The Dipolar Origin of Protein Relaxation

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1. A set of parameters is proposed to check the interpretation of the dielectric behaviour of protein solutions as a rigid-dipole relaxation of prolate ellipsoids of revolution in the frequency range between 20 kHz and 10 MHz. Besides the δ_p -function of Scheraga, another analogous function (δ_a) is presented to establish size and shape of globular proteins. A study of the influence of solvent viscosity on the dielectric dispersion also gives strong evidence in favour of rigid-dipole relaxation. 2. Measurements of the dielectric dispersion of monomer solutions of bovine serum albumin and transferrin are reported. Monomers of bovine serum albumin were obtained by fractionation on Sephadex G-150. Low-conductivity solutions of both proteins are obtained by passage through an ion-exchange resin. 3. Computer analysis of the experimental dispersion curves by use of a two-term Debye dispersion gives valuable information about transferrin and leads to an axial ratio 4.5 for a prolate ellipsoid of revolution. The dielectric increment of bovine serum albumin is very low and no conclusive results have yet been obtained.

The usefulness of measurements of dielectric dispersion in the study of the quaternary structure of globular proteins in solution depends on the validity of the model used for explaining their dielectric behaviour.

Oncley (1942) and his co-workers reviewed their work on the dielectric investigation of protein solutions. In an extensive series of papers they indicated the possibility of obtaining information about the size and shape, the permanent dipole moment and the dipole direction with respect to the ellipsoidal axes of the macromolecule. Their interpretation was based on the assumption that the dielectric increments were due solely to an orientation of permanent dipoles, considering the protein molecules as mechanically rigid ellipsoids of revolution. Other experimental studies (Dintzis, Oncley & Fuoss, 1954; Dintzis, 1952) suggest that the motion of counterions may also cause a dielectric dispersion. Proton fluctuation and ionic polarization were also proposed in several theoretical and experimental papers to describe the dielectric behaviour of protein solutions (O'Konski, 1960; Kirkwood & Shumaker, 1952).

After these studies a critical revision of the interpretation of the dielectric behaviour of protein solutions became necessary. The first extensive study on isoionic BSA* solutions considering the various theoretical proposals was published by

Moser, Squire & O'Konski (1966). Their measurements of dielectric dispersion and Kerr effect on a monodisperse albumin solution suggest that the protons of the isoionic protein are not sufficiently mobile to contribute to a large ionic polarization or to a fluctuation polarization at the dispersion frequency. They tend to reinstate dielectric-dispersion measurements for the determination of size and shape of globular proteins in solution. It was an important feature of this study to establish that a well-defined monodisperse sample is required for a reliable quantitative interpretation of the results.

The interpretation of the dielectric behaviour of a protein solution as a rigid-dipole relaxation is also supported by the experiments of Tolstoi, Spartakov & Trusov (1966), with a circular rotational electrical field, and also by the experiments on the depolarization of fluorescence by Weber (1953, 1964). The study of dielectric dispersion of proteins in viscous solvents (Takashima, 1962) supports the rigid-dipole behaviour for several proteins, whereas for catalase the results did not fit into this interpretation.

THEORETICAL

We assume the protein molecules in solution to behave hydrodynamically as rigid ellipsoids of revolution with a permanent dipole having a given direction with respect to the ellipsoidal axes.

* Abbreviation: BSA, bovine serum albumin.

According to calculations by Perrin (1934), extensively related by Cohn & Edsall (1943), the dielectric-dispersion curves for such a system can be described by two Debye relaxations:

$$\frac{\epsilon - \epsilon_0}{\epsilon_\infty - \epsilon_0} = \frac{A}{1 + (f/f_a)^2} + \frac{1-A}{1 + (f/f_b)^2} \quad (1)$$

in which A is a constant term, f_a and f_b are the two relaxation frequencies, ϵ the electrical permittivity at the frequency f , ϵ_0 the electrical permittivity for $f \ll f_a$ and f_b and ϵ_∞ the electrical permittivity for $f \gg f_a$ and f_b .

From f_a and f_b the rotational diffusion coefficients θ_a and θ_b of the hydrodynamic equivalent ellipsoid of revolution can be calculated:

$$\theta_a = \pi(2f_b - f_a)$$

$$\theta_b = \pi f_a$$

According to Perrin (1934):

$$\theta_a = \frac{kTJ_a}{6\eta_0 V_e} \quad (2)$$

with:

$$J_a = \frac{3(1+p^2) - \frac{3p^2(1+p^2)}{\sqrt{(1-p^2)}} \cdot \ln\left(\frac{1+\sqrt{(1-p^2)}}{p}\right)}{2(1-p^4)}$$

for prolate ellipsoids and:

$$J_a = \frac{\frac{3p^2(p^2+1)}{p^2-1} \cdot \tan^{-1}[\sqrt{(p^2-1)}] - 3(p^2+1)}{2(p^4-1)}$$

for oblate ellipsoids.

In these equations, k is the Boltzmann constant, T the absolute temperature, η_0 the solvent viscosity, V_e the effective volume of the ellipsoid and p the axial ratio b/a with a for the revolution axis of the ellipsoid. Analogous formulae can be derived for J_b and θ_b .

The rotational diffusion coefficients θ_a and θ_b are hydrodynamic quantities that are a function both of the effective volume V_e and of the axial ratio p of the equivalent ellipsoid. As suggested by Scheraga (1961) a combination of two hydrodynamic quantities is necessary to establish the two unknown parameters of the hydrodynamic equivalent ellipsoid.

We elaborated the combination of dielectric-dispersion measurements and viscosity data considering two rotational diffusion coefficients.

The intrinsic viscosity $[\eta]$ of a solution of rigid ellipsoidal particles is given by:

$$[\eta] = \lim_{C \rightarrow 0} \frac{\eta - \eta_0}{\eta_0 C} = \left(\frac{V_e}{M}\right) N \nu \quad (3)$$

with N Avogadro's number, M the molecular weight, V_e the effective volume, ν a shape factor being a function of the axial ratio p only and calculated by Simha (1940), and C the concentration in g./ml. By mathematical elimination of the effective volume V_e from the formula of the intrinsic viscosity $[\eta]$ (eqn. 3) and from θ_a (eqn. 2) and θ_b two δ -functions are obtained:

$$\delta_a = \frac{6}{Nk} \left(\frac{\eta_0 \theta_a}{T}\right) [\eta] M = J_a \nu \quad (4)$$

$$\delta_b = \frac{6}{Nk} \left(\frac{\eta_0 \theta_b}{T}\right) [\eta] M = J_b \nu \quad (5)$$

These quantities are functions of the axial ratio p only. The values of δ_a and δ_b for prolate and oblate ellipsoids as functions of the axial ratio p are given in Fig. 1 and Tables 1 and 2. These plots are calculated from the J_a and J_b values of Perrin (1934) and ν values of Simha (1940). It follows from Fig. 1 that the δ_a -function makes an unambiguous distinction between prolate and oblate ellipsoids and is more sensitive to the axial ratio p than is the δ_b -function calculated by Scheraga (1961).

Next the correlation between the relaxation times τ_a and τ_b and the solvent viscosity can be examined. θ_a and θ_b are related to τ_a and τ_b by the equations:

$$\tau_a = 1/2\theta_b$$

$$\tau_b = 1/(\theta_a + \theta_b)$$

From the δ_a - and δ_b -functions it is easy to deduce that:

$$\tau_a/\eta_0 = \frac{3[\eta]M}{\delta_b N k T} \quad (6)$$

$$\tau_b/\eta_0 = \frac{6[\eta]M}{(\delta_a + \delta_b) N k T} \quad (7)$$

As the values of $[\eta]$, δ_a and δ_b are completely determined by the axial ratio p and the effective volume V_e , there has to be a linear relation between τ_a , τ_b and η_0 for a given molecule. The straight line must go through the origin and the slope can be calculated beforehand. Considering

Fig. 1. Dependence of δ_a and δ_b on the axial ratio p for prolate and oblate ellipsoids: curve 1, δ_a , prolate; curve 2, δ_b , oblate; curve 3, δ_a , oblate; curve 4, δ_b , prolate.

the foregoing, the axial ratio can be calculated in five different ways, enabling multiple control of the proposed hydrodynamic model assuming globular proteins as rigid ellipsoids of revolution: (i) from the diagram of Perrin (1934) giving the axial ratio as a function of the ratio τ_a/τ_b ; (ii) from the δ_a -function; (iii) from the δ_b -function; (iv) from the slope of the $\tau_a(\eta_0)$ function and $[\eta]$; (v) from the slope of the $\tau_b(\eta_0)$ function and $[\eta]$.

The δ_a - and δ_b -functions are related to the Perrin (1934) nomogram by the equation:

$$\tau_a/\tau_b = (\delta_a + \delta_b)/2\delta_b$$

The δ_b -function is less sensitive than δ_a with respect to differences in axial ratio.

Calculation of the τ_a/η_0 and τ_b/η_0 ratios provides a check of the foregoing data.

Table 1. *Hydrodynamic parameters for prolate ellipsoids*

<i>p</i>	ν (Simha)	J_a	J_b	δ_a	δ_b (Scheraga)
1	2.500	1.00	1.00	2.50	2.50
1/2	2.908	1.23	0.66	3.60	1.93
1/3	3.685	1.33	0.42	4.92	1.57
1/4	4.663	1.38	0.29	6.46	1.37
1/5	5.806	1.41	0.21	8.22	1.25
1/6	7.099	1.43	0.16	10.18	1.17
1/8	10.103	1.45	0.10	14.72	1.07
1/10	13.634	1.46	0.07	20.23	1.02

Table 2. *Hydrodynamic parameters for oblate ellipsoids*

<i>p</i>	ν (Simha)	J_a	J_b	δ_a	δ_b (Scheraga)
1	2.500	1.00	1.00	2.50	2.50
2	2.845	0.70	0.88	2.02	2.52
3	3.430	0.54	0.68	1.87	2.34
4	4.059	0.44	0.54	1.80	2.20
5	4.708	0.35	0.44	1.67	2.10
6	5.367	0.32	0.37	1.73	2.03
8	6.700	0.25	0.28	1.69	1.93
10	8.043	0.20	0.23	1.67	1.87

Table 3. *Calculation of the axial ratio p by using literature values for δ_a and $[\eta]$*

The relaxation times for BSA monomers and for ESA (equine serum albumin) are from Moser *et al.* (1966) and for human mercaptalbumin from Dintzis (1952). For BSA (1) the relaxation times are determined from dielectric dispersion alone and for BSA (2) from dielectric dispersion and birefringence-decay measurements. The intrinsic-viscosity data are from Scheraga (1961) for ESA, from Takashima (1964) for BSA and from Cohn, Hughes & Weare (1947) for human mercaptalbumin.

Molecule	τ_a (μ sec.)	τ_b (μ sec.)	$[\eta]$	δ_a	δ_b	$\frac{\tau_a}{\tau_b}$	p_{δ_a}	p_{δ_b}	p_{τ_a/τ_b}
BSA (1)	0.23	0.11	0.043	4.98	1.57	2.09	3.1	3.0	3.1
ESA	0.36	0.075	0.049	9.8	1.14	4.8	6.5	5.9	5.9
BSA (2)	0.20	0.098	0.043	5.57	1.78	2.06	3.5	2.3	3.1
Mercaptalbumin	0.44	0.016	0.043	7.24	1.45	2.77	4.5	3.5	4

The proposed treatment was checked on experimental data obtained by other workers (Table 3). The axial ratios calculated from the δ_a - and δ_b -functions and from the Perrin (1934) diagram are in good agreement with each other.

MATERIALS AND METHODS

Preparation of samples. All the molecules in the solution should have the same hydrodynamic properties, and great attention was paid to fulfil this condition in particular for albumin.

Monodisperse BSA solution was prepared by gel filtration on Sephadex G-150. Separations were carried out in 50 mm-sodium acetate buffer, pH 4.75, at a flow rate of about 2 ml./cm.² hr. on a 2.5 cm. $\times$ 100 cm. column. The front part (25%) of the monomer peak was omitted. The collected sample, which proved homogeneous on recycling, was freeze-dried and kept in the refrigerator. To obtain very low conductivity and isoionic pH, the sample was first eluted with deionized water on Sephadex G-25 and then percolated through an ion-exchange column as described by Dintzis (1952).

The transferrin samples were commercial products, purchased from Pentex Inc. (Kankakee, Ill., U.S.A.) and AB Kabi (Stockholm, Sweden). The iron was removed by dialysis against 25 mm-EDTA for 48 hr. and quantitatively determined with bathophenanthroline. By this procedure the quantity of bound iron was decreased to about 10% of its original value.

Experimental techniques. To increase the accuracy of the dielectric measurements, the electrical conductivity of the samples must be very low (5×10^{-6} ohm⁻¹ cm.⁻¹). This increases the resolution of the bridge for capacity measurements (Schwan, Schwartz, Maczuk & Pauli, 1962) and reduces the need for corrections due to electrode polarization.

The electrical capacity of the permittivity cell was measured by means of a transformer ratio-arm bridge between 15 kHz and 7 MHz (Wayne Kerr type B601).

To avoid complicated corrections for the residual serial self-inductance a coaxial cell was used. For this cell only a small frequency-dependent correction had to be made at frequencies above 5 MHz and the frequency-independent correction was only necessary for very conductive solutions.

For correction of electrode polarization at low frequencies, the method proposed by Oncley (1942) was used.

The permittivity cell (Lamote, Denoo & Peeters, 1968)

was calibrated by using glycine solutions up to 2M. The electrical-permittivity data given by Cohn & Edsall (1943) were used. Because the dielectric-dispersion curves shift to lower frequencies with increasing protein concentration as a result of protein-protein interactions, extrapolation to zero concentration of the reduced-dispersion curves had to be made. This was done by using the graphical method proposed by Dintzis (1952).

The viscosity of sucrose and glycerol solutions was determined by using a conventional Ostwald viscometer. Literature values from Cohn *et al.* (1947) for BSA and from Oncley, Scatchard & Brown (1947) for transferrin were used.

RESULTS

Measurements on albumin monomers. The dielectric increment for several BSA concentrations was measured as a function of frequency (Fig. 2a). The reduced-dispersion curves (Fig. 2b) show clearly the shift to lower relaxation frequencies due to protein-protein interaction. A computer least-squares analysis of these data considering two relaxation frequencies gave an axial ratio 3:1 as

deduced from the δ_a -function (ii). There was, however, too much scattering on the lowest relaxation-frequency data owing to the very low dielectric increment of albumin to allow for a decisive calculation of the δ_b -function (iii) and of the τ_a/τ_b ratio (i). Analogous difficulties were encountered by other workers (Moser *et al.* 1966) and for this reason they introduced one relaxation time obtained by Kerr-effect measurements into the computer analysis.

As a linear relationship between relaxation times and solvent viscosity (iv and v) is a good argument in favour of rigid-dipole behaviour, the solvent viscosity was modified by the addition of glycerol. In Fig. 2(c) the dielectric increments as a function of frequency are given for a glycerol concentration of 38% and several BSA concentrations. In Fig. 2(d) the reduced-dispersion curves shift to lower frequencies when the solvent viscosity is increased from 0.889 to 3.05 centipoise. A linear relation between the relaxation time for an equivalent sphere, τ corresponding to $\epsilon = (\epsilon_0 - \epsilon_\infty)/2$, and the solvent viscosity is obtained and this is a

Fig. 2. (a) Dielectric-dispersion curves of deionized BSA monomers: curve 1, $c = 10.7\%$; curve 2, $c = 5.6\%$; curve 3, $c = 2.6\%$. (b) Reduced-dispersion curves of deionized monomer BSA: curve 1, $c = 2.6\%$; curve 2, $c = 5.6\%$; curve 3, $c = 10.7\%$. (c) Dielectric-dispersion curve of monomer BSA after addition of glycerol (38%), $\eta = 2.932$ centipoise: curve 1, $c_{\text{BSA}} = 3.2\%$; curve 2, $c_{\text{BSA}} = 1.6\%$; curve 3, $c_{\text{BSA}} = 0.8\%$. (d) Reduced-dispersion curves of BSA for various solvent viscosities: curve 1, water; curve 2, 26% glycerol; curve 3, 38% glycerol.

good indication in favour of the validity of Stokes's Law in this case. Though these results show that the dielectric increment is only slightly modified by the addition of glycerol, there are indications that a certain amount of glycerol is adsorbed on the protein molecule. At the highest glycerol concentration (38%) there appears a new dispersion at a frequency of about 2 MHz, as pointed out by arrows on Fig. 2(c). The computer analysis of the dispersion curve at 38% glycerol does not fit a two-term Debye dispersion and this suggests a third dispersion frequency. This phenomenon seems analogous to the dielectric dispersion of adsorbed water on protein molecules as studied by Grant (1966). From the viscosity measurements it appears that the intrinsic viscosity of BSA increases on the addition of glycerol. Finally, the dielectric increment for BSA after addition of glycerol is slightly higher (Fig. 3).

In a second set of experiments the solvent viscosity was modified by the addition of sucrose to the BSA solutions and the corresponding dispersion curves are given in Fig. 4. The linear relation between the relaxation time for an equivalent sphere and the solvent viscosity is excellent and there is no problem of adsorption (Fig. 5).

Measurements on transferrin. Transferrin was selected for an accurate study of the proposed hydrodynamic model because of its high dielectric increment (15/g./l.). Further, this protein is quite stable at low ionic strength, which allows desalting to very low conductivity. Measurements of dielectric dispersion for native transferrin and for transferrin dialysed against EDTA were made to investigate the difference in dielectric behaviour before and after removal of the iron.

As shown in Fig. 3 the dielectric increments for native transferrin (■) and for transferrin dialysed against EDTA (Δ) were equal within experimental errors. There is, however, a difference between the reduced-dispersion curves for zero concentration, and this can be interpreted as an increase of the molar volume and a decrease of the axial ratio after the removal of iron (Fig. 6).

The results of computer analysis of the experimental data are given here as follows. The computed axial ratio for native transferrin is 4.7 from the Perrin (1934) diagram, 4.5 from the δ_a -function and 5.5 from the δ_b -function. For the dialysed transferrin the axial ratio is 4.4 from the Perrin (1934) diagram, 4.2 from the δ_a -function and 5.3 from the δ_b -function.

Owing to its low slope the δ_b -function gives only a rough estimate of the axial ratio, but there is good agreement between the axial ratios from the Perrin (1934) diagram and the δ_a -function. These values indicate a lowering of the axial ratio after removal of the iron from the transferrin molecule.

In Fig. 7 the measurements of dielectric dispersion are given after the addition of sucrose for solvent viscosities between 0.93 and 13.75 centipoise. The shift of the dispersion curves to lower frequencies at increasing solvent viscosity is observed as with

Fig. 3. Dielectric increment of transferrin (curve 1), BSA after addition of glycerol (26%), $\eta = 1.870$ centipoise (curve 2), and BSA in aqueous solution (curve 3).

Fig. 4. Reduced-dispersion curves of BSA in solutions with increasing sucrose concentration: curve 1, $c_{\text{BSA}} = 4.1\%$, $\eta_{\text{solvent}} = 1.09$ centipoise; curve 2, $c_{\text{BSA}} = 3.4\%$, $\eta_{\text{solvent}} = 1.81$ centipoise; curve 3, $c_{\text{BSA}} = 3.1\%$, $\eta_{\text{solvent}} = 2.45$ centipoise; curve 4, $c_{\text{BSA}} = 2.9\%$, $\eta_{\text{solvent}} = 3.69$ centipoise; curve 5, $c_{\text{BSA}} = 2.8\%$, $\eta_{\text{solvent}} = 7.82$ centipoise.

Fig. 5. Relaxation time for an equivalent sphere [τ for $\epsilon = (\epsilon_0 - \epsilon_\infty)/2$] for BSA (curve 2) and transferrin (curve 1) in function of the solvent viscosity.

Fig. 6. Reduced-dispersion curves for zero concentration of transferrin native (curve 1) and after dialysis against EDTA (curve 2).

Fig. 7. Dispersion curves of transferrin in solutions of various sucrose concentrations: curve 1, $c_{T+t} = 0.94\%$, $\eta_{\text{solvent}} = 0.93$ centipoise; curve 2, $c_{T+t} = 0.67\%$, $\eta_{\text{solvent}} = 2.66$ centipoise; curve 3, $c_{T+t} = 0.63\%$, $\eta_{\text{solvent}} = 5.29$ centipoise; curve 4, $c_{T+t} = 0.53\%$, $\eta_{\text{solvent}} = 13.75$ centipoise.

albumin. In Fig. 5 the linear relation between the relaxation time of an equivalent sphere and the solvent viscosity is given. This is again in favour of rigid-dipole behaviour.

DISCUSSION

The reported data as well as the measurements of the Kerr effect, depolarization of fluorescence and other experimental techniques mentioned in the introduction are a good indication in favour of rigid-dipole behaviour as a means of interpretation of the dielectric behaviour of proteins in solution. The treatment of the rigid-dipole theory applied to protein solutions enables a multiple experimental control as a check of this theory.

Simha's (1940) equation giving the shape factor ν and Perrin's (1934) equation for θ lead to the δ_a - and δ_b -functions, which are independent from each other. Both of them must lead to the same value for the axial ratio p , though δ_a is more sensitive to variations of p . A further control by comparing p values of literature can also be achieved. As the δ_a - and δ_b -functions contain the

solvent viscosity η_0 and the temperature T as parameters, their influence on these functions enables a complementary control of the theory. In the Perrin (1934) diagram (i) these data are eliminated by division as:

$$\tau_a/\tau_b = (\delta_a + \delta_b)/2\delta_b$$

For transferrin the axial ratio was calculated from measurements in aqueous solutions, and a lowering after removal of iron was observed. Analogous measurements performed on BSA monomers were more difficult to interpret. The reason for this difficulty is to be found in the lower dielectric increment of this protein and this requires still more accurate measurements.

Elimination of the bridge parameters by substitution measurements involving the use of standard capacitor and resistor (Schwan *et al.* 1962) and the determination of more experimental points will enable us to improve further on these results.

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