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Neutron Small-Angle Scattering Studies of Ribonuclease in Mixed Aqueous Solutions and Determination of the Preferentially Bound Water†

M. S. Lehmann* and G. Zaccai

ABSTRACT: Neutron small-angle measurements of ribonuclease A in mixed solutions of ethanol–water and glycerol–water have been used to estimate the region near the molecule that only contains water. When glycerol is used as probe, the region corresponds to an interaction parameter ξ of 0.23 ± 0.05 g of water/g of protein. For ethanol ξ is −0.07 ± 0.05, corresponding to the macromolecule being equally accessible to ethanol and water. The observations of the radius of gyration in the mixed solutions are used to show that the volume excluding glycerol is found on the exterior of the protein.

The biological activity of soluble proteins is most often studied in an aqueous medium. It is therefore of interest to study the water in the immediate vicinity of the molecule to estimate how much is required for it to function and the minimum amount necessary for the protein to retain its three-diminsional structure. In a recent study (Lehmann & Zaccai, 1982) small angle neutron scattering was used to estimate the region around the enzyme papain that is inaccessible to ethanol and dimethyl sulfoxide. It was found that a large part of the protein surface was directly in contact with the probe molecules. The most simple and direct explanation of this is that both ethanol and dimethyl sulfoxide are small molecules that have the ability for either hydrogen-bond donation or hydrogen-bond acceptance similar to water and can thus replace this in the immediate environment of the macromolecule. Following this observation it was decided to do further studies of the same kind on ribonuclease A, and in addition to include glycerol as probe molecule, as this has been used recently in a study of preferential hydration of proteins in glycerol–water mixtures (Gekko & Timasheff, 1981). This not only allowed the comparison of observations using two different techniques, which in principle should lead to similar results (Eisenberg, 1981), but also provided the opportunity to compare the behavior of two solvent molecules of similar nature but of different size.

Materials and Methods

Ribonuclease A preparation X A was from Sigma (lot 39C-8035); it was dialyzed 3 times in a D_2O buffer (50 mM phosphate and 100 mM NaCl, pD 7.2) for a total period of 2 days at room temperature. Transmission measurements on the last buffer using neutrons showed the D_2O content to be higher than 99%. The probe molecules CH_3CH_2OD and $CH_2ODCHODCH_2OD$ were purchased from the Service des Molécules Marquées, CEA, Gif-sur-Yvette, and were enriched more than 99% and 97% in D, respectively. The glycerol contained less than 1% water. Mixing to the required volume percentages was done in microliter quantities using high-precision pipettes. For glycerol a mixture of glycerol and buffer was prepared before mixing with the sample in order to reduce the viscosity. The mixing ratio for sample and buffer was checked by using neutron transmission measurements, which are very sensitive to the hydrogen content. For the

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sample in H_2O buffer, the preparation was done in a similar manner.

The concentrations were determined from absorbance measurements at 278 nm using the values of $A^{1\%}_{1cm}$ given by Gekko & Timasheff (1981); concentration values were typically in the range from 3 to 7 mg/mL. In order to ensure that the observations were concentration independent, the small-angle measurement for the sample in D_2O buffer was done at concentrations up to 15 mg/mL.

Measurements were done on the D11 small-angle camera at the Institut Laue-Langevin by using a neutron wavelength of 7.0 Å with a maximum momentum transfer Q [$4\pi\sin(\theta/\lambda)$] of 0.09 Å^{-1}.

The data analysis using Guinier plots (Jacrot, 1976) gave two quantities, the forward scattered intensity, $I(0)$, and R_G, the radius of gyration of excess scattering length in the particle. All measurements were put on an absolute scale by using as reference the scattering from a 1-mm sample of water at 7 Å (Jacrot & Zaccai, 1981).

Following Eisenberg (1981), $I(0)$ is expressed in terms of the scattering density increment of the solution $(\partial\rho/\partial c_2)$

$$I(0) = c_2(M_2/N_A)(\partial\rho/\partial c_2)^2_{\mu\neq\mu_2} \quad (1)$$

where subscript 2 refers to the macromolecule, c is the mass concentration, M_2 is the molecular weight, N_A is Avogadro's number, and μ is the chemical potential. In all that follows the constant chemical potential of all solvent components will be assumed, and $\mu \neq \mu_2$ will be dropped from the notation. The correspondance between $(\partial\rho/\partial c)$ and the more familiar particle notation [used in Jacrot & Zaccai (1981)] has been reviewed by Zaccai & Jacrot (1983). In particular

$$\sum(b_i - \rho^0 v_i) = (M_2/N_A)(\partial\rho/\partial c_2) \quad (2)$$

where b_i is the scattering length of a volume v_i in the particle and ρ^0 is the scattering length density of the solvent. The summation should be taken over the entire volume of the particle and includes solvent that has been perturbed by the presence of the macromolecule. A model is required to evaluate $\sum(b_i - \rho^0 v_i)$ whereas $(\partial\rho/\partial c_2)$ can be expressed in terms of thermodynamic variables (Eisenberg, 1981). For a three-component solution (component 1, water; component 2, macromolecule; component 3, alcohol or glycerol)

$$(\partial\rho/\partial c_2) = B_2 + \xi_1 B_1 - \rho^0(\bar{v}_2 + \xi_1\bar{v}_1) \quad (3)$$

where the subscripts refer to the component, B is the scattering length in centimeters per gram, $\bar{v}$ is the partial specific volume (cm^3/g), ξ is an interaction parameter (g of component/g of macromolecule), and ρ^0 is the scattering length density of the solvent (cm^{-2}).

In general, ξ and $\bar{v}$ are functions of the solution composition so that $(\partial\rho/\partial c_2)$ need not be linear with ρ^0, if the latter were varied by varying the concentration of component 3 (contrast variation by the third component). Tardieu et al. (1981), however, have discussed the case where $(\partial\rho/\partial c_2)$ does vary linearly with ρ^0 when the third component is varied. They have shown that when $(\partial\rho/\partial c_2)$ in the absence of the third component falls on the same straight line, the equivalent particle is the macromolecule with a constant volume of water associated with it that cannot be penetrated by the third component.

There is no thermodynamic interpretation of the value corresponding to R_G, the radius of gyration of contrast, and a model of the particle is required:

$$R_G^2 = \frac{\sum(b_i - \rho^0 v_i) r_i^2}{\sum(b_i - \rho^0 v_i)}$$

where r_i is to the center of mass of the $b_i - \rho^0 v_i$ distribution in the particle.

Table I: Radius of Gyration, R_G, and Scattering Density Increment, $(\partial\rho/\partial c)_{obsd}$, for the Sample in the Different Mixtures[a]

solution	ρ^0 ($\times10^9$ cm^{-2})	R_G (Å)	$(\partial\rho/\partial c)_{obsd}$ ($\times10^9$ cm/g)
D_2O	64.0	14.4 (4)[b]	−22.2 (1)[b]
H_2O	−5.6	17.2 (23)	17.2 (8)
70.5% D_2O in H_2O	43.5	9.9 (39)	−10.8 (3)
79.3% D_2O in H_2O	49.7	11.9 (9)	−13.9 (1)
4.8% Et in D_2O	60.2	14.1 (10)	−20.2 (1)
11.1% Et in D_2O	56.9	14.6 (10)	−17.9 (3)
16.7% Et in D_2O	54.0	13.0 (20)	−15.8 (3)
20.0% Et in D_2O	52.5	11.2 (25)	−14.7 (3)
8.6% gly[c] in D_2O	61.6	13.0 (5)	−20.0 (1)
32.8% gly in D_2O	55.1	12.3 (5)	−13.5 (1)
41.4% gly in D_2O	52.8	8.1 (16)	−11.9 (2)
55.1% gly in D_2O	48.7	14.7 (64)	−8.4 (6)

[a] Volume ratios are obtained from the mixing, and scattering densities, ρ^0, for the deuterated solutions are calculated by assuming these to have the same behavior as the nondeuterated compounds. Scattering lengths are taken from Jacrot (1976). [b] Errors are given in parentheses in units of the last digit. [c] gly, glycerol.

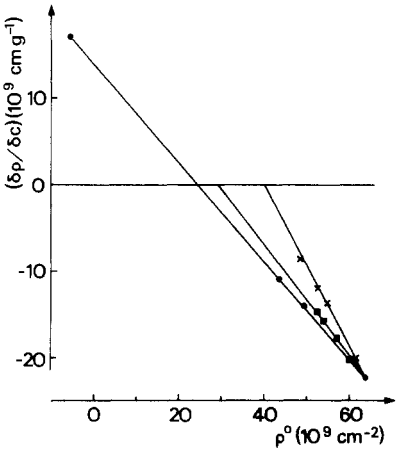

FIGURE 1: Scattering density increment as a function of bulk scattering of the solvent. Black dots are normal H_2O/D_2O contrast variation, squares are alcohol contrast variation, and crosses are glycerol contrast variation.

Contrast variation using small molecules in the solvent has been used before in neutron scattering experiments to study hydration in ferritin (Stuhrmann et al., 1976) and the hydrophilic parts of chromatin subunits (Baldwin et al., 1978).

Results and Discussion

The small-angle neutron scattering was measured for ribonuclease solutions in water–alcohol and water–glycerol mixtures. Except for the point in H_2O buffer in the absence of the third component, all experiments were in D_2O buffer with third-component molecules that were deuterated in their exchangeable hydrogen atoms. Exchanged hydrogen atoms in the protein, therefore, did not vary with solvent composition. The intensity $I(0)$ was converted to a $(\partial\rho/\partial c)_{obsd}$ by using eq 1.

The results are summarized in Table I, which gives the R_G and $(\partial\rho/\partial c)_{obsd}$ values for the different samples, and in Figure 1, which shows the scattering density increment for the same observations as a function of scattering density of the bulk solution. Three linear relationships are observed with the point in D_2O buffer in the absence of the third component falling on the same lines. The line to the left is for the standard H_2O/D_2O contrast variation and gives a match point for the

Table II: Calculation of Interaction Parameters from the Data[a]

solution	match point[b] ($\times 10^9$ cm^{-2})	B_2[c] ($\times 10^9$ cm/g)	B_1[d] ($\times 10^9$ cm/g)	$\bar{v}_1$[d] (cm^3/g)	$\bar{v}_2$[e] (cm^3/g)	ξ_{1,D_2O}[c,f] (g/g)	ξ_1[f] (g/g)
glycerol mixtures	40 ± 1	22.6	57.6	0.900	0.697	0.25 ± 0.05	0.23 ± 0.05
ethanol mixtures	29 ± 1	22.6	57.6	0.900	0.697	−0.07 ± 0.05	−0.06 ± 0.05

[a] The quantities are defined in connection with eq 1-3. [b] Match point is scattering density, ρ^0, for $(\partial\rho/\partial c)_{obsd} = 0$. Experimental values. [c] Calculated from eq 3 and experimental values. [d] Calculated from the chemical composition and density of D_2O. [e] From Gekko & Timasheff (1982); the mean value of $\bar{v}_2$ for the glycerol conditions is taken. [f] Last two columns are values for grams of D_2O per gram of protein and grams of H_2O per gram of protein, respectively.

scattering of the molecule at $(24 \pm 1) \times 10^9$ cm^{-2}. The contrast variation using CH_3CH_2OD gives a match point of $(29 \pm 1) \times 10^9$ cm^{-2}. The match point for the glycerol mixtures is $(40 \pm 1) \times 10^9$ cm^{-2}.

Figure 1 shows that for each set of solutions the particle can be considered as a macromolecule with a constant volume of water associated with it that excludes the third component. The different gradients, however, show the volumes of the particles to be different.

We can interpret $(\partial\rho/\partial c)_{obsd}$ using eq 3 and values for $\bar{v}_2$ given by Gekko & Timasheff (1981). There are no values of $\bar{v}_2$ available for ribonuclease A in alcohol-containing solvents, but it is not unreasonable to take the same value as for glycerol. First, we determine B_2 to be 22.6×10^9 cm/g from the data point of 100% D_2O. This value corresponds to 75% exchange of labile H, in reasonable agreement with crystallographic work (Wlodawer & Sjölin, 1982). Following this the interaction parameter ξ is derived from the match point, i.e., the condition $(\partial\rho/\partial c)_{obsd} = 0$, and the results are summarized in Table II. The value found for ξ for glycerol solutions agrees reasonably well with the determination of Gekko & Timasheff (1981).

The measurements show a marked difference between the hydration found with ethanol and that with glycerol as probes. For ethanol the match point observed is very near the hypothetical match point for the protein itself including the exchanged H atoms without any hydration, giving an interaction parameter value near zero. This will occur if the surface is equally accessible to ethanol and water, but how the regions of accessibility are distributed cannot be inferred from the present measurements. For glycerol a hydration of ~0.23 g/g is observed, and it remains to determine the location of this water. This can be done by assuming that the protein itself is of the same shape in all cases.

We can then use the parallel axis theorem (Engelman & Moore, 1975; Zaccai, 1978) and write R_G as

$$R_G^2 = \frac{m_1}{m_1 + m_2} R_1^2 + \frac{m_2}{m_1 + m_2} R_2^2 \qquad (4)$$

where R_G refers to the particle observed in glycerol mixtures, m_1 and R_1 refer to the excess scattering length and radius of gyration of the protein, and m_2 and R_2 are the corresponding values for the water of hydration. It is assumed that the protein and its water of hydration have identical centers of gravity.

Rearranging, we get

$$R_G^2 - xR_1^2 = (1 - x)R_2^2 \qquad (5)$$

with $x = m_1/(m_1 + m_2) = [(\rho_1 - \rho^0)V_1]/[(\rho_G - \rho^0)(V_1 + V_2)]$. ρ_1, ρ_G, and ρ^0 are scattering densities of the protein, the protein plus hydration layer, and the bulk solution, respectively. $V_1/(V_1 + V_2)$ is known from the above considerations. We can now obtain R_2 from a plot of $R_G^2 - xR_1^2$ vs. x, where R_1^2 has been obtained by interpolation from the observed values in ethanol, which only vary a little. Figure 2 shows the points for 0, 9, 33, and 41% glycerol. At least-squares line going through the point for 0% and using unit weights for the other points gives a R_2 of 20 ± 1.5 Å. As the particle itself has a R_G around 14 Å, this bound water is clearly found on the outside. Again we cannot from our measurement determine the distribution of this water, but it would be natural to assume it to be reasonably homogenously distributed over the whole surface. If so, the amount observed corresponds to approximately one layer of water molecules.

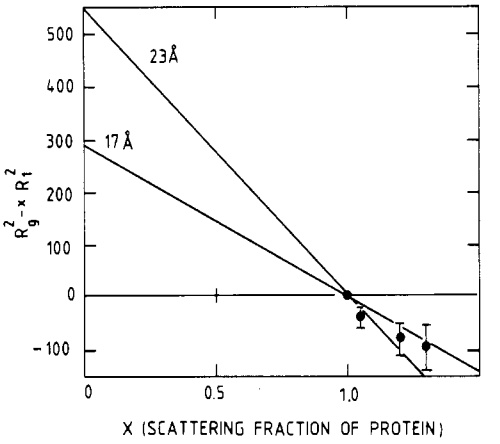

FIGURE 2: $R_G^2 - xR_1^2$, where R_G is the radius of gyration as measured in glycerol and R_1 as measured in ethanol. X is the scattering fraction of protein. The intercept gives R_2^2, where R_2 is the radius of gyration of the preferentially bound water.

Finally we must try to understand why there is such a difference between the behavior of the two probes. Glycerol is generally used because it is "mild" to the protein, creating the above discussed water layer around the molecule. The reason for this as, for example, given by Gekko & Timasheff (1981) is the favorable interaction of glycerol with water combined with a repulsion of nonpolar groups found on the surface. A similar argument could be used for ethanol, especially concerning the first point. Indeed, the molar heat of solvation is nearly twice as large for ethanol as for glycerol. Special arrangement of water by glycerol leading to a stabilization of the water structure has as well been invoked. Again one would expect ethanol to act in a similar manner, as it is known to be structure forming (Cennamo & Tartaglione, 1959).

Looking at the water–probe interaction does not seem to give any clue to the difference in behavior, and we must therefore turn our attention to the hypothetical interaction of the probe with the macromolecule. Indeed, if the two molecules are to approach each other, they should be able to bind to each other in a reasonable way by using the various usual noncovalent interactions. This is clearly easier the smaller the molecules. For glycerol, for example, several hydrogen bonds must be formed, while one would satisfy ethanol. The simplest explanation therefore is simply to relate the interaction to the size of the probe and its shape. In fact this explanation is merely an extension of the above argument of surface repulsion, and cases are known, for example, ribonuclease in 50%

2-methyl-2,4-pentanediol (Pittz & Timasheff, 1978), where the water interaction parameter is as high as 1 g/g. Here the reason for the large water layer seems to be "salting out" of the probe by the charges on the surface of the molecule. For ethanol it is even possible that there might be some preferential binding. In the present analysis we have only measured samples up to 20% ethanol content, and in this case it is quite possible that some of the surface binds ethanol, while part of the rest is inaccessible. Further measurements with higher ethanol concentrations are therefore being planned. It is clear, though, that a molecular picture of these interactions cannot be obtained by any of the methods discussed in this paper and that only a three-dimensional analysis based on single crystal data might eventually give us this information.

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Registry No. Ribonuclease A, 9001-99-4; neutron, 12586-31-1.

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Actin–Actin and Actin–Deoxyribonuclease I Contact Sites in the Actin Sequence†

Kazuo Sutoh

ABSTRACT: Actin subunits in F-actin were cross-linked with *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS). Peptide maps of the cross-linked actin dimer have revealed that the attachment sites of the MBS cross-link in actin are Cys-373 and a lysine residue in the CB-17 segment (Lys-191, Lys-213, or Lys-215). Since MBS spans ~8 Å, the result indicates that Cys-373 in an actin subunit is within the distance of ~8 Å from the lysine residue in the neighboring actin subunit. Therefore, it seems that Cys-373 and the lysine residue in the CB-17 segment are close to the regions of the actin–actin contact sites. The actin–DNase I complex was cross-linked with 1,5-difluoro-2,4-dinitrobenzene (FFD). Peptide maps of the actin–DNase I cross-linked complex have shown that the attachment site of the FFD cross-link in actin is in its CB-10 segment. The CB-10 segment of actin contains Lys-50, Lys-61, Lys-68, Tyr-53, and Tyr-69 as candidates for the attachment site. FFD can span only 3 Å, and therefore it is most likely that one of these residues is in the region of the binding site of DNase I in actin.

It has been well established that actin is present both in muscle and in nonmuscle cells. In muscles, actin molecules self-associate to form a stable filamentous structure (F-actin), in which actin subunits are arranged on two helical strands. For muscle contraction, many proteins must interact with F-actin. For example, ATP-dependent interaction of myosin with F-actin is the basis of muscle contraction and calcium-sensitive interactions of regulatory proteins such as troponin and tropomyosin with actin are essential for the contraction–relaxation cycle of muscles.

Unlike in muscles, F-actin is not a stable structure in nonmuscle cells. Many types of proteins interact with actin to control its higher order structure. Actin-depolymerizing factors such as DNase I (Lazarides & Lindberg, 1974; Hitchock et al., 1976) and profilin (Carlsson et al., 1976) are known to stabilize the monomeric form of actin by forming a 1:1 complex with actin monomer. Many other actin-binding proteins that regulate the higher order structure of actin have been isolated from various tissues and cells.

All the proteins capable of binding to actin would be expected to have specific binding sites in actin. Since primary sequences of actins from various sources (from *Physarum* plasmodia to rabbit skeletal muscle) are highly conserved (Elzinga et al., 1973; Collins & Elzinga, 1975; Vandekerckhove & Weber, 1978a–d), it seems likely that the surface of any actin is covered with a large number of areas responsible for binding these actin-binding proteins.

The studies in this and previous papers (Sutoh, 1982) were undertaken to identify these binding sites in the actin sequence. By employment of C-terminal labeling of actin, cross-linking of the labeled actin with myosin, and finally peptide maps of

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