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Study of α-Crystallin Structure by Small Angle Neutron Scattering with Contrast Variation

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Abstract—The structure of the oligomeric protein α-crystallin from bovine eye lens was investigated by small-angle neutron scattering (SANS) with contrast variation. Based on the SANS curves, the match point for α-crystallin (43% D_2O) and its average scattering length density at this point ($2.4 \cdot 10^{10}$ cm^{-2}) were evaluated. The radius of gyration and the distance distribution functions for α-crystallin were calculated. On the basis of these calculations, it was concluded that α-crystallin is characterized by homogeneous distribution of scattering density in the domains inaccessible for water penetration, and all polypeptide subunits in α-crystallin oligomers undergo equal deuteration. The latter indicates that all α-crystallin subunits are equally accessible for water and presumably for some other low molecular weight substances. These conclusions on the α-crystallin structure (homogeneous distribution of scattering density and equal accessibility of all subunits for low molecular weight substances) should be taken into account when elaborating α-crystallin quaternary structure models.

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Key words: α-crystallin, quaternary structure, small-angle neutron scattering, contrast variation

α-Crystallin is one of the main structural and functional proteins of the crystalline lens of vertebrates [1]. It is an oligomer polydisperse protein consisting of noncovalently bound polypeptide subunits of two types, αA and αB, with molecular weight of about 20 kDa each. The mean molecular weight of native α-crystallin of mammals is about 700-800 kDa according to the data of different researchers [1-3].

α-Crystallin can prevent aggregation of proteins with destabilized structure and form complexes with them (chaperone-like activity of α-crystallin) [1, 4-6]. Protein aggregation in the lens may be one of the reasons of its opacity (cataract). It is considered that the chaperone-like activity of α-crystallin promotes the maintenance of lens transparence through the whole life [1].

The spatial structure of α-crystallin oligomers and subunits is unknown. Computer modeling of the tertiary structure of α-crystallin subunits was performed [7], and a few different models of its quaternary structure were proposed [2, 7, 8].

Abbreviations: SANS, small-angle neutron scattering.

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The methods of small-angle X-ray and neutron scattering make it possible to study the structure of biological macromolecules in solutions [9, 10]. A great advantage of the method of small-angle neutron scattering is the possibility of changing the difference between the scattering densities of macromolecules and solvent (contrast) in a wide range by varying the content of usual (light) water (H_2O) and heavy water (D_2O) in the solvent. It provides valuable information about the peculiarities of internal structure of macromolecules.

However, in the presence of D_2O many of the hydrogen atoms in the regions easily accessible for the solvent are replaced by deuterium, and such deuteration may lead to a change in the structure of macromolecules. This is a serious impediment to contrast variation [10]. Therefore, for correct application of the method of contrast variation, it is necessary to analyze the effect of deuteration on the structure of the macromolecules under study.

Such analysis can be performed by the method of small-angle X-ray scattering. The hydrogen and deuterium scattering lengths for X-radiation are the same. Therefore, substitution of D_2O for H_2O in the buffer when using X-radiation does not result in contrast variation, and

comparison of the patterns of small-angle X-ray scattering for macromolecules in the buffer prepared with light water (H_2O buffer) and in the D_2O-containing buffer allows one to judge about the effect of deuteration on the structure of the studied macromolecules. In the case of similar patterns of small-angle X-ray scattering for macromolecules in the H_2O buffer and in the D_2O-containing buffer, it can be considered correct to use the method of small-angle neutron scattering with contrast variation in the study of the structure of such macromolecules.

In the present work, first the influence of deuteration on α-crystallin structure was analyzed by small-angle X-ray scattering, and then the quaternary structure of this protein was studied in solution by small-angle neutron scattering with contrast variation.

MATERIALS AND METHODS

α-Crystallin was isolated from bovine eye lens using gel-permeation chromatography and concentrated by ultrafiltration as in [3]. The buffer solution (buffer) contained: Na_2HPO_4, 20 mM; NaH_2PO_4, 20 mM; NaCl, 100 mM; NaN_3, 3 mM; EDTA, 1 mM; pH 6.8. α-Crystallin concentration in solutions was determined using spectrophotometry by the absorption at 280 nm (A_{280}) with the specific absorption coefficient $A_{280}(1\%, 1\ cm)$ equal to 8.2, which was obtained from measurement of absorption A_{280} of the solutions of lyophilized α-crystallin.

For the study of α-crystallin by small-angle neutron scattering, α-crystallin solutions (8.8 mg/ml) were prepared in the buffer containing a mixture of H_2O and 0, 23, 68, and 90% D_2O (vol. %). The background scattering for each α-crystallin solution was measured using the respective buffer solutions with the same content of D_2O. Small-angle neutron scattering was measured with a YuMO spectrometer (IBR-2, Joint Institute for Nuclear Research) in a two-detector variant [11] at 20°C within 12 h after preparing the deuterated solutions of the protein. Experimental spectra were converted into the dependence of coherent differential scattering cross-section on the scattering vector magnitude, taking into account sample transmission and the incoherent component of scattering. Experimental spectra were normalized using a vanadium scatterer by the standard procedure with SAS software [12]. The measurements of small-angle X-ray scattering by α-crystallin and the primary processing of results were carried out at the Institute of Biochemical Physics, Russian Academy of Sciences, as described in [5] (CuK_α radiation, $\lambda = 0.154$ nm).

Further, the small-angle neutron and X-ray scattering data were processed by GNOM software using indirect Fourier transform and regularization of the solution [13, 14]. As a result of such processing, a distance distribution function $P(r)$ was obtained for each α-crystallin sample; the mean radius of gyration R_g and the zero angle scattering intensity $I(0)$ were calculated on the basis of this function:

$$R_g^2 = \int_0^D P(r) r^2 dr \Big/ \int_0^D P(r) dr,$$

$$I(0) = 4\pi \int_0^D P(r) dr,$$

where D was the upper limit of domain of function $P(r)$ (parameter r_{max} in the GNOM software).

Thereby obtained $P(r)$, R_g, and $I(0)$ values are integral characteristics of polydisperse α-crystallin solution and associated with the structural parameters of individual α-crystallin oligomers by the ratios:

$$R_g^2 = \frac{1}{I(0)} \sum_{i=1}^{N} (R_g)_i^2 (\overline{\rho}_i - \rho_s)^2 V_i^2, \quad (1)$$

$$I(0) = \sum_{i=1}^{N} (\overline{\rho}_i - \rho_s)^2 V_i^2, \quad (2)$$

$$P(r) = \sum_{i=1}^{N} P_i(r). \quad (3)$$

Here, $(R_g)_i$, $\overline{\rho}_i$, V_i, and $P_i(r)$ are the radius of gyration, the mean scattering length density (mean scattering density), partial volume, and distance distribution function for a single oligomer i of α-crystallin. N is the number of such oligomers in the scattering volume. $\overline{\rho}_s$ is the mean scattering density of solvent (buffer). The values of $(R_g)_i$, $\overline{\rho}_i$, and $P_i(r)$ are expressed in terms of scattering density distribution in oligomer i of α-crystallin $\rho_i(\vec{r})$ as:

$$(R_g)_i^2 = \int_{V_i} (\rho_i(\vec{r}) - \rho_s) r^2 d\vec{r} \Big/ \int_{V_i} (\rho_i(\vec{r}) - \rho_s) d\vec{r},$$

$$\overline{\rho}_i = \frac{1}{V_i} \int_{V_i} \rho_i(\vec{r}) d\vec{r},$$

$$P_i(r) = r^2 \left\langle \int_{V_i} (\rho_i(\vec{r}') - \rho_s)(\rho_i(\vec{r}' - \vec{r}) - \rho_s) d\vec{r}' \right\rangle.$$

Here symbol $\langle\ \rangle$ means the averaging by all orientations of vector $\vec{r}$; the origin of coordinates in the expression for $(R_g)_i$ is selected in the center of masses of oligomer i.

RESULTS AND DISCUSSION

Analysis of effect of deuteration on α-crystallin structure by small-angle X-ray scattering. The influence of deuteration on α-crystallin structure was analyzed by

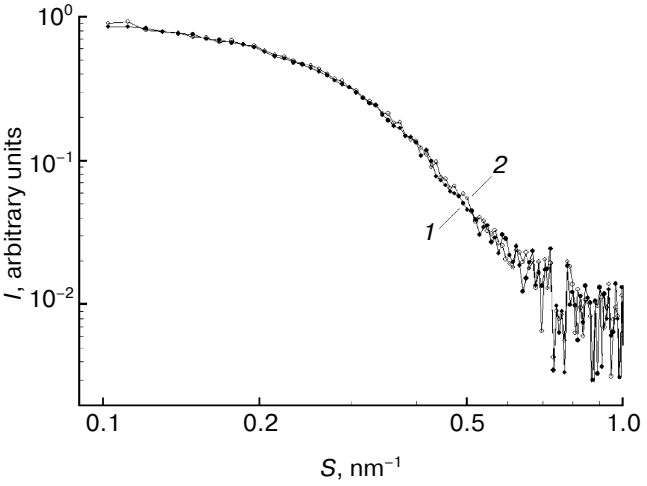

Fig. 1. Experimental curves of small-angle X-ray scattering for α-crystallin in H_2O buffer (*1*) and in 90% D_2O buffer (*2*). Protein concentration is 8.8 mg/ml.

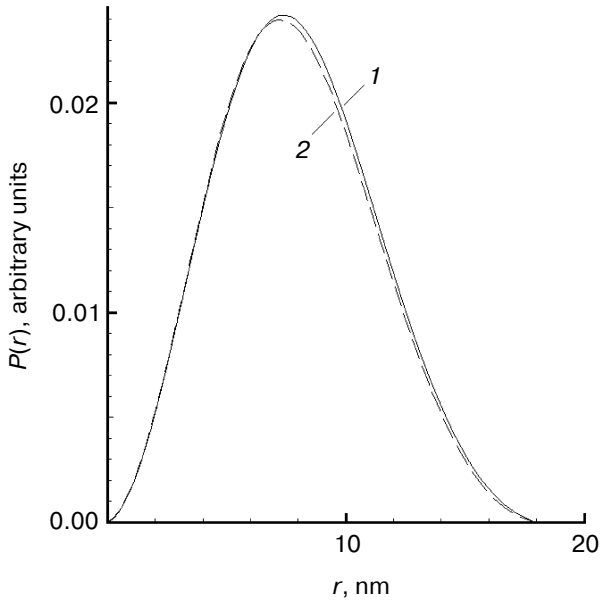

Fig. 2. Distance distributions functions $P(r)$ for α-crystallin in H_2O buffer (*1*) and in 90% D_2O buffer (*2*) calculated by the curves of small-angle X-ray scattering presented in Fig. 1.

comparative study using the small-angle X-ray scattering of α-crystallin structure in H_2O buffer and in the buffer with 90% D_2O. Small-angle X-ray scattering was measured in two α-crystallin preparations obtained as a result of two different isolations of this protein and in different periods of time (1, 3, 10, and 30 days) after preparing deuterated α-crystallin solutions. The results of α-crystallin measurements in deuterated buffer within the accuracy of experiment did not differ from the results of measurement of α-crystallin samples in H_2O buffer.

As an example, Fig. 1 shows the experimental curves of small-angle X-ray scattering for α-crystallin in H_2O buffer and in the buffer with 90% D_2O measured in 3 days after deuteration of the solutions ($S = (4\pi\sin\theta)/\lambda$ is scattering vector magnitude, 2θ is scattering angle, λ is radiation wavelength). Figure 2 shows distance distribution functions $P(r)$ calculated by these curves.

As one can see from Figs. 1 and 2, the curves of small-angle scattering and $P(r)$ functions for α-crystallin in the H_2O buffer and in the 90% D_2O buffer are practically identical. The maximum size of α-crystallin oligomers, as judged by the r value when the $P(r)$ functions go to zero, is about 18 nm (Fig. 2). For the radius of gyration of α-crystallin in these samples, the values were 6.07 ± 0.03 nm for the H_2O buffer and 6.01 ± 0.03 nm for the 90% D_2O buffer. The differences between these R_g values are within the accuracy of the experiment.

Thus, deuteration of the solution has no considerable influence on α-crystallin structure (of course as concerns the structural level to which the methods of small-angle scattering are susceptible, i.e. especially quaternary structure). Consequently, contrast variation by means of D_2O can be used for the study of α-crystallin structure by the method of small-angle neutron scattering.

Study of α-crystallin structure by small-angle neutron scattering with contrast variation. The experimental curves of small-angle neutron scattering for α-crystallin in the buffer with different contents of D_2O are shown in Fig. 3.

The values of the radius of gyration R_g and the zero angle scattering intensity $I(0)$ calculated by these curves and the errors of their determination are given in the table. The distance distribution functions $P(r)$ corresponding to the R_g and $I(0)$ values given in the table are shown in Fig. 4. All these $P(r)$ functions and the R_g and $I(0)$ values pre-

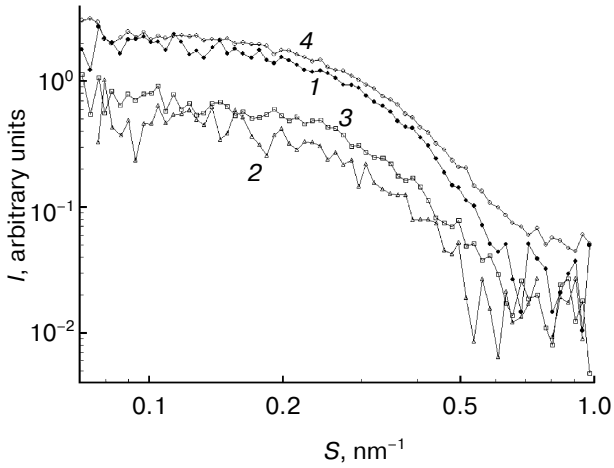

Fig. 3. Experimental curves of small-angle neutron scattering for α-crystallin in the buffer solutions with D_2O volume fractions of 0, 23, 68, and 90% (*1-4*, respectively). α-Crystallin concentration in the solutions is 8.8 mg/ml.

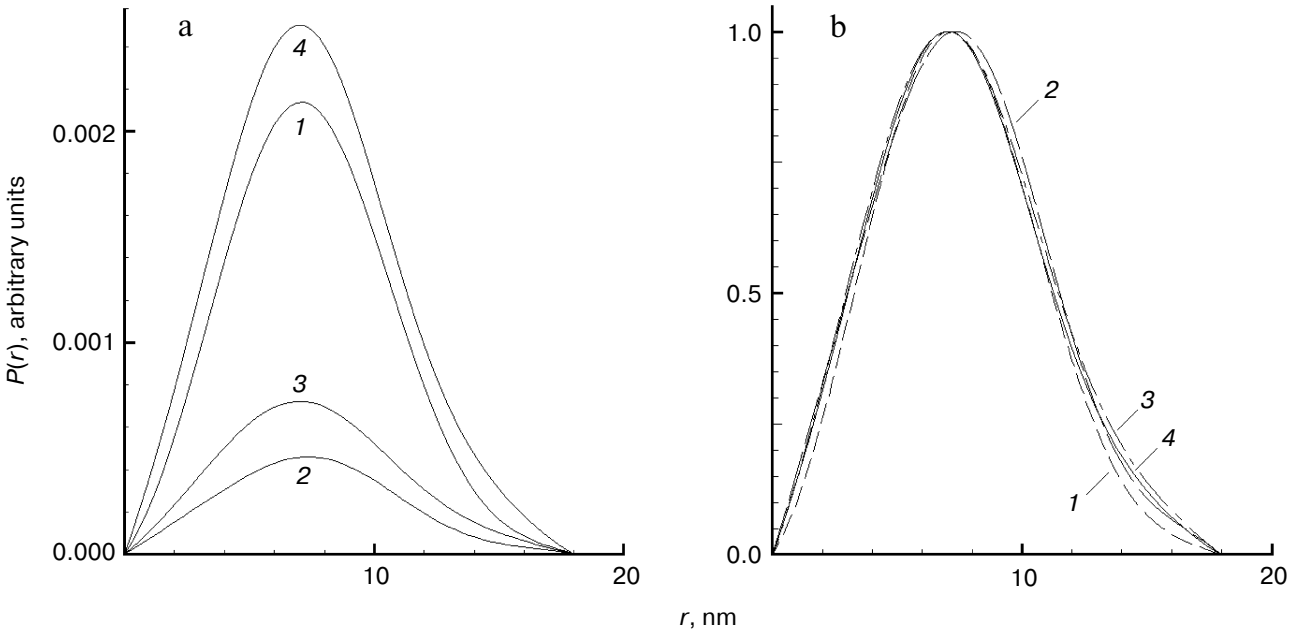

Fig. 4. Distance distribution functions $P(r)$ for α-crystallin in buffer solutions with D_2O volume fractions of 0, 23, 68, and 90% (*1-4*, respectively). a) Results of calculation from experimental curves of small-angle neutron scattering; b) the same functions after normalization by the maximum values.

sented in the table have been calculated using the GNOM software for the interval of $S = 0.077\text{-}0.975$ nm^{-1}, at $r_{max} = 18$ nm, condition $P(r_{max}) = 0$, and regularization parameter ALPHA = 1.

As is obvious (see table), the zero angle scattering intensity $I(0)$ for α-crystallin significantly depends on D_2O content in the solution. Let us consider this question in more detail, taking into account the deuteration of proteins. Let us assume, as in [10], that proteins are deuterated in proportion to the D_2O volume fraction in the solution, which will be denoted as Y. Then, for each protein particle i (in our case, for α-crystallin oligomer i)

$$\overline{\rho_i} = a_i Y + b_i. \quad (4)$$

Values of radius of gyration R_g and zero angle scattering intensity $I(0)$ for α-crystallin in buffer solutions with different volume fractions of D_2O

D_2O volume fraction, %	R_g, nm	$I(0)$, arbitrary units
0	5.74 ± 0.13	2.20 ± 0.06
23	5.85 ± 0.23	0.50 ± 0.03
68	5.90 ± 0.14	0.80 ± 0.03
90	5.83 ± 0.08	2.70 ± 0.05

Let us take into account that for the solvent containing H_2O and D_2O mixture

$$\rho_s = a_s Y + b_s. \quad (5)$$

In Eqs. (4) and (5) a_i, b_i, a_s, and b_s are constants. Substituting (4) and (5) into (2), we will obtain a quadratic dependence $I(0)$ on Y:

$$I(0) = Y^2 \sum_{i=1}^{N} (a_i - a_s)^2 V_i^2 +$$

$$+ 2Y \sum_{i=1}^{N} (a_i - a_s)(b_i - b_s) V_i^2 + \sum_{i=1}^{N} (b_i - b_s)^2 V_i^2. \quad (6)$$

If the mean scattering density $\overline{\rho_i}$ is same for all protein particles, being equal to

$$\overline{\rho} = aY + b, \quad (7)$$

then the expression (6) is simplified:

$$I(0) = ((a - a_s)Y + (b - b_s))^2 \sum_{i=1}^{N} V_i^2. \quad (8)$$

In this case, there exists a match point $Y = (b_s - b)/(a - a_s)$, where $I(0)$ takes on a value equal to zero. If the mean scattering density of protein particles is different, there will be no true match point with $I(0) = 0$. In this case, one

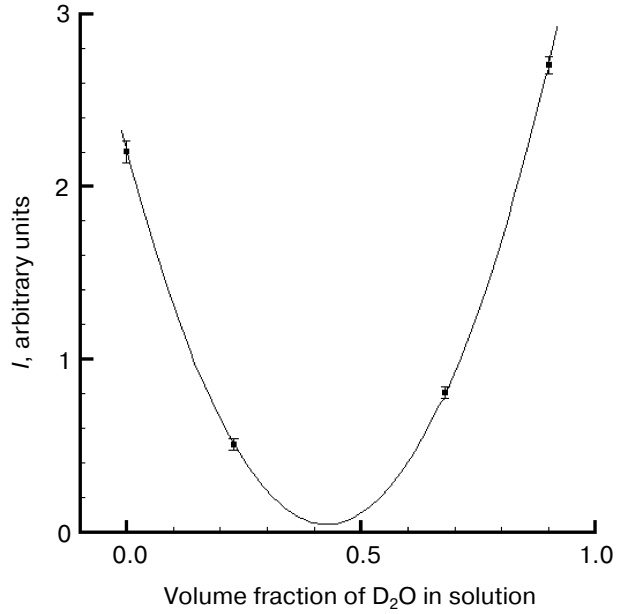

Fig. 5. Experimental dependence of zero angle neutron scattering intensity for α-crystallin $I(0)$ on volume fraction of D_2O in buffer solution and its approximation by a quadratic function.

may speak about some conditional (effective [15]) match point, where $I(0)$ takes on the minimal value.

Experimental values $I(0)$ obtained for α-crystallin at different D_2O volume fractions in solution (see table) are well approximated by the quadratic function $AY^2 + BY + C$, where A, B, and C are constants (Fig. 5). This confirms the assumption that α-crystallin is deuterated in proportion to the D_2O volume fraction in solution.

The parabola in Fig. 5 takes on a minimal value close to zero with the D_2O volume fraction in solution $Y = 0.427$. Slight excess of the zero level in the minimum point of this function may be due both to experimental errors and to minor differences in the mean scattering density of individual α-crystallin oligomers (further studies are needed to elucidate this question).

Thus, the match point (true if $I(0) = 0$ in this point, or effective if $I(0)$ in this point takes on a minimal value not equal to zero) must be observed for α-crystallin at approximately 43% D_2O in solution. The mean scattering density for α-crystallin in this point is $\overline{\rho} = \rho_s = 2.4 \cdot 10^{10}$ cm^{-2}. Here, ρ_s is calculated using the ratio (5), which, taking into account the scattering density values for D_2O and H_2O ($6.34 \cdot 10^{10}$ and $-0.56 \cdot 10^{10}$ cm^{-2} [9]), can be written as:

$$\rho_S = (6.9Y - 0.56) \cdot 10^{10} \text{ cm}^{-2}. \quad (9)$$

It should be noted that the changes in interval S, value r_{max}, and regularization parameter ALPHA within due limits during $I(0)$ calculation in the GNOM program do not result in variation of the match point by more than ±0.5%. Therefore, the match point and $\overline{\rho}$ values obtained for α-crystallin are quite reliable. They are close to the values obtained for other proteins [10].

Using the $\overline{\rho}$ value in the match point obtained for α-crystallin and the $\overline{\rho}$ value for nondeuterated proteins ($1.9 \cdot 10^{10}$ cm^{-2} [9]), we will obtain, instead of Eq. (7), a numerical dependence of α-crystallin mean scattering density on the D_2O volume fraction in solution

$$\overline{\rho} = (1.16Y + 1.9) \cdot 10^{10} \text{ cm}^{-2} \quad (10)$$

and will find the mean contrast

$$\Delta\rho = \overline{\rho} - \rho_S = (-5.74Y + 2.46) \cdot 10^{10} \text{ cm}^{-2}. \quad (11)$$

Now let us consider the conclusions that can be drawn from the values of radius of gyration R_g obtained for α-crystallin in solutions with different D_2O content (see table). The R_g values for α-crystallin at varying D_2O content in solution differ insignificantly, and these differences are within the accuracy of experiment (see table). Hence it follows that, even if the α-crystallin structure possesses some density inhomogeneities, they are low.

Let us quantitatively assess possible α-crystallin density inhomogeneities based on the obtained R_g values (see table). We will assume that the mean scattering density of α-crystallin oligomers $\overline{\rho}$, though varying due to deuteration, is the same for all oligomers at each H_2O/D_2O ratio in solution. This assumption follows from the fact that the scattering intensity in the expected compensation point is close to zero (Fig. 5). Let us use the ratio [9, 16]:

$$R_g^2 = R_c^2 + \frac{\overline{\rho}}{\Delta\rho}\left(R_p^2 + L^2 - R_c^2\right) - \frac{\overline{\rho}^2}{\Delta\rho^2}L^2. \quad (12)$$

Here, R_p is the radius of gyration of the region of a particle (α-crystallin oligomer) inaccessible for the solvent, R_c is the radius of gyration of the form of this region (the radius of gyration that this region would have under homogeneous distribution of scattering density in the latter), L is the distance between the centers of masses of these regions, $\overline{\rho}$ is the mean density of particle scattering length, and $\Delta\rho$ is a contrast. For particles with homogenous distribution of scattering density, $R_c = R_p$. In the presence of nonhomogeneities in scattering density distribution in a particle, $R_c \neq R_p$.

Ratio (12) can be applied also to polydisperse systems including α-crystallin. In this case, R_c, R_p, and L in Eq. (12) are the mean (effective) values, which are connected with the corresponding parameters of separate particles $(R_p)_i$, $(R_c)_i$, and L_i with the following ratios:

$$R_c^2 = \sum_{i=1}^{N} (R_c)_i^2 V_i^2 / \sum_{i=1}^{N} V_i^2, \quad (13)$$

$$R_p^2 = \sum_{i=1}^{N} (R_p)_i^2 V_i^2 / \sum_{i=1}^{N} V_i^2, \quad (14)$$

$$L^2 = \sum_{i=1}^{N} L_i^2 V_i^2 / \sum_{i=1}^{N} V_i^2, \quad (15)$$

where V_i is the volume of a single particle and N is the number of particles in the sample scattering volume.

As one can see from Eq. (12), the dependence of R_g^2 on $\overline{\rho}/\Delta\rho$ is generally expressed by a quadratic function.

Experimental values of R_g^2 (see table) as a function of $\overline{\rho}/\Delta\rho$ and their approximation to formula (12) are presented in Fig. 6 (R_c, R_p, and L are approximation parameters). The values $\overline{\rho}$ and $\Delta\rho$ were calculated by formulas (10) and (11). As a result of such approximation, the values $L = 0$, $R_c = 5.81 \pm 0.07$ nm, and $R_p = 5.77 \pm 0.08$ nm were obtained. Thus, the values R_c and R_p coincide within the accuracy of their determination. The maximum possible difference in the values R_c and R_p with regard to the above errors does not exceed 0.2 nm, which is about 3% of the radius of gyration of α-crystallin oligomer.

Comparison of the distance distribution functions $P(r)$ obtained at different D_2O content in solution leads to the same conclusions about the homogeneity of α-crystallin structure (Fig. 4). The shapes of these distributions are very much alike, which can be well seen after their normalization by the maximum value (Fig. 4b). This fact allows us to speak about the identical scattering density distribution in α-crystallin oligomers at different contrasts, which is possible only in the case of homogeneous density distribution. For $P(r)$ functions calculated from the small-angle neutron scattering data (Fig. 4), like in the case of small-angle X-ray scattering (Fig. 2), the maximum at $R \approx 7$ nm and a smooth decline to zero at $R \approx 18$ nm are observed. Such type of the $P(r)$ functions is typical of the particles with low degree of anisometry.

Thus, the results of analysis of the values of radius of gyration and distance distribution functions $P(r)$ for α-crystallin at different H_2O/D_2O ratios in solution show that α-crystallin is characterized by homogeneous distribution of scattering density in the regions inaccessible to a solvent (water) and is uniformly deuterated throughout the oligomer.

Of course, the homogeneity of scattering density distribution and the uniformity of deuteration here should be interpreted with regard to resolution of the method of small-angle scattering (1-2 nm). From the results it does not follow that deuteration of each individual α-crystallin subunit must be also uniform all over. It seems that mainly the surface atomic groups that are easily accessible for water are deuterated in each subunit. However, in the case of identical deuteration of all subunits, deuteration on average throughout the α-crystallin oligomer consisting of ~40 such subunits may look uniform, taking into account the resolution of small-angle scattering.

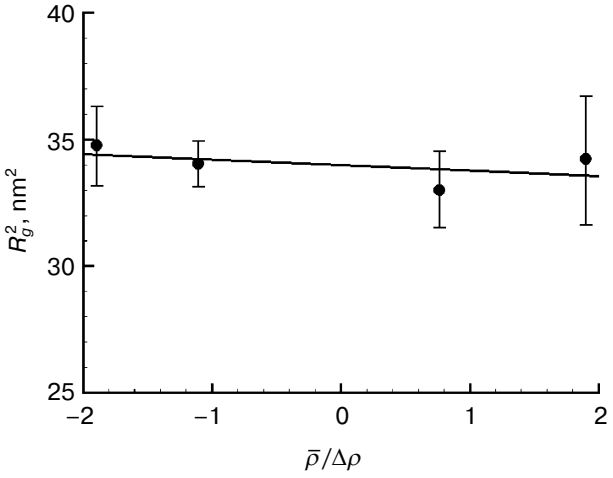

Fig. 6. Experimental dependence of R_g^2 on $\overline{\rho}/\Delta\rho$ obtained by small-angle neutron scattering for α-crystallin in the buffer solutions with different D_2O volume fractions and its approximation by formula (12).

Identical deuteration of all subunits in α-crystallin oligomer may lead to a conclusion that all of these subunits are equally accessible for water molecules and, probably, for other low molecular weight compounds, including biologically active substances. From this standpoint, the model of α-crystallin quaternary structure with three-layered location of subunits [2] seems to be less probable than the models where all subunits have equivalent positions [7, 8].

It should be noted that the regions of α-crystallin oligomer inaccessible for water penetration seem to be, first of all, polypeptide subunits themselves (their internal part). Besides, one may imagine some intersubunit regions in α-crystallin oligomer (e.g. a closed internal cavity) that may also be inaccessible for water penetration. The results obtained in this work show that the scattering density distribution is homogenous in the regions of α-crystallin oligomer where the water does not penetrate. Hence it follows, in particular, that α-crystallin oligomer has no cavities inaccessible for water penetration. This fact is in agreement both with the presence of free spaces between α-crystallin subunits that should exist according to the micelle-like model of this protein structure [7] and with the existence of an open cavity in its central part, according to the structural models proposed in other works [8, 17].

The mean radius of gyration R_g obtained for α-crystallin in this work using small-angle neutron scattering is about 5.8 nm. It is less by 4-8% than the values that we have obtained by the small-angle X-ray scattering in the present work and in the works [3, 6]. Such differences in the radii of gyration of proteins obtained by small-angle neutron and X-ray scattering, as is shown [18], may be due to protein hydration.

In conclusion, the method of small-angle X-ray scattering has demonstrated that deuteration does not influence significantly the quaternary structure of α-crystallin. This fact substantiates the correctness of using the method of contrast variation in the study of α-crystallin structure by small-angle neutron scattering.

Small-angle neutron scattering has been measured for α-crystallin solutions with the D_2O volume fraction of 0, 23, 68, and 90%. The match point (43% D_2O) and the mean scattering density ($2.4 \cdot 10^{10}$ cm^{-2}) in this point have been determined for α-crystallin from the zero angle neutron scattering intensity.

The values of the radius of gyration and the distance distribution functions calculated for α-crystallin by small-angle neutron scattering data for solutions with different D_2O/H_2O ratios have led to a conclusion about the homogeneous distribution of scattering density in α-crystallin regions inaccessible for water penetration and about the uniform deuteration of α-crystallin throughout the oligomer. Consequently, α-crystallin does not contain any cavities into which water cannot penetrate, and all subunits of this polydisperse oligomer protein are equally accessible to water molecules and, probably, to other low molecular weight compounds, including biologically active substances. These conclusions about α-crystallin structure should be taken into consideration when constructing the models of α-crystallin quaternary structure.

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