Characterization of a New Sarcoplasmic Calcium-Binding Protein with Magnesium-Induced Cooperativity in the Binding of Calcium†

Jos A. Cox and Eric A. Stein*

ABSTRACT: The sarcoplasmic calcium-binding protein (SCP) from the annelid *Nereis diversicolor* (sandworm) has been purified to electrophoretical homogeneity. The protein has a molecular weight of 20 000 as determined both by gel filtration and by electrophoresis in the presence of dodecyl sulfate. The isoelectric point is 4.8. Equilibrium dialysis studies indicate that, in the absence of Mg^{2+}, three atoms of calcium bind to the protein in a noncooperative way, with an intrinsic binding constant of 1.7×10^8 M^{-1}. In the absence of Ca^{2+}, three atoms of Mg^{2+} bind to the protein with positive cooperativity, as indicated by increasing intrinsic binding constants [$(0.43–6.6) \times 10^4$ M^{-1}]. In the presence of 0.9 mM Mg^{2+}, the binding of Ca^{2+} to the protein occurs with strong positive cooperativity (Hill coefficient = 2.0), and the binding constants decrease by about 1 order of magnitude. Thus Nereis SCP has three Ca–Mg mixed sites, and Mg^{2+} induces cooperativity in the binding of Ca^{2+}. Whereas Nereis SCP is devoid of β-sheet structure, the α-helical content is around 43% for the Ca- and Mg-saturated protein and 34% for the metal-free SCP. The far-UV circular dichroic spectra of the Ca and the Mg form are not identical. Near-UV circular dichroism also confirms that the Ca and the Mg forms are different: the chromophores Tyr and Phe are more structured in the Mg form than in the Ca form. Trp fluorescence suggests too that the Mg form has a more ordered conformation than the Ca form which in turn shows more order than the metal-free form. Trp fluorescence kinetics indicate that the conformational change induced by release of Ca^{2+} from Nereis SCP is a slow process, with a half-life of about 10 s, whereas exchange of Ca^{2+} for Mg^{2+} and vice versa is at least 10-fold faster. Nereis SCP does not activate calmodulin-deficient bovine brain phosphodiesterase, nor does it form calcium-dependent complexes with rabbit muscle troponin I or troponin T. The destabilization of the protein that occurs when Ca^{2+} replaces Mg^{2+} on the metal-binding sites could be instrumental in the function of this SCP.

Low molecular weight sarcoplasmic calcium-binding proteins (SCP's)[1] have been isolated from muscle extracts of different animal species [for review, see Wnuk et al. (1981)]. Vertebrate SCP's, generally called parvalbumins, constitute a homogeneous group of acidic proteins with a mol wt of ~12 000 and two high-affinity calcium-binding sites (Benzonana et al., 1972). The latter also bind magnesium competitively with calcium (Cox et al., 1977; Haiech et al., 1980; Moeschler et al., 1980); both metals are bound in a noncooperative fashion. Parvalbumins are much more abundant in the phasic white muscle (~1 g/kg in rabbit) than in the tonic red muscle (Blum et al., 1977), but no functional relationship could be established from this distribution (Wnuk et al., 1981).

Invertebrate SCP's have been found in considerable amounts (~2.5 g/kg) in fast striated muscle of crustacea and protochordates. They form a group less homogeneous than parvalbumins (Wnuk et al., 1981). Crayfish SCP is a dimer of identical subunits of 22 000 mol wt (Cox et al., 1976) and contains six high-affinity calcium-binding sites, among which only four can bind magnesium in a competitive way (Wnuk et al., 1979). The binding of calcium displays positive cooperativity, which is enhanced in the presence of magnesium. Magnesium binding also is slightly cooperative. The SCP of the protochordate amphioxus is monomeric with a mol wt of 22 000 and binds strongly two calciums (Kohler et al., 1978). Magnesium competes with calcium for only one metal-binding stite and induces positive cooperativity in calcium binding.

Since hitherto SCP was found in abundant amounts only in fast striated muscle, it was of interest to investigate obliquely striated muscle, which is found, for instance, in annelids. As in the case of mollusc muscle (Kendrick-Jones et al., 1976), but in contrast to the case of fast striated muscle of vertebrates and invertebrates, actomyosin from annelid muscle is calcium regulated by way of a M_r 25 000 myosin light chain which cannot be phosphorylated; furthermore, an additional regulatory mechanism seems to function on the thin filaments of annelid muscle, which contain troponin C (D'Haese, 1980).

Experimental Procedures

Materials. Analytical grade KCl, $CaCl_2$, $MgCl_2$, EGTA, Tris, and "Suprapure" HCl were obtained from Merck (Darmstadt, Germany). Phenylmethanesulfonyl fluoride was from Sigma (St Louis, MO), pepstatin A from Protein Research Foundation (Osaka, Japan). Live sandworms (*Nereis diversicolor*) were obtained from Zeeland (The Netherlands). Rabbit skeletal muscle troponin I was provided by Dr. E. H. Fischer, University of Washington, Seattle, WA. Rabbit skeletal muscle troponin C was prepared as previously described (Cox et al., 1981). Bovine brain calmodulin and calmodulin-free phosphodiesterase were prepared according to the method of Watterson et al. (1976). All buffers and protein solutions for the determination of metal binding were prepared with bidistilled water from an all-quartz apparatus.

Phosphodiesterase Activity. The assay was carried out according to the method of Boudreau & Drummond (1975).

Electrophoresis. Polyacrylamide disc gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate, or of 1 mM EGTA, or of 1 mM $CaCl_2$, was carried out according to the method of Laemmli (1970). The complex formation between Nereis SCP and troponin I was monitored by electrophoresis as described by Head & Perry (1974).

Ca Content and Specific Absorption Coefficient. A solution of 10 mg/mL Nereis SCP was extensively dialyzed against

† From the Department of Biochemistry, University of Geneva, P.O. Box 78 Jonction, 1211 Geneva 8, Switzerland. *Received March 6, 1981.* This work was supported by Swiss National Science Foundation Grant 3.237.77.

[1] Abbreviations used: SCP, sarcoplasmic calcium-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/81/0420-5430$01.25/0

bidistilled water brought to pH 7.0 with $(NH_4)HCO_3$ and containing 10 μM calcium. A fraction was used for spectral analysis and determination of the calcium content (see below); dry weight was determined on the remaining solution as previously described (Cox et al., 1976). The whole determination was carried out in duplicate.

Isoelectric Point Determination. Isoelectric focusing was carried out on agarose IEF (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method recommended by Pharmacia.

Metal-Binding Measurements. Binding of Ca^{2+} and Mg^{2+} to SCP was measured by equilibrium dialysis starting with the calcium-saturated protein. Samples (0.5 mL) containing ~6 mg/mL protein were dialyzed against 100 mL of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EGTA, 1000 cpm of ^{45}Ca/mL, and increasing amounts of $CaCl_2$ in the presence or absence of 0.9 mM $MgCl_2$. For Mg binding, similar experimental conditions were used except that the concentration of EGTA was 0.5 mM. After equilibrium was reached (48 h with one change of dialysis buffer), inside and outside metal ion concentrations were measured by atomic absorption or liquid scintillation counting. The protein concentration was measured spectrophotometrically at 280 nm. The amount of metal bound to the protein and the free metal concentration were calculated as described previously (Wnuk et al., 1979). The results obtained with atomic absorption and with ^{45}Ca scintillation counting were statistically indistinguishable, indicating that all protein-bound calcium is exchangeable.

Analysis of the Binding Data. The existence of multiple binding sites on the protein and cooperativity in binding required an analysis of the data by means of the Adair equation (Adair, 1925):

$$r = \frac{K_1[\mathrm{Me}] + 2K_1K_2[\mathrm{Me}]^2 + 3K_1K_2K_3[\mathrm{Me}]^3}{1 + K_1[\mathrm{Me}] + K_1K_2[\mathrm{Me}]^2 + K_1K_2K_3[\mathrm{Me}]^3} \quad (1)$$

where r is the amount of metal bound per mole of protein, [Me] is the free metal concentration, and K_1, K_2, and K_3 the stoichiometric binding constants of the different sites. The latter values were calculated with a curve-fitting procedure (Wnuk et al., 1979). The intrinsic association constants K_1' were obtained from the stoichiometric constants by correction for the statistical factors $^1/_3$, 1, and 3 for the respective binding sites (Cornish-Bowden & Koshland, 1975).

Circular Dichroism. Spectra were recorded at room temperature on a Jasco J-20 A spectropolarimeter with 1-nm slit. The instrument was calibrated with *d*-10-camphorsulfonate (Eastman, Rochester, NY). The protein was dissolved in 100 mM Tris-HCl buffer, pH 7.5, to a final concentration of 0.5 mg/mL in a 0.05-cm cell used below 250 nm and 5 mg/mL in a 0.5-cm cell used above 250 nm. The mean residue molecular weight of Nereis SCP was calculated from the amino acid composition and found to be 114. The circular dichroic data were expressed in terms of ellipticity per decimole of amino acid residue, $[\theta]$, or in the case of circular dichroism of aromatic residues in terms of difference molar absorptivity, $\Delta\epsilon$, which is more useful when the intensity of dichroic bands with model compounds is compared. The α-helical content was estimated by curve fitting according to Greenfield & Fasman (1969), using the data above 210 nm only. The basic spectra chosen for this analysis were the ten residues α-helix and β-sheet spectra of Chen et al. (1974). For the basic random-coil spectrum, the experimental spectrum in the presence of 6 M guanidine hydrochloride was used.

Fluorescence Measurements. Following excitation of the sample at 274 nm (insuring maximal emission), emission fluoresence spectra were recorded at room temperature on a Baird Atomic spectrofluorimeter FC 100 with a slit of 1 nm. The protein (60 μg/mL) was dialyzed against 100 mM Tris-HCl buffer, pH 7.5, containing 10 μM Ca^{2+} or 100 μM EGTA and 1 mM Mg^{2+}, alternatively. Measurements were performed in a 1 × 1 cm quartz cell. EGTA or EDTA were added to prepare the metal-free form. The kinetics of Trp fluorescence changes were measured at 335 nm.

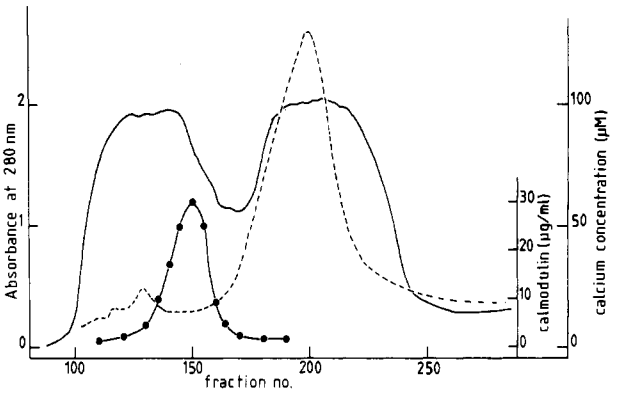

FIGURE 1: Sephadex G-100 chromatography of the albumin fraction of Nereis muscle extract. The column (5 × 80 cm) was equilibrated in 15 mM imidazole–HCl buffer, pH 7.0; fraction volume 5.5 mL. Absorbance (—). Calmodulin was monitored by the phosphodiesterase test (●), calcium by atomic absorption (---).

Results

Isolation of the Calcium-Binding Protein. Sandworm body walls were separated from the digestive tract, rinsed extensively with tap water, and homogenized in the presence of four volumes of 20 mM Tris-HCl, pH 7.5, 20 μM phenylmethanesulfonyl fluoride, 0.1 μg/mL pepstatin, and 50 μM $CaCl_2$. After centrifugation for 15 min at 130000*g*, the supernatant was heated for 3 min at 60 °C; denatured proteins and high molecular weight hemoglobins were removed by centrifugation for 3 h at 300000*g*. The solution was saturated with solid ammonium sulfate and the resulting precipitate dialyzed against the extraction buffer. The protein solution was chromatographed on Sephadex G-100 equilibrated in the same buffer. The elution profile (Figure 1) shows Ca^{2+} mainly associated with a protein of low molecular weight. Its position is distinct from that of endogenous calmodulin, which activates bovine brain phosphodiesterase; the separation is due to the abnormal high Stokes radius of Nereis calmodulin, as was documented in the case of bovine brain calmodulin (Cox et al., 1981). The SCP containing fractions, i.e., the main calcium peak of Figure 1, were pooled and passed through a column of DE-52 cellulose in the presence of 5 μM Ca^{2+}. The resulting profile (Figure 2) shows one protein-bound calcium peak which elutes at a conductivity of 4.5 mΩ^{-1}. Some remaining endogenous calmodulin elutes from the DE-52 cellulose at a conductivity of 11 mΩ^{-1}, far beyond the elution position of Nereis SCP. The pooled calcium-containing fractions show a faint yellow pigment with a maximum at 410 nm. The latter contaminant can easily be separated from SCP by DE-52 chromatography in the presence of 1 mM EDTA, since calcium-free SCP elutes at a conductivity of 8 mΩ^{-1} instead of 4.5 mΩ^{-1} for both the calcium-saturated protein and the pigment. The purity of the final product was checked by disc gel electrophoresis in the presence of $CaCl_2$ or EDTA (Figure 3). About 500 mg of pure SCP can be obtained from 1 kg of body walls.

Molecular Weight and Electrophoretic Properties. The apparent molecular weight was estimated from Sephadex

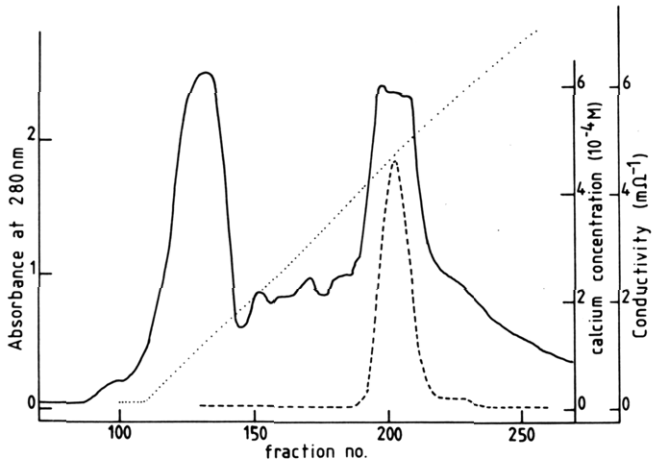

FIGURE 2: DE-52 cellulose chromatography of the calcium-containing peak of Figure 1. The column (2 × 35 cm) was equilibrated in 5 mM Tris-HCl buffer, pH 8.0, and eluted with a linear KCl gradient (0–200 mM). Absorbance (—); calcium concentration (- - -); conductivity (···).

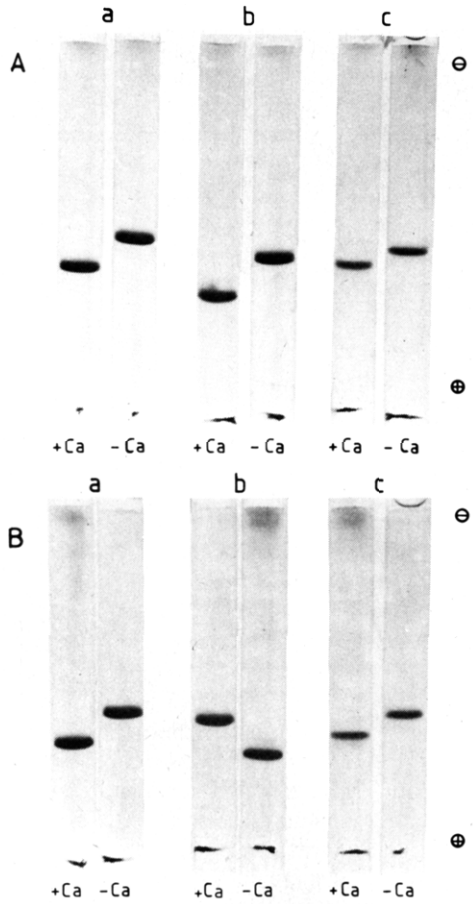

FIGURE 3: Disc gel electrophoresis on 15% gel columns of Nereis SCP (a), calmodulin (b), and troponin C (c). Samples were run in the presence (A) or absence (B) of sodium dodecyl sulfate and in the presence of 1 mM $CaCl_2$ or 1 mM EDTA.

G-100 chromatography with the following protein markers: bovine serum albumin (M_r 67 000), ovalbumin (45 000), chymotrypsinogen (25 000), myoglobin (17 800), and cytochrome *c* (12 400) and equals 20 000. The molecular weight was also estimated by polyacrylamide disc gel electrophoresis in 0.1% sodium dodecyl sulfate according to the method of Weber & Osborn (1969). However, Nereis SCP shows a calcium-dependent change in mobility (Figure 3A). The apparent molecular weight equals 17 000 in the presence of 1

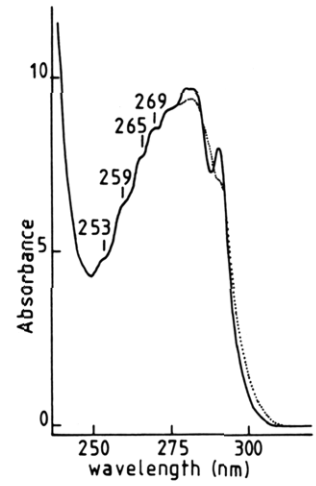

FIGURE 4: Absorption spectrum of a 1% solution of pure Ca-saturated (—) and metal-free (···) Nereis SCP in 50 mM Tris-HCl buffer, pH 8.0.

mM $CaCl_2$ and 19 700 in the presence of 1 mM EDTA. This behavior is reminiscent of bovine brain calmodulin (Burgess et al., 1980) and rabbit skeletal muscle troponin C (Figure 3A), which under our experimental conditions migrate with respective apparent molecular weights of 15 300 and 17 000 in the presence of Ca^{2+} and of 18 600 and 19 000 in the presence of EDTA. The value of 19 700 for the molecular weight of Nereis SCP is in excellent agreement with the molecular weight estimated from gel filtration and with the minimal molecular weight calculated from the amino acid composition.

The electrophoretic mobility of Nereis SCP in the absence of sodium dodecyl sulfate is also calcium dependent (Figure 3B). Calcium-saturated SCP migrates faster toward the cathode than the Ca-free protein, although the latter is expected to be more acidic; this suggests that Ca-saturated SCP has a more compact structure. In this respect, Nereis SCP behaves as troponin C; calmodulin, however, migrates as if the difference in charge is more important than a possible difference in shape.

UV Spectrum and Specific Extinction Coefficient. The UV spectrum of pure Nereis SCP (Figure 4) shows, besides the main peak at 280 nm, a peak at 290 nm which is characteristic of a high content of Trp. The Phe bands at 253, 259, 265, and 269 nm are also visible. The UV spectrum of metal-free SCP shows small differences at the level of the Trp absorption bands at 280 and 290 nm. The specific extinction coefficient $A^{1\%}_{280nm}$ of 9.70, based on dry weight, agrees well with the value calculated from the contribution of three Trp and two Tyr residues per 19 700 mol wt (see below), using the extinction coefficients for the aromatic chromophores of Edelhoch (1967). The protein sample, prepared for determination of the specific extinction coefficient (see Experimental Procedures), contains 150 μM Ca^{2+} per unit of optical density at 280 nm, which corresponds to 3 mol of Ca^{2+} per mol of Nereis SCP.

Amino Acid Composition and Isoelectric Point. The amino acid composition of Nereis SCP (Table I) shows an unusual ratio of Trp over Tyr, which explains the UV absorption peak at 290 nm. Cys is absent, as shown by performic acid oxidation (Hirs, 1967) and by thiol titration with 5,5′-dithiobis(2-nitrobenzoic acid) according to the procedure of Habeeb (1972). By use of the index of composition divergence (Cornish-Bowden, 1977), significant sequence homology can be postulated between the SCP's of Nereis and of Amphioxus as well as between Nereis SCP and the myosin light chains

Table I: Amino Acid Composition of Nereis SCP

amino acid	residue/M_r 20 000[a]	mol integer
aspartic acid	27.7	28
threonine[b]	9.6	10
serine[b]	11.8	12
glutamic acid	15.8	16
proline	5.0	5
glycine	13.5	13–14
alanine	13.3	13
valine	10.5	10–11
methionine	8.8	9
isoleucine	7.9	8
leucine	12.0	12
tyrosine	1.8	2
phenylalanine	14.8	15
histidine	1.0	1
lysine	12.2	12
arginine	4.8	5
tryptophan[c]	2.9	3
cysteine[d]	0	0
total no. of residues		174–176

[a] Mean for samples hydrolyzed for 24, 48, and 72 h unless otherwise stated. [b] Extrapolation to zero time hydrolysis. [c] Determined by the method of Liu & Chang (1971). [d] Determined as cysteic acid according to Hirs (1967).

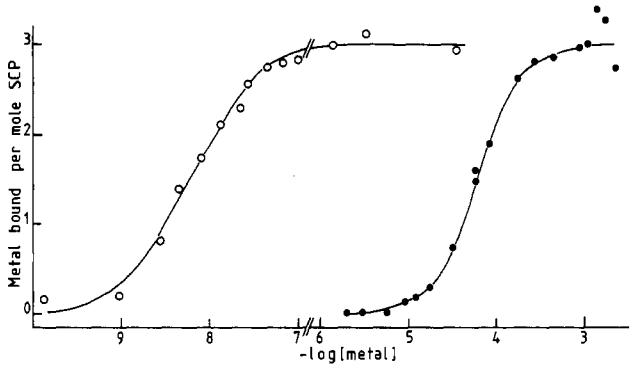

FIGURE 5: Calcium (○) and magnesium (●) binding to Nereis SCP as determined by equilibrium dialysis in EGTA-containing buffers.

from rabbit and scallop (Wnuk et al., 1981). The isoelectric point in the presence as well as in the absence of 6 M urea equals 4.8.

Metal Binding. Figure 5 depicts the saturation curves for Ca^{2+} and Mg^{2+} of Nereis SCP. For Ca^{2+} the three sites have the same intrinsic affinity ($K'_{Ca} = 1.66 \times 10^8\ M^{-1}$) without cooperativity between the sites. Magnesium binding in the absence of Ca^{2+} also occurs to three sites with half-maximal saturation at 60.2 μM free Mg^{2+}. In contrast to Ca^{2+}, Mg^{2+} binding shows positive cooperativity with a Hill coefficient of 1.73. The positive cooperativity is further demonstrated by increasing intrinsic binding constants (Table II).

Since Nereis SCP possesses an equal number of sites for Ca^{2+} and Mg^{2+}, the question arises whether these sites are common for both metals, as was demonstrated for parvalbumin (Moeschler et al., 1980). Hence calcium-binding measurements were carried out in the presence of 0.9 mM Mg^{2+}. The calcium-binding curve is shifted toward higher free Ca^{2+} concentrations by more than 1 pCa unit (Figure 6, as compared to Figure 5). Furthermore, Mg^{2+} induces positive cooperativity in the calcium binding as demonstrated by the Hill coefficient of 2.0 (calculated from Figure 6) and by the affinity relation $K_1' < K_2' < K_3'$ (Table II). The extent of positive cooperativity in Ca^{2+} binding becomes even more important at higher free magnesium concentrations: at 2 mM free Mg^{2+}, the Hill coefficient for Ca^{2+} binding reaches 2.4 (not shown).

Table II: Binding of Ca^{2+} and Mg^{2+} to Nereis SCP by Equilibrium Dialysis[a]

constants	Ca binding, no Mg^{2+}	Mg binding, no Ca^{2+}	Ca binding, 0.9 mM Mg^{2+}
K_1	5.1×10^8	1.3×10^4	8.7×10^6
K_2	1.7×10^8	1.6×10^4	1.3×10^7
K_3	5.5×10^7	2.2×10^4	1.5×10^7
K_1'	1.7×10^8	4.3×10^3	2.9×10^6
K_2'	1.7×10^8	1.6×10^4	1.3×10^7
K_3'	1.7×10^8	6.6×10^4	4.5×10^7

[a] The values of the stoichiometric binding constants K_i and intrinsic binding constants K_i' were determined as described under Experimental Procedures.

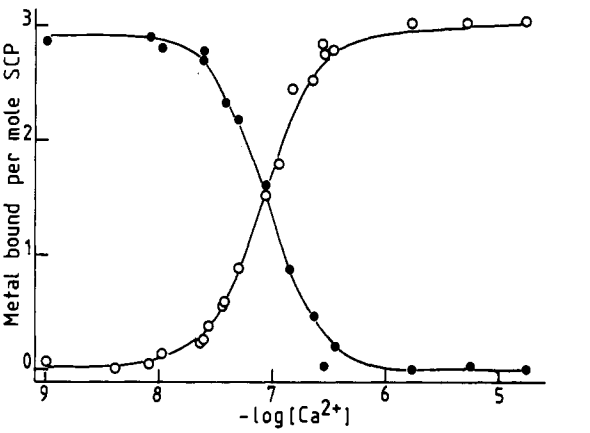

FIGURE 6: Calcium binding (○) and simultaneous magnesium release (●) in the presence of 0.9 mM free Mg^{2+}.

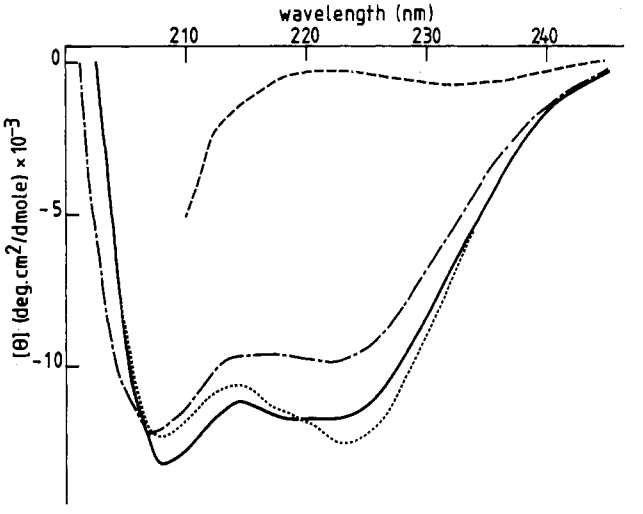

FIGURE 7: Far-UV circular dichroic spectra of Nereis SCP: 0.01 mM $CaCl_2$ (—); 1 mM $MgCl_2$ + 1 mM EGTA (···); 1 mM EDTA (- - -); 6 M guanidine hydrochloride (- -).

It can be seen in Figure 6 that calcium-free SCP binds 3 mol of magnesium per mol. Calcium uptake is accompanied by a release of Mg^{2+} in such a way that SCP saturated with three Ca^{2+} ions no longer binds Mg^{2+}. Furthermore, the Mg^{2+}-release curve is inversely symmetrical with the calcium-binding curve, demonstrating that Mg^{2+} competes with Ca^{2+} for the same metal-binding sites.

Circular Dichroism. The circular dichroic spectra below 250 nm of the Ca^{2+}- and Mg^{2+}-saturated protein as well as of the metal-free SCP, depicted in Figure 7, show a high ellipticity at 222 nm, which is characteristic of all known SCP's (Closset & Gerday, 1975; Cox et al., 1979; Kohler et al., 1978). The ellipticity values for Nereis SCP are somewhat lower than those previously published (Cox et al., 1977), since

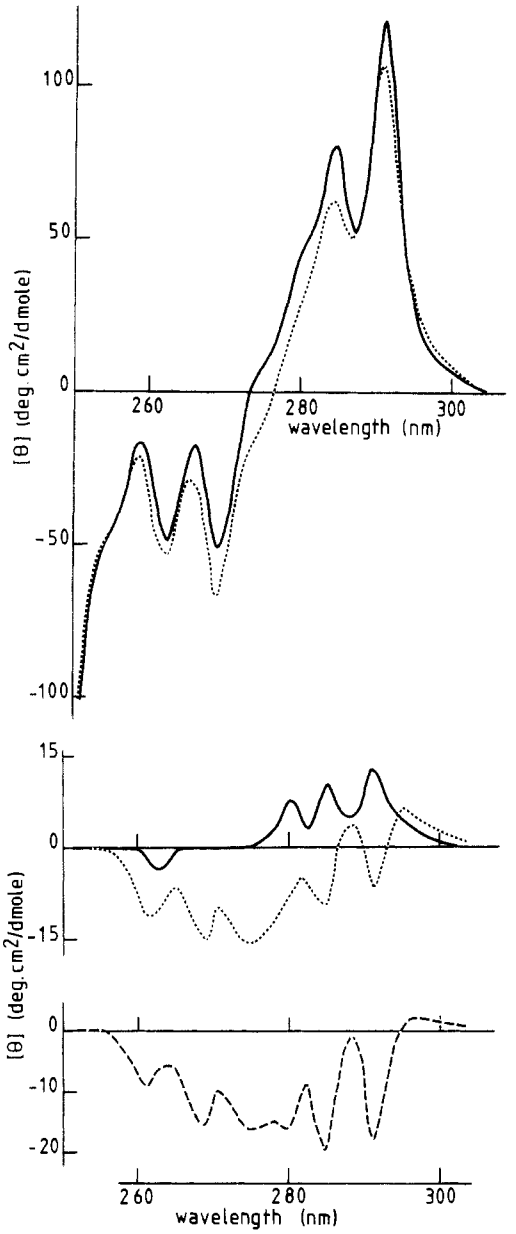

FIGURE 8: Near-UV circular dichroic spectra of Nereis SCP: (A) 0.01 mM $CaCl_2$ (—); 1 mM $MgCl_2$ + 1 mM EGTA (···). (B) Difference spectra: Ca form − metal-free form (—); Mg form − metal-free form (···). (C) Difference spectrum: Mg form − Ca form (- - -).

the latter were calculated on the basis of an overestimated specific extinction coefficient. Calculation of the standard conformations from the CD spectra reveals that Nereis SCP is devoid of β-sheet structure. The α-helical content amounts to 41.5–44.6% for the Ca- and Mg-saturated protein and to 34.0% for the metal-free SCP. These data indicate that only 13 to 18 amino acids in the molecule become incorporated in the α-helical segments upon saturation of the protein by Ca^{2+} or Mg^{2+}. It should be noted that, in contrast with parvalbumin (Cox et al., 1979), circular dichoric spectra of the calcium and magnesium forms of Nereis SCP are not identical, suggesting differences in the secondary structure. Addition of 6 M guanidine hydrochloride destroys nearly all α-helical structure (Figure 7). In contrast, the addition of 0.1% sodium dodecyl sulfate to calcium-saturated or metal-free SCP does not affect the ellipticity at 222 nm or the shape of the spectra; 0.2% $NaDodSO_4$ lowers the ellipticity at 222 nm by 4% for metal-free and by 2% for Ca-saturated SCP (not shown). The resistance of SCP to denaturation by $NaDodSO_4$ explains the

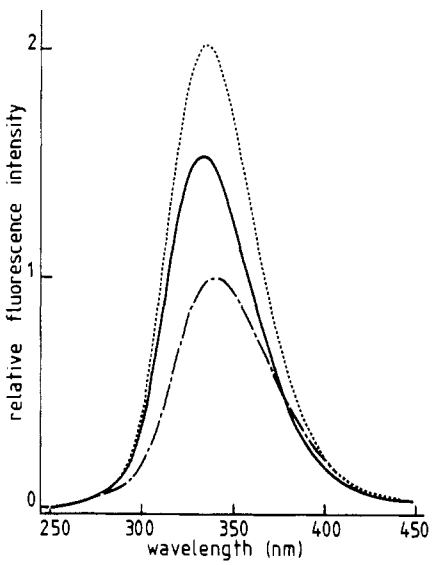

FIGURE 9: Tryptophan fluorescence spectra of Nereis SCP: 0.01 mM $CaCl_2$ (—); 1 mM $MgCl_2$ + 1 mM EGTA (···); 1 mM EDTA (- · -). The fluorescence intensity of the metal-free form at 340 nm is taken as 1.0.

calcium-dependent change in mobility upon disc gel electrophoresis in the presence of the denaturing agent (Figure 3A).

The circular dichroic spectrum between 250 and 300 nm of SCP (Figure 8A) reveals the fine structure of the aromatic side chains. Nereis SCP contains relatively high amounts of Phe (15 residues), more Trp than Tyr (3 and 2 residues respectively), and no cysteine; the interpretation of the spectrum is thus facilitated. The large positive dichroic bands with maxima at 291 and 284 nm as well as the shoulder at 280 nm can be attributed to the $0 + 0\ ^1L_b$ transition of Trp (Strickland, 1974). The high $\Delta\epsilon$ value of 1.38 for the 284.5-nm band suggests that the Trp residues are juxtaposed to aromatic amino acids (Strickland, 1974). Apparently, the two Tyr residues present in SCP do not contribute much to the ellipticity of Ca-saturated SCP and must be considered as mobile on the surface of the protein. The two negative peaks at 262.5 and 269 nm and the shoulder at 255 nm coincide with the vibronic fine structure of the Phe residues.

The difference spectra between the Ca or Mg form and the metal-free protein (Figure 8B) are of small amplitude as compared to those of parvalbumin (Closset & Gerday, 1975) or of crayfish SCP (unpublished results from this laboratory). However, distinct changes occur in the immediate environment of Trp (positive maxima at 291, 285, and 280 nm) when Ca^{2+} occupies the binding sites (Figure 8B), suggesting that the presence of Ca^{2+} reinforces the conjugation of Trp to aromatic residues. No noticeable changes are visible in the Tyr and Phe region between metal-free and calcium-saturated SCP. When magnesium saturates the protein (Figure 8B), Trp becomes less conjugated to aromatic residues as inferred from the decrease of the positive bands at 285 and 291 nm. Furthermore, one of the two Tyr and about two Phe residues are stabilized (Figure 8B). The difference spectrum between the Mg and Ca form (Figure 8C) demonstrates the conformational differences at the level of all three aromatic chromophores. Thus the chromophores Tyr and Phe have apparently a higher degree of order, and Trp a lesser one, when Nereis SCP is in the Mg form rather than in the Ca form.

Trytophan Fluorescence. Upon excitation at 284 nm, Ca- and Mg-saturated SCP display a maximum of fluorescence at 335 nm (Figure 9). In metal-free SCP, the maximum is red-shifted to 340 nm, suggesting that the Trp residues become

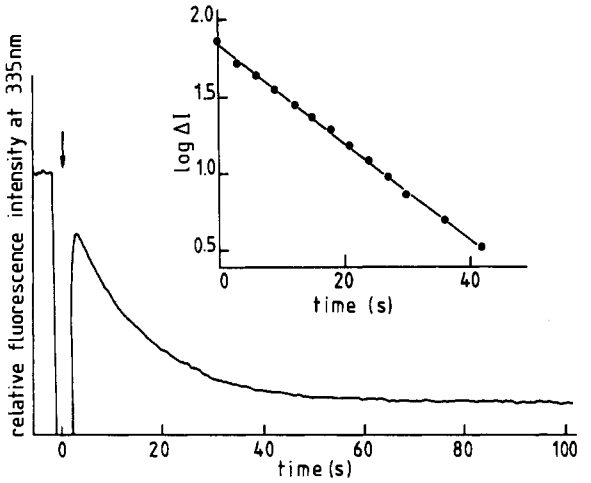

FIGURE 10: Kinetics of Trp fluorescence changes induced by dissociation of Ca^{2+} from Nereis SCP. The relative fluorescence intensity falls to zero when the cell compartment containing the protein sample is open in order to add EDTA (arrow) to a final concentration of 1 mM. The dead-time corresponds to ~ 3 s. Inset: Logarithmic expression of the first-order reaction. ΔI: difference between relative Trp fluorescene intensity at time t and after completion of the conformational change (5 min).

more exposed to a hydrophilic environment (Konev, 1967). If the relative fluorescence intensity at 340 nm of metal-free SCP is taken as 1.0, Ca-saturated SCP shows a fluorescence intensity of 1.60 and Mg-saturated SCP a value of 2.05. Mg-SCP shows a higher fluorescence intensity than Ca-SCP, which points to an energy transfer from the more structured Tyr and Phe to Trp in Mg-SCP.

The importance of the Trp fluorescence intensity changes between the three forms of Nereis SCP led to a study of the kinetics of conformational changes induced by metal dissociation. Perturbations of the Trp fluorescence caused by Ca^{2+} or Mg^{2+} binding to metal-free SCP, or by Mg^{2+} dissociation from the protein, are completed within the time necessary for mixing (~3 s). The conformational changes related to calcium dissociation from SCP are much slower: they follow first-order kinetics with an off-rate constant of about 0.06 s^{-1}, which corresponds to a decay time constant of ~10 s (Figure 10). In contrast, the conformational changes induced by the exchange of Ca^{2+} for Mg^{2+} upon addition of 1 mM EGTA to Ca^{2+}-saturated protein in the presence of 1 mM $MgCl_2$ are too fast to be followed by fluorescence measurements with conventional equipment, indicating a reaction rate with a decay time constant shorter than 0.6 s.

Functional Properties of Nereis SCP. Disc gel electrophoresis according to Head & Perry (1974) of mixtures of SCP and rabbit troponin I does not allow detection of any complex formation between the two proteins. Furthermore, Nereis SCP does not stimulate calmodulin-free phosphodiesterase, even at concentrations 1000-fold higher than those of bovine brain calmodulin that yield half-maximal activation of the enzyme. The possibility that SCP corresponds to one of the myosin light chains is unlikely, since SCP is extracted in the presence of Ca^{2+} whereas the myosin light chains do not dissociate from the heavy chain of myosin unless 10 mM EDTA is present (D'Haese, 1980).

Discussion

A remarkable feature of this new sarcoplasmic calcium-binding protein is the induction of positive cooperativity in Ca binding by Mg^{2+}. Such a phenomenon has not been described for other proteins except for Amphioxus SCP (Kohler et al., 1978). Since the molecular weight of the Mg^{2+} form of Nereis SCP is not known, monomer–polymer equilibria cannot be ruled out as a source of this cooperative behavior. Alternatively, this cooperativity could be explained according to the model of Koshland et al. (1976), which involves the existence of two different conformational states of the protein in the presence of Mg^{2+} and displacement by Ca^{2+} of the equilibrium between the two conformations.

With respect to molecular weight, UV spectrum, amino acid composition, and cooperativity in metal binding, Nereis SCP resembles the SCP of crayfish and Amphioxus more than that of parvalbumin. However, as with parvalbumin, Nereis SCP contains exclusively Ca–Mg mixed sites. This allows two important physiological deductions: (1) if the concentrations of free Ca^{2+} and Mg^{2+} in annelid muscle at rest are similar to those in vertebrates, i.e., about 1 mM for Mg^{2+} (Gupta & Moore, 1980) and 0.05 μM for Ca^{2+} (Hasselbach, 1976), then all the metal-binding sites of Nereis SCP are saturated with Mg^{2+}; (2) Ca–Mg mixed sites exchange their metal at a slow rate, as has already been documented for the Ca–Mg sites of parvalbumin (Potter et al., 1978), troponin C (Johnson et al., 1979), and myosin (Bagshaw & Reed, 1977). The kinetics of conformational changes induced in Nereis SCP upon removal of calcium by EDTA is indeed slow (decay time constant ~10 s). Apparently not only the conformational change but also the dissociation of calcium are slow for the following reasons: (a) the conformational change induced by Mg^{2+} dissociation, which is of distinctly higher amplitude, is fast, as it is completed within the time of mixing; (b) assuming that the association rate of Ca^{2+} to SCP is diffusion controlled with a rate constant of 1×10^8 M^{-1} s^{-1} (Johnson et al., 1978), a decay time constant of 1.5 s can be calculated, using the association constant for Ca^{2+} to SCP; this is of the same order as the conformational changes induced by Ca release. Similar calculations show that in troponin C and parvalbumin the speed of conformational changes is comparable to that of metal dissociation (Johnson et al., 1979; Potter et al., 1978).

For the Ca–Mg mixed sites of parvalbumin and troponin C, it has been proposed that the rate of Ca–Mg exchange is limited by the speed of dissociation of Ca^{2+} (Johnson et al., 1978; Potter et al., 1978; Cox et al., 1979). However, one should be cautious not to mistake the exchange rate of Ca^{2+} by Mg^{2+} for the off-rate of Ca^{2+}. Indeed, a comparison of the decay time constant of Ca dissociation in the absence of Mg^{2+} (1.5 s when calculated from the affinity constant) with that of the conformational changes in the presence of Mg^{2+} (0.6 s) suggests that the presence of Mg^{2+} enhances the dissociation of Ca^{2+} from the Ca-saturated protein. This raises the question as to whether the Ca–Mg exchange on Nereis SCP is sufficiently fast to respond to the speed of contraction and relaxation in muscle.

In the case of parvalbumin, the overall conformation is essentially the same whether the protein is in the Ca or in the Mg form (Cox et al., 1979; Haiech et al., 1979), although small conformational differences may exist in the immediate environment of the metal-binding sites (Moeschler et al., 1980). Hence metal exchange itself, rather than the appearance of clefts on the surface of the protein, is thought to be instrumental in the function of parvalbumin. Three different techniques, circular dichroism in the near-UV and in the far-UV and Try fluorescence, indicate that the Ca and the Mg forms of Nereis SCP have not the same conformation: the Mg form, predominant in muscle at rest, is apparently more structured than the Ca form. This is unusual: in no other calcium-binding protein studied as yet, e.g., in parvalbumin (Cox et al., 1979), crayfish SCP (Wnuk et al., 1981), troponin

C (Nagy & Gergely, 1979), calmodulin (Walshet al., 1979), does the Mg form possess a higher degree of order than the Ca form. This situation may explain why Ca binding to Mg-saturated SCP displays positive cooperativity. The fast structural changes occurring in Nereis SCP upon Ca binding in the presence of physiological levels of Mg may be involved in Ca-activated processes during the contraction–relaxation cycle.

Acknowledgments

This work was carried out in cooperation with Dr. E. H. Fischer, Department of Biochemistry, University of Washington, Seattle. We are grateful to Dr. W. Wnuk for stimulating discussions and to Michelle Comte for skilful technical assistance.

References

Adair, G. S. (1925) *J. Biol. Chem. 63*, 529.
Bagshaw, C. R., & Reed, G. M. (1977) *FEBS Lett. 81*, 386.
Benzonana, G., Capony, J. P., & Pechère, J.-F. (1972) *Biochim. Biophys. Acta 278*, 110.
Blum, H. E., Lehky, P., Kohler, L., Stein, E. A., & Fischer, E. H. (1977) *J. Biol. Chem. 252*, 2834.
Boudreau, R. J., & Drummond, G. I. (1975) *Anal. Biochem. 63*, 388.
Burgess, W. H., Jemolio, D. K., & Kretsinger, R. H. (1980) *Biochim. Biophys. Acta 623*, 257.
Chen, Y.-H., Yang, J. T., & Chan, K. H. (1974) *Biochemistry 13*, 3350.
Closset, J., & Gerday, C. (1975) *Biochim. Biophys. Acta 405*, 228.
Cornish-Bowden, A. (1977) *J. Theor. Biol. 65*, 735.
Cornish-Bowden, A., & Koshland, D. E., Jr. (1975) *J. Mol. Biol. 95*, 201.
Cox, J. A., Wnuk, W., & Stein, E. A. (1976) *Biochemistry 15*, 2613.
Cox, J. A., Wnuk, W., & Stein, E. A. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., & Siegel, F. L., Eds.) p 266, North-Holland, New York.
Cox, J. A., Winge, D., & Stein, E. A. (1979) *Biochimie 61*, 601.
Cox, J. A., Comte, M., & Stein, E. A. (1981) *Biochem. J. 195*, 205.
D'Haese, J. (1980) *FEBS Lett. 121*, 243.
Edelhoch, H. (1967) *Biochemistry 6*, 1948.
Gupta, R. K., & Moore, R. D. (1980) *J. Biol. Chem. 255*, 3987.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457.
Haiech, J., Derancourt, J., Pechère, J.-F., & Demaille, J. G. (1979) *Biochemistry 18*, 2752.
Hasselbach, W. (1976) in *Molecular Basis of Motility* (Heilmeyer, L. M. G., Jr., Rüegg, J. C., & Wieland, Th., Eds.) p 81, Springer-Verlag, Berlin.
Head, J. F., & Perry, S. V. (1974) *Biochem. J. 137*, 145.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59.
Johnson, J. D., Chalrton, S. C., & Potter, J. D. (1979) *J. Biol. Chem. 254*, 3497.
Kendrick-Jones, J., Szentkiralyi., E. M., & Szent-Györgyi, A. G. (1976) *J. Mol. Biol. 104*, 747.
Kohler, L., Cox, J. A., & Stein, E. A. (1978) *Mol. Cell. Biochem. 20*, 85.
Konev, S. V. (1967) in *Fluorescence and Phosphorescence of Proteins and Nucleic Acids* (Udenfriend, S., Ed.) p 61, Plenum Press, New York.
Koshland, D. E., Jr., Nemethy, G., & Filmer, D. (1976) *Biochemistry 5*, 365.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Liu, T.-Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842.
Moeschler, H. J., Schaer, J.-J., & Cox, J. A. (1980) *Eur. J. Biochem. 111*, 73.
Nagy, B., & Gergely, J. (1979) *J. Biol. Chem. 254*, 12732.
Potter, J. C., Johnson, J. D., & Mandel, F. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1608.
Strickland, E. H. (1974) *C.R.C. Rev. Biochem. 2*, 113.
Walsh, M., Stevens, F. C., Orkawa, K., & Kay, C. M. (1979) *Can. J. Biochem. 57*, 267.
Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251*, 4501.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4405.
Wnuk, W., Cox, J. A., Kohler, L. G., & Stein, E. A. (1979) *J. Biol. Chem. 254*, 5284.
Wnuk, W., Cox, J. A., & Stein, E. A. (1981) in *Calcium and Cell Function* (Cheung W. Y., Ed.) Vol. 2, Academic Press, New York (in press).