CYANOGEN BROMIDE PEPTIDE FROM BOVINE CARDIAC MYOSIN CONTAINING TWO ESSENTIAL THIOLS

Evidence for sequence homology with skeletal myosin in the region of the active site

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1. Introduction

In cardiac and skeletal myosin, modification of one essential thiol group per myosin head, the so-called SH_1 thiol, produces an increase in Ca^{2+}-ATPase activity and a simultaneous loss of K^+(EDTA)-ATPase activity [1,2]. From these data, together with changes in the rate of hydrolysis of ATP analogs after thiol modification, it has been concluded that the SH_1 thiol may be involved in the binding of nucleotide to the active site [2,3]. Furthermore, Seidel [4] and Mendelson et al. [5] have suggested on the basis of spin- and fluorescence-labeling experiments that the SH_1 thiol groups are at or near the actin binding site of myosin.

A second thiol group, the so-called SH_2 thiol, also has been implicated in the regulation of myosin ATPase activity [6]. Seidel [7] showed that selective modification of the SH_2 thiols produces changes in ATPase activities and nucleotide specificity that are similar to those observed following modification of the SH_1 thiols. Burke et al. [8] have found that when MgATP is bound to myosin the two essential thiol groups, SH_1 and SH_2, are brought into close spatial proximity since in this situation they can be cross-linked by the bifunctional reagent, *N,N'-p*-phenylenedimaleimide, which has a cross-linking span of 12–14 Å.

Very little information has been available about the primary structure of the myosin heavy chains in the region of the two essential thiol groups. Yamashita et al. [9] have summarized the studies on the peptides from skeletal myosin that contain SH_1 and SH_2 thiols and have concluded that the sequence around SH_2 is Arg–Cys–Asn–Gly–Val–Leu and that the sequence around SH_1 is Glu–Gly–Ile–Arg–Ile–Cys–Arg. Recently, Elzinga and Collins [10] have reported the isolation of a cyanogen bromide peptide of 92 residues from skeletal myosin that contains the two essential thiols in exactly these sequences. Here we report the isolation of a cyanogen bromide fragment from bovine cardiac myosin that contains these two thiols in a sequence closely homologous to the skeletal myosin peptide. This structural similarity between skeletal and cardiac myosins in the region at or near the active site may explain many of the similarities in their enzymatic properties.

2. Experiments and results

Myosin was isolated from about 600 g bovine left ventricle by the method of Banerjee et al. [3]. The most rapidly reacting class of thiols in myosin, the so-called SH_1 thiols, were alkylated using iodo-[^{14}C]-acetamide according to the method [11]. After

dialysis to remove the labeled alkylating reagent, the protein was denatured by addition of dry urea to a final concentration of 8 M. Dithioerythritol was added in 100-fold excess over the number of moles of cysteine in myosin. The mixture was stirred overnight under nitrogen and the unreacted thiols were blocked using non-radioactive iodoacetamide. Myosin preparations contained about 1.2–1.4 mol [^{14}C]carboxymethyl cysteine/mol. Values obtained from amino acid analysis varied from 38–42 mol carboxymethyl cysteine/mol, indicating that the cysteinyls had reacted almost quantitatively.

Figure 1 shows the electrophoretic pattern obtained with a cyanogen bromide digest of cardiac myosin in which the rapidly reacting thiols were [^{14}C]carboxymethylated. The major radioactive peak was associated with a band having an apparent molecular mass of about 8300.

Fractionation by gel filtration of a cyanogen bromide digest of cardiac myosin in which the rapidly reacting thiols were labeled is shown in fig.2. The fractions indicated were pooled, lyophilized and applied to a column of SP–Sephadex in 8 M urea (fig.3). Fractions under the major radioactive peak were pooled and lyophilized. Removal of small amounts of contaminating non-radioactive peptides was accomplished by passage over a 1 × 10 cm column of Dowex 1-X8 which had been equilibrated with 8 M urea and 0.1 M Na_2CO_3–$NaHCO_3$ buffer, pH 8.9. Under these conditions, the radiolabeled thiol

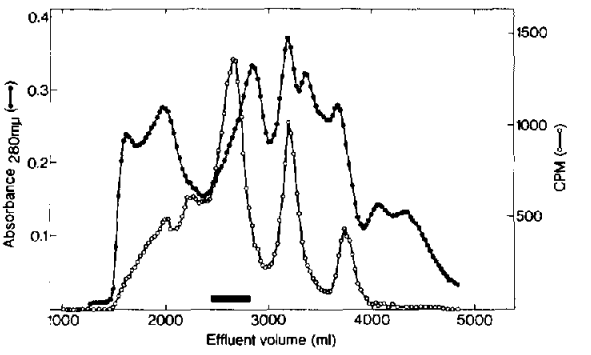

Fig.2. Chromatography of a cyanogen bromide digest of bovine cardiac myosin in which the rapidly reacting thiols were [^{14}C]carboxymethylated. About 2.5 g protein digest was dissolved in 25% acetic acid. The solution was clarified by centrifugation at 27 000 × *g* for 30 min. The supernatant solution containing about 90% of the radioactivity was placed on a 8.5 × 90 cm column of Sephadex G-75 in 25% acetic acid at 23°C and developed at a flow rate of 40 ml/h. About 10 ml fractions were collected and analyzed for absorbance and radioactivity. Fractions in the major radioactive peak were pooled as indicated and lyophilized.

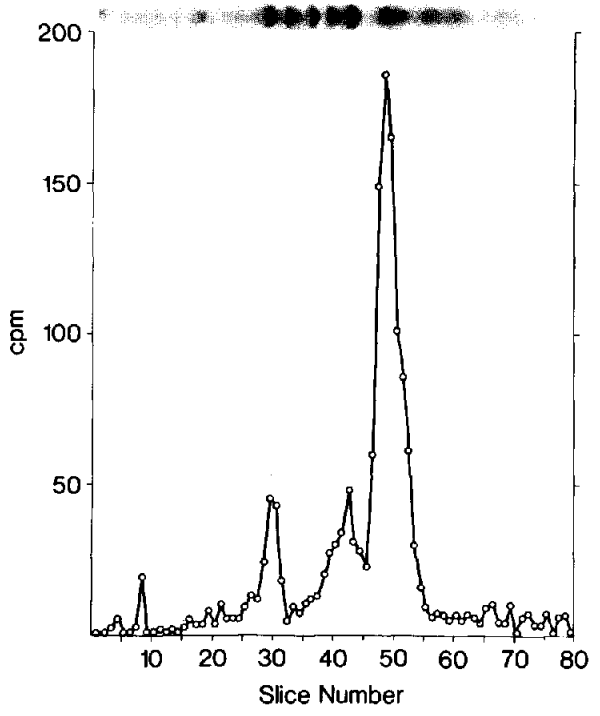

Fig.1. SDS–urea polyacrylamide gel electrophoresis of a cyanogen bromide digest of cardiac myosin in which the rapidly reacting thiols were [^{14}C]carboxymethylated. About 100 μg protein containing about 1600 cpm ^{14}C were applied to 0.5 × 12 cm disk gels prepared as described [20], except that *N,N'*-diallyltartardiamide was substituted for *N,N'*-methylene bisacrylamide [21]. The cyanogen bromide fragments of myoglobin were run simultaneously as a guide to the approx. molecular mass of the peptides. Gels were stained with Coomassie brilliant blue and destained in a mixture of 7.5% acetic acid and 25% methanol. Destained gels were frozen in dry ice, sliced at 1 mm intervals and dissolved by incubation in 0.7 ml 2% periodic acid for 1 h at 37°C. About 10 ml Aquasol (New England Nuclear) were added to each vial and the radioactivity was measured by liquid scintillation counting.

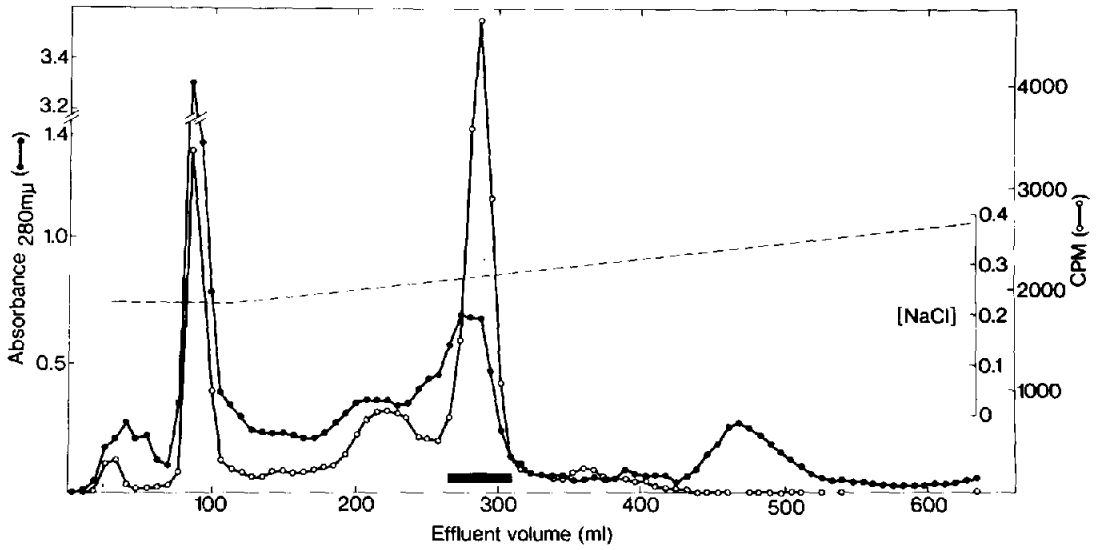

Fig.3. SP–Sephadex chromatography of the pooled major radioactive peak from the initial filtration on Sephadex G-75. About 25×10^6 ^{14}C cpm were applied to a 1 × 60 cm column of SP–Sephadex previously equilibrated at 55°C in 8 M urea and 0.01 M ammonium formate, pH 2.5. A linear 0.22–0.38 M NaCl gradient was applied and 4 ml fractions were collected at a flow rate of about 25 ml/h. Fractions were analyzed for absorbance and radioactivity and the major radioactive peak was pooled and lyophilized.

peptide was not retained on the column, suggesting that it carried an overall positive charge.

The procedure described above resulted in recovery of the radiolabeled thiol peptide with a yield of about 62%. The peptide was homogenous in SDS–urea polyacrylamide gels (fig.4) and co-electrophoresed with the major labeled peak in myosin digests. The apparent molecular mass of this peptide as determined from SDS–urea polyacrylamide gels and the amino acid composition (table 1) appeared to be similar to that of the SH_1–SH_2 thiol peptide recently isolated from skeletal myosin [10]. The skeletal myosin peptide also contains the unusual amino acid residue, N^{τ}-methylhistidine, which is found in skeletal myosin, but not in cardiac myosin [12].

A partial sequence was determined by automated Edman degradation of the intact cardiac myosin peptide in a Beckman 890C sequencer. Identification of the PTH amino acids obtained from the sequencer was made by thin layer chromatography [13] and amino acid analysis following regeneration of the amino acids by hydroiodic acid hydrolysis [14]. In table 2 a partial sequence of the cardiac peptide is compared with that of a similar region of the skeletal myosin SH_1–SH_2 thiol peptide. The cardiac peptide is shown with Met next to the NH_2-terminal His

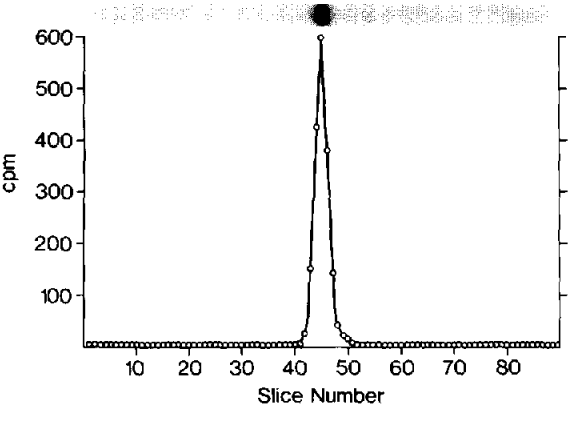

Fig.4. SDS–urea polyacrylamide gel electrophoresis of the purified labeled thiol peptide. About 1900 ^{14}C cpm labeled peptide were applied to a 0.5 × 12 cm disk gel which was prepared and analyzed for radioactivity as described in fig.1.

Table 1
Amino acid composition of SH_1-SH_2 thiol-containing peptides from bovine cardiac myosin and rabbit skeletal myosin

	Cardiac peptide		Skeletal peptide
Cysteine	1.48	(2)	2
Aspartic acid	9.14	(9)	6[a]
Threonine	2.13	(2)	2
Serine	2.69	(3)	5
Glutamic acid	13.40	(13)	11[b]
Proline	2.15	(2)	2
Glycine	6.33	(6)	8
Alanine	5.73	(6)	5
Valine	2.81	(3)	5
Isoleucine	4.75	(5)	6
Leucine	11.14	(11)	12
Tyrosine	1.77	(2)	3
Phenylalanine	4.07	(4)	6
Lysine	8.43	(8)	9
Histidine	3.44	(3)	3
N^{τ}-Methylhistidine	–		1
Arginine	6.14	(6)	5
Homoserine	0.85	(1)	1
		86	92

[a] Includes two residues asparagine
[b] Includes four residues glutamine

Each value on the cardiac peptide is the average of six determinations. The cysteine content was determined as carboxymethyl cysteine. The composition of the peptide from skeletal myosin has been published [10]

because the adjacent peptide would have to end in Met in order for cyanogen bromide cleavage to occur. It is evident that the first 28 residues of the cardiac peptide correspond almost exactly to positions 7–34 of the skeletal peptide. The cardiac and skeletal peptides differ at only three positions and all of the substitutions are conservative.

Determination of the radioactivity at each step during Edman degradation of the cardiac peptide revealed that about 94% of the counts were associated with the carboxymethyl cysteinyl in step 15. This corresponds to the cysteinyl residue at position 21 in the skeletal peptide (table 2), which was identified previously as the SH_1 thiol [9]. About 6% of the counts were found at step 5 in association with the carboxymethyl cysteine residue which corresponds to the cysteinyl at position 11 in the sequence of the skeletal myosin peptide. This cysteinyl has been

Table 2
Comparison of the partial sequence of the SH_1-SH_2 thiol peptide from bovine cardiac myosin with residues 1–34 of a similar peptide from rabbit skeletal myosin [10]

	1	2	3	4	5	6	7	8
Bovine cardiac						(Met)		
Rabbit skeletal	Glu	His	Glu	Leu	Val	Leu	His	Gln

	9	10	11	12	13	14	15	16	17	18	19	20	21
	Leu	Arg	Cys	Asn	Gly	Val	Leu	Glu	Gly	Ile	Arg	Ile	Cys

	22	23	24	25	26	27	28	29	30	31	32	33	34
	Arg	Lys	Gly	Phe	Pro	Asn / Ser	Arg	Ile	Leu	Tyr	Gly / Ala	Asp	Phe

previously identified as the SH_2 thiol [9]. Thus the two essential thiols in both cardiac and skeletal myosin are separated by only 9 residues. Moreover, the striking differences in reactivity of the two thiol groups in cardiac myosin are consistent with earlier observations on the reactivity of these thiols in skeletal myosin [15].

If the two thiol residues are at or near the nucleotide binding site, it seems likely that this region of the molecule would contain both a hydrophobic pocket for the nucleoside ring and one or more basic residues, probably Arg [16], that would electrostatically interact with the polyphosphate side chain. The Arg residues at positions 10, 19 and 22 (skeletal myosin peptide numbering) are all close to either SH_1 or SH_2, providing possible binding sites. The amino acid residues at the NH_2-terminus of the peptide (4–31 in the skeletal peptide and 9–31 in the cardiac peptide) include a high proportion of hydrophobic side chains that could form a hydrophobic pocket.

Although the binding of adenine nucleotides to cardiac myosin has not been studied extensively, Banerjee and Morkin [17] have found that about 1.5 mol MgADP are bound/mol bovine cardiac myosin in 0.5 M KCl, 4°C, with an association constant of 1.8×10^5 M^{-1}, which is similar to values reported recently for binding of MgADP to rabbit skeletal myosin [18,19]. These findings would be consistent with the strong preservation of sequence homology between possible nucleotide binding regions of these proteins noted in this report.

Acknowledgements

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