising from aldehyde in the bioluminescent reaction.

Experimental Section

Materials. Bacterial luciferase was isolated from *Photobacterium fisheri*, strain MAV, and purified according to the procedure of Gunsalus-Miguel *et al.* (1972). A specific activity of 0.8×10^{13} quanta/sec per mg of luciferase was measured from the maximal light intensity after injection of 1.0 ml of 5×10^{-5} M $FMNH_2$ (catalytically reduced with H_2 over platinized asbestos) into 1.0 ml of 0.02 M phosphate–0.2% bovine serum albumin (pH 7.0), containing luciferase and 10 μl of 0.1% dodecanal suspension. The protein concentration was determined spectrophotometrically on the basis of a specific absorbance coefficient of 0.94 (0.1%, 1 cm) at 280 nm.

FMN reductase was partially purified (100-fold) from *P. fisheri*, strain MAV. Activity measurements were based on the change in optical density at 340 nm on addition of the enzyme to 1×10^{-4} M NADH–5×10^{-6} M FMN, in 0.01 M phosphate buffer–0.1% bovine serum albumin (pH 7.0). One unit of enzyme activity is defined as the μmoles of NADH oxidized per min at 22–24°. The partially purified enzyme had a specific activity of 0.2 unit per unit absorbance at 280 nm. The enzyme was stored at 4° in 10^{-3} M dithiothreitol–0.1 M phosphate buffer (pH 7.0).

Horse liver alcohol dehydrogenase was purchased from Sigma Chemical Co. and dissolved in 0.1 M phosphate buffer (pH 7.0) prior to use. A specific absorption coefficient of 0.42 (0.1%, 1 cm) at 280 nm was used to calculate its concentration in mg/ml (Dalziel, 1958).

NAD and FMN were obtained from Sigma. Decanol (Sigma), decanal (Aldrich), and decanoic acid (Aldrich) migrated as single spots on thin-layer chromatograms (0.2 mm

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