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The in vivo phosphorylation sites of bovine αB-crystallin

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Phosphate content determinations established that in αB-crystallin two phosphate groups can be present in vivo in bovine lenses. Comparison of tryptic digests of phosphorylated and unphosphorylated αB chains, revealed the location of the two phosphorylation sites in tryptic peptides T2 and T3. Thermolytic digestion and gas-phase sequencing demonstrated that Ser-19 and Ser-45 are the in vivo phosphorylation sites of bovine αB-crystallin. This pattern of phosphorylation differs from the previously reported in vitro obtained results.

Phosphorylation; Lens protein; Crystallin, αB-

1. INTRODUCTION

α-Crystallin is an abundant protein in the eye lenses of most vertebrates. It occurs as large aggregates, composed of two types of 20 kDa subunits, αA and αB, which show a sequence homology of 55% [1]. α-Crystallin is evolutionarily related with the small HSPs [2], which makes it conceivable that these proteins share certain functional properties, probably related with stress resistance [3]. It has recently been found that αB-crystallin is also present in certain non-lenticular tissues, notably in heart [4,5], and at increased levels in scrapie-infected hamster brains [6] and in astrocytes of patients with Alexanders disease [7]. In mammals, both α-crystallin and small HSPs can be phosphorylated in vivo [8–11], which may relate to their biological functioning. In vivo and in vitro, the sole phosphorylation site in αA is Ser-122 [9,12]. In αB-crystallin, at least two different serine residues can be phosphorylated by incubation of lenses with [^{32}P]orthophosphate [13]. We identified the in vivo phosphorylation sites of bovine αB-crystallin and established a difference between this in vivo phosphorylation pattern and previously reported in vitro obtained results.

2. MATERIALS AND METHODS

2.1. *Isolation and characterization of α-crystallin subunits*

α-Crystallin was isolated from bovine lenses (4–5 years old) by gel permeation chromatography on Ultrogel AcA 34 (LKB), and subunits were separated by ion exchange chromatography on CM-52 [14] or by chromatofocussing on MonoP (Pharmacia) [15]. Purity of the peaks was checked by alkaline urea slab gel electrophoresis [16]. The different αB chains were digested with trypsin (Worthington), and the resulting peptides were separated by paper electrophoresis and chromatography [14]. Peptides were located with fluorescamine and eluted with 5% acetic acid for amino acid analysis. Preparative isolation of the large 'core' peptide T3 for phosphate content determination was performed by gel filtration over Sephadex G50 superfine, where T3 coelutes with T16 [14]. Pure T3, for subdigestion and sequence analysis, was obtained by RP-HPLC of tryptic digests, using a 300-RP-8 column (Merck), eluting at a flow rate of 0.8 ml/min with a gradient of 0.1% TFA versus acetonitrile (Rathburn) (0 to 100% in 100 min) containing 0.1% TFA (Pierce). T3 was further digested with thermolysin and the resulting peptides were separated by RP-HPLC, using an RP-18 column (Merck) and a gradient of acetonitrile as indicated in fig.2.

Sequence determinations to identify the phosphorylated serine residues, were performed on an Applied Biosystems model 470A gas-phase sequencer equipped with an on-line PTH amino acid analysis system. The almost quantitative conversion of phosphoserine residues into the PTH-DTT-dehydroalanine adduct during gas phase sequencing [17] was used for positive identification of the exact position of the phosphorylated serine residues.

2.2. *Miscellaneous methods*

Cyanogen bromide cleavage was carried out according to standard procedures. The resulting fragments were separated by gel permeation chromatography of Sephadex G50 superfine [14]. The phosphate contents of proteins, CNBr-fragments and peptides were determined according to the procedure of Fiske-SubbaRow, modified by Broeckhuyse [18]. Amino acid analyses after hydrolysis in 6 N HCl were carried out on an LKB Alpha Plus amino acid analyser.

3. RESULTS AND DISCUSSION

The in vivo occurrence in bovine lenses of monophosphorylated αB-crystallin (αB[p] or αB_1, according to older nomenclature) had already been established by phosphate content determinations [8,9]. In the latter

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Abbreviations: RP-HPLC, reversed-phase high-performance liquid chromatography; TFA trifluoroacetic acid; HSP, heat shock protein; PTH-DTT, phenylthiohydantoin-dithiothreitol

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study in vitro phosphorylation of bovine lens homogenates also revealed a minor, apparently double phosphorylated αB chain (αB[2p]). A putative αB[2p] protein can actually be observed in 4–5-year-old bovine lenses, corresponding with the αB_0 chain reported by Van Kleef et al. [19]. We isolated this protein by chromatofocussing, and found indeed approximately 2 mol phosphate/mol polypeptide (table 1).

The αB chain can thus be phosphorylated in vivo at two different sites, which we set out to identify by comparative peptide mapping of αB, αB[p] and αB[2p] (fig.1A). The only difference between the peptide maps of αB and αB[2p] is the anodal shift of peptide T2 in αB[2p], in agreement with a change in charge induced by phosphorylation of a serine residue. Indeed, gas phase sequencing of T2 of αB[2p] revealed the sequence Arg-Pro-Phe-Phe-Pro-Phe-His-Ser-Pro-Ser-Arg and demonstrated the presence of a phosphoserine at position 19 (underlined). In αB[p], T2 is present in both charge forms, indicating that the single phosphate moiety in αB[p] can be attached at different serine residues.

The second phosphorylation site might be present in T3, which is the only peptide lacking on the peptide maps, owing to its insolubility in the electrophoresis buffer. Phosphate content determinations revealed that the phosphate moiety in αB[p] is exclusively present in the N-terminal CNBr fragment, comprising residues 1–68, whereas the mixture T3/T16, isolated by gel filtration, contains approximately 0.7 mol P/mol T3 (table 1). Combining these results it is clear that most of T3 is indeed present in a phosphorylated form in αB[p]. T3 could not be isolated from αB[2p] in sufficient amounts for phosphate determinations.

Because T3 contains 5 serine residues, further identification of the phosphorylation site was necessary. We therefore isolated this peptide from total tryptic digests of the three αB chains by RP-HPLC (fig.1B). A clear difference in elution time exists between T3 of αB (peak A) and of αB[2p] (peak B), whereas T3 of αB[p] contains both peaks A and B. This suggests that peak

Table 1

Phosphate content of αB-crystallin subunits, CNBr fragments and tryptic peptide mixture T3/T16

	Phosphate content
αB	0.04
αB[p]	1.06
αB[2p]	2.13
CNBr I αB[p]	0.99
CNBr II αB[p]	0.03
T3/T16 αB[p]	0.67

The amount of protein or fragments used for phosphate measurements was determined by amino acid analysis. Values are presented as the molar ratio of phosphate to protein subunit or CNBr fragment. In the case of T3/T16, phosphate content is given in mol phosphate/mol T3.

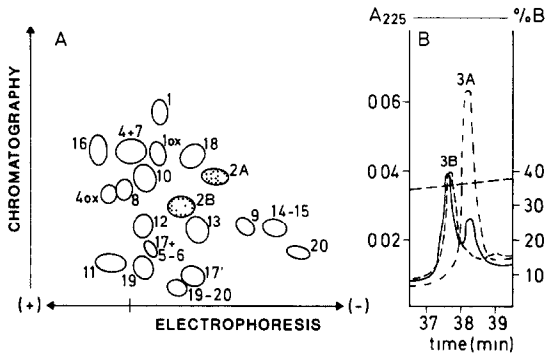

Fig.1. (A) Peptide map of tryptic digest of bovine αB-crystallin. All peptides were identified by amino acid analysis. Numbers indicate the order of the tryptic peptides in the bovine αB chain [14]. 2A corresponds with unphosphorylated T2 and is present in αB and αB[p], whereas 2B corresponds with the phosphorylated form of T2 and is present in αB[p] and αB[2p]. (B) Part of the elution profiles by RP-HPLC of digests from αB (– · –), αB[p] (—) and αB[2p] (---). 3A corresponds with unphosphorylated T3, 3B with the phosphorylated form of T3.

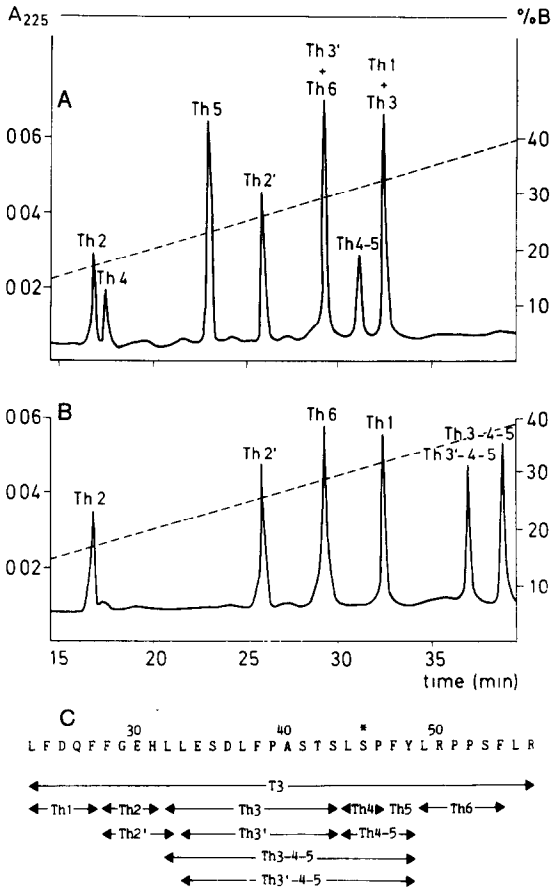

Fig.2. Elution profiles of thermolytic peptides from the unphosphorylated (A) and phosphorylated (B) form of tryptic peptide T3, and its amino acid sequence (C) according to Van der Ouderaa et al. [14]. Thermolytic peptides were fractionated by RP-HPLC and identified by amino acid analysis. Peptides Th3-4-5 and Th3'-4-5, instead of Th3, 3', 4, 4–5 and 5, were only obtained from phosphorylated T3, indicating hindrance of cleavage due to the presence of phosphoserine (*).

A represents the unphosphorylated form of T3, and peak B the phosphorylated T3. Both forms of T3 were further digested with thermolysin and the resulting peptides isolated by RP-HPLC on an RP-18 column (fig.2). The differences in elution profiles can be attributed to incomplete cleavage of T3 from peak B at residues Leu-44 and Phe-47. The presence of P-Ser is indeed known to hinder the enzymatic cleavage of adjacent sites [9]. Since Th1, Th2 and Th6 from peaks A and B have identical retention times, the phosphorylation site obviously must be located in the large peptides Th3-4-5 and Th3'-4-5. Gas phase sequencing of these peptides from the αB[p] chain revealed unambiguously a phosphorylated serine residue at position 45 in both cases (fig.2). αB-Crystallin can thus be phosphorylated at two sites in vivo, namely at Ser-19 and at Ser-45. αB[2p] is completely phosphorylated at both sites, whereas αB[p] is a mixture of αB[p45] and αB[p19], with Ser-45 being the major phosphorylated residue in this subunit.

In contrast to αA-crystallin, where the in vivo phosphorylation site is identical to the major in vitro one [9,12], a difference exists between the in vivo and in vitro phosphorylation sites of αB-crystallin. Chiesa et al. [13] identified two phosphorylation sites in αB after lens incubation in medium containing [^{32}P]orthophosphate: a major site at Ser-59 and a minor site at Ser-43 and/or Ser-45. Upon in vitro incubations of αB-crystallin in the presence of cAMP-dependent protein kinase, we also detected major labeling of tryptic peptide T4, comprising Ser-59 (Voorter, C.E.M. and De Jong, W.W., unpublished results). However, in the native αB[p] chain, no phosphorylation of Ser-59 could be detected. Differences between in vivo and in vitro sites of phosphorylation have also been demonstrated in other proteins [20,21].

Interestingly, immunoblot analysis of mouse and bovine heart extracts revealed the presence of a more acidic minor charge form of αB (data not shown), comparable with αB[p] in the lens. Strikingly enough, this form is lacking in chicken heart, like in chicken lens [9]. The functional implication of the phosphorylation of α-crystallin, if any, is still completely unclear. The fact that dephosphorylation of α-crystallin can occur in bovine lens extracts, favours the notion that the phosphorylation of α-crystallin is a metabolically controlled reversible process relating to its biological function [22,23]. It should, however, be noted that phosphorylation of α-crystallin is by no means a universal characteristic among vertebrates [9].

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