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Lens crystallins and oxidation: the special case of γS

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Abstract

Among lens crystallins, γ-crystallins are particularly sensitive to oxidation, because of their high amount of Cys and Met residues. They have the reputation to induce, upon ageing, lens structural modifications leading to opacities. A combination of small angle X-ray scattering and chromatography was used to study the oxidation of γ-crystallins. At pH 7.0, all the γ-crystallins under study were checked to have the same structure in solution. Under gentle oxidation conditions at pH 8.0, human γS (hγS) and bovine γS (bγS) formed disulfide-linked dimers, whereas the other bγ-crystallins did not. Cys20 was shown to be responsible for dimer formation since the C20S mutant only formed monomers. The hγS dimers were stable for weeks and did not form higher oligomers. In contrast, monomeric γS-crystallins freshly prepared at pH 8.0, and submitted to more drastic oxidation by X-ray induced free radicals, were rapidly transformed into higher oligomers. So, only extensive oxidation causing partial unfolding could be detrimental to the lens and linked to cataract formation. The γS-crystallins lack the temperature-induced opacification observed with the other γ-crystallins and known as cold cataract. The oxidation-induced associative behaviour and cold cataract are therefore demonstrated to be uncoupled.

Keywords: Lens crystallin; γS-crystallin; Oxidation; Cataract

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1. Introduction

Human lens crystallins need to keep their native structure and interactions to maintain lens transparency [1]. These proteins are exceptionally stable, since they are synthesised to last as long as human life. The major age related modifications found in the soluble crystallin fraction seem to be deamidations and disulfide bonds [2,3]. Yet, oxidation of lens crystallins has often been found associated with human cataract (see review in Spector [4]). Oxidation could contribute to the pathological events leading to cataract by promoting aggregation.

Because of their high level of thiol groups, γ-crystallins are usually considered likely candidates for oxidation induced oligomerisation. γ-crystallin oxidation has been studied through a variety of in vitro systems including for instance ageing at room temperature [5], vitamin C-induced oxidation [6], or H_2O_2-induced cataract on calf lenses in culture [7]. All these studies confirmed the sensitivity of γ-crystallins to oxidation with, possibly, a special sensitivity of γS, within the γ-crystallin family.

Interestingly, a point mutation in the human γD-crystallin (hγD) gene, R14C, recently shown to be associated with an hereditary cataract [8], was demonstrated to promote in vitro the formation of disulfide-linked oligomers [9].

γS belongs to the β/γ superfamily, where the polypeptide chain consists of two domains connected by a linker peptide. Each domain comprises two Greek key motifs forming a sandwich of two four-stranded, antiparallel β-sheets. The domains are always found to be associated pairwise, by intermolecular contacts in oligomeric γ-crystallins or intramolecular associations in monomeric γ-crystallins. Sequence comparison shows that γS is more closely related to the monomeric γ-crystallins (53% identity) than to the oligomeric β-crystallins (35%). Compared to the other γ-crystallins, γS has a four amino-acid N-terminal extension, lacks two C-terminal amino acids and the linker peptide has an additional amino acid (Table 1). Functionally γS also appears different from the other γ-crystallins since it is essentially expressed in the post-natal period and is therefore preferentially located in the lens cortex (peripheral part of the lens) [10,11].

From a structural standpoint, only a few studies have been devoted to bγS. Sedimentation equilibrium has shown that it was monomeric in solution at physiological pH [12]. In addition, the bγS C-terminal domain has been crystallised and the three-dimensional structure was found to be similar to that of the other γ-crystallins [13]. Therefore, the γS-crystallin three-dimensional structure has been modelled [12] on the basis of bγB [14] and bβB2 structure [15]. Only recently, the thermodynamic characterisation of equilibrium unfolding transitions, at varying pH, of recombinant hγS and bγS and their isolated N- and C-terminal domains has been published [16]. Compared to γB, γS was found less stable.

All these results prompted us to study γS-crystallin structure, interactions and oxidative behaviour in solution. We have used for our studies recombinant hγS, a C20S hγS mutant, and native bγS. When necessary, bγB and bγD were used for the sake of comparison.

2. Materials and methods

2.1. Expression of hγS

The construction of the expression plasmid for the hγS has been previously described [16]. In short, the coding sequence was amplified from a human lens cDNA library and cloned NdeI/BamHI in the pET3a vector. The expression construct was introduced into the BL21(DE3) pLysS bacterial strain. The LB medium (400 ml) containing ampicillin (100 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}) was inoculated with 1 ml of an overnight culture. Cells were grown until an $OD_{600\ nm}$ between 0.3 and 0.6 was reached. Protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 5 mM at 37°C for 4 h under vigorous shaking. The cells were harvested by centrifugation at 6000 rev. min^{-1} for 10 min at 4°C and resuspended in 20 ml buffer: 50 mM Tris–HCl (pH 7.0), 100 mM glucose, 10 mM EDTA, 1.5 mM phenylmethylsulfonyl fluoride

Table 1

```
N-arm
Bovine γB      ----
Bovine γD      ----
Human  γS      SKTG
Bovine γS      SKTG


N-terminal domain
               1                  20                  40                  60                  80
               |                  |                   |                   |                   |
Bovine γB      GKITFYEDRGFQGHCYECSSDCPNLQPFSRCNSIRVDSGCWMLYERPNYQGHQYFLRRGDYPDYQQWMGFNDSIRSCRLIP
Bovine γD      GKITFYEDRGFQGRHYECSSDHSNLQPLGRCNSVRVDSGCWMIYEQPNYLGPQYFLRRGDYPDYQQWMGLNDSIRSCRLIP
Human  γS      TKITFYEDKNFQGRRYDCDCDCADFHTLSRCNSIKVEGGTWAVYERPNFAGYMYILPQGEYPEYQRWMGLNDRLSSCRAVH
Bovine γS      TKITFFEDKNFQGRHYDSDCDCADFHMLSRCNSIRVEGGTWAVYERPNFAGYMYILPRGEYPEYQHWMGLNDRLSSCRAVH

Bovine γB      QHT-GT
Bovine γD      -HAG-S
Human  γS      LPSGGQ
Bovine γS      LSSGGQ


C-terminal domain
                          100                 120                 140                 160
                           |                   |                   |                   |
Bovine γB      FRMRIYERDDFRGQMSEITDDCPSLQDRFHLTEVHSLNVLEGSWVLYEMPSYRGRQYLLRPGEYRRYLDWGAMNAKVGSLRRVM
Bovine γD      HRLRLYEREDYRGQMIEITEDCSSLQDRFHFNEIHSLNVLEGSWVLYELPNYRGRQYLLRPGEYRRYHDWGAMNAKVGSLRRVI
Human  γS      YKIQIFEKGDFSGQMYETTEDCPSIMEQFHMREIHSCKVLEGVWIFYELPNYRGRQYLLDKKEYRKPIDWGAASPAVQSFRRIV
Bovine γS      YKLQIFEKGDFNGQMHETTEDCPSIMEQFHMREVHSCKVLEGAWIFYELPNYRGRQYLLDKKEYRKPVDWGAASPAVQSFRRIV


C-terminal extension
                174
                 |
Bovine γB      DFY
Bovine γD      DIY
Human  γS      E
Bovine γS      E
```

(PMSF). The suspension was sonicated then centrifuged at 17 000 rev. min^{-1} for 20 min at 4°C. The supernatant was clarified by another centrifugation at 30 000 rev. min^{-1} for 90 min at 4°C and dialysed overnight, at 6°C, against the selected buffer: 50 mM Tris–HCl, pH 7.0 (8.0 or 8.5), 1.5 mM PMSF or 150 mM phosphate (22 mM Na_2HPO_4, 28 mM KH_2PO_4, 200 mM KCl, 1.3 mM EDTA, 3 mM NaN_3 and 3 mM DTT) (pH 6.8), 1.5 mM PMSF. Ethylene Imine Polymer (PEI from Hampton Research) was added to a final concentration of 0.12% and left for 10 min at room temperature before centrifugation at 17 000 rev. min^{-1} for 10 min at 6°C. All steps were checked by SDS-PAGE electrophoresis.

2.2. Purification of hγS

To purify the C-terminal domain of the recombinant bγS, Basak et al. [13] have used a MonoQ ion exchange chromatography. We found, however, that gel filtration chromatography was as efficient to isolate hγS (note that *E. coli* proteins with a molecular weight approx. 20 kDa are rare). So, after the last centrifugation of the protein extracts, the supernatant was filtered and applied to a Sephacryl S-200 HR column, on a FPLC system (Pharmacia), equilibrated with either 50 mM Tris–HCl at the selected pH or 150 mM phosphate (pH 6.8). The γS containing fractions were collected and concentrated by ultrafiltration using an Amicon cell (Biomax-5 Millipore membranes). The purity of the recombinant protein was checked by SDS-PAGE electrophoresis and mass spectrometry.

2.3. The C20S hγS

The Cys20 → Ser mutation was introduced using the QuickChangeTM site — directed mutagenesis kit (Stratagene). The sense sequence of the mutation primers was CTA TGA CTG TGA TTC CGA CTG TGC AG. The mutation changes the Cys codon TGC to the Ser codon TCC and introduces a *Hinf* I site. This was used to screen the clones for the presence of the mutation. One of the mutant clones was picked and sequenced to ensure the absence of other mutations. The mutated plasmid was used to transform Bl21(DE3) pLysS competent cells from Stratagene. The protocol for the expression and purification of the C20S recombinant crystallins was the same as for the wild-type protein.

2.4. Purification of bγ-crystallins

bγ-crystallins were prepared following the main lines of the method described in Tardieu et al. [17]. They were extracted from the cortex of young calf lenses. The cortex was separated from the nucleus (central part of the lens) by gentle stirring of the lens in 150 mM phosphate buffer (pH 6.8) for 15 min at 6°C. The cortex mixture added with 43 mM PMSF and adjusted to 60 mg ml^{-1} was then crushed with a Potter crusher. The solution was then centrifuged at 30 000 rev. min^{-1} for 60 min at 6°C. The protein concentration, c_p (g ml^{-1}), was measured with an Abbe refractometer using $n = 1.3342 + 0.184\ c_p$ and adjusted to 50 mg ml^{-1}. γ-Crystallins were prepared from the clear supernatant fraction by gel filtration, using a FPLC system and a Superdex S-200PG column eluted with the phosphate buffer at a flow rate of 3 ml min^{-1}. The γ-crystallin fractions were concentrated by ultrafiltration (Amicon cell with Biomax-5 Millipore membranes).

The various γ-crystallins were then purified by cation exchange chromatography on a MonoS HR 10/10 column. To exchange the phosphate buffer against the buffer used with the MonoS column, the total γ fractions were applied to a Fast Desalting column eluted with 50 mM MES (pH 6.1) (buffer A) at a flow rate of 8 ml min^{-1}. The MonoS column was equilibrated with buffer A and then eluted at a flow rate of 4 ml min^{-1}. A gradient from 0 to 10% buffer B (buffer A + 1 M NaCl) was applied. γ-Crystallin concentrations were measured by UV absorption at 280 nm using an extinction coefficient of 1.85 cm^2 mg^{-1} for γS and 2 cm^2 mg^{-1} for the other γ-crystallins.

2.5. Electrospray mass spectrometry

The mass spectrometry measurements were made using an atmospheric pressure ionisation, single quadrupole, electrospray ionisation mass

spectrometer (ESI-MS) from Micromass. The continuous phase was 50% CH_3CN/H_2O run at 10 μl min^{-1} and samples were dissolved in the continuous phase containing 0.5% v/v HCOOH. Samples which have been run on a reversed phase column were in an elution buffer mixture of CH_3CN/H_2O containing 0.1% v/v TFA and these samples were applied directly to the mass spectrometer. Data were collected in a series of 10-s scans over an m/z range of 750–1150 for a period of 2 min. The raw data were processed using the MassLynx 3.0 software from Micromass.

2.6. Determination of oligomeric states

Protein size, i.e. oligomerisation, was measured by gel filtration on a FPLC system. On a Superdex S-75 HR 10/30 column, 50 μl of protein solutions at 20–25 mg ml^{-1} were applied and eluted at 1 ml min^{-1} with different buffers.

2.7. Small angle X-ray scattering (SAXS)

The intensity scattered by one particle as a function of the scattering vector s (where $s = 2\lambda^{-1}\sin\theta$ and 2θ is the scattering angle), usually called the particle form factor, is the Fourier transform of the spherically averaged auto-correlation function of the electron density contrast associated with the particle [18]. When the solution is ideal, i.e. in the absence of interactions, the total scattering, $I(0,s)$, is the sum of the scattering of the individual particles. With solutions of monodisperse spherical particles, and in the presence of interactions, departure from ideality may simply be accounted for by a multiplying factor or interference term, $S(c,s)$, which is a function of the scattering angle:

$$I(c,s) = I(c,0) \cdot S(c,s)$$

$S(c,s)$ is usually called the solution structure factor. With repulsive interactions, the particles are evenly distributed and $S(c,0)$ is lower than 1. With attractive interactions, fluctuations in the particle distribution are observed which lead to a $S(c,0)$ value larger than 1.

The experiments were carried out using the small angle instrument D24 using the synchrotron radiation emitted by the storage ring DCI at the Laboratory for Synchrotron Radiation, L.U.R.E. (Orsay). Data were collected using a linear position-sensitive detector with a delay line readout. The sample-to-detector distance was 1.6 m, yielding an s-increment per channel of 0.0002454 $Å^{-1}$ and the average recorded s range was $0.2 \times 10^{-2} < s < 4 \times 10^{-2}$ $Å^{-1}$. The wavelength of the X-rays was 1.488 Å (K-edge of Ni). The solution experiments were performed with a specially designed quartz cell operated under vacuum [19] that could be filled and rinsed in situ. Several successive frames, from 1 to 6 according to the protein concentration, each with a duration of 300 s, were recorded for each sample and for the corresponding buffer. The curves were scaled to the transmitted intensity and averaged when no radiation damage was observed. The intensity curves, $I(c,s)$, were subtracted for background and, within each series, scaled on the same relative value with a normation for concentration. When the X-ray spectra were unchanged as a function of time, the six spectra were averaged and one value of R_g was deduced. Intensities at the origin and radii of gyration were obtained using Guinier plots, i.e. plots of log $I(c,s)$ vs. s^2 [20].

3. Results

3.1.1. γS-crystallin three-dimensional structure in physiological conditions

Small angle X-ray Scattering was used to characterise the structures in solution of hγS and bγS and to compare them to that of bγB, whose three-dimensional structure is known. The experiments shown in Fig. 1 were performed in physiological-like conditions, i.e. in phosphate buffer (pH 6.8). The γS radii of gyration were found to be 19.5 ± 0.5 Å in this buffer (Table 2), which is consistent with the presence of monomers in solution. This result is in agreement with the ultracentrifugation experiments performed on bγS in phosphate buffer (pH 7.0) [12]. Moreover, as can be seen on the figure, the three curves superimpose which means that the three-dimensional structure in solution is identical in all three cases.

Table 2
Averaged radius of gyration (Å) of γ-crystallins as a function of X-ray exposure time (six successive frames of 5 min each) in different buffers[a]

	150 mM phosphate (pH 6.8) w DTT	50 mM Tris (pH 8.0) w DTT	50 mM Tris (pH 8.0) w/o DTT
b γS	19.0	19.7	21.1; 24.8[b]
h γS	19.9	20.4	21.7; 25.5[b]
h γS C20S	19.4	20.5	21.2; 27.9[b]
b γB	17.6[c]	16.0	17.0
b γD	18.6[c]	18.8	19.1

[a]Averaged R_g values are indicated when no significantly differences between the six frame spectra are observed.
[b]R_g values in the first frame spectra (5 min exposure) and the last frame spectra (5 min after 25 min exposure) are respectively indicated.
[c]Results from Finet [23].

The coincidence confirms that the relative position and orientation of the N- and C-terminal domains of γS-crystallins is the same as that of the other γ-crystallins, as postulated by Zarina et al. [12]. Indeed, since X-ray scattering curves probe the overall shape of the particles, a different domain pairing would have resulted in visible differences in the shape of the X-ray curves.

3.2. Oligomeric state as a function of pH

A comparative analysis of the oligomeric state of the bγB, bγS and hγS as a function of pH, by gel permeation chromatography, showed a specific behaviour of γS (Fig. 2). Whereas bγB remained essentially monomeric in the pH range between 7.0 and 8.5, both γS preparations yielded different species depending on the pH value. At pH 7.0, hγS and bγS preparations gave only monomers, whereas a mixture of monomers and dimers was observed at pH 8.0 as shown in Fig. 2. The same result was obtained by mass spectrometry (not shown). At pH 8.5, only hγS was studied; the amount of dimers was higher than at pH 8.0. The kinetics of hγS dimer formation was studied at pH 8.0. When the gel filtration was done immediately after the *E. coli* protein purification, only monomers were observed. The dimers began to appear after one day of storage at 6°C (Fig. 3a). The amount of dimers continued to increase during the next few days (between 3 and 5 days) and then remained identical, without any further oligomer formation, for at least 3 weeks (Fig. 4) in agreement with a monomer–dimer equilibrium. On the other hand, bγB and bγD, either prepared at pH 8.0 or pH 8.5 and then kept at 6°C in the same buffers, only yielded monomers, stable in time for at least 3 weeks, as also shown in Fig. 4.

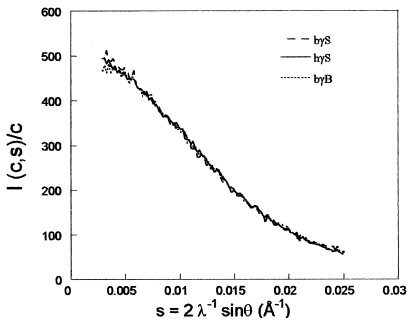

Fig. 1. SAXS patterns of bγS (…), hγS (—) and bγB (⋯) at 10 mg ml^{-1} in phosphate buffer 150 mM (pH 6.8) at 20°C.

3.3. A C20–C20 disulfide responsible for intermolecular dimer formation in the γS-crystallin

Since the cysteine *p*K is between pH 8.0 and 8.5, dimer formation around pH 8.0 often results

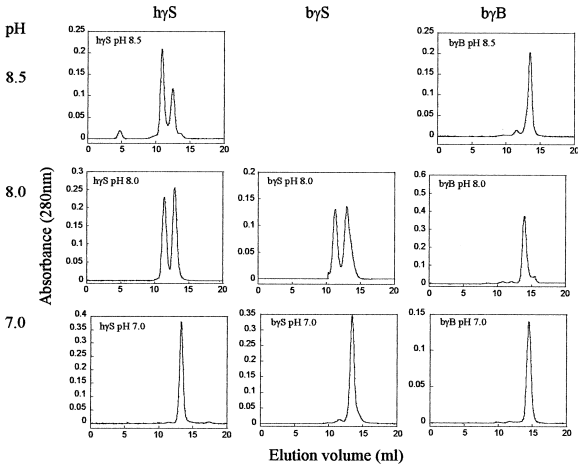

Fig. 2. pH-dependent oligomeric states of bγS, hγS and bγB as observed by gel filtration chromatography. All samples are in Tris buffer 50 mM either pH 8.5, 8.0 or 7.0.

from disulfides. In order to check this point, hγS was prepared at pH 8.0 with DTT and the protein was found only as monomers (data not shown). In addition, the dimer peak of the pH 8.0 preparation without DTT was incubated overnight with DTT. The dimers were found to be reversed to monomers (Fig. 3b).

To explain the oligomeric behaviour of the γS, we looked for the residues able to form disulfides and in particular for a surface accessible cysteine that could form an intermolecular disulfide in γS but not in the other γ-crystallins. Among the

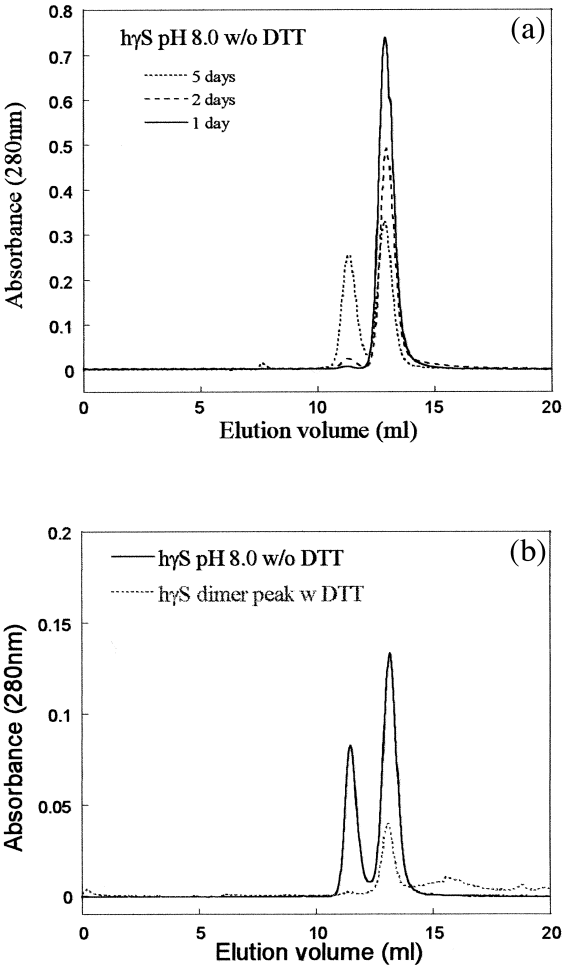

Fig. 3. (a) Kinetic of hγS dimer formation at pH 8.0 (Tris 50 mM without DTT) as a function of time. (b) Effect of dithiothreitol on the hγS dimer peak.

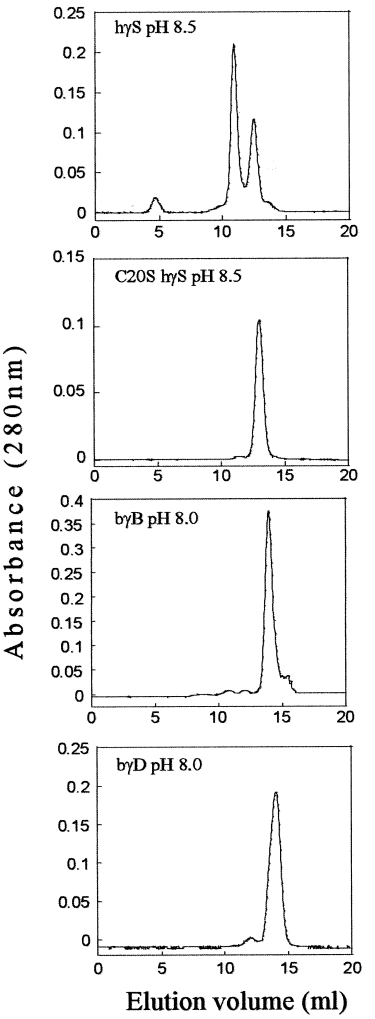

Fig. 4. From top to bottom, hγS*, C20S hγS*, bγB and bγD equilibration state after three weeks in Tris buffer 50 mM pH 8.0 or 8.5* as observed by gel filtration chromatography.

γ-crystallins that we have studied — bγB, bγD, bγS and hγS-crystallins (see Table 1) — three Cys residues are in common: Cys32, Cys78 and Cys109 (numbered 108 in γD); two residues are unique to bγS and hγS: Cys20 and Cys124, and hγS has an additional one in position 18. Previous studies of the C-terminal bγS domain [13] and our results indicate that the γS structure is identical to that of the other γ-crystallins. From the available three-dimensional structure of bγB [14] and from the accessibilities listed in Table 3,

only a few of these residues are partially accessible to solvent — Cys15, Cys18, Ser20 (which is Cys20 in γS) and Cys22 — and could be candidates for oxidation. Cys15, only present in bγB, was presented as a likely candidate for intermolecular cross-linking in other studies [5], but no dimers are formed in our conditions. In the cysteine rich domain (17–22), the hγS contains the sequence DCDCDC — which has not been observed in any other known protein sequence — the bγS contains DSDCDC whereas bγB contains ECSSDC and bγD ECSSDH. In the three-dimensional structure of bγB [14], around 50% of Cys18 and Cys22 were found engaged in an intra-disulfide bridge. Since the originally crystallised protein did not contain any disulfide, the study indicated that oxidation may occur without unfolding. Moreover, the disulfide bridge was readily reduced in the presence of DTT. The similarity in the three-dimensional structures of the γ-crystallins allows the inference that the equivalent disulfide is possible between Cys18 and Cys22 of hγS, leaving the highly accessible Cys20 free for intermolecular disulfide formation. Consequently, we hypothesised that Cys20 was the likely candidate for the specific dimer formation in the γS-crystallins and made, in this view, the hγS C20S mutant.

3.4. A hγS C20S mutant to check the hypothesis

If the hypothesis is correct, the hγS C20S mutant protein should remain monomeric at pH 8.0 or higher. The mutant was made, the protein expressed in *E. coli,* purified according to Section 2 and controlled by mass spectrometry experiments. The mutant protein prepared either at pH 8.0 or 8.5 without DTT resulted in a single peak corresponding to the monomeric species. The chromatography was repeated as a function of time. Approximately 3 weeks later, the preparation was still in a monomeric form, as shown in Fig. 4.

3.5. Sensitivity to oxidation of γS-crystallins: oligomer formation in the X-ray beam

During the course of this work, we noticed that the samples sensitive to oxidation were even more sensitive to the oxidative conditions due to the free radicals produced by X-ray irradiation. The effect was systematically studied. For that, X-ray spectra were systematically recorded in fractionated periods of time, in order to detect any gradual change. Six spectra of 5 min each were sequentially recorded for a total exposure time of 30 min.

The samples prepared in phosphate buffer at pH 6.8, which contains DTT, did not present any clue of aggregate formation during the period of measurement, in agreement with previous work [17]. On the other hand, the series recorded with the different γS at pH 8.0 without DTT, present an irreversible increase of the scattered intensity in the low angle part of the scattering curves which indicates the formation of an increasing amount of oligomeric species of increasing size (Fig. 5). The corresponding radii of gyration are shown in Table 2. The initial value of 20 ± 0.5 Å for the γS-crystallin is consistent with only monomers in the solution. Instead the final values of the extrapolated intensity at the origin and of the radii of gyration (approx. 28 Å) indicate that oligomers higher than dimers are formed. The addition of DTT, three hours before the measure-

Table 3
Solvent accessibility of γB-crystallin (PDB*n*4GCR) cysteines calculated as Connoly surface with Insight II[a]

Residue	Contact area (Å^2)	Reentrant area (Å^2)	Total area (Å^2)
15	17.38	28.58	45.96
18	2.37	21.30	23.67
20*	20.23	20.75	40.98
22	5.12	18.13	23.25
32	0.00	0.00	0.00
41	2.61	17.07	19.68
78	0.00	0.00	0.00
109	0.09	4.87	4.96
124*	0.19	6.37	6.66

[a]All residues are cysteine except those with * which are Ser20 and Lys124 in bγB-crystallin, but Cys20 and Cys124 in hγS and bγS-crystallin. bγS Cys124 was checked to be buried in the three-dimensional C-terminal domain structure (PDB*n*1A7H).

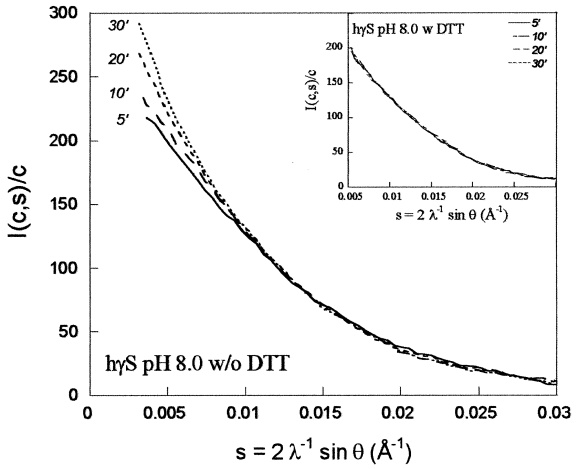

Fig. 5. hγS-crystallin SAXS patterns, in Tris 50 mM (pH 8.0) without DTT, as a function of X-ray exposure time (six successive frames of 5 min each). In the insert, identical sample with DTT added before X-ray experiment.

ments, was sufficient to prevent such an oligomer formation in the X-ray beam (insert in Fig. 5).

X-Ray induced oligomer formation therefore also seems to be associated to thiol oxidation. The X-ray induced oxidation, however, seems much more drastic than the oxidation occurring in storage conditions, since high MW oligomers are formed in 30 min. Moreover, hγS C20S oligomers were also observed in 30 min. It thus appears that new residues, probably Cys or Met, become accessible to oxidation, indicating partial unfolding in these more drastic oxidation conditions.

3.6. The reason why γS-crystallins do not opacify at low temperature

It is known for a long time that bγS does not display the cold cataract phenomenon. The cold cataract is a phase separation induced by decreasing the temperature. It is the consequence of the short-range attractive interactions between γ-crystallins at physiological pH [21,22]. X-Rays can be used to analyse the overall nature, attractive or repulsive, of the interactions in solution. Intensity curves recorded as a function of pH and protein concentration with total bovine γ-crystallin extracts are shown in Fig. 6a. As can be seen on the figure, the intensity at low angles increases with increasing protein concentration at pH 6.8, indicating attractive interactions, and decreases at pH 4.5 when the interactions are repulsive. A similar X-ray study recently performed with purified species, bγB, bγD, bγE [23] indicated also that the interactions are attractive at physiological pH and still attractive at pH 8.0 (note that in some cases, it may be difficult to distinguish the attractive interactions responsible for phase separation from oligomer formation leading to precipitates. In the present study the distinction was easy to do since the oligomer formation was irreversibly increasing with time).

A comparative analysis of the interactions in solution of bγS, hγS and hγS C20S was done at pH 6.8 and pH 8.0. All the samples were freshly prepared in order to only have monomers in solution and only 5-min spectra were recorded to avoid possible X-ray damage. As shown in Fig. 6b, the hγS-crystallin curves recorded in physiological-like buffer (phosphate buffer pH 6.8) as a function of protein concentration are superimposed, which means that hγS do not display any significant interaction, neither attractive nor repulsive. The same result was obtained in 50 mM Tris buffer pH 8.0 as well as with the bγS and hγS C20S samples in both buffers (data not shown).

4. Discussion

4.1. Three-dimensional structure and oligomerisation

We have shown that the γS structure in solution was similar to that of the other γ-crystallins, thus confirming the model built by Zarina et al. [12], and we have demonstrated that limited γS oxidation leads to the formation of dimers. This dimer formation does not seem to be accompanied by any other conformational modification. This point is important. It is supported by the observations: (1) that the dimer is reversible, almost totally, either by adding some reducing agents or by decreasing the pH; (2) that the equilibrium ratio of the species (dimers/mono-

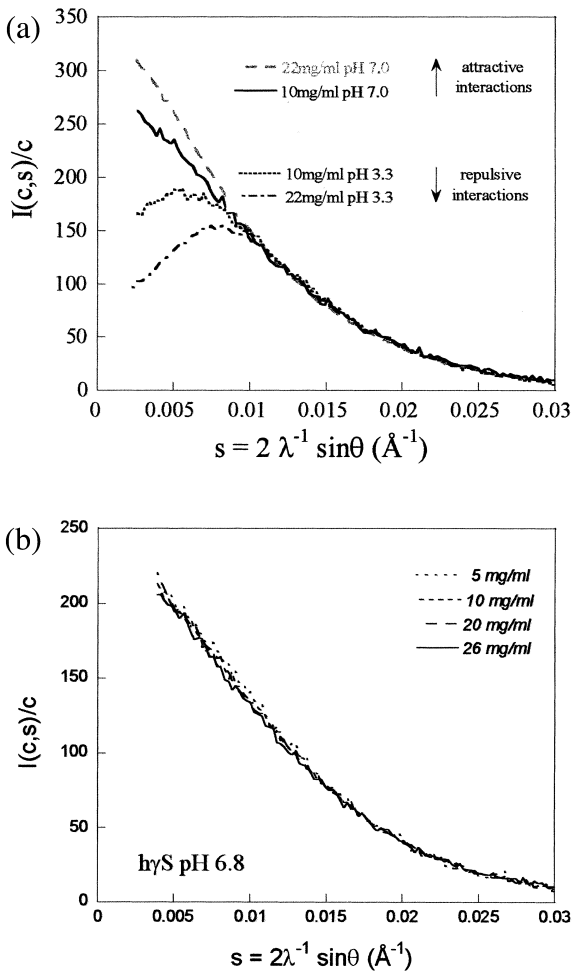

Fig. 6. (a) SAXS patterns of total bγ-crystallins from lens nuclei, in phosphate 10 mM at pH 7.0 (attractive interactions) and pH 3.3 (repulsive interactions) at two protein concentrations [30]; (b) hγS-crystallins SAXS patterns in phosphate buffer 150 mM (pH 6.8) as a function of protein concentration.

mers) is maintained as a function of time and that no higher molecular weight species is formed. The involvement of Cys20, that is exposed to the solvent, in intermolecular disulfide bridge formation, was likely. It has been confirmed by the results obtained with the hγS C20S mutant that does not form dimers in our gentle oxidative conditions.

A study of Pande et al. [5] describes the formation of bγB-crystallin dimers as a function of time, at least in part through direct disulfide bonds, whereas in the present study we only observed bγB monomers. Since Cys20 is not present in bγB, other residues have to be involved, possibly Cys15, which is absent in hγS. The absence of bγB dimers in our conditions might be due to differences in storage conditions — 6°C in our case vs. room temperature — and seems to indicate that the dimer formation of bγB, after long storage at room temperature without reducing agent [5], could also be accompanied by the beginning of an unfolding process.

The results obtained here confirm that the various γ-crystallins species may respond rather differently to an oxidative stress and that the extent of the oxidative stress may lead to different consequences. Oxidation limited to one amino acid located at the surface, leading to reversible dimers via disulfides like for hγS, without three-dimensional structural changes, would not be dangerous for the lens; it could even play a protective role (in acting as an oxidation 'buffer'). The opportunity to make an internal disulfide in bγB and hγS, could also be a stabilising factor. Such a limited oxidation could, however, become dangerous if, as demonstrated by the recent study of the hγD R14C mutant [9], a second reactive site is present at the surface, allowing the formation of large aggregates. In this respect, it may be interesting to note that the second site that seems to be reactive in hγD is Cys110, which is absent in bγD.

On the other hand, more extensive oxidation as observed during X-ray exposure, seems to be able to reach partially buried residues and therefore to involve local denaturations. Such an oxidation could be the first step of non-specific aggregate formation possibly leading to cataract.

4.2. Interactions and phase separation

The experiments presented here clarify the origin of some specific properties of γS. Since the presence of a phase separation as a function of temperature is associated to the strength of the attractive interactions, the fact that the hγS and bγS solutions in physiological conditions do not display attractive interactions, like the other γ-

crystallin solutions [17], readily accounts for the absence of a phase separation as a function of temperature. It also explains why the presence of γS in concentrated solutions of other γ-crystallins, γB and γE, may lower the phase separation temperature as reported in Liu et al. [24]. Moreover, since in the eye lens, the γ-crystallins that present attractive interactions are preferentially located in the centre of the lens (nucleus) whereas the γ-crystallins that present repulsive interactions are preferentially located at the periphery (cortex), the absence of attractive interactions is also coherent with the γS location in the cortex rather than in the nucleus [25].

In previous studies of other bγ-crystallins [5,26] the phase separation temperature was also shown to be critically dependent upon the oligomeric state. The increase in phase separation temperature could, however, rather be associated to a non-native structure of the oligomers. Indeed, we did not find such an increase of the attraction with the hγS mixture of monomers/dimers obtained at pH 8.0 (data not shown). The experiments presented here demonstrate instead that, with γS-crystallins, the interactions in solution that control the phase separation are uncoupled from the chemical reactions that govern the formation of covalently linked oligomers.

Finally, the reason why the different γ-crystallins, that have the same three-dimensional structure [14,27] may have different interactions in solution and different critical temperatures, remains unclear. The point has been extensively investigated by Norledge et al. [28], the surface charge distribution has been analysed by Chirgadze and Tabolina [29], the small yet true differences in interactions between the different γ-crystallin have been measured recently by Finet [23], yet without providing us with any clear answer. This intriguing question therefore remains a topic to investigate in further studies.

5. Nomenclature

hγD (or S): human γD (or S)-crystallin
bγS (B or D): bovine γS (B or D)-crystallin
FPLC: fast liquid protein chromatography
SAXS: Small angle X-ray scattering

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