CROSSLINKING OF α-CRYSTALLIN WITH BISIMIDOESTERS

Evidence for polyamidine formation at pH 8 from an increase in positive charges on the polypeptide chains

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1. Introduction

Mono- and bi-functional imidoesters (alkylimidates) have been widely used for the specific chemical modification of amino groups in proteins, e.g., the former for quantification of amino groups by isotope incorporation, and the latter for intermolecular crosslinking of polypeptide chains in oligomeric proteins [1–6]. By varying the chainlength of bisimidoesters intersubunit distances and symmetry relations have been determined [7–11].

The desired product of the reaction of a monofunctional imidoester (VII: for scheme of reactions and numbering of compounds see fig.2 in [6]) with an amino group is an amidine (II), thereby retaining the single positive charge. However, rapid and efficient formation of amidine has been shown to occur only near pH 10 [12,13]. Complications arise at pH 8 due to side reactions, notably an increased hydrolysis rate of imidoester, and the very rapid, essentially quantitative, formation of a relatively stable N-alkyl imidate intermediate (VIII) [12,13]. This intermediate then slowly converts to amidine (II) through reaction with ammonia, or back to free amine (XIII) by hydrolysis. If these are the only end products the overall amidination reaction is merely slower and less efficient at pH 8. As pointed out [12,13] however, the N-alkyl imidate (VIII) could also react with another protein amino group, resulting in an *N,N'*-disubstituted amidine (III). A monofunctional reagent could thus produce a one carbon atom crosslink via this mechanism, or a similar one [14], and such crosslinking has now been observed [14–17]. Finally, double modification of a single protein amino group could occur by reaction of the initially formed N-alkyl intermediate (VIII) with imidoester (VII) [18], as outlined in fig.1. This reaction could repeat itself several times (XVI → XVII), with the addition of one positive charge and 2.7 Å chainlength per step. Termination of multiple amidination could then occur through hydrolysis, or by reaction of XVII with ammonia or amino group to form conjugated polyamidines (XVIII). All of the above reactions will occur at either end of the bifunctional imidoester, and clearly, an enormous number of different products are conceivable, with severe implications for the interpretation of crosslinking experiments. Polyamidine formation has been ignored in the amidination and crosslinking literature thus far, probably due to the absence of experimental verification.

Here the crosslinking of A and B subunits of α-crystallin with bisimidoesters at pH 8 is shown to be accompanied by the formation of modified A and B polypeptide chains with a range of increasing positive charges. This presents strong evidence for the formation of polyamidine structures.

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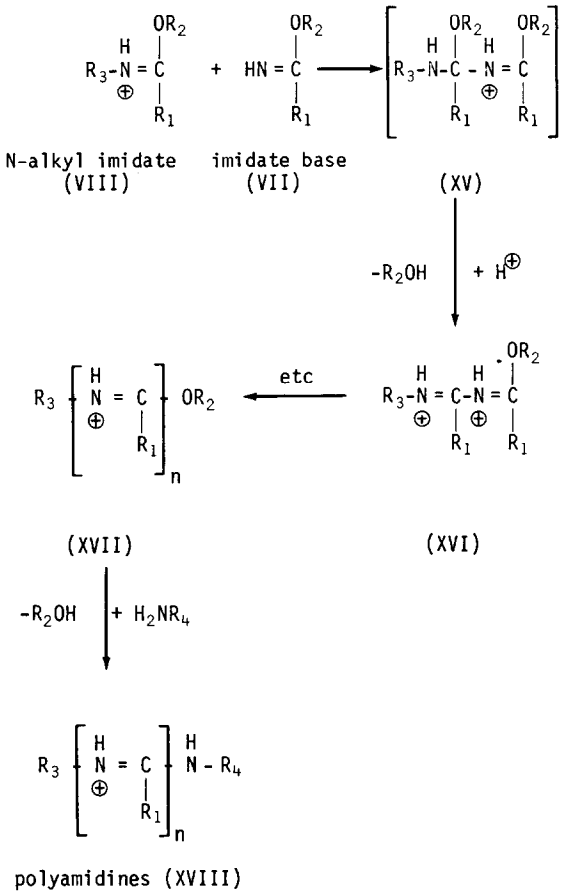

Fig.1. Proposed mechanism of polyamidine formation. The scheme and the numbering of compounds XV–XVIII are a continuation of fig.2 in [6], starting from their compounds VII and VIII. It incorporates the reactions proposed [18] for imidate trimerization (see also p. 331 of [19]). In protein crosslinking, substituents R_3 and R_4 are alkyl sidechains of lysyl residues. For dimethylsuberimidate $R_2 = -CH_3$ and $R_1 = -(CH_2)_6-C(=NH^+)OCH_3$.

2. Experimental

2.1. *Materials*

α-Crystallin from calf lens cortex was obtained by fractionation of lens extracts on Biogel A5M (Biorad) or Ultrogel AcA22 (LKB) as in [20,21]. A_2 and B_2 chains were purified by DEAE-cellulose (DE-52, Whatman) chromatography in the presence of 7 M urea [22]. Dimethylsuberimidate and dimethyladipimidate were from Pierce, whereas dimethylsebacimidate and dimethyldodecimidate were synthesized from the corresponding dinitriles by the method in [3].

2.2. *Crosslinking*

Protein was either dialyzed against 0.2 M triethanolamine–HCl (pH 8.0) overnight at 4°C, or directly dissolved in this buffer at 20°C. Crosslinking reagent was dissolved in the same buffer, at slightly higher pH, and within 1 min of preparation it was mixed with protein solution to final conc. 0.2–25 mM, final pH 8.0 and final protein conc. 1 mg/ml (0.35 mM and 0.50 mM ε-amino groups of A_2 and B_2 chains, respectively). Crosslinking was carried out at 20°C and the reaction was quenched after 2 h by addition of glycine to 50 mM. The crosslinked protein was then exhaustively dialyzed against demineralized water at 4°C and freeze dried.

2.3. *Electrophoresis*

Isoelectric focusing in the presence of 6 M urea at pH 3.5–10 was performed essentially as in [23], at a lower gel concentration (3.3% instead of 6.9% acrylamide) to facilitate penetration of crosslinked protein. After focusing the tube gels (12 × 0.6 cm) were stained [24] or frozen at −20°C until sodium dodecyl sulphate (SDS)–gel electrophoresis in the second dimension. The former gels had been loaded with 200 μg protein and the latter with 400 μg.

SDS–polyacrylamide gel electrophoresis was done by the modification [3] of the method in [25], in 4% acrylamide tube gels (7 × 0.6 cm) or 5% acrylamide slab gels (16 × 16 × 0.3 cm). The acrylamide/methylene–bisacrylamide ratio was 37.5. Protein was dissolved at 1 mg/ml in sample buffer (0.1 M borate/Na-acetate (pH 8.5), 1% SDS, 1% dithioerythritol, 10% sucrose, 0.002% bromophenol blue) and denatured at 100°C for 2 min, after which 20 μg was applied to each gel. Electrophoresis was in 0.1 M borate/Na-acetate (pH 8.5), 0.1% SDS, at 7 mA/tube gel for 1.5 h or 15–30 mA/slab gel for 16 h. The complete first-dimension isoelectric focusing gel was thawed and soaked for 1 h at 50°C in 3 changes of 20 ml sample buffer to remove Ampholine (LKB) and urea, then laid on top of the slab gel and sealed in place with a layer of 4% acrylamide gel. Staining was with Coomassie brilliant blue R250 [25].

3. Results and discussion

α-Crystallin from bovine eye lens is a spherical-shaped oligomeric protein, composed of about 30 A-subunits and about 10 B-subunits [20,21]. Both have mol. wt ~20 000 as determined from their primary sequences [26,27] and both occur in a deamidated form (A_1, B_1) and a non-deamidated form (A_2, B_2), the latter predominating in vivo.

In a study of the subunit arrangement in α-crystallin, the protein was crosslinked with dimethylsuberimidate (DMS) in 0.2 M triethanolamine buffer at pH 8.0 for 2 h at 20°C. Although a higher pH is recommended for maximum formation of amidine crosslinks and a minimum of side products [12,13], the native α-crystallin structure is unstable above pH 8 (R. J. S., J. G. Bindels and H. J. Hoenders, unpublished). SDS–polyacrylamide gels illustrate that crosslinked products were formed, all multiples of 20 000 mol. wt and the extent of crosslinking increased with DMS concentrations from 0.2–25 mM (fig.2a–e). Although this SDS–gel electrophoresis system does not distinguish between the individual A_1, A_2, B_1 and B_2 chains, nor their crosslinking products, isoelectric focusing in 6 M urea clearly separates the monomeric subunits (fig.3a), which have isoelectric points of 5.6, 5.9, 7.1 and 7.4, respectively [28]. Since no change in the charge of the protein should ensue following reaction with imidoester, it was reasoned that hybrid dimers (e.g., A_1–A_2, B_1–B_2, A_2–B_2) and possibly trimers might be identified from their isoelectric points. Surprisingly, the isoelectric focusing patterns of crosslinked α-crystallin (fig.3b–e) were not at all as anticipated, but instead a characteristic pattern of equidistant bands, increasing in number and isoelectric point with increasing concentration of DMS, was observed. Similar molecular weight and charge patterns were found when α-crystallin was crosslinked with dimethyladipimidate (DMA), dimethylsebacimidate or dimethyldodecimidate. Apparently, each band initially present in fig.3a is converted to a series of equidistant bands, with the A_2 and B_2 derivatives dominating the overall pattern.

To explore this point further, purified A_2 chains (fig.2f,3f) and B_2 chains (fig.2h,3h) were crosslinked with 25 mM DMA. Under the conditions of pH and ionic strength employed, the purified subunits are known to exist as spherical-shaped aggregates of 20 polypeptide chains [20,21] and this was checked by sedimentation velocity analysis. SDS–gel electrophoresis demonstrates that both A_2 and B_2 chains crosslink to multiples of 20 000 (fig.2g,i) and isoelectric focusing shows that concomitantly a series of more positively charged derivatives of both A_2 and B_2 are formed (fig.3g,i). The distance between bands is 0.2–0.3 pH units, which corresponds to an increase of one positive charge per step. Up to 5 extra charges on A_2 chains and 6 extra charges on B_2 chains have been found, with the +1, +2 and +3 charge derivatives dominating, and very little unmodified A_2 and B_2 remaining at the highest concentration of crosslink reagent used (fig.3e,g,i).

These results suggest a correlation between oligomer formation and increase in positive charge, and this possibility was investigated by two-dimensional slab-gel electrophoresis (fig.4). First dimension isoelectric focusing of crosslinked A_2 chains (as in fig.3g) was followed by SDS–gel electrophoresis in the second dimension. It is immediately apparent from fig.4 that each oligomer occurs in various charge states and vice versa, which argues against any direct correlation. The A_2 monomer is found in the 0, +1, +2 and +3 charge states, with the +1 and +2 states prevailing, and these components must account for most of the stain in the first dimension gel. The A_2 dimer appears to consist of the 0, +½, +1, +1½ and +2 charge states, the +1 state prevailing, and the +½ and +1½ derivatives presumably arising from crosslinks between the 0, +1 and +2 components. A similar two-dimensional pattern was found with crosslinked B_2 chains (not shown).

4. Conclusions

Crosslinking of α-crystallin with bisimidoesters at pH 8 does not leave the protein charge unaltered. Polypeptide chains with 1–6 additional positive charges are found, their relative amounts depending on the concentration of reagent used. These results strongly suggest that polyamidine structures are formed via the pathway outlined in fig.1. Since the A and B polypeptide chains of α-crystallin have 7 and 10 lysine ε-amino groups [26,27], it is impossible at this stage to distinguish whether one lysine ε-amino

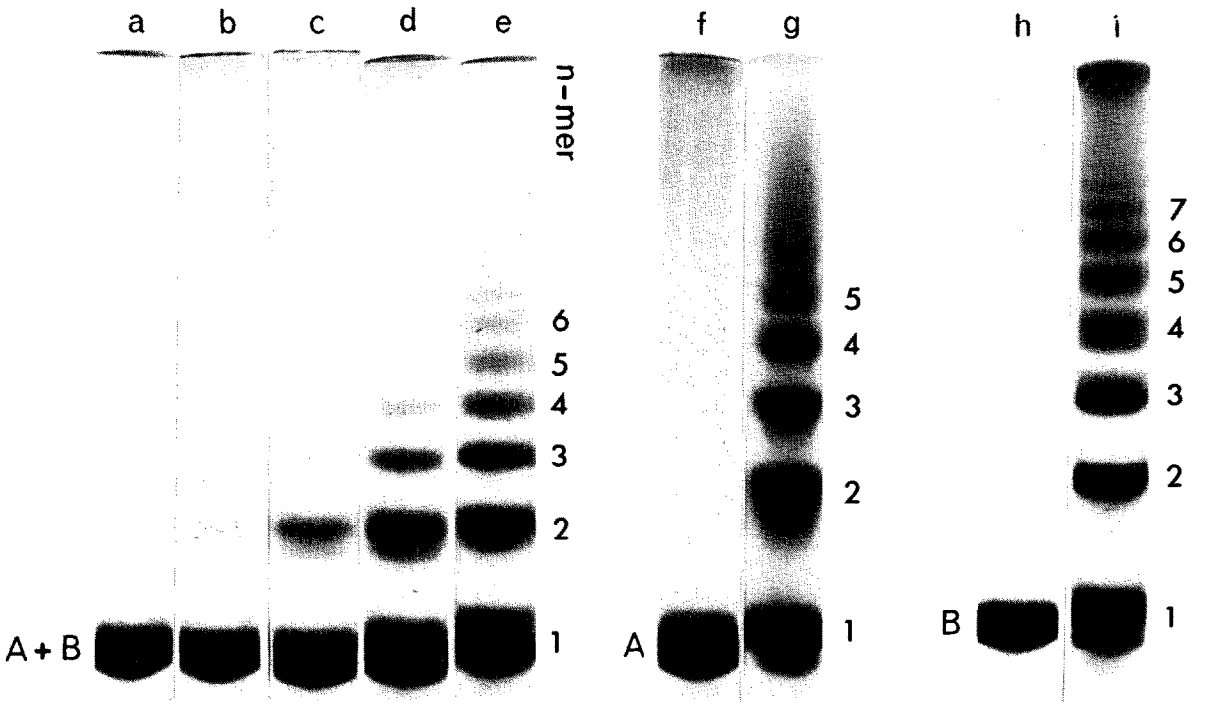

Fig.2. SDS–gel electrophoresis of crosslinked α-crystallin (a–e); A_2 chains (f,g); B_2 chains (h,i). Crosslinking was done in 0.2 M triethanolamine–HCl (pH 8.0) for 2 h at 20°C, 1 mg/ml protein, with DMS at (a) 0 mM; (b) 0.2 mM; (c) 1 mM; (d) 5 mM; (e) 25 mM; and DMA at: (f,h) 0 mM; (g,i) 25 mM. The *n*-mers are all multiples of 20 000 mol.wt, as determined with crosslinked marker proteins [7].

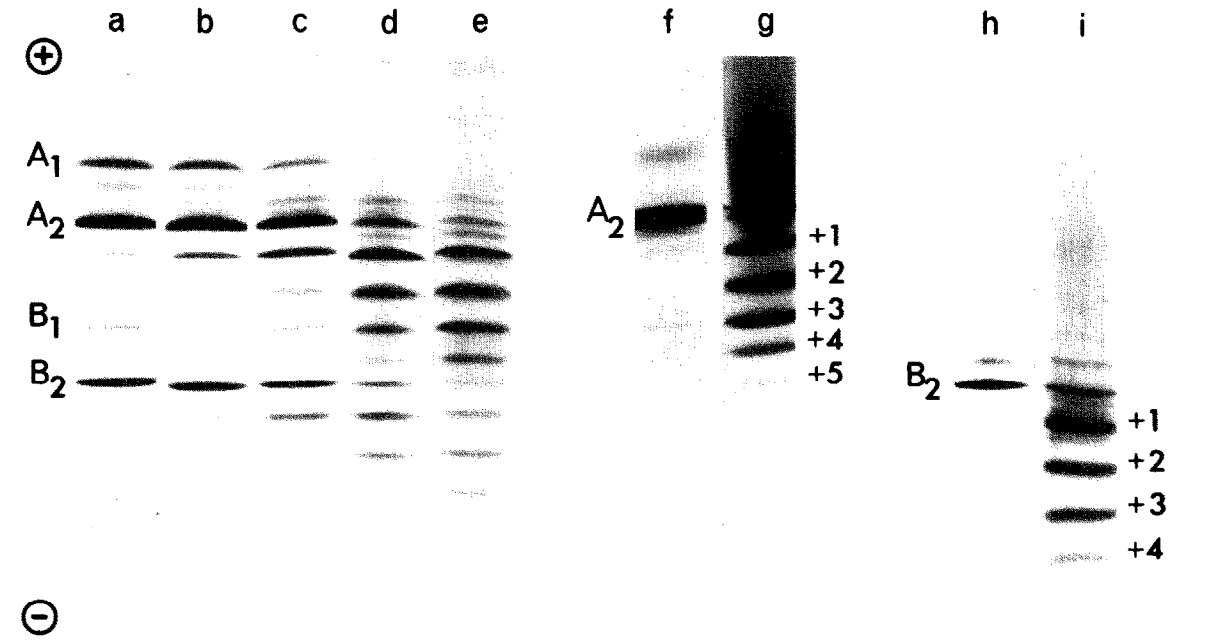

Fig.3. Isoelectric focusing in 6 M urea (pH 3.5–10) (top to bottom). Further details as in fig.2. The increasing background stain is caused by higher mol. wt bligomers which have failed to focus at their isoelectric point.

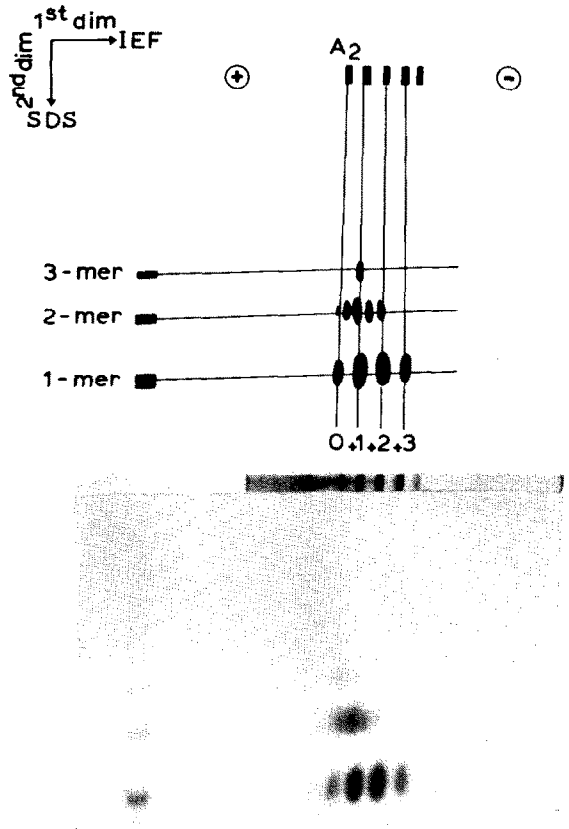

Fig.4. Two-dimensional electrophoresis of crosslinked A_2 chains. First dimension isoelectric focusing (IEF) was as in fig.3g, but now in the range pH 5–8 (left to right) to improve band separation. SDS–gel electrophoresis was performed in the second dimension. For reference purposes separation by molecular weight only is shown in the left track, and a stained duplicate of the IEF gel is shown at the top.

group has incorporated 6 additional amidine units ($n = 7$ for compound XVIII), or whether several different lysines have added one or more positive charges, although the latter seems more likely. Likewise, it is uncertain whether these polyamidine structures have participated in crosslinking (i.e., whether R_4 of compound XVIII is H or lysyl residue), since the observed oligomers could still all be crosslinked via the normal amidination reaction.

Nevertheless, the importance of various side reactions, which are known to produce disubstituted amidine crosslinks [14–17] and now polyamidines with a range of charges, must not be underestimated when interpreting results of modification reactions with mono- and bi-functional imidoesters. Doubt has been cast [12,13] on several conclusions drawn by others from studies of lysine reactivity with monofunctional reagents. It is observed polyamidine formation will interfere with the use of isotope incorporation as a measure of amino group modification, and disubstituted amidination will interfere with the use of cleavable crosslinking reagents (for incomplete cleavage see, e.g. [16,29,30]). In addition, we now note that the uncertainty in the lengths of crosslink bridges, as a result of both polyamidination and disubstituted amidination, will interfere with the method of varying chain lengths of bisimidoesters to determine inter-residue distances and symmetry relations. Furthermore, charge variations due to polyamidination can affect protein conformation, quaternary structure, substrate/effector binding and enzymatic activity.

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