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Temperature dependence of chaperone-like activity and oligomeric state of αB-crystallin

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Abstract

The chaperone-like activity and the oligomeric state of αB-crystallin were studied at different temperatures and in the presence of urea and thiocyanate. The activity, assessed measuring the ability of αB-crystallin to prevent the aggregation of denatured insulin, strongly depends on temperature. While a significant activity increase was detected at 42 °C, the presence of urea and thiocyanate does not affect the protein activity in an irreversible way. In-solution SAXS measurements performed in the same experimental conditions showed that αB-crystallin forms near-spherical, hollowed, polydisperse oligomers, whose dimensions change above 42 °C. Moreover, in the presence of urea and thiocyanate, a global fit analysis confirms the high stability of αB-crystallin assemblies in relationship with their variable quaternary structure. In particular, the changes in the inner radius as well as the thickness and dispersion of the protein shell, account for the preservation of the chaperone-like activity.

Keywords: αB-crystallin; SAXS; Chaperone activity; Heat shock protein; Protein aggregation; Quaternary structure

1. Introduction

α-crystallin is a major structural protein of the mammalian lens, where it occurs as a large macromolecular assembly of average molecular mass of 800 kDa. α-crystallin is composed by two 20-kDa subunits, designated as αA and αB [1]. Both monomers can independently form aggregates comparable in size to α-crystallin, and both of them have been found in several tissues, such as thymus and retina (αA-crystallin), brain, spleen, kidney, skeletal muscle, and heart (αB-crystallin) [2,3].

The structural function of the α-crystallin is to assist in maintaining transparency in the lens [4], but it has been observed that α monomers show a considerable sequence homology with the small heat shock proteins (sHSP). Accordingly, it has been established that α-crystallins show *chaperone-like* activity by binding partially denatured proteins and preventing further denaturation and aggregation [5,6]. Remarkable is the fact that the synthesis of αB-crystallin, but not that of αA-crystallin, can be induced by heat, osmotic shock, drugs and oncogenes [7].

Even if the protective role of αB-crystallin is clearly indicated by data showing that a missense mutation of αB-crystallin gene causes a desmin-related myocardiopathy [8,9], the mechanism of its chaperone activity is not fully understood. As for the other sHSP, clear indications that the structure of the chaperonine interacting in the protein complexes is in a polymeric form have been obtained [10]. For example, interaction studies on protein-sHSP complexes have shown that their size exceeds the predictable sum of the two monomers, while a further supporting evidence was furnished by the high sHSP/protein ratio in the complexes [11–14]. In addition, a very poor chaperone activity was detected in the dimeric form of HSP20, an analog of αB-crystallin [15]. Moreover, it remains unclear if temperature activation is necessary for triggering the chaperone-like activity of αB-

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crystallin: some reports claim that the chaperone activity starts at 30 °C, while others indicate that this is not the case [16–18].

Due to this essential role, the structural properties of αB-crystallin and the conformational changes eventually induced by temperature are under extensive investigation. However, the large protein polydispersity observed in solution has precluded both protein crystallization and decisive in-solution structural analysis, so that the obtained conclusions are questionable [19]. In fact, if some agreement concerns the tertiary structure, the main debate is related to the protein quaternary structure. The prevailing idea is that the αB-crystallin subunit shows the Ig fold, and that its structure is similar to the structure of the subunit of sHSP16.5 from *Methanococcus jannaschii* (MjHSP16.5) [20–22]. This protein shows a 20.7% sequence identity with the α-crystallin C-terminal domain, and forms a multimer composed of 24 monomers. However, as reported by Haley in 1999, there are several differences between the two proteins [23]. First, sHSP16.5 forms homogeneous assemblies, with exactly 24 subunits and a total molecular mass of 400 kDa, while α-crystallin forms assemblies that are heterogeneous, with a total molecular mass ranging from 300 kDa to over 1 MDa [24]. Second, the crystal structure of sHSP16.5 reveals an octahedral symmetry, while cryoelectron microscopy (cryo-EM) reconstruction indicates that αB-crystallin does not have one predominant symmetrical state [25]. Finally, it has recently been demonstrated that the αB-crystallin and MjHSP16.5 dimers, which can be considered the building block of the quaternary structure, are in fact structurally different [26].

Different hypothetical models have been proposed for the quaternary structure of α-crystallin, including the three layered concentric-shell model, with a variable subunit number, the dynamic protein micelle model, and the cubic and rhombic dodecahedron models [27–31]. It should be observed that other models have been proposed, as the elongated subunit micelle model, the pitted-flexiball model, the chaperon-like annulus or toroid model and the open-loose model (see [23] for a review and for references): we think, it is relevant to observe that the differences in the proposed models are in agreement with the general conclusion that αB-crystallin, as well as α-crystallin and sHSP, have a variable quaternary structure [32]. More recently, cryo-EM experiments have revealed that αB-crystallin forms large globular assemblies, containing a central cavity [23,25]. The assemblies are heterogeneous in mass, but an average value of 32 subunits per particle has been derived by image analysis. Nevertheless, it remains unclear whether this arrangement is unique and what are the correlations with the chaperone activity. A recent X-ray structure analysis of the HSP16.9 [33] has opened new questions: HSP16.9, an analog of αB-crystallin, is formed by an assembly of dodecameric double disks, but this structure is different from the assembly proposed for both MjHSP16.5 [21,22] and αB-crystallin [25].

The aim of the present study is, therefore, to evaluate the chaperone-like activity of αB-crystallin as a function of temperature and to compare the activity with its structural and aggregation properties determined in solution in the same conditions. The activity has been tested measuring the interaction with the insulin B-chain, destabilized by reducing the intra-molecular cysteine bonds, while protein structure and aggregation state have been determined by SAXS measurements. Finally, the relationship between activity and dimensions of the oligomer has been also studied in the presence of the disaggregating agents (urea and thiocyanate) at different temperatures.

2. Materials and methods

2.1. Expression and purification of human αB-crystallin

The *Escherichia coli* BL21 (DE3) cells, transformed with pET24dαB-crystallin vector, were a gift from Professor W. W. de Jong. The human αB-crystallin cloned in BL21 cells was purified, according to previously reported methods [12]. SDS-PAGE was performed to check the purity of the protein. The approximate molecular weight of the protein was determined by gel-filtration using a calibrated Sephacryl S-300 HR. The αB-crystallin complex was apparently polydisperse, showing a mean molecular weight of about 420 kDa, comprised in the range 300–800 kDa. The αB-crystallin molarity calculated through this work is based on the molecular mass of αB-crystallin monomer.

2.2. Chaperone-like activity measurements

The chaperone-like activity of αB-crystallin has been assayed by measuring its ability to prevent the aggregation of a substrate protein, insulin, denatured by reduction of disulfide bonds. The aggregation of protein upon denaturation was monitored by measuring the apparent absorption at 360 nm due to the scattering of the insulin B-chain homo-aggregates vs. time. The measurements were performed on a Beckman DU-60 spectrophotometer, equipped with a thermostated holder. Data were collected and analyzed with the software Microcal Origin 5.0.

The final composition of the analyzed samples was phosphate buffer 25 mM, pH 7.2; insulin 35 μM (corresponding to about 0.2 mg/ml) (see below for the stock solution preparation); αB-crystallin 0.05–2.5 μM (corresponding to about 1–50 μg/ml); dithiothreitol (DTT) or β-mercapto-ethanol 20 mM. The relative chaperone-like activity of αB-crystallin was calculated as the percentage of protection against the insulin B-chain aggregation using the formula:

$$\%\ \text{protection} = 100\frac{\Delta I - \Delta I_{\alpha B}}{\Delta I} \quad (1)$$

where ΔI and $\Delta I_{\alpha B}$ represent the apparent saturation absorption at 360 nm (usually after 1 h) in the absence and presence of αB-crystallin, respectively.

Insulin stock solutions were prepared according to the method previously published by Horwitz et al. [12] with the following modifications. 1 mg of insulin (I-5500, Sigma-Aldrich) was dissolved in NaOH 0.1 M (150 μl) and the pH was rapidly brought to 7.2, adding sodium phosphate buffer 0.5 M, pH 6.8 (100 μl).

The volume was then adjusted to 1 ml with bidistilled water. Since the insulin solution was not stable and sometimes the protein precipitated before the DTT addition, another procedure was developed for the insulin stock solution preparation. Insulin was dissolved in HCl (10 mM), with a volume of water of 100 μl per mg of weighed insulin. This solution was immediately diluted 10 times with bidistilled water, to get 1 mg/ml of insulin in HCl (1 mM). This method stabilized the protein in a reproducible way and the insulin B-chain aggregation, after the DTT addition, did not significantly differ with respect to the former method. The insulin stock solution and the DTT solution were daily prepared and kept over ice before the use.

As for the measurements of the influence of denaturing agents on chaperone-like activity, since high concentrations of denaturants interfere with the assay of activity, the measurements were taken diluting 20 times the αB-crystallin samples incubated with urea or thiocyanate for 2 h. The same measurements were taken for non-incubated αB-crystallin. This means that possible influence of the denaturants on the activity of αB-cystallin could be established only if the effects are irreversible or slowly reversible.

2.3. Molecular model building

A theoretical model for the dimer of αB-crystallin was obtained by homology building from the crystal structure of wheat HSP16.9 using InsightII