The Determination of the Scattering Density Distribution of Polydisperse Solutions by
Contrast Variation: A Neutron Scattering Study of Ferritin

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From the dependence of zero-angle scattering of polydisperse solutions on the scattering density of the solvent, information about the mean scattering density and the width of the scattering density distribution of different dissolved molecules can be obtained. This method has been tried with a commercial solution of native ferritin. The root-mean-square deviation of the scattering density was 0.35 x 10^-2 with respect to the mean value of 3.15 x 10^-2 cm^-2. From these data the mean saturation of native ferritin with iron is estimated to about 0.6 and the root-mean-square fluctuation of the iron contents of different ferritin molecules to about 0.4. The neutron scattering curves agree with the results from X-ray small-angle scattering.

I. Introduction

Ferritin is widely distributed throughout the various organs of mammals, especially in liver, spleen and bone marrow. It represents a depot in which surplus of iron can be stored in a non-toxic form within the cell and from which it can be mobilized when required. ‘Ferritin’ refers to the protein from horse spleen, about which most is known at present (Crichton, 1974). The ferritin molecule consists of a spherical protein shell (apoferritin) with an outer diameter of 122 Å surrounding a mineral iron oxide core (diameter 74 Å), the exact composition of which is thought to be (FeOOH)_8FeO·OPO_3H_2 (Michaelis, Coryell & Granick, 1943; Granick & Hahn, 1944).

Solutions of ferritin have been extensively studied by X-ray small-angle scattering (Bielig, Kratky, Rohns & Wawra, 1964; Fischbach & Anderegg, 1965). By adding sucrose to solutions of ferritin, the protein shell had been masked, and the iron core could be investigated separately. This was one of the most successful applications of the contrast-variation technique.

The variation of contrast is still more easily achieved in neutron scattering work. Contrary to X-rays, neutrons are scattered by nuclei. The scattering lengths of the nuclei are relatively low and comparable to the form factors of the ‘light atoms’ in X-ray scattering. However some nuclei have negative scattering lengths. The most prominent example is ^1H: b = -0.372 x 10^-12 cm. Its heavier isotope ^2H or D is characterized by b = +0.66 x 10^-12 cm. The scattering lengths of carbon and oxygen are rather close to that of deuterium. Nitrogen and iron exhibit a somewhat higher b. From these data it is seen that mixtures of H_2O/D_2O cover a much wider range of scattering densities than do sugar solutions in X-ray work. It is therefore interesting to know to what extent neutron scattering experiments can reproduce the results from X-ray scattering and where new domains of investigations can be opened.

As we used an unfractionated ferritin solution during our first test experiments at the High Flux Reactor of the Institute Max von Laue-Paul Langevin, we happened to find a strong influence of the polydispersity on the small-angle scattering pattern, especially at low contrasts. This is going to be discussed in this paper.

II. Experimental

Native horse spleen ferritin, prepared by the procedure of Granick (1942) was obtained from Nutritional Biochemicals (N.B.C.) Cleveland (Ohio, U.S.A.), batch number 2470. Concentrated solutions of ferritin in H_2O or D_2O were prepared by dialysis of stock solutions (100 mg/ml) against H_2O or D_2O respectively, and then mixed to give ten ferritin solutions with different H_2O:D_2O ratios. Series of decreasing concentrations were prepared in H_2O/D_2O mixtures containing 98%, 60% and 0% D_2O. All scattering curves were extrapolated to infinite dilution. The volume fraction of ferritin was estimated from the non-linear dependence of zero-angle scattering on the dilution. The corresponding solvents were obtained by mixing the H_2O and D_2O fractions from the dialysis in the same proportions as for the ferritin solutions. Series of decreasing concentrations were prepared in H_2O/D_2O mixtures containing 98%, 60% and 0% D_2O. All scattering curves were extrapolated to infinite dilution. The volume fraction of ferritin was estimated from the non-linear dependence of zero-angle scattering on the dilution. The neutron source was the High Flux Reactor of the Institute Max von Laue-Paul Langevin. The
neutrons were moderated by liquid deuterium ('cold source') in order to obtain a high intensity of long-wavelength neutrons \((3 \, \text{Å} < \lambda < 20 \, \text{Å})\). The small-angle scattering instrument is connected to the cold source by means of totally reflecting neutron guides. The monochromatization is performed by a mechanical slot selector, a rotating drum with helically curved slits on its surface. At 2400 rotations per minute a wavelength spectrum centred at \(3.5 \, \text{Å}\) with a full half width of \(1.5 \, \text{Å}\) was cut out of the Maxwellian spectrum of the incoming neutrons. A neutron flux of \(10^8 - 10^9 \, \text{n s}^{-1}\) passed the sample area of \(2 \, \text{cm}^2\), depending on the desired angular resolution. The thickness of the sample was \(2 \, \text{mm}\). The scattering pattern was registered by a large \(\text{BF}_3\)-ionization chamber consisting of a matrix formed by crossing arrays of 64 anode wires and 64 cathode strips. Each of the resulting 4096 detection elements has a size of \(1 \, \text{cm}^2\) (Schmatz, Springer, Schelten & Ibel, 1974). About 200 to 5000 neutrons per channel were recorded within a measuring period of eight minutes.

III. Results and discussion

Neutron scattering of ferritin in 10 different \(\text{H}_2\text{O}/\text{D}_2\text{O}\) mixtures has been measured. Changing the solvent actually changes the excess scattering density \(\rho(r)\) of the solute. \(\rho(r)\) can be approximated (Stuhrmann & Kirste, 1967) as:

\[
\rho(r) = \rho_{\text{solute}} - \rho_{\text{solvent}} = \rho_2 - \rho_1
\]

where the solvent is regarded as a homogeneous continuum. A similar equation holds for the mean values of the terms of equation (1). The mean excess scattering density, or contrast, \(\bar{\rho}\), is given by the difference between the mean scattering density of the solute \(\rho_2\) and the scattering density of the solvent \(\rho_1\):

\[
\bar{\rho} = \bar{\rho}_{\text{solute}} - \bar{\rho}_{\text{solvent}} = \rho_2 - \rho_1.
\]

We chose as a reference state for the description of the excess scattering density that state of the solute in the \(\text{H}_2\text{O}/\text{D}_2\text{O}\) mixture in which its mean scattering density equals that of the solvent. This excess scattering density is denoted by \(\rho_s(r)\) because it describes the details of the structure. For monodisperse systems, the zero-angle scattering intensity will vanish when the solute is in the reference state.

On adding \(\text{H}_2\text{O}\) to a ferritin solution in a \(\text{H}_2\text{O}/\text{D}_2\text{O}\) mixture, the excess scattering density will increase, in those regions of the solute macromolecules which are inaccessible to the solvent molecules, by exactly the same amount as the scattering density of the solvent is lowered. However in the case of proteins two phenomena are known to occur and modify the dependence of \(\rho(r)\) on the scattering density of the solvent: the H/D exchange of dissociating protons, and the hydration state of the protein molecule, which gives rise to \(\text{H}_2\text{O}/\text{D}_2\text{O}\) exchange. Both phenomena will lower the increase of the excess scattering density for a given decrease of the solvent scattering density, in those regions of the macromolecule which are accessible to the solvent. This effect is formally taken into account by describing the contrast \(\varphi(r)\) as:

\[
\varphi(r) = \varphi_s(r) = \varphi_2 + \varphi_s(r)
\]

![Fig. 1. Neutron small-angle scattering of ferritin in various \(\text{H}_2\text{O}/\text{D}_2\text{O}\) mixtures: \(\bigcirc\) 77\% \(\text{D}_2\text{O}; \bullet 58\% \text{D}_2\text{O}; \bigotimes 49\% \text{D}_2\text{O}; \bigotimes 30\% \text{D}_2\text{O}.\) The scattering curves were corrected for the distortions due to the wavelength distribution of the incoming neutrons, and extrapolated to infinite dilution.](image)

![Fig. 2. The square root of the extrapolated zero-angle scattering intensity of the ferritin solutions is plotted versus the volume fraction of \(\text{D}_2\text{O}\) of the solvent. Further explanation is given in the text.](image)
where \( q(r) \), which assumes values between 0 and 1, defines the effective local increase of the contrast in the different regions of the solute molecule.

From equation (2) the Fourier transform of \( g(r) \) is given by:

\[
\hat{A}(\mathbf{k}) = \bar{q} \cdot \hat{A}_1(\mathbf{k}) + \hat{A}_2(\mathbf{k});
\]

(3)

\( \mathbf{k} \) is a vector in reciprocal or momentum space. Its absolute value is \( \kappa = (4\pi/\lambda) \sin \theta \), where \( 2\theta \) and \( \lambda \) are the scattering angle and the wavelength respectively. At a resolution of 20 Å the ferritin molecule appears to be spherical and therefore small-angle scattering is described by:

\[
I(\mathbf{k}) = \langle \bar{q} \cdot \langle A_1(\mathbf{k}) \rangle + \langle A_2(\mathbf{k}) \rangle \rangle^2.
\]

(4)

The brackets \( \langle \ldots \rangle \) denote integration with respect to the solid angle. Fig. 1 shows some scattering curves of ferritin at various positive and negative contrasts.

(a) Zero-angle scattering

The validity of equation (4) can readily be tested by plotting the square root of the extrapolated zero-angle scattering intensity \( I_0 \) versus the scattering density of the solvent. With monodisperse solutions this plot should show a straight line. Fig. 2 shows that ferritin behaves in a different way. Starting from ferritin in \( \text{H}_2\text{O} \) the square root of zero-angle scattering intensity decreases almost linearly with increasing volume fraction of \( \text{D}_2\text{O} \). The minimum value of zero-angle scattering – observed in a \( \text{H}_2\text{O}/\text{D}_2\text{O} \) mixture containing 53\% \( \text{D}_2\text{O} \) – is not zero. With still higher \( \text{D}_2\text{O} \) contents of the solvent, \( I_0 \) again increases.

In order to explain this observation we must admit that the scattering density of different ferritin molecules is not equal because of the variable iron contents. The scattering density distribution will be described by \( W(q^2) \). \( q_2 \) and \( q_1 \) are the scattering densities of a ferritin molecule and of the solvent respectively. Zero-angle scattering \( I_0 \) is proportional to the sum of the squared contrasts multiplied by their probability \( W \):

\[
I_0(q_1) = \int W(q_2) \cdot (q_2 - q_1)^2 \cdot dq_2
\]

\[
= \int W(q_2) \cdot q_2^2 \cdot dq_2 - 2q_1 \cdot \int W(q_2) \cdot q_2 \cdot dq_2 + q_1^2.
\]

The integral over the distribution function has been normalized to one. From this equation the minimum value of \( I_0(q_1) \) is found for:

\[
q_1 = \int W(q_2) \cdot q_2 \cdot dq_2 = \bar{q}_2
\]

which defines experimentally the mean value of the distribution. From the scattering density of the \( \text{H}_2\text{O}/\text{D}_2\text{O} \) mixture with 53\% \( \text{D}_2\text{O} \) the mean scattering density of our ferritin sample is found to be \( 3.15 \times 10^{10} \, \text{cm}^{-2} \). Reference values would be \( 2.65 \times 10^{10} \, \text{cm}^{-2} \) for apoferritin and \( 3.45 \times 10^{10} \, \text{cm}^{-2} \) for iron-saturated ferritin; these values have been estimated from the percentage of \( \text{D}_2\text{O} \) in the solvent at vanishing contrast for apoferritin (41\% \( \text{D}_2\text{O} \)) and iron-saturated ferritin (58\% \( \text{D}_2\text{O} \)) respectively (Stuhmann, H. B., Haas, J., Ibel, K., Koch, M. H. G. & Crichton, R. R., in preparation). The mean iron contents of our sample is therefore about 60\% of that of full ferritin.

The root-mean-square (r.m.s.) deviation of the distribution \( W(q_2) \) can be derived from the minimum value of the zero-angle scattering intensity. From the definition of \( I_0(q_1) \), the minimum value of \( \sqrt{I_0(q_1)} \), obtained for \( q_1 = \bar{q}_2 \) (see above), is:

\[
\text{Min} \{ \sqrt{I_0(q_1)} \} = \left[ \int W(q_2) \cdot (q_2 - \bar{q}_2)^2 \cdot dq_2 \right]^{1/2}
\]

which is the definition of the r.m.s. deviation of the distribution \( W(q_2) \). After scaling the ordinate in units of scattering density, at contrasts high enough for the polydispersity to become negligible, we can read from Fig. 2:

\[
\text{Min} \{ \sqrt{I_0(q_1)} \} = (0.35 \pm 0.1) \times 10^{10} \, \text{cm}^{-2}.
\]

Compared with the mean value \( \bar{q}_2 = 3.15 \times 10^{10} \, \text{cm}^{-2} \), this shows a very broad distribution, which on the basis of a Gaussian distribution would lead to incompatibilities with the limiting scattering density values of ferritin cited above. About 30\% of the spectrum would be outside the possible scattering density range. The presence of larger amounts of
apoferritin together with rather highly saturated ferritin has been observed in sedimentation runs of native ferritin (Crichton, 1974). The resulting distribution with a sharp peak due to apoferritin and a broad peak due to the variable iron contents of ferritin would explain the high r.m.s. deviation of the distribution.

(b) The radius of gyration

If we introduce equation (2) into the definition of the radius of gyration we obtain (Stuhrmann & Kirste, 1967):

\[
R^2 = R_c^2 + \frac{1}{\bar{c}} \cdot \frac{\int \varrho(r) r^2 d^3r}{\int \varrho(r) d^3r} = R_c^2 + \frac{1}{\bar{c}} \cdot \alpha. \tag{5}
\]

\(R_c\) is the radius of gyration of the dissolved molecules at infinitely high contrast. As it has become clear that our ferritin sample is polydisperse, equation (5) will hold only at high contrasts \(\bar{c}\) (i.e. small \(1/\bar{c}\)). Then the distribution of ferritin molecules with different individual contrasts can be approximated by a mean contrast \(\bar{c}\). At infinitely high contrast (i.e. \(1/\bar{c} = 0\)), interpolation of the data of Fig. 3 yields \(R_c = 49\ \text{Å}\). This \(R_c\) value is 2.7 Å smaller than \(R\) of apoferritin as determined by X-ray small-angle scattering (Fischbach & Anderegg, 1965), and 2 Å higher than \(R\) of full ferritin as calculated from the dimensions of the ferritin molecule.

From the slope of the straight line in Fig. 3, \(\alpha = -1.14 \times 10^{-3}\). For full ferritin \(\alpha = -1.5 \times 10^{-3}\) is expected, and if the scattering density of the internal hole is smaller than that of the protein shell (as in apoferritin or low-iron ferritin) \(\alpha\) can be assumed to be less than \(0.05 \times 10^{-3}\). These extreme values are derived respectively from a ferritin model with a homogeneous core (Fischbach & Anderegg, 1965) and from \(\alpha\) values of other proteins (Stuhrmann, 1974). The \(\alpha\) value of our ferritin sample corresponds to an iron content of 80% of that of full ferritin. The presence of a larger number of almost saturated ferritin molecules is therefore evident. As already pointed out, ferritin molecules with low iron contents will not notably contribute to \(\alpha\). The \(\alpha\) value is practically not affected by polydispersity, since it is obtained mainly from the measurements at high contrasts.

There is another point which deserves attention. In a solvent containing about 41% \(D_2O\), which corresponds to the mean scattering density of apoferritin, the protein shell is masked, and mainly the mineral core is made visible in neutron small-angle scattering. The radius of gyration measured in these conditions (29 Å) is in good agreement with the values reported by Fischbach & Anderegg (1965).

(c) Neutron scattering of ferritin at wider angles

The polydispersity of the ferritin solution influences small-angle scattering also at wider angles. The linear...
dependence of the square root of the intensity at an arbitrary momentum transfer $\kappa$ on the scattering density of the solvent is annihilated by the presence of a rather broad distribution of scattering densities of the ferritin molecules. We therefore have to analyse the intensities rather than their square roots as a function of the mean contrast. Equation (4) can be rewritten as:

$$I(\kappa) = \bar{\rho}^2 \cdot \langle |A_x(\kappa)|^2 \rangle + \bar{\rho} \cdot \langle A_x(\kappa) \cdot A_x^*(\kappa) + A_x^*(\kappa) \cdot A_x(\kappa) \rangle + \langle |A_x(\kappa)|^2 \rangle$$

$$= \bar{\rho}^2 \cdot I_x(\kappa) + \bar{\rho} \cdot I_{cs}(\kappa) + I_s(\kappa). \quad (6)$$

This equation holds for monodisperse systems. For polydisperse systems, like the ferritin solutions, we have the same representation for each type of molecule; however, in a given solvent the contrast $\bar{\rho}$ and the coefficients $I_x, I_{cs}$ and $I_s$ will be different from one ferritin molecule to another.

However at high enough contrasts, the variation of contrast from one molecule to the other can be neglected, and $\bar{\rho}$ can be approximated by its averaged value over all molecules $\bar{\rho}_m$, whereas $I(\kappa)$ will now be $\bar{\rho}_m^2 \cdot I_x(\kappa)$, in which only $I_x(\kappa)$ is affected by the polydispersity. Different $I_x(\kappa)$ will originate from $\bar{\rho}(r)$ of ferritin molecules with different iron contents. In the extreme cases of full ferritin and apoferritin, $\bar{\rho}(r)$ will describe a full sphere and a hollow sphere respectively. In Fig. 4 the experimental $I_x(\kappa)$ is compared with the corresponding scattering curves calculated for the hollow sphere of apoferritin and the full sphere of saturated ferritin. For $\kappa < 0.2$ the experimental $I_x(\kappa)$ lies in between both extreme cases, whereas at higher $\kappa$ both models fail to match the experimental curve. This is partially due to the subunit structure of the protein moiety, which has not been taken into account in the calculation.

On the other hand, at low contrast we have to consider the influence of polydispersity on the contrast $\bar{\rho}$ and on the basic scattering functions $I_x, I_{cs}$ and $I_s$. At minimum contrast $\bar{\rho}_m$ and $\kappa = 0$, the non-zero scattering intensity has already been discussed in terms of the r.m.s. deviation of the scattering density distribution of the ferritin molecules. At higher angles (0.06 < $\kappa$ < 0.25) the $I_x(\kappa)$ calculated from the experimental data fits reasonably well the $I_x(\kappa)$ calculated from a two-phase model of the ferritin molecule, a homogeneous core with a radius of 37 Å and a protein shell with an outer radius of 61 Å (Fischbach & Anderegg, 1965) (Fig. 5). The shape of the calculated curve $I_x(\kappa)$ does not depend on the iron contents of the model, and its absolute height is mainly determined by the iron-rich fraction of our ferritin sample. Apoferritin would not give rise to any $I_x(\kappa)$ in this range. At still higher angles the deviation of the experimental data from the model curve is due to the detailed structure of the protein.

IV. Conclusions

Contrast variation is a suitable method for the determination of the scattering-density distribution of polydisperse solutions. Neutron scattering offers good conditions for the application of this technique.

The scattering pattern is fairly well resolved, though a neutron beam with a rather broad wavelength distribution has been used. Further investigations of well characterized ferritin samples are in progress.

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