Volume 311, number 2, 143–149 FEBS 11655 October 1992

Identification by ^{1}H NMR spectroscopy of flexible C-terminal extensions in bovine lens α-crystallin

John A. Carver[a], J. Andrew Aquilina[a], Roger J.W. Truscott[a] and Gregory B. Ralston[b]

[a]*Australian Cataract Research Foundation, The University of Wollongong, NSW, Australia* and [b]*Department of Biochemistry, The University of Sydney, Sydney, Australia*

Received 20 July 1992; revised version received 1 September 1992

Two-dimensional ^{1}H NMR spectroscopy of bovine eye lens α-crystallin and its isolated α_A and α_B subunits reveals that these aggregates have short and very flexible C-terminal extensions of eight (α_A) and ten (α_B) amino acids which adopt little preferred conformation in solution. Total α-crystallin forms a tighter aggregate than the isolated α_A and α_B subunit aggregates. Our results are consistent with a micelle model for α-crystallin quaternary structure. The presence of terminal extensions is a general feature of those crystallins, α and β, which form aggregates.

Lens; Crystallin; NMR; Conformation; Aggregation

1. INTRODUCTION

The eye lens contains a high concentration of proteins (ranging from approximately 200 to 400 gl^{-1}) whose particular structural arrangement is required for the transmission and focussing of light onto the retina. The water-soluble proteins are known collectively as the crystallins and, in the mammalian lens, are divided into three classes, α, β, and γ. The γ-crystallins are monomeric proteins whereas the α- and β-crystallins are oligomeric (reviewed in [1]). α-Crystallin is composed of two homologous subunits, α_A and α_B, each of molecular weight approximately 20 kDa and 173 and 175 residues respectively [1]. Under usual isolation conditions, α-crystallin exists as an aggregate of average molecular weight of 800 kDa [2] but α-crystallin of size 320 kDa can be isolated at 37°C [3]. The size of α-crystallin aggregates depends also on ionic strength and pH [4].

Unlike the β- and γ-crystallins, for which X-ray crystal structures are available [5,6], little is known about the tertiary and quaternary structures of α-crystallin. Indeed, the structure of the aggregated state of α-crystallin has provided much controversy with two structures being proposed; a three-layered model [7] and a micelle model [8].

We present here a ^{1}H NMR study of α-crystallin and its isolated α_A and α_B subunits. From analysis of the two-dimensional ^{1}H NMR spectra of these large aggregated proteins, we are able to assign resonances that arise almost exclusively from short C-terminal extensions of both subunits. These extensions have great flexibility compared with the bulk of the protein and adopt little preferred conformation.

Correspondence address: J.A. Carver, Australian Cataract Research Foundation, Department of Chemistry, The University of Wollongong, Locked Bag 8844, South Coast Mail Centre, NSW 2521, Australia. Fax: (61) (42) 214 287.

2. EXPERIMENTAL

α-Crystallin was isolated from bovine lenses via gel chromatography at 20°C [9]. The individual α_A and α_B subunits were separated from α-crystallin by gel filtration in 0.1 M glycine at pH 2.5 on a column (70 × 1.4 cm) of Sephadex G-75 [10]. Sample purity was checked by SDS-PAGE and IEF-PAGE and gave results identical to those of [10] and [11], respectively. The α_A and α_B fractions were also characterised by electrospray mass spectrometry on a VG Quattro mass spectrometer. For α_A, peaks of molecular weight 19,832 and 19,913 were observed which corresponded exactly to expected molecular weights for the native and singly-phosphorylated species. The α_B sample gave peaks of molecular weight 20,084 and 20,161. The total α-crystallin sample revealed all four molecular weight species. A slight cross- contamination of the α_A- and α_B-crystallin samples by their respective other α subunit was observed. The samples were dialysed exhaustively against water prior to lyophilisation.

Sedimentation equilibrium studies were performed at 25°C using a Beckman Model E analytical ultracentrifuge fitted with electronic speed control and RTIC unit. Samples of solution depth 1–3 mm were at concentrations of 0.5–2 gl^{-1}, pH 4.2 and 25°C and were centrifuged at 4,000, 6,000 and 10,000 rpm. The concentration distribution at sedimentation equilibrium was measured with the aid of interference optics. Some experiments were performed at ~50 gl^{-1} with the aid of schlieren optics.

Samples for NMR were prepared by dissolving ~40 mg of sample in 0.8 ml of either 99.5% D_2O or 10% D_2O in water at pH ~4 (to give a subunit concentration of ~2 mM). ^{1}H NMR spectra were accumulated at 400 MHz and 25°C on a Varian Unity 400 NMR spectrometer. 2D NMR spectra were acquired with the pulse sequences and parameters described in [12].

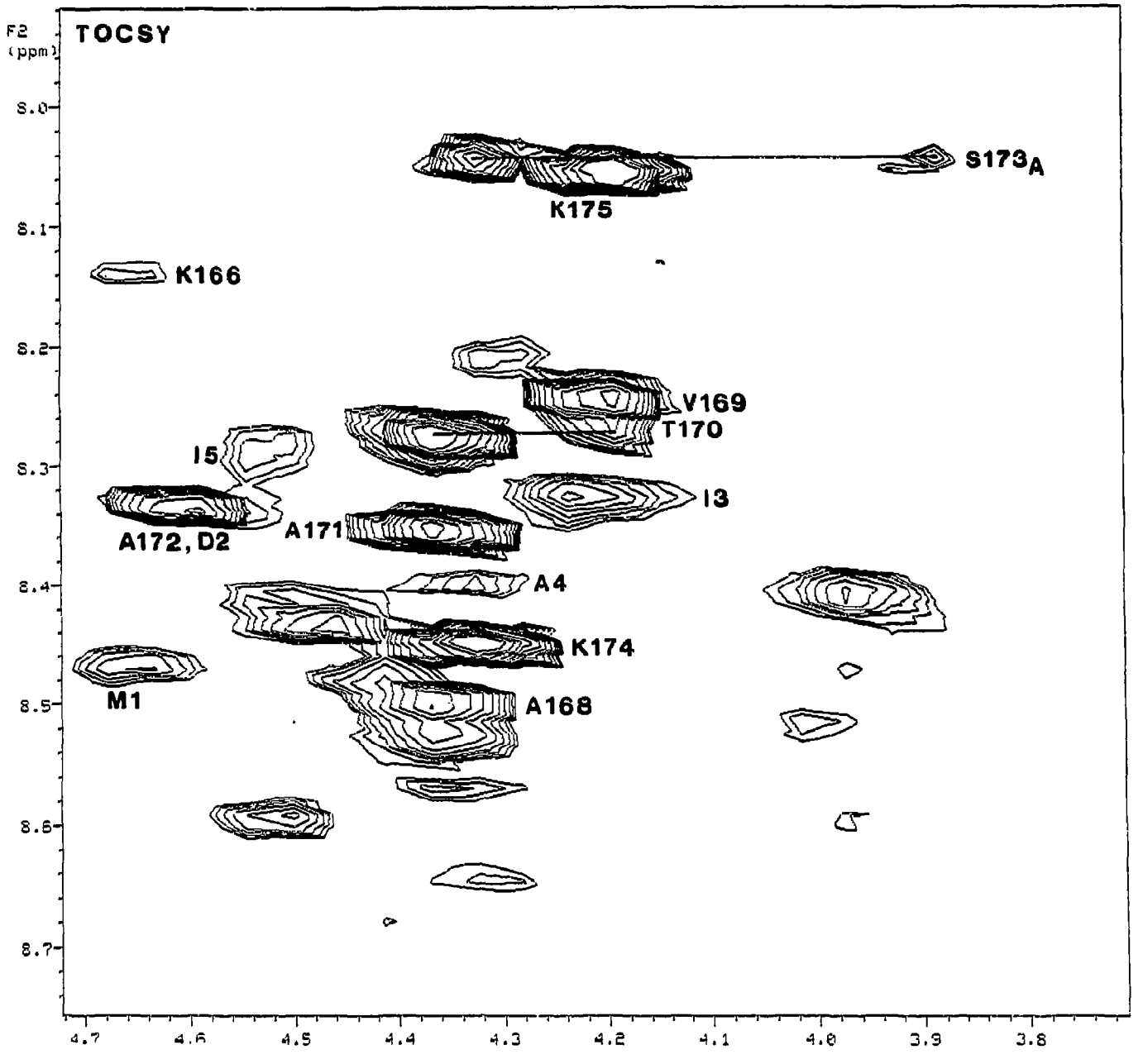
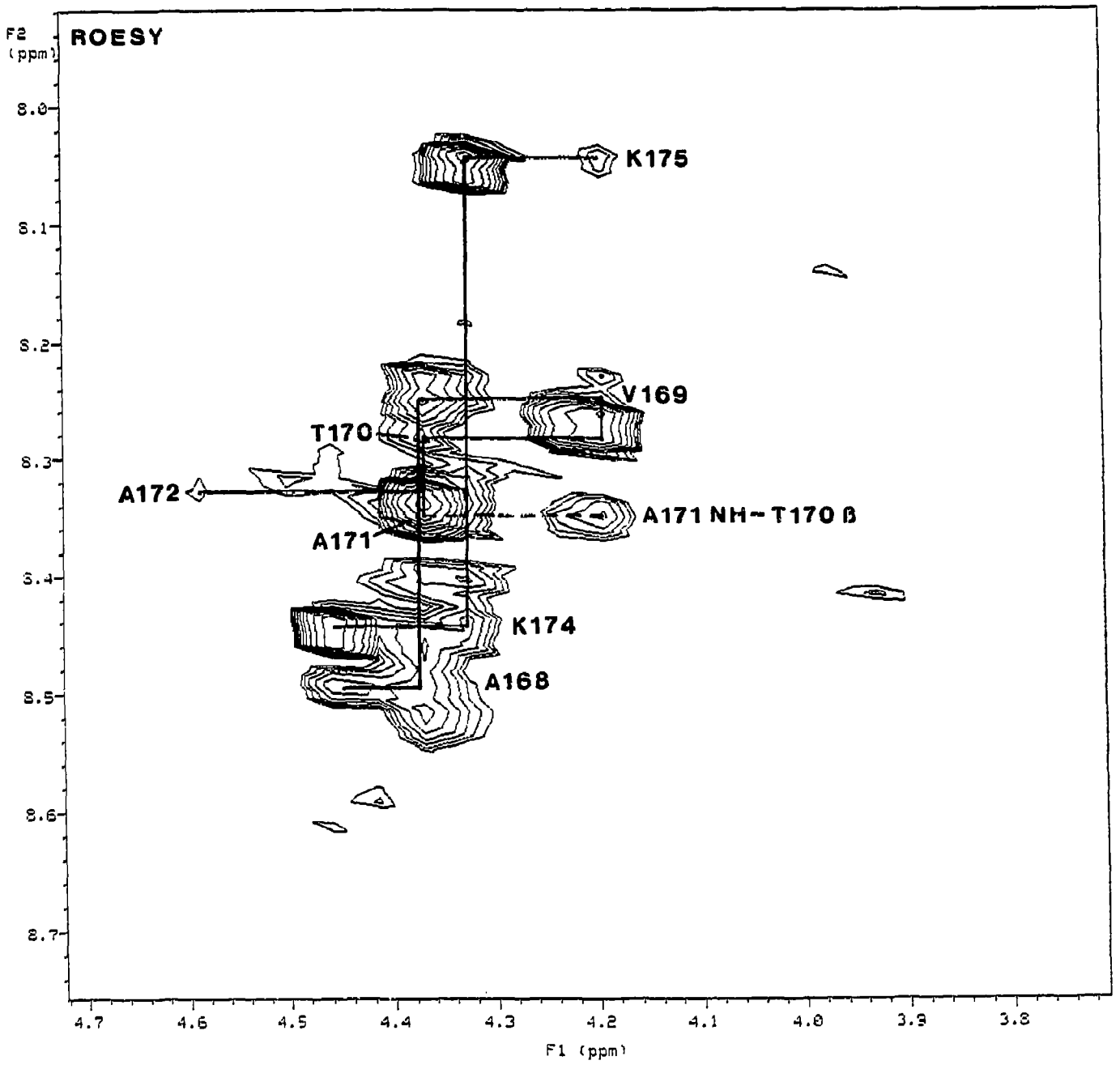


Fig. 1. NH to α-CH region of a TOCSY spectrum (spin lock time = 30 ms) and a ROESY spectrum (mixing time = 200 ms) of α_B-crystallin in H_2O, pH 4.4. Sequential $d_{\alpha N}$ connectivities [16] are traced out in the ROESY spectrum with intra-residue nOe's labelled. The connectivities between Pro^{173} and Ala^{172} and Pro^{167} and Lys^{166} were determined from $d_{\alpha\delta}$ nOe's [16]. A small amount of α_A-crystallin was present in the sample which gave rise to resonances from Ser^{173}_A. The assignments for the N-terminal amino acids are tentative.

3. RESULTS

Sedimentation equilibrium experiments on α_A, α_B and total α-crystallin at pH ~4 (the pH at which the NMR experiments were acquired) indicated that all three samples existed as heterogeneous but highly aggregated species. Thus, for α_A, species with a molecular weight range from ~200 kDa to ~800 kDa were present; for α_B, species from 135 to 660 kDa were observed whilst total α was even more aggregated in having species from 600 kDa to several million Daltons present. The molecular weight distribution for total α is in excellent agreement with recent light scattering studies [13]. At the concentrations used for NMR measurements (~50 gl^{-1}), the α samples gelled in the ultracentrifuge, so samples were run at lower concentrations (0.5–2 gl^{-1}). Little concentration dependence of the molecular weight distribution was observed for the three samples suggesting that the



(For legend see facing page.)

above results could be extrapolated to the NMR samples. Previous sedimentation equilibrium studies at different pH values, concentrations, buffer conditions and rotor speeds from those used here have also shown that total α and the isolated α_A and α_B subunits exist as highly aggregated species [14,15].

Being such large aggregates, it would be expected that the NMR spectra of α-crystallin and its α_A and α_B subunits would display little, if any, structural information. It was surprising, therefore, that the ^{1}H NMR 1D spectra exhibited a number of well-resolved resonances (not shown), indicating that the protein does have regions of enhanced flexibility. The NMR studies were undertaken on intact α samples which had been purified by gel chromatography and characterised by gel electrophoresis and mass spectrometry. Thus, the well-resolved resonances arise from the intact protein and not from any proteolysed fragments. A series of two-dimensional ^{1}H NMR experiments were acquired on the isolated α_A, α_B and total α samples to elucidate the identity of the resonances.

Fig. 1 shows the NH to α-CH region of TOCSY and ROESY spectra of α_B in H_2O. The majority of the observed resonances were assigned by sequential assign-

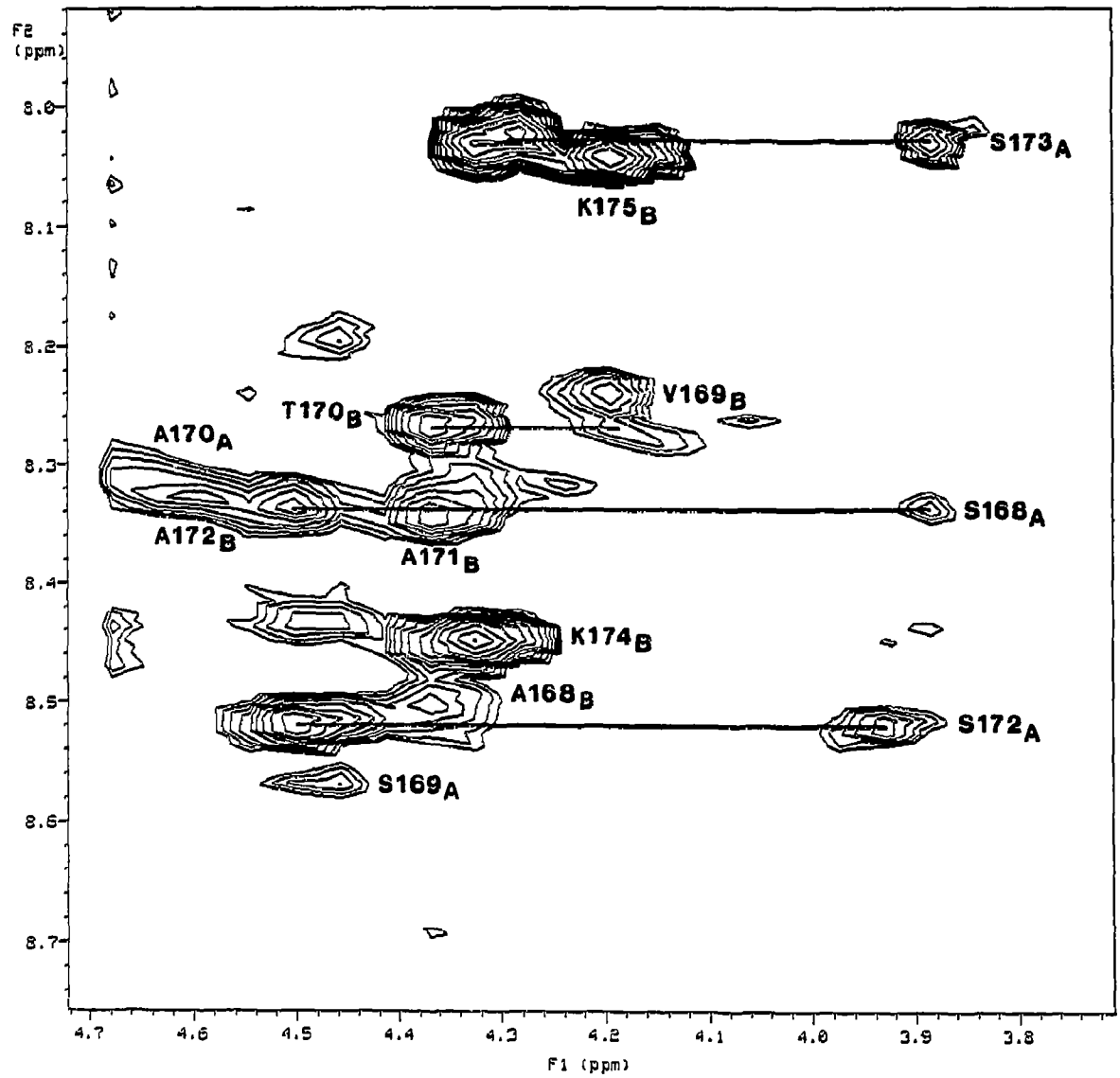


Fig. 2. NH to α-CH region of a TOCSY spectrum (spin lock time = 30 ms) of total α-crystallin in H_2O, pH 4.2. Assignments are indicated; A and B subscripts refer to α_A and α_B subunits, respectively.

ment methods using the ROESY spectrum [16]. The strongest cross-peaks arise from resonances in the C-terminal end of the molecule and it was straight forward to assign from Lys^{175} to Lys^{166} via nOe's from the NH to the α-CH ($d_{\alpha N}$) and/or β-CH resonances of the preceding residue. The cross-peaks for Lys^{166} were weak and it was not possible to assign any further back along the polypeptide chain. The remaining cross-peaks in the TOCSY spectrum of Fig. 1 are weaker, and some are tentatively assigned to residues at the N-terminal end of the molecule since resonances from two Ile, an Ala and Asp residues were observed. It was not possible, however, to assign these resonances sequentially due to overlap and weak cross-peaks in the ROESY spectrum. Cross-peaks for Ser^{173} of α_A were also observed in Fig. 1 which arise from the small amount of α_A present.

The spectra for α_A were similar to those for α_B in that the strongest cross-peaks observed were for the C-terminal region of the protein and it was possible to assign sequentially from Ser^{173} to Pro^{167} (data not shown). Again, there were extra cross-peaks apart from the C-terminus that were present but it was not possible to assign these resonances due to overlap. The first five N-terminal amino acids in α_A and α_B are identical but, unlike the case for the α_B spectra, no resonances suggestive of this portion of the molecule were observed.

The TOCSY spectrum of total α also gave rise to a well-resolved spectrum with the NH to α-CH region (Fig. 2) and the aliphatic region (Fig. 3) exhibiting almost exclusively resonances from the C-terminal regions of α_A and α_B. The assignments were again confirmed by sequential assignment methods. Thus, reso-

nances from all but the C-terminal portions of the α_A and α_B subunits are absent in total α spectra implying that the C-terminal portions of the molecule have great flexibility compared with the bulk of the protein. Furthermore, the chemical shifts of the α_A and α_B individual subunit aggregates were identical to those observed in total α, implying that the C-terminal portions of the subunits in α_A and α_B are independent of, and do not interact with, each other. The assignments of the C-terminal portions of α_A and α_B in total α-crystallin are given in Table I.

4. DISCUSSION

From analysis of the 2D NMR spectra of α-crystallin and its subunits we have demonstrated that the last 8 amino acids in the α_A subunit and the last 10 amino acids in the α_B subunit have great conformational flexibility and move independently of each other in the α-crystallin aggregate. From the little variation in chemical shifts of their resonances and their similarity to random coil chemical shifts [16] these extensions have little preferred or ordered conformation. The individual α_A and α_B subunit C-terminal extensions do not interact with each other within the aggregate nor do they interact with extensions from other α-crystallin aggregates. The latter point is in agreement with the results of Siezen and Owen [17] who investigated the interactions of total α-crystallin aggregates via exclusion chromatography.

The β-crystallins are not homologous to the α-crystallins and exist as aggregated species ranging in size from dimers through to octomers [1]. They also have terminal extensions; both N- and C-terminal extensions in the basic β-crystallins and only N-terminal extensions in the acidic β-crystallins. Our recent NMR studies on the major β-crystallin, βB2, have shown that these extensions also have enhanced conformational flexibility compared with the bulk of the protein [18]. The monomeric γ-crystallins, apart from γ_s-crystallin [1], do not contain extensions; thus, the presence of highly flexible terminal extensions is a general feature of the aggregated α- and β-crystallins. The extensions are most likely involved in the binding together of the various crystallins in the lens and/or interaction with membrane components [19]. Indeed, it has recently been demonstrated that α_A interacts specifically with lens membrane [20]. The arrangement of the crystallin proteins is crucial to the maintenance of lens transparency and its disruption can lead to opacification of the lens (i.e. cataract formation).

The enhanced flexibility and solvent accessibility of the C-terminal extensions would suggest that they are amenable to proteolytic cleavage. Proteolysis studies on total α-crystallin in vitro are in excellent agreement with this proposal [21,22]. The Thr^{170}-to-Ala^{171} bond in α_B is highly susceptible to cleavage by a variety of proteases and the C-terminal region prior to and including the C-terminal extensions of α_A is accessible to proteolytic enzymes [21]. The endogenous lens protease, calpain, also specifically degrades α_A and α_B in the C-terminal regions [22]. Age-related degradation of α-crystallin in vivo is accompanied by similar cleavage of C-terminal fragments [23]. Likewise, Ca^{2+}-activated proteolysis of N-terminal β-crystallins in murine lenses is accompanied by cortical cataract [24]. The ubiquity of terminal extensions in α- and β-crystallins and their ready solvent accessibility could mean that their proteolysis is a general phenomenon in cataract formation.

From the sedimentation equilibrium studies, the α_A and α_B aggregates have lower molecular weights compared to the total α aggregate. The NMR spectra show that α_A and α_B have more conformationally flexible regions (e.g. possibly the N-terminal extension in α_B) compared to total α (compare Figs. 1 and 2) where almost exclusively only the C-terminal extensions are observed. Thus, the total α aggregate is a larger and tighter structure than those of the subunit aggregates with only the C-terminal extensions being freely accessible to solvent in total α-crystallin.

Circular dichroism studies suggest that the secondary structure of the α-crystallin subunits is predominantly β-sheet with a small amount of α-helix [25]. Siezen and Argos [25,26] have used structure prediction methods to propose that the α subunits adopt a similar four-motif

Table I

^{1}H NMR chemical shifts in ppm of α-crystallin in 90% H_2O/10% D_2O, pH 4.2, 25°C[a]

	NH	α-CH	β-CH	Other
αA				
Lys^{166}	n.o.	4.66	1.78, 1.86	γ-CH 1.50 δ-CH_2 1.73 ε-CH_2 3.29 ε-NH_3^+ 7.30
Pro^{167}	–	4.36	2.01, 2.35	γ-CH_2 2.10 δ-CH_2 3.71
Ser^{168}	8.34	4.51	3.89	
Ser^{169}	8.55	4.47	3.98	
Ala^{170}	8.31	4.63	1.39	
Pro^{171}	–	4.50	2.04, 2.35	γ-CH_2 2.04 δ-$CH_2$3.70, 3.85
Ser^{172}	8.52	4.50	3.93	
Ser^{173}	8.04	4.31	3.89	
αB				
Lys^{166}	8.14	4.64	1.73, 1.83	γ-CH_2 1.47 δ-CH_2 1.73 ε-CH_2 3.27 ε-NH_3^+ 7.30
Pro^{167}	–	4.46	1.95, 2.34	γ-CH_2 2.08 δ-CH_2 3.71, 3.88
Ala^{168}	8.50	4.37	1.42	
Val^{169}	8.25	4.19	2.13	γ-CH_3 0.98
Thr^{170}	8.28	4.36	4.22	γ-CH_3 1.26
Ala^{171}	8.36	4.37	1.40	
Ala^{172}	8.34	4.60	1.39	
Pro^{173}	–	4.46	2.08, 2.36	γ-CH_2 2.05 δ-CH_2 3.70, 3.87
Lys^{174}	8.45	4.33	1.77, 1.86	γ-CH_2 1.51 δ-CH_2 1.77 ε-CH_2 3.06 ε-NH_3^+ 7.59
Lys^{175}	8.06	4.20	1.73, 1.83	γ-CH_2 1.42 δ-CH_2 1.73 ε-CH_2 3.01 ε-NH_3^+ 7.59

[a] Chemical shifts are accurate to ± 0.02 ppm. n.o = not observed.

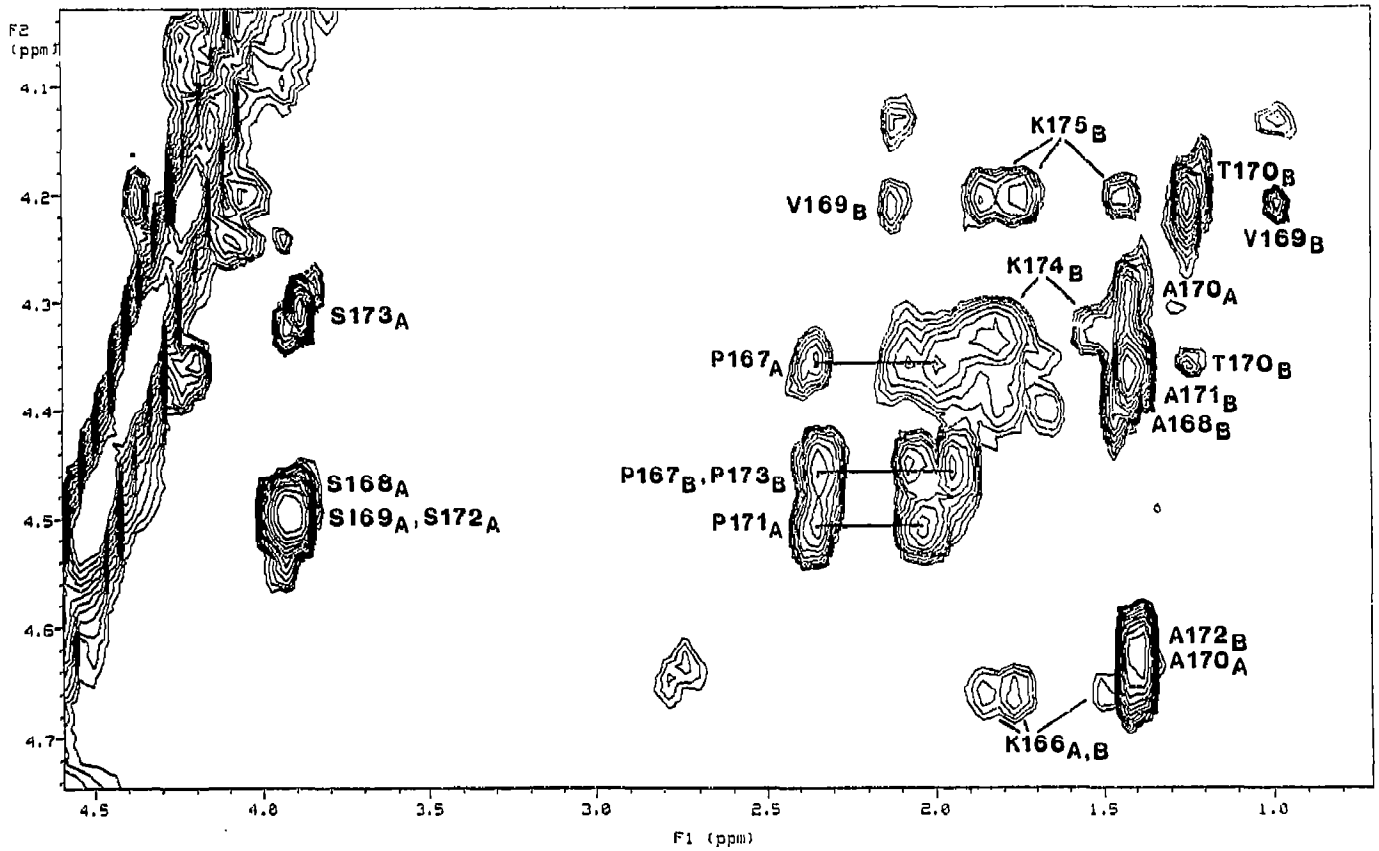


Fig. 3. A portion of the aliphatic region of a TOCSY spectrum (spin lock time = 30 ms) of total α-crystallin in D_2O, pH 4.2. Assignments are indicated; A and B subscripts refer to α_A and α_B subunits, respectively.

folding pattern to that in the β- and γ-crystallins. Furthermore, they propose that α_A and α_B have C-terminal extensions of eight (from Lys^{166} to Ser^{173}) and ten (from Lys^{166} to Lys^{175}) amino acids, respectively. These regions encompass exactly those observed in the NMR spectrum of α_A and α_B and our results are therefore good supporting evidence for the proposed structure of Siezen and Argos [25,26]. A similar structure for all the crystallins would enable each component of the heterogeneous mixture of crystallins in the lens to have similar light-refracting capabilities. The two-domain model for α-crystallin tertiary structure also proposed that the individual subunits have C-terminal extensions [27].

Two models for the α-crystallin quaternary structure have been proposed: the three-layered model of Tardieu et al. [7], which has recently been modified by Walsh et al. [28], and the micelle model of Augusteyn and Koretz [8]. Radlick and Koretz [29] have recently provided strong experimental evidence for the micelle hypothesis. The micelle model is based around the variation in amphilicity of the α subunits, i.e. the N-terminal third of the molecule is strongly hydrophobic with the remainder being hydrophilic [30]. The observation of only C-terminal extensions of α_A and α_B in the NMR spectrum of total α is consistent with the N-terminal portion of the molecule being buried and not accessible to solvent. Indeed, the start of the C-terminal extensions in α_A and α_B is part of one of the proposed four sequential antigenic regions in the α-crystallins [30], whereby antigenic determinants are located on solvent-exposed regions of the molecule that are charged and hydrophilic [31]. Furthermore, the C-terminal extensions, as discussed above, are readily susceptible to proteolytic attack [21,22].

The original three-layered model of Tardieu et al. [7] does not take into account the amphiphilic nature of the α_B subunits although the model of Walsh et al. [28] suggests that the first two layers of the α-crystallin aggregate have a micelle-like structure with the first layer containing 12 α_A subunits, the second layer six α_A and α_B subunits and the third layer 24 subunits which are weakly bound via polar–polar interactions, leaving the hydrophobic N-terminal portions of the subunits exposed to solvent. The micelle model is much simpler. It requires no specificity in the location of the individual α_A and α_B subunits with the quaternary structure and size of the aggregate being determined purely by hydrophobic and hydrophilic interactions. Our results would

favour the micelle model because only C-terminal extensions of α_A and α_B are observed in the NMR spectrum, i.e. no resonances from the N-terminal regions are present and there is no specificity in the arrangement of α_A and α_B subunits in the total α-crystallin aggregate.

Acknowledgements: We thank Greg Kilby, Larry Hick and Dr. Margaret Sheil for acquisition and interpretation of the mass spectra and Philip Cooper for helpful discussions. This work was supported by a grant from the National Health and Medical Research Council of Australia. The NMR spectrometer was purchased through grants from the Australian Research Council and the Ramaciotti Foundation.

REFERENCES

[1] Harding, J.J. (1992) in: Cataract: Biochemistry, Epidemiology and Pharmacology, pp. 30–63, Chapman and Hall, London.
[2] Bloemendal, H. (1977) Science 197, 127–138.
[3] Thomson, J.A. and Augusteyn, R.C. (1983) Exp. Eye Res. 37, 367–377.
[4] Van den Oetelaar, P.J.M., Clauwaert, J., Van Laehem, J. and Hoenders, H.J. (1988) J. Biol. Chem. 260, 14030–14034.
[5] Blundell, T., Lindell, P., Miller, L., Moss, D., Slingsby, C., Tickle, T., Turnell, B. and Wistow, G. (1981) Nature 289, 771–777.
[6] Bax, B., Lapatto, R., Nalini, V., Driessen, H., Lindley, P.F., Mahadevan, D., Blundell, T.L. and Slingsby, C. (1990) Nature 347, 776–780.
[7] Tardieu, A., Laporte, D., Licinio, P., Krop, B. and Delaye, M. (1986) J. Mol. Biol. 192, 711–724.
[8] Augusteyn, R.C. and Koretz, J.F. (1987) FEBS Lett. 222, 1–5.
[9] Kleiman, N.J., Chiesa, R., Kolks, M.A.G. and Spector, A. (1988) J. Biol. Chem. 263, 14978–14983.
[10] Stevens, A. and Augusteyn, R.C. (1987) Curr. Eye Res. 6, 739–740.
[11] Thomson, J.A. and Augusteyn, R.C. (1989) Biochem. Biophys. Acta 994, 246–252.
[12] Esposito, G., Pessi, A. and Verdini, A.S. (1989) Biopolymers 28, 225–246.
[13] Augusteyn, R.C., Parkhill, E.M. and Stevens, A. (1992) Exp. Eye Res. 54, 219–228.
[14] Augusteyn, R.C., Ellerton, D.H., Putilina, T. and Stevens, A. (1988) Biochim. Biophys. Acta 957, 192–201.
[15] Thomson, J.A. and Augusteyn, R.C. (1989) Biochim. Biophys. Acta 994, 246–252.
[16] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, J. Wiley, New York.
[17] Siezen, R.J. and Owen, E.A. (1983) Biophys. Chem. 18, 181–194.
[18] Carver, J.A., Cooper, P. and Truscott, R.J.W. (1992) Biochemistry, submitted for publication.
[19] Berbers, G.A.M., Hockman, W.A., Bloemendal, H., De Jong, W.W., Kleinschmidt, T. and Braunitzer, G. (1984) Eur. J. Biochem. 139, 467–479.
[20] Ifeanyi, F. and Takemoto, L. (1990) Curr. Eye Res. 9, 259–265.
[21] Siezen, R.J. and Hoenders, H.J. (1979) Eur. J. Biochem. 96, 431–440.
[22] Yoshida, H., Yumoto, N., Tsukahara, I. and Murachi, T. (1986) Invest. Ophthalmol. Vis. Sci. 27, 1269–1273.
[23] Van Kleef, F.S.M., De Jong, W.W. and Hoenders, H.J. (1975) Nature 258, 264–266.
[24] Shearer, T.R., David, L.L., Anderson, R.S. and Azuma, M. (1992) Curr. Eye Res. 11, 357–369.
[25] Siezen, R.J. and Argos, P. (1983) Biochim. Biophys. Acta 748, 56–67.
[26] Argos, P. and Siezen, R.J. (1983) Eur. J. Biochem. 131, 143–148.
[27] Wistow, G. (1985) FEBS Lett. 181, 1–6.
[28] Walsh, M.T., Sen, A.C. and Chakrabarti, B. (1991) J. Biol. Chem. 266, 20079–20084.
[29] Radlick, L.W. and Koretz, J.F. (1992) Biochim. Biophys. Acta 1120, 193–200.
[30] Puri, N., Augusteyn, R.C., Owen, E.A. and Siezen, R.J. (1983) Eur. J. Biochem. 134, 321–326.
[31] Hopp, T.D. and Woods, K.P. (1981) Proc. Natl. Acad. Sci. USA 78, 3824–3828.