Amino Acid Sequence of Two Sarcoplasmic Calcium-Binding Proteins from the Protochordate *Amphioxus*†

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ABSTRACT: Two isotypes of sarcoplasmic Ca^{2+}-binding proteins (SCP) have been purified from muscle of the protochordate *Amphioxus*. Both proteins have three functional Ca^{2+}-binding sites, one of which can also bind Mg^{2+} in a competitive way with Ca^{2+}. Antibodies raised against each of the two proteins react indistinguishably with each of the two antigens. The amino acid sequences of the two SCP's were determined. The proteins possess 183 amino acid residues, and the N-terminus is free Gly. The two types differ in only seven residues from each other; microheterogeneity (one substitution of a Leu for a Met residue) was found in SCP II. The three functional Ca-binding domains were localized on the basis of homology with known Ca-binding domains and internal homology and appear to be domains I, II, and III. Domain II contains an Asp instead of a Gly residue at position 6 of the Ca^{2+}-binding loop, which is exceptional for the whole family of intracellular Ca^{2+}-binding proteins, but does not prevent the domain from binding effectively Ca^{2+} with high affinity. Comparison of the sequence of SCP's from four different invertebrate phyla shows that only domains I and III have always been conserved, with a sequence homology of 25–44% in domain I and 17–30% in domain III.

Low molecular weight sarcoplasmic calcium-binding proteins (SCP's),[1] which are different from calmodulin, troponin C, or parvalbumin, have been isolated from muscle extracts of different invertebrates (Cox et al., 1976; Kohler et al., 1978; Cox & Stein, 1981; Collins et al., 1983). Like parvalbumins, SCP's seem to be more abundant in fast contracting muscles, but no functional relationship could be established from this distribution [for review, see Wnuk et al. (1981)]. Amino acid sequence analyses on the SCP's of crustacea, molluscs, and annelids (Takagi & Konishi, 1984; Takagi et al., 1984; Kobayashi et al., 1984) revealed that they belong to the family of Ca^{2+}-binding proteins that has evolved from a four-domain ancestor. In the light of the sequence homology of the Ca-binding sites of these SCP's with those of proteins of known tridimensional structure such as parvalbumin, vitamin D dependent calcium-binding protein, troponin C, and calmodulin, it can be inferred that the Ca-binding domains of SCP's possess the so-called EF-hand structure (Moews & Kretsinger, 1975). Interestingly, divergent evolution was extensive in the SCP family: only domains I–III retained the ability to bind Ca^{2+} in crustacean SCP's, domains I, III, and IV in sandworm SCP, and only domains I and III in scallop SCP. The overall sequence homology of SCP's from these three different classes is between 14 and 20%, indicating that it is not a conservative protein.

Another invertebrate class rich in SCP (Kohler et al., 1978) is that of the cephalochordates, close to the common ancestors of vertebrates. Previously, we distinguished only one calcium-binding protein in the muscle extract of *Amphioxus*. In the present work, we isolated and characterized two isoforms of SCP and describe their amino acid sequences.

Materials and Methods

Reduction and Alkylation. Pure protein (see Results) was dissolved in 6 M guanidine hydrochloride containing 0.2 M Tris, pH 8.5, and 0.01 M EDTA. It was reduced with 10 mM dithiothreitol and then alkylated with 15 mM iodoacetic acid. Excess reagents were removed by dialysis against water, and the protein (Rcm-SCP) was then lyophilized.

CNBr Treatment on a Large Scale. Twenty milligrams of Rcm-SCP II was dissolved in 2 mL of 70% formic acid, and 200 mg of CNBr was added. Digestion took place in a sealed tube at room temperature for 18 h. After addition of water, the reaction mixture was evaporated and lyophilized. CNBr peptides were separated by gel filtration on Sephadex G-50 superfine (2 × 90 cm), which was equilibrated and eluted with 0.05 M NH_4HCO_3. The peptides were monitored by their absorbance at 225 nm. CNBr peptides were further purified by high-performance liquid chromatography (HPLC) using a column (4 × 250 mm) of Lichrosorb RP-8 (Merck, Darmstadt, Germany). Solvant A was 0.05 M ammonium acetate, pH 6.8, and solvent B was 90% acetonitrile in 0.05 M ammonium acetate. The column was equilibrated with solvent A and appropriate amounts of solvent B. It was eluted by raising the concentration of solvent B linearly.

CNBr Treatment on a Small Scale. To 75 nmol of protein in 200 μL of 70% formic acid was added a small crystal of

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[1] Abbreviations: SCP, sarcoplasmic Ca-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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CNBr. After a 4-h treatment at room temperature, it was lyophilized. CNBr peptides were dissolved in 150 μL of 0.1 M NH_4HCO_3 and submitted to HPLC. For this purpose, an ERC WP300C4 (C4 with pore size 300 Å, Erma Optics, Tokyo) column (6 × 250 mm) was used and the elution carried out as described.

Enzymatic Digestion. Large CNBr peptides were further digested with Tos-Phe-CH_2Cl- (Worthington, Freehold, NJ) treated trypsin, chymotrypsin (Worthington), staphylococcal protease (V8 strain, Miles, Elkhart, IN), or thermolysin (Daiwakasei, Osaka). Peptides (ca. 80 nmol) were digested in 170 μL of 0.1 M NH_4HCO_3, pH 8.5, and 20 μL of enzyme (1 mg/mL) at 37 °C for 4 h. To cleave both glutamyl and aspartyl bonds by staphylococcal protease, 0.1 M phosphate buffer, pH 7.8, was employed instead of NH_4HCO_3, and the reaction was carried out for 18 h at 37 °C. The reaction mixture was subjected to HPLC on the Lichrosorb RP-8 column. Some of the hydrophilic peptides were further purified by using an Asahipak GS-320 column (7.6 × 500 mm) (Asahi Chemicals, Kawasaki) and eluted with 0.05 M ammonium acetate, pH 6.8. More hydrophobic peptides were purified on a small column (4 × 150 mm) of Lichrosorb RP-18, equilibrated with solvent C, 0.1% trifluoroacetic acid (F_3AcOH), and appropriate amounts of solvent D (90% acetonitrile in 0.1% F_3AcOH), and developed by increasing the concentration of solvent D linearly.

Tryptic Digestion of Entire Protein. Rcm-protein (2 mg) was digested in 350 μL of 0.1 M NH_4HCO_3, pH 8.5, and 50 μL of trypsin (1 mg/mL in 1 mM HCl) at 37 °C for 4 h. Half the volume (200 μL) of the reaction mixture was subjected to HPLC, and the remaining was kept at −20 °C until use.

Amino Acid Analysis and Sequence Determination. Protein and peptides (1–3 nmol) were hydrolyzed with 50 μL of F_3AcOH/HCl (1:2 v/v) containing 0.02% phenol in vacuum-sealed tubes at 170 °C for 20 min. The hydrolysate was analyzed with a Hitachi 850-50 amino acid analyzer.

The amino acid sequences of peptides were determined by the manual Edman method according to Tarr (1977) and modified as described previously (Takagi & Konishi, 1984). Pth derivatives were identified by HPLC using a column (4.6 × 250 mm) of ERC-ODS (Erma Optics, Tokyo) at 60 °C with isocratic elution by 30% acetonitrile and 3% methanol in 0.01 M sodium acetate buffer, pH 4.5, at a flow rate of 1.0 mL/min. The absorbance at 269 nm was monitored. The C-terminal amino acids of some of the peptides were determined by carboxypeptidase P (Peptide Institute, Osaka) digestion. Peptide (1–2 nmol) was digested in 100 μL of 0.1 M pyridine–acetate buffer, pH 5.5, and 10 μL of carboxypeptidase P (1 mg/mL) at 37 °C for the appropriate time. The reaction was stopped by lyophilization. After solubilization, the samples were analyzed with the amino acid analyzer.

Nomenclature of Peptides. The primary peptides are numbered from the amino-terminus, and the subpeptides are numbered in order with the parent peptide. In this paper, the prefix CN indicates a cyanogen bromide peptide; T, a tryptic peptide; C, a chymotryptic peptide; V, a staphylococcal protease peptide digested in 0.1 M NH_4HCO_3; VP, a staphylococcal protease peptide digested in 0.1 M phosphate buffer; and Th, a thermolytic peptide.

Immunochemical Techniques. For the production of polyclonal antisera, rabbits were injected subcutaneously with 0.2 mg of either SCP I or SCP II in 0.75 mL of 0.145 M NaCl, emulsified with 0.75 mL of Freund's complete adjuvant. Blood was processed further as described by Harboe and Ingild (1973) and the immunoglobulin fraction passed over a column of Sepharose 4B–conjugated SCP II. Immunodiffusion experiments were performed on 1.5% agarose in 0.3 M potassium phosphate buffer, pH 8.0. Western blotting was performed according to the instruction of Bio-Rad Laboratories, Richmond, CA.

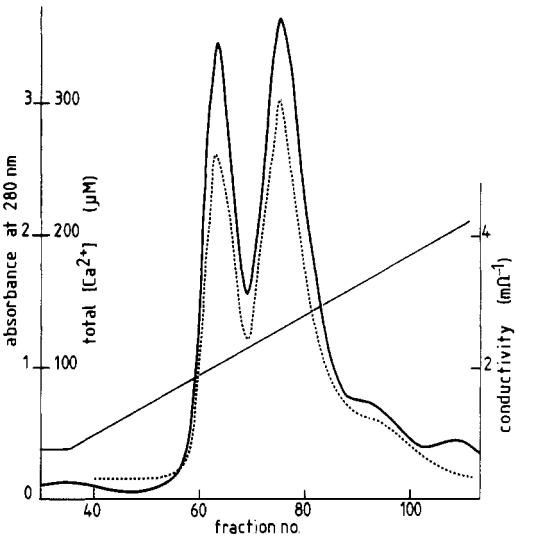

FIGURE 1: DE-52 chromatography of the SCP-containing fractions after Sephadex G-100 chromatography as in Kohler et al. (1978). The column (2 × 35 cm) was equilibrated in 20 mM Tris-HCl, pH 7.5, 0.05% mercaptoethanol, and 15 μM $CaCl_2$ and eluted with a linear KCl gradient (0–200 mM). Absorbance (—); calcium concentration (···).

Miscellaneous. Polyacrylamide disc gel electrophoreses were carried out according to the method of Laemmli (1970). Ca^{2+}- and Mg^{2+}-binding studies were done as previously described (Cox & Stein, 1981). Ca^{2+} and Mg^{2+} concentrations were determined with a Perkin-Elmer 2380 atomic absorption spectrophotometer. The rate of reaction of sulfhydryl groups with DTNB (Calbiochem, San Diego, CA) was determined according to Habeeb (1972).

RESULTS

Isolation of SCP I and SCP II from Amphioxus Muscle Extract. The first steps of the purification, including gel filtration on Sephadex G-100, were carried out as previously described (Kohler et al., 1978) and yielded an electrophoretically pure component under sodium dodecyl sulfate denaturing conditions. However, upon 12.5% gel electrophoresis without sodium dodecyl sulfate in the presence of Ca^{2+}, two major bands with R_f values of 0.33 and 0.38 of about equal intensity are visible, as well as a minor component with an R_f of 0.43. The three components undergo Ca^{2+}-dependent shifts in electrophoretic mobility, suggesting that all three interact with Ca^{2+} ions. The two major components were purified by repeated DEAE-52 cellulose chromatography (Figure 1) to homogeneity as checked by electrophoresis and electrofocusing experiments. Figure 1 also shows that, at 15 μM free Ca^{2+}, both display a mean value of 71 μM Ca^{2+} bound per unit of optical density at 280 nm.

General Characteristics of the Isoforms of SCP. The UV spectra of SCP I and SCP II were similar to the ones published previously (Kohler et al., 1978), but with a better A_{280} to A_{250} ratio (3.1 as compared to 2.5 previously). The specific extinction coefficient $A^{1\%}_{280nm}$ on the basis of dry weight, which was determined as previously described for other SCP's (Cox et al., 1976; Cox & Stein, 1981), amounted to 18.4 for SCP II. Independent determinations of the extinction coefficients

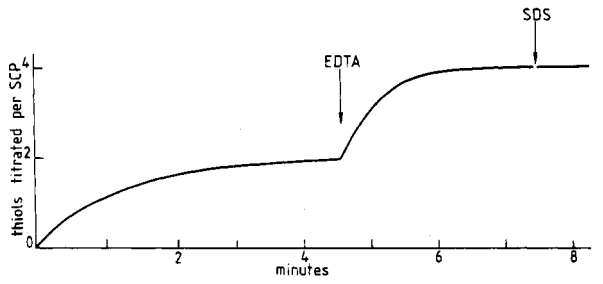

FIGURE 2: Reactivity of thiols in SCP I toward DTNB in 30 mM Tris–glycine buffer, pH 8.0. EDTA to a final concentration of 2 mM was added as indicated.

on the basis of amino acid analyses revealed values, $A^{1\%}_{280nm}$, of 17.6 for SCP I and 18.4 for SCP II. The Lowry method, using bovine serum albumin as a standard, showed a relative color yield of 1.2 for SCP II. The content of bound Ca^{2+} after extensive dialysis against bidistilled water was 66–68 μM per unit of optical density. With a molecular weight of 21 000 (see below), this corresponds to 2.5 mol of Ca^{2+}/mol of SCP I and II. Equilibrium dialysis experiments in 60 mM TES, pH 7.0, 50 mM NaCl, 0.05 mM DTT, and 0.2–2 mM EGTA with various concentrations of $CaCl_2$ yielded for both SCP's simple binding isotherms without any cooperativity with a plateau value at 2.94 mol of Ca^{2+}/mol of SCP and a K_{diss} of $10^{-7.78}$ M. The latter value is in almost perfect agreement with the dissociation constant determined on the mixture of SCP's (Kohler et al., 1978). Since the stoichiometry of Ca binding to SCP is different from the one published previously (Kohler et al., 1978), we also redetermined the stoichiometry of Mg binding to SCP. Equilibrium dialysis experiments in 50 mM PIPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, and 200 mM $MgCl_2$ yielded a value of 0.95 mol of Mg^{2+} bound per mole of SCP II. From this and from the data of Kohler et al. (1978), it can safely be concluded that SCP of *Amphioxus* displays three functional high-affinity Ca-binding sites, two of which are specific for Ca^{2+} and one which can also accommodate Mg^{2+}, but with an affinity 1000-fold lower.

Both SCP's contain four titrable thiols: in the presence of Ca^{2+}, only two react rapidly with DTNB (half-life ca. 50 s); the remaining two thiols react only after addition of EDTA (Figure 2).

The antibodies raised against *Amphioxus* SCP I and II do not cross-react with SCP's from crayfish or sandworm in immunodiffusion and Western blotting experiments, thus complementing the observation of Kohler et al. (1978) that antibodies against the latter SCP's do not cross-react with *Amphioxus* SCP's. The antibodies against SCP I cross-react with SCP II and vice versa in an indistinguishable way in diffusion experiments (unpublished observations). Furthermore, all the antibodies againt SCP I were retained on immobilized SCP II. Lastly, immunoblotting experiments after electrophoresis in Ca-containing gels in the absence of sodium dodecyl sulfate showed that both antibodies react with both proteins with equal intensity (not shown). Together these data indicate that SCP I and SCP II contain the same immunogenic determinants.

Sequence Determination of SCP II. The summary of data used for sequence determination of SCP II is shown in Figure 3. The details are described in the supplementary material (see paragraph at end of paper regarding supplementary material). *Amphioxus* SCP II was treated with CNBr in a large-scale experiment (20 mg), and the resulting fragments were separated on a column of Sephadex G-50. Five fractions (I–V) were pooled separately. Fraction V contained one tetrapeptide (Gly-Gln-Lys-Pro), and since it has no homoserine, it was placed at the C-terminus of SCP II. Fraction IV was subjected to HPLC and separated into three peptides (CN2, CN3, and CN5). The elution profile of Sephadex G-50, HPLC patterns of each fraction, and the amino acid compositions of CNBr peptides and their subpeptides are summarized in the supplementary material. CN2 was sequenced from the N-terminus to the C-terminus and the sequence at the C-terminal portion confirmed by using its thermolytic peptide. The sequence of CN3 was also determined from the N-terminus to the C-terminus, and the C-terminal part was confirmed by carboxypeptidase P digestion. The N-terminal 16 residues of CN5 were sequenced, and the sequence was established by using its tryptic peptides.

Fraction III was purified by HPLC and yielded three main peaks. The last two peaks had the same amino acid composition. Their separation may be due to the presence of homoserine or homoserine lactone. The first peak corresponds to peptide CN2–3, which was not cleaved between the Met–Met–Thr bond. The sequence of CN2–3 was confirmed by using its tryptic peptides (data not shown). The Met–Thr bond is known to be resistant to CNBr digestion. The above results were revealed after the overlapping of CNBr peptides was established (see below). The amino acid sequence of CN4 was established by using its tryptic and staphylococcal protease peptides as shown in Figure 3.

In fraction II, no clearly identified peptide was obtained: most of the peptides were the same as those derived from fraction I, and a small amount originated from fraction III.

Fraction I was purified by HPLC, and mainly two peptides were obtained (CN6 and CN6′). CN6′ has no homoserine and ended with Tyr-Arg, which was identified by carboxypeptidase P digestion. Analysis of its tryptic peptides revealed that CN6′ is identical with CN6 minus the C-terminal 15 residues, i.e., cleaved between Arg (165) and Leu (166) in Figure 3. The sequence of CN6 was determined by using its tryptic and staphylococcal protease peptides as shown in Figure 3. By these procedures, mainly five CNBr peptides (CN2–CN6) were isolated and their sequences determined.

To align the CNBr peptides, Rcm-SCP II was digested with trypsin, and the tryptic peptides were subjected to HPLC. The elution profile is shown in Figure 4A. Some of the peptides were eluted as a mixture and further purified by HPLC under the different conditions described under Materials and Methods. Among them, we found four peptides (T1, T2, T3, and T3′) which were not expected from the study of the CNBr peptides. T1 and T2 were sequenced from the N-terminus to the C-terminus. The sequences of 19 residues of T3 and T3′, determined from the N-termini, were identical except that the eighth residue was Met in T3 and Leu in T3′. Starting from the tenth residue of T3 or T3′, the amino acid sequence was the same as the N-terminal sequence of CN2. Although the N-termini of SCP's of other species so far sequenced were blocked by an acetyl group, in the case of *Amphioxus* SCP II the N-terminus of none of the CNBr peptides was blocked, and all newly found tryptic peptides could be sequenced from the N-terminus. Thus, we tried to determine the N-terminal sequences of intact *Amphioxus* SCP's: both SCP I and SCP II started with Gly-Leu-Asn-Asp-Phe. Therefore, the N-terminus of *Amphioxus* SCP was not blocked. On the basis of sequence identity, T1 had to be placed at the N-terminus of SCP II.

Since we found no sequence of CNBr peptides starting with Gly-Leu-Asn-Asp-Phe, we reexamined very carefully all fractions of the Sephadex G-50 column (see above) by HPLC using stronger elution conditions, as the missing peptide(s) was

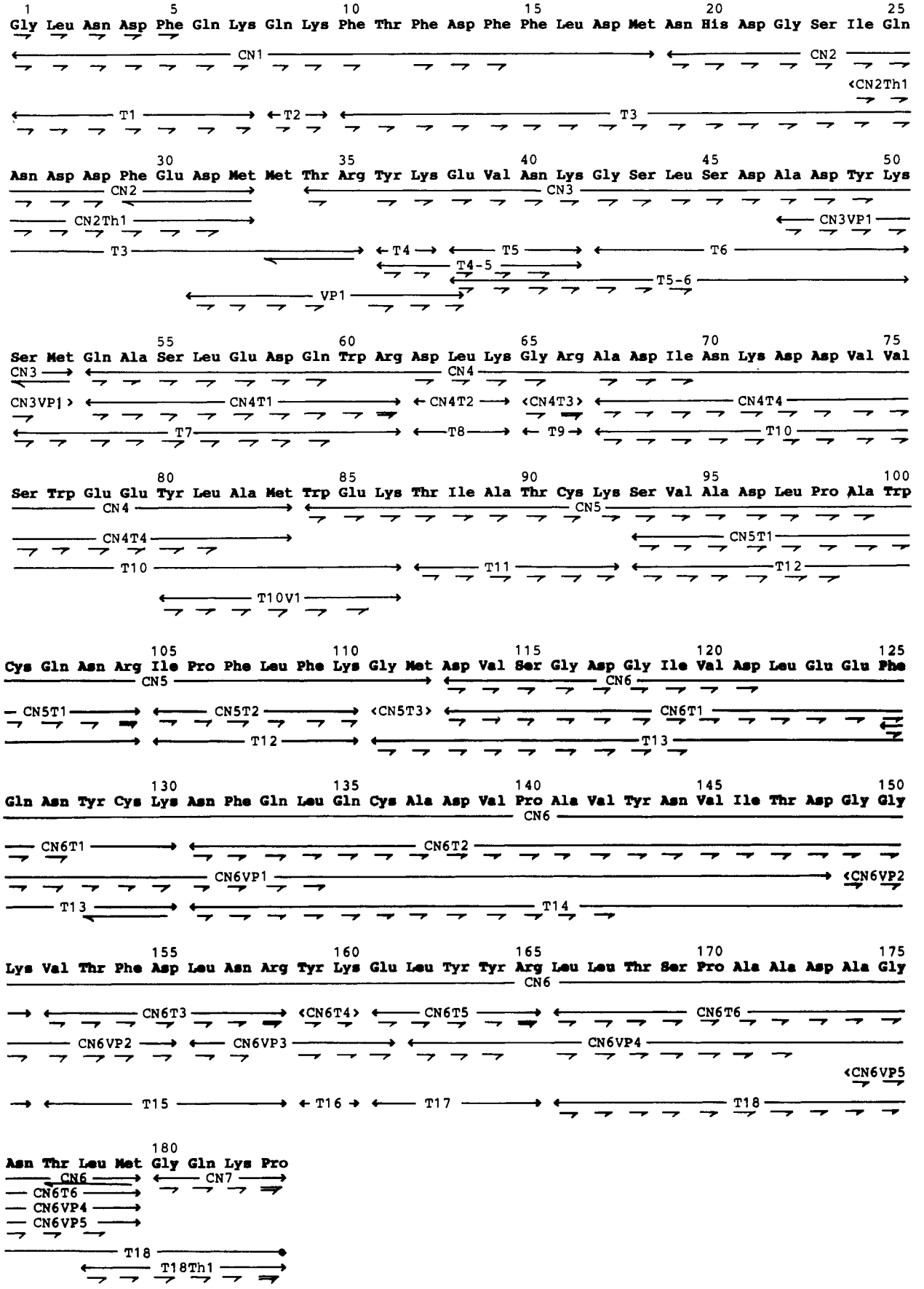

FIGURE 3: Methodology for sequence determination of *Amphioxus* SCP II. (—›) Manual Edman method; (⟹) from amino acid analysis after completion of the sequence; (—|) carboxypeptidase P digestion. CN, cyanogen bromide; T, tryptic; Th, thermolytic; V, staphylococcal protease; VP, staphylococcal protease peptides digested in the presence of phosphate buffer. The typical yields of each of the peptides as well as their actual amino acid compositions are given in Tables 1S and 2S of the supplementary material.

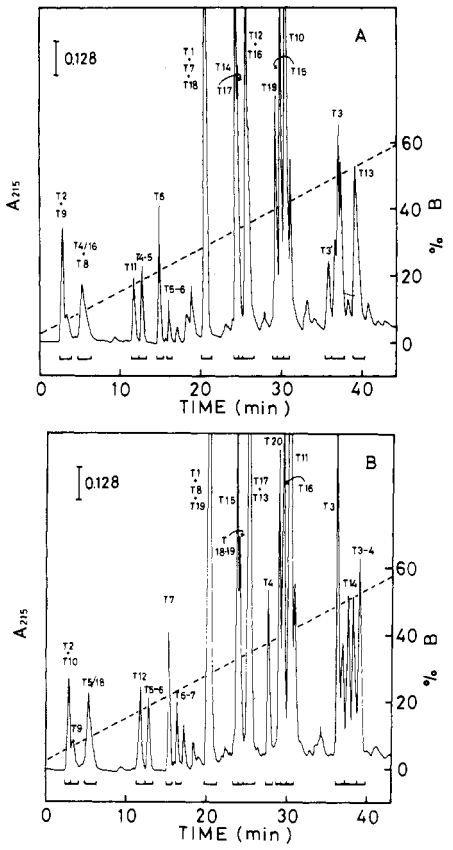

FIGURE 4: Separation of tryptic peptides by HPLC. A column (4 × 250 cm, Lichrosorb RP-8) was equilibrated with 98% of solvent A (0.05 M ammonium formate, pH 6.8) and 2% of solvent B (90% acetonitrile in 0.05 M ammonium formate) and eluted by increasing the concentration of solvent B linearly as indicated by the dashed line. Flow rate: 1 mL/min. Absorbance was monitored at 215 nm. Sample: 50 nmol. (A) SCP II; (B) SCP I.

(were) expected to be very hydrophobic. However, no other peptide was found. Therefore, SCP II (75 nmol) was treated with CNBr, and the CNBr peptides were directly applied to HPLC. The elution profile is shown in the supplementary material. The N-terminal sequence of the last peak indicated that CN1 corresponds to Gly-Leu-Asn-Asp-Phe-Gln-Lys-Gln-Lys-Phe-(Thr)-Phe-Asp-Phe, i.e., the same as the N-terminal sequence of intact SCP, and corresponds also to the overlapping sequence of T1, T2, and T3. The reason for CN1 not being eluted from the Sephadex column is not clear but could be due to its very hydrophobic character. All tryptic peptides from the N- to the C-terminus were aligned with the CNBr peptides, as shown in Figure 3. The amino acid composition of tryptic peptides used for overlapping of CNBr peptides are shown in the supplementary material.

When staphylococcal protease digestion of entire SCP II was used, a peptide (VP1) overlapping CN2 and CN3 was found to contain one extra methionine between CN2 and CN3; curiously, we never found free homoserine in the total CNBr digest.

Sequence Determination of SCP I. As we knew from the sequence determination of SCP II that using large amounts of sample did not improve the results, 2 mg (ca. 100 nmol) of Rcm-SCP I was digested with trypsin, and half of it was subjected to HPLC. As shown in Figure 4B, the elution pattern of SCP I was the same as that of SCP II, except above a 35-min elution time; the amino acid compositions and sequences of peptides eluted within 35 min were exactly the same as those of SCP II, except that SCP I had one extra peptide T4. Further separation profiles of mixtures of peptides and

Table I: Differences in Amino Acid Sequences of SCP I and SCP II

position from N-terminus	SCP I	SCP II
18	Tyr	Met[a]
20	Lys	His
26	Gln	Asn
27	Glu	Asp
31	Glu	Asp
33	Ile	Met
34	Lys	Thr

[a] In SCP II, some of the Met at position 18 is replaced by Leu, indicating the presence of still another genetically different isoform of SCP II.

amino acid compositions are shown in the supplementary material. The sequences of some large peptides were determined by using their staphylococcal or chymotryptic peptides. The sequences of T1 (N-terminus), T2, and T5–T20 (C-terminus) were identical with the corresponding peptides of SCP II; thus, they were placed according to the sequence of the latter. T3, T4, and the peptide (T3–4), which was not cleaved between T3 and T4, were different from those of SCP II. The N-terminal sequence of T3 was identical with that of T3 in SCP II; at the C-terminus, Met and His were replaced by Tyr and Lys, respectively. The C-terminal part of T4 was different from the corresponding sequence of T2 in SCP II. To confirm and overlap the sequences of tryptic peptides, SCP I was treated with CNBr, and the CNBr peptides were separated by HPLC. As expected from the sequence, six CNBr peptides were eluted, although CN4 and CN5 were obtained as a mixture. CN1, CN2, and CN6 were sequenced from the N-termini, and CN3 and a mixture of CN4 and CN5 were digested with staphylococcal protease. From the amino acid compositions and partial sequences of tryptic peptides, the sequences of CN3, CN4, and CN5 were already expected to be identical with those of SCP II, and this was verified by establishing the sequences of their staphylococcal protease peptides. The summary of sequence determination of SCP I is shown in Figure 5.

Discussion

The two isoforms of *Amphioxus* SCP are composed of 183 amino acid residues with relative molecular masses of 21 119 daltons for SCP I and 21 044 daltons for SCP II. In contrast to SCP's of the three other phyla, whose N-terminus is blocked by an acetyl group, the two isoforms of *Amphioxus* have a free N-terminal Gly. Table I summarizes the seven differences in the sequence of SCP I and SCP II. At neutral pH, SCP I possesses between one and two positive charges more than SCP II. The seven differences are restricted to a short region in the N-terminal part of the protein. Although one Ca^{2+}-binding domain is involved, the amino acid substitutions do not affect the metal-binding properties. Antibodies raised against each of the two isoforms react indistinguishably with the two antigens, indicating that the substitutions occur in a region that is not immunogenic or that is buried inside the protein.

The two *Amphioxus* SCP's have three Ca-binding sites of high affinity: two calcium-specific ones and one Ca–Mg site. From sequence data alone, it is not yet possible to assign the domains that differentially bind the two ions, but we can reasonably exclue domain IV as a functional Ca-binding site, on the basis of the considerations developed below. According to Gariepy and Hodges (1983) and Haiech and Sallantin (1985), the 12-residue-long central loop in each Ca-binding site displays strong constraints in positions 1, 3, 5, 6, 8, and 12, much lower constraints in positions 4, 9, 10, and 11, and no constraints in 2 and 7. Figure 6 shows that, in the first

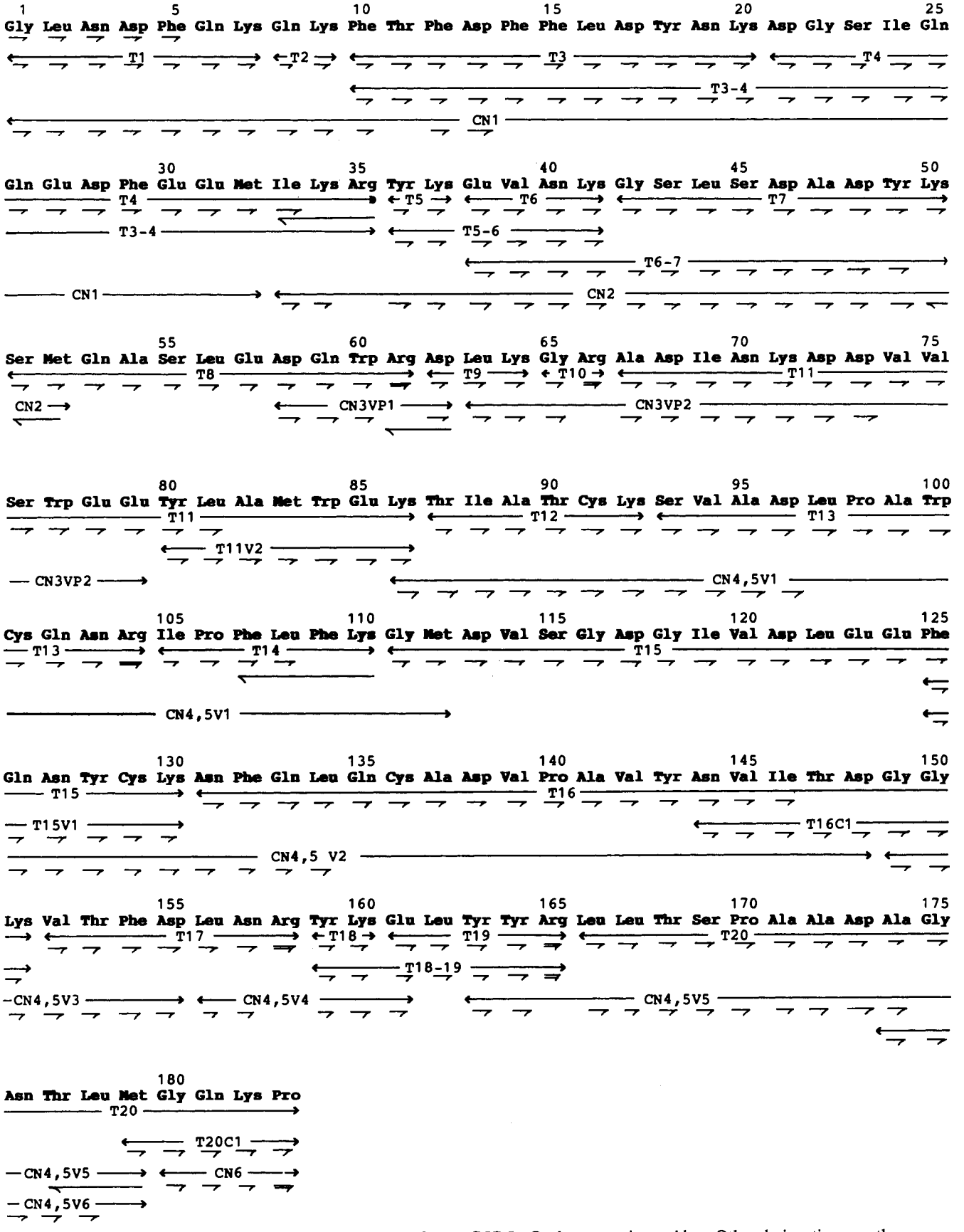

FIGURE 5: Methodology for sequence determination of *Amphioxus* SCP I. C, chymotryptic peptides. Other designations are the same as in the legend for Figure 3. The typical yields of each of the peptides as well as their actual amino acid compositions are given in Tables 3S and 4S of the supplementary material.

three domains of *Amphioxus*, one finds Asp in position 1, Asn in position 3 (except for domain III which displays a Ser), Asp in position 5, Gly in position 6 (except for domain II; see below), a highly hydrophobic residue (Ile or Val) in position 8, Glu in position 11, and an Asp or Glu in position 12. A particularity is the Asp residue in domain II, position 6: in

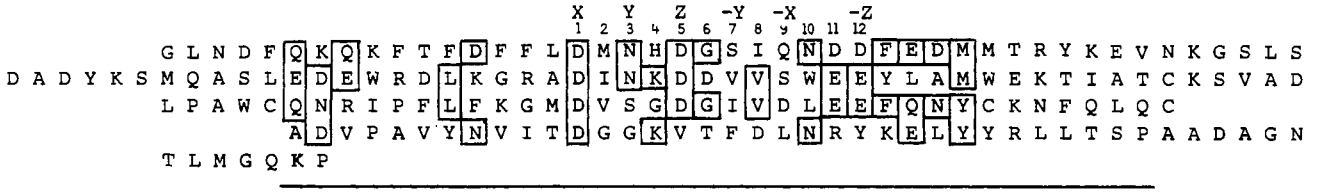

FIGURE 6: Sequence alignment of SCP II according to the Ca^{2+}-binding domains (bar) predicted by the Kretsinger model (1975). Homologous residues are boxed. The positions of the Ca-coordinating loops are presented as in Gariepy and Hodges (1983).

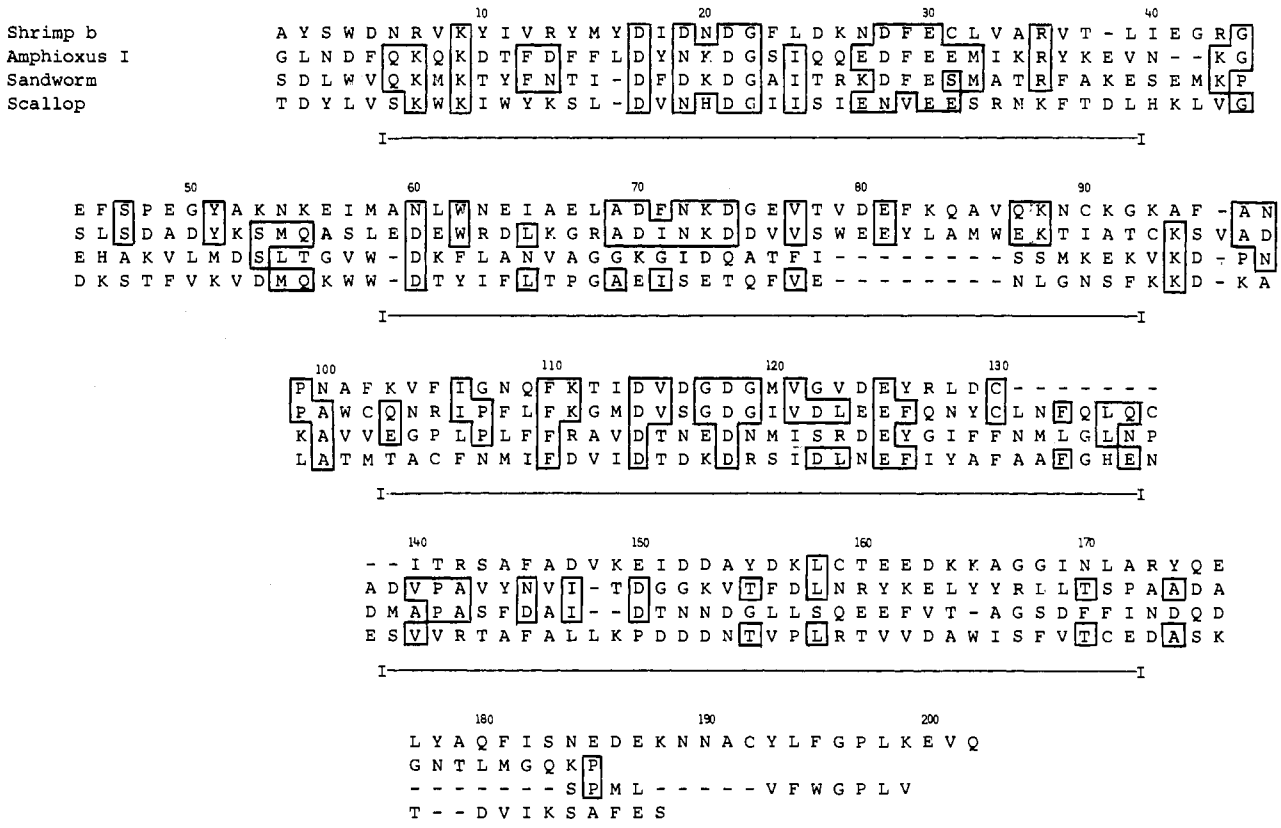

FIGURE 7: Comparison of amino acid sequences of SCP's from four invertebrate phyla. Deletions (–) are introduced to obtain maximal homology. Residues homologous with those of *Amphioxus* SCP I are boxed.

more than 80 sequences of Ca-binding loops, position 6 is occupied by Gly, except in two parvalbumins of lower vertebrates (coelacanth and thorn ray) and in three SCP's (sandworm, scallop, and *Amphioxus*). Our Ca-binding data, presented here and previously (Cox & Stein, 1981), clearly indicate that the replacement of Gly in position 6 by Asp or Asn does not alter the Ca-binding capacity. The central loop of domain IV has been the subject of quite drastic nonconservative amino acid replacements in positions 3, 5, 6, 8, 11, and 12 and must have lost its Ca-binding properties. Thus, the first three EF-hand domains retained functional characteristics. The three functional domains of *Amphioxus* SCP have retained 24% of sequence homology with one another without significant distinction between any of the three pairs. The fourth domain shows only 8–12% of sequence homology with any of the other domains.

In Figure 7, the sequences of SCP's of representative members of four invertebrate phyla have been compared. Although the overall sequence homology between SCP's is not as pronounced as in the case of calmodulin and troponin C, and there is no immunological cross-reactivity between SCP's of different phyla, it is striking that some EF-hand domains (including the α-helices) remained conservative: in domain I, the homology between *Amphioxus* SCP and shrimp, sandworm, or scallop SCP is 25, 44, and 31%, respectively, and in domain III these values are 30, 17, and 22%. The other two domains and especially the fourth have not been conserved. Crustacean SCP's occur as homo- or heterodimers (Cox et al., 1976; Wnuk & Jauregui-Adell, 1983), a particularity that is not shared by SCP's of other phyla. Figure 7 shows that the C-terminus of crayfish SCP is about 15 residues longer than any other SCP; this region is very well conserved in three isoforms of crustacean SCP's and may well be involved in the dimerization properties of this protein. In addition to the four EF-hand domains and the extra C-terminal stretch in crustacean SCP, the SCP's of the four phyla contain a 19-residue-long stretch between domains I and II, which might possess some common functional properties, e.g., be a catalytic center. However, the mean homology between each pair of SCP in this domain is about 10%, making it unlikely that it serves a structural or functional role common to the different SCP's.

As a whole, sequence studies on different SCP's did indicate that they still contain the four EF-hand domains, some still functional and some degenerated, tied together by short (or longer) stretches, which themselves are not sufficiently conserved to point to some biological function. The fast rate of evolution of SCP's away from the ancestral four-domain polypeptide may be explained by the fact that their function is weakly defined and likely to be not very specific.

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SUPPLEMENTARY MATERIAL AVAILABLE

Four tables containing amino acid compositions of CNBr peptides and their subpeptides of *Amphioxus* SCP I and SCP II and seven figures showing the elution profile of CNBr peptides of SCP II and HPLC separation patterns for CNBr peptides in fractions I, III, and IV, of SCP I and SCP II, and of mixtures of tryptic peptides (20 pages). Ordering information is given on any current masthead page.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4; SCPI, 101858-44-0; SCPII, 101858-45-1.

REFERENCES

Collins, J. H., Johnson, J. D., & Szent-Györgyi, A. G. (1983) *Biochemistry 22*, 341–345.
Cox, J. A., & Stein, E. A. (1981) *Biochemistry 20*, 5430–5436.
Cox, J. A., Wnuk, W., & Stein, E. A. (1976) *Biochemistry 15*, 2613–2618.
Gariepy, J., & Hodges, R. S. (1983) *FEBS Lett. 160*, 1–6.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457–464.
Haiech, J., & Sallantin, J. (1985) *Biochimie 67*, 555–560.
Harboe, N., & Ingild, A. (1973) in *A Manual of Quantitative Immunoelectrophoresis* (Axelsen, N. H., Kroll, J., & Weeks, B., Eds.) p 161, Universitetsforlaget, Oslo.
Kobayashi, T., Takasaki, Y., Takagi, T., & Konishi, K. (1984) *Eur. J. Biochem. 144*, 401–408.
Kohler, L., Cox, J. A., & Stein, E. A. (1978) *Mol. Cell. Biochem. 20*, 85–93.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Moews, P. C., & Kretsinger, R. H. (1975) *J. Mol. Biol. 91*, 201–228.
Takagi, T., & Konishi, K. (1984) *J. Biochem. (Tokyo) 95*, 1603–1615.
Takagi, T., Kobayashi, T., & Konishi, K. (1984) *Biochim. Biophys. Acta 787*, 252–257.
Tarr, G. E. (1977) *Methods Enzymol. 47*, 335–357.
Wnuk, W., & Jauregui-Adell, J. (1983) *Eur. J. Biochem. 131*, 177–182.
Wnuk, W., Cox, J. A., & Stein, E. A. (1982) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. II, pp 243–278, Academic, New York.

Anomalous Asymmetric Kinetics of Human Red Cell Hexose Transfer: Role of Cytosolic Adenosine 5′-Triphosphate†

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ABSTRACT: Cytosolic adenosine 5′-triphosphate (ATP) modifies the properties of human red cell sugar transport. This interaction has been examined by analysis of substrate-induced sugar transporter intrinsic fluorescence quenching and by determination of Michaelis and velocity constants for D-glucose transport in red cell ghosts and inside-out vesicles lacking and containing ATP. When excited at 295 nm, human erythrocyte ghosts stripped of peripheral proteins display an emission spectrum characterized by a scattering peak and a single emission peak centered at about 333 nm. Addition of sugar transport substrate or cytochalasin B and phloretin (sugar transport inhibitors) reduces emission peak height by 10% and 5%, respectively. Cytochalasin B induced quenching is a simple saturable phenomenon with an apparent K_d (app K_d) of 60 nM and a capacity of 1.4 nmol of sites/mg of membrane protein. Quenching by D-glucose (and other transported sugars) is characterized by at least two (high and low) app K_d parameters. Inhibitor studies indicate that these sites correspond to sugar efflux and influx sites, respectively, and that both sites can exist simultaneously. ATP induces quenching of stripped ghost fluorescence with half-maximal effects at 20–30 μM ATP. ATP reduces the low app K_d and increases the high app K_d for sugar-induced fluorescence quenching. D-Glucose transport in intact red cells is asymmetric (K_m and V_{max} for influx < K_m and V_{max} for efflux). In addition, two operational K_m parameters for efflux are detected in zero- and infinite-trans efflux conditions. Protein-mediated sugar transport in ghosts and inside-out vesicles (IOVs) is symmetric with respect to K_m and V_{max} for entry and exit, and only one K_m for exit is detected. Addition of millimolar levels of ATP to the interior of ghosts or to the exterior of IOVs restores both transport asymmetry and two operational K_m parameters for native efflux. A model for red cell hexose transport is proposed in which ATP modifies the catalytic properties of the transport system. This model mimics the behavior of the sugar transport systems of intact cells, ghosts, and inside-out vesicles.

In spite of extensive study, the properties of human red cell sugar transport "defy simple description" (Naftalin & Roselaar, 1985). The properties of erythrocyte hexose transfer appear to be consistent with Widdas' original suggestion (the mobile carrier hypothesis; Widdas, 1952) that a single sugar binding/transport site is alternately accessible at each surface of the plasma membrane (Krupka & Deves, 1981; Gorga & Lienhard, 1981). Nevertheless, the observation of asymmetry in Michaelis and velocity constants for sugar influx and efflux and the discovery of two operational sugar transport sites at

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