Protons Induce Calsequestrin Conformational Changes

Cecilia Hidalgo,*# Paulina Donoso,*# and Patricio H. Rodriguez§
*Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Santiago 7; #Centro de Estudios Científicos de Santiago, Santiago 9; and §Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

ABSTRACT Calsequestrin, a high-capacity, intermediate-affinity, calcium-binding protein present in the lumen of sarcoplasmic reticulum, undergoes extensive calcium-induced conformational changes at neutral pH that cause distinct intrinsic fluorescence changes. The results reported in this work indicate that pH has a marked effect on these calcium-induced intrinsic fluorescence changes, as well as on calorimetric changes produced by the addition of Ca^{2+} to calsequestrin. The addition of Ca^{2+} at neutral pH produced a marked and cooperative increase in calsequestrin intrinsic fluorescence. In contrast, at pH 6.0 calsequestrin's intrinsic fluorescence was not affected by the addition of Ca^{2+}, and the same intrinsic fluorescence as that measured in millimolar calcium at neutral pH was obtained. The magnitude and the cooperativity of the calcium-induced intrinsic fluorescence changes decreased as either $[H^+]$ or $[K^+]$ increased. The evolution of heat production, determined by microcalorimetry, observed upon increasing the molar ratio of Ca^{2+} to calsequestrin in 0.15 M KCl, decreased markedly as the pH decreased from pH 8.0 to pH 6.0, indicating that pH modifies the total heat content changes produced by Ca^{2+}. We propose that protons bind to calsequestrin and induce protein conformational changes that are responsible for the observed proton-induced intrinsic fluorescence and calorimetric changes.

INTRODUCTION

Calsequestrin is a high-capacity, intermediate-affinity, calcium-binding protein (Ikemoto et al., 1972, 1974; MacLennan and Wong, 1971; Ostwald et al., 1974; Damiani et al., 1986) present in the lumen of sarcoplasmic reticulum (SR) of skeletal, cardiac, and smooth muscle (for a review, see Yano and Zarain-Herzberg, 1994). Calsequestrin is specifically localized in the SR terminal cisternae regions (Meissner, 1975; Jorgensen et al., 1985). It is anchored to the junctional SR membrane in the vicinity of the feet (Saito et al., 1984; Franzini-Armstrong et al., 1987) through interactions with other SR proteins (Mitchell et al., 1988; Damiani and Margreth, 1990; Guo and Campbell, 1995) such as junctin (Jones et al., 1995).

Calsequestrin has a dual functional role, acting both as a luminal calcium buffer that reduces the amount of free Ca^{2+} inside the SR (Ikemoto et al., 1972, 1974; MacLennan and Wong, 1971), and as a modulator of SR calcium release (Ikemoto et al., 1989, 1991; Gilchrist et al., 1992; Kawasaki and Kasai, 1994).

All calsequestrin isoforms are highly acidic proteins (Yano and Zarain-Hersberg, 1994) that at neutral pH have a large calcium-binding capacity (40–55 mol/mol) and which have an intermediate affinity for calcium in the range of 1 mM in 0.1 M KCl (MacLennan and Wong, 1971; Ikemoto et al., 1974; Ostwald et al., 1974). The nature of calsequestrin calcium-binding sites remains unknown. Primary sequence determinations show that calsequestrin contains most of its negatively charged residues localized near the C-terminal region (Fliegel et al., 1987), suggesting that this region is involved in calcium binding. It has been proposed that the net charge density rather than distinct calcium-binding sites determine the large calcium-binding capacity of calsequestrin (Ohnishi and Reithmeier, 1987).

The physicochemical properties of skeletal and cardiac calsequestrins have been studied using tryptophan fluorescence, circular dichroism, Raman spectroscopy, ^1H NMR, sedimentation, and proteolytic digestion. These studies indicate that calsequestrin undergoes extensive conformational changes upon cation binding (Ikemoto et al. 1972, 1974: Ostwald et al., 1974; Cala and Jones, 1983; Aaron et al., 1984; Cozens and Reithmeier, 1984; Mitchell et al., 1988; Williams and Beeler, 1986; Ohnishi and Reithmeier, 1987; Slupsky et al., 1987; Krause et al., 1991); only some of these changes are specific for Ca^{2+} (Mitchell et al., 1988; Krause et al., 1991). After cation binding, calsequestrin increases its α-helical content and internalizes aromatic residues and hydrophobic domains. Calsequestrin aggregates at high calcium and protein concentration; the calmodulin antagonists trifluoroperazine and melittin inhibit calcium-induced folding, and aggregation, of calsequestrin (He et al., 1993).

Other cations, such as Zn^{2+}, Sr^{2+}, Tb^{3+}, bind to calsequestrin and cause changes in fluorescence and circular dichroism spectra analogous to those effected by Ca^{2+} (Ikemoto et al., 1974; Ostwald et al., 1974; Ohnishi and Reithmeier, 1987). Potassium ions, in analogy to Ca^{2+}, also produce changes in circular dichroism and fluorescence spectra, albeit at much higher concentrations than Ca^{2+}; in the absence of Ca^{2+} the spectral changes are completed at concentrations of $K^+ \geq 0.5$ M (Ikemoto et al., 1974; Ostwald et al., 1974). Mg^{2+} binds to different sites on calsequestrin than Ca^{2+}; Ca^{2+}-binding affinity is not affected by the Mg^{2+} concentration, and fluorescence determinations

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Address reprint requests to Dr. Cecilia Hidalgo, Centro de Estudios Científicos de Santiago, Casilla 16443, Santiago 9, Chile. Tel.: 56-2-206-0092; Fax: 56-2-233-8336. E-mail: ch@cecs.cl.

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indicate that the conformation of tryptophans in the Ca^{2+} + Mg^{2+}-saturated protein is not different from the calcium-saturated protein (Krause et al., 1991).

All of these studies have been carried out in the vicinity of pH 7.0; there is only one report indicating that at pH 3.0, in the absence of Ca^{2+}, calsequestrin undergoes circular dichroism spectral changes of a magnitude and shape similar to those induced by Ca^{2+} at pH 7.5 (Ostwald et al., 1974). In this work we studied the effect of systematically decreasing pH on calcium-induced calsequestrin conformational changes, with the rationale that in analogy with other ions, protons may also induce conformational changes in this protein. We found that protons produced marked changes in calsequestrin conformation, as evidenced by the effect of pH on calcium-induced changes in protein intrinsic fluorescence and in solution heat content.

EXPERIMENTAL PROCEDURES

Isolation and purification of calsequestrin

Calsequestrin was purified by phenyl-Sepharose chromatography from a soluble Na_2CO_3 extract of triads (Cala and Jones, 1983). Triads were isolated from rabbit skeletal muscle as described previously (Hidalgo et al., 1993).

Fluorescence measurements

Emission fluorescence spectra of purified calsequestrin were recorded in a Shimadzu RF540 spectrofluorometer or in a Perkin-Elmer LS-50 spectrofluorometer. The excitation wavelength was 295 nm; excitation and emission slits of 5 nm were used. To prevent protein precipitation after the addition of calcium, calsequestrin concentration was always kept below 30 μg/ml; at this low protein concentration, changes in light scattering were negligible. All spectra were recorded in 20 mM buffer, with or without KCl as indicated, using 2-[*N*-morpholino]-ethane sulfonic acid (MES) tritrated with Trizma base (MES/Tris) in the pH range 5.5 to 6.25, 3-[*N*-morpholino]-propane sulfonic acid (MOPS)/Tris in the pH range 6.8 to 7.5, and HEPES/Tris at pH 8.0. The Ca^{2+} concentration was modified by sequential additions of small volumes of concentrated stock solutions of $CaCl_2$ to give the desired final [Ca^{2+}]. The lowest [Ca^{2+}] used was 0.01 mM, because the same fluorescence spectra were obtained at this [Ca^{2+}] as in 1 mM EGTA.

Titration calorimetry

Titrations of purified calsequestrin with calcium were carried out on a MicroCal MCS ultrasensitive isothermal titration calorimeter (MicroCal, Northampton, MA), using Observer software for instrument control and data acquisition. The procedure, instrumentation, and data analysis followed in these experiments have been described previously (Wiseman et al., 1989). The solution contained calsequestrin (0.7 to 1 mg/ml) in 0.15 M KCl; the pH of the solution was adjusted using 20 mM MES/Tris for pH 6.0, 20 mM MOPS/Tris for pH 7.0, and 20 mM Tris/HEPES for pH 8.0. During the titration, the protein solution was kept at a constant temperature of 25°C in a stirred (400 rpm) reaction cell (1.5 ml). To increase Ca^{2+}, 25 mM $CaCl_2$ was added in serial injections (5 μl per injection).

Other procedures

Protein was determined according to the method of Hartree (1972), using bovine serum albumin as the standard.

Materials

All reagents used were of analytical grade. Phenyl-sepharose and the protease inhibitors used for triad isolation (leupeptin, pepstatin A, benzamidine, and phenylmethylsulfonyl fluoride) were obtained from Sigma.

RESULTS

Effects of calcium and pH on calsequestrin intrinsic fluorescence spectra

In this work we examined the effect of increasing [Ca^{2+}] on calsequestrin intrinsic fluorescence spectra as a function of pH, in solutions containing either 0.15 M KCl or no KCl. Spectra measured in a range of [Ca^{2+}] from 0.01 to 2 mM are illustrated in Fig. 1 *A* for experiments done in 0.15 M KCl, and in Fig. 1 *B* for experiments done without KCl. The top panels illustrate spectra measured at pH 6.0, the middle panels, at pH 7.0, and the bottom panels, at pH 8.0.

Incremental additions of calcium to solutions containing calsequestrin in 0.15 M KCl produced a marked increase in protein intrinsic fluorescence at pH 7.0 and at pH 8.0, as well as a blue shift in the emission maxima (Fig. 1 *A*, *middle* and *bottom*). The changes saturated in the mM [Ca^{2+}] range and were even more pronounced in the absence of KCl (Fig. 1 *B*, *middle* and *bottom*).

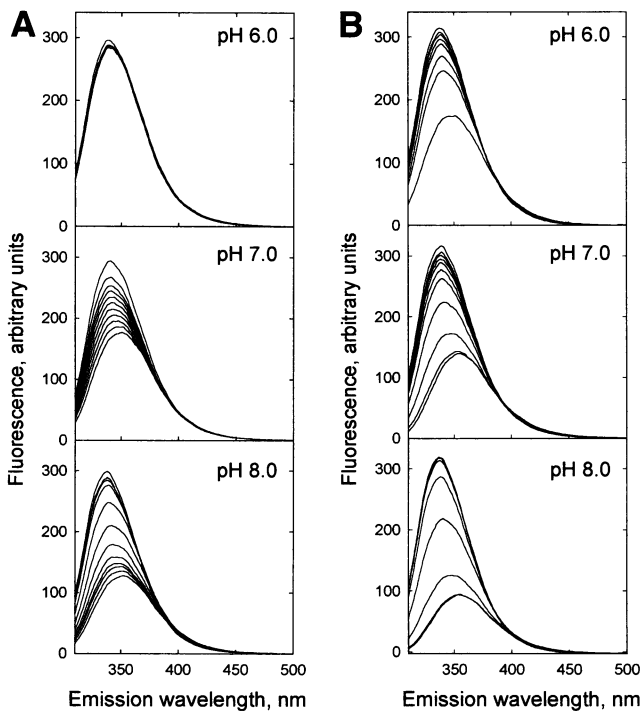

FIGURE 1 Effect of Ca^{2+} on calsequestrin intrinsic florescence spectra measured in 0.15 M KCl (*A*) and without KCl (*B*). Top panels: spectra were measured at pH 6.0. Middle panels: spectra were measured at pH 7.0. Lower panels: spectra were measured at pH 8.0. The concentrations of Ca^{2+} (mM) used to record the spectra in order of increasing fluorescence, were as follows: pH 6.0: 0.01, 0.1, 0.2, 0.3, 0.4, 0.8, 1.0, 2.0; pH 7.0: 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 2.0; pH 8.0: 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0.

At pH 6.0 the addition of Ca^{2+} to calsequestrin solutions in 0.15 M KCl had no effect on the intrinsic fluorescence spectra (Fig. 1 *A*, *top*). All spectra were similar to the spectra recorded in 2 mM Ca^{2+} at pH 8.0 (Fig. 1 *A*, bottom). The addition of Ca^{2+} in the absence of KCl at pH 6.0 produced some spectral changes (Fig. 1 *B*, *top*), although they were significantly less pronounced than those observed at higher pH values (Fig. 1 *B*, *middle* and *bottom*).

The wavelengths at which maximum fluorescence intensities were obtained, λ_{max}, changed as a function of both pH and Ca^{2+}. The largest blue shift, observed at pH 8.0 in the absence of KCl after calcium was increased to 2 mM (Fig. 1, *bottom panels*) was 17 nm (λ_{max} decreased from 354 nm to 337 nm). This blue shift was negligible at pH 6.0 for calsequestrin solutions in 0.15 M KCl; in these conditions a constant λ_{max} of 338 nm was obtained for all [Ca^{2+}] studied (Fig. 1 *A*, *top*).

The maximum and minimum fluorescence intensities, defined as the fluorescence obtained in 2 mM Ca^{2+} and in 0.01 mM [Ca^{2+}], respectively, were measured from spectra such as those shown in Fig. 1. The maximum fluorescence values, F_{max}, were essentially the same in zero KCl as in 0.15 M KCl, and in the pH range 5.5 to 8.0 (Fig. 2, *open symbols*).

In contrast, pH and KCl had a marked effect on the minimum fluorescence values, F_{min} (Fig. 2, *solid symbols*). At pH 8.0 F_{min} was considerably lower than F_{max}. At lower pH values the differences decreased, becoming negligible at pH 6.0 in 0.15 M KCl (Fig. 2, *solid squares*). The pH-dependent changes in the F_{min} values measured in 0.15 M KCl followed a titration curve with a pK of 6.4 (Fig. 2, *solid squares*). In the absence of KCl, lower values of F_{min} than F_{max} were obtained, even at pH 5.5, the lowest pH studied; the line drawn corresponds to a hypothetical titration curve, fitted to the data assuming that at a pH of <5.5, F_{min} would eventually reach F_{max}; this fit yielded a pK of 5.4.

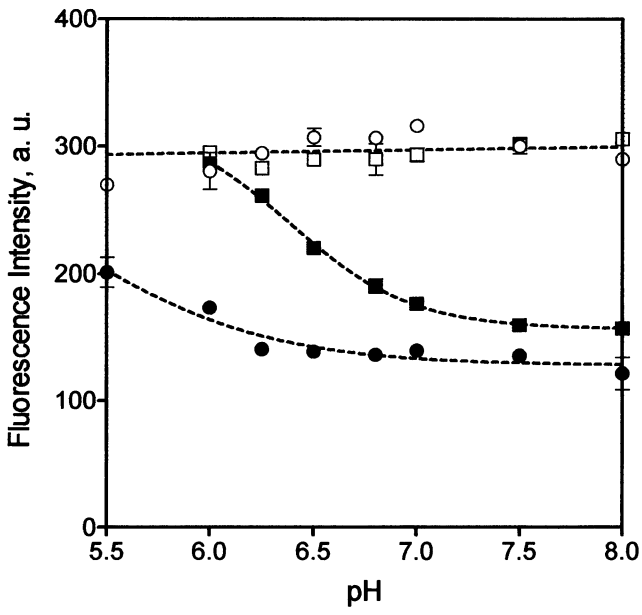

FIGURE 2 Effect of pH on calsequestrin intrinsic fluorescence measured in 2 mM Ca^{2+}, defined as F_{max} (○, □), and on the fluorescence measured in 0.01 mM [Ca^{2+}], F_{min} (●, ■). □, ■, Values measured in 0.15 M KCl; ○, ●, data obtained in zero KCl.

Effect of pH on the cooperativity of the calcium-induced changes in calsequestrin intrinsic fluorescence

Experiments done in 0.15 M KCl

Solution pH had a remarkable effect on the cooperativity of the calcium-induced changes in calsequestrin intrinsic fluorescence. At pH 7.5, the addition of Ca^{2+} produced a highly cooperative change in fluorescence intensity (Fig. 3 *A*, *open squares*); these experimental points were scaled making F_{max} = 100% and F_{min} = 0, and the resulting values, fitted to the Hill function $F = (100\,x^n)/([K_H]^n + x^n)$, gave a best nonlinear fit with n = 2.9 and K_H = 0.57 mM (Fig. 3 *B*, *open squares*). The changes in fluorescence intensity observed at pH 6.8 (Fig. 3 *A*, *open circles*) showed less cooperativity than at pH 7.5; the best fit to the above

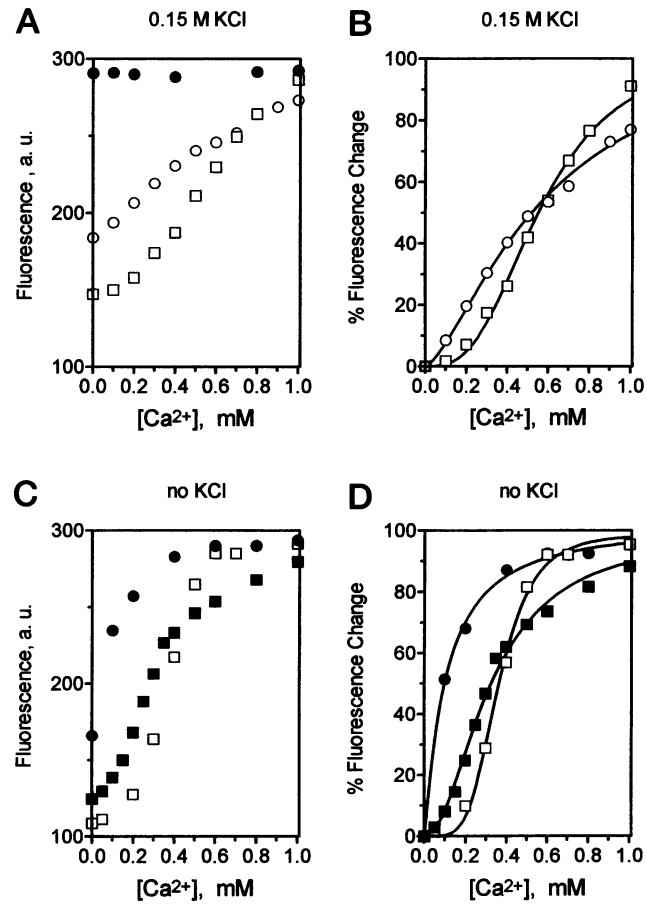

FIGURE 3 Changes in intrinsic fluorescence of calsequestrin as a function of added Ca^{2+} measured at different pH values. (*A* and *B*) 0.15 M KCl. (*C* and *D*) 0 KCl. The left panels correspond to the experimental data, and the right panels to the corresponding normalized data fitted to a Hill equation (*solid lines*). ●, pH 6.0; ■, pH 6.25; ○, pH 6.8; □, pH 7.5.

Hill function gave $n = 1.4$, $K_H = 0.60$ mM (Fig. 3 *B*, *open circles*). At pH 6.0, maximum intrinsic fluorescence was observed in 0.01 mM Ca^{2+}, so that further fluorescence changes did not take place after increasing Ca^{2+} to 1 mM (Fig. 3 *A*, *solid circles*).

Experiments done in the absence of KCl

Calcium-induced changes in calsequestrin intrinsic fluorescence were more cooperative at a given pH in the absence of KCl (Fig. 3 *C*) than in 0.15 M KCl (Fig. 3 *A*). At pH 7.5 the experimental points (Fig. 3 *C*, *open squares*), scaled and fitted to the above Hill equation, gave a highly cooperative fluorescence change with Ca^{2+} (Fig. 3 *D*, *open squares*), with $n = 4.4$ and $K_H = 0.36$ mM. The values obtained at pH 6.25 (Fig. 3 *C*, *solid squares*), scaled and fitted to the above equation, yielded a Hill coefficient of $n = 2.0$ and $K_H = 0.32$ mM (Fig. 3 *D*, *solid squares*). Even at pH 6.0 and pH 5.5, where no changes in fluorescence were observed in 0.15 M KCl, the calcium-induced changes in fluorescence intensity, although small, were measurable and made possible a reliable fit of the data to the Hill equation. The changes obtained at pH 6.0 (Fig. 3 *C*, *solid circles*) gave for this experiment values of $n = 1.2$ and $K_H = 0.1$ mM.

These results show that decreasing pH reduced markedly the cooperativity of the calcium-induced fluorescence changes, as evidenced by the decrease in Hill coefficients observed on lowering pH (Fig. 4 *A*). Lower Hill coefficients were consistently obtained at a given pH in 0.15 M KCl than in 0 KCl, indicating that both H^+ and K^+ reduce the cooperativity of the calcium-induced fluorescence changes. Cooperativity was lost at pH $\leq$ 6.0 in the absence of KCl (Fig. 4 *A*, *open squares*) and at pH 6.5 in 0.15 M KCl (Fig. 4 *A*, *solid squares*).

The pH dependence of the corresponding K_H values shows that in the absence of KCl, K_H values fluctuated around 0.3 mM in the pH range 8.0 to 6.25, and increased to 0.85 mM at pH 5.5 (Fig. 4 *B*, *open circles*). In 0.15 M KCl, higher K_H values were obtained that increased markedly when the pH of the solution decreased below 7.0 (Fig. 4 *B*, *solid circles*).

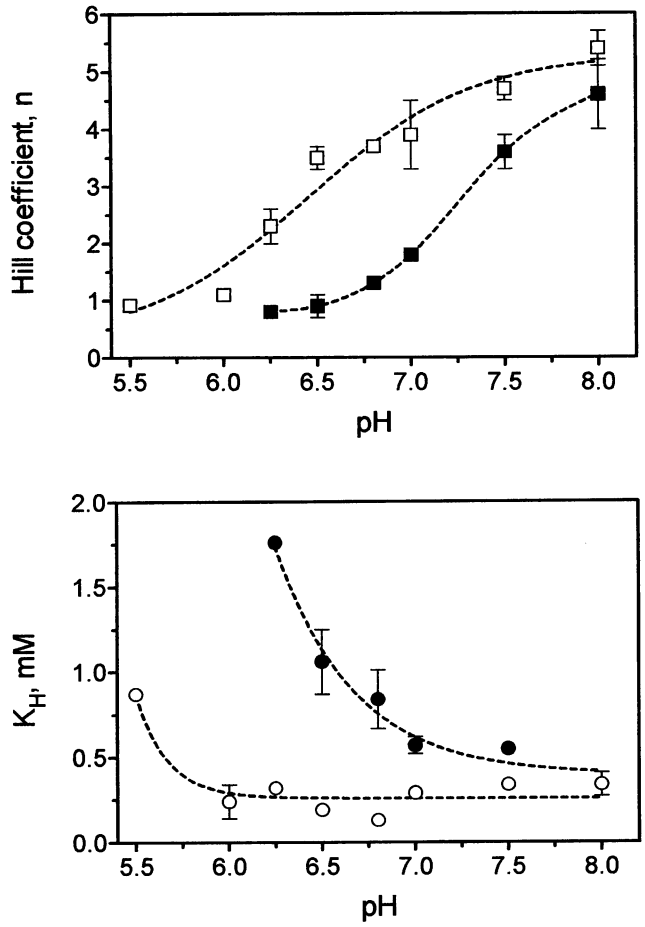

FIGURE 4 Effect of pH on the cooperativity of the calcium-induced fluorescence changes of calsequestrin. (*Top*) Hill coefficient, *n*, versus pH with 0.15 M KCl (■) or without KCl (□). (*Bottom*) K_H versus pH in 0.15 M KCl (●) and 0 KCl (○). The dotted lines have no theoretical meaning.

Effect of KCl on the cooperativity of the calcium-induced changes in calsequestrin intrinsic fluorescence, measured at pH 7.0

The effect of systematically increasing the concentration of KCl on calcium-induced fluorescence changes was investigated. At pH 7.0 the largest fluorescence changes were observed in the absence of KCl ($F_{max}/F_{min} = 2.7$; 100% change), and the best fit to the Hill equation yielded the highest cooperativity with $n = 3.5$ (Table 1). Both the absolute magnitude of the changes in intrinsic fluorescence, as well as their cooperativity, decreased in proportion to the KCl concentration of the solution. At concentrations of KCl in the physiological range, calcium-induced fluorescence changes were partially prevented and were completely abolished by 0.5 M KCl (Table 1). Similar effects of KCl were detected at pH 6.8 and at pH 7.2 (not shown).

Calorimetric experiments

As a complement to the fluorescence studies, we studied by microcalorimetry Ca^{2+} binding to calsequestrin in 0.15 M KCl at three different pH values, 6.0, 7.0, and 8.0. Microcalorimetry gives an understanding of thermodynamic changes associated with the binding process and allows determinations of low-affinity binding constants.

The evolution of heat production upon increasing the molar ratio of Ca^{2+} to calsequestrin in 0.15 M KCl varied markedly at the three pH values studied (Fig. 5), indicating that pH modifies the total heat content changes produced by Ca^{2+}. The largest exothermic changes were observed at pH 8.0 (Fig. 5, *solid circles*). At this pH the titration profile showed a complex pattern: a heat increase close to linear was observed up to a molar ratio of 15 Ca^{2+} per calsequestrin, the solution heat content decreased somewhat up to a

TABLE 1 Effect of KCl on the Ca-induced fluorescence change determined at pH 7.0

KCl (mM)	F_{max}/F_{min}	Hill coefficient, n	K_H (mM)
0	2.7 (100%)	3.5 ± 0.5 (3)	0.26 ± 0.02 (3)
0.10	2.2 (81%)	3.2 ± 0.6 (2)	0.51 ± 0.08 (2)
0.15	1.8 (6.7%)	2.4 ± 0.2 (5)	0.58 ± 0.04 (5)
0.25	1.3 (48%)	1.5 ± 0.2* (1)	0.57 ± 0.04* (1)
0.50	1.0 (0%)	#	#

F_{max} and F_{min} correspond to the fluorescence intensities obtained in 2 mM Ca^{2+} and in 0.01 mM Ca^{2+}, respectively.
Data are given as mean ± SEM. The number of experiments is given in parentheses.
*For these values, the error of the nonlinear fit to the Hill equation is given.
#Calcium-induced fluorescence changes did not occur in 0.5 M KCl (F_{max}/F_{min} = 1).

molar ratio of 30, and it increased again in a nearly linear fashion up to a molar ratio of 40, saturating at molar ratios of >40. Because of their complexity, our attempts to fit the calorimetric changes obtained at pH 8.0 with a theoretical Ca^{2+} binding function were unsuccessful.

Lower exothermic heat changes were observed at pH 7.0 (Fig. 5, *solid triangles*; see *inset* for an amplification of the values). At pH 7.0 the titration profile showed an increase in solution heat content that saturated above a molar ratio of 50. A smooth curve was obtained by fitting the data to a model considering a single set of n identical binding sites; this fit yielded n = 29.6 ± 0.2 sites, ΔH = −0.589 ± 0.037 kcal/mol and K_{eq} = 12,800 ± 410 M^{-1}. Calcium-induced changes were essentially abolished after the addition of 1 mM trifluoroperazine at pH 7.0 (data not shown).

The smallest calorimetric changes were observed at pH 6.0 (Fig. 5, *solid squares*). The addition of up to 15 mol Ca^{2+} per mol of calsequestrin produced a small endothermic change, which was reversed by further Ca^{2+} addition. As in the case of pH 8.0, the complexity of the changes prevented a straightforward fit to a theoretical binding equation.

The molar calcium-induced heat changes decreased at lower concentrations of protein (data not shown). Thus, in addition to heat changes due to calcium binding to individual molecules, calorimetric changes presumably reflect a significant contribution of calcium-induced calsequestrin aggregation. A quantitative determination of the relative contributions of intermolecular and intramolecular calcium binding to the calorimetric changes, and of the effects of pH upon them, is outside the scope of the present work.

DISCUSSION

The results reported in this work show for the first time that pH has a marked effect on calcium-induced changes in calsequestrin intrinsic fluorescence and solution heat content, and indicate that protons, in the absence of calcium ions, can elicit significant calsequestrin conformational

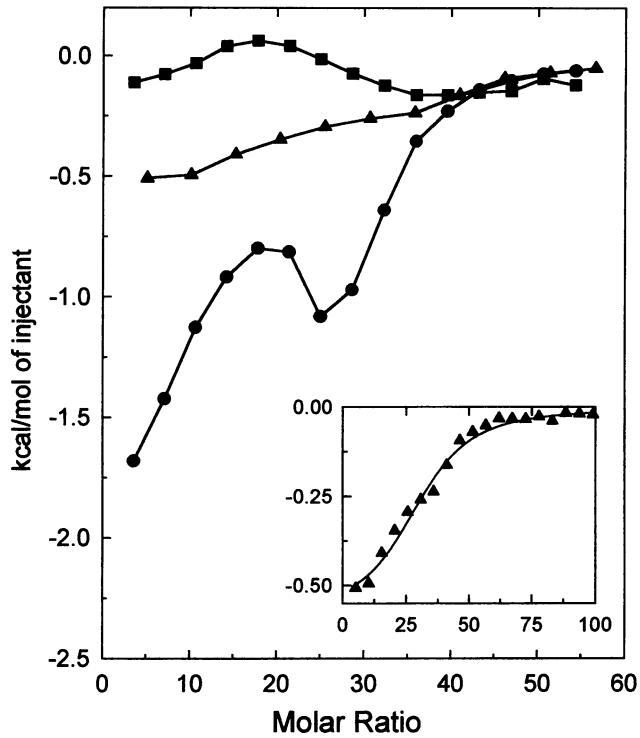

FIGURE 5 Effect of pH on the production of heat upon addition of calcium to calsequestrin. Data were obtained using 22 μM calsequestrin in 0.15 M KCl at pH 6.0 (■), pH 7.0 (▲ in main figure and inset), and pH 8.0 (●). For further details, see text.

changes in the pH range 6 to 8 and in the presence of physiological concentrations of KCl.

Calsequestrin intrinsic fluorescence changes

It is has been known since the earlier studies that the addition of Ca^{2+} to calsequestrin, at pH 7.0 to 7.5, produces an increase in its intrinsic fluorescence, as well as a blue shift in its emission maxima (Ikemoto et al., 1972, 1974; Mitchell et al., 1988; Slupsky et al., 1987). These changes are due to modifications of the local environment of its tryptophan chromophores, and take place as a consequence of the conformational changes of calsequestrin produced by Ca^{2+} binding, which transfer tryptophan residues from the polar surface to the hydrophobic interior of the protein (Ikemoto et al., 1972, 1974).

Other cations produce intrinsic fluorescence changes similar to those induced by Ca^{2+} (Ikemoto et al., 1974; Ostwald et al., 1974; Ohnishi and Reithmeier, 1987), but the effect of H^+ on intrinsic calsequestrin fluorescence has not been described.

In this work we have found that protons in 0.15 M KCl produced changes in calsequestrin intrinsic fluorescence similar to those produced by calcium ions. Thus the highest intrinsic fluorescence of calsequestrin was obtained either by the addition of 2 mM Ca^{2+} at pH 8.0, or by the addition of protons to pH 6.0 in 0.15 M KCl and 0.01 mM [Ca^{2+}].

This effect of protons was enhanced by the presence of potassium ions; in 0.01 mM [Ca^{2+}] increasing [H^+] to pH 5.5 in 0 K^+ did not produce the same high intrinsic fluorescence as that observed at pH 6.0 in 0.15 M KCl, indicating that in the absence of K^+, a significantly higher [H^+] is needed to produce the same fluorescence changes. Furthermore, at pH 7.0 in 0.01 mM [Ca^{2+}], increasing [K^+] to 0.5 M elicited the same maximum intrinsic fluorescence changes as increasing [Ca^{2+}] to 1 mM. These combined observations indicate that either H^+ or K^+, or both ions jointly, can transfer tryptophans to the hydrophobic protein interior and can replace Ca^{2+} in eliciting the intrinsic fluorescence changes of calsequestrin. The residues involved in the pH-induced protein conformational changes had an apparent pK of 6.4 in the presence of 0.15 M KCl.

Cooperativity of the intrinsic fluorescence changes

The present results also show that the addition of Ca^{2+} induced highly cooperative fluorescence changes at pH $\geq$ 7.0, indicating that in these conditions calcium binding causes cooperative conformational changes in the calsequestrin regions that elicit the intrinsic fluorescence changes. The cooperativity of the calcium-induced intrinsic fluorescence changes has not been reported in previous studies, although highly cooperative effects of Ca^{2+} on increasing the protein α-helical content have been described at pH 7.5 (Aaron et al., 1984). In contrast, Ca^{2+} binding to calsequestrin is noncooperative, either when measured to calsequestrin in solution at pH 7.5 (Krause et al., 1991), or when measured at pH 6.8 to calsequestrin that is still forming part of isolated triads (Donoso et al., 1995).

We propose that this difference in cooperativity between calcium-induced intrinsic fluorescence changes and calcium binding to calsequestrin is due to the presence of additional calcium-binding sites in calsequestrin that do not participate in generating the intrinsic fluorescence changes. According to this view, Ca^{2+} at low concentrations would bind first to a few discrete sites, causing highly cooperative conformational changes in the protein region that contains all five tryptophan residues that are clustered near the highly negatively charged N-terminal domain (Fliegel et al., 1987). These conformational changes would bury these tryptophans, giving rise to cooperative intrinsic fluorescence changes. Further addition of Ca^{2+} would promote binding to additional sites present in individual protein molecules, as well as to intermolecular sites involved in protein aggregation (He et al., 1993), without further increasing calsequestrin intrinsic fluorescence. As a consequence, the overall Ca^{2+} binding to calsequestrin would be noncooperative. In support of our proposal, in 0.15 M KCl lower [Ca^{2+}] is needed to effect fluorescence changes ($K_H \approx 0.3$ mM in the pH range 8.0 to 7.0) than to saturate all Ca^{2+}-binding sites ($K_d \approx$ 1–2 mM at pH 7.5; Krause et al., 1991). The observations, confirmed in the present work, that 0.5 M KCl at pH 7.0 abolishes the calcium-induced intrinsic fluorescence changes of calsequestrin (Ikemoto et al., 1974; Ostwald et al., 1974), whereas significant calcium-induced calsequestrin conformational changes still take place at pH 7.0 in the presence of 0.5 M monovalent ions (Cala and Jones, 1983), further indicate that calsequestrin has calcium-binding sites in addition to those responsible for the intrinsic fluorescence changes.

Calorimetric determinations

Thermodynamic studies indicate that binding of calcium to calsequestrin is mainly entropy driven and is accompanied by a release of H^+; metal-free calsequestrin would contain carboxyl-carboxylate bonds that would be disrupted by Ca^{2+} but not by Mg^{2+} (Krause et al., 1991).

We found that pH had a marked effect on calorimetric changes observed after the addition of Ca^{2+} to calsequestrin. The significant exothermic changes obtained at pH 8.0 and pH 7.0 in 0.15 M KCl were not observed at pH 6.0. These results indicate that the calcium-induced changes in the conformation of calsequestrin that lead to these calorimetric changes do not take place or are strongly reduced at pH 6.0, in analogy to the complete lack of effect of Ca^{2+} on the intrinsic fluorescence of the protein measured at pH 6.0 in 0.15 M KCl.

Only the experimental data obtained at pH 7.0 were amenable to theoretical analysis of binding (Wiseman et al., 1989). At pH 7.0 and at a concentration of calsequestrin of 22 μM, the theoretical Ca^{2+} binding curve yielded 29.6 $\pm$ 0.2 equal Ca^{2+} binding sites, $\Delta H = -0.589 \pm 0.037$ kcal/mol. Although not corrected for contributions from calcium-induced H^+ release from calsequestrin, these values are essentially equal to the corrected values of 31 Ca^{2+}-binding sites and $\Delta H = -0.359$ kcal/mol (-1.5 kJ/mol) reported by Krause et al. (1991) when measuring Ca^{2+} binding to 4.2 μM calsequestrin in 0.15 M KCl, pH 7.5.

Physiological implications

From the results reported in this work, we propose that protons bind to calsequestrin and produce the same burying of tryptophan residues caused by Ca^{2+} (Ikemoto et al., 1974; Ostwald et al., 1974; Aaron et al., 1984). Protons (pH 6.0) also inhibited the calcium-induced conformational changes detectable by microcalorimetry. It remains to be determined, however, whether protons produce the same overall calsequestrin conformational changes as Ca^{2+}.

The physiological implications of the effects of Ca^{2+} and H^+ on calsequestrin conformation may lie in the proposed role of calsequestrin as a modulator of the function of the calcium release channel/ryanodine receptor of junctional SR. It has been shown that decreasing luminal [Ca^{2+}] to 0.1 mM produces at pH 6.8 a marked inhibition of caffeine-induced (Ikemoto et al., 1989) or of ATP-induced cal-

cium release rates in triads (Donoso et al., 1995), concomitant with a marked decrease in calcium binding to calsequestrin (Donoso et al., 1995). The results showing that conformational changes of the release channel protein occur after calcium binding to calsequestrin, and are reversibly abolished by dissociation of the calsequestrin-calcium channel complex (Ikemoto et al., 1989), plus the finding that transient increases in intravesicular [Ca^{2+}] precede calcium release (Ikemoto et al., 1991), suggest that calsequestrin and the channel protein are mutually coupled, so that conformational changes of one protein are transmitted to the other. From these and other studies (see Hidalgo and Donoso, 1995, for a review), it has been proposed that calsequestrin modulates the activity of the calcium release channels through calcium-induced conformational changes.

We have found recently that in 0.1 mM [Ca^{2+}] increasing the luminal [H^+] of SR to pH 6.0 in 0.1 M KCl can effectively replace luminal Ca^{2+} in producing release channel activation (Donoso et al., manuscript submitted for publication). From the present results, we propose that in these conditions luminal protons maintain the calsequestrin conformation that activates the calcium release channels.

In resting muscle, SR luminal pH is presumably at equilibrium with cytoplasmic pH, because the SR membrane is very permeable to protons (Meissner and Young, 1980), whereas luminal calcium concentrations in the millimolar range have been estimated (Maylie et al., 1987). Thus, at rest calsequestrin should be saturated with calcium, giving rise to its observed compacted configuration in a quasicrystalline array (Saito et al., 1984). If there is a significant counterflow of protons (Somlyo et al., 1981; Pape et al., 1990) to compensate for the loss of Ca^{2+}, SR luminal pH should decrease transiently during calcium release. As a consequence, the unfolding of calsequestrin due to calcium dissociation would be prevented, at least partially, by proton binding. This proposed mechanism may have a physiological regulatory role, keeping the channel activated by calsequestrin even after substantial release of Ca^{2+} from the SR.

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