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Determination of the Complete Amino Acid Sequence of Bovine Cardiac Troponin C†

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ABSTRACT: The amino acid sequence of bovine cardiac troponin C has been completely determined. The protein was cleaved by cyanogen bromide and the resulting peptides were isolated. All of the 161 residues of the protein could be accounted for in 12 cyanogen bromide peptides. Overlapping peptides were generated by tryptic digestion of citraconylated troponin C and isolation of the resulting five peptides. The primary structure of cardiac troponin C was elucidated by sequential manual Edman degradation of these peptides. It consists of four homologous regions, one of which probably has lost the ability to bind calcium ions. By comparing the amino acid sequence of cardiac troponin C with the sequence of skeletal troponin C, it was found that the mutation rate of the region that does not bind calcium is almost twice as high as the mutation rate of the three homologous regions that do bind calcium.

Troponin plays an important role in the regulation of muscular contraction (Ebashi and Endo, 1968). One of its subunits (troponin C) has a strong affinity for calcium (Hartshorne and Pyun, 1971) and upon calcium binding it undergoes a large conformational change (van Eerd and Kawasaki, 1972) which triggers a set of events resulting in muscular contraction.

The almost complete amino acid sequence of rabbit skeletal troponin C has been reported by Collins et al. (1973). Troponin C is homologous to parvalbumins, a group of calcium binding proteins with a molecular weight around 12 000 (Pechère et al., 1971b, Collins et al., 1973). More recently Collins has indicated that troponin C is also homologous to the "alkali light chains" of myosin (Collins, 1974). The three dimensional structure of a parvalbumin of carp muscle has been determined by x-ray diffraction analysis,

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allowing the exact localization of the two calcium ions (Kretsinger and Nockolds, 1973). By comparing the amino acid sequence of this parvalbumin with the sequence of skeletal troponin C, Collins et al. (1973) tentatively indicated four calcium binding sites in troponin C. Troponin C shows internal homology consisting of four homologous regions, each containing one calcium binding site. From this they deduced that troponin C has evolved through gene duplication from a precursor protein of 38–40 amino acid residues containing one calcium binding site. Internal homology of the amino acid sequence of the "alkali light chains" of myosin is more speculative (Weeds and McLachlan, 1974). The "alkali light chains" do not bind calcium. It is assumed that in the evolutionary process they have lost the ability to bind calcium (Weeds and McLachlan, 1974).

Tsukui and Ebashi (1973) have published a method to obtain pure cardiac troponin in good yield and to isolate troponin C from it. In a short communication we have reported the amino acid sequence of bovine cardiac troponin C and we have mentioned that one of the four homologous regions has probably lost the ability to bind calcium (van Eerd and Takahashi, 1975). In this paper we present a detailed report on the determination of the amino acid sequence of bovine cardiac troponin C. From the comparison of the sequence of this protein with the sequences of skeletal troponin C, parvalbumins, and the "alkali light chains" of myosin, it may also be concluded that the mutation rate of these proteins is considerably increased when the ability to bind calcium is lost.

Experimental Section

Materials

Bovine cardiac troponin C prepared according to Tsukui and Ebashi (1973) was generously provided by Professor S. Ebashi.

Extra pure reagents, specially purified for sequencing, were bought from Tokyo Kasei Kogyo Co. Ltd. and Wako Pure Chemical Industries Co. Ltd.

Methods

Carboxymethylation. Troponin C was carboxymethylated essentially according to Crestfield et al. (1963). Troponin C (33 mg), dissolved in a solution of 8 M urea, 0.15 M Tris-HCl, pH 8.6, and 0.3 mM EGTA[1], was flushed with nitrogen. β-Mercaptoethanol (20 μl) was added and the solution was kept stirring under nitrogen. After 4 h at room temperature, 100 μl of a freshly prepared solution of neutralized iodoacetic acid was added, making sure that the iodoacetic acid was slightly less on a molar basis than the amount of β-mercaptoethanol. After 15 min reaction time, while the solution was protected from light, the carboxymethylated troponin C was desalted on a column (2.1 × 30 cm) of Sephadex G-25. It was eluted with distilled water. The desalted protein was subsequently freeze-dried.

Cyanogen Bromide Cleavage and Isolation of Fragments. Cyanogen bromide cleavage was essentially as described by Gross and Witkop (1962) with the modifications of Steers et al. (1965). Freeze-dried carboxymethylated troponin C (33 mg) was suspended in 3.5 ml of 70% formic acid. A 50-fold molar excess of cyanogen bromide over methionine residues was added, and the reaction was allowed to proceed at room temperature for 24 h. Subsequently the reaction mixture was diluted with 10 volumes of water and freeze-dried.

Gel Filtration. The freeze-dried material after cyanogen bromide cleavage was dissolved in a minimum volume of 0.1 N ammonia, applied to a column (0.9 × 400 cm) of Sephadex G-50 fine, and eluted with 10 mM NH_4HCO_3.

High-Voltage Paper Electrophoresis. The small peptides (≤10 residues) were further purified by high-voltage electrophoresis. Peptide mixtures were streaked on Whatman 3MM paper (0.1 μmol of peptide per 2 cm of width). Electrophoresis was performed at 2500 V/60 cm using pH 3.5 buffer (pyridine–acetic acid–water, 1:10:89 v/v/v). Peptides were localized by staining a guide strip with a solution of 0.2% ninhydrin in acetone. Peptides were eluted with 10% acetic acid and stored at −20 °C.

Dowex 50-X2 Chromatography. Cyanogen bromide peptides of intermediate size (between 10 and 20 residues) were concentrated by rotatory evaporation, acidified to pH 2.5, and applied to a column (0.9 × 45 cm) of Dowex 50-X2 (200 mesh) maintained at 26 °C. The resin was equilibrated with starting buffer of 0.2 M pyridine–acetic acid, pH 3.1. The column was developed with 1 column volume of starting buffer, 5 column volumes of a gradient of starting buffer to 2 M pyridine–acetic acid, pH 5.1, and finally with 3 column volumes of 4 M pyridine–acetic acid, pH 5.6. The eluate was monitored for peptides by alkaline hydrolysis and ninhydrin color development of aliquots of every other fraction (Hirs et al., 1956). The peptide-containing fractions were pooled and stored at −20 °C.

DEAE-Sephadex A-25 Chromatography. Larger peptides (≥20 residues) were concentrated by rotatory evaporation and applied to a column (0.8 × 25 cm) of DEAE-Sephadex A-25 equilibrated with 20 mM Tris-HCl, pH 8.2. The column was developed with 5 column volumes of a KCl gradient (0–1.0 M KCl) in starting buffer. Peptides were localized by reading the absorbance at 230 or 210 nm. Peptides were pooled and desalted by passing though a column of Sephadex G-25 (2.1 × 30 cm, eluent 10 mM NH_4HCO_3). The desalted peptides were stored at −20 °C.

Further Fragmentation of Cyanogen Bromide Peptides. Peptides too big for direct sequencing were digested further by trypsin or chymotrypsin. The nomenclature used for the peptides is explained at the end of the Experimental Section.

Fragment CB2. CB2 (300 nmol) in 1 ml of 0.1 M NH_4HCO_3 was digested with 60 μg of trypsin (Worthington TRTPCK) for 1 h at 37 °C. After digestion the sample was directly applied to a Sephadex G-25 column (0.9 × 200 cm) and eluted with 10 mM NH_4HCO_3. The two peptides (CB2-T2 and CB2-T3) eluting at the void volume were separated by DEAE-Sephadex A-25 chromatography using a gradient from 0–1.0 M KCl. The three smaller peptides eluting from the Sephadex G-25 column as one broad peak were separated by high-voltage electrophoresis at pH 3.5 (CB2-T1, CB2-T4, and CB2-T5).

Fragment CB2-T3. This peptide was further digested with chymotrypsin: 190 nmol in 0.8 ml of 0.1 M NH_4HCO_3 was digested with 30 μg of chymotrypsin (Worthington CDI) for 3 h at 40 °C. The digestion mixture was applied to a column (0.9 × 200 cm) of Sephadex G-25 and eluted with 10 mM NH_4HCO_3. Two peaks appeared. The first was a dodecapeptide (CB2-T3-C2), and the second a hexapeptide (CB2-T3-C1).

[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Pth, phenylthiohydantoin; PAM, minimum accepted point mutations per 100 residues.

Fragment CB5. This peptide (130 nmol) in 1.0 ml of 0.1 M NH_4HCO_3 was digested with 15 μg of chymotrypsin (Worthington CDI) for 3 h at 37 °C. The digestion mixture was applied to a column (0.9 × 200 cm) of Sephadex G-25 and eluted with 10 mM NH_4HCO_3. A large peptide eluted at the void volume and was well separated from the COOH-terminal tripeptide (CB5-C1).

Isolation of Overlapping Peptides. In order to be able to align the cyanogen bromide peptides, the lysyl groups of carboxymethylated bovine cardiac troponin C were reversibly blocked with citraconyl groups. Then the protein was digested by trypsin so that the peptide chain could only be cleaved at the four arginine residues. The resulting five peptides were isolated.

Citraconylation of Lysyl Residues. Citraconylation was essentially according to Atassi and Habeeb (1972) with minor modification. Lyophilized carboxymethylated bovine cardiac troponin C (23 mg) was dissolved in 3 ml of 6 M guanidine hydrochloride containing 0.5 mM EGTA[1]. The pH was adjusted with 6 N NaOH on a pH-stat to pH 8.2. Citraconic anhydride (120 μl) was added in 5–10 μl amounts, making sure that the pH did not drop below pH 7. After completion of the addition, the solution was kept at pH 8 for 30 min and subsequently deslated on a column (2.1 × 30 cm) of Sephadex G-25. The citraconylated protein was eluted with 0.1 M NH_4HCO_3, pH 8.2.

Tryptic Digestion of Citraconylated Troponin C. The citraconylated protein in 19 ml of 0.1 M NH_4HCO_3 and 0.3 mM EGTA was digested with 0.25 mg of trypsin (Worthington TRTPCK) at 40 °C. The digestion mixture was gently shaken by hand every 10–15 min. After 2 h, 0.50 mg of soybean trypsin inhibitor (Worthington SI) was added.

Tryptic peptides of citraconylated troponin C were sized into three fractions by chromatography on a column (0.9 × 200 cm) of Sephadex G-50 fine. The citraconyl groups of the three fractions (I, II, and III) were removed by lowering the pH with formic acid to pH 2.0 and incubating the solution at room temperature for 2 h. Fractions I and II were separated into two fractions each, by DEAE-Sephadex A-25 chromatography. Fraction III contained only one peptide.

Amino Acid Analysis. Samples were hydrolyzed in evacuated sealed tubes with constant boiling HCl for 24 h at 110 °C. Amino acid analysis was performed on a JEOL JLC-5AH automatic amino acid analyser using a two-column system. Maximum sensitivity per column was about 2 nmol. Homoserine eluted between serine and glutamic acid. The values for threonine and serine were corrected by 5 and 15%, respectively, for hydrolytic losses.

Sequence Determination. Manual Edman degradation was essentially according to Edman (1970), using the apparatus described by Iwanaga and Samejima (1970) and using some of the experimental modifications of Peterson et al. (1972). A peptide (100–300 nmol) in a glass-stoppered 3-ml centrifuge tube was dried in a stream of nitrogen at 50 °C. The peptide was dissolved in 100 μl of a buffer solution of 0.4 M dimethylallylamine in 1-propanol–H_2O (60:40 v/v) adjusted to pH 9.5 with trifluoroacetic acid. Phenyl isothiocyanate (5 μl) was added and, after flushing the tube with nitrogen and mixing the reagents, the coupling was allowed to proceed for 30 min at 50 °C. After coupling, the solution was twice extracted with 0.3 ml of benzene. The aqueous phase was subsequently freeze-dried, using the apparatus described by Iwanaga and Samejima (1970), and remaining reagents were removed by sublimation in vacuo (≤50 mTorr) at 50 °C for 20 min. Cleavage was performed by dissolving the dried peptide in 50 μl of trifluoroacetic acid and incubating it at 50 °C under nitrogen. After 10 min the trifluoroacetic acid was removed in a stream of nitrogen, 0.2 ml of water was added, and the thiazolinone was removed from the water layer by extracting it three times with 0.5 ml of peroxide-free and aldehyde-free ethyl acetate. The next coupling could be started after freeze-drying the peptide-containing water layer.

Conversion and Identification of Pth[1]-Amino Acids. The ethyl acetate extract was dried in a stream of nitrogen at 50 °C, and conversion was carried out by heating the thiazolinone for 10 min at 80 °C in 0.2 ml of 1 N HCl under nitrogen (Ilse and Edman, 1963). The Pth-amino acids were extracted with ethyl acetate (3 × 0.5 ml) except Pth-Arg which remained in the water layer. Pth-amino acids were identified by a combination of gas–liquid chromatography (Pisano et al., 1962) and thin-layer chromatography (Kulbe, 1974). For gas–liquid chromatography, a JEOL JGC-20KFP gas chromatograph was used with a 6 ft × 2 mm glass column filled with 80–100 mesh Gaschrome Q coated with 10% SE-30 as liquid phase. Chromatography was performed isothermally at 240 °C. Thin-layer chromatography was performed on 5 × 5 cm polyamide layer plates (Schleier and Schüll) according to Kulbe (1974). In this way all Pth-amino acids extracted in the ethyl acetate phase could be identified except Pth-Ile and Pth-Leu. To discriminate between these two, HI hydrolysis, followed by amino acid analysis, was performed (Smithies et al., 1971). Pth-Arg was identified in the water phase by thin-layer chromatography using Kulbe's (1974) system.

Braunitzer Treatment. One tryptic peptide (CB2-T3-C2) was treated with Braunitzer reagent I (4-sulfophenyl isothiocyanate, Pierce Comp.) to modify the COOH-terminal lysine in order to make the peptide more hydrophilic (Braunitzer et al., 1970). This reagent was used in the first step of the Edman degradation instead of phenyl isothiocyanate. About a fivefold molar excess was used.

Carboxypeptidases A and B Digestion. COOH-terminal residues were identified by digesting the peptides with carboxypeptidase A and/or B (Worthington COADFP and COBDFP). Experimental conditions were essentially those of Ambler (1972). Samples of the digestion mixture were taken at time intervals, taken up in pH 2.2 citrate buffer, and directly applied on the amino acid analyser.

Determination of Amino-Terminal Acetyl Group. The NH_2-terminal acetyl group was determined by hydrazinolysis followed by dansylation at pH 3 according to Schmer and Kreil (1969).

Nomenclature of Peptides. The peptides are numbered starting from the NH_2-terminal of the complete amino acid sequence. CB stands for cyanogen bromide peptide, T for tryptic peptide, C for chymotryptic peptide, and Tc for tryptic peptide of citraconylated troponin C. If a peptide was isolated after tryptic digestion of a cyanogen bromide peptide, it was named CBnumber-Tnumber.

Results

Isolation of Cyanogen Bromide Peptides. In Figure 1 is shown how the 12 cyanogen bromide peptides were isolated. The peptides were first fractionated according to size by Sephadex G-50 chromatography (see Figure 1a) and four peaks were further purified.

Peak I. This contained the largest cyanogen bromide peptide (CB2, 44 residues). It was purified on DEAE-Sepha-

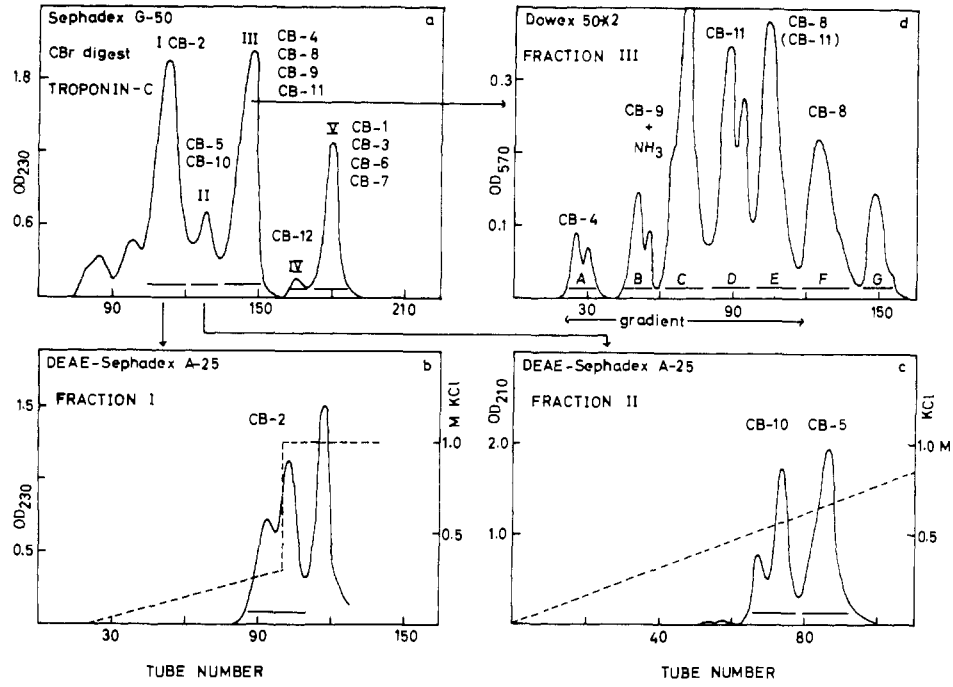

FIGURE 1: Isolation of the cyanogen bromide peptides of carboxymethylated bovine cardiac troponin C. Peaks were pooled as indicated by horizontal bars. The volume of one tube was 1.3 ml in Figure 1a, 1.2 ml in Figure 1b, 1.4 ml in Figure 1c, and 1.8 ml in Figure 1d. The gradient of pyridine-acetic acid [0.2 (pH 3.1) to 2 M (pH 5.1)] in Figure 1d was started at tube 20 and was finished at tube 100.

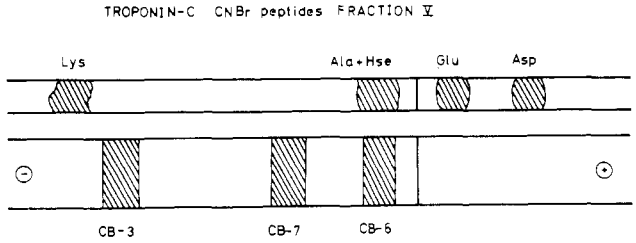

FIGURE 2: Separation of the cyanogen bromide peptides of fraction V of Figure 1a by high-voltage electrophoresis. Electrophoresis was performed for 45 min at 2500 V using a buffer of pH 3.5. The samples were applied on the vertical line. The migration distance of lysine was 14.0 cm.

dex A-25 (see Figure 1b). The pure peptide eluted as a double peak (homoserine and homoserine lactone form). Contaminating partially cleaved peptides were eluted as one single peak due to the sudden increase in ionic strength from 0.3 to 1.0 M KCl.

Peak II. The two cyanogen bromide peptides in peak II were separated on DEAE-Sephadex A-25 (see Figure 1c). The first one (CB10) eluted as a double peak. The second one (CB5) did not elute as a double peak but as an asymmetrical one.

Peak III. The purification of the peptides in peak III was more troublesome. Upon acidification before Dowex 50 chromatography, a precipitate formed. It was removed by centrifugation and washed with starting buffer. Amino acid analysis showed that the precipitate consisted of a pure peptide (CB9). The acid-soluble peptides were chromatographed on Dowex 50-X2 (see Figure 1d). The peptides also eluted as double peaks (homoserine and homoserine lactone form). Peak A contained pure CB4. Peak B did not contain any peptide and was probably a ninhydrin-positive contaminant. Peak C had a high color intensity but most of it was due to ammonia that eluted at this position. Peak C also contained peptide CB9 but it was not pure. So CB9 was found not only in the precipitate formed upon acidification before Dowex 50 chromatography but also in peak C. Probably this peptide has a low solubility at low pH and is partially precipitated. The next double peak, peak D, contained one pure peptide (CB11). Peak E contained CB8 but was contaminated with CB11 which could be removed by DEAE-Sephadex A-25 chromatography. Peak F contained pure CB8 and peak G did not contain any peptide.

Peak IV. This peak contained one tetrapeptide in pure form. It did not contain homoserine. Therefore it was concluded that this was the COOH-terminal peptide (CB12).

Peak V. A number of small peptides was present in this peak. The absorption at 230 nm was higher than expected from the amount of peptide material present. Probably some cyanogen bromide or formic acid used during the cyanogen bromide cleavage was not completely removed during lyophilization and eluted at this position. Three peptides (CB3, CB7, and CB6) were separated by high-voltage electrophoresis (Figure 2). Amino acid analysis of a sample from peak V without prior hydrolysis showed the presence of free homoserine (CB6). The peptide Ac-Hse (CB1) was overlooked at first because it is ninhydrin negative. However, amino acid analysis after acid hydrolysis of the unfractionated peptides from peak V showed 4 homoserine residues, and hydrazinolysis followed by dansylation revealed the presence of an acetylated peptide. This suggested the presence of Ac-Hse (CB1).

The amino acid composition, number of residues, and the overall yield of the 12 cyanogen bromide peptides of bovine cardiac troponin C are listed in Table I. The combined amino acid composition of the peptides agrees well with the amino acid composition of the original troponin C.

Subfractionation of Some Cyanogen Bromide Peptides. Because some of the larger cyanogen bromide peptides could not be sequenced completely, smaller fragments were generated by tryptic and chymotryptic digestion. CB2 was digested with trypsin and its tryptic peptides were isolated.

Table I: Amino Acid Composition of the Cyanogen Bromide Peptides of Carboxymethylated Bovine Cardiac Troponin C.[a]

	CB1	CB2	CB3	CB4	CB5	CB6	CB7
Lys		4.6 (5)					
Arg			1.0 (1)				1.0 (1)
Cys (cm)		0.8 (1)					0.5 (1)
Asp		5.2 (5)		1.1 (1)	4.9 (5)		
Thr		1.9 (2)		0.9 (1)	0.9 (1)		
Ser		1.8 (1)			1.2 (1)		
Hse[b]	1[f]	0.9 (1)	0.8 (1)	1.0 (1)	0.8 (1)	1[f]	0.7 (1)
Glu		8.0 (8)		4.8 (5)	3.1 (3)		
Pro				2.0 (2)			
Gly		3.3 (3)		1.2 (1)	2.2 (2)		
Ala		4.8 (5)					
Val		2.7 (3)			2.7 (3)		1.1 (1)
Met							
Ile		2.6 (3)			1.1 (1)		
Leu		3.0 (3)		2.0 (2)	0.9 (1)		
Tyr		0.9 (1)					
Phe		2.7 (3)			1.8 (2)		
No. of residues	(1)	(44)	(2)	(13)	(20)	(1)	(4)
Yield (%)		64	25	50	42		22

	CB8	CB9[d]	CB10	CB11	CB12	Total	Expected[c]
Lys	3.4 (3)	2.0 (2)		2.0 (2)	0.9 (1)	(13)	12.7
Arg	0.9 (1)			1.0 (1)		(4)	4.0
Cys (cm)						(2)	1.6
Asp	3.0 (3)	4.1 (4)	2.0 (2)	6.6 (7)		(27)	27.4
Thr			2.8 (3)			(7)	7.0
Ser	2.9 (3)					(5)	4.6
Hse[b]	0.8 (1)	1.0 (1)	0.8 (1)	0.9 (1)		(11)	
Glu	3.1 (3)	2.1 (2)	5.0 (5)	2.1 (2)	1.0 (1)	(29)	29.3
Pro						(2)	2.0
Gly	1.3 (1)	1.4 (1)	1.2 (1)	2.0 (2)	1.0 (1)	(12)	12.6
Ala		1.1 (1)	1.1 (1)			(7)	7.2
Val					1.1 (1)	(8)	8.0
Met							10.1
Ile		1.7 (2)	2.0 (2)	1.0 (1)		(9)	8.6
Leu	1.9 (2)	1.9 (2)	2.1 (2)	1.2 (1)		(13)	13.0
Tyr		0.8 (1)		0.6 (1)		(3)	2.3
Phe	0.9 (1)	1.1 (1)		1.8 (2)		(9)	8.9
No. of residues	(18)	(17)	(17)	(20)	(4)	(161)	
Yield (%)	39	49[e]	45	37	88		

[a] The numbers in parentheses are the numbers of residues found by sequencing. [b] Homoserine plus homoserine lactone. [c] Amino acid composition of original carboxymethylated troponin C. [d] Amino acid composition of the precipitate before Dowex 50-X2 column chromatography. [e] 17% for precipitate, 32% for peak E of Figure 1d which was not pure. [f] For details, see text.

One of the tryptic peptides (CB2-T3) was further digested with chymotrypsin. CB5 was also digested with chymotrypsin. For experimental details, see Experimental Section. The amino acid composition and the yields of these tryptic and chymotryptic peptides can be found in Table II.

Isolation of Tryptic Peptides of Citraconylated Troponin C. In Figure 3 is shown how the tryptic peptides of carboxymethylated and citraconylated troponin C were isolated. The citraconylated peptides were fractionated first on Sephadex G-50 (see Figure 3a). Fraction III contained one single peptide and, from the absence of arginine, this peptide was understood to be the COOH-terminal peptide Tc5. The citraconyl groups of fractions I and II were removed and the peptides were further purified by DEAE-Sephadex A-25 chromatography. Chromatography was performed in the presence of 2 mM β-mercaptoethanol to prevent the formation of methionine sulfoxide and consequently to prevent the peptides from eluting as multiple peaks (Harris, 1967). However, when the peptides from fraction II were chromatographed on DEAE-Sephadex A-25, many peaks appeared (see Figure 3c). The first one contained no peptide and had the smell of β-mercaptoethanol. Amino acid analysis of the other peaks revealed the presence of only two peptides (Tc2 and Tc3). Tc3 eluted as four distinct peaks. Apparently the time for the removal of the citraconyl groups had been too short and Tc3 was still partially citraconylated. A similar multiple peak pattern was evident upon chromatography of fraction I from the G-50 column on DEAE-Sephadex A-25 (see Figure 3b). This fraction contained only two peptides (Tc1 and Tc4). In one region both peptides overlapped.

Because cardiac troponin C contains only four arginine residues, five peptides were expected after citraconylation and tryptic digestion. A total of five peptides was found in fractions I, II, and III. Their amino acid composition is listed in Table III, and the combined composition accounts for the amino acid composition of the original cardiac troponin C.

Amino Acid Sequence Determination. The complete amino acid sequence of bovine cardiac troponin C, together with the individual peptides isolated and the amino acid sequence determination of a number of these peptides, is presented in Figure 4. A combination of the sequence data and a knowledge of the nature and the amino acid composition of the peptides establish the complete sequence and provides the necessary overlaps to align the peptides. Some points in Figure 4 which might need further explanation are described below.

NH_2-Terminal Ac-Met-Asp. An attempt to determine

Table II: Amino Acid Composition of Cyanogen Bromide Peptide Fragments of Carboxymethylated Bovine Cardiac Troponin C.[a]

	CB2-T1	CB2-T2	CB2-T3-C1	CB2-T3-C2	CB2-T4	CB2-T5	CB5-C1
Lys	1.0 (1)	1.9 (2)		1.0 (1)	1.1 (1)		
Cys (cm)				0.6 (1)			
Asp	2.1 (2)	1.1 (1)	1.2 (1)	1.2 (1)			
Thr		1.0 (1)		0.9 (1)			
Ser				1.0 (1)			
Hse[b]						0.8 (1)	1.0 (1)
Glu		6.0 (6)		1.4 (1)	0.9 (1)		
Gly				2.0 (2)	1.1 (1)		
Ala		2.1 (2)	2.1 (2)	1.0 (1)			
Val		1.0 (1)		0.9 (1)		1.0 (1)	0.9 (1)
Ile	1.0 (1)		0.9 (1)	1.0 (1)			
Leu		1.0 (1)		1.1 (1)	1.0 (1)		1.1 (1)
Tyr	0.9 (1)						
Phe		0.9 (1)	1.9 (2)				
No. of residues	(5)	(15)	(6)	(12)	(4)	(2)	(3)
Yield (%)[c]	32	62	38	44	30	21	25

[a] The numbers in parentheses are the number of residues found by sequencing. [b] Homoserine plus homoserine lactone. [c] Based on original cyanogen bromide peptide.

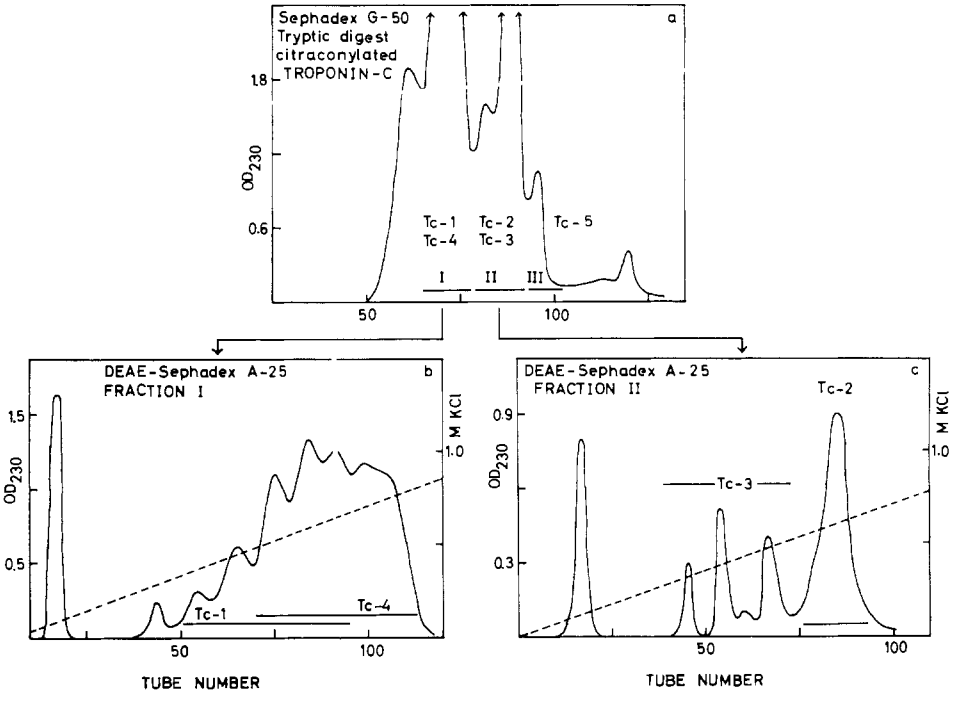

FIGURE 3: Isolation of the tryptic peptides of carboxymethylated and citraconylated bovine cardiac troponin C. Tube volumes were 1.2 ml in Figure 3a, 1.4 ml in Figure 3b, and 1.4 ml in Figure 3c.

the NH_2-terminal amino acid of cardiac troponin C using the sodium dodecyl sulfate dansyl method (Weiner et al., 1972) failed, suggesting that the NH_2-terminal is blocked.

It was concluded that Tc1 is the NH_2-terminal peptide because no amino acids were released in an attempt to sequence this peptide by the manual Edman degradation method. The amino acid composition of Tc1 and CB2 is very similar, suggesting that they originate from the same part of the troponin C molecule. Tc1 contains two more amino acids than CB2, one arginine residue that has to be placed at the COOH-terminal and one methionine residue. Because Tc1 is blocked, the extra methionine was most likely near the NH_2-terminal. To get evidence for this, the NH_2-terminal and the COOH-terminal tryptic peptides of Tc1 (Tc1-T1 and Tc1-T3) were isolated in the same way as the tryptic peptides of CB2 (see Methods). The NH_2-terminal peptide (Tc1-T1) has the same amino acid composition as CB2-T1 except for one extra methionine residue. Tc1-T1 was found to be acetylated and therefore it was concluded that the NH_2-terminal sequence of Tc1 is Ac-Met-Asp-.

CB1. The above mentioned results show that there must be a cyanogen bromide peptide Ac-Hse, which had been overlooked. It should be present in fraction V of the cyanogen bromide peptides fractionation on Sephadex G-50 (Figure 1a). Analysis of this fraction indeed revealed the existence of an acetylated peptide and a more careful analysis of the homoserine residues showed that one homoserine residue had been overlooked. This establishes the existence of CB1.

Residues 38–48. Alignment of the Tryptic Peptides of CB2. Digestion of CB2 with trypsin yielded five peptides which could be readily aligned. The amino acid sequences

Table III: Amino Acid Composition of the Tryptic Peptides of Carboxymethylated and Citraconylated Bovine Cardiac Troponin C.[a]

	Tc1	Tc2	Tc3	Tc4	Tc5	Total
Lys	4.7 (5)		2.9 (3)	4.2 (4)	1.0 (1)	(13)
Arg	1.0 (1)	0.7 (1)	0.9 (1)	1.0 (1)		(4)
Cys (cm)	0.7 (1)		0.7 (1)			(2)
Asp	5.3 (5)	6.1 (6)	3.1 (3)	11.0 (11)	2.1 (2)	(27)
Thr	2.0 (2)	1.9 (2)		2.5 (3)		(7)
Ser	1.3 (1)	1.2 (1)	2.6 (3)			(5)
Glu	7.8 (8)	8.2 (8)	3.0 (3)	8.3 (7)	2.8 (3)	(29)
Pro		2.1 (2)				(2)
Gly	3.4 (3)	3.2 (3)	0.9 (1)	4.3 (4)	0.9 (1)	(12)
Ala	4.7 (5)			2.3 (2)		(7)
Val	2.6 (3)	3.6 (4)		0.8	0.9 (1)	(8)
Met	1.7 (2)	3.4 (4)	0.8 (1)	2.7 (3)	0.9 (1)	(11)
Ile	2.6 (3)	1.0 (1)		3.5 (4)	0.8 (1)	(9)
Leu	2.8 (3)	2.7 (3)	1.9 (2)	3.7 (4)	1.1 (1)	(13)
Tyr	0.9 (1)			1.2 (1)	0.8 (1)	(3)
Phe	2.5 (3)	1.7 (2)	0.9 (1)	2.0 (1)	1.7 (2)	(9)
No. of residues	(46)	(37)	(19)	(45)	(14)	(161)
Yield (%)	16[b]	72	81	41[b]	30	

[a] The numbers in parentheses are the number of residues found by sequencing. [b] The nonoverlapping parts of Figure 3b.

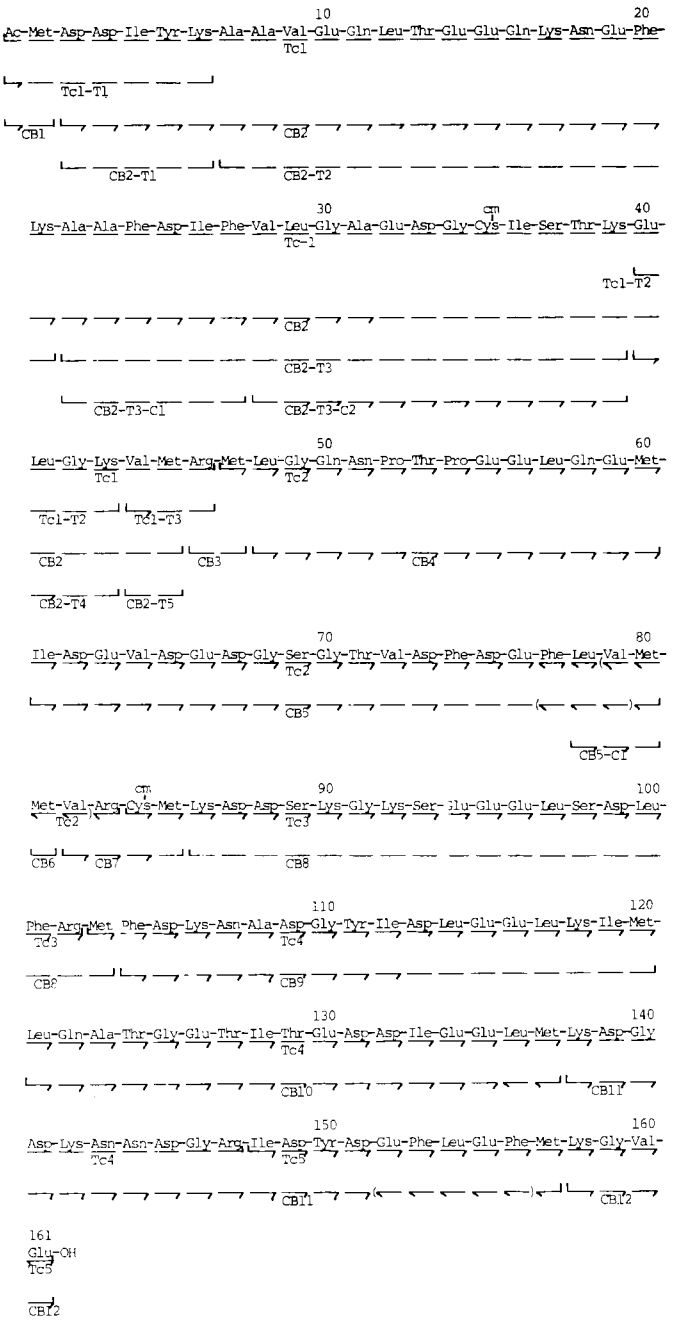

FIGURE 4: Amino acid sequence determination of cardiac troponin C. —→means sequence determined by manual Edman degradation. — means sequence determined by carboxypeptidase A and/or B digestion. ( ) indicates that the sequence within brackets was not established. CB means cyanogen bromide peptide, T means tryptic peptide, C means chymotryptic peptide, and Tc means tryptic peptide of citraconylated troponin C.

of the first two peptides (CB2-T1 and CB2-T2) were known from the sequence of the first 30 amino acid residues of CB2. The sequence of one peptide (CB2-T3) was partially known. Another peptide was the homoserine-containing COOH-terminal peptide (CB2-T5), so the remaining peptide (CB2-T4) had to be positioned between the peptide with the partially known sequence and the COOH-terminal one.

CB2-T3-C2. This peptide was treated with Braunitzer reagent I (Braunitzer et al., 1970) and sequenced. The first amino acid could not be identified because it was split off as the 4-sulfothiazolinone. For the same reason the COOH-terminal amino acid lysine could not be determined.

CB2-T4. This tryptic tetrapeptide was not treated with Braunitzer reagent I before sequencing. The first two amino acids were determined by Edman degradation starting with 40 nmol of peptide. The third amino acid residue could not be determined because of extraction losses. The peptide is a tryptic peptide, so lysine has to be the COOH-terminal and this establishes the sequence Glu-Leu-Gly-Lys.

CB2-T5. Its amino acid composition is Val 1.0, Hse 0.8, and, because it is a cyanogen bromide peptide, homoserine must be the COOH-terminal. Thus the sequence is Val-Hse.

Tc1-T3. The composition of the COOH-terminal peptide of Tc1 is Val 0.9, Met 1.0, and Arg 1.2. A one-step Edman degradation showed valine to be the NH_2-terminal amino acid.

Overlap between Tc1 and Tc2. The COOH-terminal part of Tc1 is Met-Arg. Therefore CB2 should be followed by a peptide having arginine as NH_2-terminal. Only one cyanogen bromide peptide has arginine as NH_2-terminal, viz. CB3 (Arg-Hse). The tryptic peptide of citraconylated troponin C which follows Tc1 should have methionine as NH_2-terminal. Two peptides (Tc2 and Tc4) have methionine as NH_2-terminal. Tc4 is followed by the COOH-terminal peptide Tc5. If we choose Tc4 there is no room for Tc2 and Tc4. Therefore Tc1 must be followed by Tc2.

Residues 80–82. The COOH-terminal part of Tc2 could be determined by combined carboxypeptidase A and B digestion. It released one arginine, two valine, two methionine, one leucine, and one phenylalanine residues. Because Phe-Leu-Val-Hse is the COOH-terminal part of CB5, the COOH-terminal amino acid sequence of Tc2 must be Phe-Leu-Val-Met-*Met-Val-Arg* or Phe-Leu-Val-Met-*Val-Met-Arg*. The second sequence is impossible because in that case there should be two cyanogen bromide peptides with arginine as the NH_2-terminal residue. In the other case free homoserine and a cyanogen bromide peptide with Val-Arg as the NH_2-terminal part are expected. This was indeed found. Therefore Phe-Leu-Val-Met-Met-Val-Arg is the COOH-terminal part of Tc2, and CB5 is followed by free

Table IV: Manual Edman Degradation of Tc2 Yields of Pth-Amino Acids Identified by Gas Chromatography

Residue No.	Amino Acid	Yield (nmol)	Overlap (nmol)
1	Met	205	
2	Leu	163	
3	Gly	210	
6	Pro	141	
8	Pro	102	
11	Leu	86	
12	Gln[a]		16 (Leu)
14	Met	86	
15	Ile	61	
16	Asp[a]		19 (Ile)
18	Val	59	
19	Asp[a]		20 (Val)
22	Gly	34	
24	Gly	21	
26	Val	19	
27	Asp[a]		9 (Val)
28	Phe	11	
31	Phe	4.5	
32	Leu	2	

[a] Identified by thin-layer chromatography.

homoserine (CB6) which is followed in turn by the tetrapeptide Val-Arg-Cys(cm)-Hse (CB7).

Residues 102–104. The amino composition of Tc3 and CB8 is almost the same. The only difference is the presence of one residue of *S*-carboxymethylcysteine in Tc3. From this and from the nature of the peptides, it can be concluded that the COOH-terminal part of CB8 is Arg-Hse. Tc3 should be followed by a peptide having methionine as NH_2-terminal. Of the two tryptic peptides isolated from citraconylated troponin C, with NH_2-terminal methionine (Tc2 and Tc4), Tc2 had been placed already. Therefore Tc3 is followed by Tc4.

Residues 137–139. The last residues that could be determined with certainty in Tc4 were Met-Lys. Therefore CB10 should be followed by a cyanogen bromide peptide with NH_2-terminal lysine. Two cyanogen bromide peptides have lysine at the NH_2-terminal but, because CB12 does not contain homoserine, it was placed as the COOH-terminal peptide.

Discussion

The calculated molecular weight of bovine cardiac troponin C is 18 459. The protein is very rich in acidic residues which usually come in clusters of two or three. Tryptophan and histidine are absent.

Yields of the Manual Edman Degradation. The Pth-amino acids after manual Edman degradation were identified using a combination of gas chromatography and thin-layer chromatography. The approximate yields of the Pth-amino acids identified by gas chromatography can be used to calculate the repetitive yields and the percentage overlap of the long stretches sequenced by the manual Edman degradation method. The approximate yields for Tc2 are listed in Table IV. It can be calculated from this table that the repetitive yield was 92% for the first 26 residues, thereafter it dropped to 73%. In a similar way it was calculated that the repetitive yield was 84% in the degradation of Tc3 and 95% in the degradation of Tc4. The overlap in the degradation of Tc2 as calculated from Table IV was 2.1% per step. In the degradation of Tc4, the overlap was 2.5% per step. Overlap values were only calculated for Pth-Leu, Pth-Ile, and Pth-Val because they could be determined most accurately.

Sensitivity of the Identification of Pth-Amino Acids. In Table IV it can be seen that the Pth-amino acids could be identified at the nanomole level. Amounts less than 0.5 nmol could be identified by gas chromatography and by thin-layer chromatography on polyamide sheets. The background of other Pth-amino acids due to random cleavage of the peptide chain was so low that it never interfered with the sequence determination. The gas chromatograms always showed a few small peaks which did not correspond to any Pth-amino acid: one peak just before the position of Pth-Ala; a second one just before Pth-Phe; and a third one after Pth-Phe. The occurrence of these peaks (reagent impurities?) did not interfere with the identification of the Pth-amino acids. No foreign spots were observed on the thin-layer plates.

Amides Assignment. Cardiac troponin C contains several clusters of acidic residues, e.g., Glu-Glu-Gln (residues 14–16), Asp-Glu-Asp (residues 65–67), Glu-Glu-Glu (residues 94–96), and Asn-Asn-Asp (residues 143–145). The determination of such sequences, while using the dansyl-Edman technique, is difficult. No particular problems were experienced by using Edman degradation followed by direct identification of the Pth-amino acids. The Pth-derivatives of Gln and Asn are well separated on the thin-layer plates from Pth-Glu and Pth-Asp.

Manual Edman Degradation Procedure. Cardiac troponin C was completely sequenced using only 60 mg of protein. This was possible because of the high repetitive yield of the manual Edman degradation, making it possible to determine the sequence of up to 36 residues using 200 to 300 nmol of starting material (CB2, 30 residues, 300 nmol; Tc2, 32 residues, 200 nmol; Tc4, 36 residues, 200 nmol). These results compare well with many results obtained with an automatic sequence analyser. At first the method of Edman (1970) was followed for the sequence determination of most cyanogen bromide peptides. This allowed the determination of the sequence of some 15 residues. Later on some modifications were made according to Peterson et al. (1972), resulting in the above mentioned results. An important feature in the method used here is that, after the cleavage and removal of the trifluoroacetic acid, water was added followed by extraction of the thiazolinone by ethyl acetate. This method was first described by Iwanaga and Samejima (1970) who recommended this modification to be used in the case of hydrophobic peptides. In this study it has been the standard procedure, and it was found that it resulted in very low mechanical losses during each step of Edman degradation. Another reason for the high repetitive yield might be the low extraction losses due to the highly hydrophilic nature of the peptides.

Internal Homology. Collins et al. (1973) indicated that skeletal troponin C shows internal homology and, when the sequence of cardiac troponin C is arranged in a similar way, it is not surprising to see that this protein also contains internal homology (see Figure 5). The best homology is between regions II and IV (29% identity). The homology between region I and the other regions is markedly lower; 18% of the amino acid sequence of region I is identical with the sequence of region III and a mere 13% is identical with the sequence of region II.

Mutation Rate. The mutation rate of a family of proteins is fairly constant. Skeletal troponin C consists of four homologous regions, each containing one calcium binding site.

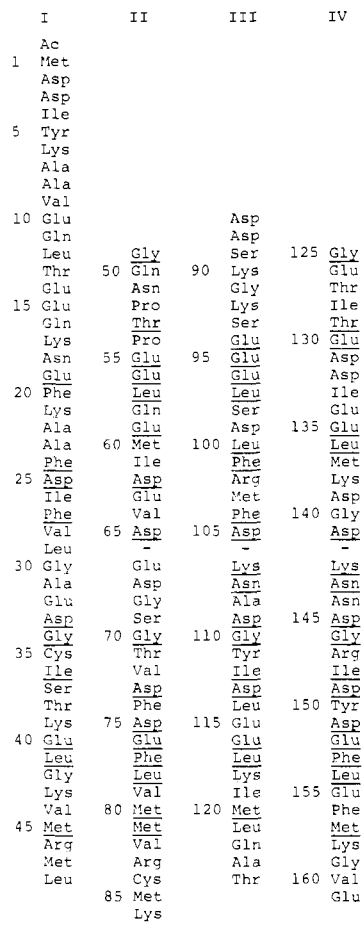

FIGURE 5: Proposed homologous regions in cardiac troponin C. Alignment is on the basis of maximum sequence similarity. Identical amino acids which occur at the corresponding position in another homologous region are underlined.

Because of this, the mutation rate of the four regions is probably very similar. However, in the case of cardiac troponin C, it is likely that region I has lost the ability to bind calcium (van Eerd and Takahashi, 1975), and this might influence the mutation rate of this region. Therefore the number of amino acid replacements between bovine cardiac troponin C and rabbit skeletal troponin C are listed in Table V for the four regions separately. The percentage difference is converted into minimum accepted point mutations per 100 residues (PAM)[1] using the table of Dayhoff et al. (1972). It can be seen that the mutation rate of the region that probably lost the ability to bind calcium (region I) is almost twice as high as the mutation rate of the other regions (75 PAM as against an average of 40 PAM).

When the primary structure of carp parvalbumin (Coffee and Bradshaw, 1973) and hake parvalbumin (Pechère et al., 1971a) are compared, it is found that the two calcium binding regions are less different (residues 34–108, 21 PAM) than the region without calcium binding properties (residues 1–33, 45 PAM). In other words, when the ability to bind calcium with high affinity was lost, the number of acceptable mutations increased and consequently the mutation rate became about twice as high. This also might explain why in carp parvalbumin the internal homology between the part that does not bind calcium and the parts that do bind calcium cannot be detected, while a distant relationship can be detected between the two calcium binding regions (McLachlan, 1972).

Table V: Relative Mutation Rates of the Four Homologous Regions of Cardiac and Skeletal Troponin C.

Region	I	II	III	IV
Residues	1–48	49–86	87–124	125–161
Replacements	23	9	14	11
% replaced	48%	24%	37%	30%
PAM[a]	75	30	51	39

[a] Minimum accepted point mutations per 100 residues.

The "alkali light chains" of myosin are homologous to troponin C, but they have lost the property to bind calcium (Collins, 1974). Therefore from the above considerations it is not surprising that the internal homology of the "alkali light chains" of myosin is less pronounced than in the case of troponin C (Weeds and McLachlan, 1974).

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Human α-Crystallin: Characterization of the Protein Isolated from the Periphery of Cataractous Lenses†

Debdutta Roy and Abraham Spector*

ABSTRACT: α-Crystallin has been isolated from the peripheral region of old cataractous lenses. It was found to be closely related to bovine α-crystallin and to human newly synthesized α-crystallin in terms of its amino acid composition, the size of its polypeptide chains and the lack of free NH_2-terminal groups. However, in contrast to the simple urea gel electrophoretic polypeptide patterns obtained with the reference proteins, 11 polypeptides were detected in the preparation. Ten of the polypeptides were isolated and shown to be either A or B chains on the basis of their amino acid compositions and comparison of the peptide maps of their tryptic hydrolysates. The four B chains as well as the six A chains were closely related, with most of the tryptic peptides being common to all members of their respective group. A nomenclature based upon the urea gel electrophoretic mobilities of the polypeptides has been proposed to define each chain. It was found that this α-crystallin preparation is composed of at least two populations of macromolecules, one of which contains macromolecules greater than 5×10^6 daltons on the basis of gel filtration with Bio-Gel A-5m. The compositions of the two fractions were found to be essentially identical.

Bovine α-crystallin has been more thoroughly investigated than any other lens protein. There are a number of reasons for such attention. The protein is easily isolated, has an unusual macromolecular structure, and displays an age-dependent increase in molecular weight which may be related to cataract formation (Spector, 1972). Only the newly synthesized protein is physically homogeneous with a weight average molecular weight (M_w[1]) of approximately 7×10^5 (Spector et al., 1968). Within a short period of time, the macromolecular population becomes heterogeneous with an increase in M_w to approximately 1×10^6 (Spector and Katz, 1965) and with further aging, primarily in the interior region of the tissue, macromolecular aggregates greater than 50×10^6 daltons have been observed (Spector et al., 1971; Jedziniak et al., 1972; Spector, 1972).

A change in the composition of the polypeptides accompanies the development of size heterogeneity. While bovine NSα contains essentially two chemically different polypeptides designated A_2 and B_2 (Stauffer et al., 1974), post-translational changes quickly produce two additional polypeptides, A_1 arising from A_2 (Palmer and Papaconstantinou, 1969; Bloemendal et al., 1972; Delcour and Papaconstantinou, 1972) and B_1 from B_2 (Stauffer et al., 1974). With further aging, the complexity of the polypeptide population increases further, particularly in the nuclear region of the lens (Stauffer et al., 1974). A number of modified A and B chains having different charge densities, lower molecular weights, and elimination of some of their carboxy-terminal amino acids have been observed (van Kleef et al., 1974).

Investigation of human lens α-crystallin has progressed slowly because normal lens material has been difficult to obtain and the relationship of the human protein to the well characterized bovine α-crystallin has not been defined. However, recently a human NSα has been isolated and characterized (Spector et al., 1976). Sedimentation equilibrium studies indicated a relatively homogeneous preparation with an M_w of 7.5×10^5. Gel electrophoresis in alkaline urea buffers revealed essentially three polypeptide chains, one corresponding to bovine B_2 and two polypeptides with mobilities in the A chain region. The molecular weights of these polypeptides were similar to the bovine A and B chains. With a few exceptions, the amino acid composition of the newly synthesized human protein corresponded to its bovine counterpart.

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[1] M_w, weight average molecular weight; NSα, newly synthesized α-crystallin; DTE, dithioerythritol; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.