Isolation and Properties of a Sarcoplasmic Calcium-Binding Protein from Crayfish†

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ABSTRACT: The sarcoplasmic calcium-binding protein from crayfish muscle has been purified to homogeneity. The protein has a molecular weight of 44 000, as determined by sedimentation equilibrium and Sephadex chromatography. It dissociates in the presence of sodium dodecyl sulfate, 8 M urea, or, after succinylation, into two subunits of 22 000 molecular weight. The protein is free of carbohydrate and phosphorus but contains 4 g-atoms of calcium/44 000 at a free calcium concentration of 0.1 μM. Approximately 45% of the polypeptide backbone appears to be α-helical. The amino acid composition reveals a high proportion of alanine and acidic amino acids, a normal content of aromatic amino acids, and the absence of histidine. The isoelectric point, as determined by isoelectric focusing, is 5.1. The protein contains a free threonyl NH_2 terminal. Two thiols react rapidly in the native protein, six in the calcium-free form. Immunochemically, there is no difference between the protein from tail, claw, and heart muscle. In these three crayfish tissues, the concentrations of calcium-binding protein, as determined by rocket immunoelectrophoresis, are markedly different: 2.73 g/kg in tail, 0.72 in claw, and 0.073 in heart muscle. A functional analogy with the parvalbumins of vertebrates can be postulated.

In crustaceans, such as crayfish (Benzonana et al., 1974b) and lobster (Regenstein and Szent-Györgyi, 1975), the regulation of the muscular contraction by calcium is similar to that of vertebrates. Release of calcium from the sarcoplasmic reticulum triggers contraction by interaction of the metal ion with the troponin–tropomyosin complex. The morphology (Jahromi and Atwood, 1966) and activity (Benzonana et al., 1974b) of the sarcoplasmic reticulum of crayfish muscle are very similar to that of vertebrates. Whereas the concentration of the regulatory complex in whole crustacean muscle is nearly the same as in mammals, four times less calcium is bound by the former during contraction (Regenstein and Szent-Györgyi, 1975). These findings and the fact that the total calcium content in muscle is nearly the same for both species (Cox et al., 1976) have prompted a comparison of the sarcoplasmic calcium stores.

In vertebrate skeletal muscle, the sarcoplasmic calcium is bound to parvalbumins (Pechère et al., 1971b; Lehky et al., 1974). These are low-molecular-weight acidic proteins of unknown physiological role that are more abundant in the phasic white than in the tonic red muscle, but scarce in heart (Gosselin-Rey, 1974). In crayfish muscle, the absence of parvalbumins and the presence of a different sarcoplasmic calcium-binding protein (CMCP)[1] have been demonstrated (Benzonana et al., 1974a). In this manuscript, the isolation of the latter protein and some physical and chemical characteristics are described and compared with those of parvalbumins, with special attention to the concentration of this calcium-binding protein in skeletal and heart muscle.

Experimental Procedure

Chemical Analyses. Protein concentration was determined by the biuret technique with reference to a standard curve established with pure CMCP or by spectrophotometry at 278 nm (see below). Metals were determined with a Perkin-Elmer 303 atomic absorption spectrophotometer. Phosphate was assayed according to Fiske and Subbarow (1925), and sugars after disc gel electrophoresis according to Zacharius et al. (1969).

Circular Dichroism and Molar Absorption Coefficient. Circular dichroism measurements were done with a Jasco J-20A automatic recording spectropolarimeter. For determination of the specific absorption coefficient, the protein was dialyzed against distilled water until the conductivity of the dialyzate had dropped to 0.2 $\mu\Omega^{-1}$. An aliquot was used for spectral analysis; the remaining solution, containing about 20 mg of protein, was dried to constant weight at reduced pressure over phosphorus pentoxide in an Aberhalden dryer at 110 °C (boiling point of toluene).

Disc gel electrophoresis in 10% polyacrylamide gels was carried out either in Tris–glycine buffer, according to Davis (1964), or in the presence of 0.1% sodium dodecyl sulfate, according to Weber and Osborn (1969). Staining was performed with 0.25% G-250 Coomassie brilliant blue.

Molecular Weight Determination. The following protein markers, from Serva, were used for molecular weight determination by Sephadex chromatography or by disc gel electrophoresis: bovine serum albumin (mol wt 67 000), ovalbumin (45 000), chymotrypsinogen (25 000), myoglobin (17 800), and cytochrome *c* (12 400). Chromatography in the absence of urea was performed in a 1 × 150 cm column of Sephadex G-100; in the presence of urea (6 or 8 M), an upward flow column (1 × 90 cm) of Sephadex G-200 equilibrated in 1% mercaptoethanol was used.

Ultracentrifugation analyses were made at 20 °C with a Model E Spinco ultracentrifuge fitted with Schlieren and Rayleigh interference optics.

Amino acid analyses were carried out according to Moore and Stein (1963) with a norleucine internal standard. Hydrolyses were performed in sealed evacuated tubes with 5.7 N HCl at 108 °C for periods of 24, 48, and 72 h. Half-cystine was oxidized by performic acid. The hydrolyzed samples were analyzed with a Beckman Model 120 C automatic amino acid analyzer. For the analysis of tryptophan, the spectrophotometric methods of Edelhoch (1967) and of Bredderman (1974)

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[1] Abbreviations used are: CMCP, crayfish muscle calcium-binding protein; dansyl, 8-dimethylamino-1-naphthalenesulfonyl; pCMB, *p*-chloromercuribenzoic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

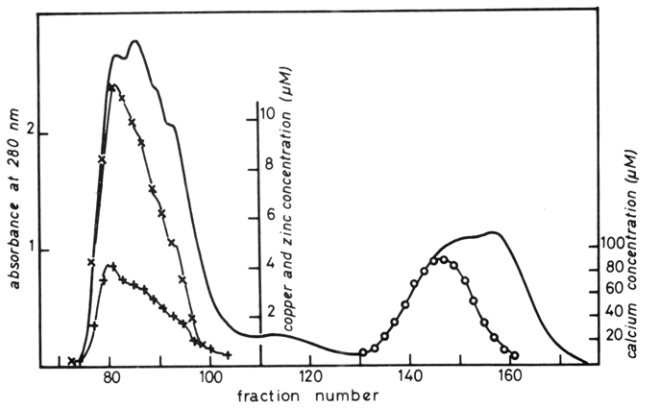

FIGURE 1: Sephadex G-100 chromatogram of the albumin fraction of crayfish myogen. The column (5 × 90 cm) was equilibrated with a 15 mM imidazole buffer, pH 7.0, and eluted at 60 ml/h, 3-ml fractions. Optical density at 280 nm (—); concentration of copper (x), zinc (+), and calcium (○), as determined by atomic absorption.

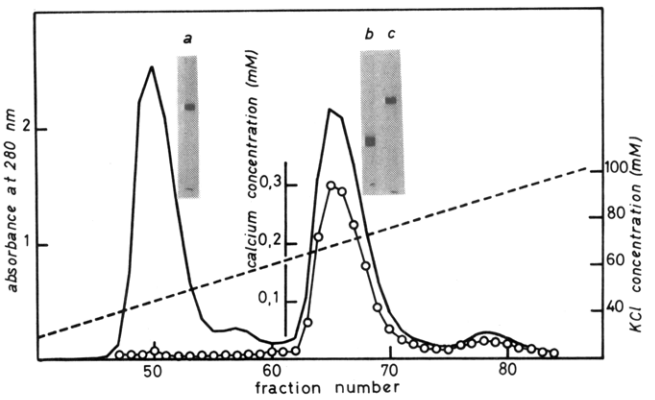

FIGURE 2: Elution profile after chromatography on DEAE-cellulose (2 × 20-cm column) of the calcium-containing peak of Figure 1. Elution was carried out at 25 ml/h with a linear KCl gradient (0–150 mM) in imidazole buffer, pH 7.0, 3-ml fraction. Optical density at 280 nm (—); calcium concentration (○); KCl concentration measured by conductivity (- - -). Inset: polyacrylamide (10%) disc gel electrophoresis of fraction 50 in the presence of dodecyl sulfate (a) and of fraction 65 in the presence (b) and absence (c) of the detergent.

were used, as well as the hydrolysis technique of Liu and Chang (1971).

The NH_2-terminal amino acid was determined by the dansylation method of Gros and Labouesse (1969). The dansyl amino acids were chromatographed on silica gel G with the solvents described by Seiler and Wiechmann (1964) and compared to reference dansyl amino acids from Serva.

Free Thiol Groups. Thiol titrations with recrystallized pCMB (Serva) were carried out according to Boyer (1954). The kinetics of the thiol reaction with Nbs_2 from Calbiochem was carried out according to Habeeb (1972).

Isoionic and Isoelectric Point. To determine the isoionic point, pure CMCP (5–16 mg) was dissolved in 600 μl of water and passed through a 1.5 × 35-cm column of Sephadex G-25 fine type, equilibrated with CO_2-free distilled water with a conductivity of 0.2 $\mu\Omega^{-1}$. The conductivity, pH, and optical density at 280 nm were continuously monitored in the eluate. The isoelectric point of the native protein was determined by gel electrofocusing according to Wrigley (1971). Polyacrylamide gels 7.5% containing pH 4–6 Ampholine (LKB) were polymerized in quartz tubes in the presence of 100 μg of CMCP/gel. After 2 h at 300 V, the protein was localized by uv scanning with a Zeiss PMQ2 spectrophotometer fitted with a ZK4 gel scanner. The CMCP segment was eluted with 1 ml of water and used for pH measurement. For isoelectric focusing of urea-denatured CMCP, where a high voltage is required for extended periods of time, the latter technique does not provide sufficient cooling and the flat bed method of Vesterberg (1972) was used. Electrofocusing was done in a LKB Multiphor apparatus for 24 h at 900 V. Staining was carried out according to Vesterberg (1972).

Immunochemical Techniques. For the production of antiserum, rabbits (2.5–3 kg) were injected subcutaneously with a first dose of about 20 mg of CMCP in 2 ml of 0.145 M NaCl, pH 7.0, emulsified with 2 ml of Freund's complete adjuvant (Difco Laboratories). After 21 days, three additional injections of 5 mg of CMCP in Freund's incomplete adjuvant were given at weekly intervals. The rabbits were bled via the *arteris carotis interna* 10 days after the last injection. Immunoglobulins were isolated by salting out and ion-exchange chromatography, as described by Harboe and Ingild (1973). Immunodiffusion by double diffusion method (Ouchterlony and Nillson, 1974) was performed on 1.5% agarose in 0.3 M potassium phosphate buffer, pH 8.0. The patterns were allowed to develop during 1–6 days at room temperature. Quantitative estimations of CMCP were carried out by rocket immunoelectrophoresis according to Laurell (1966). Diluted muscle extracts were applied in 1.5% agarose gels containing antibodies in barbital buffer $I = 0.02$, pH 8.6; electrophoresis was performed for 18 h at 15 °C in a LKB Multiphor apparatus at 2 V/cm in the gel. The slides of double diffusion and immunoelectrophoresis were washed, dried, and stained, as described by Weeke (1973). Protein concentrations were calculated with reference to a standard curve established with pure CMCP.

Results

Isolation of the Calcium-Binding Protein. Live freshwater crayfish (*Astacus pontastacus leptodactylus leptodactylus*) tail muscle (ca. 60 g) was homogenized at 0 °C with a Sorvall Omnimixer (2 × 30 s) in 4 volumes of a 50 mM potassium phosphate buffer adjusted to pH 7.2 with HCl and containing 30 mM NaCl, 1 mM $MgCl_2$ and 0.3 mM $CaCl_2$, and centrifuged. The supernatant containing approximately 4 g of protein was dialyzed overnight against distilled water, clarified by centrifugation, and saturated at 4 °C with solid ammonium sulfate. The precipitate was dissolved during dialysis against a 15 mM imidazole buffer, pH 7.0, and chromatographed on Sephadex G-100. The elution profile (Figure 1) yielded two major peaks. The first one, corresponding to the void volume, contained proteins which firmly bind zinc and copper. The latter metal was shown to be associated with the respiratory pigment hemocyanin, which contaminates myogen extracts from crayfish (Cox et al., 1976). The second peak, containing CMCP, was passed through a column of DE-52-cellulose. The resulting profile is shown in Figure 2, where the first peak emerging at 45 mM KCl corresponds to pure crayfish arginine kinase. Crayfish muscle contains about 10 g of arginine kinase/kg of fresh muscle. The second major peak, eluted at 70 mM KCl (Figure 2), coincides with the calcium-containing fractions: the ratio of calcium to absorbancy at 280 nm is constant in all the fractions. From 1 kg of tail muscle, about 2.5 g of CMCP could be obtained, which corresponds to 80% of the total amount of this protein in crude muscle extracts (see below). At the end of the DEAE chromatography, elution at 89 mM KCl yielded a protein with essentially the same properties as those of CMCP except for a more acidic isoelectric point (4.9); it represents, however, only 5% of the amount of CMCP.

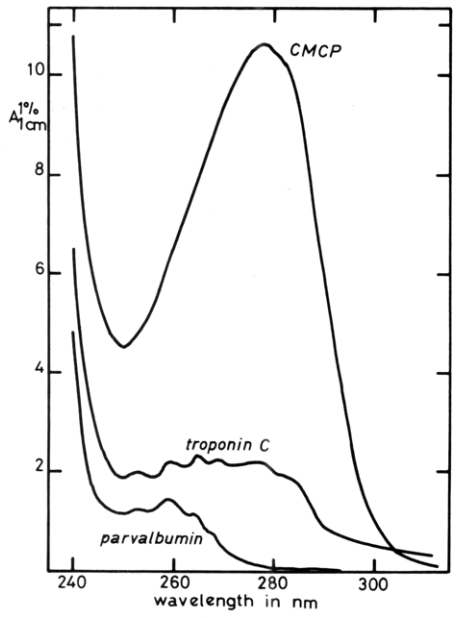

FIGURE 3: Ultraviolet spectrum of CMCP in 50 mM Tris-HCl buffer, pH 8.0, compared to those of rabbit troponin C and parvalbumin.

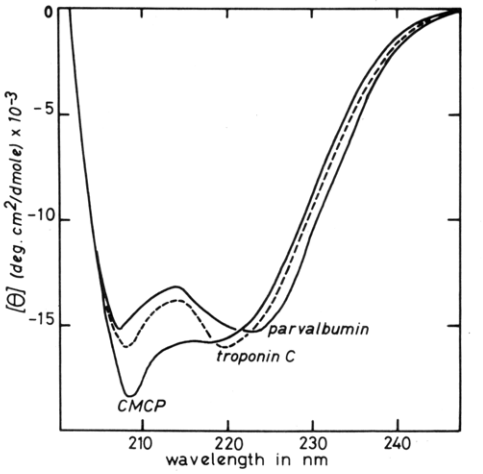

FIGURE 4: Far-ultraviolet circular dichroism spectra of CMCP and carp component 3 parvalbumin in 50 mM Tris, pH 7.6, compared to that of rabbit troponin C.

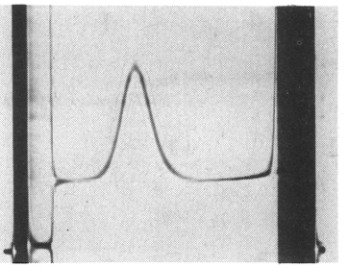

FIGURE 5: Ultracentrifugation pattern of a 1.67% solution of CMCP in phosphate buffer, I = 0.1 and pH 7.3, at 59 780 rev/min; picture taken after 96 min.

CMCP has also been isolated from crayfish claw muscle. The purification yields similar elution profiles except that: (a) in sodium dodecyl sulfate disc electrophoresis of the whole myogen and during Sephadex G-100 chromatography, a protein with a molecular weight of about 11 000 was observed; since this protein has a normal uv spectrum, binds zinc but not calcium, it cannot be related to parvalbumins; (b) a blue colored protein (ca. 100 mg/kg of fresh claw muscle) with a maximal extinction at 590 nm was eluted from the DEAE-cellulose column between arginine kinase and CMCP. This chromoprotein, with a molecular weight of about 45 000 in the native state, was devoid of any Zn, Ca, Mg, Co, Ni, or Cu. It might be related to the carapace pigment crustacyanin (Kuhn and Kuhn, 1967).

Spectral Studies. Pure CMCP has a normal uv spectrum (Figure 3) with a 260:280-nm absorbance ratio of 0.5 and a specific extinction coefficient $A_{278nm}{}^{1\%}$ of 10.6, based on dry weight. In this respect, CMCP differs markedly from parvalbumins and troponin C, both of which are characterized by a very low absorption at 278 nm (Pechère et al., 1971b; Greaser and Gergely, 1973). The circular dichroic spectrum, below 250 nm (Figure 4), shows a minimum at 209 nm and a shoulder at 221 nm with $[\theta]_{221nm}$ of -15 300 deg cm^2/dmol. From the latter value, the apparent α-helical content, estimated by comparison with polylysine according to Greenfield and Fasman (1969), is about 45%. This amount is comparable with the 47% α helix of parvalbumin (Donato and Martin, 1974) and the 43% value of troponin C (Murray and Kay, 1972), both determined by circular dichroism; on the other hand, 53–59% helicity has been reported for parvalbumin by x-ray diffraction studies (Kretsinger and Nockolds, 1973).

Molecular Weight. The ultracentrifugation pattern of CMCP in phosphate buffer, I = 0.1, pH 7.3, shows one symmetrical peak (Figure 5), indicating that the protein is homogeneous. The sedimentation coefficient, $s_{20,w}{}^0$, is 3.6 S with a concentration dependence of 0.04 S per g/l. Sedimentation equilibrium analyses at a protein concentration of 0.3 and 0.5% give additional evidence of a pure protein, as can be inferred from the strict linearity of concentration distribution as a function of distance within the cell. The mean molecular weight thus obtained is 44 000, which is in excellent agreement with the value estimated from chromatography on Sephadex G-100 (not shown) and thus indicates that the protein is essentially globular.

Subunit Structure. CMCP incubated in 0.1% sodium dodecyl sulfate and 4 M urea migrates during electrophoresis as a single component with a molecular weight of 21 000. A similar molecular weight was observed upon Sephadex chromatography when CMCP was succinylated according to Gounaris and Perlmann (1967) or incubated in 8 M urea in the presence of 1% mercaptoethanol. These results show that the native CMCP is composed of two subunits of identical molecular weight. Furthermore, by isoelectric focusing in 8 M urea of native or alkylated CMCP, only one single band could be detected, indicating that both subunits have also an identical isoelectric point. The strength of the bonds involved in the association of the two CMCP subunits is demonstrated by the urea treatment: in the presence of 1 μM free calcium, no dissociation occurs at 6 M urea, but only at 8 M.

Chemical Analyses. The protein hydrolysed with concentrated nitric acid is free of phosphorus and of Mg, Zn, Fe, Ni, or Cu. No positive reaction could be observed by the sugar test of Zacharius et al. (1969), a procedure which would have demonstrated the presence of 0.1 residue/protein molecule.

Calcium Content. The protein emerges from the preparative ion-exchange chromatography, which is performed at 5 μM free calcium, with 5.7 g-atoms of bound calcium/mole (Figure 2). After equilibrium dialysis against water of 0.2 $\mu\Omega^{-1}$ conductivity or passage through a 1.5 × 35 cm column of Sephadex G-25 equilibrated in water of the same purity, the free calcium concentration drops below 0.1 μM and 3.7 g-atoms of calcium/mol of protein are retained.

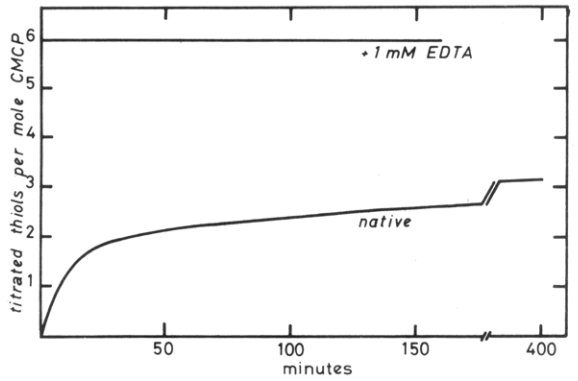

FIGURE 6: Reactivity of thiols in CMCP towards Nbs_2 in 0.1 M phosphate buffer, pH 8.0, with or without 1 mM EDTA.

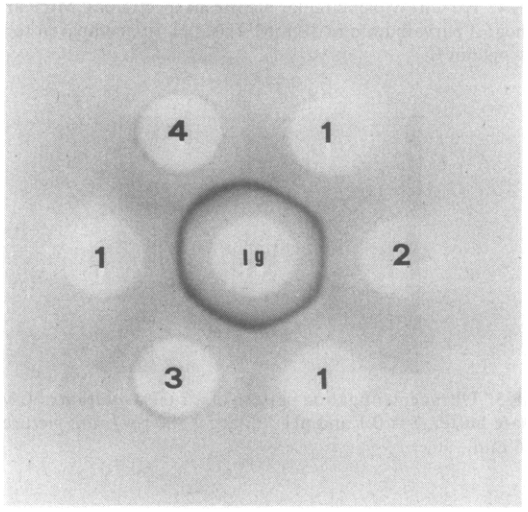

FIGURE 7: Immunodiffusion in 1.5% agarose plate. Ig, rabbit immunoglobulin against tail muscle CMCP: (1) purified tail CMCP; (2) purified claw CMCP; (3) tail muscle sarcoplasm; (4) claw muscle sarcoplasm.

Amino Acid Composition. Table I gives the amino acid composition of the monomer of tail CMCP, as both subunits are most probably identical. It accounts for 95.4% of the protein determined by weight of the lyophilized powder. The tryptophan determination by the spectrophotometric methods gave values which increased from 1.8 to 2.2 residues/subunit within 24 h. Therefore, the value for tryptophan (2.0) obtained by hydrolysis with *p*-toluenesulfonic acid was retained. Special features of the amino acid composition are the high content of aspartic and glutamic acids, accounting together for 29% of the total number of residues, and of alanine (12%). CMCP shows neither the unusually high ratio of lysine to arginine nor the absence of tryptophan (and often of tyrosine) characteristic of both parvalbumin and troponin C. The dansylation method revealed threonine as the NH_2-terminal amino acid, whereas in parvalbumin and troponin C the terminal group is acetylated (Pechère et al., 1971a; Collins et al., 1973).

Thiol Content. Figure 6 shows that in the native protein only 2 thiols react rapidly with a half-life of about 7 min, whereas the other thiols react very slowly (3.1 thiols titrated after 7 h). In the presence of EDTA (1 mM) or sodium dodecyl sulfate (1%), 5.9 thiols were titrated/molecule of CMCP with Nbs_2. This accounts for all the cysteine residues present in the protein, as determined by amino acid analyses (Table I), and thus excludes the presence of disulfide bridges. In these incubation media, the protein is decalcified and the thiols react instantaneously after mixing. Similarly, removal of calcium enhances the reactivity of the single thiol of parvalbumins (Donato and Martin, 1974). When native CMCP was incubated with pCMB, 5.4 thiols could be titrated/mole of protein after 24 h of reaction.

TABLE I: Amino Acid Composition of CMCP.

Amino Acid	Residue/22 000 mol wt	Mol Integer
Glycine	11.8	12
Alanine	22.9	23
Valine[a]	13.1	13
Leucine[a]	13.0	13
Isoleucine[a]	10.8	11
Serine[b]	7.1	7
Threonine[b]	5.3	5
Methionine	2.0	2
Cysteine[c]	2.9	3
Proline	3.1	3
Phenylalanine	13.1	13
Tyrosine	9.5	9–10
Tryptophan[d]	2.0	2
Aspartic acid	35.2	35
Glutamic acid	20.8	21
Histidine	0.0	0
Lysine	13.2	13
Arginine	6.9	7
Amino sugars	0.0	0
Total number of residues		192–193

[a] Value of 72-h hydrolysis. [b] Extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid according to Hirs (1967). [d] Determined by the method of Liu and Chang (1971).

Isoionic and Isoelectric Point. The appearance of the protein in the eluate of the Sephadex G-25 column was accompanied by a conductivity rise from 0.2 to no more than 6 $\mu\Omega^{-1}$, which indicates that the protein is essentially free of extraneous ions. By this method, an isoionic point of 5.16 was obtained; an isoelectric point of 5.06 was determined according to Wrigley (1971).

Immunodiffusion of CMCP from Tail and Claw Muscle. Immunoglobulins against tail CMCP gave a single precipitation line with the antigen and also with claw CMCP (Figure 7). Both tail and claw CMCP are immunochemically indistinguishable. The double diffusion patterns of the crude extracts of tail and claw, as shown in Figure 7, demonstrate the specificity of the antibodies and the absence of contamination by antibodies to other muscle proteins. CMCP cross-reacts neither with carp parvalbumin components 2, 3, and 5 nor with the major and minor components of turtle parvalbumin (not illustrated).

Content of CMCP in Muscle. The above monospecific antibodies were used for the separate determination by rocket immunoelectrophoresis of CMCP in whole muscle from tail, heart, and claw (crusher and cutter). Fresh muscles were homogenized at 0 °C in the presence of 10 volumes of water. After centrifugation for 30 min at 300 000*g*, 90% of the total CMCP could be extracted. An additional 3–10% CMCP could be obtained by a second extraction; a third extraction gave supernatants with CMCP concentrations below the limit of detection of the method. The results are presented in Table II and show a ratio of approximately 1:10:40 for heart, claw, and tail muscle CMCP, respectively.

Discussion

CMCP shares some of the characteristic features of the cytoplasmic calcium-binding proteins, such as parvalbumins

TABLE II: Concentration of CMCP in Different Muscles (g/kg of Fresh Tissue).

Tissue	1st Extraction	2d Extraction	Sum
Tail muscle[a]	2.62 ± 0.36	0.11 ± 0.06	2.73 ± 0.42
Claw muscle[a]	0.67 ± 0.30	0.05 ± 0.02	0.72 ± 0.32
Heart muscle[b]	0.066	0.007	0.073

[a] Mean value for seven animals and dispersion. [b] Value of seven pooled muscles.

(Pechere et al., 1971b), vitamin D-induced calcium-binding proteins (Fullmer and Wasserman, 1975), nerve tissue calcium-binding proteins (Siegel et al., 1974), and the phosphodiesterase activator described by Wang et al. (1975). Although the isoelectric point of these proteins varies from 4.3 to 7.0, their acidic amino acids (including the corresponding amides) comprise more than one-fourth of the total residues, whereas the basic amino acids amount to no more than one-tenth. In contrast to these monomeric proteins, CMCP is a dimer in the native state. It is likely that its subunits are identical, since they have the same molecular weight (22 000), isoelectric point, and NH_2-terminal amino acid. Furthermore, the amino acid composition shows integers for all residues except tyrosine. Although the subunits of CMCP are strongly held together, disulfide bridges are not involved: the cysteine residues found by amino acid analysis (Table I) can all be titrated with pCMB, or with Nbs_2 in the presence of EDTA.

CMCP is easily extracted from muscle by centrifugation in a physiological salt solution or in water, and the recovery corresponds fairly well to the expected one (90–97% of total CMCP extracted from an 11 times diluted muscle homogenate; see Table II); it is therefore unlikely that CMCP binds to the particulate structure of muscle. As no other sarcoplasmic proteins with strong affinity for calcium such as parvalbumins could be detected in crayfish myogen extracts (Cox et al., 1976), it is tempting to hypothesize that CMCP replaces in crustaceans the parvalbumins found in vertebrates. A possible functional analogy of CMCP and parvalbumins is supported by the similarity of their distribution within morphologically different muscles. In fish, the concentration of parvalbumin is much less in red than in white muscle, and only traces are detected in heart (Gosselin-Rey, 1974). With respect to tension development, contraction speed, and mitochondria content, the crustacean tail muscle is analogous to vertebrate white muscle; in the claw of crayfish, the large crusher muscle has the characteristics of a red, the minor cutter those of a white muscle (Atwood, 1970).

It is interesting that parvalbumins, which have exhibited considerable invariance in the course of vertebrate evolution (Lehky et al., 1974), are absent in crustacean sarcoplasm. Distinct amino acid compositions and absence of immunological cross-reactivity between parvalbumins and CMCP do not rule out resembling fragments in the primary structure, or an analogous physiological function; although the intestinal calcium-binding proteins from mammals and birds show no structural homology, they both are synthetized in answer to the same physiological compound, vitamin D.

CMCP binds approximately 6 g-atoms of calcium/mol at 1 μM free calcium, which means that the protein can bind tightly 370 μmol of calcium/per kg of crayfish tail muscle. This represents 10% of the total amount of calcium present in this tissue (Cox et al., 1976). Thus, three times more calcium can be bound to CMCP than to troponin C, which in crustaceans has only one binding site (Regenstein and Szent-Györgyi, 1975). This implies that about 75% of the calcium released from the sarcoplasmic reticulum is taken up by CMCP. In the instance of rabbit, the proportion of calcium taken up by parvalbumin is much smaller (Lehky et al., 1974). The higher calcium-buffering capacity of crustacean sarcoplasm is in agreement with the fact that the crustacean regulatory response requires 3–4 times less calcium than that of rabbit (Regenstein and Szent-Györgyi, 1975).

Recently, Lehman and Szent-Györgyi (1975) isolated from the scallop striated muscle a sarcoplasmic calcium-binding protein with the same monomer molecular weight and uv spectrum as CMCP. The presence of similar sarcoplasmic calcium-binding proteins in invertebrates as different as molluscs (that possess exclusive myosin calcium control) and crustaceans (with actin control) suggests a fundamental function for the protein described here.

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Proteolytic Fragmentation of *Helix pomatia* α-Hemocyanin: Structural Domains in the Polypeptide Chain†

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ABSTRACT: α-Hemocyanin from the Roman snail *Helix pomatia* is composed of polypeptide chains with a molecular weight of 360 000 ± 30 000. The cylindrically shaped hemocyanin molecule contains 20 of these large chains. The polypeptide chain has been split into components with molecular weights of: 210 000, 154 000, 147 000, 112 000, 120 000, 98 000, 55 000, and 50 000, by gentle proteolysis with enzymes of different specificities. Most of the fragments have molecular weights which are about 50 000 or a multiple of 50 000. Departure from these values, as found in the 112 000 and 120 000 fragments, is probably caused by the high carbohydrate content of these components. A mixture of these fragments has the same oxygen binding properties as the nondigested protein. Subtilisin converts the hemocyanin polypeptide chain, under appropriate conditions, almost completely into fragments of 50 000 and 55 000 daltons with conservation of the oxygen binding properties of the nondigested protein. We conclude from these studies that the polypeptide chain of *Helix pomatia* α-hemocyanin is folded into about seven compact tertiary structures, which are covalently interconnected. This chain of structural domains has been visualized (Siezen and Van Bruggen (1974), *J. Mol. Biol. 90,* 77–89) by electron microscopy, which shows 1/20 hemocyanin molecules to be flexible structures consisting of 7–8 apparently spherical units of 55–60 Å diameter.

The α-hemocyanin of the Roman snail, *Helix pomatia,* is a copper containing, oxygen binding protein, with a molecular weight of approximately 8–9 × 10^6 at neutral pH. A stepwise dissociation of the native molecule into successively 1/2-, 1/10-, and 1/20-size molecules occurs on mild changes in pH, ionic strength, etc. (Konings et al., 1969a; Elliot et al., 1972; Siezen and Van Driel, 1974). Brouwer and Kuiper (1973), who studied the molecular weight of the hemocyanin polypeptide chain, have suggested that this chain consists of separated, compact tertiary structures, so-called structural domains, covalently interconnected by more exposed stretches of the polypeptide chain, as has been found for immunoglobulins (Edelman et al., 1970; Poljak et al., 1972; Schiffer et al., 1973) and bovine serum albumin (Adkins and Foster, 1965; Pederson and Foster, 1969; King and Spencer, 1970). This hypothesis was supported by Siezen and Van Bruggen (1974) who showed by electron microscopy 1/20 hemocyanin molecules to occur as a linear chain of seven to eight globules. Each structural domain is thought to contain one oxygen binding site. If this hypothesis is correct, it may be possible to prepare biologically

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