Near-infrared Fourier transform Raman and conventional Raman studies of calf γ-crystallins in the lyophilized state and in solution

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ABSTRACT We present in this report a detailed structural study of calf γ-crystallins both in the solid state and in solution by the newly developed technique of near-infrared (IR) Fourier transform (FT)-Raman spectroscopy as well as by the conventional Raman method. In comparison with conventional laser Raman spectroscopy, the near-IR FT-Raman approach exhibits several attractive features such as fluorescence rejection capability, frequency accuracy, and the FT's multiplex and throughput advantages. These distinct characteristics combined form the basis for the particular suitability of FT-Raman in crystallin structural analysis and elucidation. We have thus obtained evidence in support of the view that native calf γ-II crystallin does not contain a disulfide bond either in the lyophilized state or in solution. In addition, conventional Raman spectra are examined for all four γ-crystallin fractions, γ-S, γ-II, γ-III, and γ-IV, and the results indicate a high degree of structural similarities among them. It is also found that the sulfhydryl groups in all four γ-crystallins are highly resistant to air oxidation and are capable of maintaining their reduced state during isolation in the absence of added reductants or such chelating agents as EDTA.

INTRODUCTION

Among the three major structural proteins (commonly designated α-, β-, and γ-crystallins) of the eye lens (1–3), the γ-crystallins (γ-S, γ-II, γ-III, and γ-IV) exhibit several interesting characteristics: (*a*) they are composed of closely related, monomeric polypeptides with molecular weights of ~20,000 D (4, 5); (*b*) they are unusually rich in sulfhydryl (SH) groups (4, 6); and (*c*) they are deposited predominantly in the lens nucleus as a result of their synthesis in early development (7, 8). Extensive amino acid sequencing (9, 10) and x-ray crystallographic studies (11, 12) of this unique family of eye lens proteins (especially, γ-II, the most abundant γ-crystallin fraction) have greatly enhanced our understanding of the fundamental aspects of lens transparency and cataract formation. The γ-II crystallin is remarkably stable against cryoprecipitation, denaturation, and other forms of structural modifications (13); such stability has been postulated to arise from its exceptionally high internal symmetry (a two-domain β-structure with four similar "Greek key" motifs) and the presence of ion pairs and hydrophobic patches on the protein surface (11, 12). The γ-crystallins as a family are also known to be vulnerable to inter- and intramolecular structural alterations under oxidative stress or severe dehydrating conditions (14, 15). For example, covalent changes of the γ-crystallin polypeptide chain have been shown to occur during human lens aging and brunescent cataract formation (16, 17).

The precise structure and behavior of the monomeric γ-crystallins, however, still remain incompletely elucidated. The fact that bovine γ-II crystallin is so stable and yet contains a large number of reactive sulfhydryl groups (11, 12) needs to be adequately explained, especially in view of the well documented involvement of thiol groups in human cataractogenesis (18, 19). The spatial locations of the seven cysteine residues in calf γ-II crystallin are now known through x-ray studies (12); the SH reactivities have also been estimated on the basis of their accessibilities toward organomercurial/gold compounds (20) and their reaction with reduced or oxidized glutathione to form protein-glutathione adducts (21). On the other hand, a general consensus is still lacking as to the oxidation state of the sulfhydryl groups in native calf γ-II crystallin and to the possible structural/metabolic functions of the sulfur atoms. X-Ray crystal data obtained for calf γ-II at 1.9 Å resolution indicated that cys 18 and 22 were spatially close enough to form a disulfide (SS) bond (12), whereas a later investigation with freshly grown crystals showed the absence of a disulfide linkage and the sulfur atoms of cys 18 and 22 pointing in opposite directions (22). In an attempt to resolve this uncertainty, laser Raman spectroscopy has been applied to the analysis of SH and SS groups in γ-crystallins. The Raman results reported by two research groups, however, appear conflicting: whereas Yu, N.-T. and co-

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workers presented evidence for the complete absence of disulfide vibrational signals in the Raman spectrum of calf γ-II crystallin in the lyophilized state (23), Spector, A. and co-workers argued for the presence of a disulfide bridge in the same protein on the basis of spectrophotometric assay (24) and solution Raman data (25). Resolving this apparent incongruence can only be made through a direct comparison of solid-state and solution Raman data, because these two studies were carried out with lyophilized lens proteins and crystallin solutions, respectively. Furthermore, the previous Raman spectra reported by Yu et al. (23) and by Pande et al. (25) are generally poor in signal-to-noise ratios, and the conclusions drawn from such noise-laden spectra should be treated with caution. Clearly, it is necessary to acquire high-quality Raman spectra of γ-crystallins both in the lyophilized state and in solution (under the same instrumental conditions) before this conflict can be resolved.

Toward this end, we have applied the newly developed technique of near-IR FT-Raman to investigate the detailed structure of lens crystallins. The conventional Raman method (i.e., dispersive scanning spectrometer with PMT photon counting and visible laser excitation) is also employed to study lyophilized protein powders because the conventional approach yields equally excellent results for solid samples that are highly purified and are essentially free of fluorescence.

As has been amply demonstrated in the literature (26, 27), the application of Raman spectroscopy in lens research has provided valuable molecular information unobtainable through any other existing methodologies, such as conformation determination of lens crystallins *in situ,* visual axis profiles of various chemical constituents, and in vivo studies of precataractous lenses. However, the conventional Raman method frequently suffers from the drawbacks of fluorescence interference, poor spectral reproducibility, and long periods of data acquisition time. The new technique of near-IR-excited Fourier transform (FT) Raman can eliminate all these problems (28, 29). As will be shown in this study, high-quality FT-Raman spectra, free from fluorescence interference, can be readily obtained for ocular lens proteins both in the lyophilized state and in aqueous solution. This technical improvement has thus made it possible to carry out a more thorough study of the state of sulfhydryl groups in calf γ-crystallins. Clear spectroscopic evidence has been obtained in this study, supporting the complete lack of a disulfide bond in native γ-II crystallin. Conventional Raman spectra are also measured for the four γ-crystallin fractions, γ-s, γ-II, γ-III, and γ-IV, for purposes of structural comparison and evaluation of the stability of sulfhydryl groups in γ-crystallins.

MATERIALS AND METHODS

Separation of calf γ-crystallin fractions

Calf γ-crystallins were isolated from the core regions of calf lenses in the absence of a protectant according to two slightly different procedures as described in the literature (4, 21, 24, 30).

(*a*) *The procedure of Slingsby and Miller* (21). Immediately before use, three or four frozen calf lenses were thawed and homogenized in pH = 7.2 buffer solution (0.05 M tris-HCl, 0.02% NaN_3). The homogenate was separated into soluble and insoluble proteins by centrifugation at 135,000 *g* for ~1 h at 4°C. The monomeric γ-crystallins in the soluble portion were then fractionated from the α- and β-crystallins by gel chromatography on a Sephadex G-75 column (Pharmacia, Inc., Piscataway, NJ) with the same pH = 7.2 buffer as the eluent. The γ-crystallin mixture solution was concentrated to ~10 ml by ultrafiltration (Immersible CX-10; Millipore Corp., Bedford, MA) and was dialyzed first against deionized distilled water and then against 0.2 M sodium acetate buffer (pH = 5.0). Further separation of the mixture into γ-s, γ-II, γ-III, and γ-IV crystallins was carried out by ion-exchange chromatography on SP-Sephadex C-50 (Pharmacia, Inc.) in sodium acetate buffer with a 2 liter linear sodium acetate gradient (from 0.2 to 0.5 M). The separated four γ-crystallin fractions were then pooled, dialyzed, and lyophilized for storage at −80°C.

(*b*) *The procedure of McDermott et al.* (24). This procedure is similar to (*a*) except that pH = 7.4 buffer solution (0.05 M tris-HCl, 0.2 M NaCl, 1 mM EDTA) was used in lens homogenization and gel filtration, and that SP-Sephadex C-25 (Pharmacia, Inc.) in a pH = 5.0 sodium acetate buffer (0.1 M NaCl, 1 mM EDTA) with a 3 liter sodium acetate gradient (from 0.01 to 0.5 M) was employed in ion-exchange chromatography.

Denaturation and oxidation of γ-II crystallin

The γ-II crystallin solution (~30 ml) collected from the chromatographic separation was dialyzed against deionized distilled water and was then exposed to pH = 8.2 buffer solution (0.05 M tris-HCl, 0.02% NaN_3) for several days. The crystallin was then dialyzed, lyophilized, and stored at −80°C, pending Raman analysis.

For denaturation and oxidation studies of the γ-II crystallin, the protein (~10 mg) was incubated for 48 h in 8 M urea tris-acetate (0.05 M) buffer with and without the presence of 2.5 mM oxidized glutathione, respectively. Dialysis of the protein solutions against distilled water led to precipitation. The precipitated part was then separated from the soluble part by centrifugation, and both parts were lyophilized and stored at −80°C.

Conventional and Fourier transform (FT)-Raman spectroscopy

Conventional Raman spectra were obtained on a model 1401 spectrometer (SPEX Inds. Inc., Edison, NJ) equipped with an RCA 31034 photomultiplier tube, as described previously (31). Visible excitation at 514.5 nm was provided by an Innova-70 Ar^+ laser (Coherent Inc., Palo Alto, CA). Lyophilized protein samples were packed into a conical depression at the end of a stainless rod which was put in a freeze-drying flask containing one drop of deionized distilled water. Excitation powers at the sample were normally 40–60 mW.

FT-Raman spectra of γ-crystallins were obtained in the lyophilized form and in aqueous solution (~100 mg protein/ml) by using a Bruker

IFS 66/FRA 106 FT-spectrophotometer Bruker Instruments, Inc. (Billerica, MA) (32). Continuous-wave (CW) near-IR excitation at 1.064 μm was provided by a diode laser pumped Nd:YAG laser (Adlas, DPY 301; Bruker Instruments). FT-Raman spectra reported in this study were all original and were not smoothed through data manipulation.

RESULTS

Fig. 1 shows FT-Raman spectra of calf γ-total crystallin both in the lyophilized state and in solution specifically in a spectral region where the SS stretching vibration (~510 cm^{-1}) is expected to appear. The FT-Raman spectra are far more superior (as measured by signal-to-noise ratio) than the conventional Raman spectra obtained previously for ocular lens proteins (23, 26). This dramatic improvement arises from two main features: first, the use of near-IR laser excitation at 1.064 μm, which not only eliminates the problem of fluorescence interference from impurities but also reduces the high inelastic scattering background frequently encountered for freeze-dried biological samples with visible laser excitations; second, the use of a Michelson interferometer for detection, which affords multiplex and energy throughput advantages over a dispersion-based Raman system (33).

Clearly, the FT-Raman spectra exhibit no vibrational lines that might be attributable to the disulfide stretching vibration (~510 cm^{-1}). Accordingly, the SH Raman signals are expected to be intense; this is indeed the case as evidenced by the FT-Raman detection of prominent sulfhydryl lines at 2,560 and 2,578 cm^{-1} in Fig. 2. It is also interesting to note that the solution and solid-state FT-Raman spectra are almost identical (except at ~547 cm^{-1}) in spectral relative intensities and frequencies, indicating that γ-total crystallin is structurally rather similar in the lyophilized state and in solution.

High-quality FT-Raman data can also be readily acquired for the individual γ-crystallin fractions. Figs. 3 and 4 depict solid-state and solution FT-Raman spectra of calf γ-II crystallin in the SS stretching frequency region and in the SH spectral region, respectively. Quite analogous to the case of γ-total, the FT-Raman spectra of γ-II show the lack of disulfide Raman signals and similarities for this protein in the freeze-dried state and in aqueous solution. In view of the demonstration that the conventional Raman method is capable of unambiguously detecting one disulfide bond in a ~20,000 mol wt protein (23, 25), the complete absence of SS Raman signals in the superior FT-Raman spectra should be considered as conclusive evidence for the lack of a disulfide bond in native calf γ-II crystallin (mol wt ~20,000).

It is now generally accepted that certain isolation procedures (25) can introduce one disulfide bond in the cysteine-rich calf γ-II crystallin and that reduction with dithiothreitol (but not β-mercaptoethanol) is able to convert the disulfide to its reduced state (24). We have examined conventional Raman spectra of the calf γ-II crystallin isolated according to the procedures of McDermott et al. (24) and that of Slingsby and Miller (21) at

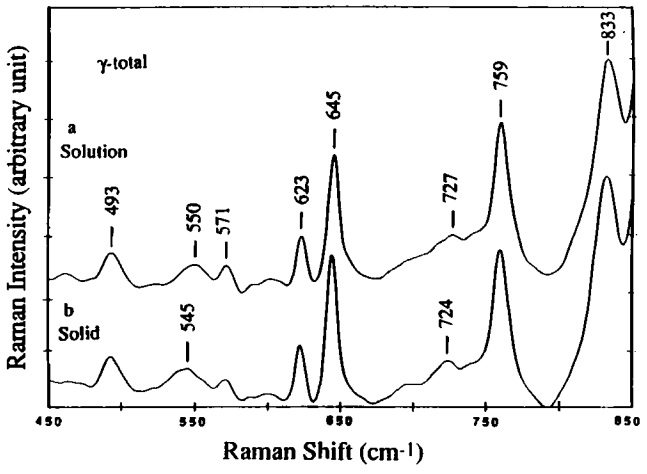

FIGURE 1 Comparison of FT-Raman spectra in the S–S region obtained for calf γ-total crystallin (*a*) in aqueous solution (~100 mg protein/ml water) and (*b*) in the lyophilized state. Date acquisition conditions: excitation wavelength = 1.064 μm; laser power = 300 mW; spectral resolution = 4.0 cm^{-1}; coadded scans = 1,000 (~45 min).

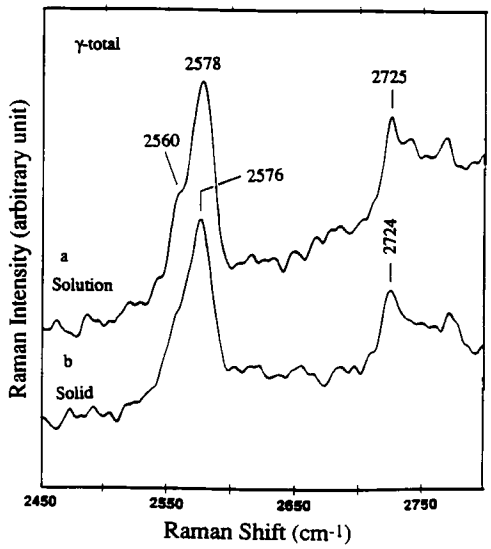

FIGURE 2 Comparison of FT-Raman spectra in the S-H region for calf γ-total crystallin (*a*) in aqueous solution and (*b*) in the lyophilized state. Data acquisition conditions were the same as in Fig. 1.

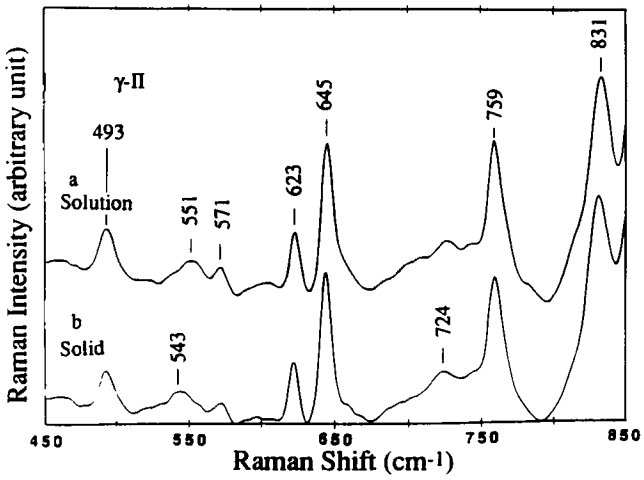

FIGURE 3 FT-Raman spectra of calf γ-II crystallin in the S–S region obtained (*a*) in aqueous solution (~100 mg protein/ml water) and (*b*) in the solid state. Data acquisition conditions were the same as in Fig. 1.

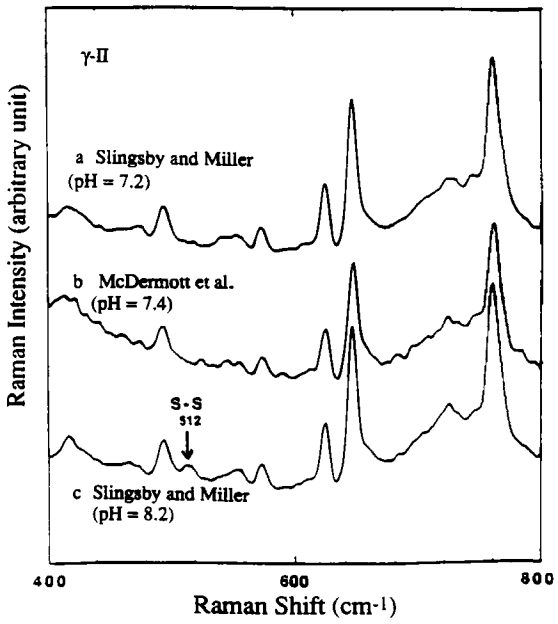

FIGURE 5 Effects of isolation and incubation conditions on the state of sulfhydryl groups in calf γ-II crystallin. The conventional Raman spectra were obtained from lyophilized γ-II crystallin that was isolated by (*a*) the procedure of Slingsby and Miller (1985 [reference 21]), (*b*) the procedure of McDermott et al. (1988 [reference 24]), or (*c*) by incubation pH = 8.2 after isolation. See text for details. Data acquisition conditions: excitation wavelength = 514.5 nm; laser power = 50 mW; scan rate = 1.0 cm^{-1}/s; integration time = 1.0 s; bandpass = 4.0 cm^{-1}; number of repetitive scans = 4.

different pH's (see Fig. 5). The detection of a moderate Raman line at 512 cm^{-1} (Fig. 5 *c*) clearly shows disulfide formation upon incubation of γ-II crystallin at pH = 8.2 for a prolonged period of time. A careful measurement of the SH Raman signal intensities indicates that only one disulfide bond is generated per protein at pH = 8.2, that is, five cysteine residues still remain in the reduced

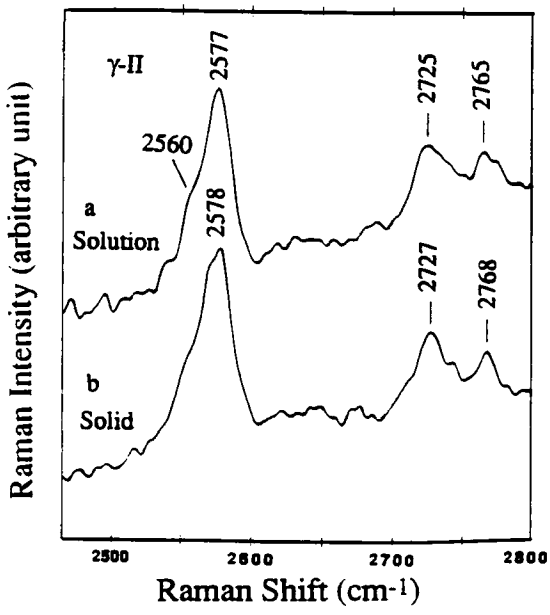

FIGURE 4 FT-Raman spectra of calf γ-II crystallin in the S-H region (*a*) in aqueous solution (~100 mg protein/ml water) and (*b*) in the solid state. Data acquisition conditions were the same as in Fig. 1.

state. As suggested by x-ray studies (11, 12), the intramolecular S–S linkage is most likely to be between cys 18 and 22. At lower pHs, however, the calf γ-II crystallin is stable enough to resist air oxidation even in the absence of protecting agents such as 2-mercaptoethanol.

By employing the conventional Raman technique, we have carried out a comparative study of the SH groups in all the crystallin fractions. The results are displayed in Fig. 6 in the SS stretching region and in Fig. 7 in the SH spectral region. The lack of SS Raman signals for γ-s, γ-III, and γ-IV agrees with the report (24) that these γ-crystallin lack cys 22 and are thus unable to form intramolecular disulfides. For γ-II crystallin, on the other hand, formation of an intramolecular disulfide bond occurs by incubation at pH = 8.2 (Fig. 5 *c*). When γ-II crystallin is unfolded in 8 M urea, incomplete SS formation also takes place (Fig. 6 *e*). As evidenced by the complete disappearance of the SH Raman signals in Fig. 7 *f*, all seven sulfhydryl groups in γ-II are converted to mixed disulfides in the presence of 8 M urea and an excess amount of oxidized glutathione (G-S-S-G). In view of fluorescence and optical circular dichroism (CD) data which indicate that mixed disulfide formation can

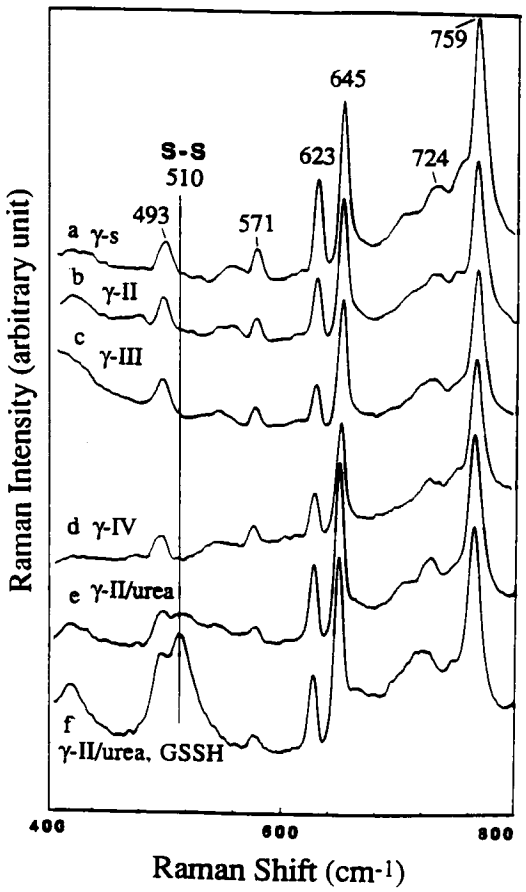

FIGURE 6 Comparison of conventional Raman spectra of the four γ-crystallin fractions in the S–S region and effects of added urea and oxidized glutathione on the state of sulfhydryl groups in calf γ-II crystallin. Data acquisition conditions were the same as in Fig. 5.

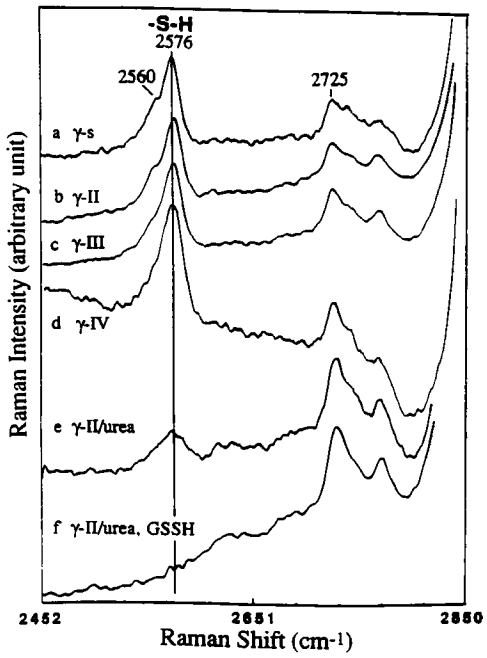

FIGURE 7 Conventional Raman spectra of the four γ-crystallin fractions in the S–H region and effects of added urea and oxidized glutathione on the state of sulfhydryl groups in calf γ-II crystallin. Data acquisition conditions were the same as in Fig. 5.

lead to partial protein unfolding or to a destabilized protein conformation (34, 35), the presence of glutathione is thus expected to facilitate the denaturation of γ-II crystallin through the formation of protein-glutathione SS bonds. This leads eventually to the complete conversion of the seven SH groups to disulfides.

It should be noted that the isolation and purification of γ-crystallin fractions have nearly always been carried out by incorporating a protectant against air oxidation such as 2-mercaptoethanol. In this study, however, the γ-crystallins were prepared in the absence of reducing agents. The lack of disulfide Raman signals thus indicates that all sulfhydryl groups in γ-s, γ-II, γ-III, and γ-IV are resistant to air oxidation and are capable of maintaining their reduced state without added reducing agents during isolation. McDermott et al. (24) have also noted the stability of the SH groups in γ-crystallins. This finding is important in explaining the known stability of the calf γ-crystallins *in situ* which maintain all their SH groups in the reduced state for many decades of existence (11, 12).

DISCUSSION

Techniques for the detection of SH/SS groups in lens proteins

Due to the widely recognized importance of SH and SS groups in lens aging and cataract formation (19), an array of physical techniques has been applied to examine their oxidation states and concentrations in intact ocular lenses as well as in separate protein fractions. As for the nature of the sulfhydryl groups in native γ-II crystallin, previous investigations using x-ray crystallography, spectrophotometric assay, chemical oxidation/HPLC, and laser Raman spectroscopy have not produced a consistent picture. In this paper we reported a novel application of the new FT-Raman technique in the analysis of fractionated lens proteins. It is now appropriate to consider the advantages and limitations of these techniques in more details.

The x-ray method provides important structural information as to the location and arrangement of cysteine residues, but extensive x-ray studies of the calf γ-II

crystallin could not establish the presence or absence of a disulfide link (11, 12). In fact, the x-ray data are rather inaccurate in the cys 18 and 22 region due to distortions in the local electron density caused by nearby heavy atom binding sites. The utility of the x-ray method is further hampered by the fact that few proteins have been crystallized to x-ray standards. Spectrophotometric assay based on the coloration reaction of SH and DTNB [5,5′-dithiobis(2-nitrobenzoic) acid] has been widely employed to measure exposed SH groups in various proteins (36). This wet chemical approach, however, is prone to nonprotein SH interference and measurement inaccuracies. Furthermore, disulfide bonds, either mixed or intraprotein, cannot be detected by this method. As has been demonstrated by Lou et al. (37, 38), chemical oxidation of SH groups and mixed disulfides in conjunction with HPLC is well-suited for analyzing mixed-disulfide bonds and is also able to differentiate glutathione SH from cysteine SH. This approach, however, is destructive in nature (release of GSO_3H and $CysO_3H$ through oxidation, where G = glutathione and Cys = cysteine) and is unable to detect intraprotein disulfides or deeply buried SH groups.

Laser Raman spectroscopy is a nondestructive optical technique, and it is in principle applicable to the study of intact ocular lenses, lyophilized protein powders and protein solutions. Whereas the Raman technique is sensitive enough to detect one disulfide bond in a 20,000 mol wt protein (23, 25), its most attractive feature is perhaps its singular ability to measure the total sulfhydryl and disulfide contents, that is, SH groups that are either exposed or buried and S–S bonds that are either intra- or intermolecular. The recently developed technique of near-IR-excited FT-Raman significantly enhances the utility of Raman scattering in ocular lens research because it combines fluorescence rejection capability and the multiplex and throughput advantages associated with interferometric detection (28, 29). As has been demonstrated above, the technique of near-IR FT-Raman is especially well suited for analyzing the S-H and S–S groups in lens proteins both in the lyophilized state and in solution. It should also be pointed out that the conventional dispersive approach may yield superior results for those highly purified samples that exhibit a low level of fluorescence interference and background scattering.

Stability of SH groups in γ-crystallins

The stability of reduced protein thiols toward disulfide formation has important implications in the normal function and some pathological processes of the ocular lens. The nucleus of certain rodent lenses (viz, mouse and rat lenses) becomes hardened as a result of an early and rapid conversion of SH to SS in the normal aging process (39–41). Such a conversion is also known to occur in human senile cataractogenesis (42). Bird lenses do not develop the hard nucleus of the rodent lenses because in the bird lens the nucleus γ-crystallin is replaced with δ-crystallin, which is practically devoid of reactive SH groups due to the general lack of cysteine residues in this avian lens protein (43, 44). Mammalian γ-crystallins contain more exposed SH groups than any other lens crystallins, and cytoplasmic γ-crystallins have been shown to form disulfide cross-links with membrane proteins in human cataract (45). It is therefore interesting that the calf γ-II crystallins are able to maintain all the SH groups in the reduced state and are remarkably resistant to air oxidation. Because there is little or no capacity for protein regeneration in the lens core region (46), the γ-crystallins laid down in early development must also be chemically and structurally stable enough to perform their role as one of the major components in maintaining lens transparency in an environment that becomes increasingly dehydrated during lens maturation/aging. Critical insights into the stability of γ-crystallins have been provided by high-resolution x-ray crystallographic studies of bovine γ-II crystallin, which reveal that the protein has a two-domain, globular β-structure with four similar folding motifs (11, 12). Whereas x-ray data obtained for other γ-crystallin fractions are less accurate than those for γ-II (47, 48), they reveal striking structural similarities among all the γ-crystallins. The high stability of all SH groups in γ-s, γ-II, γ-III, and γ-IV against disulfide formation in the absence of a protectant thus appears to be correlated with the high degree of internal symmetry found for all four γ-crystallins.

The present demonstration of the absence of a disulfide linkage in native γ-II crystallin is also important in ascertaining whether lens crystallins contribute only to the structural characteristics of the lens or they are also metabolically active (24). The presence of such a linkage in γ-II crystallin would imply its involvement in oxidation/reduction and possibly in free-radical quenching reactions (49, 50). Although γ-crystallins scavenge hydrated electrons more efficiently than α-crystallin (25), the unusual stability of γ-crystallins against oxidative SH to SS conversion argues against their role in redox reactions. On the other hand, the sulfur atoms in γ-crystallins are all situated in close proximity to aromatic side chains (12, 22); they may thus be involved in interactions that stabilize inter- and intramolecular contacts as well as acting to protect aromatic residues from photooxidation (51).

Structural comparison of γ-crystallin fractions

The striking Raman spectral similarities observed for all four fractions confirm the report (52) that the dominant antiparallel β-sheet structure is common to all the γ-crystallins. In the SH stretching region, however, the Raman spectra of γ-s and γ-II crystallins exhibit a more pronounced shoulder at 2,560 cm^{-1} compared to the γ-III and γ-IV spectra. On the basis of the effects of mixed-disulfide bond formation on sulfhydryl Raman signals, Yu et al. (23) have shown that both accessible reactive cysteines and buried residues contribute to the major Raman line at 2,580 cm^{-1}, whereas the 2,560 cm^{-1} line arises from two accessible cysteine residues that are located in a different environment. The lack of a distinguishable 2,560 cm^{-1} line suggests that the multiple microenvironments for cysteine residues found in the γ-s and γ-II fractions do not exist in the γ-III, and γ-IV fractions. Furthermore, appreciable differences exist in the cryoprecipitation and denaturation behaviors of different γ-crystallin fractions and in their proteolysis rate (13, 53). Apparently, the detailed tertiary structures of the closely related monomeric γ-crystallins are markedly different.

Finally, we note that in the revision process of this paper, a related article on the Raman spectroscopic study of the four major protein constituents of calf γ-crystallin appeared in the literature (54). In that report, Raman spectra were obtained by using a dispersive spectrometer coupled with visible laser excitation (488 nm) and OMA (optical multichannel analyzer) detection. Whereas the reported strong similarities for all four fractions are consistent with the present study, the OMA-detected spectra exhibit a series of weak features in the 2,500–2,600 cm^{-1} region that are absent in the FT-Raman spectra. In view of the absence of such weak features also in the extensive Raman spectra of lens crystallins reported in the past (26, 27), they are likely to be spectral artifacts generated during spectral subtraction or correction.

CONCLUSIONS

The results presented in this report for calf γ-crystallins clearly demonstrate that the newly developed technique of near-IR FT-Raman is particularly advantageous as a noninvasive and fingerprinting method for the analysis of various lens proteins. As compared to the conventional Raman approach, near-IR-excited FT-Raman spectroscopy completely eliminates the fluorescent interference problem frequently encountered with visible laser excitations and yields significantly improved Raman spectra of lens crystallins both in the lyophilized state and in solution. The ability to obtain high-quality Raman spectra should thus facilitate a more detailed structural analysis of ocular lens components.

The much improved FT-Raman results are in support of the absence of a disulfide link in native calf γ-II crystallin and hence its absence in calf lenses in vivo. An intramolecular disulfide bond, however, can be generated by exposing the protein to pH = 8.2 solutions for a long period of time. Denaturation of the γ-II crystallin in 8 M urea leads only to incomplete disulfide formation, but in the presence of excess oxidized glutathione, all the seven thiols in γ-II crystallin are converted to mixed-disulfide bonds. In the absence of a strongly denaturing environment, however, all sulfhydryl groups in the four γ-crystallins (i.e., γ-s, γ-II, γ-III, and γ-IV) are sufficiently oxidation-resistant to maintain their reduced state without added reducing agents during isolation. This finding supports the belief that the calf γ-crystallins, deposited primarily in the lens nucleus, must be structurally stable enough against sulfhydryl oxidation and structural alterations throughout life.

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