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How α-Crystallin Prevents the Aggregation of Insulin

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Abstract—Using steady-state, polarized, and phase-modulation fluorometry, the dithiothreitol-induced denaturation of insulin and formation of its complex with α-crystallin in solution were studied. Prevention of the aggregation of insulin by α-crystallin is due to formation of chaperone complexes, i.e. interaction of chains of the denatured insulin with α-crystallin. The conformational changes in α-crystallin that occur during complex formation are rather small. It is unlikely that N-termini are directly involved in the complex formation. The 8-anilino-1-naphthalenesulfonate (ANS) is not sensitive to the complex formation. ANS emits mainly from α-crystallin monomers, dimers, and tetramers, but not from oligomers or aggregates. The possibility of highly sensitive detection of aggregates by light scattering using a spectrofluorometer with crossed monochromators is demonstrated.

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Key words: α-crystallin, insulin, chaperone complex, aggregation, light scattering, fluorescence

Insulin is a small protein composed of only 51 amino acid residues and formed by two chains connected by three S–S bridges stabilizing its structure. It easily denatures and loses its physiological properties upon heating or reduction with dithiol compounds. Native insulin in aqueous solution is present mainly as a monomer. Denatured insulin forms aggregates.

Bovine α-crystallin (the main protein of the crystalline lens) is composed of two similar subunits, α and β. The protein has no disulfide bonds. The structural features, domain organization, dissociation, and oligomerization of α-crystallin subunits are described in [1, 2]. The subunits are associated in spherical complexes. In aqueous solutions, α-crystallin is preferably in the form of dimers, tetramers, and oligomers. The molecular mass of oligomers reaches 540 kD (about 26 subunits) [2]. Oligomerization of α-crystallin is not necessary for chaperone functions [1, 2]. Chaperones are known to bind to denatured proteins providing their proper folding [3], delivery into corresponding cell compartments [4], and preventing aggregation [5]. For example, the chaperone activity of α-crystallin in renaturation of citrate synthase was studied in [6].

An interesting feature of α-crystallin is its ability to interrupt thermal aggregation of various proteins. This ability is manifested not only upon heating, but also at room temperature upon dithiothreitol (DTT)-induced denaturation. In particular, the prevention of DTT-induced aggregation of insulin by α-crystallin was studied in [7]. Of the two insulin chains, α and β, formed upon reduction of S–S bonds, the β-chain is the best in binding with α-crystallin [7], and the complex formed is near equimolar [8].

Protein aggregation occurring upon denaturation can be detected by light scattering (commonly using a spectrophotometry) [7-11]. This light scattering is eliminated, when chaperone protein–protein complexes are formed. The disaggregating effect of α-crystallin on various proteins was also studied using tryptophan and 8-anilino-1-naphthalenesulfonate (ANS) fluorescence [9-11]. In particular, ANS interacting with α-crystallin was thought to be sensitive to complex formation with denatured insulin. Researchers supposed that changes in ANS fluorescence intensity resulted from conformational changes in the complex formed. However, other parameters of fluorescence were not measured.

To determine the mechanism of the prevention of insulin aggregation by α-crystallin, more exhaustive investigation of fluorescence is necessary, including determination of not only fluorescence intensity, but also polarization degree (*P*) and lifetime of excited state (τ) of both components.

The goal of this work was to study the DTT-induced denaturation of insulin and formation of its complexes

Abbreviations: ANS) 8-anilino-1-naphthalenesulfonic acid; DTT) dithiothreitol.

with α-crystallin in solution using the steady-state, polarized, and phase-modulating fluorometry of tryptophan and tyrosine protein residues and ANS. Moreover, unlike previously reported studies, in which the light scattering was determined on a spectrophotometer by optical density, the author determined light scattering in a more sensitive way—by intensity of scattered exciting light entering the registration channel with crossed monochromators of a spectrofluorometer.

MATERIALS AND METHODS

Bovine α-crystallin and insulin (Serva, Sweden; Fluka, Germany) were used in the experiments. The proteins were dissolved in 50 mM sodium phosphate (pH 8.4). Unlike study [11], in which both proteins were taken at concentration of 2 mg/ml, the initial concentration of α-crystallin in our experiments was 4 mg/ml corresponding to the subunit concentration of $2 \cdot 10^{-4}$ M (molecular weight of each α-crystallin subunit is about 20 kD), and the initial concentration of insulin was 2 mg/ml corresponding to $3.3 \cdot 10^{-4}$ M (molecular weight of insulin is about 6 kD). In most experiments 150 μl of the initial α-crystallin solution was mixed with 100 μl of the initial insulin solution, i.e. at nearly equimolar ratio ($1.2 \cdot 10^{-4}$ M α-crystallin and $1.3 \cdot 10^{-4}$ M insulin). Insulin denaturation was initialized by addition of DTT (10 μl of the initial 0.5 M solution). All control samples (150 μl of α-crystallin in 100 μl of the buffer; 100 μl of insulin in 150 μl of the buffer; and the same solutions with 10 μl of DTT) were prepared in the same way.

The tested solutions were placed at 22°C into mirror microcuvettes allowing several times increase of signal on a spectrofluorometer and the use of small solution volumes (to save protein preparations) [12]. The cells were closed by lids for prevention of evaporation. Most measurements were carried out on an SLM-4800 spectrofluorometer (SLM Inc., USA). All measurements were corrected for the light scattering of the buffer solution.

Polarization degree (P) of the emission of α-crystallin tryptophan residues and ANS (Sigma, USA) was measured in accordance with [12] using Glan–Thompson prisms in four positions with correction for the light scattering of the buffer to eliminate both the contribution of artifact polarization of the light by the monochromators and the contribution of light scattering. The final concentrations of α-crystallin and insulin used in most experiments were $1.2 \cdot 10^{-4}$ and $1.3 \cdot 10^{-4}$ M, respectively. Concentration of ANS in experiments was generally 10^{-5} M, one order lower than the protein concentration, to exclude self-association of the probe, ensure its complete binding with the proteins, and substantially reduce effects of light screening and reabsorption.

Fluorescence lifetime (τ) was determined by the phase modulation method at modulation frequencies of 30 MHz (for tryptophan residues) and 18 MHz (for ANS). Signal accumulation was 30 sec for one point. The τ value was calculated as the arithmetic mean of values measured by phase and by modulation from four independent measurements.

Effective volumes (V) of proteins were determined using the modified Levshin–Perrin equation [12], proceeding from τ and P of the ANS probe:

$$V = 8498\tau/(1/P - 2.381). \quad (1)$$

This equation is reasonably accurate for room temperatures and aqueous solutions. Herein τ is expressed in nanoseconds and V in cubic angstroms.

Intense light scattering was determined by the absorption value in transmitted light at 310 nm (neither insulin nor α-crystallin absorb at this wavelength) in a standard compartment of an M-40 spectrophotometer in 0.5-cm quartz cells.

Weak light scattering was determined by intensity of the scattered light registered by an SLM-4800 photomultiplier tube at right angle to the exciting light; in this case, both monochromators (before and after the microcuvette) were set to the same wavelength (310 nm; slit width was 1 nm).

RESULTS AND DISCUSSION

Light scattering. Light scattering in the UV region is known to be significantly higher than in the visible. Tyrosines and tryptophans absorb virtually no light at 310 nm. So, the light scattering by protein aggregates can be conveniently observed at this wavelength. Highly sensitive detection of the aggregates can be carried out on a spectrofluorometer (with narrow slits of both monochromators crossed at 310 nm). Exciting light being scattered from the aggregates gets through into the detection channel. This gives large signal (Fig. 1).

Solutions of native insulin are absolutely transparent even at high concentration (more than 10^{-4} M), and their optical density is very low at 310 nm. Nonetheless, a direct highly sensitive measurement of light scattering on a spectrofluorometer indicates that a small portion of molecules is in an aggregated state, because the light scattering level is not zero (Fig. 1), and this level depends on the insulin concentration. When DTT is added to the insulin solution, highly intense scattering develops (Fig. 1), which becomes apparent to the naked eye. DTT reduces disulfide bonds resulting in dissociation of the molecule into two separate chains, α and β. As this takes place, insulin degrades into a denatured state, in which some of the hydrophobic protein groups are on the surface. As a result, the solubility in water drastically decreases, and individual chains stick together to form large aggregates, which leads [7] to high scattering.

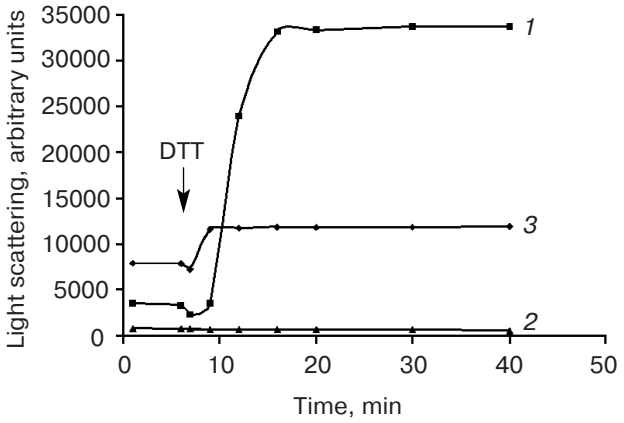

Fig. 1. Changes in the light scattering of insulin (*1*), α-crystallin (*2*), and their mixture (*3*) upon addition of DTT. The light scattering was excited and detected on an SLM-4800 spectrofluorometer with crossed monochromators at the same wavelength of 310 nm. Concentrations: insulin, $1.3 \cdot 10^{-4}$ M; α-crystallin, $1.2 \cdot 10^{-4}$ M.

DTT added to α-crystallin has no effect on its light scattering (Fig. 1). This means that DTT under the conditions of the control experiment does not cause denaturation and aggregation of α-crystallin. Low level of light scattering in the case of α-crystallin signifies that this protein does not form large aggregates at concentrations about 10^{-4} M and lower.

Addition of α-crystallin to insulin undergoing DTT-induced denaturation leads to suppression of the light scattering of insulin (Fig. 1). This means that α-crystallin taken at near equimolar (per subunit) ratio to insulin effectively prevents the aggregation of insulin. However, the light scattering is still slightly increases after DTT addition. This can be due to both incomplete prevention of insulin aggregation and formation of very large crystallin–insulin complexes. Indeed, in excess of α-crystallin in relation to insulin, the binding achieves almost 100%, but light scattering does not vanish (not shown).

It is worth noting that the light scattering level of the mixture of the two proteins without DTT, when measured in the highly sensitive way on the spectrofluorometer with crossed monochromators (customary spectrophotometry fails in detection), was somewhat higher than the sum of light scattering levels of these proteins taken separately (Fig. 1). This means that mixing of two native proteins can lead to formation of large, even if small in number, aggregation complexes. They seem to be formed due to nonspecific hydrophobic interactions between some molecules.

Tryptophan fluorescence of α-crystallin. The α-crystallin molecule contains three tryptophans (one in the first subunit and two in the second). Although all three tryptophans are localized at the *N*-termini, they are faced to the inner space of the protein globule rather than to water. Their emission spectrum has a maximum near 338 nm [13]. The tryptophan fluorescence of α-crystallin is very little affected by addition of insulin or DTT. This concerns its intensity, degree of polarization, *P*, and lifetime, τ (table). When insulin and DTT were added together followed by incubation for 40 min, the intensity decreased by only 12%, in comparison to that of the mixture of proteins without DTT. Both *P* and τ also varied only slightly. This suggests that the α-crystallin conformation is little affected upon crystallin–insulin complex formation, and the *N*-termini of its subunits are not likely to be directly involved in the complex formation. Moreover, the polarization degree of tryptophan fluorescence did not increase. According to the Eq. (1), this means that the binding of insulin with α-crystallin does not lead to marked increase of the particle volume, that is, the situation does not appear when one molecule of α-

Intensity, degree of polarization, and lifetime of fluorescence of tryptophan residues of α-crystallin and ANS in the presence and absence of insulin as well as before and after 40-min incubation with DTT

In solution	F_{trp} (%)	P_{trp}	τ_{trp} (nsec)	F_{ANS} (%)	P_{ANS}	τ_{ANS} (nsec)
α-Crystallin	100	0.284	3.9	100	0.36	15.1
α-Crystallin + DTT	92	0.290	4.4	122	0.35	16.9
α-Crystallin + insulin	97	0.283	3.9	86	0.35	16.9
α-Crystallin + insulin + DTT	88	0.281	4.3	80	0.36	17.0
The same after 40 min	85	0.279	4.6	95	0.34	17.0
Insulin	–	–	–	3	0.32	12.7
Insulin + DTT	–	–	–	2	0.34	12.6
The same after 40 min	–	–	–	18	0.34	12.5

Note: Excitation of tryptophans, at 300 nm; emission, at 338 nm. Excitation of ANS, at 370 nm; emission, at 465 nm. Accuracy of measurement of phase τ of α-crystallin tryptophans was ±0.1 nsec and that of phase modulation τ of ANS was ±0.4 nsec. Accuracy of measurement of *P* of α-crystallin tryptophans was ±0.002 and that of ANS was ±0.01.

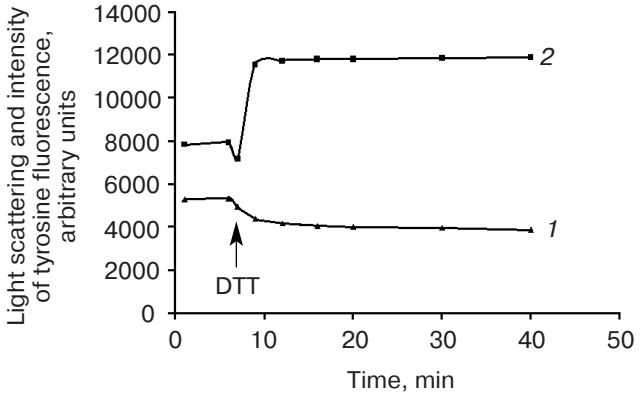

Fig. 2. Changes in tyrosine fluorescence (*1*) and light scattering (*2*) of insulin in the process of its DTT-induced aggregation. Tyrosine fluorescence was excited at 275 nm and detected at 310 nm. Light scattering was measured on an SLM-4800 spectrofluorometer with crossed monochromators at 310 nm. Insulin concentration was 10^{-5} M.

crystallin binds many molecules of insulin. Minor decrease of *P* after 40-min incubation is apparently caused by increase of τ. Since tryptophans of α-crystallin are localized at the flexible *N*-end, they possess some rotary mobility [12, 13], which partially depolarizes their radiation, thus impeding application of the equation for exact calculation of particle sizes.

Tyrosine fluorescence of insulin. Insulin does not contain tryptophans, but it has four tyrosines. Tyrosine fluorescence of insulin substantially decreases with its denaturation and aggregation (Fig. 2). This is due to two causes: strong conformational changes and aggregation-caused light scattering. As one can see in Fig. 2, a decrease of tyrosine fluorescence corresponds to drastic increase of the scattering. Observation of complex formation between α-crystallin and insulin by tyrosine fluorescence of the latter is hampered because of the obstructive effect of α-crystallin tyrosines (each subunit of α-crystallin contains two tyrosines).

Fluorescence of ANS in insulin aggregates. The ANS probe binds very little with native insulin. So, the probe fluorescence is very weak. Incubation of insulin for 40 min in the presence of DTT led to the protein denaturation and sixfold increase in intensity of the ANS fluorescence (table). However, the absolute value of the intensity remained low. The observed sixfold increase of the intensity results from high increase of the probe binding by insulin aggregates; some contribution from elongation of the optical path of exciting light in a scattering medium may also occur (similar increase in intensity was reported elsewhere [7]). As this takes place, both lifetime and polarization degree of ANS remain virtually unchanged: τ = 12.5-12.7 nsec and *P* = 0.32-0.34. Note that free ANS molecules in water have τ = 3.8 nsec and *P* < 0.06 [12]. The calculation by the Levshin–Perrin equation (1) has shown that the mean effective volume of insulin aggregate in presence of ANS is about 164,000 Å^3, that is, its diameter is 69 Å. It should be stressed that we are dealing with precisely those insulin aggregates ANS binds with and from which depth it emits rather than with all aggregates (ANS hardly penetrates into large aggregates).

Fluorescence of ANS in α-crystallin. ANS binds with α-crystallin not instantly, but rather slowly [13], for about 10 min under the conditions of the experiments. The probe gradually penetrates into the protein, between subunits (the quickest penetration occurs into dimers and tetramers, not into oligomers), which is accompanied by many-fold intensification of the probe fluorescence and shift of the emission spectrum to shorter wavelengths [12, 13]. The ANS lifetime on binding with α-crystallin is 15.1 nsec. This value is slightly greater than that in the case of insulin. The intensity change is much greater, i.e., it increases 33-fold (table). This is due to complete transition of the probe from the polar aqueous phase into the hydrophobic areas of the complex. The polarization degree of ANS in α-crystallin (*P* = 0.36) is slightly higher than in insulin aggregates. The *P* value for the bound ANS mainly depends on rotation of the probe together with the aggregate; to a first approximation, self-rotation of the probe is negligible [12]. The mean volume of α-crystallin molecules calculated by the Levshin–Perrin equation with parameters of ANS radiation is 379,000 Å^3, diameter 90 Å [13], that is, ANS emits preferentially from dimers and tetramers rather than from large oligomers.

Fluorescence of ANS in the presence of α-crystallin upon denaturation of insulin. Incubation of α-crystallin with insulin in the presence of DTT is accompanied by noticeable change in intensity of ANS fluorescence (Fig. 3). Addition of DTT to α-crystallin alone (in the absence of insulin) results in first increase in intensity of the probe fluorescence followed by its decrease to a level below the

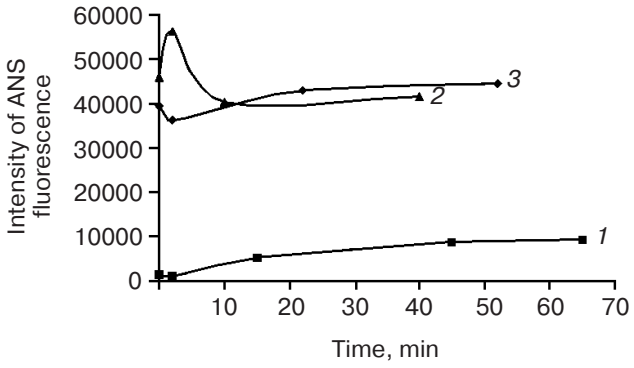

Fig. 3. Change in intensity of ANS fluorescence in solutions of insulin (*1*), α-crystallin (*2*), and their mixture (*3*) immediately after addition of DTT. The fluorescence was excited at 370 nm and recorded at 465 nm. Concentrations: ANS, 10^{-5} M; insulin, $1.3 \cdot 10^{-4}$ M; α-crystallin, $1.2 \cdot 10^{-4}$ M.

initial one. This seems to reflect kinetics of conformational changes of protein subunits. Since both kinetics and magnitude of the increase in ANS fluorescence intensity in the mixture of two proteins in response to addition of DTT are close to those in the absence of α-crystallin (DTT added to insulin with ANS), one can suppose that the main cause of gradual increase in the intensity is enhancement of ANS binding in insulin aggregates. If this is the case, no appreciable response of the probe to the crystallin–insulin complexes occurs, that is, ANS does not "feel" the complex formation. This is also evident from the very small changes of P and τ values of ANS (table) after incubation of the protein mixture with DTT. Calculation by the Levshin–Perrin equation show that the volumes of all protein particles (α-crystallin, insulin, and their mixture) determined from the ANS parameters did not increase after incubation with DTT.

The binding of ANS with α-crystallin was studied in detail in work [14]. The authors demonstrated that even in a 24-subunit oligomer only one binding site is present (with the dissociation constant of $9 \cdot 10^6$ M^{-1}). The binding of the probe occurs in a hydrophobic area localized near the surface. It is not very sensitive to the number of subunits, that is, ANS responds little to the interactions between subunits. No difference was found in binding of the probe to α and β subunits of this protein. The increase in ANS fluorescence intensity in the mixture of α-crystallin and insulin denatured by DTT was found to be caused by the enhancement of binding of the probe to insulin (primarily to its β-chain), but not to α-crystallin [7]. These data are in agreement with the data reported here. The intensity of the probe fluorescence in the mixture of α-crystallin and native insulin was equal to the sum of the intensities from the two individual proteins [7], so the authors concluded that the proteins did not form a complex. It is of interest that the overall intensity in the mixture with denatured insulin was almost the same as in the case of the individual proteins, which is confirmed by the data of the present study.

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