Amino Acid Sequences Around the Cysteine Residue of Calf Lens α -Crystallin

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1. Calf lens α -crystallin was carboxymethylated with radioactive sodium iodoacetate to label the thiol group. 2. The protein was then digested with trypsin or alternatively fractionated in urea to obtain the acidic (A) chains, which were then digested with trypsin. Either procedure gave two radioactive peptides containing carboxymethylcysteine. 3. These two peptides were closely related: the longer form contained 28 amino acid residues, and the shorter lacked two residues at the *N*-terminal end of the longer form. 4. The amino acid sequence of the peptides have been determined. 5. No evidence for the presence of more than one cysteine residue /chain was found. 6. The question of the molecular weight of the chains is discussed.

The vertebrate lens is rich in characteristic proteins (Waley, 1969a). One of these is α -crystallin, a large protein built up of many subunits (Bloemendal, Bont, Jongkind & Wisse, 1962). Calf lens α -crystallin contains chains of two kinds, called A chains and B chains (Waley, 1969b). Each of these kinds may be further sub-divided: there are A₁ and A₂ chains, and B₁ and B₂ chains. Embryonic calf lenses are deficient in the A₁ chain (Schoenmakers & Bloemendal, 1968; Palmer & Papaconstantinou, 1968, 1969), as are epithelial cells in the adult animal (Delcour & Papaconstantinou, 1970). Formation of the A₁ chain, probably from the A₂ chain (Palmer & Papaconstantinou, 1969), apparently accompanies aging and differentiation. Spector, Wandel & Li (1968) have isolated a 'highly-labelled' protein; this is monodisperse, whereas α -crystallin is polydisperse, and Spector *et al.* (1968) suggest that the 'highly-labelled' protein is a precursor of α -crystallin. Thus it appears that various changes are gradually taking place in the α -crystallin molecule in the lens. The interpretation of these events demands information about the structures of the chains.

The A₁ and A₂ chains of calf lens α -crystallin are very closely related, and indeed they may only differ by the A₁ chains having a glutamic acid residue where the A₂ chains have a glutamine residue (Schoenmakers, Gerding & Bloemendal, 1969). For many purposes the distinction between the A₁ and A₂ chains can be ignored, and they are collectively referred to as A chains in what follows. Scarce residues are useful in structural studies.

The three least abundant amino acids in the A chains (and in α -crystallin) are cysteine, methionine and tryptophan. There are two residues of methionine (Schoenmakers *et al.* 1969; Palmer & Papaconstantinou, 1969) and probably only one residue of cysteine (this is discussed in more detail below) and of tryptophan. The sequence of the first 11 residues in the A chain had been determined (Corran & Waley, 1969), and this sequence includes one methionine and one tryptophan residue. Limited sequences around the cysteine residue have also been reported (Waley, 1965; Mok & Waley, 1967); we now report an extended sequence of 28 residues around this residue. This sequence includes the second residue of methionine in the chain. Preliminary results suggest that cleavage at this methionine residue with cyanogen bromide gives two fragments of unequal size, the larger fragment being derived from the *N*-terminal part of the chain.

A sequence of 28 residues may be long enough to reveal homology. At some stage of evolution, lens proteins were presumably being formed from some other protein, and this ancestral protein may have given rise to other tissue proteins, which may still bear a resemblance to lens proteins. Knowledge of the sequence is necessary to detect any such resemblance.

Previously, unfractionated α -crystallin had been used in studying the sequence around the cysteine residue. Since the B chains contain no cysteine, the information obtained relates to the A chains. In fact, some of the work to be described was done

with α -crystallin, which is available in larger amounts. But the tryptic peptide was easier to purify when the A chain, rather than α -crystallin, was used.

MATERIALS AND METHODS

α -Crystallin. Decapsulated calf lenses were extracted with water and the α -crystallin precipitated at pH 5.1 as described by Mok & Waley (1967). The precipitate was dissolved in buffer (40 mM- NaHCO_3 , m -NaCl, 0.1 M-EDTA, pH 8, 0.02% in sodium azide), dialysed against this buffer, and the solution then made 5% (w/v) in sucrose and layered on to a column (44 cm high $\times$ 7 cm diam.) of Sephadex G-200, equilibrated with the buffer. The first peak in the eluate is α -crystallin (Schoenmakers, Hoenders & Bloemendal, 1968), and the protein was isolated by ultrafiltration, dialysis and then freeze-drying.

Carboxymethylation of α -crystallin. α -Crystallin (500 mg) was added to 5 ml of 0.5 M-tris-HCl buffer, pH 8.7, 5 M in guanidine hydrochloride and 5 mM in dithiothreitol, under N_2 . After 2 h, 0.15 ml of 0.5 M-iodo[2- ^{14}C]acetic acid (0.2 $\mu\text{Ci}/\mu\text{mol}$) was added, and after a further 1 h the solution was acidified with formic acid, diluted with 2 vol. of water and desalted on a column (50 cm high $\times$ 4.5 cm diam.) of Sephadex G-25 in 0.5% formic acid. The protein, recovered by freeze-drying, had a specific activity of 1.0–1.3 μmol of $^{14}\text{C}/20$ mg.

Separation of A and B chains. The procedure of Schoenmakers & Bloemendal (1968) was used, and the A chains were isolated and digested with trypsin as previously described (Corran & Waley, 1969).

Enzymes. Trypsin (twice-crystallized, salt-free), crystalline bovine chymotrypsin and carboxypeptidase A treated with di-isopropyl phosphorofluoridate were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Thermolysin was from Cugai Boyaki Co. Ltd. (P.O. Box Higashi, no. 106, Osaka, Japan). Subtilisin was from BDH Chemicals Ltd., Poole, Dorset, U.K.

Paper electrophoresis and paper chromatography. These were carried out as before (Corran & Waley, 1969); if chromatography was being used peptides were usually purified in solvent BAWP.*

Sequence methods and amino acid analysis. These were carried out as described by Miller & Waley (1971).

Peptide nomenclature. The tryptic peptide containing Cmc is called peptide 2, as the *N*-terminal tryptic peptide from α -crystallin was called peptide 1 (Corran & Waley, 1969). Different samples are, where necessary, distinguished as shown in Table 1. Further cleavage of peptide 2 with thermolysin, chymotrypsin, cyanogen bromide or acid gave peptides denoted by Th, C, X or H respectively.

RESULTS AND DISCUSSION

Isolation of peptide 2

The isolation of peptide 2 depended mainly on its large size and its radioactivity (Table 1). When carboxymethylated A chains were used, and the

tryptic digest fractionated on Sephadex G-50, peptide 2 was obtained in an adequate state of purity from the first (main) radioactive peak (sample A2, Table 1). Further purification was attended by severe losses, and so most of the work was done with sample A2. Peptide 2 was also prepared from carboxymethylated α -crystallin, but more extensive purification was then required (Table 1), and the samples obtained were less satisfactory. The compositions are in reasonable agreement with that obtained from the sequence finally arrived at, except that the losses of serine were rather larger than expected. Two forms of the peptide were obtained, the proportions varying in different experiments. The forms could be separated by electrophoresis, preferably in a ridge-pole apparatus (at about 10 V/cm), where the evaporation of buffer and large liquid flow seemed to diminish the tendency of the peptides to streak. The 'long form' had an extra residue of tyrosine and of arginine (Table 1). Apart from these two residues, the structures seemed to be the same and so are discussed together.

Sequence of peptide 2

By far the most useful procedure in the work on the sequence of peptide 2 was cleavage by thermolysin; the mixtures of peptides obtained by the action of the other enzymes tried (chymotrypsin, pepsin, subtilisin) were very difficult to fractionate. It is convenient to start by describing peptides derived from the *N*-terminal region of the shorter form of peptide 2, i.e. the form represented by residues 3–28 (Fig. 1).

Residues 3–15. Peptide Th1 (Table 2) was neutral, and so contains asparagine rather than aspartic acid; the sequence was determined by the dansyl-Edman method as Leu-Pro-Ser-Asn. The dansyl-Edman method on peptide A2 gave Leu-Pro and the only other residue of proline is near the *C*-terminus (see below), and so peptide Th1 represents residues 3–6. Peptide Th2 on treatment with carboxypeptidase gave alanine, then serine; the sequence determined by the dansyl-Edman method was: Val-Asx-Glx-Ser-Ala.

The electrophoretic mobility (Offord, 1966) shows the presence of one amide group, which was located by examining the mobility of the DNS-peptides at each stage of the degradation (Gray, 1967); this showed that the amide group was in the third position (Fig. 1). The composition of peptide Th3 suggested that it was built up from peptides Th1 and Th2, joined (from the nature of the *N*-terminal residues) in this order, and thus represents residues 3–11. Peptide Th4 was the only radioactive peptide in the digest. Carboxypeptidase released serine and a little carboxymethylcysteine; the sequence of the first three

* Abbreviations: solvent BAWP, butan-1-ol-acetic acid-pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953); Cmc, *S*-carboxymethylcysteine.

Table 1. *Amino acid composition of tryptic peptides containing carboxymethylcysteine*

Sample $\alpha 2$ was prepared from a tryptic digest of carboxymethylated α -crystallin and purified by chromatography on Sephadex G-50 in aq. 20 mM-NH₃, electrophoresis at pH 4 and then at pH 1.9. Sample A2 was prepared from carboxymethylated A chains; the tryptic digest was fractionated on Sephadex G-50 in 0.5% formic acid, and the peptide in the first radioactive peak is called A2. Some of the peptide was further fractionated at pH 6.5, and pH 1.9 [sample A2(6)]. The amino acid compositions are given as molar ratios, and were calculated on the assumption that the total number of residues of those amino acids listed (omitting Cmc, Tyr, Arg and Ile) was 25. The yield is based on the radioactivity. The last columns give the compositions derived from the sequence (Fig. 1).

Amino acid	Peptide		A2(6)	From sequence	
	$\alpha 2$	A2		'Long form'	'Short form'
Cmc	0.3	0.5	0.2*	1	1
Asp	3.3	3.1	3.2	3	3
Thr	1.3	1.0	1.1	1	1
Ser	5.0	5.5†	5.3†	6	6
Glu	1.3	1.4	1.4	1	1
Pro	2.2	2.1	2.2	2	2
Gly	2.1	2.1	2.0	2	2
Ala	2.2	2.2	1.7	2	2
Val	1.5	1.1	1.3	1	1
Met	0.6	0.8	0.8	1	1
Ile	0.6	0.2	0	0	0
Leu	3.7	3.8†	4.0†	4	4
Tyr	0.8†	0.2	0	1	0
Phe	0.8	0.9	1.0	1	1
Lys	1.1	0.9	0.8	1	1
Arg	0.8	0.3	0	1	0
Total no. of residues				28	26
Yield (%)	22	48	7		

* Value from radioactivity: 1.0.

† Corrected for estimated loss of 10%.

‡ *N*-terminal residue.

residues was obtained by the dansyl-Edman method, and together these results gave the sequence as Leu-Ser-Cmc-Ser.

When less thermolysin was used, there was another radioactive peptide, Th5 (Table 2). The composition of peptide Th5 suggested that it was built up from peptides Th3 and Th4; thus if peptide Th3 represents residues 3–11, peptide Th5 represents residues 3–15. These suggestions are borne out by the cleavages in acid, and by cyanogen bromide, described below.

Residues 16–28. Peptide Th6 gave methionine on treatment with carboxypeptidase and contains aspartic acid rather than asparagine (Table 2). The sequence was determined by the dansyl-Edman method as Leu-Ser-Ala-Asp-Gly-Met. The last four residues were placed in the same sequence by the dansyl-Edman method on the chymotryptic peptide C3 (Table 2). Peptide Th7 (Table 2) was Leu-Thr. Peptide Th8 contains the only lysine residue, and its sequence was determined by the dansyl-Edman method as Phe-Ser-Gly-Pro-Lys. Since peptide 2 is a tryptic peptide, the lysine residue is expected to be *C*-terminal, and on this

basis peptide Th8 represents residues 24–28. The experiments described do not settle the order of peptides Th6 and Th7, but the fact that the residue succeeding methionine in the chymotryptic peptide C3 was leucine gives the order Th6–Th7 (Fig. 1), and this was confirmed by cleavage with cyanogen bromide. Of the other chymotryptic peptides in Table 2, peptide C1 was impure, but was derived from the *N*-terminal region, and peptide C4 from the *C*-terminal region; peptide C2 was the tetrapeptide isolated by Mok & Waley (1967). Another point, namely the order at the junction of residues 11–12, was confirmed by cleavage in acid. These experiments are now described.

Cleavage of peptide 2 with cyanogen bromide and with acid. Cleavage with cyanogen bromide gave the acidic peptide X1 and the basic peptide X3 (Table 3). Peptide X2 appears to be uncleaved peptide 2. The sequence of the basic peptide X3 was determined by the dansyl-Edman method as Leu-Thr-Phe-Ser-Gly-Pro-Lys. Digestion of peptide X3 with subtilisin gave two peptides; one contained leucine, threonine and phenylalanine, and the other contained serine, proline, glycine

Table 2. *Peptides obtained by digestion of peptide 2 with thermolysin and with chymotrypsin*

Peptide 2 (0.5 μ mol) was digested with 10 μ g of thermolysin in 0.2 ml of 1% NH_4HCO_3 for 4 h at 37°C; peptide 2 Th5 was obtained from a digest in which only 1 μ g of thermolysin was used. The digests were fractionated by chromatography in solvent BAWP, followed by electrophoresis at pH 3.5 and at pH 1.9, or by electrophoresis at pH 6.5, then at pH 1.9, and then (if necessary) chromatography in solvent BAWP. Sample A2 (Table 1) was used for all but the last two thermolysin peptides. Sample $\alpha 2$ was used for the last two thermolysin peptides, and for the chymotrypsin peptides, which were obtained from a digestion of 0.5 μ mol with 0.05% chymotrypsin at 37°C for 5.5 h; the digest was fractionated by chromatography in solvent BAWP followed by electrophoresis at pH 3.5. The electrophoretic mobilities (m) refer to pH 6.5, and are calculated (Offord, 1966) relative to aspartic acid -1.00 . The N -terminal amino acid is shown in *italics*. The last column gives the inferred position of the peptide in the sequence (Fig. 1) in residue numbers.

Peptide	Amino acid composition (molar ratios)	m	Inferred position
Th 1	Asp (1.0), Ser (1.1), Pro (0.9), <i>Leu</i> (0.9)	0	3- 6
Th 2	Asp (0.9), Ser (0.9), Glu (1.0), Ala (1.0), <i>Val</i> (0.9)	-0.41	7-11
Th 3	Asp (2.0), Ser (1.8), Glu (1.0), Pro (1.2), Ala (1.0), Val (1.0), <i>Leu</i> (1.0)	-0.27	3-11
Th 4	Cmc (0.9), Ser (2.1), <i>Leu</i> (0.9)	-0.45	12-15
Th 5	Cmc (0.3), Asp (2.0), Ser (3.6), Glu (1.1), Pro (0.7), Ala (1.1), Val (1.0), <i>Leu</i> (2.0)	-0.38	3-15
Th 6	Asp (1.0), Ser (1.0), Gly (1.0), Ala (1.0), Met (0.7), <i>Leu</i> (0.9)	-0.38	16-21
Th 7	Thr (1.0), <i>Leu</i> (1.0)	0	22-23
Th 8	Ser (1.1), Pro (1.0), Gly (1.1), <i>Phe</i> (1.0), Lys (0.9)	$+0.35$	24-28
Th 9	Asp (1.0), Ser (1.0), Pro (1.1), <i>Leu</i> (1.0), <i>Tyr</i> (0.4), Arg (0.8)	$+0.27$	1- 6
C 1	Asp (2.1), Ser (2.4), Glu (1.1), Gly (0.5), Ala (1.0), Pro (0.5), Val (1.1), <i>Leu</i> (2.1), Arg (0.5)	0	2-12
C 2	Cmc (0.5), Asp (0.4), <i>Ser</i> (2.0), <i>Leu</i> (1.0)	-0.43	13-16
C 3	Asp (1.0), Ser (1.0), Gly (1.0), Ala (0.8), Val (0.3), Met (0.5), <i>Leu</i> (1.0)	Acidic	17-22
C 4	<i>Ser</i> (0.8), Pro (1.0), Gly (1.1), Lys (1.0)	$+0.47$	25-28

Table 3. *Peptides obtained by cleavage of peptide 2 with cyanogen bromide and with acid*

Cleavage with cyanogen bromide was carried out in 50% (v/v) formic acid, under N_2 , for 18 h at 20°C; the reagent/methionine molar ratio was 40. The solution was diluted with 3 vol. of water and fractionated on Sephadex G-25 in 5% (v/v) formic acid, followed by electrophoresis at pH 6.5. The peptides are referred to as X1, X2 and X3. Cleavage with acid was carried out in 0.03 M-HCl for 6.5 h at 110°C, and the mixture fractionated by electrophoresis at pH 1.9, and then at pH 6.5; the main radioactive peptide (H) had zero net charge at pH 1.9, and was ninhydrin-negative; no N -terminal acid was detected. Both the above experiments were carried out with sample A2 (Table 1). The N -terminal amino acid is shown in *italics*.

Peptide	Amino acid composition (molar ratios)	Mobility at pH 6.5
X1	Cmc (0.5), Asp (3.1), Thr (0.4), Ser (4.1), Hsr (0.2)*, Glu (1.2), Pro (1.5), Gly (2.0), Ala (2.2), Val (0.7), <i>Leu</i> (2.6)	-0.34
X2	Cmc (0.4), Asp (3.1), Thr (1.0), Ser (5.0), Hsr (0), Glu (1.0), Pro (2.1), Gly (2.2), Ala (2.3), Val (0.7), <i>Leu</i> (3.9), Lys (present)	-0.26
X3	Thr (1.0), Ser (1.1), Pro (1.3), Gly (1.3), <i>Leu</i> (0.9), Phe (1.0), Lys (0.8)	$+0.33$
H	Cmc (0.5)†, Ser (3.6), Glu (1.3), Ala (1.9), <i>Leu</i> (2.1)	-0.55

* Hsr: homoserine.

† Cmc (1.0) from radioactivity.

and lysine. Hence this peptide represents residues 22-28 (Fig. 1), and confirms the placing of the methionine residue at position 21. The composition of the acidic peptide X1 is in fair agreement with that expected for residues 3-21 (Fig. 1).

Tsung & Fraenkel-Conrat (1965) have pointed out that cleavage in acid is often largely restricted to splitting at both sides of a residue of aspartic acid,

and so we thought that this might be useful in providing an '11-12 overlap' peptide, as there are residues of aspartic acid at positions 8 and 19. We expected also that the N -terminal glutamine (residue 9) would cyclize and so the desired peptide would be uncharged at pH 1.9. These expectations were fulfilled, and peptide H (Table 3) represents residues 9-18 (Fig. 1).

'Long form' of peptide 2. The sample $\alpha 2$ (Table 1) had *N*-terminal tyrosine and also contained arginine; after digestion with thermolysin, peptide Th9 (Table 2) was isolated. The sequence of the first four residues were determined by the dansyl-Edman method as Tyr-Arg-Leu-Pro. The next residue was thought to be serine, but this was not firmly established. Since the hexapeptide is basic, it contains asparagine rather than aspartic acid, and so represents residues 1-6 (Fig. 1). Thus the 'long form' of peptide 2 has two extra residues, tyrosine and arginine, in positions 1 and 2 respectively; it is present when trypsin fails to cleave the Arg-Leu bond. The rest of the sequences of the two forms are likely to be the same, as most of the thermolysin peptides have been prepared from both forms, and no differences detected between them.

Number of thiol groups

Values for the number of thiol groups in α -crystallin, and in A chains, are given in Table 4 (the B chains contain no thiol groups). The values for α -crystallin are not very useful, because they only represent an average, and after separation the recovery of A and B chains is only about 60%. If the thiol groups in α -crystallin are not protected, oxidation occurs during the fractionation in urea, and estimates of the thiol groups originally present in the A chains are based on the cysteic acid obtained after further oxidation and hydrolysis (Table 4). The interpretation of the value (1.5 groups/chain) is not clear. If the thiol groups are protected by carboxymethylation, and the carboxymethyl protein is fractionated, the carboxymethyl-A chains obtained contain 1 mol.prop. of carboxymethyl-cysteine/20000 g of chain (Table 4).

Schoenmakers *et al.* (1969) suggested that alkylation might be incomplete, and so we sought conditions that would lead to higher labelling. These were found (Table 4) (see the Materials and Methods section) but about 30% of the radio-

activity was now present in a peptide that was eluted from the Sephadex G-50 column later than peptide 2. This radioactive peptide was further purified by chromatography on Sephadex G-25, followed by electrophoresis at pH 6.5, 3.5 and 1.9. The purified peptide contained tryptophan and had the following analysis (molar ratios): Asp (1.0), Glu (1.1), Pro (0.8), Ala (0.9), Met (0.6), Ile (2.4), Phe (0.8), His (1.1), Lys (1.0); no *N*-terminal residue was detected. This is the composition of the *N*-terminal peptide (1A) from the A chain (Corran & Waley, 1969), and comparison with a sample showed that they had the same electrophoretic mobilities at the three pH values mentioned. It seems likely that the methionine residue in peptide 1A had been alkylated. Peptides containing carboxymethylated residues of methionine have the same mobility as the parent peptide (Vithayathil & Richards, 1960; Jörnvall & Harris, 1970). Apparently the carboxyl group is still ionized, even at pH 1.9, and the carboxylate balances the sulphonium ion. Higher labelling, then, leads to reaction with a methionine residue. It thus seems unlikely that there is a second thiol group in the A chains: we have found only one kind of cysteine sequence and higher labelling leads only to reaction elsewhere.

Molecular weight of A chains

The molecular weight of the chains has hitherto been taken to be about 20000. Earlier, values of about 40000 were reported, from measurements in the ultracentrifuge in 7M-urea (Björk, 1964; Spector & Katz, 1965), but the molecular weight dropped to about 20000 in 5M-guanidine hydrochloride in the presence of mercaptoethanol (Spector & Katz, 1965). Thus the values of about 40000 for the molecular weight were ascribed to incomplete disaggregation or formation of disulphides (or both). Rabbit lens α -crystallin has chains of molecular weight about 20000, according to electrophoresis in polyacrylamide gel in the

Table 4. *Estimations of cysteine in α -crystallin and in A chains*

The values are expressed as no. of cysteine residues/20000 g of protein (calf lens α -crystallin); A chains were prepared by ion-exchange chromatography (in the presence of urea) of either α -crystallin or carboxymethyl α -crystallin.

Method	α -Crystallin	A chains	Reference
Oxidation, and determination of cysteic acid	1.2		Wisse <i>et al.</i> (1966);
		1.5	Schoenmakers <i>et al.</i> (1969)
Carboxymethylation, and (i) determination of radio-activity (ii) determination of Cmc	1.2	0.82, 1.25*	This work
		1.0	Schoenmakers <i>et al.</i> (1969)

* In this sample there was also some labelling of the terminal methionine residue (see the text).

presence of sodium dodecyl sulphate (Shapiro, 1968). Chemical methods on bovine α -crystallin supported the value of 20 000 (Mok & Waley, 1968; Hoenders, Schoenmakers, Gerding, Tesser & Bloemendal, 1968; Schoenmakers *et al.* 1969). However, slow disaggregation to chains of molecular weight about 10 000 has recently been reported (Hoenders, de Groot, Gerding & Bloemendal, 1969; de Groot, Hoenders, Gerding & Bloemendal, 1970). The results from 'peptide mapping' and calculations from amino acid analysis by the method of Katz (1968) were taken to support the view that the molecular weight of the chains is about 10 000 (Leon, de Groot & Bloemendal, 1970). The first procedure is often unreliable (Harris & Hindley, 1965), and the second, too, can be misleading when one is trying to distinguish between molecular weights of x and $2x$. Our results are more readily accommodated by the presence of one residue of cysteine and two of methionine in a chain of molecular weight about 20 000. We think that the molecular weight of the chains in α -crystallin is still an open question.

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