The conformational stability of α-crystallin is rather low: Calorimetric results

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Abstract The eye lens protein and chaperonin, α-crystallin, was studied by differential scanning microcalorimetry, spectroscopy and size exclusion chromatography. The thermal transition of α-crystallin proceeds at $T_{trs} = 59.8 \pm 0.6$°C with an enthalpy change of $\Delta H = 336 \pm 9$ kJ per mol subunit. Disagreement between previous ΔH values could be attributed to a side reaction that leads, depending on the scan rate, to the formation of a non-productive folding form. The conformational stability of α-crystallin is rather low ($\Delta G = 24 \pm 5$ kJ/mol of subunit). The minimal cooperative unit of α-crystallin is the monomeric subunit.

Key words: α-Crystallin; Heat shock protein; Scanning calorimetry; Conformational stability; Non-productive folding form

1. Introduction

α-Crystallin is a major structural protein of the eye lens. It is a multimeric protein (~800 kDa) composed of αA and αB subunits, each of 20 kDa molecular mass. Besides its main function in light focusing, αB-crystallin shares a similarity with heat shock proteins. In this respect, αB-crystallin belongs to the so-called junior chaperones [1]. Probably, the dual function makes the protein important in both maintaining the refractive index and preventing loss of lens transparency due to unspecific aggregation of other lens proteins [2].

Its biophysical properties, which could be helpful in understanding structure and function of α-crystallin, are still disputed. While X-ray structure analysis of β- and γ-crystallins is possible, no crystallographic data for α-crystallin are available. Models proposed for the quaternary structure of α-crystallin remain controversial [3]. The conformational stability of α-crystallin could not yet be quantified, although it is supposed to have crucial importance in supporting structural models. Even thermal denaturation led to divergent results. No thermal transition below 100°C could be found by means of circular dichroism [4]. In contrast, a thermal transition at about 60°C was observed by scanning microcalorimetry, however, the enthalpy change remained divergent [5–7].

The present paper aims to determine the conformational stability of α-crystallin by differential scanning microcalorimetry, and to identify the minimal cooperative unit of the macromolecular complex.

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2. Materials and methods

2.1. Purification of α-crystallin

α-Crystallin from bovine eye lens was purchased from Sigma. The protein was further purified by gel filtration chromatography at 4°C on a HiLoad high-resolution Sephacryl S 300 column (1.6×60 cm) to remove high molecular weight fractions. Elution was undertaken in 20 mM potassium phosphate buffer, pH 7.2. H-α-Crystallin from Sigma was used without further purification.

2.2. Differential scanning calorimetry

Scanning calorimetric measurements were carried out on a MicroCal MC-2D instrument (MicroCal Inc., Northampton, MA) and a DASM-1M calorimeter (Biopribor, Pushchino), modified for automated data acquisition. Protein concentrations were in the range of 0.7–7.5 mg/ml. Data treatment was carried out using the MicroCal DA-2 and Origin software packages and a home-made program for two-state transitions that approximates pre- and postdenaturational slope by polynomial expressions (O. Ristau, unpublished). Additionally, manual treatment of calorimetric curves was performed according to Privalov and Khechinashvili [8] to determine calorimetric and effective heat.

α-Crystallin concentration was determined spectrophotometrically using an extinction coefficient of $\varepsilon = 0.8$ ml mg^{-1} cm^{-1} and a molecular mass of $M = 800$ kDa. Partial specific volume was calculated from the amino acid composition as 0.71 ml/g. All protein solutions were degassed at room temperature before calorimetric measurement.

2.3. Spectroscopy

Melting curves monitored by optical absorption at $\lambda = 286.5$ nm were measured as described in [9]. The α-crystallin concentration in 20 mM potassium phosphate pH 7.2 was 2.7×10^{-4} mM, and the heating rate 1 K/min.

2.4. Stokes radius

The determination of the Stokes radius of α-crystallin was performed by gel-exclusion chromatography on a HiLoad high-resolution Sephacryl S 300 column (1.6×60 cm) using 20 mM potassium phosphate buffer, pH 7.2, containing 200 mM potassium chloride. Calibration was performed with the standard proteins anhydrase, serum albumin, aldolase, catalase, ferritin, thyroglobulin, DNP-L-α-alanine and blue dextran (substances from Pharmacia). K_{av} is defined by $K_{av} = (V_e - V_o)/(V_c - V_o)$ with elution volumes (V_e) of protein and standards. For further details, see [10].

3. Results

α-Crystallin is capable of preventing protein aggregation [11]. However, α-crystallin has itself a strong tendency towards aggregation on thermal denaturation [12]. This phenomenon, which is also well known from previous calorimetric studies, affects the postdenaturational baseline and renders any quantitative determination of thermodynamic quantities difficult.

A representative scanning calorimetric recording of α-crystallin is shown in Fig. 1. The curves shown in Fig. 1 represent results of a systematic search for suitable experimental conditions aimed to minimize formation of insoluble aggregates

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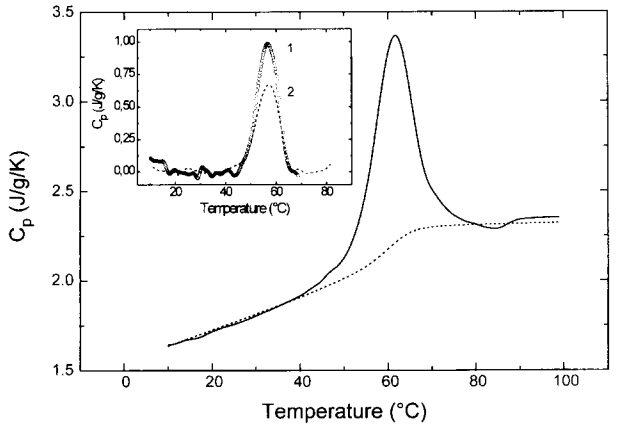

Fig. 1. Scanning calorimetric recording of α-crystallin (1.32 mg/ml) in 20 mM potassium phosphate pH 7.4 at a scan rate of 2 K/min (solid line). The data are normalized to 1 mg/ml protein. (Dashed line) Baseline drawn according to a two-state treatment (see Section 2). Results of the single scan: $T_{trs} = 61.4°C$, $\Delta H^{cal} = 303$ kJ/mol, $\Delta H^{eff} = 299$ kJ/mol, and $\Delta C_p = 4$ kJ/mol per K. (Inset) Reversibility of the thermal transition checked by reheating. Given is the specific excess heat capacity of α-crystallin versus temperature. 1, first scan; 2, reheating. Scan rate, 1.5 K/min; protein concentration, 0.59 mg/ml. Buffer: 20 mM potassium phosphate, pH 9.3, containing 0.1 mM EDTA and 1 mM DTT.

and to optimize reproducibility of calorimetric scans. The following parameters were varied: pH from 7.2 to 9.5, KCl concentration from 0 to 100 mM, EDTA from 0 to 10 mM, DTT from 0 to 1 mM, and protein concentration from 0.7 to 7.5 mg/ml. To check for aggregation, samples were taken from the calorimetric cell after completing the thermal transition and routinely supplied to turbidity measurements and rechromatography.

The experimental conditions chosen in Fig. 1 enable calorimetric scans with reproducible pre- and post-denaturational slope as well as minimal production of insoluble aggregates. At the same time, the thermal transition is partly reversible, i.e. about 65% of the peak area can be obtained on reheating (Fig. 1, inset).

At a heating rate of 1 K/min the transition temperature is $T_{trs} = 59.8 \pm 0.6°C$ in fair agreement with independent findings [5–7]. The calorimetric heat based on the peak area and subunit molecular mass of 20 kDa amounts to $\Delta H^{cal} = 231 \pm 27$ kJ/mol under the specific buffer conditions and heating rate. At the same time, the effective heat based on van't Hoff treatment of the thermal transition amounts to $\Delta H^{eff} = 315 \pm 26$ kJ/mol (mean of 4 measurements). Thermodynamic quantities determined by van't Hoff treatment from heating curves monitored by UV absorption at 286.5 nm ($T_{trs} = 59.9°C$ and $\Delta H^{eff} = 285$ kJ/mol) are within experimental uncertainties in agreement with the calorimetric data.

The non-coincidence of calorimetric heat and effective heat is remarkable. To elucidate the reason for this, the dependence of the thermodynamic quantities on the following dependencies was analyzed:

(a) Dependence on α-crystallin concentration:

no significant changes in T_{trs} and ΔH^{cal} could be found. At higher protein concentration ΔH^{cal} could not be determined due to increasing aggregation;

(b) Assumption of alternative denaturation models including higher subunit molecular mass or unfolding coupled with subunit dissociation:

no convincing model could be deduced (data not shown),

(c) Dependence on heating rate:

ΔH^{cal} turned out to be scan rate dependent whereas T_{trs} and ΔH^{eff} remained unchanged (Fig. 2).

When ΔH^{cal} is extrapolated to infinite scan rate, $\Delta H^{cal,\infty} = 336 \pm 9$ kJ/mol is obtained (Fig. 2), a value that approaches the mean value of the van't Hoff heat ($\Delta H^{eff} = 329 \pm 31$ kJ/mol, data for scan rate from 0.5 to 2 K/min).

Rechromatography of α-crystallin samples after completing the calorimetric scan shows renatured α-crystallin and a portion of the protein (depending on the experimental conditions) being in a still soluble higher associated form. The Stokes radii of various proteins, including α-crystallin, are shown in Fig. 3. The value for native α-crystallin corresponds to the expected value for an 800 kDa protein [13,14]. The soluble associate exhibits about twice the molecular mass of native α-crystallin. With respect to this, the Stokes radius of the soluble associate is within experimental uncertainty the same as the value for high molecular weight α-crystallin (H-α-crystallin) known as a by-product in α-crystallin preparation [13]. A distinct decrease in the formation of the higher associated soluble form was found with increasing scan rate.

Since the high molecular weight form produced during heating could be similar to H-α-crystallin, the melting pattern of the latter was determined (Fig. 3, inset). In fact, H-α-crystallin does not show a significant heat absorption peak as native α-crystallin. Accordingly, H-α-crystallin content in α-crystallin samples will reduce the denaturational enthalpy ΔH^{cal}. These findings suggest at finite heating rate a side reaction leading to a non-productive, higher associated form of α-crystallin (probably H-α-crystallin), thus reducing the effective concentration of the 'meltable' protein. The slow and irreversible side reaction leading to the non-productive folding form can be suppressed by increasing the scan rate whereas the formation of insoluble aggregates after thermal denaturation can be suppressed by the proper choice of buffer conditions and protein concentration as shown above.

4. Discussion

There is virtually no protein turnover in the eye lens. Ac-

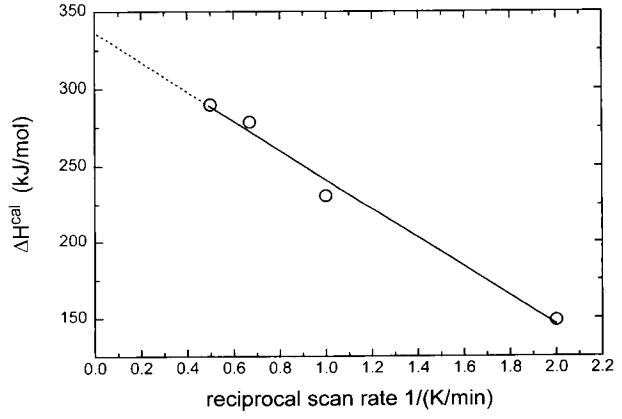

Fig. 2. Molar enthalpy change at thermal denaturation of α-crystallin versus reciprocal scan rate. The data points represent mean values of each determination. Extrapolation to infinite scan rate gives $\Delta H^{cal,\infty} = 336 \pm 9$ kJ/mol.

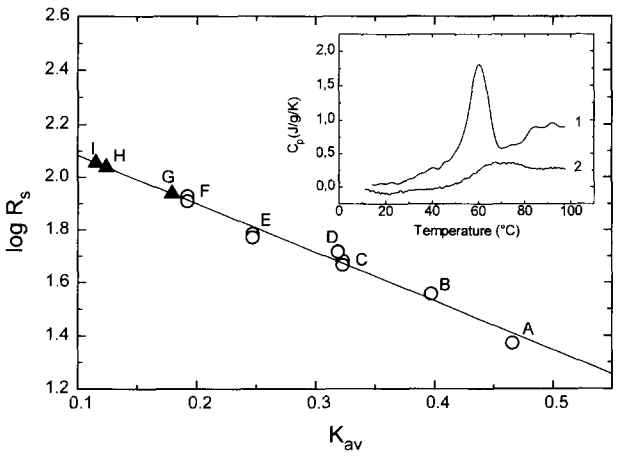

Fig. 3. Logarithm of Stokes radius (R_s) of various proteins versus K_{av} determined by size exclusion chromatography (see Section 2). (▲) Native α-crystallin (G), H-α-crystallin (H), and main fraction of thermally denatured α-crystallin after completing a calorimetric scan (I). Reference proteins: carbonic anhydrase (A), serum albumin (B), aldolase (C), catalase (D), ferretin (E), and thyroglobulin (F). (Inset) Calorimetric scans of (1) native α-crystallin and (2) H-α-crystallin measured at a scan rate of 1.5 K/min. The specific conditions for H-α-crystallin were: 20 mM potassium phosphate, pH 7.2, 1.7 mg/ml protein as determined by dry weight.

cordingly, unusual stability of crystallins was proposed. However, scanning calorimetry, which is well established for quantifying protein stability, led to a rather low transition temperature and enthalpy change [5–7].

Reconsidering recent papers on α-crystallin melting, remarkable agreement can be found with respect to the thermal transition temperature at about 60°C, and the effective heat of about 335 kJ/mol, even if determined by non-calorimetric methods [5–7]. On the other hand, ΔH^{cal} values calculated per subunit ranging from 60 to 140 kJ/mol were reported. As shown in the present paper, ΔH^{cal} (calculated per mol subunit) is scan rate dependent and amounts at infinite scan rate to $\Delta H^{cal,\infty} = 336 \pm 9$ kJ/mol, i.e. it approaches the effective heat $\Delta H^{eff} = 329 \pm 31$ kJ/mol. $\Delta H^{cal,\infty}$ will be considered in the following as the true value which is not influenced by a side reaction (see below).

From the cooperative ratio $CR = \Delta H^{cal}/\Delta H^{eff}$ which equals 1.0 at infinite scan rate, it follows that the monomeric subunit represents the minimal cooperative unit in α-crystallin melting. This finding is not trivial since α-crystallin is composed of αA and αB subunits. Therefore, it can be assumed that αA- and αB-crystallin that share 57% sequence identity [15] do not differ greatly with respect to the thermal transition.

The thermal transition of α-crystallin is accompanied by a heat capacity change as usually observed in protein unfolding [16]. In fact, the thermal transition is accompanied by massive loss of secondary structure according to a recent Fourier-transform infrared study [7]. The heat capacity change ΔC_p amounts to 5.7 ± 3.0 kJ/mol per K (mean from 9 single scans). Therefore, all data necessary to estimate the conformational stability of α-crystallin by Eq. 1 are available.

$$\Delta G(T) = \Delta H^{cal,\infty}(1 - T/T_{trs}) - \Delta C_p[(T_{trs} - T) + T \ln(T/T_{trs})] \quad (1)$$

Eq. 1 yields $\Delta G = 24 \pm 5$ kJ/mol per subunit at 25°C. Usually, conformational stability of proteins expressed by ΔG amounts to 25–65 kJ/mol [16]. Thus, the conformational stability of α-crystallin is unexpectedly low. The previous calorimetric studies [5–7] and the present findings do not support the view of extraordinary thermal stability of α-crystallin [4].

A specific feature of α-crystallin is the formation of a non-productive folding form produced during slow heating:

native α-crystallin ⇔ thermally denatured α-crystallin [→ insoluble aggregates]

↓ aggregation

[non-productive, soluble high molecular form]

The non-productive folding form seems to be similar to the well known heavy form of α-crystallin that has a similar Stokes radius and no significant thermal transition. In this manner, a proportion of the non-productive folding form will reduce the calorimetric enthalpy change ΔH^{cal}. On the other hand, the effective heat ΔH^{eff} remains unaffected since it is based on van't Hoff analysis of the calorimetric transition curve irrespective of protein concentration. Therefore, the formation of the non-productive form will increase the disagreement between ΔH^{cal} and ΔH^{eff} as is observed on lowering the scan rate.

It is worth mentioning that no unfolding-like thermal transition could be found up to 75°C in NMR experiments [17,18]. Probably the concentration and heating rate under NMR conditions favour the formation of the non-productive folding form of α-crystallin.

The chaperone properties of α-crystallin are of particular interest. In this respect, αB-crystallin is indistinguishable from small heat shock proteins (Hsp25, Hsp27, and plant sHsp) [2,11,19,20]. Therefore, the question arises if the calorimetric melting profiles provide further arguments for the similarity within this group of proteins. As shown recently [21], Hsp25 and Hsp27 show a main transition at higher temperature (about 70°C) and, additionally, characteristic transitions at 32 and 38°C, respectively. Whereas the cooperative subunit in α-crystallin is the monomer, the minimum cooperative subunit of the Hsps was found to be a dimer. Do these differences point to different spatial organization of the multi-subunit complexes? Subunits of α-crystallin consist mainly of β-structure and a flexible C-terminal extension [18,22]. Various models for subunit packing have been proposed (for a review see [3]). The present calorimetric results are not suited to rule out definitely any particular structure model. However, our results are consistent with a micelle model rather than with those models that propose layer arrangements. The layer arrangements imply thermodynamic non-equivalence of the subunits, which is not supported by the present results. The micelle model was originally proposed by Augusteyn and Koretz [23] and further supported by independent findings [17,24,25].

There is a hope that calorimetry could become a valuable tool in studying junior chaperones. As an example, the complex of Hsp27 and α-crystallin which was first described by Zantema et al. [26] shows a specific endothermal transition at 27°C which is not detectable in the isolated components (W. Pfeil and M. Gaestel, unpublished results). These observation might open a way for thermodynamic studies on interactions in such complex systems.

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