Rates of Carbamylation of Specific Lysyl Residues in Bovine α-Crystallins*

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Previous investigations indicate that some forms of cataract may be due to the reactions of isocyanate with lens proteins. The present investigation was directed toward identifying the products of these reactions and determining rate constants for their formation. Bovine α-crystallins were incubated with isocyanate and separated into αA- and αB-crystallins by reversed-phase HPLC (high-performance liquid chromatography). Products of the reaction of isocyanate with α-crystallins were analyzed by mass spectrometry and isoelectric focusing. Proteolytic digests of carbamylated αA-crystallins were analyzed by HPLC and fast atom bombardment mass spectrometry to determine the extent of reaction of each of the 7 lysyl residues present in αA. These results demonstrate that incubation of α-crystallins in 0.1 M KNCO leads to partial carbamylation of all 7 lysyls of αA-crystallin. The extent of modification after 24 h of incubation varied from 7% at Lys 88 to 61% at Lys 11. Rate constants for the reaction of specific lysyl residues with isocyanate ranged from 5 to 54 x 10^-2 M^-1 h^-1. The distribution of reaction products, as determined by isoelectric focusing, indicates that the physiologically relevant initial stages of carbamylation of the 7 lysyl residues of αA proceed in a noncooperative manner.

The reaction of isocyanate with primary amino groups of proteins (Stark et al., 1960; Stark, 1965) to form carbamylated products, as illustrated below,

\[ \text{NCO}^- + \text{protein-NH}_2 \rightarrow \text{protein-NHCONH}_2 \]  

(Eq. 1)

has proved useful in experiments as diverse as probing the conformation of hemoglobin (Nigen et al., 1976), examining the relationship of the toxicity and basicity of snake venom (Yang et al., 1981), and determining the pK_a of the active site lysyl residue of aspartate transaminase (Slebe and Martinez-Carrion, 1976). The reaction of isocyanate with hemoglobin inhibits the erythrocyte sickling process characteristic of sickle cell anemia (Cerami, 1972) and was proposed as a possible method for detecting conformational changes occurring as a result of modification of specific residues. Isolation and Carbamylation of Lens Crystallins—Bovine αA-crystallins were isolated and identified as previously reported (Smith et al., 1976). Development of cataract following treatment with isocyanate was attributed to the possible carbamylation of the lens proteins.

Several post-translational modifications of the lysyl residues of lens proteins have been implicated in cataractogenesis. The reaction of isocyanate with the lysyl residues is of interest because it may be responsible for the high incidence of cataract common to diseases that are accompanied by elevated levels of isocyanate. Isoxazole is endogenous to lens because it is in equilibrium with urea. Under physiological conditions, the level of isocyanate is approximately 1% that of urea (Dirnhuber and Schultz, 1948). It has been suggested that the increased incidence of cataract accompanying severe diarrhea or chronic renal failure may be due to the reaction of isocyanate with the lens proteins since both diarrhea and renal failure are associated with elevated levels of blood urea (Harding and Rixon, 1980; van Heyningen and Harding, 1986). Because carbamylation of the ε-NH_2 removes the positive charge on lysyl residues, conformational changes may occur which might disrupt the close packing required for transparency and cause increased aggregation and lens opacity (Beswick and Harding, 1984). This hypothesis is supported by a correlation between the severity of nuclear cataract and a decrease in the number of free amino groups in lens proteins (Garcia-Caño, 1983).

The principal objectives of the present investigation were to isolate and identify the products of the reaction of isocyanate and α-crystallins, and to determine rate constants for formation of these products. Of particular interest were the rate constants for carbamylation of each of the 7 lysyl residues in αA-crystallin, and whether these reactions may be accelerated through synergistic effects. These objectives were accomplished through a new type of application of fast atom bombardment mass spectrometry (FABMS), which was used to determine the extent of carbamylation of each of the lysyl residues. The FABMS results were compared with isoelectric focusing (IEF) results to determine if rates of carbamylation of α-crystallins are affected by carbamylation of other lysyl residues in the same molecule. In addition to their relevance to the reactions of isocyanate and α-crystallins, these results suggest that this combination of mass spectrometry and isoelectric focusing may be the basis of a new approach for detecting conformational changes occurring as a result of modification of specific residues.

EXPERIMENTAL PROCEDURES

Isolation and Carbamylation of Lens Crystallins—Bovine αA-crystallins were isolated and identified as previously reported (Smith et al., 1991). Homogenates of whole bovine lenses were separated by gel filtration into the α-, β-, and γ-crystallin fractions using Sephadex G-200. The α-crystallins (500 μg/ml) were carbamylated by incubation in 0.1 M KNCO, 0.2 M NaN_3 at pH 7. The abbreviations used are: FABMS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; m/z, mass/charge; IEF, isoelectric focusing; TEMED, N,N,N',N'-tetramethylethylenediamine.
The same buffer, but with 6 M guanidine hydrochloride, at 37 °C for 24 h. A control was prepared by incubating α-crystallins in the same buffer with 0.1 M KCl in place of the 0.1 M KNCO. After incubation, the samples were dialyzed against water to remove salts. The acidic (αA) and basic (αB) forms of the α-crystallins were separated by reversed-phase HPLC (Rainin Instrument Co., Woburn, MA) using a Vydac 0.46 × 25-cm C18 column with UV detection at 280 nm. A linear gradient of 30–60% CH3CN in H2O, both with 0.1% of trifluoroacetic acid, was used to elute the proteins. The gene products, αA2 and αB2, coelute with their respective phosphorylated forms αA1 and αB1 (Smith et al., 1991, 1992). The fractions corresponding to αA-crystallin were collected, freeze-dried, and stored at −10 °C until analyzed.

**Isoelectric Focusing of the Carbamylated Proteins**—The carbamylation of αA-crystallin was investigated by IEF in a vertical slab gel apparatus (Hoefer SE 400, San Francisco, CA). The 0.75-mm-thick gel contained 8 M urea, 2% Triton X-100, 3% pH 4–6.5 amorpholyte, 4% acrylamide, 0.03% ammonium persulfate, and 0.1% TEMED. The αA-crystallin samples, incubated in isocyanate or KCl (control), were dissolved in a buffer containing 8 M urea, 8% Triton X-100, and 1% amorpholyte (pH 4–6.5) and placed in the wells. Isoelectric focusing was continued for 18 h with a voltage of 400 V. The gel was fixed with a solution of CH3OH/HOAc/H2O (25:15:60%), and the amorpholyte was removed with a solution containing CH3OH/HOAc/H2O (50:10:40%). The gel was stained with Coomassie Blue. The quantities of protein in each band were determined by scanning the gels at 590 nm.

**Proteolysis of αA-Crystallins**—The freeze-dried αA-crystallins (150 μg) were dissolved in 500 μl of either 0.03 N HCl for peptic (Sigma) digestion, 0.1 M Tris-HCl, 0.01 M CaCl2, pH 8.3, for chymotryptic (Worthington) digestion, or 0.2 M Tris, pH 8.2, for Asp-N (Boehringer-Mannheim) digestion with an enzyme to substrate ratio of 1:50. The solutions were incubated at 37 °C for 1 h, for the peptic and chymotryptic digestions, or for 7 h for the Asp-N digestion. The resulting peptides were separated by reversed-phase HPLC using a linear gradient of 5–60% CH3CN in H2O, both with 0.1% trifluoroacetic acid. The carbamylated and uncarbamylated peptides were collected in the same fraction, freeze-dried, and analyzed by FABMS.

**Mass Spectrometric Methods**—The FABMS analyses were performed with a Kratos MS-50 mass spectrometer (Kratos Analytical, Manchester, United Kingdom) equipped with an RF magnet and a DS-90 data acquisition system. The mass range (m/z 400–2000) was calibrated with CsI. Lyophilized HPLC fractions were dissolved in 50% formic acid and analyzed using a 1:1 mixture of glycerol and thioglycerol as the matrix. A scan rate of 30 s per decade was used to record the FAB mass spectra. For the instrument conditions used in this investigation, 1 nmol of peptide usually gave an excellent spectrum from which the molecular weight of the peptide and the intensity could be determined. Mass spectrometric data were processed with Kratos MACH3 software on a SUN workstation. Peptide identifications were confirmed from the molecular weights of the residues present after incubation of the peptide in carboxypeptidase Y (Sigma) or B (Worthington) in 0.1 M NH4OAc, pH 7.0, at 37 °C for 10–70 min (Caprioli and Fan, 1986; Qin et al., 1992). Peak intensities for the modified and unmodified peptides, averaged over at least 10 scans, were used to determine the extent of carbamylation of each lysyl residue.

**Determination of Isocyanate Concentration**—To determine the extent of hydrolysis of the isocyanate during the incubation period, a solution of 0.1 M KNCO was incubated in the same buffer that was used for the carbamylation experiments. Samples (10 μl) were removed at various time intervals, and the concentration of KNCO determined by reacting the remaining NCO− with a peptide, Ile-Ser-Bradykinin (16 nmol in 20 μl of a buffer, 0.1 M pyrophosphate, pH 8.0, with 6 M guanidine hydrochloride) at 45 °C for 30 min. The carbamylated and uncarbamylated peptides were separated by reversed-phase HPLC and quantified by their UV absorbance. The HPLC response was calibrated with solutions of 0.02–0.10 M KNCO.

**RESULTS**

**HPLC Analysis of Carbamylated α-Crystallins**—After incubation, the isocyanate and buffers were removed from the proteins by dialysis and the α-crystallins were separated into αA- and αB-crystallins by reversed-phase HPLC (Fig. 1).

![FIG. 1. Reversed-phase HPLC chromatograms of α-crystallins incubated in 0.1 M KNCO, 0.2 M NH4OAc, pH 7.2, at 37 °C for (A) 0 h and (B) 24 h.](image-url)

Previous studies demonstrated that αB elutes at 39–43% acetonitrile and αA elutes at 44–50% (Smith et al., 1991). Similar results were obtained in the present study for analysis of α-crystallins incubated in isocyanate. However, it was noted that the peaks became broader with a larger portion of both αA and αB eluting later as the incubation time increased, as illustrated in Fig. 1A for unmodified α-crystallin and in Fig. 1B for α-crystallin incubated in 0.1 M KNCO for 24 h. This chromatographic behavior is consistent with formation of a mixture of α-crystallins that are carbamylated at different sites and to different extents. When the crystallins were incubated under denaturing conditions with 6 M guanidine hydrochloride, the original peak disappeared and a new sharp peak with a longer retention time appeared, suggesting that a uniform fully carbamylated product had been formed. αA-crystallin to be used for further analyses was collected in an elution volume that included uncarbamylated as well as all forms of the carbamylated protein.

**Modifications to αA-Crystallins**—The products of the reaction of isocyanate with αA-crystallin were identified by analyzing proteolytic digests of the protein incubated for 24 h in isocyanate by a combination of HPLC and FABMS. Asp-N was used to fragment the protein, because it gave peptides that could be assigned to the entire 173 amino acids in the α-crystallin. Nearly all of the peptides detected by FABMS could be assigned to unmodified segments of αA. The FAB mass spectra of the unidentified segments could be assigned to...
peptides with lysyl residues with a +43 mass unit modification, indicating that all the lysyl residues had been partially carbamylated. No evidence was found for other modifications, such as the carbamylated cysteinyl residues which have been detected in γH-crystallin carbamylated under similar incubation conditions.2

Quantification of Carbamylation by IEF—The extent of carbamylation was determined as a function of time by isoelectric focusing. Since carbamylation lowers the isoelectric point in a predictable manner, α-crystallins carbamylated to different extents give a series of discrete IEF bands. Separation of carbamylated and uncarbamylated αα-crystallin by IEF is illustrated by the bands in lane 1 of Fig. 2 for a sample prepared by combining unincubated αA with αA that had been incubated in 6 M guanidine hydrochloride and isocyanate. The four bands present in lane 1 are due to αA that is not carbamylated (αA0(0) and αA1(0)), and αA that is fully carbamylated (αA2(0) and αA1(0)). The numbers in parentheses indicate the number of carbamylated lysyl residues per molecule, while αA2 and αA1 refer to nonphosphorylated and phosphorylated αα-crystallin, respectively.

Lanes 2–5 in Fig. 2 show the extent of carbamylation of αα-crystallins after 2, 4, 9, and 24 h of incubation in 100 mM KNCO. Although the phosphorylated and nonphosphorylated forms of uncarbamylated αα-crystallin were easily separated by IEF (lane 1), some of the partially carbamylated forms were not separated. To identify species present in these bands, αA2 and αA1 were isolated by preparative IEF, partially carbamylated, and analyzed by analytical IEF (results not shown). From these experiments, it was evident that αA2 with one site carbamylated had an isoelectric point between αA1 and αA2. For αA with more than two sites of carbamylation, the isoelectric points of the carbamylated αA2 proteins overlapped with αA1 with one less carbamylation site. The three bands in lane 6, obtained for αA-crystallin that had been extensively carbamylated in 6 M guanidine hydrochloride, are in order of decreasing pH αA2(6) + αA1(5), αA2(7) + αA1(6), and αA1(7). Densitometer measurements of lanes 2–5 indicated that an average of 1.2, 1.4, 2.3, and 2.7 lysines per molecule were carbamylated after 2, 4, 9, and 24 h of incubation, respectively.

Quantification of Carbamylation by FABMS—To determine rate constants for carbamylation of each of the 7 lysyl residues in αα-crystallin, it was necessary to quantitatively assess the extent of carbamylation at each position. This was achieved by enzymatically fragmenting the protein and determining the relative abundances of uncarbamylated and carbamylated peptides by FABMS. As indicated in Table I, three different enzymes were used to generate a set of peptides that included the 7 lysyl residues in αA. Selection of proteases was important, because large errors could be introduced if carbamylation of the lysyl residue affected production of the diagnostic peptide. For example, trypsin could not be used, because it cleaves COOH-terminal to Lys but does not cleave at carbamylated Lys.

Not only must the carbamylated and noncarbamylated peptides be formed at the same rate, the FABMS response to the modified and unmodified forms of the lysine-containing peptides must also be known. Because modification of a single sidechain may change the FABMS response, the effect of carbamylation was investigated. Oxidized B-chain of insulin was used as a model peptide. Incubation of this peptide in isocyanate led to carbamylation of the NH2 terminus and Lys.29 The fully carbamylated peptide, as indicated by FABMS, was prepared by incubating the peptide with isocyanate in 6 M guanidine hydrochloride for 24 h. Equal amounts of uncarbamylated and carbamylated B-chain were combined, separated from reagents by HPLC, and digested with V8-protease to give three peptides corresponding to segments 1–13, 14–21, and 22–30. The digest was fractionated by HPLC. The uncarbamylated and carbamylated forms of the 22–30 segment, which were expected to be equimolar, were collected in the same fraction and analyzed by FABMS. The spectrum (Fig. 3) has two peaks, corresponding to the molecular ions of the uncarbamylated (m/z 1087) and the carbamylated (m/z 1130) peptides. The ratio of the intensities of the ions averaged over 10 scans was 0.99, indicating that the FABMS response was not substantially affected by carbamylation of 1 lysyl residue. Results of similar investigations with several model peptides in which both the NH2 terminus as well as 1 lysyl residue were carbamylated indicated that carbamylation of 1 lysyl residue changes the FABMS response by less than 10%.

This combination of peptide mapping and FABMS was

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2 W. Qin, J. B. Smith, and D. L. Smith, unpublished observations.

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**Table I**

| Lysine-containing peptides used to quantify the extent of carbamylation of specific lysyl residues in αα-crystallin | Lys Residues | Sequence | Protease | m/z* |
|---|---|---|---|---|
| 11 | 11–22 | KRTLPGFYPSDL | Pepsin | 1434.8 |
| 70 | 64–71 | VRSRDFKE | Pepsin | 1022.5 |
| 78 | 75–85 | LDKHFPESDL | Pepsin | 1299.6 |
| 88 | 86–92 | TVKQED | Pepsin | 818.4 |
| 99 | 92–104 | DFFEIIHGNERQ | Asp-N | 1608.8 |
| 145 | 142–157 | SGPKPSGVDAGHSER | Chymotrypsin | 1593.8 |
| 166 | 158–173 | AIPVSVKEFPPASS | Chymotrypsin | 1641.8 |

*a* The m/z values of the carbamylated peptides are 43 mass units higher.
used to determine the extent of carbamylation of all 7 lysyl residues in αA-crystallin as a function of the time the protein was incubated in isocyanate. Results of this investigation are presented in Fig. 4, which is a plot of percent modification versus incubation time. These data show that all 7 lysyl residues are carbamylated, some more rapidly than others. The initial rapid increase of modification was followed by a plateau region, where the extent of modification varied from 7% for Lys66 to 61% for Lys1 following 24-h incubation. Since the extent of modification did not increase significantly for incubation to 48 h, the possibility that the isocyanate was undergoing hydrolysis was considered. Analysis of a 100 mM isocyanate solution, incubated using the same conditions as were used to incubate the protein showed that the level of isocyanate decreased by 50% within the first 24 h of incubation (Fig. 4). Because the concentration of isocyanate was much greater than that of the protein, the decrease in isocyanate was attributed to hydrolysis (Kemp and Kohnstam, 1956). By combining the extent of modification of individual residues, it was determined that 1.2, 1.7, and 2.4 lysyl residues of αA-crystallin were carbamylated after 4, 9, and 24 h, respectively.

Since the combination of peptide mapping and FABMS is a new approach for determining the extent of modification at specific residues in proteins, the reliability of the method was investigated using different peptides that contain the same lysyl residue. For example, the extent of carbamylation of Lys196 was determined from the 142–157 chymotryptic peptide (m/z 1593) as well as from the 136–150 Asp-N peptide (m/z 1505). Similarly, the extent of carbamylation of Lys78 was determined from the 75–85 peptic peptide and the 76–83 Asp-N peptide; and the extent of carbamylation of Lys86 was determined from the 86–92 peptic peptide and the 85–92 Asp-N peptide. Results for these duplicate messenger peptides suggest that the uncertainty in these measurements is usually less than 10%.

Rate constants for the carbamylation at each lysyl residue were calculated assuming that the reaction was first order with respect to the concentration of unmodified lysine, which was determined from the percent modification after adjustment for hydrolysis of isocyanate during the incubation (Fig. 4). The second-order rate constants for the reaction at each lysine (Table II) were calculated from the slopes of plots of ln[Lys] versus incubation time (Fig. 5) divided by the concentration of isocyanate (0.1 M).

DISCUSSION

Bovine α-crystallin, one of the three classes of structural proteins found in the eye lens, is composed of approximately 40 subunits, as isolated in this study (van den Oetelaar et al., 1985; Tardieu et al., 1986). These subunits consist of two gene products, αA and αB, which have 0, 1, or 2 serine residues phosphorylated (Spector et al., 1985; Voorter et al., 1986; Smith et al., 1991, 1992). The quaternary structure of α-crystallin is of current interest (van den Oetelaar et al., 1990; Walsh et al., 1991) because of its probable relation to cataract, as well as the remarkable temperature stability of α-crystallins (Maiti et al., 1988). According to the three-layer micellar model (Tardieu et al., 1986; Walsh et al., 1991) there is a stable core of 18 αA subunits surrounded by an outer layer of approximately 24 αA or αB subunits. Rates of chemical modification of specific residues likely depend on quaternary structure, and may therefore be used as a probe of this structure. For example, the reactivity of the single cysteiny1 residue in bovine αA suggests that there are three groups of αA subunits, leading to the proposal of the three-layer model. If the subunits in the core are tightly bound by hydrophilic surfaces of the subunits, as proposed by Walsh et al. (1991), one would expect considerable variation in the exposure of lysyl residues in different positions in the sequence, as well as lysyl residues in the same position but on different molecules. In apparent contrast to the three-layer model, Schurtenberger and Augusteyn (1991) have presented results consistent with polymerization of monomeric units into linear chains.

One of the goals of this investigation was to determine whether some lysyl residues in αA-crystallin are particularly reactive nucleophiles or whether all lysyl residues have similar reactivities. As indicated in Table II, the rate constants for carbamylation of the 7 lysyl residues in αA vary from 5 to 54 $\times 10^{-2}$ M$^{-1}$ h$^{-1}$. These results indicate that all of the lysyl residues in αA-crystallin can be carbamylated readily and must therefore have access to the solvent. As models for the quaternary structure of α-crystallin evolve, it will be important to include solvent accessibility for lysyl residues in all seven positions of αA-crystallin. The range of rate constants suggests that Lys11, which is most reactive, is on the surface and has direct exposure to the solvent, while Lys86, the least reactive, has less access to the solvent. This observation is

![Fig. 4. Percent carbamylation of the lysines of αA-crystallin after incubation in 0.1 M KNCO, 0.2 M NH$_4$OAc, pH 7.2, for various time periods. The dotted line indicates the change in the concentration of isocyanate as a function of incubation period.](image)

![Fig. 5. Plot of ln[Lys] versus time used to calculate the rate constants for carbamylation at each lysine of αA-crystallin.](image)
consistent with the limited proteolysis results of Siezen and Hoenders (1979) which demonstrated that Arg\textsuperscript{12} is especially prone to tryptic cleavage. The variation in reactivities of the lysyl residues may also reflect the different pK\textsubscript{a} values of the protonated ε-amines. Whether these different reactivities are due to tertiary or quaternary structure cannot be ascertained from the present results. It is significant however, that plots of \ln[\text{Lys}] versus time of carbamylation are linear (Fig. 5), indicating that specific lysyl residues on different molecules have the same reactivity.

The present investigation has also used isoelectric focusing electrophoresis to examine the reaction of isocyanate with \(\alpha\)-crystallin. Since carbamylation of lysine decreases the positive charge on a protein by one unit, \(\alpha\)-A-crystallin subunits with different numbers of carbamylated lysyl residues can be separated, as illustrated in Fig. 2. The density of these bands is a direct measure of the distribution of the number of modified lysyl residues per molecule of \(\alpha\), and may be used to examine the possibility that carbamylation of 1 residue alters the conformation causing an increased reactivity of other lysyl residues, a scenario that may be relevant to the role of isocyanate in some forms of cataractogenesis (Beswick and Harding, 1984; Crompton et al., 1985). For example, carbamylation of Lys\textsuperscript{11}, the most reactive lysyl residue, might induce a conformational change that causes the remaining lysyl residues to be rapidly carbamylated. For limited reaction times, this model would lead to two populations of molecules, one unmodified and another highly modified. Analysis by IEF would give a bipolar distribution of bands. The relative abundances of the variously modified \(\alpha\)-A-crystallin was determined by scanning the IEF gel presented in Fig. 2. The product distribution obtained for the 24-h incubation, given in Fig. 6, shows that the experimental data are consistent with a uniform progression of carbamylation (noncooperative reaction) and that the distribution of products is not bipolar, as would be expected for a cooperative model.

Although IEF gives no information about carbamylation of specific lysyl residues, it does give information that complements and corroborates results obtained by mass spectrometry. For example, the number of carbamylated lysine residues per subunit after incubation in isocyanate for 4, 9, and 24 h determined by the two methods is in good agreement (see "Results"). A more stringent test of the internal consistency of the IEF and mass spectrometric results was conducted by assuming that a noncooperative reaction model dominates and using the mass spectrometric product distribution to calculate the IEF pattern. This calculated IEF pattern is given in Fig. 6 where it can be compared with the product distribution obtained by scanning the IEF gel. These product distributions are remarkably similar, further demonstrating the internal consistency of the IEF and mass spectrometric results and supporting the idea of noncooperative reactions of the lysyl residues of \(\alpha\)-A-crystallin.

The high reactivity of Lys\textsuperscript{11} near the NH\textsubscript{2} terminus suggests that this region is exposed to solvent, in spite of the fact that the region is hydrophobic. Studies by Ifeanyi and Takemoto (1991) indicate that the NH\textsubscript{2} terminus is involved in membrane recognition, a reaction that would be favored by both accessibility and hydrophobicity of the NH\textsubscript{2} terminus. The high reactivity of Lys\textsuperscript{11} also offers an explanation for the change in circular dichroism that occurs with carbamylation of \(\alpha\)-crystallins. The circular dichroism in some of cataractogenesis (Beswick and Harding, 1984) showed that carbamylation affected tryptophan and tyrosine chromophores. However, the effects of isocyanate incubation on tryptophan and tyrosine chromophores could not be determined independently. Since there are 6 tyrosines in \(\alpha\)-A-crystallin, none very close to the lysines, and only 1 tryptophan (Trp\textsuperscript{9}), close to the highly reactive Lys\textsuperscript{11}, it seems likely that the altered circular dichroism spectrum following carbamylation is due to a change in the environment of Trp\textsuperscript{9} caused by carbamylation of Lys\textsuperscript{11}.

The high reactivity of Lys\textsuperscript{11} reported here also supports observations made in studies of aging bovine crystallins. Using antisera to nine regions of \(\alpha\)-A-crystallin, Takemoto et al. (1989) showed that changes occurred with aging which made the NH\textsubscript{2}- and COOH-terminal regions less reactive toward their antibodies. They suggested that the decreased recognition of the COOH terminus might be due to degradation, and decreased recognition at the NH\textsubscript{2} terminus might be due to lower availability of the NH\textsubscript{2}-terminal epitope. Our data showing that lysines in both the COOH-terminal and NH\textsubscript{2}-terminal regions, Lys\textsuperscript{11} and Lys\textsuperscript{10}, are highly reactive suggest the possibility that the decrease in antibody recognition at both termini might be due to age-related modification of the lysines in these regions. The low reactivity of Lys\textsuperscript{10} and Lys\textsuperscript{9} supports their conclusion that this region is less accessible to the solvent. However, the present results do not indicate that carbamylation leads to increased exposure (reactivity) of these internal lysyl residues. In a study of human lens tissue, Takemoto et al. (1990), again using antisera to the COOH-terminal region of \(\alpha\)-A-crystallin, found evidence suggesting that cataractous lenses have modifications in the region 164–173. Our results suggest the possibility that these modifications may be due to the high reactivity of Lys\textsuperscript{10}.

Previous investigations of carbamylation of lens proteins have been severely restricted by the experimental methods used. In some investigations, the carbamylated protein has been hydrolyzed by acid followed by amino acid analysis. Carbamylated lysine residues were detected as homocitrullin (Harding and Rixon, 1980). Unfortunately, acid hydrolysis also hydrolyzes the carbamylated lysine (Stark et al., 1960) and gives no information about the location of carbamylated residues. Other investigators (Garcia-Castineiras and Miranda-Rivera, 1983) have circumvented acid hydrolysis by using the reaction of 2,4,6-trinitrobenzenesulfonic acid with
lysyl residues to quantify the free lysyl residues. However, addition of SDS to prevent precipitation of the protein-trinitrobenzenesulfonic acid derivative may cause errors, because SDS can also bind to the free amino groups, preventing the reaction of the amino group with trinitrobenzenesulfonic acid (Habeeb, 1966). Furthermore, this method only indicates whether the lysyl group is free; it gives no information about the nature or location of the modification. Radiolabeled isocyanate has also been used to investigate the reaction of isocyanate with a-crystallin (Harding and Rixon, 1980; Crompton et al., 1985). Although this approach indicated the uptake of radiolabel, neither the products nor their locations were determined.

The use of mass spectrometry in the present investigation of the reactions of isocyanate with a-crystallin gives a particularly detailed account of the reactivities of individual lysyl residues in aα-crystallin. All of the major products of this reaction were isolated, identified, and located with respect to the primary structure of the protein. Upon determining the relative response of FABMS to uncarbamylated and carbamylated peptides, the extent of carbamylation of specific lysyl residues was determined from the FAB mass spectra. This approach is of general significance because it should be applicable to different types of modifications, to modifications of different types of residues, and to different proteins.

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