The hydrophobic probe 4,4′-bis(1-anilino-8-naphthalene sulfonic acid) is specifically photoincorporated into the N-terminal domain of αB-crystallin

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Received 12 April 1997

Abstract Photoincorporation of the fluorescent probe 4,4′-bis(1-anilino-8-naphthalene sulfonic acid) (bis-ANS) can be used to locate solvent-exposed hydrophobic regions in proteins. We show that bis-ANS is specifically incorporated into the putative N-terminal domain of αB-crystallin. This incorporation diminishes the chaperone-like activity of αB-crystallin, suggesting that hydrophobic surfaces in the N-terminal domain are involved in the binding of unfolding proteins.

Key words: αB-Crystallin; Small heat shock protein; Chaperone-like activity; Photolabeling

1. Introduction

The small heat shock proteins (hsp) form a large and diverse family of multimeric proteins, most of which can be induced by heat or other stress conditions [1–3]. The physiological role of these proteins is rather unclear, but the fact that they share a chaperone-like property in vitro, i.e. the ability to stabilize aggregation-prone proteins [4,5], may be the key to gain insight into their in vivo functioning. Amongst the family of small hsp is the major eye lens protein α-crystallin, a macromolecular complex comprised of two homologous types of subunits, αA- and αB-crystallin. Formerly, α-crystallin was believed to be strictly lens-specific, but now it is known that especially αB-crystallin is expressed in a variety of non-lenticular tissues [6,7]. In addition, increased levels of αB-crystallin have been found in several neurodegenerative disorders [8–11].

In the absence of any X-ray crystal data, little is known about the tertiary and quaternary structure of α-crystallin. Intron positions in the α-crystallin genes suggested a tertiary structure consisting of two domains and an extending C-terminal arm [12]. Experimental support for this structure comes from unfolding studies and NMR-analysis [13,14]. The arrangement of the subunits in the multimeric complex is subject of controversy, because the experimental data are equivocal and contradictory.

Probe-binding studies have shown that α-crystallin, unlike β- and γ-crystallins, is very rich in solvent-exposed hydrophobic regions [15]. These regions are apparently responsible for the ability of α-crystallin to prevent protein aggregation [16,17], by having affinity for the molten globule state of unfolding proteins [18,19]. Heterocomplexes formed between α-crystallin and molten globules are even more hydrophobic than α-crystallin itself, but nevertheless they are stable and do not precipitate [19,20]. The high solubility of these heterocomplexes may be attributable to the solvent exposed C-terminal arms of the α-crystallin subunits [21]. Deletion of the C-terminal 17 residues from human αA-crystallin results indeed in a marked reduction of chaperone-like activity [22]

The amino acid residues of α-crystallin which constitute the hydrophobic surface regions are unknown. The fluorescent probe 4,4′-bis(1-anilino-8-naphthalene sulfonic acid) (bis-ANS) has frequently been used to monitor changes in hydrophobic surfaces in various proteins, including α-crystallin [17,23]. Recently this probe was also used to determine the actual location of solvent-exposed hydrophobic regions in the bacterial chaperone GroEL [24]. It was shown that photoinduced incorporation of bis-ANS specifically occurs in the apical domain of GroEL-subunits, which is indeed the binding site for misfolded proteins [25,26]. In this paper we similarly use bis-ANS to determine whether the N-terminal or C-terminal domain of αB-crystallin is potentially involved in the binding of unfolding proteins.

2. Materials and methods

2.1. Materials

A pET3a cDNA-construct encoding rat αB-crystallin was transformed in the host *Escherichia coli* B BL21(DE3)pLysS [27]. Induction, purification and reconstitution of αB-crystallin were performed as described before [28]. Cyanogen bromide and bovine pancreas insulin were obtained from Sigma. Sephadex G-50 gel permeation material was purchased from Pharmacia Biotech and 4,4′-bis(1-anilino-8-naphthalene sulfonic acid) dipotassium salt was obtained from Molecular Probes.

2.2. Photoincorporation

A solution of αB-crystallin (300 μg) and bis-ANS (50 nmol) in 1 ml of 20 mM NaP_i, 100 mM Na_2SO_4, pH 6.9, was exposed to laboratory strip lighting for 30 min at ambient temperature. A control sample without bis-ANS was given the same treatment. Salts and non-bound bis-ANS were removed by applying the samples to a Sephadex G-50 spin column (pre-equilibrated with water) followed by dialysis against water. Subsequently, the solutions were lyophilized. The color of αB-crystallin incubated with bis-ANS was yellow, indicating incorporation of the fluorescent probe. The incorporation efficiency could be slightly increased by using a 366 nm UV light source (Ultra Violet Products, UVL-56) instead of strip lighting. However, exposure to long wave UV radiation also resulted in a certain amount of undesired protein crosslinking.

2.3. Cyanogen bromide cleavage

A cyanogen bromide solution was prepared by adding a few crystals of cyanogen bromide to 200 μl of formic acid. The lyophilized protein samples were dissolved in 30 μl of the cyanogen bromide solution and incubated overnight at room temperature. To check whether the acidic conditions alone induce protein cleavage, control

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Abbreviations: bis-ANS, 4,4′-bis(1-anilino-8-naphthalene sulfonic acid); hsp, heat shock protein(s)

PII S0014-5793(97)00498-5

samples were incubated in formic acid without cyanogen bromide [29]. Afterwards, the formic acid was evaporated in a Speed Vac Concentrator (Savant, SVC-100H) and the protein residues were dissolved in tricine-SDS-PAGE sample buffer.

2.4. Chaperone assay

Samples of αB-crystallin (900 μg in 1 ml), incubated with and without bis-ANS (150 nmol), were applied to a Sephadex G-50 spin column (pre-equilibrated with 20 mM NaP_i, 100 mM Na_2SO_4, pH 6.9). Afterwards, the volume of the samples was measured and adjusted to precisely match the protein concentrations. Chaperone-like activity was determined essentially as described by Farahbakhsh et al. [30]. Briefly, αB-crystallin (54 or 216 μg) was pre-incubated with 245 μg bovine pancreas insulin for 3 min at 40°C. Denaturation of insulin was initiated by addition of 20 μl of 1 M dithiothreitol, and scattering was monitored for 15 min at 360 nm using a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer equipped with a thermostated circulating water bath at 40°C and a thermocouple to register the sample temperature. Total volume was 1.0 ml, and all solutions were in 20 mM NaP_i, 100 mM Na_2SO_4, pH 6.9.

2.5. Miscellaneous methods

The concentration of bis-ANS stock solutions in 20 mM NaP_i, 100 mM Na_2SO_4, pH 6.9, was determined by measuring the absorbance at 385 nm ($\varepsilon_{385} = 16790\ M^{-1}\ cm^{-1}$) [31]. Protein concentrations were determined in triplicate using the Bradford assay [32]. Gel permeation analysis was performed on a Superose 6 HR 10/30 prepacked column (Pharmacia Biotech) as described before [28]. High molecular mass standards (Pharmacia Biotech) were used for calibration. Protein samples were analyzed by tricine–SDS-PAGE [33]. Photolabeled proteins were visualized on a UV light box equipped with a video camera (Biorad Geldoc 1000). The data were processed with Molecular Analyst 1.2 software (Biorad).

3. Results and discussion

Rat αB-crystallin has a length of 175 amino acid residues. The putative N-terminal domain comprises residues 1–67 [12] The putative C-terminal domain and C-terminal arm are formed by residues 68–175. The demarcation of the C-terminal arm is not completely clear, but according to NMR-analysis only residues 166–175 have great flexibility [34].

Hydropathy analyses suggested that the N-terminal domain is on average more hydrophobic than the C-terminal domain [35,36]. However, this does not imply that the N-terminal domain is directly involved in protein binding. The apolar residues of this domain may constitute a non-exposed hydrophobic core contributing to structural stability rather than a solvent-accessible hydrophobic surface. Furthermore, as illustrated by the Kyte-Doolittle plot in Fig. 1, the C-terminal domain may comprise a few regions which are at least as hydrophobic as the N-terminal domain.

To discriminate between the N-terminal and C-terminal domain as potential substrate binding region, purified rat αB-crystallin was incubated with bis-ANS and photolabeling was induced by exposure to laboratory strip lighting. Subsequently, the non-bound bis-ANS was removed and the photolabeled αB-crystallin was subjected to cleavage with cyanogen bromide. Rat αB-crystallin contains three methionine residues, one at the N-terminus and the other two precisely at the boundary between the two putative domains, i.e. at positions 68 and 70. Therefore, reaction with cyanogen bromide should result in a 8 kDa fragment comprising the N-terminal domain and a 12 kDa fragment comprising the C-terminal domain and C-terminal arm. The cleavage products were analyzed via tricine–SDS PAGE. The protein patterns after Coomassie Brilliant Blue staining are shown in Fig. 2A. Lane 2 displays control αB-crystallin and lane 1 shows that

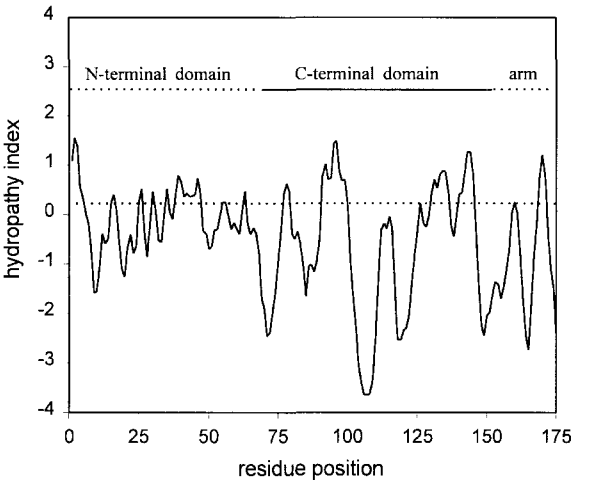

Fig. 1. Hydropathy plot of rat αB-crystallin. The hydropathy index was calculated for segments of six residues according to the method of Kyte and Doolittle [42]. The hydropathy index of each segment is plotted as a function of its location in the primary structure. The hydropathy profile of the C-terminal domain is highly conserved in the small hsp family [2].

reaction of this protein with cyanogen bromide results in the formation of two new polypeptide bands. The estimated molecular masses of these cleavage fragments were as expected, i.e. 8 and 12 kDa. Furthermore, lane 1 also exhibits some uncleaved αB-crystallin indicating that the reaction did not proceed to completion. Lane 4 displays αB-crystallin incubated with bis-ANS and lane 3 shows that cleavage of this sample proceeds nearly as efficiently as cleavage of control αB-crystallin (compare lanes 3 and 1). The fluorescence gel shown in Fig. 2B corresponds to the stained gel in Fig. 2A and visualizes the polypeptides which have incorporated the bis-ANS probe. As expected, cleaved and uncleaved control αB-crystallin are not discernible on this gel (lanes 1 and 2, respectively). However, the fluorescent band in lane 4 clearly demonstrates that αB-crystallin becomes photolabeled upon incubation with bis-ANS. In addition, lane 3 shows that this photolabeling is restricted to the N-terminal domain, because only the 8 kDa cleavage fragment is fluorescent. Thus the N-terminal domain, but not the C-terminal domain exposes hydrophobic surfaces which are able to covalently bind the hydrophobic probe bis-ANS.

The bis-ANS probe is a small aromatic molecule with a molecular mass of only 673 Da. In contrast, the usual protein substrates of α-crystallins have molecular masses which are at least several kilodaltons. Hence, the binding sites for bis-ANS in αB-crystallin may not or only partially coincide with the binding sites for protein substrates. However, if the bis-ANS probe is actually bound to hydrophobic surfaces involved in protein binding, photolabeling of αB-crystallin is likely to diminish chaperone-like activity. That is to say, the binding sites may loose their affinity for unfolding substrates upon irreversible crosslinking to bis-ANS. To determine the influence of bis-ANS incorporation on chaperone-like activity, photolabeled and control αB-crystallin were assayed for their ability to prevent the chemically-induced aggregation of insulin B-chains. Reduction of the disulfide bonds between the A- and B-chain of insulin, results almost instantaneously in a precipitation of the B-chains. Fig. 3 shows that this event

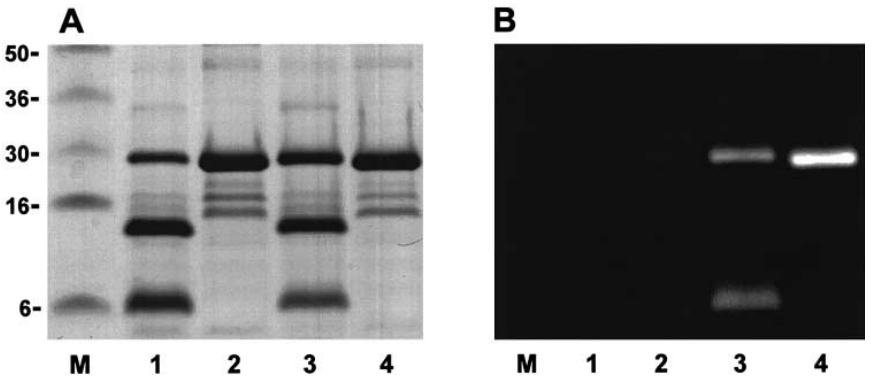

Fig. 2. Photoincorporation of bis-ANS into αB-crystallin. A: Coomassie Brilliant Blue stained tricine–SDS gel. B: Corresponding fluorescent gel. Molecular mass markers (M in kDa) are indicated. Samples are: control αB-crystallin cleaved with cyanogen bromide (lane 1), uncleaved control αB-crystallin (lane 2), photolabeled αB-crystallin cleaved with cyanogen bromide (lane 3), uncleaved photolabeled αB-crystallin (lane 4). The uncleaved samples (lanes 2 and 4) display two minor components below the main band. These components are most probably products of truncation caused by the incubation in formic acid (see Section 2).

can easily be monitored by measuring the absorbance at 360 nm. Insulin was reduced at αB-crystallin to insulin mass ratios of 22% and 88%, respectively. Comparison of the aggregation profiles in Fig. 3 clearly demonstrates that at both ratios the activity of photolabeled αB-crystallin is considerably lower than that of control αB-crystallin. Thus, incorporation of bis-ANS into αB-crystallin decreases its ability to suppress aggregation of insulin B-chains. Although this result suggests that bis-ANS indeed interacts with functional protein binding sites, other explanations cannot be excluded. For example, incorporation of bis-ANS may affect the structure of αB-crystallin, resulting in a lower functional activity. However, gel permeation analysis does not support that a major conformational change is induced by bis-ANS incorporation. Like control αB-crystallin, photolabeled αB-crystallin elutes as a regular multimeric complex with a molecular mass of about 670 kDa (data not shown).

The specific binding of bis-ANS to the N-terminal domain of αB-crystallin is in agreement with the hydropathy analyses [35,36], that is, the N-terminal domain is indeed more hydrophobic than the C-terminal domain. Furthermore, binding of the soluble bis-ANS probe suggests that the N-terminal domain is at least partially solvent-exposed in the multimeric αB-complex. Other evidence for an accessible N-terminal domain comes from phosphorylation studies. The phosphorylation sites in bovine αB-crystallin are located in the N-terminal domain at serine positions 19, 45 and 59 [37,38]. These sites are conserved in rat αB-crystallin and indicate that the N-terminal domain is sufficiently exposed to enable interaction with kinases. Our data suggest that the binding sites for bis-ANS and substrate proteins at least partially coincide. It thus appears that the N-terminal domain of αB-crystallin is involved in substrate binding, and therefore essential for activity. This conclusion is in agreement with the findings of Smith et al. [39], who propose on basis of hydrogen–deuterium exchange experiments that residues 28–34 in bovine αB-crystallin constitute a possible substrate binding region. Recently, hsp18.1 from pea was subjected to photolabeling, and incorporation of bis-ANS was found in the extreme N-terminal region as well as in a region of the C-terminal domain [40]. The latter region was proposed to be critical for chaperone-like activity because it is highly conserved among plant small hsp. However, we show that the corresponding region in rat αB-crystallin, i.e residues 79–96, does not incorporate bis-ANS at a detectable level.

Members of the small hsp family share two important features; they occur as dynamic multimeric complexes and they can bind unfolding proteins. These features are likely to be related, i.e. multimerization of small hsp subunits may be nature's solution to create a very stable and soluble complex with a high capacity for preventing substrate proteins from aggregation. The C-terminal domain of small hsp subunits is much more conserved during evolution than the N-terminal domain [2,12]. Although it seems contradictory to associate functional activity with a relatively non-conserved region, it should be realized that chaperone-like activity is a non-specific phenomenon. The functional sites of small hsp must be able to bind a variety of substrate proteins, and therefore their amino acid sequence may be subject to variation as long as hydrophobic surfaces are preserved. In contrast, multimerization may require rather defined structural elements that do

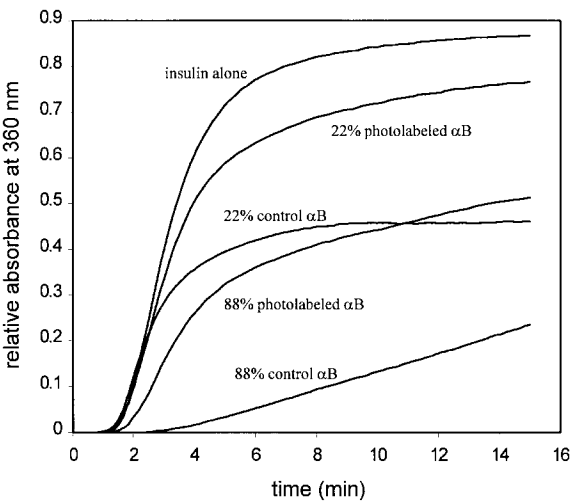

Fig. 3. Chaperone-like activity of control and photolabeled αB-crystallin. Reduction-induced aggregation of insulin (245 μg/ml) as a function of time in the absence and presence of αB-crystallin (54 or 216 μg/ml; 22% or 88% mass ratios). The incubation temperature was 40°C and the reduction of insulin was initiated by addition of 20 μl of 1 M dithiothreitol.

not allow much sequence change, as is the case in the C-terminal domain.

The present results indicate that the N-terminal domain of αB-crystallin has solvent-accessible hydrophobic regions. Moreover, other studies have shown that the flexible C-terminal arm as well as parts of the C-terminal domain are susceptible for enzymatic post-translational modifications [41]. Thus, whatever the quaternary structure of αB-crystallin may be, the subunits are likely to have an orientation which allows both domains to be accessible for either unfolding proteins or modifying enzymes.

Acknowledgements: We thank Perry Overkamp for purifying recombinant αB-crystallin. This investigation was carried out under auspices of the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and NIH Grant EY09683.

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