Randerath, K., Janeway, C. M., Stephenson, M. L., & Zamecnik, P. C. (1966) *Biochem. Biophys. Res. Commun. 24*, 98-105.

Rapaport, E., & Zamecnik, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3984-3988.

Rapaport, E., Svihovec, S. K., & Zamecnik, P. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2653-2657.

Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T., & Britten, R. J. (1957) in *Studies of Biosynthesis in Escherichia coli*, Carnegie Institution of Washington Publication, Washington, DC.

Rouf, M. A. (1964) *J. Bacteriol. 88*, 1545-1549.

Ryan, L. D., & Vestling, C. S. (1974) *Arch. Biochem. Biophys. 160*, 279-284.

Scherer, P., Lippert, H., & Wolff, G. (1983) *Biol. Trace Elem. Res. 5*, 149-163.

Sillero, M. A. G., Villalba, R., Moreno, A., Quintanilla, M., Lobaton, C. D., & Sillero, A. (1977) *Eur. J. Biochem. 76*, 331-337.

Traut, T. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2809.

Vallejo, C. G., Infante, J. M., Renart, J., & Sillero, A. (1973) *Biochem. Biophys. Res. Commun. 51*, 113-118.

Vallejo, C. G., Lobaton, C. D., Quintanilla, M., Sillero, A., & Sillero, M. A. G. (1976) *Biochim. Biophys. Acta 438*, 304-309.

Weinmann-Dorsch, C., Hedl, A., Grummt, I., Albert, W., Ferdinand, F. J., Friis, R. R., Pierron, G., Moll, W., & Grummt, F. (1984) *Eur. J. Biochem. 138*, 179-185.

Zamecnik, P. C. (1983) *Anal. Biochem. 134*, 1-10.

Zamecnik, P. C., & Stephenson, M. L. (1968) in *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells* (San Pietro, A., Lambory, M. R., & Kenney, P. T., Eds.) pp 3-16, Academic Press, New York.

Zamecnik, P. C., & Stephenson, M. L. (1969) in *The Role of Nucleotides for the Function and Conformation of Enzymes* (Kalckar, H. M., Klenow, H., Munch-Petersen, G., Ottesen, M., & Thuysen, J. M., Eds.) pp 276-291, Alfred Benzon Symposium I, Munksgaard Copenhagen.

Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., & Randerath, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 91-97.

Zamecnik, P. C., Janeway, C. M., Randerath, K., & Stephenson, M. L. (1967) in *Regulation of Nucleic Acid and Protein Biosynthesis* (Koningsberger, V. V., & Bosch, L., Eds.) pp 169-176, Elsevier, Amsterdam.

Enzyme Phosphorylation with Inorganic Phosphate Causes Ca^{2+} Dissociation from Sarcoplasmic Reticulum Adenosinetriphosphatase†

Leopoldo de Meis*

Centro de Ciencias da Saude, Instituto de Ciencias Biomedicas, Departamento de Bioquimica, Universidade Federal do Rio de Janeiro, Cidade Universitaria, 21910 Rio de Janerio, Brazil

Giuseppe Inesi

Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

Received May 4, 1984

ABSTRACT: Sarcoplasmic reticulum ATPase is phosphorylated by ATP in the presence of calcium, with a consequent reduction of the affinity of the binding sites for calcium and dissociation of the divalent cation from the enzyme. ATPase phosphorylation with P_i, on the other hand, requires prior removal of calcium from the enzyme, indicating that the energy requirement for phosphorylation of the enzyme–calcium complex can be met by ATP but not by P_i. We find that when the energy yield of the P_i reaction with the enzyme is increased by the addition of dimethyl sulfoxide to the medium, ATPase phosphorylation with P_i occurs even in the presence of calcium, and the binding sites undergo a reduction in affinity with consequent dissociation of Ca^{2+} from the enzyme, in analogy to the effect of ATP. It is thereby demonstrated experimentally that an essential step in the coupling of catalytic and transport activities is an interdependence and mutual ligand exclusion of the phosphorylation and calcium sites, in which ATP does not play a direct role. An important difference between the effects of ATP and P_i is that the former produces dissociation of Ca^{2+} inside the vesicles as the result of advancement of the catalytic cycle in the forward direction, while P_i produces dissociation of calcium into the outer medium as a consequence of equilibration of enzyme states producing a shift in the reverse direction of the enzyme cycle. These observations demonstrate how equilibration of intermediate enzyme states determines extent and direction of overall reaction flow. It is also found that in the presence of dimethyl sulfoxide ATP can undergo hydrolysis (at very low rates) even in the absence of calcium, indicating that the coupling rules are not absolute, and it is possible for the reaction flow to proceed through shunted pathways depending on the experimental conditions.

It is well established that active transport of Ca^{2+} in sarcoplasmic reticulum (SR) vesicles requires a change in orientation and a reduction of the affinity of the ATPase sites for Ca^{2+} (Inesi et al., 1978a; de Meis & Vianna, 1979). This transformation occurs as a consequence of phosphoryl transfer from ATP to an aspartyl residue of the ATPase catalytic site (Yamamoto & Tonomura, 1967; Makinose, 1969; Bastide et al., 1973; Degani & Boyer, 1973), from which the free energy required for this transformation is derived. The phosphory-

† This work was supported by grants from the National Institutes of Health (HL 27867) and from the Brazilian FINE P-CN Pq.

0006-2960/85/0424-0922$01.50/0 © 1985 American Chemical Society

lation reaction with ATP requires the presence of calcium at the activation (and transport) sites of the enzyme. This bound calcium is translocated from the outer surface of the membrane and released inside the vesicles following enzyme phoshorylation and related transformation of the binding sites (Inesi et al., 1978b).

The same aspartyl residue of the catalytic site can also be phosphorylated by P_i (Masuda & de Meis, 1973). In contrast to the phosphorylation with ATP, the P_i reaction requires removal of calcium since calcium occupancy of the binding sites inhibits enzyme phosphorylation by P_i. This suggests that the free-energy requirement for the phosphorylation reaction and related transformation of the binding sites is much greater when these sites are occupied by calcium then when they are not. Normally, this requirement is met by ATP. However, we find now that when the interaction of the enzyme with P_i is favored by the addition of dimethyl sulfoxide (Me_2SO), the phosphorylation occurs (with a much lower P_i concentration dependence) even in the presence of Ca^{2+} and is also accompanied by a reduction of the affinity of the binding sites for Ca^{2+}.

Materials and Methods

SR vesicles (Eletr & Inesi, 1972) and ATPase assembled in leaky vesicles (Meissner et al., 1973) were prepared as previously described. Protein was determined by the Folin method standardized with bovine albumin.

Enzyme phosphorylation was obtained by incubation with [^{32}P]P_i or [γ-^{32}P]ATP in appropriate reaction mixtures (see legends to figures). The reaction was quenched with an equal volume of 0.25 M PCA and 2 mM P_i, and a volume of the quenched reaction mixture corresponding to 0.1 mg of protein was placed on a 0.45-μm Millipore filter under vacuum. Following repeated washing with 0.125 M PCA and 1 mM P_i, the filter was dissolved in 1 mL of dimethylformamide, and the radioactivity remaining with the filter was determined by scintillation counting.

Calcium binding was measured in reaction mixtures identical with those used for enzyme phosphorylation, except that instead of radioactive phosphate $^{45}CaCl_2$ and [^{3}H]glucose were added for determination of calcium and medium volume in the filters, as previously described (Wantanabe et al., 1981). The excess calcium over medium calcium was assumed to be bound to the SR vesicles. Contaminant calcium in SR vesicles (15–20 nmol/mg of protein) and reaction medium was measured with the use of murexide, as previously described (Dani et al., 1979). ATPase activity was assayed by measuring the release of [^{32}P]P_i from [γ-^{32}P]ATP. After precipitation with PCA, the [^{32}P]P_i was extracted as the phosphomolybdate complex with a mixture of benzene and isobutyl alcohol (de Meis et al., 1974).

Results and Discussion

In preliminary experiments, we confirmed that addition of Me_2SO to an aqueous medium (40% v/v) decreases the P_i concentration (from $\sim 10^{-2}$ to $\sim 10^{5}$ M) required for phosphorylation of SR ATPase in the absence of Ca^{2+} and increases the equilibrium constant of the phosphorylation reaction (de Meis et al., 1980). Furthermore, we found that while in totally aqueous media enzyme phosphorylation with P_i is inhibited by low (micromolar) Ca^{2+}, maximal phosphoenzyme levels are obtained in the presence of ~ 40 μM Ca^{2+} when Me_2SO is added (Figure 1). However, higher concentrations of Ca^{2+} inhibit the phosphorylation reaction even in the presence of Me_2SO, and reduction of the phosphoenzyme to half-maximal levels is produced by 100–400 μM Ca. These effects of higher

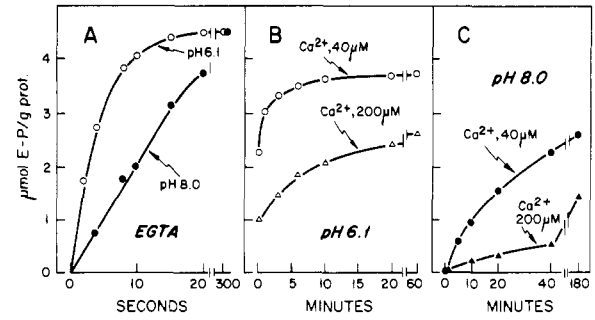


FIGURE 1: Enzyme phosphorylation by P_i in the absence and in the presence of Ca^{2+}, at pH 6.1 and 8.0. The assay medium consisted of 20 mM $MgCl_2$, 1 mM [^{32}P]P_i, 40% (v/v) dimethyl sulfoxide, and either of the following: (A) 0.5 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 100 mM either MES [4-morpholineethanesulfonic acid] buffer, pH 6.1 (○), or Tris-HCl [tris(hydroxymethyl)aminomethane], pH 8.0 (●); (B) 100 mM MES buffer, pH 6.1, and 40 (○) or 200 μM (△) $CaCl_2$; (C) 100 mM Tris-HCl buffer (pH 8.0) and 40 (●) or 200 μM (▲) $CaCl_2$. The reaction was started by the addition of vesicles to a final concentration of 0.5 mg of protein/mL and quenched at serial time intervals. temperature was 35 °C.

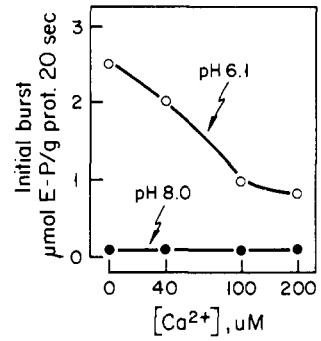


FIGURE 2: Initial burst of enzyme phosphorylation with P_i in the presence of Ca^{2+}. Vesicles (0.5 mg of protein/mL) were preincubated for 10 min in a medium containing 20 mm $MgCl_2$, 40% (v/v) dimethyl sulfoxide, Ca^{2+} at the concentrations shown in the figure, and 100 mM of either MES buffer, pH 6.1 (○), or Tris-HCl buffer, pH 8.0 (●). The phosphorylation reaction was then started by the addition of [^{32}P]P_i (final concentration 1 mM) and quenched after 20-s incubation at 35 °C.

Ca^{2+} concentrations (Figure 1) retain a pH dependence (between 6 and 7), while the pH dependence of the P_i concentration requirement for the phosphorylation reaction is lost when Me_2SO is present. Therefore, calcium binding is affected by ionization of specific residues that are distinct from those affecting the P_i concentration dependence of the phosphorylation reaction.

When P_i is added to SR vesicles in the presence of Me_2SO and in the absence of Ca^{2+}, the phosphoenzyme level increases monotonically with a rate constant of 0.005–0.3 s^{-1}, depending on the pH (Figure 1). On the other hand, if P_i is added to SR vesicles preincubated with 40–200 μM Ca^{2+}, the phosphorylation reaction (at pH 6.1) includes a slow kinetic component (0.1 min^{-1}) in addition to the fast component (Figure 1B). The fraction of enzyme phosphorylated at a fast rate decreases as the Ca^{2+} concentration is raised (Figure 2). At alkaline pH (Figure 1C), the slow component is largely prevalent, due to the higher affinity of the binding sites for Ca^{2+} at low H^+ concentrations (Hill & Inesi, 1982). We attribute the fast kinetic component to phosphorylation of enzyme molecules with binding sites *free of calcium* and the slow component to a slow step related to calcium release and preceding phosphorylation of enzyme molecules *occupied by calcium*. In fact, when we measured calcium binding in

Table I: Extent of "Rapid" and "Slow" Enzyme Phosphorylation by P_i and Calcium Binding before and after Enzyme Phosphorylation[a]

	E-P (μmol/g of protein) incubation time		Ca bound (μmol/g of protein)	
pH	20 s	60 min	before P_i	after P_i
6.1	2.48 ± 0.05 (4)	3.89 ± 0.07 (4)	4.59 ± 0.56 (6)	1.82 ± 0.47 (6)
7.0	0.05 ± 0.03 (10)	1.97 ± 0.10 (10)	9.30 ± 1.03 (15)	4.14 ± 0.50 (15)

[a] Note that stoichiometry of calcium release is approximately twice that of the "slow phosphorylation". Conditions were 100 mM buffer (MES, MOPS, or Tris-HCl), 20 mM $MgCl_2$, 5 mM [^{3}H]glucose, 40 μM $^{45}Ca^{2+}$ (measured), 1 mM P_i, 40% Me_2SO, and 0.5 mg of SRV protein/mL. Assay performed at 35 °C for calcium binding. ^{45}Ca, [^{3}H]glucose, and nonradioactive P_i were used. For phosphoenzyme formation, [^{32}P]P_i and nonradioactive Ca^{2+} and glucose were used. The vesicles were preincubated 10 min with Ca^{2+} before the addition of P_i. Values are average ± SE of the number of experiments shown in parentheses.

Table II: Levels of Enzyme Phosphorylation by ATP and Calcium Binding before and after Phosphorylation in Intact and Leaky Vesicles[a]

	intact vesicles (SRV)			leaky vesicles (Meissner)		
		Ca bound (μmol/g of protein)			Ca bound (μmol/g of protein)	
ATP (mM)	E-P (μmol/g of protein)	before ATP	after ATP	E-P (μmol/g of protein)	before ATP	after ATP
0.1	2.15 ± 0.07 (6)	8.13 ± 0.38 (16)	7.88 ± 0.65 (10)	1.82 ± 0.08 (6)	9.36 ± 0.46 (16)	4.56 ± 0.52 (16)
1.0	2.87 ± 0.11 (12)		7.35 ± 0.35 (14)	1.98 ± 0.15 (12)		4.13 ± 0.23 (13)

[a] Note that no calcium is released into the outside medium from the ATPase of intact vesicles, while 2 mol of calcium/mol of phosphorylated enzyme is released onto the outside medium from the ATPase of leaky vesicles. Conditions were 100 mM MOPS, pH 7.0, 20 mM $MgCl_2$, 40 μM Ca^{2+}, 5 mM glucose, 40% Me_2SO, and 0.5 mg/ml SRV or Meissner enzyme. Temperature was 35 °C. The enzyme was preincubated with ^{45}Ca for 10 min before addition of ATP—samples were filtered (Ca binding) or quenched with perchloric acid (E-P) after 30 s or 2 min. Essentially the same values of calcium binding and E-P were obtained at both incubation intervals. Values are the average ± SE of the number of experiments in parentheses.

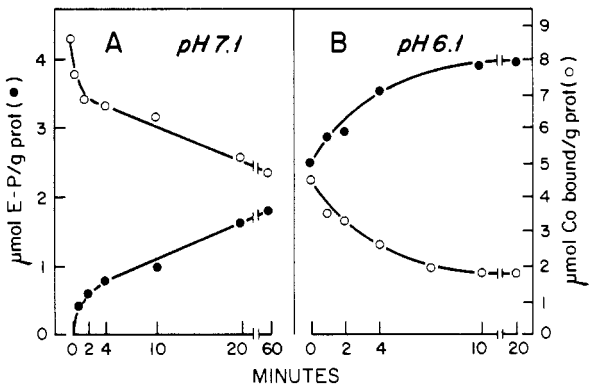


FIGURE 3: Phosphorylation by P_i and Ca^{2+} dissociation. Calcium binding (○) was measured in a medium containing 100 mM MOPS buffer, pH 7.1 (A), or MES buffer, pH 7.1 (B), 20 mM $MgCl_2$, 40% (v/v) dimethyl sulfoxide, 5 mM [^{3}H]glucose, vesicles to a final concentration of 0.5 mg of protein/mL, and $^{45}CaCl_2$ to a final concentration of 45 μM. Contaminant calcium in the medium and SR vesicles was measured before the addition of ^{45}Ca. The reaction was started by the addition of P_i to a final concentration of 1 mM, and 0.3-mL samples were filtered to Millipore filters after different incubation intervals at 35 °C. In the figure, zero time refers to ^{45}Ca bound to the vesicles before the addition of P_i. Enzyme phosphorylation of P_i (●) was measured using the same reaction medium except that [^{32}P]P_i and nonradioactive $CaCl_2$ and glucose were used.

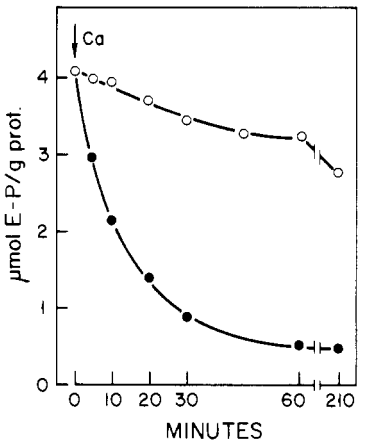
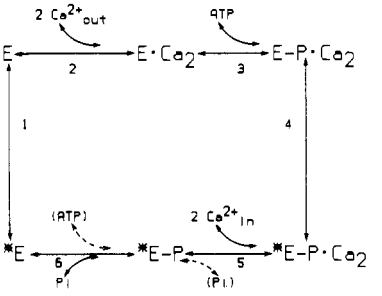


FIGURE 4: Calcium-induced decay of phosphoenzyme formed with P_i. SR vesicles (0.5 mg of protein/mL) were phosphorylated in a medium containing 50 mM Tris–maleate buffer (pH 6.0), 20 mM $MgCl_2$, 1 mM [^{32}P]P_i, 40% (v/v) dimethyl sulfoxide, and 0.1 mM EGTA. After 5 min of incubation at 35 °C, $CaCl_2$ was added to a final concentration of 0.3 (○) or 5.1 mM (●). The reaction was quenched at serial times following the addition of $CaCl_2$.

parallel with enzyme phosphorylation with P_i, we found that 2 mol of calcium is released into the outside medium per each molecule of enzyme undergoing slow phosphorylation (Figures 3 and 4 and Table I). Concentrations of Ca^{2+} sufficiently high to keep the divalent cation bound to the enzyme ($K \simeq$ 400 μM at pH 6; $K \simeq$ 100 μM at pH 8) effectively reduce the levels of phosphorylated enzyme (Figure 1).

If the ATPase of native SR vesicles is phosphorylated with ATP instead of P_i, no Ca^{2+} release into the *outside* medium is observed (Table II). On the other hand, when phosphorylation with ATP is carried out on purified ATPase (Meissner et al., 1973), which is reassembled in leaky vesicles, Ca^{2+} release from the enzyme is observed (Table II). This indicates that, following phosphorylation with ATP, Ca^{2+} is released *inside* the vesicles and can be observed in the outside medium only if the vesicles are leaky. On the contrary, enzyme phosphorylation with P_i produces Ca^{2+} dissociation from the enzyme into the outside medium directly. These observations can be explained with Scheme I in which the binding sites are considered to be either in a high-affinity and outward-oriented state (E) or in a low-affinity and inward-oriented state (*E). We suggest that free-energy input of ATP of reaction 3 favors the forward direction of the cycle with consequent dissociation of Ca^{2+} inside the vesicles (reactions 5 and 6). On the other

Scheme I



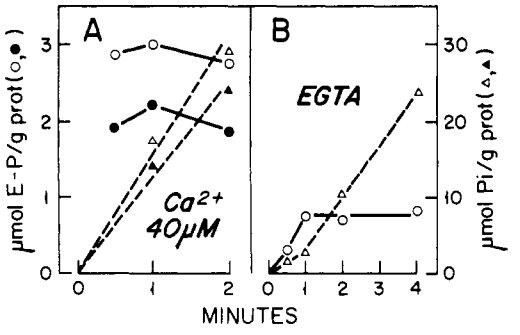


FIGURE 5: ATPase activity and phosphorylation by ATP in the presence and in the absence of Ca^{2+}. The assay medium consisted of 100 mM MOPS buffer (pH 7.0), 20 mM $MgCl_2$, 40% (v/v) dimethyl sulfoxide, 5 mM glucose, 1 mM [γ-^{32}P]ATP, and either 40 μM $CaCl_2$ (A) or 1 mM EGTA (B). The reaction was started by the addition of intact or leaky vesicles, to a final concentraion of 0.5 mg of protein/mL. The assay was performed at 35 °C. (○, ●) phosphoenzyme; (△, ▲) ATPase activity; (○, △) intact vesicles; (●, ▲) leaky vesicles.

hand, Me_2SO increases the free-energy yield of enzyme phosphorylation with P_i (reaction 6), thereby favoring the reverse direction of the cycle and permitting Ca^{2+} release into the outside medium by reequilibration of reactions 1 and 2. When high Ca^{2+} is added in these conditions, the binding sites of *E (low-affinity state) are saturated with calcium, and the phosphoenzyme level decays (Figure 4), since *E Ca cannot be phosphorylated by P_i. If thermodynamic limitations are overcome then, it is possible to demonstrate experimentally that ATP is not strictly necessary, but the presence of phosphate is sufficient for the basic step in the coupling mechanism between catalysis and transport. This is related to an interdependence and mutual ligand exclusion of the phosphorylation and calcium sites, as suggested by experiments with vanadate (Medda & Hasselbach, 1983; Inesi et al., 1984). Theoretical principles regarding energy utilization for reciprocal effects on distinct binding sites have been discussed extensively (Wyman, 1964; Weber, 1972; Jencks, 1980; Hill & Eisenberg, 1981; Tanford, 1982).

A final point of interest in our experimentation is that in the presence of Me_2SO significant phosphoenzyme levels and P_i production (Figure 5) are obtained with ATP as a substrate even in the absence of Ca^{2+}. This is in agreement with previous observations on the utilization of furylacryloyl phosphate (Inesi et al., 1980) and ATP (Carvalho-Alves & Scofano, 1983) in the absence of Ca^{2+}, and suggests that substrate flow can occur in special conditions through shunted pathways (dotted lines in Scheme I) bypassing calcium activation and transport. Therefore, the coupling rules are not absolute, and the Ca to ATP coupling ratios observed in steady-state transport are frequently less than a perfect 2. The contribution of different intermediate states (resulting from microscopic equilibria) to the overall reaction flow of SR ATPase has been considered in detail by Inesi & Hill (1983).

Registry No. ATPase, 9000-83-3; ATP, 56-65-5; P_i, 14265-44-2; Ca, 7440-70-2.

REFERENCES

Bastide, F., Meissner, G., Fleischer, S., & Post, R. L. (1973) *J. Biol. Chem. 248*, 8385–8391.

Carvalho-Alves, P., & Scofano, H. (1983) *J. Biol. Chem. 258*, 3134–3139.

Chaloub, R., Guimaraes-Motta, H., Verjovski-Almeida, S., de Meis, L., & Inesi, G. (1977) *J. Biol. Chem. 254*, 9464–9468.

Dani, A., Cittadini, A., & Inesi, G. (1979) *Am. J. Physiol. 237*, 147–155.

Degani, C., & Boyer, P. (1973) *J. Biol. Chem. 248*, 8222–8226.

de Meis, L., & Carvalho, M. (1974) *Biochemistry 13*, 5032–5038.

de Meis, L., & Vianna, A. (1979) *Annu. Rev. Biochem. 48*, 275–292.

de Meis, L., Martin, O., & Alves, E. (1980) *Biochemistry 19*, 4252–4261.

Eletr, S., & Inesi, G. (1972) *Biochim. Biophys. Acta 282*, 174–179.

Hill, T., & Eisenberg, E. (1981) *Q. Rev. Biophys. 14*, 1–49.

Hill, T., & Inesi, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3978–3982.

Inesi, G., & Hill, T. (1983) *Biophys. J. 44*, 271–280.

Inesi, G., Coan, C., Verjovski-Almeida, S., Kurzmack, M., & Lewis, D. (1978a) *Frontiers of Biological Energetics*, Vol. 2, pp 1129–1136, Academic Press, New York.

Inesi, G., Kurzmack, M., & Verjovski-Almeida, S. (1978b) *Ann. N.Y. Acad. Sci. 307*, 224–227.

Inesi, G., Kurzmack, M., Nakamoto, R., de Meis, L., & Bernhard, S. (1980) *J. Biol. Chem. 255*, 6040–6043.

Inesi, G., Lewis, D., & Murphy A. (1984) *J. Biol. Chem. 259*, 996–1003.

Jencks, W. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol. 51*, 75–106.

Makinose, M. (1969) *Eur. J. Biochem. 10*, 74–82.

Masuda, H., & de Meis, L. (1973) *Biochemistry 12*, 4581–4585.

Medda, P., & Hasselbach, W. (1983) *Eur. J. Biochem. 137*, 7–14.

Meissner, G., Conner, G., & Fleischer, S. (1973) *Biochim. Biophys. Acta 298*, 246–269.

Tanford, C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6527–6531.

Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C., & Inesi, G. (1981) *Biochemistry 20*, 6617–6625.

Weber, G. (1972) *Biochemistry 11*, 864–878.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.

Yamamoto, T., & Tonomura, Y. (1967) *J. Biochem. (Tokyo) 62*, 558–575.