LIMITED TRYPTIC DIGESTION OF α-CRYSTALLIN FROM CALF EYE LENS:

Possible correlation between in vivo and in vitro degradation

Roland J. SIEZEN and Herman J. HOENDERS
Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands

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1. Introduction

α-Crystallin comprises more than 30% of the water-soluble eye lens proteins of mammals [1,2]. Low molecular weight α-crystallin (LMW-α) from the outer part (cortex) of the bovine lens is a mixture of apparently spherical aggregates with molecular weights ranging from 6×10^5 to 9×10^5 and sedimentation coefficients ranging from 17 to 21 Svedbergs [3–5]. An age-dependent increase in molecular weight to more than 50×10^6 occurs, primarily in the inner part (nucleus) of the lens [6,7].

Bovine α-crystallin is composed of two main types of polypeptide chains, the acidic A chains and the more basic B chains, occurring in a ratio of about 2:1. The amino acid sequences of the bovine A_2 and B_2 chains, the primary gene products, have been established and were found to show 57% homology [8,9]. The A_2 chain has 173 residues and mol. wt 19 830, whereas the B_2 chain has 175 residues and mol. wt 20 070. Other A- and B-type chains arise from post-synthetic deamidation and degradation of A_2 and B_2 in vivo. The relative amount of C-terminal shortened A chains (loss of 5, 22 or 72 residues) and B chains (loss of 5 residues) increases from cortex to nucleus of the lens, suggesting an age-dependent degradation process [10–12]. Deamidation of A_2 to A_1 and B_2 to B_1, in both cases presumably due to loss of two amide groups, is often more pronounced in the cortex and is not considered to result from aging [12].

Although both shortening of polypeptide chains and increase of molecular weight seem to be age-related, the relationship (if any) between these processes is not clear. To establish a link, knowledge is required of the spatial arrangement of the polypeptide chains and in particular the topography of the α-crystallin aggregate. The exposed segments of the chains should be more susceptible to attack by proteolytic enzymes, either in vivo or in vitro, than the buried segments.

In this paper we show that short tryptic digestion of LMW-α leads to a very specific nicking of A chains at the Arg(157)–Ala(158) bond, close to the main in vivo degradation site, the Asp(151)–Ala(152) bond. The resulting C-terminal peptide is shown to be essential for correct subunit interactions.

2. Materials and methods

LMW-α-crystallin from calf lens cortex was obtained by fractionation of lens extracts on a Biogel A5M column as described before [6] except that the buffer used throughout was 20 mM Tris–HCl, 80 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, pH 7.3.

Limited digestion of α-crystallin (5 mg/ml) was performed in 0.1 M NH_4HCO_3 pH 8.0 at 30°C by addition of trypsin (bovine, TPCK-treated, Merck). Digestion was stopped after varying periods of time by addition of soybean trypsin inhibitor (Sigma).

Sedimentation analysis was performed in a Beckman Spinco E analytical ultracentrifuge using Schlieren optics, at 60 000 revs./min.

SDS-gelelectrophoresis was performed according to Laemmli [13] using 15% gels. Hemoglobin, myoglobin, α-chymotrypsin B chain and α-crystallin A(1–173) chain and A(1–151) chain were used as MW markers. After Coomassie Brilliant Blue R250

staining, and destaining, the gels were scanned at 580 nm in a Gilford spectrophotometer with gel-scanner.

Isoelectric focusing in the presence of 6 M urea was performed as described [6] using 3.3% gels.

Peptides were fractionated on a Sephadex G-100 column (3.0 × 185 cm) in 10% formic acid/7M urea, desalted on Sephadex G-25 or G-10 in 5% formic acid, and lyophilized. Peptide mapping and amino acid analysis were performed according to V.d. Ouderaa et al. [8].

3. Results and discussion

3.1. *Tryptic digestion*

Digestion products of α-crystallin, incubated with trypsin (0.1%, w/w) for 10 and 60 min, were analysed by SDS-gelelectrophoresis (fig.1). Although A and B chains both have a molecular weight of about 20 000, they are well separated on SDS-gels, since B chains run with an apparent molecular weight of 21 000 presumably due to incomplete unfolding [14].

After 10 min only one main digestion product (T-I) of apparent mol. wt 18 100 is found, with a concomitant decrease of mainly A chains (table 1), suggesting a single specific nick in A chains. On isoelectric focusing gels in 6 M urea (fig.2a,b) no changes are seen in the region of the A bands, and only some minor new bands in the B band region. No change is observed in the sedimentation behavior (fig.3a,b), indicating that no dissociation of the α-crystallin aggregates occurs at this stage.

After 60 min a second digestion product (T-II) of apparent mol. wt 16 800 arises, with further decrease of A chains (fig.1, table 1). At the same time α-crystallin begins to dissociate (fig.3c) and the dissociation products are rapidly broken down, since no other intermediates of lower molecular weights were observed

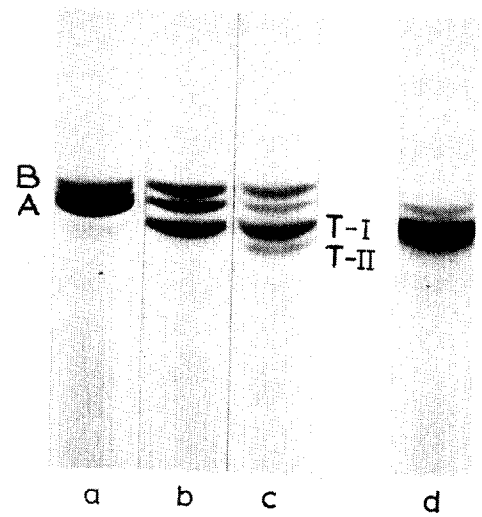

Fig.1. SDS-polyacrylamide gel electrophoresis of α-crystallin after short tryptic digestion (0.1 M NH_4HCO_3, 30°C, 0.1% trypsin w/w); (a) untreated, (b) 10 min, (c) 60 min and (d) T-I digestion product purified from 10 min digest by Sephadex G-100 chromatography in 7 M urea/10% formic acid.

Table 1
Kinetics of tryptic digestion of α-crystallin

	Percentage[a]			
	B	A	T-I	T-II
0 min	32	68		
10 min	25	27	48	
60 min	23	13	51	14
Purified T-I		∿ 5	∿ 90	∿ 5
Apparent mol. wt[b]	21 000	19 800	18 100	16 800

[a]The amount of protein in each band was determined by scanning the gels and converted to mol% of polypeptide chain using the apparent molecular weight.
[b]Apparent molecular weights were obtained from a plot of log mol. wt versus R_f obtained with marker proteins.

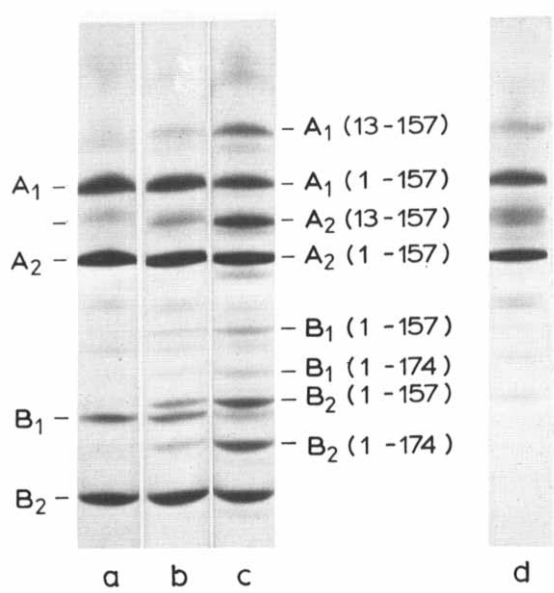

Fig.2. Isoelectric focusing (pH range 5–8, top to bottom) in 6 M urea of α-crystallin after short tryptic digestion, as in fig.1; (a) untreated, (b) 10 min, (c) 60 min and (d) T-I digestion product isolated from 10 min digest.

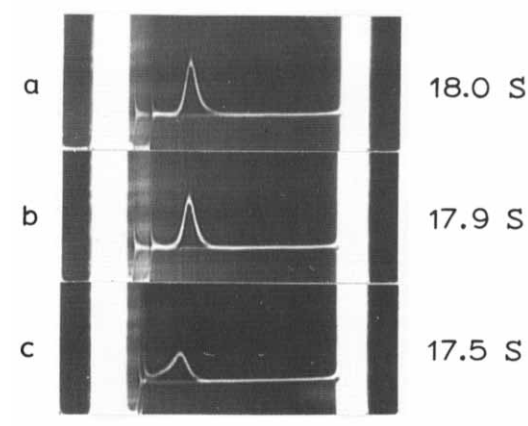

Fig.3. Sedimentation analysis of α-crystallin after short tryptic digestion, as in Fig.1; 60 000 revs./min, 20°C, 5 mg/ml, left to right; (a) untreated, (b) 10 min and (c) 60 min.

after longer incubation. At this stage isoelectric focusing show new bands in both the regions of A and B bands (fig.2c).

After 6 h dissociation and digestion were complete, as no bands were seen at all on SDS-gels.

The same two digestion products were found using other enzyme : substrate ratios (0.001–1% trypsin, w/w) and appropriate digestion times.

3.2. *Isolation and characterisation of T-I*

The T-I component was isolated from a 10 min digest (as in fig.1b) by molecular sieving on Sephadex G-100. The SDS-gel of purified T-I shows only traces of contaminating A chain and T-II (fig.1d, table 1). Isoelectric focusing shows that T-I consists mainly of two components of isoelectric point identical to A_2 and A_1 chains, occurring in the same ratio in which A_2 and A_1 occur in the undigested α-crystallin (fig.2d).

Peptide mapping of T-I, after complete tryptic digestion, shows a typical A chain map from which only the two C-terminal peptides T-19 and T-20 are missing (figs.4a and 5). Therefore T-I is a mixture of A_2 (1–157) and A_1 (1–157) chains, which have a calculated molecular weight of 18 210 and are formed by specific cleavage of the Arg(157)–Ala(158) bond.

3.3. *Isolation and characterisation of small peptides*

The smaller peptides which were split off by trypsin were isolated using the G-100 column in 7 M urea and mapped directly without additional complete tryptic digestion (fig.4b). In fact, a much faster way of finding these peptides proved to be direct peptide mapping of the complete 10 min trypsin digest, leading to a map identical to fig.4b.

One major spot was found which was identified by amino acid analysis to be A(158–173), the complete piece of A chain C-terminal to the specific nicking site. Six other peptides are found, in 10–20-fold lower concentration, and five of these have been identified (fig.4b). Apparently a small amount of A(158–173) is subsequently split, resulting in about equal amounts of A(158–163) and A(164–173).

The occurrence of B(158–175) shows that some nicking of B chains occurs in the equivalent position, the Arg(157)–Thr(158) bond, in addition to loss of C-terminal Lys(175). The resulting shortened chains B(1–174) and B(1–157) are more acidic by 1 and 2 charges respectively, which explains the new bands on IEF-gels in the B region (fig.2b). After 60 min digestion these new bands increase and it is clear that both shortened B_2 and B_1 chains are formed at about equal rates (fig.2c).

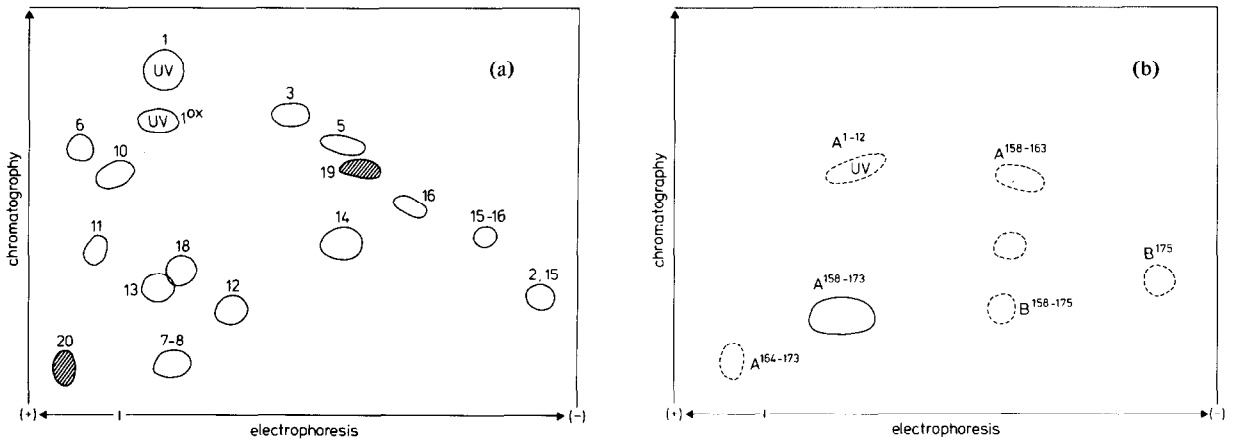

Fig.4. Peptide maps. High-voltage electrophoresis (pH 6.5) was carried out at 50 V/cm for 90 min and followed by descending chromatography for 18 h in *n*-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.). Peptides were stained with 0.02% ninhydrin in buffered acetone. Spots marked UV were fluorescent before staining, indicating the presence of Trp. (a) Tryptic hydrolysate of T-I, isolated from 10 min digest. The striped spots are present in A chains but absent in T-I. All other spots are present in both A chains and T-I. The tryptic peptides are numbered according to their order in the A_2 chain [8]. (b) Peptides found in a 10 min tryptic digest of α-crystallin (see text). Spots were identified by amino acid analysis.

Finally, the presence of some A(1–12) indicates that the N-terminus may also be slightly exposed (fig.4b and 5). After this second nick the N- and C-terminal shortened chain A(13–157) is formed, which has a calculated molecular weight of 16 644 and may be product T-II observed on SDS-gels (fig.1c, table 1). The A(13–157) would be more acidic than A(1–157) by one charge, which could explain the two new prominent bands on IEF-gels in the A band region after 60 min digestion (fig.2c), namely A_2 (13–157) and A_1 (13–157). If this interpretation is correct, A_2 (13–157) and A_1 (13–157) seem to be formed at about equal rates.

3.4. *Role of digestion products in maintaining quaternary structure*

Nicking at specific sites in the α-crystallin aggregate does not necessarily mean that all resulting small peptides are lost from the aggregate. The sedimentation coefficient did not change after 10 min digestion (fig.3), but this is not a very sensitive parameter for loss of mass.

Preliminary experiments indicate that after extensive dialysis or gel filtration of the 10 min tryptic digest, the peptides A(158–173), A(164–173) and B(158–175) cannot be removed from the aggregate.

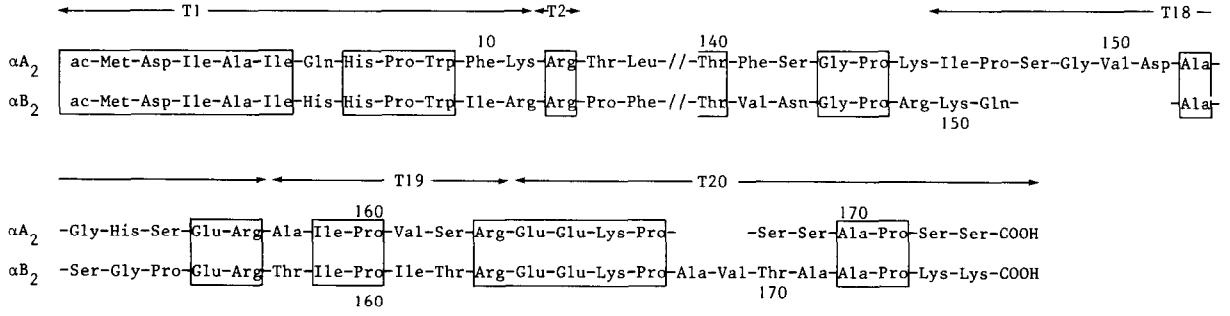

Fig.5. Primary structure of the N-terminal and C-terminal ends of the A_2 and B_2 chains of bovine α-crystallin [8,9]. Identical residues are enclosed in rectangles to illustrate homology. The numbering of some of the tryptic peptides of the A_2 chain is indicated.

These peptides could only be removed after dissociation of α-crystallin in 7 M urea.

An attempt to reassociate isolated A(1–157) chains, by dissolving them in pH 7.3 buffer, was unsuccessful. Either these shortened chains did not dissolve at all or immediately formed very high molecular weight aggregates and precipitated from solution. Under these conditions undigested A_2 and/or A_1 are known to reassociate to 12 S aggregates [15,16].

4. Conclusion

No clear difference was observed in the rate of tryptic digestion of deamidated or regular A and B chains, indicating that by this criterion deamidated chains are not exceptionally exposed on the surface, as has been proposed [17].

At least 50% of the polypeptide chains in the α-crystallin aggregate can be nicked by trypsin without change in quaternary structure. The first nick occurs almost exclusively at the Arg(157)–Ala(158) bond of A chains. Since at least two-thirds of the A chains were nicked at this site, they must be in (semi–equivalent positions. A section of the A chain from about residues 146 to 158 possibly forms a surface loop (fig.6), because it contains (a) the primary tryptic nicking site, (b) the primary in vivo degradation site [10], and (c) a high degree of sequence variability, as shown from homology studies of A chains from various mammals [18].

Nicking of B chains at the equivalent Arg(157)–Thr(158) bond hardly occurs, even though the surrounding amino acids are very similar to those in the A chain. In vivo degradation of B chains in this region has not been observed either. Perhaps a similar loop in the B chain is less exposed because it is 4 residues shorter than the A chain in this region (fig.5). The C-terminus of B chains is certainly exposed, since trypsin splits off Lys(175), and several other proteases specifically remove small peptides from the C-terminal end (R. J. Siezen, unpublished results).

The second points of attack in the A chain after longer tryptic digestion are the Arg(163)–Glu(164) bond and the Arg(12)–Thr(13) bond. Nicking at one or both of these sites may disturb subunit interactions, since dissociation of α-crystallin begins at this stage.

The C-terminal peptides A(158–173), A(164–173) and B(158–175), which are formed after one or two nicks by trypsin, are still firmly attached to the α-crystallin

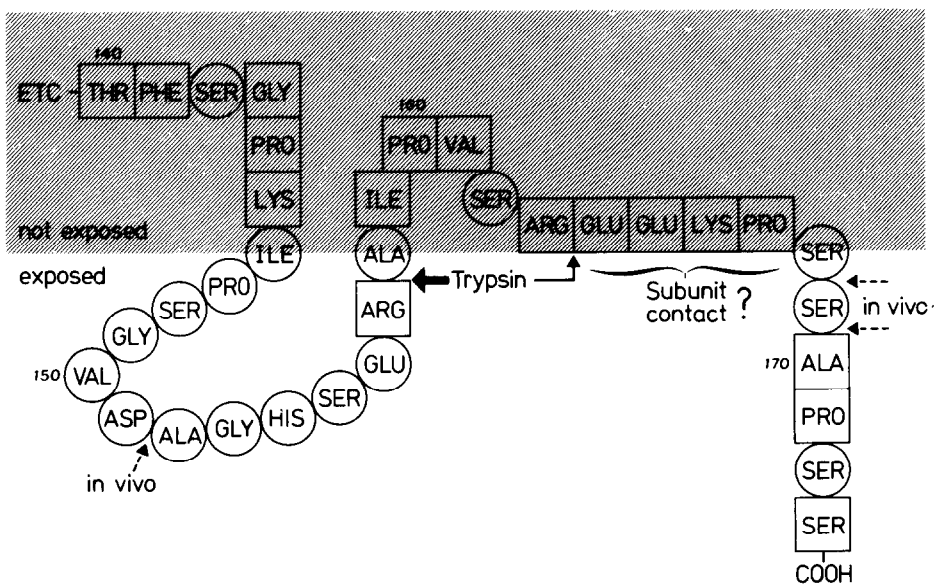

Fig.6. Model of the C-terminus of A chains in the α-crystallin aggregate. Residues in squares were found to be invariant in A chains of seventeen mammalian species [18]. The exposed sections are based on sequence variability and susceptibility to degradation in vivo and in vitro (trypsin).

aggregate by non-covalent bonds. These fragments seem to be essential for subunit contacts. Without these peptides, reassociation of isolated A(1–157) chains leads to insoluble aggregates instead of the soluble 12 S aggregates which are formed when complete A chains are reassociated. The sequence Arg–Glu–Glu–Lys–Pro in these peptides may be the essential contact region since it is invariable, occurring in all known sequences of A and B chains from mammals [18,19].

It is tempting to speculate that a similar situation occurs in vivo. Upon aging A chains are nicked at the Asp(151)–Ala(152) bond, and in analogy with the results above the C-terminal peptide A(152–173) would remain attached to the α-crystallin aggregate. Subsequent slow degradation of this peptide from its N-terminal end by lens leucine aminopeptidase may eventually lead to loss of the essential contact region, followed by aggregation to higher molecular weight aggregates. This hypothesis is presently under investigation.

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