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Age-dependent deamidation of αB-crystallin

Patricia J.T.A. Groenen, Maria J.P. van Dongen, Christina E.M. Voorter, Hans Bloemendal and Wilfried W. de Jong

Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Bovine and human αB-crystallin undergo deamidation upon aging in the lens. In bovine αB-crystallin, the specific site of deamidation has been identified by peptide mapping after tryptic digestion. Asn-146 was found to be subject to deamidation, whereas the only other asparagine residue, at position 78, is not affected. Asn-146 is flanked at the carboxylic side by a glycyl residue. Yet, the rate of in vivo deamidation is low. In vitro studies reveal that the deamidation is accompanied by significant racemization, indicating that the deamidation proceeds via formation of a succinimide intermediate.

Lens; αB-Crystallin; Deamidation; Racemization; Molecular aging

1. INTRODUCTION

One of the most abundant structural proteins in the eye lens is α-crystallin. This protein occurs as large water-soluble aggregates (up to 10^6 Da) and is composed of two types of related subunits, αA- and αB-crystallin. The absence of protein turnover in the inner part of the lens makes it necessary that the crystallins are extremely stable and long-living proteins [1]. α-Crystallin is therefore an excellent model for the study of in vivo aging of proteins. α-Crystallins also occur in several cell types and organs outside the lens [2,3] and increased levels of αB-crystallin have been observed in several neurological disorders [4,5] and in cell lines under stress conditions [6]. There is a striking sequence homology between α-crystallin and the small heat-shock proteins [7,8]. Recent studies demonstrate that they also have functional similarities [6] and that both α-crystallin and the small heat shock proteins can function as molecular chaperones [9–11].

From this functional point of view it is relevant to increase the understanding of the stability and in vivo modification of α-crystallin. It has been shown that both αA- and αB-crystallin undergo various posttranslational modifications, both enzymatic and nonenzymatic [1,12,13]. Deamidation is one of the major nonenzymatic modifications in aging proteins [14] and constitutes indeed a problem of considerable concern in protein engineering [15]. Deamidation was proposed at an early stage to contribute to the observed charge heterogeneity of the α-crystallin chains [16]. Only more recently could the specific sites of deamidation in chicken and bovine αA-crystallin [17,18] be established. In the present study we describe the age-dependent site-specific deamidation of bovine αB-crystallin at Asn-146.

Correspondence address: W.W. de Jong, Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: (31) (80) 540525.

2. EXPERIMENTAL

Bovine eye lenses of different ages were obtained through the Central Animal Facilities of the University of Nijmegen, School of Medicine. Human lenses were provided by Dr. G. Vrensen (the Netherlands Ophthalmic Research Institute, Amsterdam) and by Dr. M. Hogeweg (Academic Hospital, Leiden).

α-Crystallin from calf lens cortex (6-month-old), bovine lens nucleus (5-year-old) and human lenses (75- to 85-year-old) was isolated from the water-soluble fraction by gel permeation chromatography on Ultrogel AcA-34 (Pharmacia LKB). Bovine αB-crystallin was purified by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) and was digested with trypsin (1%, w/w). The resulting peptides were isolated by high-voltage paper electrophoresis at pH 6.5 followed by descending chromatography [19]. After visualization with fluorescamine, peptides were identified by amino acid analysis. The T16 peptide was further analyzed by thermolytic digestion (2%, w/w) at 50°C in 0.1 M NH_4HCO_3, pH 8.9, using a peptide concentration of 0.5 nmol/μl. The resulting thermolytic peptides were separated again by high-voltage electrophoresis at pH 6.5.

In vitro deamidation of α-crystallin and purified αB-subunits from 6-month-old calves was performed under vacuum in 0.1 M sodium phosphate buffer, pH 7.4, at 70°C, using a protein concentration of 10 nmol/150 μl [20]. Incubations were terminated at different time intervals by short dialysis and subsequent lyophilization. D/L-Aspartic acid ratios (or % D-Asp) were determined after isolation of Asp-containing peptides as described [21,22].

Alkaline urea gel electrophoresis was carried out at pH 8.5 [19]. Two-dimensional gel electrophoresis was performed according to O'Farrell [23].

3. RESULTS

Analysis by two-dimensional gel electrophoresis of α-crystallin isolated from the water-soluble fraction of

Fig. 1. Coomassie brilliant blue-stained two-dimensional gel pattern of α-crystallin, isolated from the water-soluble fraction from 75- to 85-year-old human lenses. The superscripts ^{d,p} and ^{pp} indicate the presumed deamidated, mono- and di-phosphorylated α-crystallin subunits.

75- to 85-year-old human lenses reveals a characteristic pattern, composed of αA- and αB-crystallin and several posttranslational modification products (Fig. 1). It is known from earlier studies that bovine αA- and αB-crystallin both undergo phosphorylation [24–26] and that αA-crystallin in addition undergoes site-specific deamidation of Asn-101, which is accompanied by isomerization, racemization and peptide bond cleavage [18]. Also in human αA-crystallin conspicuous deamidation of Asn-101 has been observed [27]. No evidence has been provided yet for the occurrence of deamidation in αB-crystallin, although Kramps [27] isolated from human, but not from bovine α-crystallin, an αB-subunit that he suspected to be deamidated. Indeed, the two-dimensional gel pattern of human α-crystallin shows a spot with one more negative charge than αB-crystallin, responding positively to an antiserum specific for αB-crystallin (data not shown), indicative for a deamidated αB-crystallin product. There are only 4 amino acid differences between bovine and human αB-crystallin [28,29], not involving the sequences surrounding the only two asparaginyl residues. For this reason and because bovine lenses were more easy to obtain, the in vitro and in vivo deamidation of αB-crystallin was studied using bovine α-crystallin.

Bovine αB-crystallin undergoes a single charge difference upon incubation in 100 mM phosphate buffer pH 7.4 at 70°C, conditions that favor succinimide formation [20,30,31] (Fig. 2, lanes 2,3). This presumed deamidation process of αB-crystallin proceeds very quickly, the $t_{1/2}$ value is 8 h. To find out if this charge difference is indeed due to the deamidation of an asparaginyl residue, tryptic peptide mapping of the native and modified αB-crystallin was performed. The tryptic peptide maps of the two chains were identical apart from a change in mobility of a single peptide (Fig. 3). Upon analysis of the amino acid composition, this peptide was identified as T16, comprising residues 124–149 of the αB chain [28]. This peptide contains an Asn residue at position 146 that is flanked by a glycyl residue. In addition to Asn-146 there are in this peptide three Asp residues (positions 127, 129 and 140) of which Asp-140 is also followed by Gly. It is known that Asn–Gly sequences are particularly prone to deamidation via succinimide formation, which leads to isomerization and racemization as well [20]. Similarly, Asp–Gly sequences can easily form succinimide rings, again leading to enhanced isomerization and racemization. To check whether racemization occurs concomitantly during deamidation of αB-crystallin, the percentage of D-Asp in the tryptic peptide T16 and its thermolytic fragments were determined. Table I shows that the level of racemization of T16 increases from 2.2% to 17.3% during 96 h of incubation. This increase is largely accounted for by the strong racemization of deamidated Asn-146 (T16Th4^{d}) and Asp-140 (T16Th3). The level of D-Asp of other aspartyl residues hardly increases upon in vitro incubation.

To assess whether deamidation of αB-crystallin also occurs in vivo, α-crystallin was isolated from 5-year-old bovine lens nuclei. This fraction contains a band that corresponds in electrophoretic mobility with the in vitro deamidated αB-product (Fig. 2, lane 4). The amount of this product relative to the unmodified αB-chain in 5-year-old α-crystallin is estimated at about 20%, whereas in young α-crystallin this product is absent (Fig. 2, lane 1). These data clearly point out that the deamidation of αB-crystallin is an age-dependent process. The supposed deamidated αB-product was purified by means of

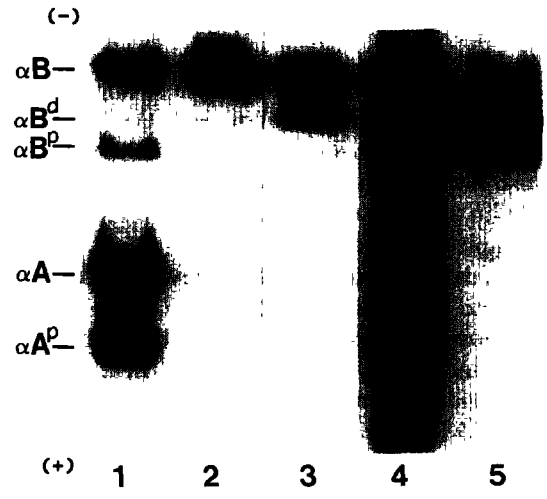

Fig. 2. Alkaline urea gel electrophoresis pattern showing deamidation of αB-crystallin. (1) α-Crystallin isolated from the water-soluble fraction of calf lens cortex (35 μg), (2) αB-crystallin isolated from calf lens cortex (5 μg), (3) αB-crystallin, in vitro deamidated for 7 h at 70°C under vacuum (12.5 μg), (4) α-crystallin isolated from the water-soluble fraction of 5-year-old bovine lens nuclei (75 μg), (5) αB^{d}-crystallin, isolated from 5-year-old bovine lens nuclei (20 μg). The in vitro and in vivo αB^{d}-product is located between αB-crystallin and the monophosphorylated αB^{p}-crystallin, demonstrating a single additional negative charge in αB^{d}- relative to αB-crystallin.

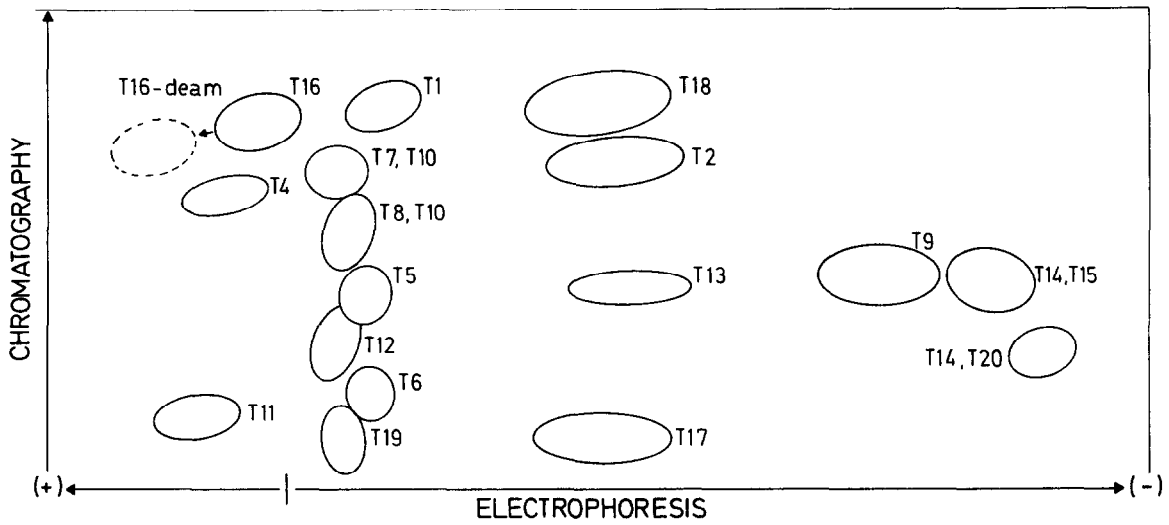

Fig. 3. Peptide map of tryptic digest of bovine αB-crystallin. The arrow indicates the displacement of T16 (dashed) in the deamidated αB-chain. This displacement was found both upon in vitro deamidation (incubation time 24 h) and in the in vivo deamidated αB-crystallin isolated from 5-year-old bovine lens nuclei.

anion-exchange chromatography (Fig. 2, lane 5). Tryptic peptide mapping revealed again the displacement of T16, demonstrating that Asn-146 of bovine αB-crystallin deamidates in vivo as well. The identification of Asn-146 as site of deamidation is supported by the finding that chicken αB-crystallin, which lacks the corresponding Asn residue, is not deamidated at all in 10-year-old lenses (see Fig. 3 in [17]).

4. DISCUSSION

The number of well-characterized in vivo deamidation sites in cellular proteins is still quite limited [32,33]. In only a few proteins, particularly the long-living lens crystallins, deamidation has been correlated with aging [34]. Besides the site-specific, age-dependent deamidation in chicken, bovine and human αA-crystallin [17,18,27], we now provide evidence that also bovine αB-crystallin undergoes site-specific deamidation at Asn-146, both during in vitro incubation and upon in vivo aging of this protein in the lens. The deamidated αB-crystallin reaches a level of at least 20% in 5-year-old bovine lens nuclei, whereas it is not detectable in 6-month-old calf lens cortex. High levels of deamidated αB-crystallin can also be observed in 75- to 85-year-old human lenses (Fig. 1), while this modification product is not detectable in fetal human lenses (see Fig. 1B in [35]).

Table I

Racemization of aspartyl residues of αB-crystallin after in vitro incubation in 0.1 M sodium phosphate buffer, pH 7.4 at 70°C. Values are the mean of two independent incubations; percentages of D-Asp were measured in triplicate.

Peptides as indicated		% D-Asp $t = 0$	% D-Asp $t = 96$ h
T16	: Ala-Asp^{127}-Val-Asp^{129}-Pro Ser-Asp^{140}-Gly Val-Asn^{146}-Gly	**2.2%**	–
T16[d]	: Asn^{146} → Asp^{146}	–	**17.3%**
T16Th1	: Ala-Asp^{127}-Val-Asp^{129}-Pro	1.5%	5.2%
T16Th3	: Ser-Asp^{140}-Gly	**2.3%**	**24.2%**
T16Th4	: Val-Asn^{146}-Gly	**2.3%**	–
T16Th4[d]	: Asn^{146} → Asp^{146}	–	**28.7%**
T4	: Ile-Asp^{62}-Thr	2.9%	6.7%
T6	: Lys-Asp^{73}-Arg	2.0%	4.1%
T12	: Gln-Asp^{109}-Glu	1.8%	2.7%

[d] Peptides T4, T6 and T12 are included for comparison.

Because αB-crystallin rapidly deamidates in vitro under conditions that favor succinimide formation and because of the pronounced racemization that was found to accompany the site-specific deamidation, we assume that the deamidation of αB-crystallin under physiological conditions in the lens also proceeds through an imide intermediate. It is known from previous studies that the rate of succinimide-mediated reactions is strongly influenced by the surrounding residues [20,36,37]. Amino acids at the amino side of Asn or Asp have little or no effect, whereas amino acids at the carboxylic side of Asn or Asp affect the imide ring formation significantly. In general, the rate of succinimide-linked reactions is decreased as the size and steric bulk of the carboxylic flanking residue increases. Hence, Asn–Gly sequences easily undergo deamidation. Here we demonstrate that deamidation of bovine αB-crystallin actually occurs at Asn-146–Gly-147. However, the rate of in vivo deamidation at Asn-146 is low, indicating conformational constraints on the deamidation process [32]. The only other asparagine residue present

in bovine (and human) αB-crystallin (Asn-78) is flanked by a bulky leucine residue and does not deamidate.

Imide ring formation at aspartic acid residues, which recently has been demonstrated to occur in vivo [38], leads to their isomerization and racemization. In previous studies, it has been inferred that this process takes place in bovine αA-crystallin of which a single aspartyl residue exhibits pronounced age-dependent racemization [22,39]. A similar modification has now been found in αB-crystallin. Among the 3 aspartyl residues in T16, only Asp-140 becomes strongly racemized upon incubation of αB-crystallin under conditions that favor succinimide formation. Like Asn-146, this Asp-140 is flanked by a glycine residue at the carboxylic side.

It is not clear whether deamidation of αB-crystallin has any influence on its functioning as a lens structural protein. Outside the lens, however, the role of αB-crystallin as a stress protein will probably not be affected by this modification because of the low deamidation rate at 37°C.

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