The Role of $\alpha$- and $\epsilon$-Amino Groups in the Glycation-mediated Cross-linking of $\gamma$B-crystallin

STUDY OF THREE SITE-DIRECTED MUTANTS

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In the previous report we demonstrated that $\gamma$B-crystallin is glycated predominantly at the N-terminal $\alpha$-amino group (Casey, E. B., Zhao, H. R., and Abraham, E. C. (1995) J. Biol. Chem. 270, 20781–20786). To investigate the possible role of $\alpha$- and $\epsilon$-amino groups of $\gamma$B-crystallin in glycation-mediated cross-linking, Lys-2 or Lys-163, or both, were mutated to threonine by site-directed mutagenesis in bovine $\gamma$B-crystallin cDNA. Wild type and mutant $\gamma$B-crystallins were expressed in Escherichia coli cells. Cross-linking studies were performed by incubating wild type and mutant $\gamma$B-crystallins with glycer-aldehyde, ribose, and galactose followed by SDS-polyacrylamide gel electrophoresis under reducing conditions. When both of the lysines of $\gamma$B-crystallin were mutated to threonines ($\gamma$B-K2T/K163T), the quantity of cross-linked products was greatly reduced, indicating that, despite the fact that the $\alpha$-amino group is a major glycated site, $\epsilon$-amino groups play a predominant role in cross-linking. Therefore, cross-linking ability depends not only upon the level of glycation but also upon which amino group is glycated. Steric hindrance may decrease the cross-linking ability of the $\alpha$-amino group. Our results also show that Lys-2 and Lys-163 play almost equal roles in cross-linking of $\gamma$B-crystallin. By incubating carbonyl anhydride, a protein with a blocked N terminus, and our novel “no lysine” $\gamma$B ($\gamma$B-K2T/K163T) with sugar, we were able to show for the first time that significant cross-linking occurs between lysines and non-lysine sites. The fact that pentosidine and imidazolysine, formed from ribose and methylglyoxal, respectively, were present in the cross-linked $\gamma$B-crystallins revealed the existence of Lys-Arg and Lys-Lys cross-linking.

Aldehyde or keto groups of reducing sugars react mainly with $\alpha$- and $\epsilon$-amino groups on proteins forming unstable Schiff base adducts, which rearrange to form more stable Amadori products (1). These Amadori products undergo a series of chemical reactions, becoming brown, fluorescent, and irreversibly cross-linked products. Vlassara et al. (2) first used the term advanced glycosylation (glycation) end products (AGEs) to describe these cross-linked products formed during the late stage of the Maillard reaction in vivo. AGE-mediated cross-linking is believed to be important in the pathogenesis of aging and in the complications of diabetes (3–5). Cross-linking of proteins is believed to occur mostly among $\epsilon$-amino groups of lysines as well as guanidinium groups of arginines. But the roles of these groups and of $\alpha$-amino groups in glycation-mediated cross-linking are still not clear because AGE structure is largely unknown. At present, AGEs are thought to be composed of diverse structures. However, studies with recently developed monoclonal and polyclonal antibodies against AGEs have suggested that they all have a common structure (6, 7). Elucidated AGE structures include $N^\epsilon$-(carboxymethyl)lysine (8), pentosidine (9), pyrraline (10), $N^\alpha$-(carboxymethyl)hydroxylysine (11), crossline (12), and imidazolysine (13, 14). Among them, pentosidine (Lys-Arg), crosslines (Lys-Lys), and imidazolysine (Lys-Lys) have cross-linking properties.

$\gamma$-Crystallin is one of the major classes of lens-soluble proteins. Bovine $\gamma$B-crystallin, the major species of bovine $\gamma$-crystallin, contains only two lysines (Lys-2 and Lys-163) among its 174 amino acid residues (15). $\gamma$-Crystallin is the only crystallin with a free N terminus (16). It is synthesized at an early stage in the development of the lens and is found mainly at the core region of the mammalian lens (17). Since there is little or no protein turnover in the core region (18), posttranslational modifications of $\gamma$B-crystallin such as glycation will accumulate in the nucleus of the lens. Indeed, it is the core region where nuclear cataract, the important form of cataract with protein cross-linking and brunescence in humans, is formed.

In a previous report, we showed that the N-terminal $\alpha$-amino group of bovine $\gamma$B-crystallin is the predominant glycation site (19), as confirmed recently by mass spectrometry analysis (20). In the present communication, the two lysines of bovine $\gamma$B-crystallin were mutated to threonine; this “no lysine” $\gamma$B-crystallin, as well as the mutants containing only Lys-2 or Lys-163, was used to study the role of the N-terminal $\alpha$-amino group of Gly-1 and the $\epsilon$-amino groups of two lysines in glycation-mediated cross-linking of $\gamma$B-crystallin.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The bovine $\gamma$B-crystallin cDNA in pBluescript (15) was kindly provided by Dr. Regine Hay (Department of Ophthalmology, Washington University). $\gamma$B-crystallin cDNA was subcloned into the NcoI/HindIII cloning site of expression vector pMON5743 (pMON-$\beta$B), and site-directed mutagenesis of Lys-2 to Thr (pMON-$\beta$B-K2T) was carried out using polymerase chain reaction as described previously (19). Creation of two new mutants, Lys-163 to Thr

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(K163T) as well as Lys-2 and Lys-163 to Thr (K2T/K163T), was accomplished with synthetic oligonucleotides. The desired base changes of Lys-163 to Thr were incorporated into the oligonucleotides (AAC-CAACTTGCCATTG and AAPGCCAGAGTGGTTTC), and mutagenesis was performed by two-stage polymerase chain reaction-based overlapping extension method (21). Using pMONB as a template led to the generation of a γB-K163T mutant, whereas when pMONB-K2T was used as the template, the double mutant γB-K2T/K163T was generated. The nucleotide sequences were checked using the sequencing kit from U. S. Biochemical Corp. Other methods used in subcloning were described previously (19).

Expression and Purification of Recombinant γB-crystallins—Clones containing pMONB, pMONB-K2T, pMONB-K163T, and pMONB-K2T/K163T were used to express recombinant γB-crystallins of wild type, mutant K2T (γB-K2T), mutant K163T (γB-K163T), and mutant K2T/K163T (γB-K2T/K163T), respectively. Expression of wild type and mutant γB-crystallins was performed according to the method of Olins and Rangwala (22). Wild type and mutant γB-crystallins in the cell lysates were purified using cation-exchange HPLC according to the method of Siezen et al. (23) with minor modifications (19). Since γB-K2T/K163T has a lower PI, buffers used in cation-exchange HPLC were adjusted to pH 5.5 instead of pH 6.0, and bacterial proteins found in the γB-K2T/K163T fraction were removed by molecular sieve chromatography on a 2 × 100-cm Sephadex G-100 (Sigma) column.

SDS-PAGE and Western Blot—SDS-PAGE was performed according to the method of Laemmli (24) using 12% separating gel and a 4% stacking gel under reducing conditions. Gels were routinely stained with Coomassie Blue, but in the case of galactose incubation a silver stain kit (Bio-Rad) was used to visualize small amounts of cross-linked proteins. Western blotting was carried out using a monoclonal antibody against rat γ-crystallin (19, 25). Bovine γ-crystallin was prepared as described previously (19).

Cross-linking of the Recombinant γB-crystallins with Sugars—Fifty microliters of wild type or mutant γB-crystallin (2 mg/ml) were incubated with or without sugar in darkness at 37 °C in phosphate-buffered saline (pH 7.4) containing 0.02% sodium azide. Since the cross-linking ability of different sugars varies (26), different sugar concentrations were used: glyceraldehyde (5 mM), ribose (100 mM), and galactose (1 mM) (all from Sigma). To investigate the ability of lysine to cross-link with non-lysine sites (e.g., α-amino group as well as arginine and histidine residues), equal concentrations of carbonic anhydrase (CA) and γB-K2T/K163T or wild type γB were incubated with 5 mM glyceraldehyde. After incubation for 10 days (glyceraldehyde and ribose) or 20 days (galactose), the samples were analyzed by SDS-PAGE under reducing conditions, and the gels were scanned on a CS-9301 PC dual wavelength Fly Spot scanning densitometer (Shimadzu Corp., Tokyo, Japan). Protein concentration was determined by the method of Lowry et al. (27).

Estimation of Pentosidine and Imidazolysine by Reversed-phase HPLC—Pentosidine (Lys-Arg cross-link) was synthesized according to the method of Sell and Monnier (9). Imidazolysine (Lys-Lys cross-link) was estimated by reversed-phase HPLC on a C18 column (Vydac, 218TP, 250 × 4.6 mm, 5 μm). The mobile phase consisted of water (A) and 70% methanol in water (B) with 0.01 M heptafluorobutyric acid (Sigma). The gradient program was 25% → 100% B in 35 min. The effluent from the column was monitored for fluorescence at 385 nm (excitation, 335 nm). Pentosidine eluted at ~33.9 min. For quantification, the peak area in the samples was compared with the peak area of a known amount of purified pentosidine.

RESULTS

Expression, Purification, and Characterization of Recombinant γB-crystallins—Wild type and mutant γB-crystallins were expressed in JM101 Escherichia coli cells. The supernatants of cell lysates were concentrated and analyzed on 12% SDS-PAGE. Heavy bands with molecular mass equal to calf γ-crystallin were detected in all lysates except in the lysate of the cells containing the vector pMON5743 without γB-crystallin cDNA (control). STD, molecular mass standards. B, SDS-PAGE and Western blot of purified recombinant γB-crystallins and recombinant calf γ-crystallin. Wild type and mutant γB-crystallins were purified by cation-exchange and molecular sieve chromatography, and the purity was checked on SDS-PAGE (right). Proteins were transferred to a polyvinylidene difluoride membrane and then probed with a monoclonal antibody to rat γ-crystallin. The bands were visualized by alkaline phosphatase stain (left).
were seen in wild type γB (Fig. 2A). Fewer cross-linked products were found in γB-K2T and γB-K163T, but heavy dimer bands could still be seen in both the mutants. If γB-K2T and γB-K163T are compared, the former seems to have slightly less cross-linked protein than the latter. Only a faint band of cross-linked product was observed with the double mutant γB-K2T/K163T despite the fact that the α-amino group of Gly-1 is the predominant glycation site of γB-crystallin. Gel scanning showed that formation of the dimer of γB-K2T/K163T decreased by 73% as compared with that of wild type γB. When other slower reacting sugars, such as ribose and galactose, were incubated with wild type and mutant γB-crystallins, similar results were obtained (Fig. 2, B and C), but no significant difference in cross-linking was seen among the wild type γB, γB-K2T, and γB-K163T, and no trimer or higher cross-linking product was observed. Again, only faint cross-linked dimer bands were found in the incubation of γB-K2T/K163T, which confirmed that α-amino groups of lysine are important in glycation-mediated cross-linking.

To investigate possible cross-linking between α-amino groups and non-lysine cross-linking sites (e.g. α-amino group as well as arginine and histidine residues), CA was incubated with γB-K2T/K163T or wild type γB in the presence of 5 mM glyceraldehyde for 10 days. CA was chosen because it has a blocked N terminus, 18 lysines (29), and a molecular mass of about 29 kDa, which facilitates distinguishing the different cross-linked dimers, e.g. γB-K2T/K163T-γB-K2T/K163T, γB-K2T/K163T-CA, and CA-CA, on SDS-PAGE. Fig. 3 shows that there are substantial cross-linked products consisting of γB-K2T/K163T and CA (lane 2), and the quantity of the cross-linked product is much higher than that made up of two γB-K2T/K163T mutants (lane 5) but less than that comprised of wild type γB-crystallin and CA (lane 3).

**Formation of Pentosidine and Imidazolysine**—To confirm the existence of Lys-Arg and Lys-Lys cross-linking, pentosidine and imidazolysine were analyzed after cross-linking recombinant γB with sugars (Fig. 4). Our data show that pentosidine and imidazolysine do exist in the cross-linking of γB-crystallin and the amounts of pentosidine and imidazolysine decrease along with the decrease of lysine content of γB. Fig. 4 also shows that approximately the same amount of pentosidine or imidazolysine was found from γB-K2T and K163T. This finding agrees with our cross-linking data, which show that γB-K2T and γB-K163T incubations contain about the same quantities...
were able to show for the first time that significant cross-linking between lysine and non-lysine cross-linking sites exists (Fig. 3). Noticeable loss of arginine and lysine after incubating lysozyme with glucose suggests that lysine and arginine are important candidates for cross-linking (31). Earlier studies (9) have shown that lysine and arginine can be cross-linked by a pentose to form a Lys-Arg cross-link, namely pentosidine. Pentosine could be formed from hexose through sugar fragmentation or from triose, tetrose, and ketose by condensation and/or reverse aldose reactions (32). Recently, Nagaraj et al. (14) showed that a three-carbon metabolite, methylglyoxal, which increases in diabetic lenses, reacts with lysines of proteins forming a Lys-Lys cross-link, namely imidazolysine. Thus, pentosidine and imidazolysine were analyzed in cross-linked γB-crystallin. The fact that pentosidine and imidazolysine are present in the cross-linking of γB-crystallin reveals the existence of Lys-Arg and Lys-Lys cross-linking. The high levels of imidazolysine (5–9 nmol/23 nmol of lysine) may have resulted from the possible lysine-rich protein contamination on crystallin preparation. Experiments with ultrapure preparations of proteins may shed light on this issue.

According to Prabhakaram and Ortweth (33), [14C]lysine was more readily incorporated into lysozyme than [14C]leucine during glycation-mediated cross-linking studies. This finding was interpreted as evidence for cross-linking principally at the ε-amino group rather than at the α-amino group. This hypothesis was confirmed by our protein-protein cross-linking model. Okitani et al. (31) show that acetylation of free amino groups of lysozyme before incubation with glucose prevents cross-linking of the protein and loss of lysine and arginine. This finding strengthens the importance of the amino group in cross-linking. By using site-directed mutagenesis, we were able to differentiate between the roles of α- and ε-amino groups and show that the ε-amino group is more important than the α-amino group in cross-linking. The present study shows for the first time that an engineered "no lysine" protein is useful in assessing the role of α- and ε-amino groups in glycation-mediated cross-linking. The advantage of site-directed mutagenesis over the chemical modification is that the roles of individual amino groups of proteins in cross-linking can be ascertained.

The reason the readily glycated α-amino group has a weaker ability to cross-link with itself or other non-lysine sites than lysine is not clear. The N-terminal α-amino group is, in fact, on the surface of γB-crystallin (34) and is thus accessible to other cross-linking sites. One possible explanation is that cross-linking at this site is greatly decreased by steric hindrance. Interestingly, an earlier study by Beswick and Harding (35) shows that changes in the conformation of γ-crystallin take place after glycation with glucose 6-phosphate, and this may prevent cross-linking at the α-amino group. Prabhakaram and Ortweth (33) observe that incorporation of [14C]leucine into protein is ~20% that obtained with lysine. We also noted that although α-amino groups have much less cross-linking ability than ε-amino groups, cross-linking studies with γB-K2T/K163T suggest that the α-amino group of Gly-1 could form some low level cross-links with itself or with arginine and histidine residues (Fig. 2).

By incubating CA, used as an ε-amino group provider, with γB-K2T/K163T, we were able to show that there was significant cross-linking between the ε-amino groups of CA and the non-lysine cross-linking sites of γB-K2T/K163T (Fig. 3). We noted that the quantity of cross-linking products found in the incubation containing CA and wild type γB (lane 3) is higher than that found in CA and γB-K2T/K163T incubation (lane 2). This increased quantity could come from the cross-linking of lysine to lysine and/or lysines of wild type γB to non-lysine sites.
of CA. When CA alone is incubated with glyceraldehyde, a clear dimer band can be observed on SDS-PAGE, but when CA is incubated with wild type yB or yB-K2T/K163T, the CA-CA dimer bands almost disappear (Fig. 3). CA seems to cross-link more readily with yB-crystallin than with itself, and thus the major cross-linking product is CA-yB rather than CA-CA.

Arginines and histidines are thought to be involved in glycation-mediated cross-linking (9, 31, 36). Even though bovine B-crystallin contains 16 arginines and 5 histidines, when both lysines were mutated to threonine, cross-linking was greatly reduced, suggesting that they act only as cross-linking “acceptors” of the glycated e-amino group.

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