Coexistence of High- and Low-Affinity Ca^{2+} Binding Sites of the Sarcoplasmic Reticulum Calcium Pump†

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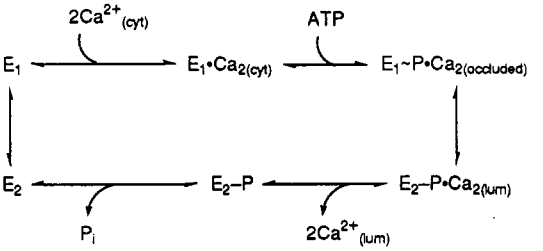
ABSTRACT: We have recently shown [Mészáros, L. G., & Bak, J. (1992) *Biochemistry 31*, 1195–1200] that, during the rapid phase of Ca^{2+} uptake into sarcoplasmic reticulum (SR), internalization and binding of Ca^{2+} to the cytoplasmic high-affinity binding sites of the Ca^{2+} ATPase occur simultaneously, resulting in a transient supernumerary Ca/ATP stoichiometry. Here we address the question of whether the cytoplasmic high-affinity and the luminal low-affinity Ca^{2+} binding sites of the SR Ca^{2+} ATPase also coexist. SR vesicles were loaded with Ca^{2+} (0–10 mM), and then the kinetics of EP formation and decomposition as well as the maximum level of EP formed from radiolabeled ATP were determined at conditions which only allow single-cycle reactions to occur: empty or Ca-loaded SR vesicles (in micromolar extravesicular Ca^{2+}) were either mixed with ATP *plus* millimolar EGTA or added in amounts that set a Ca^{2+} ATPase/ATP ratio of 80–85 at the initiation of the reaction. The rates of EP formation and decomposition were both significantly reduced in Ca-loaded, compared to empty (ionomycin-treated), vesicles. However, the value of EP_{max} was unaltered by Ca-loading, suggesting the existence of the enzyme intermediate, $E{\cdot}Ca_{2(cyt)}{\cdot}Ca_{2(lum)}$, i.e., the coexistence of the cytoplasmic and the luminal Ca^{2+} binding sites of the Ca-pump. These results suggest that the uphill transport of Ca^{2+} might not be based on an alternating relocation and conversion of the Ca^{2+} binding sites of the Ca^{2+} ATPase.

Alternative access (Jardetzky, 1966) models of the sarcoplasmic reticulum (SR)[1] Ca^{2+} ATPase, as first formalized by de Meis and Vianna (1979), describe the mechanism of Ca^{2+} transport in terms of two major states of the enzyme, E_1 and E_2 (Tanford, 1984; Inesi, 1985; see Scheme I). According to such an E_1–E_2 model, the high- and low-affinity Ca^{2+} binding sites of the calcium pump (Ca-pump) are exclusive: they are accessible either from the cytoplasm (E_1 high-affinity state) or from the lumen of the SR (E_2 low-affinity state). The transport of Ca^{2+} against its concentration gradient is achieved in an $E_1 \rightarrow E_2$ conversion step, during which the cytoplasmic (cyt) Ca^{2+} binding sites are first occluded (Dupont, 1980) and then relocated to the luminal (lum) side of the membrane in a process that is fueled by a parallel decrease in the chemical potential of the phosphoenzyme ($E_1{\sim}P \rightarrow E_2$–P). The existence of the cytoplasmic high-affinity [for review, see Ikemoto (1982) and Inesi (1985)] and the luminal low-affinity Ca^{2+} binding sites (Kalbitzer et al., 1978; Chaloub et al., 1979; Suko et al., 1981) of the enzyme has been documented, and their characteristics have been described in detail. However, evidence that would support alternating relocation of the Ca^{2+} binding sites during a Ca-pump cycle is lacking. Moreover, (i) Jencks and his co-workers have pointed out that certain features of an alternating access scheme, as formalized in E_1–E_2 models, are difficult to demonstrate experimentally [for a review, see Jencks, (1989)]; in particular, the existence of the E_2 kinetic state, which represents a principal species of the mechanism of Ca^{2+} binding site relocation, is not evident (Pickart & Jencks, 1982); and (ii) the supernumerary Ca/EP stoichiometry during the rapid phase of ATP-dependent Ca^{2+} uptake into sealed SR vesicles, as observed by comparing the kinetics of Ca^{2+} uptake using different rapid-mixing techniques (Mészáros & Bak, 1992), indicates that internalized and cytoplasmic Ca^{2+} binding sites are, at least transiently, occupied by Ca^{2+} simultaneously.

Scheme I



Here we report findings which suggest that the binding of Ca^{2+} to the high- and low-affinity sites, i.e., the cytoplasmic and luminal sites, can also occur simultaneously, as evidenced by determining the maximal levels of EP formed from ATP (in the presence of extravesicular Ca^{2+}) in empty and Ca-loaded sealed SR vesicles. The results shown here indicate that relocation and, thus, interconversion of Ca^{2+} sites, as proposed by E_1–E_2 models, might not occur during Ca^{2+} transport; instead, Ca^{2+} ions move through the membrane from site to site.

MATERIALS AND METHODS

Sealed SR vesicles were prepared from fast-twitch muscles of rabbit hind legs as described (de Meis & Hasselbach, 1971). The final pellet was resuspended in 0.3 M sucrose, 20 mM MOPS, pH 6.8, and a mixture of protease inhibitors (0.1 mM PMSF, 10 μg/mL aprotinin, 1 mM benzamidine, 1 μg/mL

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; cyt, cytoplasmic; lum, luminal.

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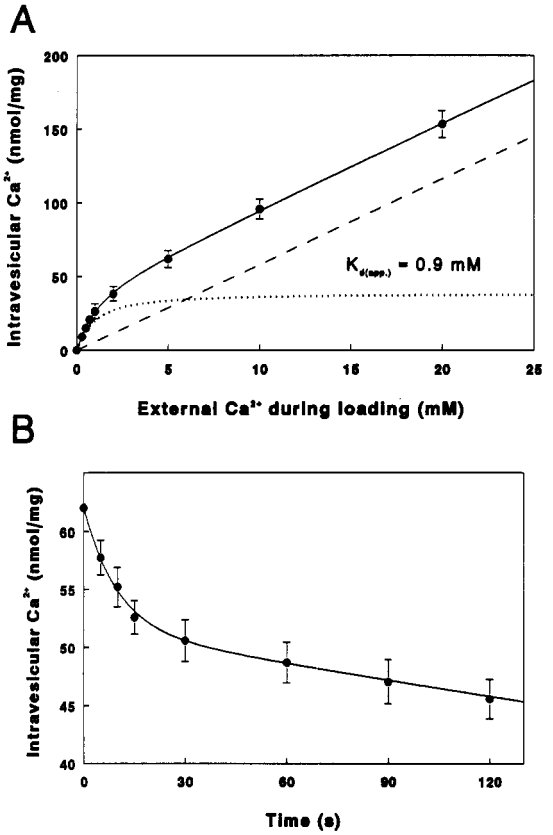

FIGURE 1: Ca^{2+} content in SR as a function of Ca^{2+} concentration during passive Ca loading (A) and release of Ca^{2+} from the vesicles (B). The vesicles were loaded with $^{45}Ca^{2+}$ (increasing concentrations in panel A and 5 mM in panel B), as described in the Materials and Methods section, and then diluted into BRS at $t = 0$. At the indicated time points, ice-cold La-quench solution was added and the samples were filtered and washed three times with 2 mL of the same solution. Intravesicular Ca^{2+} (A) is represented by the $t = 0$ time points. The solid line is the sum of the Hill equation (dotted line) and a linear equation (dashed line). The points represent the average of six determinations with SD values (bars).

leupeptin, and 2 μg/mL pepstatin A) and stored at −80 °C until use. The protein content of SR samples was determined by the Lowry method, using bovine serum albumin as standard. The Ca^{2+} ATPase content of SR was determined by densitometry of Coomassie brilliant blue-stained SDS gel electrophoretic patterns of SR and found to be about 80–85% of the total protein content.

Calcium loading of SR vesicles was carried out by incubating the vesicles (10–15 mg/mL) for 6 h on ice in the presence of $CaCl_2$ (0–10 mM ± $^{45}Ca^{2+}$ tracer) in 150 mM KCl, 5 mM $MgCl_2$, and 20 mM MOPS, pH 6.8 (basic reaction solution, BRS). The amounts of intravesicular, i.e., loaded, Ca^{2+} (Figure 1A) and the rate of Ca^{2+} efflux (Figure 1B) from Ca-loaded vesicles were determined by diluting ^{45}Ca-loaded SR (to give a final protein concentration of 0.1–0.2 mg/mL) into a reaction medium (see figure caption), after which, either at time 0 or after given time periods, an ice-cold solution of BRS containing 10 mM $LaCl_3$ was added to terminate Ca^{2+} efflux. Millimolar La^{3+}, an inhibitor of both Ca^{2+} release from SR (Ohnishi, 1979) and Ca^{2+} ATPase activity (Krasnow, 1977; Chiesi & Inesi, 1979; Highsmith & Murphy, 1984; Scott, 1984), was found to efficiently block the efflux of $^{45}Ca^{2+}$. After La-quenching, the vesicles were Millipore-filtered and washed with the above La-quench solution. The radioactivity retained on the filters was determined by liquid scintillation counting. The $t = 0$ data points represent the vesicular Ca^{2+} content of Ca-loaded SR (Figure 1A). Upon dilution of the Ca-loaded vesicles into an EGTA-containing medium ($[Ca^{2+}] < 10^{-7}$ M), about 20% of internal Ca^{2+} present at $t = 0$ was released rapidly, while the remaining Ca^{2+} leaked out of the vesicles in a slow process (Figure 1B).

The time course of the formation (and subsequent decomposition) of the phosphorylated intermediate of the Ca-pump (EP) from [γ-^{32}P]ATP (0.37 TBq/mmol; New England Nuclear, Boston, MA) was determined at room temperature in both hand-mixing and rapid-quenching experiments as described (Kurzmack et al., 1977; Mészáros & Ikemoto, 1985). Briefly, for rapid-quench experiments, the contents of syringe a (Ca-loaded or unloaded SR vesicles in BRS ± 50 μM $CaCl_2$) of the apparatus (Durrum three-syringe rapid mixer, Palo Alto, CA) were rapidly mixed with those of syringe b (labeled ATP ± 10 mM EGTA or ± 50 μM $CaCl_2$) at a 1:1 mixing volume ratio. The reaction was terminated by delivering 20% ice-cold trichloroacetic acid (TCA) from syringe c. The filling of syringe a with SR vesicles involved an unavoidable dilution into BRS, upon which Ca^{2+} release occurs (see above). Thus, the time elapsed between the dilution step and the addition of ATP was minimized and carefully kept constant at 30 s. Accordingly, each data point represents separate fillings of the syringes of the rapid mixer. In hand-mixing experiments, the reaction was initiated by the dilution of concentrated SR solutions into the reaction medium containing ATP ± EGTA (no predilution step) and was terminated as described above. Following Millipore filtration and consecutive washing with an ice-cold solution of 5% TCA and 10 mM Na_2HPO_4, the protein-bound radioactivity retained on the filter was determined.

The data points in the figures represent the average of at least three independent determinations. The solid lines are drawn to the best fits obtained by using the program NFIT (Island Products, Galveston, TX). Further details are given in the figure captions.

RESULTS AND DISCUSSION

The existence of low-affinity, luminal Ca^{2+} binding sites on the free (nonphosphorylated) Ca^{2+} ATPase is supported by a body of kinetics evidence, which demonstrates that intravesicular Ca^{2+} (in millimolar concentrations) decreases the rate of EP formation from P_i (Kalbitzer et al., 1978; Chaloub et al., 1979; Suko et al., 1981, Myung & Jencks, 1993), and also by the finding that the saturation of the low-affinity Ca^{2+} binding sites on the enzyme influences its intrinsic tryptophan fluorescence (Lüdi & Hasselbach, 1983). Accordingly, the equation which best described the dependence of luminal Ca^{2+} content of SR vesicles on extravesicular Ca^{2+} present during the Ca-loading procedure (see also Materials and Methods) was the sum of the Hill equation and a linear function (Figure 1A), indicating the existence of titratable, low-affinity (K_d of 0.9 mM) Ca^{2+} binding sites in the lumen of the SR. Since, according to E_1–E_2 models, the occurrences of the high- and low-affinity Ca^{2+} binding sites of the Ca^{2+} ATPase are exclusive, i.e., in E_1 they are in a high-affinity, outward-oriented configuration while in E_2 they have low affinity with inward orientation, the E_1:E_2 ratio is determined by the degree of the saturation of the cytoplasmic and luminal Ca^{2+} binding sites, i.e., by the concentrations of extra and intravesicular Ca^{2+}. Thus, when the extra and intravesicular Ca^{2+} sites are saturated simultaneously, two species, $E_1{\cdot}Ca_{2(cyt)}$ and $E_2{\cdot}Ca_{2(lum)}$, are expected to be present in significant concentrations. [In the absence of Ca^{2+}, the E_1:E_2 ratio, on the basis of thermodynamic considerations (Tanford, 1984), is proposed to be close to 1; thus, when both types of sites are saturated,

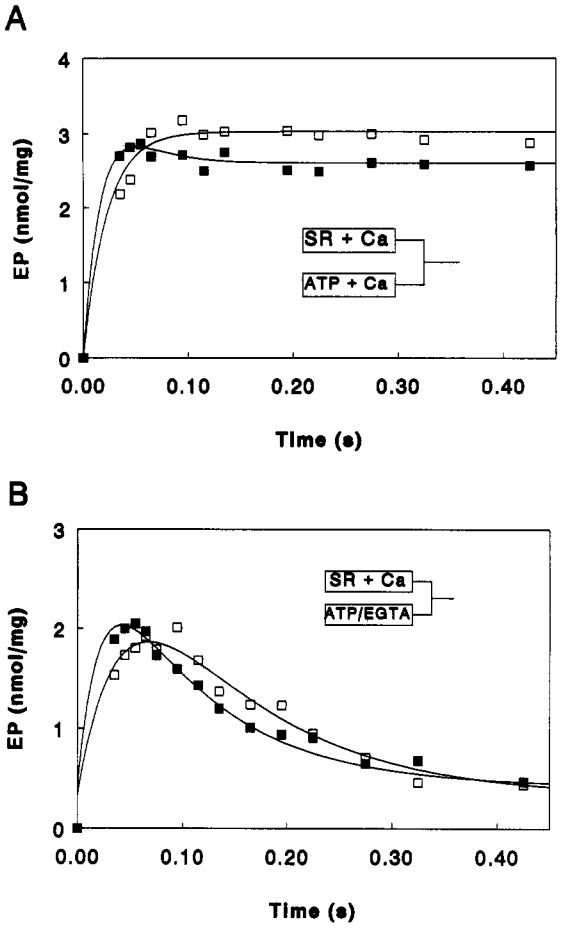

FIGURE 2: EP formation and subsequent decomposition in empty and Ca-loaded SR vesicles. SR vesicles were either treated with 2 μM ionomycin (to decrease the amounts of contaminating Ca^{2+} trapped inside the vesicles; ■) or loaded with 5 mM Ca^{2+} (□), as described in the Materials and Methods section. SR, 30 s before the initiation of the reaction, was diluted into BRS to give a final protein concentration of 0.16 mg/mL in syringe a. [Ca^{2+}] was adjusted to 50 μM by adding either $CaCl_2$ (to empty vesicles in BRS) or the appropriate concentration of EGTA (for loaded vesicles in BRS). The contents of syringe a were mixed with those of syring b, which contained either 10 μM [^{32}P]ATP in BRS (A) or ATP and 10 mM EGTA (B). The inclusion of EGTA in syringe b prevented EP formation in subsequent cycles, thus permitting us to monitor a single cycle of EP formation and decomposition [see Davidson and Berman (1988)]. The data points represent the average of three independent determinations, each with duplicates.

E_1·$Ca_{2(cyt)}$ and E_2·$Ca_{2(lum)}$ should also be in about equal concentrations.] Since, as is generally thought [for a review, see Inesi (1985)], the ATP-reactive enzyme intermediate is E_1·$Ca_{2(cyt)}$ (and not E_2·$Ca_{2(lum)}$; see also below), the kinetics of EP formation and subsequent decomposition should differ significantly in empty and Ca-loaded SR vesicles.

The saturation of the low-affinity Ca^{2+} sites (i.e., Ca loading of the vesicles) significantly decreased the rate of EP formation (Figure 2A) and that of the subsequent EP decomposition (Figure 2B), in accordance with the facts that the rate of the E_1–E_2 conversion in either direction is significantly lower than that of EP formation from ATP (Fernandez-Belda et al., 1984; Inesi et al., 1988) and that the dissociation of Ca^{2+} from E_2-P·$Ca_{2(lum)}$ is inhibited by millimolar intravesicular Ca^{2+} (Yamada & Tonomura, 1972; Chaloub et al., 1979; Yamada & Ikemoto, 1980; de Meis & Inesi, 1985). On the other hand, as seen in Figure 2B, in a single-cycle EP formation/decomposition reaction, i.e., when millimolar EGTA was added together with ATP to lower the extracellular Ca^{2+} to 10^{-8} M and thus prevent EP formation in the second cycle [see

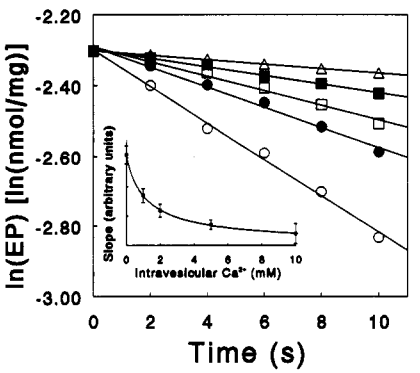

FIGURE 3: The effects of intravesicular Ca^{2+} on EP_{max} and the rate of EP decomposition. SR vesicles were loaded with 0 (○), 1 (●), 2 (□), 5 (■), or 10 mM Ca^{2+} (△) and then, at t = 0, diluted into BRS containing 0.1 μM labeled ATP and EGTA in appropriate concentrations to produce 50 μM [Ca^{2+}] for each of the conditions. The protein concentration during the reaction was 1 mg/mL (i.e., about 8–8.5 μM Ca^{2+} ATPase). The reaction was quenched by adding ice-cold TCA (final concentration, 10%). The lines were drawn to log data points by fitting a linear equation. Data points represent the average of 5 determinations. Inset: The slopes from the time courses are plotted as a function of the internal Ca^{2+} concentration at t = 0. The bars represent ±SD. The solid line was drawn by fitting the data to the Hill equation.

Davidson and Berman (1988)], the maximum level of EP (EP_{max}) was about the same for both empty and Ca-loaded vesicles. Similar results were also obtained when the internal Ca^{2+} sites were saturated by adding 5 mM Ca^{2+} *plus* 2 μM ionomycin to the reaction medium instead of loading the vesicles with Ca^{2+} (data not shown). (If the vesicles were first diluted into EGTA to decrease the extravesicular free Ca^{2+} concentration to 10^{-8} M and then, after 30 s, the reaction was initiated by the addition of ATP, no EP was formed in either empty or Ca-loaded vesicles; data not shown.) In keeping with the aforementioned rationale, the finding of the same EP_{max} values for empty and Ca-loaded vesicles indicates that the concentration of the ATP-reactive enzyme species was the same regardless of the saturation of the luminal Ca sites and thus suggests that, in Ca-loaded vesicles, the cytoplasmic high-affinity sites and the luminal low-affinity sites are simultaneously occupied by Ca^{2+}.

Figure 3 shows the time courses of EP decomposition determined in hand-mixing experiments, in which the reactions were initiated by the dilution of SR vesicles with different (0–10 mM) Ca^{2+} loads into solutions containing 0.1 μM labeled ATP. SR was added in amounts which, at t = 0, resulted in a Ca^{2+} ATPase:ATP ratio of 80–85. Under such conditions, EP formation/decomposition practically occurs in a single-cycle reaction due to the high enzyme:ATP ratio. Moreover, the time course of the first-order decomposition reaction was easily resolvable due to low EP_{max} levels. In accordance with previous reports (Ikemoto, 1975; Yamada & Ikeomoto, 1980), the rate of EP decomposition decreased as the Ca^{2+} load was increased. The apparent K_d (1.15 mM) of the luminal Ca^{2+} binding sites determined from the plot of the reduction of the EP decomposition rate as a function of the Ca^{2+} load (Figure 3, inset) was in good agreement with the value obtained from the determination of the internal Ca^{2+} content as a function of Ca^{2+} load (see Figure 1A). On the other hand, similar to that found in the rapid-mixing experiments (see Figure 2B), EP_{max} (the value at the t = 0 intercept) was virtually unchanged by increasing Ca^{2+} load.

The above results taken together indicate that the concentration of the enzyme species which reacts with ATP is the same for empty and Ca-loaded vesicles. These results can be

explained on the basis of the following: (i) ATP, through an allosteric action, shifts the E_1–E_2 equilibrium, favoring the E_1·$Ca_{2(cyt)}$ intermediate; and (ii) the cytoplasmic high-affinity and luminal low-affinity Ca^{2+} binding sites of the Ca^{2+} ATPase are simultaneously occupied by Ca^{2+}, i.e., an E·$Ca_{2(cyt)}$·$Ca_{2(lum)}$ intermedite of the enzyme exists and is able to react with ATP. Since the time course of EP formation of Ca-loaded vesicles (similar to empty vesicles) shows no initial lag phase even with 1-ms time resolution (Stahl & Jencks, 1987), the first possibility seems unlikely (although it cannot be ruled out if the ATP-induced shift is extremely fast, with rate constants exceeding several thousand per second). In the light of our recent findings (Mészáros & Bak, 1992) that, during the rapid phase of Ca^{2+} uptake, Ca^{2+} simultaneously occupies both the high-affinity cytoplasmic and the intermediate sites, the second possibility seems to be a plausible explanation. Thus, a model which would consider a Ca^{2+} transfer through a channel-type structure from Ca^{2+} sites facing the cytoplasm to those facing the lumen (instead of the relocation and the interconversion of Ca^{2+} sites, as suggested by E_1–E_2 models) is proposed here.

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REFERENCES

Chaloub, R. M., Guimaraes-Motta, H., Verjovski-Almeida, S., de Meis, L., & Inesi, G. (1979) *J. Biol. Chem. 254*, 9464–9468.

Chiesi, M., & Inesi, G. (1979) *J. Biol. Chem. 254*, 10370–10377.

Davidson, G. A., & Berman, M. C. (1988) *J. Biol. Chem. 263*, 11786–11791.

de Meis, L., & Hasselbach, W. (1971) *J. Biol. Chem. 246*, 4759–4763.

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

de Meis, L., & Inesi, G. (1985) *Biochemistry 24*, 922–925.

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

Fernandez-Belda, F., Kurzmack, F. M., & Inesi, G. (1984) *J. Biol. Chem. 259*, 9687–9698.

Highsmith, S. R., & Murphy, A. J. (1984) *J. Biol. Chem. 259*, 14651–14656.

Ikemoto, N. (1975) *J. Biol. Chem. 250*, 7219–7224.

Ikemoto, N. (1982) *Annu. Rev. Physiol. 44*, 297–317.

Inesi, G. (1985) *Annu. Rev. Physiol. 47*, 573–601.

Inesi, G., Kurzmack, M., & Lewis, D. (1988) *Methods Enzymol. 157*, 154–190.

Jardetzky, O. (1966) *Nature (London) 211*, 969–970.

Jencks, W. P. (1989) *J. Biol. Chem. 264*, 18855–18858.

Kalbitzer, H. R., Stehlik, D., & Hasselbach, W. (1978) *Eur. J. Biochem. 82*, 245–255.

Krasnow, N. (1977) *Arch. Biochem. Biophys. 181*, 322–330.

Kurzmack, M., Verjovski-Almeida, S., & Inesi, G. (1977) *Biochem. Biophys. Res. Commun. 78*, 772–776.

Lüdi, H., & Hasselbach, W. (1983) *Biochim. Biophys. Acta 732*, 479–482.

Mészáros, L. G., & Ikemoto, N. (1985) *J. Biol. Chem. 260*, 16076–16079.

Mészáros, L. G., & Bak, J. (1992) *Biochemistry 31*, 1195–1200.

Myung, J., & Jencks, W. P. (1993) *Biophys. J. 64*, A9.

Ohnishi, S. T. (1979) *J. Biochem. (Tokyo) 86*, 1147–1150.

Pickart, C. M., & Jencks, W. P. (1982) *J. Biol. Chem. 257*, 5319–5322.

Scott, T. L. (1984) *J. Biol. Chem. 259*, 4035–4037.

Stahl, N., & Jencks, W. P. (1987) *Biochemistry 26*, 7654–7667.

Suko, J., Plank, B., Preis, P., Kolassa, N., Hellmann, G., & Conca, W. (1981) *Eur. J. Biochem. 119*, 225–236.

Tanford, C. (1984) *Crit. Rev. Biochem. 17*, 123–151.

Yamada, S., & Tonomura, Y. (1972) *J. Biochem. 72*, 417–425.

Yamada, S., & Ikemoto, N. (1980) *J. Biol. Chem. 255*, 3108–3119.