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Early steps in the crystallization process of proteins: an approach by fluorescence anisotropy

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An investigation of the early steps occurring in the crystallization process of a protein has been attempted by means of fluorescence anisotropy using ribonuclease A with ethanol as precipitating agent. It is found that the apparent fluorescence anisotropy is sensitive to protein-protein interactions and increases linearly with protein concentration. The virial coefficient for rotational diffusion appears to increase sharply beyond 40% ethanol, the concentration which corresponds to crystallization conditions of the protein. The results reported here demonstrate that fluorescence anisotropy can be used to monitor directly the prenucleation phase of the crystallization process.

Nucleation; Crystallization; Fluorescence anisotropy; Ribonuclease A

1. INTRODUCTION

The recent progress in fast acquisition of crystallographic data, due to the development of area detectors, has increased the need for growth of good crystals in relatively short periods of time. This requirement has led to a new interest in the crystallization process of proteins and a careful search for general rules governing the appearance and the growth of protein crystals. Usually, crystals are obtained by the old fashioned 'trial and error' method, using visual observations under the microscope [1]; this kind of technique implies micrometric objects and cannot detect small aggregates or nuclei appearing in the early steps of the process. However, such objects are essential and govern the evolution of the system towards an amorphous precipitate or a crystal [2].

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Abbreviations: IAEDANS, *N*-{[(iodoacetyl)amino]ethyl}-5-naphthyl-amine-1-sulfonic acid; RNase A, ribonuclease A

The purpose of this work is to study the first steps of protein association under crystallization conditions. Fluorescence anisotropy appears to be a suitable method to follow the prenucleation phase; it is sensitive to any factor affecting the rate of rotational diffusion and has been successfully used in the case of polymerization of proteins such as glutamate dehydrogenase [3] or actin [4].

In this paper, we present a study using bovine pancreatic ribonuclease A (RNase A), a protein which can be crystallized under various conditions [5]. The more common procedures involve addition of an organic solvent under moderately acidic pH; the precipitant agent chosen was ethanol, the physical and chemical properties of which are well known. The fluorescent signal was obtained from a probe, *N*-{[(iodoacetyl)amino]ethyl}-5-naphthyl-amine-2-sulfonic acid (IAEDANS), covalently attached to one of the histidines of the active site; in previous studies [6,7] this probe was found to be a suitable indicator of the conformation of the protein; buried in the active site with a fluorescence lifetime around 20 ns, its fluorescence anisotropy reflects the rotational diffusion of the whole protein.

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Fluorescence anisotropy measurements of the labelled protein were performed under conditions in which ribonuclease A crystallizes. We demonstrate that such technique can be used for monitoring specific intermolecular interactions in crystallization conditions.

2. MATERIALS AND METHODS

2.1. *Materials*

RNase A (RNase type XII-A from Sigma), was highly purified by means of ion-exchange chromatography [8]; the AEDANS-labelled protein was prepared as described by Jullien and Garel [6]. Ethanol was from Carlo Erba and sodium cacodylate from Merck. All the solutions were made with bidistilled water.

Ethanol concentrations were determined by refractometry; viscometric data were taken from the CRC Handbook of Chemistry and Physics (1980).

2.2. *Fluorescence measurements*

Fluorescence anisotropy measurements were made using a Jobin Yvon spectrofluorometer equipped with polarization accessory; the wavelengths of excitation and emission were 380 nm and 480 nm. Observed anisotropies were corrected for partial transmission of the out of plane component by the polarizing filters. To avoid reabsorption of emitted light, the concentration of labelled protein was always kept less than 0.2 mg/ml and protein concentrations up to 30 mg/ml were obtained by adding unlabelled protein.

2.3. *Crystallization conditions*

In the case of organic solvent mixtures, precautions must be taken against heat of mixing and bubble formation since they can induce protein precipitation. Protein solutions were slowly brought up to the desired ethanol concentration by dialysis overnight at 4°C and clarified by centrifugation. Anisotropy measurements were then performed as soon as the solutions reached ambient temperature.

2.4. *Analysis of the data*

At infinite dilution (or very low protein concentrations), the fluorescence anisotropy for labelled protein is related to its rotational relaxation time Θ and to the excited state lifetime τ of the label by the Perrin equation [9]:

$$r_0/r = 1 + \tau/\Theta \quad (1)$$

where r_0 is the intrinsic anisotropy in absence of all motion and $\Theta = \eta V/kT$, with η, viscosity of the solvent; V, hydrodynamic volume of the protein; k, Boltzmann constant; T, absolute temperature.

At high protein concentrations, intermolecular interactions are no longer negligible and the solution deviates from the ideal. In the absence of aggregation, the apparent rotational relaxation time can be written, in first approximation, like other hydrodynamic parameters [10]:

$$\Theta_{app}/\Theta = 1 + \alpha c, \quad (2)$$

where c is the protein concentration and α an empirical virial factor due to deviation from the ideal behaviour and characteristic of the protein. In this case, an apparent value of fluorescence anisotropy is measured:

$$r_0/r_{app} = 1 + \tau/\Theta_{app}. \quad (3)$$

Combining eqn 1 and eqn 2 with eqn 3, we obtain: $r_{app}/r = (1 + \tau/\Theta)/[1 + \tau/\Theta(1 + \alpha c)]$, which can be approximated by:

$$r_{app}/r = 1 + \beta c, \quad (4)$$

with

$$\beta = \alpha\tau/(\Theta + \tau). \quad (5)$$

3. RESULTS

The crystallization of a protein is related to its solubility. In the presence of 100 mM cacodylate, at pH 6, the precipitation curve of RNase A by ethanol is highly cooperative (fig.1). For a protein concentration of 15 mg/ml, the midpoint of the transition is obtained for about 60% ethanol. This effect is slightly dependent on protein concentration; multiplying protein concentration by a factor of two translates the curve towards lower ethanol concentrations by about 2%.

The rotational diffusion of a macromolecule is function of the viscosity of the external medium. At constant temperature, the viscosity of ethanol solutions varies with the proportion of organic solvent; this behaviour is reflected by fluorescence anisotropy of labelled RNase A molecules, when ethanol is added to the buffer. The plot of r_0/r vs

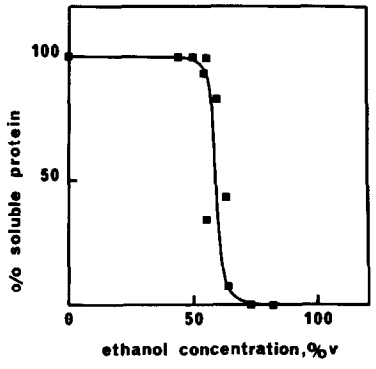

Fig.1. Precipitation curve of RNase A as a function of ethanol concentration. The data were obtained by mixing 20 μl of a 165 mg/ml stock solution of RNase A with 200 μl of the appropriate ethanol solution, the concentration of which was corrected for dilution; the solution was allowed to stand one week, centrifuged and the OD_{278} of the supernatant recorded (ϵ_{278} = 9.8 × 10^3 $M^{-1}\cdot cm^{-1}$). Buffer: 100 mM sodium cacodylate, pH 6; room temperature.

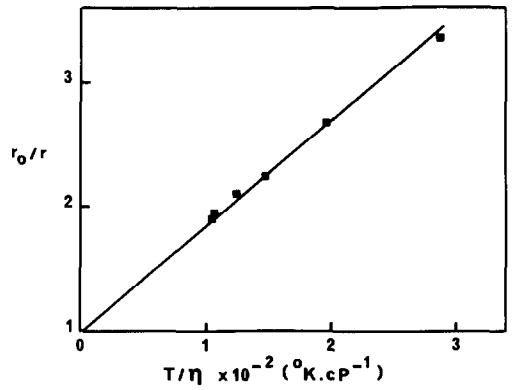

Fig.2. Perrin plot for fluorescence anisotropy of AEDANS-labelled RNase A for ethanol contents varying from 0 to 60% (v/v). Excitation wavelength: 380 nm with a bandwidth of 10 nm; emission wavelength: 480 nm with a bandwidth of 10 nm; r_0 = 0.32; temperature 20°C; protein concentration: 0.2 mg/ml; buffer: 100 mM sodium cacodylate, pH 6.

T/η (fig.2) for ethanol concentrations varying from 0 to 60% follows a straight line with a slope similar to that previously obtained by adding sucrose or varying temperature [6].

When unlabelled RNase A is added to a solution of labelled RNase in order to increase protein concentration, the apparent fluorescence anisotropy increases; in cacodylate buffer, for protein concentrations up to 25 mg/ml, the variation appears to

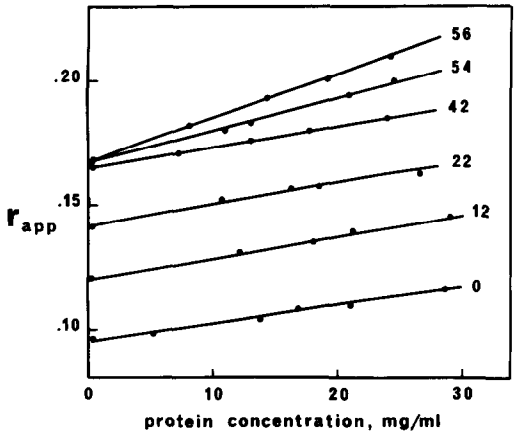

Fig.3. Dependence of the apparent anisotropy r_{app} on RNase A concentration, in the absence and presence of ethanol; the number of each line gives ethanol percentage (v/v). Note that anisotropy value for infinite dilution, r, increases with ethanol content. Precision on all anisotropy values is about 1%. For other conditions, see the legend of fig.2.

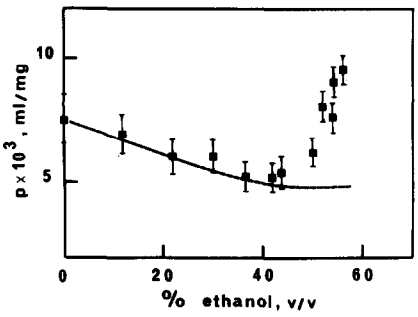

Fig.4. Effect of ethanol concentration on the slope p of the straight line obtained from r_{app}/r vs protein concentration. Broken line simulates effects of solvent viscosity on the parameter β defined by eqn 5, using a value of 10^{-2} ml/mg for the virial coefficient α. Same experimental conditions as in fig.2.

be linear (fig.3); varying the ethanol percentage from 0 to 56% (v/v) increases apparent anisotropies but a still linear dependence on protein concentration is obtained (fig.3). For ethanol concentrations greater than 56%, the solution becomes opalescent too quickly and the increasing turbidity hinders anisotropy measurements. For each ethanol content, a straight line was plotted through experimental points in order to extrapolate to an infinite dilution value, r, which was used to normalize the apparent values of the anisotropy. This procedure permitted the analysis of the effect of protein concentration alone, independently of viscosity effects due to ethanol content in the solvent. The different slopes, p, of the r_{app}/r values vs protein concentration obtained in this way are reported in fig.4 as a function of ethanol percentage. The following behaviour emerges from this representation: the slope decreases monotonously from 7.5×10^{-3} ml/mg to about 5×10^{-3} ml/mg for 45% ethanol and then sharply increases.

4. DISCUSSION

Fluorescence anisotropy is determined by events which occur during the lifetime of the excited state, so that changes in anisotropy may arise from molecular rotation as well as alteration of fluorescence lifetime. It has been observed that the mean fluorescence lifetime of labelled RNase A is about 20 ns [6] and measurements in the presence of 54% ethanol have shown that this value remains

constant within 2% (unpublished). Therefore, the straight line obtained from the Perrin plot in fig.2, for various viscosities due to increasing additions of ethanol, indicates that, in the concentration range 0–60%, ethanol does not induce any local unfolding nor significant change in the hydrodynamic volume of the protein.

The slope p of r_{app}/r vs protein concentration, is an indicator of protein-protein interactions. Light scattering experiments have shown that RNase A molecules do not aggregate in the presence of low organic solvent contents [11]; therefore, the contribution of the non-ideality of the solution to the rotational relaxation time may be deduced from the slope, p, obtained in the absence of ethanol (fig.4). With $\tau = 19$ ns and $\Theta = 8$ ns, a value of 10^{-2} ml/mg can be calculated for the parameter α, in the absence of ethanol at 20°C. Buzzel and Tanford [12] reported a value of 3.3×10^{-3} ml/mg for the intrinsic viscosity of RNase A in water; therefore, the effect of non-ideality appears to be more important on the rotational diffusion coefficient than on viscosity. To our knowledge, it is the first time that fluorescence anisotropies are measured for such high protein concentrations. On the other hand, Van Holde and Baldwin [13] have found a linear dependence on protein concentration for the translational diffusion coefficient of RNase A with a coefficient of about 7×10^{-3} ml/mg.

The different experimental values of p reported in fig.4 reflect the dependence of intermolecular interactions on ethanol concentration. We have simulated in the same figure the variation of the parameter β defined by eqn 5. It must be recalled that this parameter is related to the virial coefficient α by a weighting factor which involves the fluorescence lifetime and the rotational relaxation time at infinite dilution and which only depends on the viscosity of the solvent, as long as the fluorescence lifetime and the protein structure remain unchanged. For such simulation, the value of α obtained in the absence of ethanol was used and the weighting factor was deduced from anisotropies obtained at infinite protein dilution for each ethanol concentration. The comparison of the experimental dependence with the simulated one indicates a good agreement for ethanol concentrations up to about 40%. For ethanol contents higher than 43%, the experimental curve levels off, indicating stronger intermolecular interactions with a varying virial coefficient. This effect may be associated to a significant increase of the apparent volume of the fluorescent molecules. Nevertheless, a still linear dependence of anisotropy vs protein concentration, with a relatively small slope suggests that, if association occurs, it leads to very small and compact aggregates at the beginning of the process; this is not surprising since the association kinetics are very slow, in these low supersaturated conditions (fig.1). It must be pointed out that the effect presently observed appears only beyond a critical value of ethanol content; moreover, the range of ethanol concentrations for which the slope increases corresponds to crystallization conditions of the protein [5,14]. In fact, if the solutions used for anisotropy measurements in this ethanol concentration range were left undisturbed for several weeks, small protein crystals similar to those of native RNase A were observed: the AEDANS label does not inhibit crystallization. This result is in agreement with a study of crystal packing which shows that the active site of the protein is not involved in intermolecular contacts (to be published).

In this preliminary study, only the appearance of nuclei was tested; the characterization of these small aggregates will need further investigation. Since, at the beginning of the process, the solution is probably heterogeneous, fluorescence anisotropy decays will be more appropriate than static anisotropy which reflects the overall behaviour of the solution. Regarding polydisperse systems, the apparent anisotropy value is a number-average [15] which reflects the real distribution of species in the solution. However, when high aggregates are present, they depolarize the signal by means of scattering of excited or emitted light [16]; this effect was actually observed for ethanol concentrations higher than 56%.

We think that, using a very sensitive technique such as fluorescence, we have detected very small aggregates, appearing early in the pathway of crystallization. It must be pointed out that the specific effect of the precipitating agent on protein structure and intermolecular interactions appears only for concentrations higher than a critical value of 40%. Comparison with other precipitating agents will help in understanding the protein crystallization process.

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