Structure of C Protein Purified from Cardiac Muscle

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ABSTRACT C protein is a component of the thick filament of striated muscles. Although the function of C protein remains unknown, a variety of evidence suggests that C protein may regulate actin-myosin interaction or be involved in structural support or elasticity of the sarcomere. We have previously proposed (Hartzell, H. C., 1984, J. Gen. Physiol., 83:563–588) that C protein is involved in regulating twitch relaxation in cardiac muscle. To gain further insight into the function of C protein, we have studied the structure of C protein purified from chicken heart. C protein was purified from extracts of detergent-washed myofibrils by sequential hydroxylapatite and DEAE-Sephacel chromatography. C protein was judged >95% pure by SDS PAGE. The polypeptide subunit had a molecular weight of 155,000 and the native molecule sedimented on linear sucrose or glycerol gradients at 4–5S. For electron microscopy, purified C protein was dialyzed and diluted into a volatile buffer in 50% glycerol, aspirated onto mica, dried under vacuum, and rotary platinum-shadowed. Replicas revealed particles of relatively homogeneous overall dimensions. Over half of the particles were V-shaped. The "arm" lengths of the V-shaped particles were 22 ± 4.5 nm (SD). Gel filtration on Sephacryl S-300 demonstrated that purified C protein had a Stokes' radius of 5.07 nm. Measurements of viscosity gave an intrinsic viscosity of 16.5 cm3/g. These data are consistent with the electron microscopic data and suggest that C protein in heart muscle is asymmetric. The C protein molecule is large enough to extend from the surface of a thick filament to adjacent thin or thick filaments.

C protein is a component of the thick filament of skeletal and cardiac muscles (26, 38). The function of C protein remains unknown, but it has been suggested that C protein may regulate thick filament assembly and length (14, 33), participate in thick-filament structural support (26, 27), maintain the structure and contribute to the radial elasticity of the sarcomere (17), or regulate cross-bridge movement during contraction (26). C protein has a number of properties that make particularly attractive the suggestion that it plays a role in regulation of contractile activity. For example, C protein binds to both purified actin (22) and myosin (23, 34) and can alter the ability of actin to stimulate myosin ATPase (21, 22, 26, 38). The effects of C protein on ATPase activity, however, are complex: C proteins inhibit skeletal muscle actomyosin ATPase, but stimulate cardiac muscle actomyosin ATPase (38). It has been suggested that the physiological role of C protein may involve a calcium-regulated binding of C protein to thin filaments, because C protein binding to native thin filaments occurs only in the presence of micromolar concentrations of calcium (20). Recently, it has been shown that C protein in cardiac muscle becomes phosphorylated in response to β-adrenergic agonists and dephosphorylated in response to cholinergic agonists (5, 11, 12). The level of C protein phosphorylation correlates with the rate of relaxation of the cardiac contraction and it has been suggested that C protein regulates twitch relaxation in cardiac muscle (9).

Understanding the function of C protein requires a thorough knowledge of its structure and organization in the myofibril. C protein has been shown to be localized to seven to nine stripes perpendicular to the axis of the thick filament in the middle third of each half of the A band (2, 3, 25, 27–29). The spacing between each stripe is ~43 nm, a value very similar to the axial repeat of myosin heads on the thick filament. The significance of this spacing remains unknown, although Squire (33) has used this observation to support the idea that C protein regulates thick filament length. Hydrodynamic measurements (26) suggest that C protein is a prolate ellipsoid 3 × 35 nm, but direct measurements of the shape of C protein by electron microscopic techniques have not been published. For this reason, we have examined the structure of purified C protein in the electron microscope using low-angle rotary shadowing with platinum. This technique has been

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used successfully to reveal interesting features of the structure of a wide variety of proteins including myosin (4, 16), spectrin (32, 37), and dynein (30). We have chosen to study cardiac muscle C protein because it is reversibly phosphorylated under physiological conditions, and we would ultimately like to examine structural changes that might occur in response to phosphorylation. We describe here that a prevalent particle seen in replicas of unphosphorylated C protein from chicken cardiac muscle is V-shaped. Each arm of the V is ~22 nm long.

MATERIALS AND METHODS

Protein Purification: C protein was purified from frozen chicken heart (Tyson Foods, Cumming, GA) by methods described in detail elsewhere (10). Frozen chicken heart (250 g) was pulverized and homogenized in 750 ml of buffer A (50 mM KCl, 2 mM EDTA, 20 mM Tris-HCl, 15 mM 2-mercaptoethanol, pH 7.9) for 2 min in a Waring blender (Waring Products, New Hartford, CT). The homogenate was centrifuged at 3,000 g for 15 min. The pellet was washed three times with 750 ml of buffer A, twice with buffer A containing 1% Triton X-100, and five times in buffer A. The final pellets of myofibrils were resuspended by homogenization with a Polytron homogenizer (Brinkman Instruments, Inc., Westbury, NY) in 400 ml of EDTA-PO4 buffer, pH 5.9 (10 mM EDTA-Na2, 124 mM NaH2PO4, 31 mM Na2HPO4 [see reference 26]) and centrifuged at 10,000 g for 20 min. The pellet was extracted with an additional amount of 400 ml of EDTA-PO4, pH 5.9. The supernatant from the two extractions was pooled and concentrated by ammonium sulfate precipitation (55% saturation). The precipitated protein was dissolved in 20 ml of 400 mM NaCl, 20 mM Tris-HCl, 0.1 mM EDTA, 2 mM Na2S2O4, X-100, 0.1 mM EDTA, and 3 mM 2-mercaptoethanol and dialyzed overnight.

The extract containing C protein was then chromatographed as described by Starr and Offer (35) on a 2.5 x 35-cm column of hydroxyapatite (high-resolution powder, Calbiochem-Behring, San Diego, CA) using a 500-ml gradient from 0.3 M NaCl, 2 mM NaNO3, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 15 mM NaH2PO4, 28 mM K2HPO4, to 0.3 M NaCl, 1 mM NaNO3, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 56 mM NaH2PO4, 119 mM K2HPO4. The major protein peak was concentrated by ammonium sulfate precipitation, dissolved in 4 mM NaH2PO4, 6 mM K2HPO4, 5 mM NaH2PO4, 2 mM NaNO3, and 0.1 mM EDTA (pH 8.3) and dialyzed overnight. The hydroxyapatite-purified C protein was chromatographed on a 1.6 x 35-cm column of DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) using a 250-ml gradient from 4 mM NaH2PO4, 6 mM K2HPO4, 5 mM NaH2PO4, 2 mM NaNO3, 0.1 mM EDTA (pH 8.3) to 250 mM NaH2PO4, 5 mM NaH2PO4, 2 mM NaNO3, and 0.1 mM EDTA adjusted to pH 6 with NaOH. The major protein peak was stored in 60 mM NaCl, 25 mM Tris-HCl, 0.1 mM EDTA, 2 mM NaNO3, and 3 mM 2-mercaptoethanol, pH 8.0 at 4°C.

Myosin was purified from fresh chicken cardiac muscle by methods modified only slightly from those described by Margossian and Lowey (18).

C Protein Characterization: The purity of C protein was assessed by SDS PAGE. The SDS gels were 1.5 mm thick and composed of a 3% acrylamide stacking-gel and a 5-15% acrylamide gradient-resolving gel (15). Gels were run at a constant voltage of 45 V for 15 h.

The purity of C protein was estimated by quantitative densitometry (11). Coomassie blue-stained SDS gels were scanned with a Transydine 2500/2510 scanning densitometer (Transydine General Corp., Ann Arbor, MI) at 595 nm, and the area of the protein peaks was integrated. The area of the C protein peak was linear from ~0.1 to ~2 μg of loaded protein. Within this range, the integrated area of the C protein peak was 99% of the total area of the scan. With these loads of C protein, it was possible that the amount of contamination was underestimated because the contaminating bands were below the linear range. Thus, large loads of protein (20-40 μg) were used to estimate the concentration of contaminants and low loads of protein (1 μg) were used to estimate the amount of C protein. Under these conditions, the amount of C protein comprised 95% of the total protein.

Zonal Centrifugation: Aliquots (0.25 ml) of purified C protein (2.1 mg/ml) were layered onto linear 8-30% glycerol gradients made up in a buffer containing 0.2 M ammonium acetate or ammonium bicarbonate, 0.1 mM EDTA, and 2 mM NaNO3. In some cases linear 5-20% sucrose gradients were used. The 11.6-ml gradients were centrifuged in a Beckman SW-41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 35,000 rpm for 15.5 h (Beckman J-LS-65 centrifuge) and fractioned into 21 equal volumes. The approximate sedimentation value of C protein was determined by the methods of Martin and Ames (19) using catalase (11.3S [19]), myosin (6.4S [16]), IgG (7.6S [6]), and IgM (18S [6]) as standards. Aliquots from fractions of the gradient were prepared for SDS PAGE. In some experiments the peak C protein fraction was used for electron microscopic examination by methods described below.

Electron Microscopy: C protein was prepared for electron microscopy in several ways with very similar results: (a) C protein (20 mg/ml) was dialyzed overnight into 0.2 M ammonium bicarbonate and diluted to 40 μg/ml with 0.2 M ammonium bicarbonate in 50% glycerol; (b) C protein was centrifuged in glycerol gradients as described above, and the C protein-containing fraction from the gradient was diluted to ~30 μg/ml in a buffer composed of 0.2 M ammonium acetate in 50% glycerol. A 125-μl aliquot was loaded into a glass nebulizer, aspirated onto freshly cleaved mica, and immediately dried under vacuum in the Balzers 400 freeze-fracture machine (Balzers, Hudson, NH) (cf. reference 37). The sample was then rotary-shadowed (60 rpm, 6° shadow angle) with a platinum-carbon mixture from the electron beam gun. A quartz crystal monitor was used to reproduce platinum shadow thickness. Carbon-stabilized platinum replicas were floated onto water and picked up on formvar-coated, copper grids. Replicas were viewed at 80 kV. The magnification was calibrated by using a carbon-grating replica.

For analysis of particle shapes, every particle in eight fields of ~2 μm2 each was scored as V-shaped, elongated, or amorphous. V-shaped particles were defined as particles consisting of two asymmetric arms that were joined at their ends to form an angle. The arms were not necessarily of equal size or length. Elongated particles were composed of a single part that was asymmetric in shape. Amorphous particles were heterogeneous in size and shape. Particle dimensions and shapes were analyzed by tracing enlargements of images on a Hi-Pad Digitizing pad (Houston Instruments, Austin, TX), attached to an Apple IIIE (Apple Computers, Cupertino, CA) microcomputer. A stereometric program was used to determine the particle arm lengths. Maximum point-to-point dimensions were determined by tracing the perimeter of particles and using the stereometric program to calculate maximum diameter of the enclosed figure.

Filtration Chromatography: C protein was chromatographed on a 2.5 x 95-cm column of Sephacyrl S-300 (Pharmacia Fine Chemicals) at a flow rate of 30 ml/h in either 0.2 M NH4HCO3 or 60 mM NaCl. 25 mM Tris-HCl, 0.1 mM EDTA, 2 mM NaNO3, 3 mM 2-mercaptoethanol, pH 8.0. Results were identical for both buffers. The column was calibrated with catalase, ferritin, and aldolase (Pharmacia Fine Chemicals). Usually 10 mg of each protein in 2 ml was loaded onto the column and the elution monitored by an ISCO UA-5 absorbance monitor (Lincoln, NE). Each protein was chromatographed at least three times on the same column. Kd was calculated as Kd = (Vc - V0)/(Vt - V0) where Vc was the elution volume, V0 was the column bed volume, and Vt was the void volume of the column. Kd values varied <0.02 between replicate runs of the same protein.

Viscometry: C protein solutions were centrifuged at 150,000 g for 1 h and diluted to the appropriate concentration in 60 mM NaCl, 25 mM Tris-HCl, 0.1 mM EDTA, 2 mM NaNO3, 3 mM 2-mercaptoethanol, pH 8.0. The protein concentration was determined by A280 measurements using an extinction coefficient of ε280 = 1.09 (Hartzell, H. C., unpublished results). Reduced viscosity was determined using a Cannon-Manning Semimicro 100 capillary viscometer (Cannon Instrument Co., State College, PA) according to Schachman (31). The measurements were performed in a water bath at 20 ± 0.1°C. The transit time for buffer was 67.4 ± s. Reduced viscosity was calculated from the equation [η] = (t0/t4) - 1, where t0 was the transit time for buffer, t4 was the transit time for sample, and c was the concentration of protein in g/cm³.

RESULTS

Purity of C Protein

To interpret data on the structure of C protein in the electron microscope, it was important to know the purity of the preparation of C protein that was being used. The purity of the C protein was assessed by SDS PAGE on gradient gels which resolved proteins in the range of >200,000 to 12,000 mol wt (Fig. 1). In Fig. 1, various amounts of a typical preparation of C protein were loaded onto an SDS gel. The C protein preparation was composed of one major band migrating at 155,000 mol wt. Quantitative densitometry revealed that the C protein preparation was >95% C protein. At the lowest loads, there was no evidence of multiple bands in the 155,000 region. At the highest loads, the major contaminating band was seen at 127,000. This 127,000-mol-wt band and several of the lighter ones were thought to be proteolytic fragments of C protein because the intensity of these bands...
increased upon storage and some of these bands were labeled in immunoblots with antibody against C protein (Hartzell, H. C., unpublished results). We always used C protein preparations <1 wk old for all our structural studies to ensure that there was little proteolytic degradation. SDS gels were run on each preparation at the time the C protein was prepared for shadowing to evaluate degradation.

Because the function of C protein was unknown, it was difficult to evaluate whether it remained in its native state after isolation. Several observations, however, might be relevant. C protein was an excellent substrate for cyclic AMP-dependent protein kinase (10), and was selectively dephosphorylated by phosphatase 2A (Titus, L., and H. C. Hartzell, unpublished results). In addition, preliminary studies suggested that this C protein preparation contains 0.2 mol of phosphate per mole of C protein. Up to 2.6 additional mol of phosphate can be incorporated in the presence of cyclic AMP-dependent protein kinase and [γ-32P]ATP.

Electron Microscopy

Our initial examination of micrographs of shadowed C protein gave us the impression that the particles were quite homogeneous in size (Fig. 2). To quantify this impression we measured the maximum point-to-point dimensions of all the particles in several low-magnification fields. 97% of the particles had a maximum point-to-point dimension <44 nm. Examination of low-power micrographs revealed three basic forms of particle. Our criteria for classifying these particles are described in Materials and Methods, and these forms are illustrated in Fig. 3. The most subjectively interesting particle was V-shaped. It was composed of two arms meeting at an angle (Fig. 3, circles; Fig. 4). The V-shaped particles were relatively homogeneous in their shape: the range of shapes of these particles is illustrated in the bottom row in Fig. 4. Other particles exhibited an elongated appearance (Fig. 3, squares). These particles had dimensions similar to those of a single arm of a V-shaped particle or to a "collapsed" V-shaped particle. The remaining particles were heterogeneous in size and shape. They were usually globular or amorphous (Fig. 3, arrows). These amorphous particles exhibited no regular substructure and ranged in size from several nanometers to a maximum of 44 nm in the longest dimension. We were unable to classify these amorphous particles into discrete subclasses.

Of 299 particles counted in low-power micrographs, 58% were V-shaped, 20% were elongated, and 22% were amorphous. The V-shaped particles were always the prevalent form in 33 replicas from six separate C protein purifications. Substitution of ammonium acetate for ammonium bicarbonate or variation of the glycerol concentration from 20 to 50% had no obvious effect on the V-shaped C-protein particle.

The arm lengths of 200 different V-shaped particles were measured by three different individuals. For these measurements, only well-spread molecules such as those in Fig. 4 were measured. Each arm was measured from the vertex of the V to the tip of the arm. The average length was 22.1 ± 4.5 nm (mean ± SD, n = 400, Fig. 5). One arm of each V-shaped particle appeared to be slightly longer than the other. The average difference between the long and short arms was 4 nm, which was not statistically significant. The angle inscribed by the arms of selected particles averaged 59.1° ± 19° (mean ± SD, n = 153). We estimated the width of the arms to be 3–4 nm; however, accurate measurements were compromised by errors imposed by the size of the platinum grains and uncertainties about decoration of these molecules with platinum or glycerol. However, the width of the C protein arms appeared significantly greater than the α-helical rod portion of the myosin molecule (Fig. 3). The vertex of the V often appeared to be a slightly more bulky, globular domain.

Because of the prevalence of the V-shaped particles, we concluded that this particle represented a major form of C protein. The possibility, however unlikely, remained that the V-shaped particles were contaminants or a minor form. To eliminate this possibility, we shadowed preparations of C protein that had been mixed with a known concentration of another, well-characterized marker protein, in this case purified myosin. Counting the relative numbers of C protein and myosin particles permitted us to evaluate the approximate relative molar concentrations of myosin and C protein particles in the original solution. C protein particles were defined as particles <44 nm in their maximum point to point dimension without regard to their shape and myosin particles were defined as particles >140 nm long with two globular heads and a rodlike tail. Control experiments verified that the myosin sample contained only myosin particles. 40 μg/ml of DEAE-purified heart muscle myosin was mixed with an equal volume of 40 μg/ml C protein in 0.2 M NH₄HCO₃ in 50% glycerol and the sample prepared for shadow replication as usual. Because the molecular weights of myosin and C protein are, respectively, 458,000 (7) and 155,000, we expected a ratio...
of three C protein particles to one myosin particle if the C protein particles were monomeric C protein molecules. We found a ratio of 1.42 single C protein particles to one myosin particle within the perimeter of dried microdrops (351 particles counted). Of the particles <44 nm, most were V-shaped. These results demonstrated that the V-shaped particle was not a minor component of the C protein solution. The unexpectedly low ratio of C protein particles to myosin particles could be explained if the number of C protein particles were underestimated or if the C protein particles were dimers of 155,000-mol-wt C protein molecules. No obvious interaction between myosin and C protein was observed, probably due to the high

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FIGURE 3. Different forms of particles in replica of shadowed C protein. The predominant particle types are V-shaped (circles), elongated (squares), and amorphous (arrows). (Inset) Purified heart myosin prepared for electron microscopy in an identical manner as C protein, included for comparison. Bar, 50 nm. × 150,400.
salt concentration in the mixtures of C protein and myosin. In other experiments the density of V-shaped particles was proportional to the protein concentration of the purified C protein sample.

**Gel Filtration**

To gain further insight into the molecular size and composition of C protein, we chromatographed C protein on Sephacryl S-300. The S-300 column was calibrated using globular proteins with known Stokes' radii: aldolase (4.81 nm), catalase (5.22 nm), and ferritin (6.1 nm). Over this range of molecular size, a plot of $K_v$ vs. the log of molecular weight was linear (Fig. 6b), as was a plot of $(-\log K_v)^2$ vs. Stokes' radius ($r = 0.99$, not shown). C protein eluted from the column as a single, symmetrical peak (Fig. 6b) between aldolase and catalase, with an apparent molecular weight of 209,000 and a Stokes' radius of 5.07 nm. Greater than 95% of the protein loaded onto the column was recovered in this peak. The finding that C protein eluted more quickly than expected on the basis of its chain molecular weight in SDS (155,000) demonstrated that C protein was asymmetric and/or existed in rapid equilibrium between monomeric and dimeric forms.

**Viscosity**

A plot of reduced viscosity vs. C protein concentration extrapolated to give an intrinsic viscosity of C protein of 16.5
electron microscopy in suggesting that the C protein in solution are confident that these particles are C protein molecules for by SDS PAGE on gradient gels. In all experiments, we used these studies was at least 95% pure C protein as determined particles that are observed are homogeneous in their size. We were within a factor of two of that expected from the catalase, and ferritin and the $K_v$ plotted as a function of the log of molecular weight. (Inset) Elution profile of an experiment in which C protein and aldolase were mixed and chromatographed together. C protein elutes before aldolase. Protein identities were determined by SDS PAGE of the peak fractions.

cc/g (Fig. 7). This value was three to four times larger than that expected for globular proteins and indicated the protein was asymmetric or had a high degree of hydration (36).

DISCUSSION

This paper demonstrates that when C protein purified from cardiac muscle is viewed in the electron microscope, the particles that are observed are homogeneous in their size. We are confident that these particles are C protein molecules for several reasons: (a) The C protein preparation we used for these studies was at least 95% pure C protein as determined by SDS PAGE on gradient gels. In all experiments, we used fresh C protein preparations and assessed proteolytic degradation by SDS PAGE to be <5%. (b) In experiments with mixtures of C protein and myosin, the proportion of C protein particles was within a factor of two of that expected from the molar concentration of the proteins in the solution. A variety of different particle shapes were seen in the C protein replicas, but the most common particle was V-shaped. Many of the other particles that were seen could be explained as distorted, stretched, or collapsed V-shaped particles.

Gel filtration and viscometry data were consistent with the electron microscopy in suggesting that the C protein in solution was an asymmetric molecule. Gel filtration demonstrated that the C protein had a Stokes' radius of 5.07 nm, which was significantly larger than expected from a globular protein of 155,000 mol wt. The viscosity was intermediate between that of globular proteins and highly asymmetric proteins such as tropomyosin. The molecular dimensions of C protein estimated from electron microscopy were consistent with previous hydrodynamic studies that showed that C proteins from skeletal (26) and cardiac (38) muscles were asymmetric.

From the present data, we were unable to determine whether the C protein particles represented monomers or dimers of 155,000-mol-wt polypeptides. Offer et al. (26) and Harrington (8) reported that at low ionic strength, C protein from skeletal muscle reversibly dimerized. On gel filtration (Fig. 6), C protein eluted more slowly than if it were a stable dimer but more rapidly than if it were a stable, globular monomer. The elution of C protein could be explained if C protein were an asymmetric monomer or if C protein rapidly and reversibly dimerized during its migration through the column. Measurements of the ratio of C protein and myosin particles in a mixture were inconclusive in proving whether the C protein was in a monomeric or dimeric form because of questions about the fraction of molecules preserved in unaggregated form during the aspiration and shadowing procedure.

Organization of C Protein in the Thick Filament

Previous electron microscopic immunocytochemical studies demonstrated that C protein is localized in seven to nine 10-nm-wide transverse stripes spaced at 43 nm in the middle third of each half of the A band (2, 3, 25, 27). Furthermore, it has been estimated that there are between 37 (26) and 54 (24) C protein molecules on each thick filament. Thus, if C protein resides in 14 locations on each thick filament, there are 2.1-3.8 C protein molecules on each thick filament at each stripe.

The orientation of C protein molecules on the thick filament, however, remains unknown. Squire (33) has speculated that the C protein molecules are linear and lie parallel to the thick filament axis and has suggested that the stripes seen in cryosections (1) and antibody-stained sections (2, 25, 27) result from a bulky portion of the C protein molecule. Another possibility, however, is that C protein extends radially from the surface of the thick filament into the interfilament space. Our observations that C protein molecules have an arm length of 22 nm suggests that C protein is large enough to extend a considerable distance into the interfilament space. Inasmuch as the distance between the surfaces of thick and
thin filaments ranges between 9 and 16 nm at sarcomere lengths between 3.6 and 1.9 μm, respectively (13). C protein is large enough to span the distance between thick and thin filaments and could exert a regulatory role on the actin-myosin interaction. Alternatively, C protein could bridge the gap between adjacent thick filaments (23 nm) if it existed in an extended (rather than bent) conformation in situ. “Side-struts” observed between thick filaments have been ascribed to C protein and have been hypothesized to provide radial elasticity to the sarcomere (17).

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