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Calcium binding to *Procambarus clarkii* sarcoplasmic calcium binding protein splice variants

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ABSTRACT

Sarcoplasmic calcium binding protein (SCP) is a high-affinity calcium buffering protein expressed in muscle of crayfish and other invertebrates. In previous work, we identified three splice variants of *Procambarus clarkii* SCP (pcSCP1a, pcSCP1b, and pcSCP1c) that differ in a 37 amino acid region that lies mainly between the 2nd and 3ed EF-hand calcium binding domain. To evaluate the function of the proteins encoded by the pcSCP1 transcripts, we produced recombinant pcSCP1 and used tryptophan fluorescence to characterize calcium binding. Tryptophan fluorescence of pcSCP1a decreased in response to increased calcium, while tryptophan fluorescence of the pcSCP1b and pcSCP1c variants increased. We estimated calcium binding constants and Hill coefficients with two different equations: the standard Hill equation and a modified Hill equation that accounts for contributions from two different tryptophans. The approaches gave similar results. Steady-state calcium binding constants (K_d) ranged from $2.7 \pm 0.7 \times 10^{-8}$ M to $5.6 \pm 0.1 \times 10^{-7}$ M, consistent with previous work. Variants displayed significantly different apparent calcium affinities, which were decreased in the presence of magnesium. Calcium K_d was lowest for pcSCP1a and highest for pcSCP1c. Site-directed mutagenesis of pcSCP1c residues to the amino acids of pcSCP1b decreased the calcium K_d, identifying residues outside the EF-hand domains that contribute to calcium binding in crayfish SCP.

1. Introduction

Sarcoplasmic calcium binding protein (SCP) is an invertebrate EF-hand calcium binding protein (Hermann and Cox, 1995).[1] Similar to vertebrate parvalbumin, SCP binds calcium with high affinity (binding constants ~10^{-7} to 10^{-8} M) and is highly expressed in fast-twitch muscle (Cox et al., 1976; Wnuk et al., 1979; Heizmann et al., 1982; Wnuk and Jauregui-Adell, 1983; Leberer and Pette, 1986a,b). However, the calcium binding properties of SCP differ from those of vertebrate parvalbumin. Each ~12 kDa parvalbumin molecule contains two high-affinity calcium binding sites that bind magnesium with lower affinity (Haiech et al., 1979; Schwaller, 2010). In contrast, crayfish SCP exists as a dimer of ~22 kDa subunits with 6 high-affinity calcium binding sites. Two of these sites are calcium specific while four also bind magnesium. The calcium specific sites and two of the calcium-magnesium sites demonstrate positive cooperativity, while the other two calcium–magnesium sites demonstrate negative cooperativity (Wnuk et al., 1979).

Although the precise function of SCP remains unknown, comparisons to parvalbumin offer working hypotheses. Parvalbumin probably speeds relaxation of skeletal muscles by binding free calcium concentration in the sarcoplasm following contractions (Muntener et al., 1995; Schwaller et al., 1999; Chin et al., 2003). Additionally, parvalbumin may buffer calcium levels during sustained contractions, thereby limiting increases in calcium levels that could impair muscle function. Similar roles for SCP are plausible, but no direct evidence supports such roles. Also, Hermann and Cox (1995) suggest that SCP may serve to regulate intracellular magnesium levels.

SCP cDNAs have been cloned from several invertebrates, including shrimp, *Drosophila*, and crayfish (Takagi and Konishi, 1984; Jauregui-Adell et al., 1989; Pauls et al., 1993; Kelly et al., 1997; Gao et al., 2006). *Procambarus clarkii* SCP (pcSCP1) is expressed most highly in skeletal muscle but is also found in cardiac muscle (Gao et al., 2006). Recently, we described three splice variants of crayfish SCP (pcSCP1a, pcSCP1b, and pcSCP1c) that differ in sequence across a 37 amino acid region between the second and third EF-hand domain (White et al., 2011). All three variants are highly expressed in fast-twitch muscle. The functional significance of these variants is not understood, but comparisons to parvalbumin again offer some insight.

Fish express multiple isoforms of parvalbumin, with variation across different muscle subtypes. Trout expresses at least two parvalbumin isoforms: Parv 1 and Parv 2. Red muscle of trout parr mainly expresses

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[1] Abbreviations: SCP, sarcoplasmic calcium binding protein; pcSCP, *Procambarus clarkii* sarcoplasmic calcium binding protein.

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the Parv1 isoform, while the relative expression of Parv1 and Parv2 is approximately equal in white muscle of parr (Coughlin et al., 2007). Eight isoforms of parvalbumin have been identified in carp (Brownridge et al., 2009). Expression of some of these isoforms differs across a longitudinal gradient. For example, isoforms β4 and β5 are more prevalent in anterior axial muscle, which has faster relaxation kinetics than posterior axial muscle. Differential expression of isoforms has been observed in other fish (Schoenman et al., 2010). Additionally, functional analyses have shown that parvalbumin isoforms can display different calcium binding properties. For example, organic osmolytes have a greater influence on calcium binding by stingray PVII than on PVI (Heffron and Moerland, 2008). These studies suggest that differential expression and binding dynamics of parvalbumin isoforms may contribute to functional differences across muscles.

Manipulating the divalent cation affinities of EF-hand calcium binding proteins is an emerging therapeutic strategy (Zhang et al., 2011; Wang et al., 2013). Such work depends upon knowledge of the mechanisms that underpin divalent cation affinities. For example, recent work demonstrating that engineered troponin C can improve calcium sensitivity in diseased cardiac muscle relies upon prior work showing that conversions of hydrophobic amino acids to polar amino acids sensitize cardiac troponin C to calcium (Tikunova and Davis, 2004; Liu et al., 2012).

The crayfish SCP splice variants offer the opportunity to explore the role of SCP and to probe structure-function relationships of EF-hand calcium binding proteins. Here, we employ tryptophan fluorescence to measure conformational changes of the three splice variants in response to calcium, as alternative splicing has been shown to influence protein structure and function (Stetefeld and Ruegg, 2005; Ochoa-Leyva et al., 2013). We test that hypothesis that the splice variants have different calcium binding affinities and cooperativities.

2. Methods

2.1. Construction of expression vectors

Full length cDNAs encoding pcSCP1a (GenBank accession no. **JF692202**), pcSCP1b (GenBank accession no. **JF692203**), and pcSCP1c (GenBank accession no. **JF692204**) (White et al., 2011) were subcloned into the pET21b expression vector (Novagen, EMD Chemicals Inc., Darmstadt, Germany) using standard techniques. In brief, an NdeI restriction site was added to the 5′ untranslated region of the three pcSCP1 variants in the TOPO TA cloning vector (Invitrogen, Life Technologies, Grand Island, NY). pcSCP1 open reading frames were then transferred from the TOPO TA cloning vector to the pET21b expression vector using the NdeI and XhoI restriction sites. T7 Express Competent *Escherichia coli* (New England Biolabs, Ipswich, MA) were transformed with the resultant plasmids. Each plasmid was sequenced on forward and reverse strands (Retrogen, San Diego, CA).

Expression plasmids containing pcSCP1c with targeted mutations were generated and transformed into *E. coli* (BL21(DE3)) by a commercial service (TOP Gene Technologies, Quebec, Canada). For each point mutation, an amino acid in pcSCP1c was changed to an amino acid that is conserved in pcSCP1a and pcSCP1b (White et al., 2011). Nucleotide 250, originally A, was mutated to C to generate pcSCP1c with glutamine instead of lysine at position 84 (pcSCP1c-K84Q). Nucleotides 310 and 311, originally GT, were mutated to AC to substitute tyrosine for valine at position 104 (pcSCP1c-V104Y). Both sites were mutated to generate the double mutant pcSCP1c-K84Q-V104Y.

2.2. Expression and purification of pcSCP1 proteins

Cells were grown in LB broth to OD600 ~ 0.2 and expression was induced with IPTG. After 2 h at 37 °C with shaking at 225 rpm, cells were collected by centrifugation, resuspended in cold Tris–HCl (20 mM, pH 7.9), sonicated (50% intensity, 3 times 10 s), and centrifuged at 13,000 *g* for 10 min to remove debris. To purify SCP protein from crayfish muscles, Cox et al. (1976) used gel filtration chromatography on Sephadex® G-100 columns followed by anion exchange chromatography on DEAE cellulose columns. Because pcSCP1 protein already represented a large fraction of soluble protein in induced cells, we found chromatography on 35 × 1 cm DEAE cellulose columns to be sufficient. Soluble extracts were loaded onto a column and washed for 30 min at 2 ml/min with Tris–HCl (20 mM, pH 7.9). Proteins were eluted with a high salt buffer (1 M NaCl, 20 mM Tris–HCl, pH 7.9) applied as a gradient from 0 to 100% over 280 min at 1.75 ml/min. Fractions were collected every 3.5 min and analyzed by protein assay (BCA reagent, Thermo Scientific, Brockford, IL).

Fractions containing protein were analyzed by SDS-PAGE using 12% Tris-HCl polyacrylamide gels, Laemmli sample loading buffer, and tris/glycine/SDS running buffers (Mini-PROTEAN® Tetra System, BioRad, Hercules, CA) followed by Coomassie staining. Fractions with substantial pcSCP1 (identified by molecular weight) were concentrated and had their buffer exchanged for storage buffer (200 mM MOPS, 10 μM $CaCl_2$, pH 7.4) using centrifugal concentrators (30 kDa cutoff, EMD Millipore Corporation, Billerica, MA). Total protein concentration was determined by BCA assay and Coomassie stained gels were analyzed using ImageJ to determine the percent purity of pcSCP1. For each variant, all fractions with a high purity (at least 85%) of pcSCP1 were combined and diluted to 6.25 μM. Select fractions were evaluated by native PAGE on 12% Tris–HCl polyacrylamide gels with Tris–glycine buffer systems. Some of the purified protein was stored at −20 °C in single-use aliquots for fluorescence experiments, and the remainder was stored at −70 °C.

2.3. Tryptophan fluorescence

pcSCP1 samples were diluted to 250 nM with a MOPS/EGTA buffer (200 mM MOPS; 2 mM EGTA; 0, 1, or 10 mM MgCl2; pH 7.45; 2.5 mL final volume) in a quartz cuvette. For experiments with pcSCP1c mutants, the buffer pH was 7.15, which did not significantly alter the response of pcSCP1c wild-type protein. Fluorescence spectra were obtained using a SPEX FluoroMax-3 fluorescence spectrophotometer (Jobin Yvon, Inc, Edison, NJ) with excitation at 295 nm and emission at 303–500 nm (1 nm increments, 0.3 s/nm, 2 nm slit widths) at 21 °C. Three scans were averaged for each measurement. Fluorescence of the MOPS/EGTA buffer was measured for background subtraction. Free calcium concentrations were determined based on the pH, ionic strength, and concentration of EGTA, Ca^{2+}, and Mg^{2+} using the MaxChelator program (http://maxchelator.stanford.edu/CaMgATPEGTA-TS.htm). To explore calcium binding, spectra were obtained in fifteen increasing concentrations of free Ca^{2+} ranging from approximately 10^{-11} to 10^{-5} M for an individual sample of pcSCP1. Spectra were taken approximately 5 min after each addition of Ca^{2+}. Preliminary experiments showed no differences in integrated fluorescence values over the first 5 min after altering calcium concentration.

2.4. Analysis of fluorescence spectra

Blank-subtracted spectra were integrated and normalized based on the extreme high and low values within each experiment. To determine apparent Ca^{2+} binding affinity and cooperativity, we first applied the standard Hill model (Tables 1–3). In several instances, we were unable to obtain reasonable estimates of the Hill coefficient due to an insufficient number of data points across the calcium concentrations in the variable portion of the binding curve. Also, the standard Hill model sometimes failed to capture features of the binding curves, especially for pcSCP1c (Fig. 1) and pcSCP1b (data not shown).