·Basic Research·

Identification of the preferentially targeted proteins by carbamylation during whole lens incubation by using radio –labelled potassium cyanate and mass spectrometry

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Abstract

• AIM: To attempt to identify the primary targets of carbamylation in bovine lenses incubated under physiological condition.

• METHODS: Fresh intact bovine lenses were incubated with [^{14}C]-labelled potassium cyanate for seven days. The water-soluble proteins (WSP) of both cortex and nucleus lens were isolated by size-exclusion chromatography on a Sephacryl S-300HR column. The higher radioactive fractions were pooled and freeze-dried, and separated further by loading on an Affinity Blue column to separate some enzymes. In addition, WSP from cortex was separated directly by affinity chromatography. The most reactive fractions with higher radioactivity from [^{14}C]-cyanate were further analyzed by SDS-gels and mass spectrometry.

• RESULTS: The majority of protein incorporating [^{14}C]-labelled potassium cyanate was in the water-soluble fractions, and much more in the cortex than in the nucleus. Chromatography results demonstrated that the major incorporated [^{14}C]-carbamylated crystallins were fractions corresponding to α-crystallin, β-crystallin and ξ-crystallin in the cortex, but β-crystallin and γ-crystallin in the nucleus. The SDS gels showed that bound fractions of cortex crystallins after Affi-Gel Blue separation were abundant with 20 and 35kDa proteins. However, the bound fractions of nucleus crystallins mainly showed 20kDa proteins. Mass spectrometry analysis of these higher radioactivity fractions and a database search revealed that the proteins were originated from bovine α-crystallin A and B chains and ξ-crystallin in the cortex; βA1 and αB-crystallins with a little γB-crystallin in the nucleus respectively. Further analysis suggested the location of this carbamylation of αB-crystallin in the nucleus to be at Lys 92 and 103.

• CONCLUSION: α-and ξ-crystallin from cortex can be preferentially targeted by carbamylation during whole lens incubations. Carbamylation of these crystallins at the earlier stage may result in further unfolding and misfolding of lens proteins, leading to aggregation of crystallins and eventually to cataract formation.

• KEYWORDS: carbamylation; cyanate; cataract; identification; lens; mass spectrometry; post-translational modification; ξ-crystallin

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INTRODUCTION

Carbamylation is an irreversible process of non-enzymatic modification of proteins by the breakdown products of urea. The reaction of cyanate (isocyanate) with proteins can lead to conformational changes and loss of function. Diarrhea and renal failure have been identified as important risk factors in cataractogenesis due to protein carbamylation by cyanate derived from urea. Cyanate-induced opacities are thought to be due to carbamylation of the lysyl residues that causes a decrease in the protein charge and subsequent conformational changes that permit disulphide bonding. Carbamylation of the ε-amino group of lysine, determined as homocitrulline, has been detected in a variety of proteins, particularly from patients with renal impairment [1]. The accumulated evidence of carbamylation of lens crystallins led to a proposal that cataract in renal failure patients may be due to carbamylated crystallins. *In vivo* administration of cyanate to rats caused a significant decrease in the serum GSH and increase in calcium and phosphate level both in serum and lens. About

2% of αB-crystallin from human cataractous lens was carbamylated at Lys 92 as analyzed by mass spectrometry of tryptic digests of this modified protein[2]. Curiously this group had reported earlier that the only modification observed in the α-crystallin of renal failure patients, but not in the normal old lenses, was glutathione adducts to Cys 131 and Cys 142 rather than carbamylation.

Following the hypothesis that severe diarrhea may cause the excess of cataract in the third world, acting partly via carbamylation, this modification of lens crystallins has been studied extensively *in vitro*. Carbamylation caused α-crystallin to unfold exposing thiol groups that can become oxidized to disulphide cross-links and to lead to high molecular weight aggregates. The most reactive site of γII-crystallin by carbamylation is the N-terminal glycine residue. Under the extreme conditions all 7 lysines of αA-crystallin can be carbamylated *in vitro*. The extent of modification after 24 hours of incubation varied from 7% at Lys 88 to 61% at Lys 11. The idea that carbamylation of α-crystallin resulted in loss of its chaperone activity is controversial. The carbamylation at the cysteinyl residues of γII-crystallin is reversible. A number of enzymes have been inhibited by carbamylation *in vitro* including 6-phospho-gluconate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, esterase (Yan *et al*, unpublished results) and acetylcholinesterase. The covalent cyanate binding to protein inactivated the enzyme in a concentration-dependent fashion. In general the inactivation of enzymes by carbamylation can be achieved with lower concentrations of isocyanate than the more universal damage to structural proteins. α-Crystallin functions as molecular chaperone and protects numerous enzymes against chemically-induced inactivation. It is able to protect 6-phosphogluconate dehydrogenase against cyanate-induced inactivation, but not glyceraldehyde 3-phosphate dehydrogenase or esterase. Accumulated carbamylation of proteins may lead to conformational changes within lens proteins and inactivation of enzymes, which can subse-quently result in lens opacification and cataract [3]. However, identification of the earlier vulnerable lens protein targeted by carbamylation is particularly important to provide key evidence of mechanisms in the development of cataract. In order to approach this aim, intact bovine lens was incubated with potassium [^{14}C] cyanate as tracer under physiological condition. The proteins from lens cortex and nucleus were separated by size-exclusion chromatography following further separation of [^{14}C] cyanate-incorporated proteins by an Affinity Blue column. The rate and extent of covalent modification of lens proteins by cyanate was monitored by assaying the radioactivity incorporated into trichloroacetic acid precipitable protein. Mass spectrometric analysis was applied for identification of the primarily carbamylated proteins.

MATERIALS AND METHODS

Materials Affi Gel 601, Econo-Pac Blue prepacked Cartridge, and Silver Stain Plus Kit were obtained from Bio-Rad Laboratories (Hemel Hempstead, UK) while Sephacryl S-300HR was purchased from Pharmacia (Milton Keynes, UK). Potassium [^{14}C]-cyanate was obtained from American Radiolabelled Chemicals Inc. (Bristol, UK). Liquid Scintillation Cocktail [EcoLite(+)TM] was obtained from ICN Pharmaceuticals Ltd (Basingstoke, UK). GelCode Blue Stain Reagent was purchased from PIERCE (Tattenhall, Cheshire, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK) or Merck (Lutterworth, UK) and were of the highest grade available. The fresh bovine lenses were obtained from a local slaughterhouse and lenses dissected out within 3-4 hours of slaughter (Oxford, UK). An incision was made in the sclera under sterile conditions. Lenses were then placed, anterior face upwards.

Methods

Incubation of lens with potassium [^{14}C] cyanate and isolation of crystallins The incubation solutions were based on a conventional tissue culture medium (MEM) with the following ionic composition (mmol/L): $CaCl_2$, 1.0; KCl, 5.4; $MgSO_4$, 1.7; NaCl, 136.4; NaH_2PO_4, 1.2, was buffered at pH 7.4 with HEPES (10mmol/L). In the first incubation, two whole bovine lenses were incubated with 9.25×10^3KBq of potassium [^{14}C] cyanate in final volume of 9mL MEM that was added to 100μL antibiotic (contains penicillin, streptomycin and Amphotericin B, Sigma) solution for 7 days in a 37℃ shaking waterbath. In the second incubation, 2.96×10^4KBq of potassium [^{14}C] cyanate was used under the same incubation condition. After 7 days of incubation, lenses were separated into cortex and nucleus fractions by gentle rubbing between thumb and index finger (with gloves), which sufficed to remove the cortex from the physically larger nucleus. The cortex and nucleus of two lenses were then weighed and homogenized gently in seven times mass volume of 50mmol/L sodium phosphate buffer, pH6.7, containing 0.2mol/L KCl in a hand-operated glass homogeniser, respectively. In a second incubation, the cortex and nucleus of two lenses were homogenized gently in five times mass volume of 50mmol/L sodium phosphate buffer respectively. The homogenates were centrifuged at 22 440g for 40 minutes at 4℃ before being loaded on to a Sephacryl S-300HR column (120cm ×2.7cm). The fractions corresponding to the radioactivity peaks were pooled and dialysed against three changes of distilled water at 4℃, then freeze-dried for the next separation procedures.

Radioactivity measurements The protein-bound radioact-ivities were determined in a liquid scintillation spectrometer. Samples, 100μL (total proteins/soluble proteins) were taken

into 1.0mL of 10% trichloroacetic acid (TCA), and left overnight at 4℃ to allow the protein in them to precipitate. The precipitated protein was then recovered by filtration on Whatman GF/C microfiber filter disc under vacuum, and washed with 30mL of 5% TCA. Blanks consisted of filter papers washed with 30mL of 5% TCA. The filters holding precipitated proteins were placed into mini scintillation vials and oven dried at 70℃ for 30 minutes, cooled and then added to 4.0mL of scintillation fluid [EcoLite(+)TM]. The total amount of radioactivity covalently bound to lens proteins was then measured in a scintillation counter. Three aliquots of 50μL of the soluble proteins (supernatants) were removed and added to 4.0mL of scintillation fluid in mini scintillation vials in order to confirm the total amount of radiolabelled potassium cyanate in the solutions. In the second incubation after being run on the Blue column, 600μL of each one after the other eluted fractions was taken for scintillation counting to measure the radioactivity. The pooled fractions according to the higher radioactivity were dialysed against distilled water and then three aliquots of 200μL of the fractions were removed and added to 4.0mL of scintillation fluid for counting respectively.

Separation of enzymes by Affi-Gel blue column The freeze-dried protein fractions from both cortex and nucleus with the highest radioactivity after S-300HR chromatography were further separated on a 5mL of Econo-Pac affi-gel blue prepacked cartridge using an Econo™ Gradient Pump (Bio-Rad). Optimum conditions were established during test runs using a mixture of α-crystallin with glucose-6-phosphate dehydrogenase (G6PDH). The cartridge was equilibrated with 50mmol/L potassium phosphate buffer (pH7.1). A mixture containing bovine αL-crystallin (12mg) and commercial bovine adrenal G6PDH (100μg, Sigma) in a total volume of 1mL in 50mmol/L potassium phosphate buffer (pH7.1), was loaded onto the column at a flow rate of 60mL/h and fractions (1.5mL) were collected. 20mL of degassed application buffer (50mmol/L potassium phosphate buffer, pH7.1) was washed through the column, followed by a NaCl gradient of 50mL (0.1mol/L to 1mol/L NaCl) with application buffer. 1mol/L NaCl in 50 mmol/L potassium phosphate buffer (10mL) was then run through followed by 20mL of application buffer at 120mL/min. Absorbance was read automatically at 280nm and then the activity of G6PDH of each fraction was determined by using the method previously described[4]. The peaks of α-crystallin were in the front of fractions (4-20 tubes) and enzymes e.g. a higher G6PDH activity always came out in the middle of gradient (1mol/L NaCl). Then the cartridge was equilibrated with 50mmol/L potassium phosphate buffer (pH7.1) before running again. A 1mL sample of each fraction (10-18g/L) was loaded onto the column at a flow rate of 60mL/h and fractions (1.5mL) were collected. The elution methods were the same conditions as mentioned above. Absorbance was then read at 280nm and the radioactivity of each fraction was determined by using the method described above (Radioactive its measurements). Protein peaks and the corresponding areas with a higher radioactivity were pooled and then dialysed. After freeze-drying, the pooled fractions were kept for SDS-gel electrophoresis.

SDS-gel electrophoresis SDS polyacrylamide gel electrophoresis was performed using a Bio-Rad Mini-Protean II dual slab mini gel apparatus with 0.5mm spacers. The gels contained 120g/L acrylamide with a 120g/L running gel and a 40g/L stacking gel. Coommassie Brilliant Blue stain (R-250, Merck) was used to detect the polypeptide bands and the large range and low molecular weight markers (Sigma, Poole, UK) were used throughout the study. The protein content in the bound fractions was further analysed by mass spectrometry.

Mass spectrometry analyses After electrophoresis, the gel was placed in a clean tray and rinsed 3 times for 5 minutes each time with 100-200mL of deionised water. Then the gel was stained with 20mL of GelCode Blue Stain Reagent. Protein band development was monitored periodically during gentle shaking. After scanning the gel, the 20 and 35kDa bands from the cortex and 20 and 66 kDa bands from the nucleus were cut out and put into Eppendorf tubes. The cortex sample was the bound fraction after Affi-gel Blue column separation. The nucleus sample was the bound fraction of γ-crystallin after Affi-gel Blue column separation. The mass spectrometry was carried out on a Applied Biosystems Voyager DE-STR MALDI-TOF instrument using α-cyano- 4-hydroxycinnamic acid (CHCA) matrix for peptides and sinapinic acid matrix for proteins unless otherwise stated.

RESULTS

Separation of Proteins Incorporating Radioactivity from [^{14}C]-Cyanate in the First Incubation The uptake rate of radioactivity of lens proteins from different portions after 7 days incubation with 9.25×10^{3}kBq [^{14}C]-labelled potassium cyanate was 27.2% (32.40 ±1.93 →2.36 ±2.24kBq). After incubation the cortex and nucleus were separated from the incubated lenses, homogenised and centrifuged. The radioactivity in the total protein of homogenates was greater in the cortex (37.82±2.01kBq) than in the nucleus (19.51± 2.35kBq) indicating that the rate of incorporation by cyanate is higher in the cortex than in nucleus. A greater proportion of radiolabelled proteins became water-insoluble protein in the nucleus (2.64±0.04kBq) compared with the cortex (1.31± 0.12kBq). The supernatants from the cortex and the nucleus were loaded onto a Sephacryl S-300HR size-exclusion gel chromatography column. The eluted fractions were

monitored for radioactivity and protein. The chromatogram demonstrated that the greatest incorporation of cyanate at an early stage in lens the cortex was into the β-crystallin fraction. Less cyanate reacted in lens nucleus but the radioactivity was more widely distributed. It appears that α and β_H-crystallin fractions were the most likely primary targets for carbamylation in lens nucleus. Fractions corresponding to α-, β_H- and β_L-crystallin from cortex and α-, β_H-, β_L- and γ-crystallin from nucleus were separately pooled and dialysed against distilled water over 24 hours. The radioactivity of different fractions was then measured (Table 1). The major proteins reacting with cyanate are β_L and α-crystallin in cortex, α and β-crystallin in nucleus. It is clear that γ-crystallin is not a vulnerable protein to this insult. After further separation of each fraction on Affi-Gel Blue prepacked cartridges, the bound and unbound fractions were pooled, dialysed and freeze-dried separately before analysis by SDS-PAGE. The gels showed a normal pattern of bands for the major crystallin fractions that were not bound to the Affi-Gel Blue column. However there were extra bands from each bound fraction in lens cortex with a molecular weight about 35kDa. There were no such significant bands in nucleus fractions, suggesting that the selectivity of carbamylation may be different in cortex and nucleus. The extra 35kDa bands need to be further identified. The bound fractions demonstrated very lower radioactivity. The major radiolabelled proteins were in the unbound fractions of both cortex and nucleus. The dehydrogenases, expected to be bound by the Affi-Gel Blue column, may not be the proteins most vulnerable to carbamylation under our conditions.

Separation of Proteins Incorporating [^{14}C]-Cyanate in the Second Incubation The radioactivities of lens proteins from different portions incubated with 2.96 ×104kBq [^{14}C] -labelled potassium cyanate for 7 days and then the cortex and nucleus were separated from the incubated lenses, then homogenised and centrifuged as described above. The extent of incorporation of [^{14}C] cyanate further supported the previous observation that more cyanate were incorporated by the water-soluble proteins in the cortex compared with the nucleus (26624.89 ±19.57 →25247.50 ±422.29kBq). After separation of water-soluble proteins from the cortex and nucleus by size-exclusion chromatography, the protein concentration and radioactivity of eluted fractions were determined and plotted. The elution profile for the cortex showed radioactivity spread through all major crystallin regions.

After being run on a Sephacryl S-300HR column, the corresponding crystallin elution peaks were pooled and then dialysed against distilled water. The radioactivity of α-, β_H- and β_L-crystallin from cortex in the total was 23%, 14% and 63%, respectively. The greatest incorporation of [^{14}C] cyanate in cortex was into β_L (63%) fraction, which is consistent with above observation. Less radioactivity was incorporated into nuclear proteins and the major peak was with the γ-crystallin fraction. Examination of the pooled fractions confirmed that the major proteins β- and γ-crystallin in nucleus incorporated the greater proportion of the cyanate (The radioactivity of α-, β_H- and β_L-crystallin in the total was 12%, 37% and 51%, respectively). Comparison of elution profiles and radioactivity from nucleus between the first and second incubation suggested that γ-crystallin in nucleus may be one of the vulnerable proteins to carbamylation. If given chance (e.g. elevated concentration of cyanate), γ-crystallin in nucleus may react more with cyanates.

Table 1 The radioactivity in different fractions from incubated lenses (kBq)

	Cortex	Nucleus
Homogenates (H_1)	33.91±4.49	10.70±1.79
Homogenates (H_2)	33.13±7.31	6.590±0.18
Supernatants (S)	30.99±3.86	4.48±0.25
S/H_2	93.5%	67.9%
Washing of WIP	4.79±1.47	2.11±0.69

H_1: Before dialysis; H_2: After dialysis; WIP: Water-insoluble proteins

The freeze-dried fractions after separation on a Sephacryl S-300HR column were further separated on an Affi Gel Blue column. Nucleus fractions became partially insoluble after separation procedures. The absorbance of soluble proteins from nucleus fractions at 280nm was lower than that from cortex fractions in terms of the similar weight of proteins applied on the Blue column, indicating that proteins from lens nucleus are vulnerable to insolubilization.

The elution profile of the cortex α-crystallin showed most of the protein eluting without binding with very little absorbance showing bound protein. The elution peaks were pooled (4-6, 7-16, 17-28, 29-58) and dialysed and the radioactivity of the pooled fractions was assayed. The total radioactivity was greatest in the bound fraction of the α-crystallin fraction from cortex. Similarly most of the cortical β_H-crystallin fraction was not bound to the Affi Gel Blue column. Again a large proportion of the radioactivity was found in the bound fraction with slightly more in the unbound fraction. Rather more of the cortical β_L-crystallin was bound to the Blue column but this time the preponderance of radioactivity was unbound.

The nuclear fractions were fractionated in the same way as the cortical fractions. The α-crystallin from nucleus showed more bound fraction than was found for cortical fractions. The radioactivity was widely spread throughout the pooled fractions. The nuclear β-crystallinhad had less protein bound, but most radiolabel was bound. Finally the nuclear γ-crystallin fraction contained little protein that bound to the Blue column, with radiolabel present in both bound and unbound fractions.

Overall the bound fractions had higher radioactivity particularly in α-crystallin (as 78% of total radioactivity in α-crystallin) and β_H-crystallin (as 53% of total in β_H-crystallin fraction) from lens cortex and in β-crystallin (as 74% of total in β-crystallin fraction) and γ-crystallin (as 54% of total in γ-crystallin fraction) from lens nucleus. However, the percentage of total radioactivity in the bound fractions from cortex β_L-crystallin and nucleus α-crystallin were approx imately 31% and 24% . Although the total radioactivity of cortex β_L-crystallin was higher in both incubation experiments, it was possibly due to its greater proportion. These results suggested that the bound fractions of cortex α – and β_H-crystallin after separation by affinity chromatography, together with the bound fractions of nucleus β and γ-crystallin, include the proteins preferentially targeted by carbamylation.

After separation on the Blue column, the pooled fractions were dialysed and freeze-dried, and further analysed by SDS-PAGE. The SDS-PAGE showed that the unbound and bound fractions separated from α-crystallin in both cortex and nucleus had similar bands exception for a faint 66kDa band. However, there were extra 35kDa bands in bound fractions separated from both β-crystallin but not from the other fractions. Surprisingly, a fairly sharp band of molecular weight as 66kDa was shown in bound fraction of nucleus γ-crystallin. Moreover, a faint 66kDa band was present in bound fractions of both cortex and nucleus, though they were fairly faint exception for nucleus γ-crystallin. Mass spectrometry analysis on nucleus γ-crystallin fraction revealed that it is serum albumin (see below). In an attempt to improve the recovery of the carbamylated proteins, the supernatants from cortex and nucleus were directly loaded onto a Blue column without previous separation on Sephacryl S-300HR. The elution profile of supernatants from cortex showed that a significant amount of protein was eluted as the NaCl ionic strength increased, indicating it is bound fraction. However, there was no such a peak corresponding to bound fraction in the nucleus. The fractions corresponding to unbound and bound peaks were pooled and dialysed. Then the total radioactivity in each fraction was measured as 1.63±0.62kBq in unbound fractions and 0.16± 0.01kBq (8.9% of total radioactivity) in bound fractions from cortex supernatants, 0.54±0.07kBq in unbound fractions and 0.08±0.03kBq (13.3% of total radioactivity) in bound fractions from nucleus supernatants, respectively. These data suggest that the extent of incorporation by cyanate in cortex is higher than that in nucleus, further supporting above observations. However, the majority of carbamylated proteins are in unbound fractions not surprisingly and due to the greater proportion compared with bound fractions.

After being pooled and freeze-dried, the bound and unbound fractions from both cortex and nucleus were further analysed by SDS-PAGE. The SDS-PAGE of the bound fraction separated from cortex supernatant showed a sharp 35kDa band and abundant 20-22kDa band. However, there was no significant 35kDa band from nucleus supernatant. The bound fraction from nucleus presented a few of 20-29kDa molecular weight bands. The 35kDa (marked as C35) and 20kDa (marked as C20) of bound fractions from cortex, 20kDa (marked as N20) and 66kDa (marked as N66) of bound fractions from nucleus γ-crystallin, were further analysed by mass spectrometry. In order to provide enough purified proteins for mass spectrometry analysis, several lanes were loaded onto SDS-PAGE and then it was performed by using GelCode Blue Stain.

Identification of Carbamylated Proteins by Mass Spectrometry To identify the proteins that reacted with [^{14}C] cyanate, the major proteins on the SDS-PAGE gel were cut out, digested with trypsin and investigated by using MALDI-TOF mass spectrometry. The mass spectra of peptides from gel digests were analysed. The mass spectra of tryptic peptides of 20kDa bands on SDS-gel from cortex showed that the masses of nine of the fragments identified matched bovine α-crystallin A chains. Another nine masses matched α-crystallin B chain (Table 2). The mass spectra of tryptic peptides from the 35kDa bands of bound fractions run on SDS-gel from cortex showed that the best match of the 28 fragment masses was with bovine ξ-crystallin, also known as quinone oxidoreductase (Table 2). The mass spectra of tryptic peptides of about 20kDa bands on SDS-gel of bound fractions from nucleus showed that the fragment masses matching bovine β-crystallin A3/A1, α-crystallin B chain and γB-crystallin were identified as shown in Table 3.

The masses of the peptides that were unmatched by the software were examined for possible carbamylation which would increase the mass by 43Da. Fragments of mass 1.43 and 1.76 kDa were found, and are 43.0Da higher than that of 1.40kDa (position 91-103, peptide sequence: VKVLGDV IEVHGK) and 1.72kDa (position 93-107, peptide sequence: VLGDVIEVHGKHEER) as expected masses from α_B-crystallin. However, both are minor peaks in mass spectra. Both these sequences contain lysine as the residue most likely to be carbamylated. The mass spectra of tryptic peptides of 66kDa band on SDS-gel from nucleus showed that the masses of fragments from the 66kDa bands from nucleus are shown in Table 3. They matched bovine serum albumin, which is an unlikely component of the 'crystallin' fraction from size-exclusion chromatography. We conclude that some serum albumin used in a trial run on the blue column became bound but leached off slowly when stronger buffers were applied.

Table 2 Masses of fragment for matched bovine α-crystallin A and B chain in 20kDa and ξ-crystallin in 35kDa bands from cortex

Chain	M/z submitted	Start	End	Peptide Sequence	Modifications
α-crystallin A	980.5512	71	78	(K) FVIFLDVK(H)	
	1037.5277	13	21	(R) TLGPFYPSR(L)	
	1073.4511	104	112	(R) QDDHGYISR(E)	pyroGlu
	1090.4760	104	112	(R) QDDHGYISR(E)	
	1172.5771	79	88	(K) HFSPEDLTVK(V)	
	1175.6121	55	65	(R) TVLDSGISEVR(S)	
	1193.6209	12	21	(K) RTLGPFYPSR(L)	
	1224.5926	146	157	(K) IPSGVDAGHSER(A)	
	1300.6334	89	99	(K) VQEDFVEIHGK(H)	
α-crystallin B	872.4345	150	157	(R) KQASGPER(T)	
	986.4633	83	90	(K) HFSPEELK(V)	
	1071.4734	108	116	(R) QDEHGFISR(E)	pyroGlu
	1088.5003	108	116	(R) QDEHGFISR(E)	
	1165.6385	93	103	(K) VLGDVIEVHGK(H)	
	1192.6157	73	82	(K) DRFSVNLDVK(H)	
	1374.7074	12	22	(R) RPFFPFHSPSR(L)	
	1462.6996	57	69	(R) APSWIDTGLSEMR(L)	
	1639.7579	104	116	(K) HEERQDEHGFISR(E)	
ξ-crystallin	852.4833	35	41	(K) DHQVLIK(V)	
	899.4956	250	257	(R) GTIEINPR(D)	
	980.5524	188	196	(K) IVLENGAHK(V)	
	1166.6604	24	34	(K) LQSDVAVPIPK(D)	
	1219.6076	176	187	(K) VLGTASTEEGQK(I)	
	1221.6428	13	23	(R) VFEFGGPEVLK(L)	
	1510.7675	125	138	(K) QGAAIGIPYFTAYR(A)	pyroGlu
	1527.7909	125	138	(K) QGAAIGIPYFTAYR(A)	
	1592.7695	42	55	(K) VQACGVNPVDTYIR(S)	
	1721.8449	308	324	(K) ATQAHENIIHSSGATGK(M)	
	2470.1852	98	120	(R) TISGGYAEYALAADHTVYTLPEK(L)	
	2763.4077	217	242	(K) GVDVIIEMLANVNLSNDLNLLSHGGR(V)	
	2973.4765	98	124	(R) TISGGYAEYALAADHTVYTLPEKLDFK(Q)	

DISCUSSION

Peptide mass mapping has been widely used for protein identification and to identify post-translational modifications. Cyanate readily reacts with protein amino group to form carbamylated derivatives, which were implicated in human cataract especially in tropical countries. The potential importance of carbamylation lens crystallins to cataractogenesis has been studied extensively in our group and others. Carbamylation of lens crystallins, has been detected in human cataractous lens and may initiate aggregation and cataract [2]. Sites of reaction of cyanate with crystallins *in vivo* and *in vitro* have been identified. The more recent work used the peptide mass approach to pick out the modifications as we have in the present report. In other studies posttranslational modification proteins may produce the uninterpreted masses [5]. These studies for the first time identified the primary targets for carbamylation in lens proteins under physiological condition. Based on present data ξ-crystallin appears to be the first evidence of carbamylation in the lens and it is also a good candidate for primary target by carbamylation. The oxidoreductase activity of ξ-crystallin was inhibited irreversibly by ophthalaldehyde, a bifunctional cross-linking reagent, and pyridoxal-5'-phosphate [6]. NADPH protects the oxidoreductase against inactivation induced by heat, N-ethylmaleimide, H_2O_2, and ophthalaldehyde [7]. Interestingly, α-crystallin acting as molecular chaperone protects ξ-crystallin against thermally-induced inactivation [6] and assists unfolding and refolding of ξ-crystallin from its different urea-denatured states [8]. The N-terminal amino groups of α- and β-crystallins are acetylated in the lens, so that only the free ε-amino groups of α-, β-, and γ-crystallins and the N-terminal amino group of γ-crystallin can be modified [1]. Since α-crystallin is a major structural protein in the lens which functions as molecular chaperone, it has been extensively investigated for modified products and specific sites of modification. Several lysyl residues in both αA- and αB-crystallins are highly reactive *in vitro*. Present results suggested that αA and αB-crystallins are preferentially carbamylated, since αB-crystallin from both cortex and nucleus, and αA-crystallin from cortex,

Table 3 Masses of fragment for matched bovine crystalline and albumin from nucleus

	M/z submitted	Start	End	Peptide Sequence	Modifications
β-crystallin A3	1455.7279	33	44	(K) ITIYDQENFQGK(R)	
in 20kDa bands	1466.8072	126	137	(K) ITIFEKENFIGR(Q)	
	1561.8192	110	122	(R) LMSFRPICSANHK(E)	
	1611.8731	33	45	(K) ITIYDQENFQGKR(M)	
	1691.8351	96	109	(R) WDAWSGSNAYHIER(L)	
	1727.9130	197	211	(R) EWGSHAQTSQIQSIR(R)	
	1922.9133	163	177	(K) IQCGAWVCYQYPGYR(G)	
	1955.9069	178	193	(R) GYQYILECDHHGGDYK(H)	
α-crystallin B	872.4614	150	157	(R) KQASGPER(T)	
in 20kDa bands	986.4880	83	90	(K) HFSPEELK(V)	
	1071.5014	108	116	(R) QDEHGFISR(E)	pyroGlu
	1088.5241	108	116	(R) QDEHGFISR(E)	
	1165.6572	93	103	(K) VLGDVIEVHGK(H)	
	1192.6449	73	82	(K) DRFSVNLDVK(H)	
	1374.7380	12	22	(R) RPFFPFHSPSR(L)	
	1462.7369	57	69	(R) APSWIDTGLSEMR(L)	
	1478.7137	57	69	(R) APSWIDTGLSEMR(L)	1Met-ox
	1639.8142	104	116	(K) HEERQDEHGFISR(E)	
γB-crystallin	580.3141	92	95	(R) IYER(D)	
20 kDa bands	943.4690	3	9	(K) ITFYEDR(G)	
	1294.7011	143	152	(R) QYLLRPGEYR(R)	
	2247.9965	59	76	(R) RGDYPDYQQWMGFNDSIR(S)	
	1169.6472	623	633	(R) KAELVAVAVNR(L)	
	1462.7101	98	109	(R) IQLPDMQMIDAR(V)	
	3007.4587	148	175	(R)RGTSDADNMNGPLSMRPGEESVGPMTLR(K)	
serum albumin	927.4917	161	167	(K) YLYEIAR(R)	
in 66 kDa bands	1163.6237	66	75	(K) LVNELTEFAK(T)	
	1249.6157	35	44	(R) FKDLGEEHFK(G)	
	1305.7112	402	412	(K) HLVDEPQNLIK(Q)	
	1439.8081	360	371	(R) RHPEYAVSVLLR(L)	
	1479.7891	421	433	(K) LGEYGFQNALIVR(Y)	
	1480.4708	106	117	(R) ETYGDMADCCEK(Q)	
	1567.7357	347	359	(K) DAFLGSFLYEYSR(R)	
	1639.9490	437	451	(R) KVPQVSTPTLVEVSR(S)	
	1725.8239	469	482	(R) MPCTEDYLSLILNR(L)	
	1881.9081	508	523	(R) RPCFSALTPDETYVPK(A)	
	1908.8975	529	544	(K) LFTFHADICTLPDTEK(Q)	
	2045.0167	168	183	(R) RIIPYFYAPELLYYANK(Y)	
	2493.2122	45	65	(K) GLVLIAFSQYLQQCPFDEHVK(L)	

incorporated label from cyanate at an early stage. Possibly αB-crystallin is more vulnerable to carbamylation than αA-crystallin. As observed from the mass spectra of 20kDa bands from cortex, we detected nearly all the components of matched and unmatched masses. Among the masses of unmatched peptides from αB-crystallin, fragments of mass 1.43 and 1.76kDa were found to be 43.0Da higher than that of 1392.8209 (position 91-103, peptide sequence: VKVLGDVIEVHGK) and 1.72kDa (position 93-107, peptide sequence: VLGDVIEVHGKH EER) as matched masses of αB-crystallin. Both these peptide sequences contain internal lysine residues that constitute potential carbamylation sites: Lys 92 and Lys 103 respectively.

The finding that lysine 92 of αB-crystallin from the water-soluble fraction of bovine lens was carbamylated under our conditions is consistent with the findings from human cataractous lens *in vivo* [8], supporting the view that this residue is among the most active of the lysine residues. Present data also demonstrated that at lysine 103 of αB-crystallin is carbamylated. Discrepancies between *in vivo* and *in vitro* phosphorylation of lens crystallins has been reported [4,6], and it may support the observation on carbamylation. Mass spectrometry analysis revealed that γB-crystallin is one of the preferential targets for carbamylation. However, γ-crystallins are synthesized at an early stage in the development of the lens and are

consequently located in the lens core. The high prevalence of γ-crystallin in nucleus may contribute to the present results under the conditions described. The carbamylation of γB-crystallin may result in altered surface charges of crystallin that cause conformational changes as shown in vitro, which subsequently lead to formation of disulphide bonds and crystallin insolubility. This phenomenon has been demonstrated in deamidation among the disulphide bonded γS-crystallin from human cataractous lenses. It is notable that the predominant disulphide cross-linked species in the water-insoluble portion of nuclear cataract is γS-crystallin[9], indicating that γ-crystallin presents extensively in nucleus. γ-Crystallin was a major component of a labelled fraction after incubation with labelled isocyanate, but there was no direct evidence from mass spectrometry to show specific carbamylation and the sites of its modification, so there is only tentative support for the idea that γ-crystllin is a primary target by carbamylation, although it has been shown to be a primary target for glycation in bovine lens [10]. However, the γB-crystallin, a richer lysine residues crystallin, would be modified substantially if given time.

A number of age-related post-translational modifications have been observed in human β-crystallins including formation of disulphide bonds in βB2, truncation among βB1 and βA3/A1 and deamidation in all human crystallins with exception of α B-crystallin. Loss of both the N-terminal region of βB1 and βA3/A1 and removal of hydrophilic portions are particularly important in maintaining protein solubility. βB2-Crystallin retains its hydrophilic N-terminus with ageing, indicated that βB2-crystallin undergoes less modification than any of the other crystallins[11]. In this study, bovine βA1-crystallin from nucleus was identified as primary target of carbamylation, suggesting that it is likely nucleus for aggregation and cataractogenesis. Although serum albumin was revealed by mass spectrometry analysis in this studies, it seems unlikely that it exists in bovine lens. It has been previously reported that the lens epithelium and fibres are devoid of any serum protein constituent, due to the avascular nature of the lens after complete invagination and separation of the lens vesicle from the surface ectoderm, which occurs at a very early stage of development of the vertebrate embryo. Furthermore, lens proteins do not show any cross-reactivity with homologous serum proteins, and the slightest trace of albumin immunofluorescent staining. It has been observed that the bovine serum albumin became tightly bound to affinity Blue columns (Rixon and Harding, unpublished results). Our results were shown on SDS-PAGE, 66kDa bands in every bound fractions, so we conclude that serum albumin had contaminated the affinity Blue column and was subsequently eluted by the stronger buffer used to remove the bound fractions. This problem was probably exacerbated due to large amount of bound fraction that had to be pooled and concentrated after the separation of lens proteins on the affinity Blue column.

In conclusion, the proteins that incorporated cyanate at an earlier stage were identified in this study as ξ-crystallin, α-crystallins, βA1 and γB-crystallins. Our data present the first evidence of ξ-crystallin as a primary target for carbamylation in the lens under physiological condition *in vitro*. Mass spectrometry provided direct evidence for the sites of carbamylation as Lys 92 and Lys 103, sites previously identified as the carbamylated sites in human lenses *in vivo*. The sites of carbamylation on the other target protein need further investigation. Carbamylation of lens structural proteins could cause unfolding leading to aggregation and thus cataract. Carbamylation of the molecular chaperone, α-crystallin, could compromise its chaperone function so that it was unable to protect the unfolding proteins. Carbamylation of the quinone oxidoreductase could prevent it from eliminating harmful quinones from the lens, and they in turn could react with proteins causing crosslinking and thus cataract. Similar changes may occur in other tissues and other diseases especially in conformational diseases.

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