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Purification and Characterization of a Scallop Sarcoplasmic Calcium-Binding Protein†

John H. Collins,* J. David Johnson, and Andrew G. Szent-Györgyi

ABSTRACT: A sarcoplasmic Ca^{2+}-binding protein (SCBP) from scallop striated muscle has been purified to homogeneity by chromatography on Sephacryl S-200 and diethylaminoethylcellulose. It appeared to be greater than 95% pure on sodium dodecyl sulfate gel electrophoresis, with a mobility corresponding to M_r ~22000. The amino terminus of SCBP appears to be blocked, as judged by automated sequencer analysis. Amino acid analysis indicates that SCBP is similar to other invertebrate sarcoplasmic Ca^{2+}-binding proteins. The amino acid compositions of SCBP, scallop calmodulin, and scallop troponin C are all quite different, showing that these three Ca^{2+}-binding proteins are distinct entities. The Ca^{2+}- and Mg^{2+}-binding properties of SCBP were investigated by measuring the decreases in tryptophan fluorescence upon titration with these cations. There appears to be two classes of Ca^{2+}-binding sites, with different affinities and specifications: one class binds either Ca^{2+} (with $K > 10^8$ M^{-1}) or Mg^{2+} (with $K \sim 4 \times 10^4$ M^{-1}), and the other class specifically binds Ca^{2+} (with $K \sim 3 \times 10^6$ M^{-1}). These values are very similar to those for the Ca^{2+}-Mg^{2+} and the Ca^{2+}-specific sites found in rabbit skeletal muscle troponin [Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628-4633]. Circular dichroic studies show that SCBP contains about 35% α helix in the absence of Ca^{2+} and Mg^{2+}. The helical content can be increased to about 40% by adding Ca^{2+}. Mg^{2+} addition produces only about half of this increase in α helix, consistent with the observation that SCBP contains both Ca^{2+}-Mg^{2+} and Ca^{2+}-specific binding sites. Although the function of SCBP is unknown, the presence of Ca^{2+}-specific sites on this protein suggests that it may play a direct role in the regulation of scallop muscle contraction.

Muscle cells contain a homologous group of Ca^{2+}-binding proteins that includes troponin C, myosin light chains, and parvalbumin. Also related to these are calmodulin (which appears to be present in all cells), brain S-100 protein, and intestinal vitamin D dependent Ca^{2+}-binding proteins [for reviews, see Collins (1976), Barker et al. (1978), Kretsinger et al. (1980), and Wnuk et al. (1982)]. Although crystallizations of several of these proteins have been reported (Kretsinger et al., 1980; Mercola et al., 1975; Strasburg et al., 1980), to date, a parvalbumin from carp is the only member of this family to have its three-dimensional structure determined by X-ray diffraction studies (Kretsinger & Nockolds, 1973). The structure of the carp parvalbumin has formed the basis for many predictions and speculations regarding the structures of the related Ca^{2+}-binding proteins [e.g., see Collins et al. (1973), Weeds & McLachlan (1974), Kretsinger & Barry (1975), Reid & Hodges (1980), and Bagshaw & Kendrick-Jones (1980)].

Parvalbumins appear to be absent from invertebrate sarcoplasm, and instead a new class of muscle calcium-binding proteins has been found in crayfish (Cox et al., 1976; Wnuk et al., 1979, 1981), scallops (Lehman & Szent-Györgyi, 1975), the protochordate *Amphioxus* (Kohler et al., 1978), and

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sandworm (Cox & Stein, 1981; Gerday et al., 1981). The physiological role of these proteins has not yet been established [for a review, see Wnuk et al. (1982)]. In this paper, we describe the purification of the scallop Ca^{2+}-binding protein (SCBP) and some of its chemical and physical properties.

Experimental Procedures

SCBP preparations were obtained as described previously (Lehman & Szent-Györgyi, 1975), by Sephadex G-100 chromatography of the low ionic strength extract of scallop striated muscle. The partially purified material was lyophilized, dissolved to a volume of 20–40 mg/mL in column solvent, and chromatographed on a column of Sephacryl S-200 (see legend to Figure 1 for details). Fractions from this column containing SCBP were pooled and lyophilized. For further purification, this material was dissolved to about 5 mg/mL in starting buffer and chromatographed on a column of diethylaminoethylcellulose (DEAE-cellulose) (for details, see legend to Figure 2). Protein-containing fractions were lyophilized, dissolved in a small volume (less than 10 mL) of 50 mM NH_4HCO_3, pH 8.0 (containing 0.01% sodium azide), desalted on a 2.7 × 55 cm column of Sephadex G-25 run in the same solvent, and lyophilized again. Sodium dodecyl sulfate ($NaDodSO_4$) electrophoresis was done by the method of Laemmli (1970) (for details, see legend to Figure 3). NH_2-terminal sequence analysis was done on a Beckman 890-C sequencer, by using the standard 1.0 M Quadrol–fast protein program supplied by the manufacturer. Amino acid analyses were carried out on a Glenco MM-60 amino acid analyzer, by using standard procedures. Samples for analysis were evacuated and hydrolyzed at 110 °C for 20 h in 6 N HCl containing 0.1% phenol. Determinations of cysteine were done on preparations of SCBP that were S-carboxymethylated with iodoacetic acid prior to hydrolysis (Hirs, 1967).

Fluorescence studies were conducted in a Perkin-Elmer MPF-44A ratio-recording spectrofluorometer operated in the ratio mode. Tryptophan fluorescence was monitored by using the 285, 340 nm excitation, emission wavelength pair. Titrations were conducted in 3.0 mL total volume of 10 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.0, containing 90 mM KCl and 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or 2 mM ethylenediaminetetraacetic acid (EDTA) as previously described (Johnson et al., 1978). The free metal concentrations were calculated by using the computer program of Perrin & Sayce (1967), taking into account the level of contaminating Ca^{2+} ($\sim 10^{-6}$ M, determined by atomic absorption spectroscopy). The association constants of Sillén & Martell (1971) for metal and H^+ binding to EGTA and EDTA were used.

The circular dichroism studies were conducted on a Cary 61 recording spectropolarimeter with 1-cm quartz cuvettes. The percentage of α helix was calculated from the equation % α helix = $[([\theta]_{222} + 3300)/39\,000] \times 100$, where $[\theta]_{222}$ represents the mean residue ellipticity at 222 nm (Lux et al., 1972).

Results

In the preliminary report (Lehman & Szent-Györgyi, 1975), SCBP was purified by Sephadex G-100 chromatography and appeared by $NaDodSO_4$–polyacrylamide gel electrophoresis to be a pure protein of $M_r \sim 22\,000$. However, our subsequent experience with several preparations showed that the Sephadex G-100 fraction was often contaminated, especially with lower molecular weight material. In order to further purify our SCBP, we now routinely subject the Sephadex G-100 fraction to further chromatography on Sephacryl S-200. A typical chromatogram is shown in Figure 1. The SCBP is contained in a single major peak of 280-nm absorbance; it accounts for 65–75% of the protein recovered from the column. Further purification, if necessary, is achieved by chromatography on DEAE-cellulose (see Figure 2) under conditions very similar to those used by Cox et al. (1976) for the purification of crayfish muscle Ca^{2+}-binding protein (CMCP). The purified SCBP was >95% homogeneous, according to $NaDodSO_4$ gel electrophoresis (see Figure 3). Amino-terminal sequence

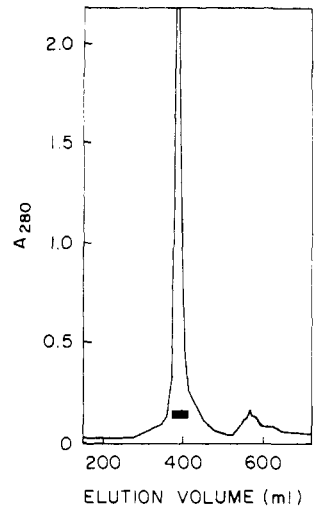

FIGURE 1: Chromatography of ~100 mg of partially purified (see text) SCBP on a 2 × 195 cm column of Sephacryl S-200 (from Pharmacia). The solvent was 50 mM NH_4HCO_3, pH 8.0, containing 0.01% sodium azide, 0.1% 1-butanol, and 1 mM 2-mercaptoethanol. Fractions of 4 mL were collected at a flow rate of 20 mL/h. The horizontal bar indicates the SCBP-containing fractions pooled.

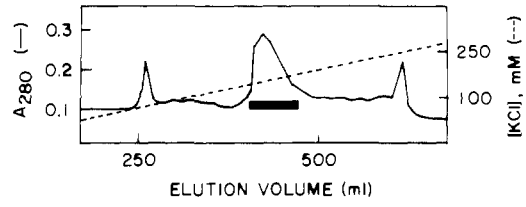

FIGURE 2: Chromatography of SCBP from Figure 1 on a 0.9 × 25 cm column of DEAE-cellulose (Whatman DE-52). The sample was applied in the initial solvent of 15 mM imidazole hydrochloride (pH 7.0) containing 0.01% sodium azide, 1 mM 2-mercaptoethanol, and 2 mM EGTA. Immediately following sample application, a linear KCl gradient was started, by using 500 mL each of the initial solvent and the same solvent containing 500 mM KCl. Fractions of 5 mL were collected at a flow rate of 30 mL/h. The horizontal bar indicates the SCBP-containing fractions pooled.

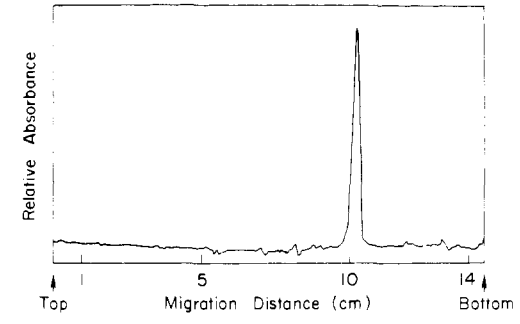

FIGURE 3: Densitometric scan of polyacrylamide gel electrophoresis (Laemmli, 1970) of SCBP from Figure 2. The gel contained 12.5% acrylamide and was loaded with 30 μg of protein. The gel was stained for 5 h with a solution containing 10% acetic acid, 50% methanol, 40% H_2O and 0.05% Coomassie Blue R-250. Destaining of the gel was carried out overnight with a solution of 87.5% H_2O, 7.5% acetic acid, and 5% methanol.

Table I: Amino Acid Compositions of SCBP and Other Calcium-Binding Proteins[a]

amino acid	SCBP[b]	CMCP[c]	*Amphioxus* CBP[d]	scallop calmodulin[e]	scallop troponin C[f]
Asp	31.2 (31)	35.2 (35)	29.7 (30)	22.0 (22)	23.0 (23)
Thr	11.0 (11)	5.3 (5)	8.3 (8)	11.5 (12)	7.1 (7)
Ser	11.0 (11)	7.1 (7)	9.2 (9)	4.8 (5)	8.7 (9)
Glu	22.6 (23)	20.8 (21)	23.8 (24)	25.3 (25)	26.9 (27)
Pro	6.59 (7)	3.1 (3)	6.0 (6)	2.1 (2)	2.2 (2)
Gly	10.2 (10)	11.8 (12)	11.9 (12)	10.6 (11)	11.8 (12)
Ala	15.1 (15)	22.9 (23)	13.2 (13)	9.7 (10)	10.8 (11)
Val	10.7 (11)	13.1 (13)	10.2 (10)	6.6 (7)	10.4 (10)
Met	3.13 (3)	2.0 (2)	5.5 (5)	8.0 (8)	3.9 (4)
Ile	12.1 (12)	10.8 (11)	7.1 (7)	7.4 (7)	7.0 (7)
Leu	11.8 (12)	13.0 (13)	15.1 (15)	8.6 (9)	16.0 (16)
Tyr	4.88 (5)	9.5 (9-10)	7.3 (7)	1.0 (1)	1.6 (2)
Phe	13.4 (13)	13.1 (13)	10.1 (10)	8.3 (8)	7.3 (7)
Lys	23.4 (23)	13.2 (13)	16.0 (16)	7.8 (8)	16.2 (16)
His	4.15 (4)	0.0 (0)	0.9 (1)	0.9 (1)	0.7 (1)
Arg	4.09 (4)	6.9 (7)	6.4 (6)	5.5 (6)	6.1 (6)
Cys	2.08 (2)	2.9 (3)	5.0 (5)		1.1 (1)
Trp	3.1 (3)	2.0 (2)	5.9 (6)	(0)	
total	(200)	(192-193)	(190-191)	(142)	(161)

[a] Expressed as residues per mole; numbers in parentheses are nearest integers. [b] Cys determined as carboxymethylcysteine; Trp determined by the spectrophotometric method of Bredderman (1974). [c] From Cox et al. (1976). [d] From Kohler et al. (1978). [e] From Yazawa et al. (1980); value for Lys includes 1.0 residue of trimethyllysine. [f] From Lehman et al. (1980).

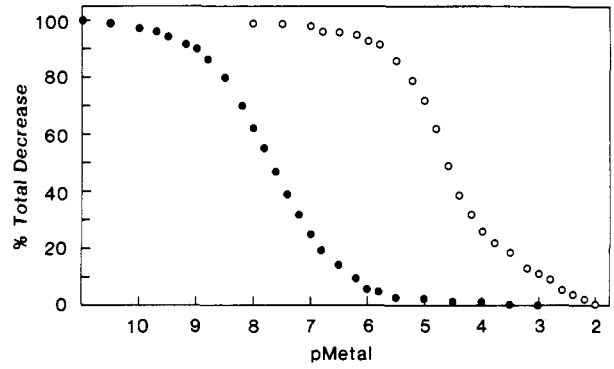

FIGURE 4: Effect of Ca^{2+} (●) and Mg^{2+} (○) on scallop calcium-binding protein tryptophan fluorescence. The percent of the total decrease is shown as a function of p_{metal}. Each solution contained 0.1 mg/mL protein in 3.0 mL total volume of 10 mM Mops, 90 mM KCl, and 2 mM EDTA at pH 7.0. Sufficient microliter amounts of Ca^{2+} or Mg^{2+} were added to achieve the desired p_{metal}. The pH was maintained at 7.0 by addition of microliter amounts of 45% KOH. Excitation and emission wavelengths were 285 and 340 nm, respectively.

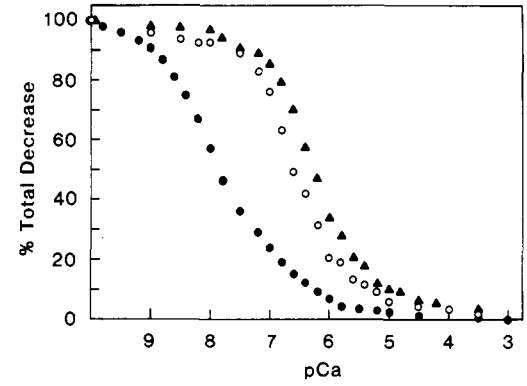

FIGURE 5: Effect of Mg^{2+} on the Ca^{2+}-dependent decrease in scallop calcium-binding protein tryptophan fluorescence. The fluorescence decrease as a function of Ca^{2+} is shown in the presence of no added Mg^{2+} (●), 3 mM Mg^{2+} (○), and 10 mM Mg^{2+} (▲). The total Ca^{2+}-induced decrease was 1.8-fold, 1.4-fold, and 1.4-fold, respectively. Titrations were performed as in Figure 4, except 2 mM EGTA was used instead of EDTA.

analysis of three different preparations yielded negative results, indicating that the NH_2-terminal amino group of SCBP is blocked.

The amino acid composition of SCBP is shown in Table I. The values shown are an average of analyses from four different samples and were normalized to a total of 200 residues. This procedure was used because the resulting calculated M_r (23 103) is close to the value of 22 000 estimated by Na-DodSO$_4$–polyacrylamide gel electrophoresis (Lehman & Szent-Györgyi, 1975) and because it yields close to integral values for those amino acids (methionine, tyrosine, histidine, arginine, cysteine, and tryptophan) which are present in lowest concentration in SCBP. It should be emphasized, however, that these calculated values are primarily based on an assumed M_r of 22 000 and should not be considered as proof of the size of the protein.

Ca^{2+} and Mg^{2+} bind to SCBP to produce a decrease in its tryptophan fluorescence. The Ca^{2+} and Mg^{2+} dependence of this fluorescence descrease is shown in Figure 4. Calcium produces an 80% reduction (initial fluorescence/final fluorescence) in SCBP fluorescence intensity without a significant change in the wavelength of its emission maximum (λ_{max}), which occurs at 328 nm. This fluorescence decrease was half-maximal near pCa 7.7 and occurs over a broad range of [Ca^{2+}] (from pCa 10 to pCa 6.0). Mg^{2+} produces a smaller (50%) fluorescence decrease (which is half-maximal near pMg 4.6), again with no change in λ_{max}.

In an effort to determine if SCBP contains any Ca^{2+}-specific sites, we conducted Ca^{2+} titrations in the presence of increasing [Mg^{2+}], using an EGTA buffering system. In the absence of Mg^{2+}, Ca^{2+} again produces an 80% decrease in SCBP tryptophan fluorescence, which is half-maximal near pCa 7.8. This is in good agreement with our results (Figure 4) in the EDTA buffering system. Addition of 3 or 10 mM Mg^{2+} to SCBP in EGTA produces a 30% decrease in its tryptophan fluorescence. Ca^{2+} titrations of SCBP in EGTA with 0, 3, and 10 mM Mg^{2+} are shown in Figure 5. With 3 and 10 mM Mg^{2+}, Ca^{2+} produces a further 40% reduction in fluorescence, which is half-maximal near pCa 6.5 and 6.3, respectively. These Ca^{2+}-induced fluorescence decreases, which occur even in the presence of high [Mg^{2+}], presumably result from Ca^{2+}

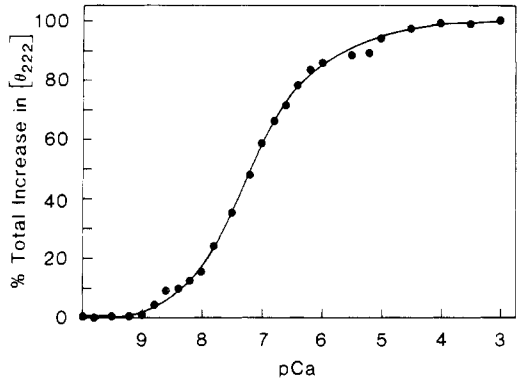

FIGURE 6: Ca^{2+} dependence of scallop Ca^{2+}-binding protein at $[\theta]_{222}$. The percentage of the total change in ellipticity is plotted as a function of free $[Ca^{2+}]$. The total change in ellipticity was from 10554 to 12520 deg cm^2/dmol, corresponding to an increase in α helix from 35% to 40%. Titrations were conducted on 0.10 mg/mL protein in 3 mL of 10 mM Mops, 90 mM KCl, and 2 mM EGTA, pH 7.0, as described previously (Johnson & Potter, 1978).

binding to Ca^{2+}-specific sites on SCBP. These sites would have a $K_{Ca} \sim (2-3) \times 10^6$ M^{-1}. If Mg^{2+} binding to Ca^{2+}–Mg^{2+} sites alone (with a K_{Mg} of 4×10^4 M^{-1}) and a direct competition of Ca^{2+} for Mg^{2+} were responsible for the observed shift in the Ca^{2+} dependence (from pCa 7.8 in the absence of Mg^{2+} to pCa 6.3 in the presence of 10 mM Mg^{2+}), then we would expect the Ca^{2+}-induced fluorescence decrease to be half-maximal at pCa 5.4 and not at pCa 6.3 as we observe. This suggests that SCBP has Ca^{2+}-specific sites.

The far-UV circular dichroic spectrum of SCBP exhibits a minimum near 222 nm which may be used to follow Ca^{2+}-induced changes in the α-helix content of this protein. Figure 6 shows the Ca^{2+} dependence of $[\theta]_{222}$ of SCBP. A 19% increase in $[\theta]_{222}$ (from 10554 to 12520 deg cm^2/dmol), indicating a total increase in α helix from ~35% to ~40%, was produced by Ca^{2+}. Addition of 3 mM Mg^{2+} (in the absence of Ca^{2+}) produced an 8% increase in $[\theta]_{222}$, increasing the α-helical content to ~37%. Subsequent additions of Ca^{2+} (in the presence of 3 mM Mg^{2+}) increased the α-helical content to ~40% with a transition midpoint near pCa 6.2 (data not shown).

Discussion

As shown in Table I, the amino acid composition of SCBP is similar to those of corresponding proteins from other invertebrate species. A comparison of the composition of SCBP with two other scallop Ca^{2+}-binding proteins, troponin C and calmodulin, shows clearly that these three proteins are different entities (see Table I). It is not surprising that no NH_2-terminal amino acid was found in SCBP, in view of the fact that troponin C, most parvalbumins, and many other muscle proteins have acetylated NH_2 termini. Gerday et al. (1981) recently reported that the NH_2 terminus of the corresponding sandworm protein is blocked by an acetyl group. On the other hand, Cox et al. (1976) found earlier that the NH_2 terminus of CMCP is not blocked and that the NH_2-terminal residue is threonine. To test the possibility that SCBP may be homologous to the troponin C family of Ca^{2+}-binding proteins [see, e.g., Wnuk et al. (1982)], we have purified and sequenced a few short cyanogen bromide and tryptic peptides from SCBP (J. H. Collins, unpublished results). The results do not provide any evidence of homology, and this question will probably not be settled until much longer segments of the sequence are determined. It should be mentioned that sequence studies on CMCP are reported to be in progress (Wnuk et al., 1979; Kretsinger et al., 1980) and that crystals of CMCP suitable for X-ray diffraction studies have been prepared (Kretsinger et al., 1980).

Calcium binding to SCBP produces a large decrease in its tryptophan fluorescence. This decrease occurs over a broad pCa range, suggesting that several Ca^{2+}-binding sites with different affinities are involved. Magnesium is able to produce part of the fluorescence decrease produced by Ca^{2+} (with a $K_{Mg} \sim 10^4$ M^{-1}). This suggests that SCBP contains Ca^{2+}–Mg^{2+} sites. Even in the presence of high $[Mg^{2+}]$ (3 and 10 mM), Ca^{2+} produces further decreases in tryptophan fluorescence [with a $K_{Ca} \sim (2-3) \times 10^6$ M^{-1}]. This indicates that SCBP also contains Ca^{2+}-specific sites.

These indirect binding studies (unlike direct $^{45}Ca^{2+}$-binding studies) do not permit us to determine the total Ca^{2+}-binding capacity or the number of Ca^{2+}-specific or Ca^{2+}–Mg^{2+} sites on SCBP. Some conclusions may, however, be obtained with regard to the different types of sites and their relative affinities. The Ca^{2+}-induced fluorescence decrease results in part from Ca^{2+} binding to Ca^{2+}–Mg^{2+} and Ca^{2+}-specific sites. The Ca^{2+}–Mg^{2+} sites have a high Ca^{2+} affinity and a K_{Mg} of ~4 $\times 10^4$ M^{-1}; the Ca^{2+}-specific sites have a Ca^{2+} affinity of (~2–3) $\times 10^6$ M^{-1}. The Ca^{2+} affinity of the Ca^{2+}–Mg^{2+} sites of SCBP cannot be determined from the Ca^{2+}-induced decrease in tryptophan fluorescence, since this decrease is presumably the result of Ca^{2+} binding to both the Ca^{2+}-Mg^{2+} and the Ca^{2+}-specific sites. However, we estimate a high Ca^{2+} affinity for these Ca^{2+}–Mg^{2+} sites ($\geq 1 \times 10^8$ M^{-1}).

In terms of its Ca^{2+}-binding sites, SCBP appears to resemble other muscle Ca^{2+}-binding proteins. Its Ca^{2+}–Mg^{2+} sites are similar to those of troponin (Potter & Gergely, 1975), parvalbumin (Potter et al., 1977), CMCP (Wnuk et al., 1979), and the corresponding proteins from *Amphioxus* (Kohler et al., 1978) and sandworm (Cox & Stein, 1981), all of which have a $K_{Ca} \sim (1-5) \times 10^8$ M^{-1} and a $K_{Mg} \sim 4 \times 10^4$ M^{-1}.

Calcium-specific sites have also been observed in troponin (Potter & Gergely, 1975), calmodulin (Dedman et al., 1977), CMCP (Wnuk et al., 1979), and the *Amphioxus* Ca^{2+}-binding protein, but not in parvalbumin. The affinity of Ca^{2+} for the Ca^{2+}-specific sites of SCBP is close to the $K_{Ca} \sim 5 \times 10^6$ M^{-1} for the Ca^{2+}-specific sites of troponin (Potter & Gergely, 1975). Thus, SCBP is similar to the corresponding proteins from crayfish and *Amphioxus* in that it has both Ca^{2+}-specific and Ca^{2+}–Mg^{2+} sites but apparently differs from the sandworm protein which has been reported to contain only Ca^{2+}–Mg^{2+} sites (Cox & Stein, 1981).

Our circular dichroism studies indicate that SCBP contains a large amount (~35%) of preformed α helix, even in the absence of Ca^{2+}. Calcium binding to the high-affinity sites in SCBP increases the α-helical content to 40%. Magnesium produces less than half of this increase in α helix. This is a further indication that SCBP contains both Ca^{2+}-specific and Ca^{2+}–Mg^{2+} sites. Ca^{2+}- and Mg^{2+}-induced increases in the α-helical content of SCBP are similar to those reported in other muscle Ca^{2+}-binding proteins (Dedman et al., 1977; Johnson & Potter, 1978; Cox & Stein, 1981; Wnuk et al., 1981).

Johnson et al. (1979, 1981) have shown that the Ca^{2+}-specific sites, but not the Ca^{2+}–Mg^{2+} sites, of troponin can exchange Ca^{2+} rapidly enough to be directly involved in the contraction–relaxation cycle of skeletal muscle. It is interesting to speculate that SCBP and other invertebrate Ca^{2+}-binding proteins that contain Ca^{2+}-specific sites may respond to rapid Ca^{2+} transients in the sarcoplasm. Parvalbumins, which contain only Ca^{2+}–Mg^{2+} sites, would not be able to function in this manner [for further discussion, see Wnuk et al. (1982)]. Since the Ca^{2+}-specific sites on troponin are the regulatory

sites of muscle contraction (Potter & Gergely, 1975), it is possible that SCBP plays a direct role in the regulation of the contraction–relaxation cycle in scallop muscle.

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Registry No. Ca, 7440-70-2; Mg, 7439-95-4; L-tryptophan, 73-22-3.

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