A Theoretical Approach to the Binding of Amphipathic Molecules to Globular Proteins

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1. A theory based on a multiple equilibrium model with stoichiometric binding constants has been formulated. It is applicable to the interaction of amphipathic molecules with charged macromolecules. 2. The theory has been applied to the binding of sodium *n*-dodecyl sulphate to ribonuclease A, β -lactoglobulin and bovine serum albumin. 3. Over the ranges of surfactant concentration where binding is non-co-operative and co-operative, the experimental data can be satisfactorily fitted with energy and co-operativity parameters which are of comparable magnitude for the three globular proteins. 4. The results imply that the energy of interaction of sodium *n*-dodecyl sulphate with the proteins is equivalent to the formation of approximately four CH₂ hydrophobic bonds.

Amphipathic molecules, such as surfactants like sodium *n*-dodecyl sulphate, disrupt the native structure of most globular proteins at very low concentrations (approx. mM). The mechanism of the denaturation of proteins by surfactants involves the binding of the surfactant ions to sites on the protein molecule. There is evidence that the initial interaction between surfactants and proteins is predominantly ionic; the surfactant ions bind to groups of opposite charge on the protein (Burkhard & Stolzenberg, 1972; Tipping *et al.*, 1974; Oakes, 1974). These initial interactions cause the protein to unfold, which results in the exposure of more binding sites, and, as the surfactant concentration is increased, binding becomes co-operative, and ultimately saturation occurs. For several globular proteins interacting with sodium *n*-dodecyl sulphate, co-operative binding occurs when the free surfactant concentration is approx. 1–3 mM. A convenient way of representing the binding of small ligands to macromolecules is by means of a semi-logarithmic plot of the average number of ligands bound per macromolecule ($\bar{v}$) against the logarithm of the free ligand concentration ($[A]_f$). For surfactant binding to proteins, such plots can, in general, be divided into three regions, as illustrated schematically in Fig. 1: (a) the initial binding of a few surfactant ions (<10) to specific strongly binding sites, for example, bovine serum albumin is known to have such sites for anionic surfactants (Reynolds *et al.*, 1967, 1968); these are independent binding sites; (b) the binding of a much larger number of surfactant ions in a non-co-operative fashion; and finally, (c) co-operative binding and ultimately saturation. Region (a) is specific to particular surfactant–protein combinations and if the number of independent binding sites is small, then the binding isotherm can be fitted

theoretically, and the association constants for each site specified. The theoretical approach to regions (b) and (c) is more difficult, since it involves a very large number of sites, each of which must have a different association constant. It might be expected, however, that once the independent binding sites in region (a) are saturated, the association constants for binding in regions (b) and (c) would change monotonically, and, if this was so, it might be possible to give a theoretical treatment of binding on the basis of a multiple-equilibrium model. In the present paper we report such a binding theory, and its application to the binding of sodium *n*-dodecyl sulphate to several globular proteins.

Fig. 1. Schematic plot of the number of bound ligands per protein molecule ($\bar{v}$) as a function of the logarithm of the free ligand concentration ($[A]_f$)

Region (a), specific binding; region (b), non-co-operative binding; region (c), co-operative binding and saturation.

Theory

The binding of a ligand, A, to a protein, P, can be represented by a series of equilibria. Two different methods of formulating multiple equilibria can be distinguished, first, in terms of stoichiometric-binding constants, and second, in terms of site-binding constants (Klotz, 1974). The two methods differ in that the stoichiometric treatment does not distinguish between species which have the same number of bound ligands, i.e. the species PA_j can be formed in several ways, depending on which particular set of j sites are occupied. We adopt here the stoichiometric formulation, since this is a rigorous thermodynamic treatment which implicitly includes interactions between successively bound ligands. The equilibria may be written as follows:

In general, the stoichiometric-binding constants are given by

$$k_j = \frac{[PA_j]}{[PA_{j-1}][A]} \quad (4)$$

For such a set of equilibria, the average number of ligands bound per protein molecule ($\bar{v}$) can be written in terms of the association constants, and the free ligand concentration, $[A]$, by the equation (Adair, 1925):

$$\bar{v} = \frac{\sum_{j=1}^n j \binom{j}{1} (\pi k_j) [A]^j}{1 + \sum_{j=1}^n \binom{j}{1} (\pi k_j) [A]^j} \quad (5)$$

where the summations extend over the total number of binding sites on the protein. To evaluate $\bar{v}$ for a given free ligand concentration we require the values of all the association constants, k_j .

The free energy of association (ΔG_a^j) of the j th surfactant ligand with a surfactant-protein complex of average net charge $\bar{Z}$ can be expressed in terms of an intrinsic free energy of association (ΔG_{int}), modified by the changes in the electrostatic free energy (W_{el}) and the hydrophobic free energy (W_{eh}) which occur on binding. ΔG_{int} represents the Gibbs free energy of association of a single *n*-dodecyl sulphate ion with a protein molecule of zero net charge. Thus we may write

$$\Delta G_a^j = \Delta G_{int} + \left(\frac{\partial W_{el}}{\partial \bar{Z}} \right) - \left(\frac{\partial W_{eh}}{\partial j} \right) \quad (6)$$

The signs in eqn. (6) take account of the fact that the ionic and hydrophobic contributions made to the free energy of a surfactant-protein complex, on binding a surfactant ion, have opposite effects on the free energy of association of subsequent ligands. To a

reasonable approximation, the electrostatic free-energy can be calculated on the assumption that the surfactant-protein complex is spherical. This would not be a good assumption for reduced proteins at high binding levels, which have more rod-like characteristics (Reynolds & Tanford, 1970), but for non-reduced proteins, in the absence of an equation for the electrostatic free-energy of an ellipsoid with a uniform charge distribution, the spherical model is adequate. [For an ellipsoid with an axial ratio of 3–4, W_{el} will be decreased by about 10% (Tanford, 1961). As shown below such a decrease in W_{el} has an almost negligible effect on the calculations.] For such a model

$$W_{el} = \frac{\bar{Z}^2 e^2}{8\pi\epsilon_0\epsilon_r R_j} \left(1 - \frac{\kappa R_j}{1 + \kappa a} \right) \quad (7)$$

where e is the electronic charge, ϵ_0 and ϵ_r are the permittivity of vacuum and the relative permittivity of the medium respectively, R_j is the radius of the complex, a is the distance of nearest approach of counterions and κ is the Debye length, given by

$$\kappa^2 = \left(\frac{2e^2 N 10^3}{\epsilon_0 \epsilon_r kT} \right) I \quad (8)$$

where N , k and T are Avogadro's number, the Boltzmann constant and the absolute temperature respectively, and I is the ionic strength of the solution. The electrostatic free energy is conveniently written in terms of the work function, w , according to the equation

$$W_{el} = \bar{Z}^2 kT w \quad (9)$$

It follows from eqn. (9) that the derivative of the electrostatic free energy of the complex, with respect to the net charge, is simply $2\bar{Z}kTw$. The net charge on the surfactant-protein complex will depend on the net charge of the native protein, the number of bound surfactant ions (j) and also the extent to which counterions are bound to the complex. It would be expected that the binding of counterions to a complex would occur to a similar extent as does counterion binding to ionic micelles. Ionic micelles are approximately 20% ionized (Hartley, 1936). If the net positive charge on the native protein is $\bar{z}$, then on binding j surfactant anions, the net charge on the complex will be approximately given by

$$\bar{Z} = 0.2(j - \bar{z}) \quad (10)$$

As binding proceeds, the radius of the complex, R_j , will increase. The radius can be calculated from the hydrodynamic volume V_h by means of the equations (Reynolds & Tanford, 1970)

$$V_h = \frac{M}{N} (\bar{v}_2 + \delta_1 v_1^0 + \delta_2 v_2^0) \quad (11)$$

$$R_j = \left(\frac{3V_h}{4\pi} \right)^{\frac{1}{3}} \quad (12)$$

where $\bar{v}_2$, v_1^0 and v_2^0 are the partial specific volume of the protein and the specific volumes of the medium and the surfactant respectively. δ_1 and δ_2 are the mass of solvent and that of the surfactant per unit mass of protein in the complex respectively. M is the molecular weight of the protein. Eqns. (8), (9), (10), (11) and (12) can be used in conjunction with eqn. (7) to calculate W_{e1} .

No theory is available for the hydrophobic free energy, W_{ch} . It is, however, reasonable to assume that the more surfactant ions are bound to the protein, the greater will be its hydrophobicity; the fact that, in the limit, binding becomes co-operative, would seem to support this suggestion. For non-co-operative binding we may write

$$W_{ch} = jE \quad (13)$$

where E is the change in the hydrophobic free energy, W_{ch} , of the complex on binding a surfactant ligand. It follows from eqns. (6), (9), (10) and (13) that

$$\Delta G'_n = \Delta G_{int} + 0.4(\bar{z} - j)NkTw - NE \quad (14)$$

From eqn. (14) it follows that the association constants, k_j , can be related to the intrinsic association constant k_{int} by the equation

$$k_j = k_{int} \left(\frac{n-j+1}{j} \right) \exp \left(\frac{E}{kT} - 0.4(j - \bar{z})w \right) \quad (15)$$

The statistical factor $(n-j+1)/j$ has been included in eqn. (15) to account for the fact that the association constants of independent identical sites are not equal.

For co-operative binding, the hydrophobic free energy must increase at a rate which is more than directly proportional to the number of ligands bound. An expression with the required characteristics is

$$W_{ch} = j^{1+x}E \quad (16)$$

where we define x as the co-operativity parameter. Hence the association constants for co-operative binding are given by the equation

$$k_j = k_{int}^c \left(\frac{n-j+1}{j} \right) \exp \left((1+x)j^x \frac{E}{kT} - 0.4(j - \bar{z})w \right) \quad (17)$$

Eqns. (15) and (17) can be used in conjunction with eqn. (5) to compute $\bar{v}$ as a function of $[A]$ for both non-co-operative and co-operative binding. Hence we may write

$$\bar{v} = \frac{\sum_1^n j \left(\frac{j}{1} k_j \right) [A]^j}{1 + \sum_1^n \left(\frac{j}{1} k_j \right) [A]^j} + \frac{\sum_1^n j \left(\frac{j}{1} k_j^c \right) [A]^j}{1 + \sum_1^n \left(\frac{j}{1} k_j^c \right) [A]^j} \quad (18)$$

The relative contributions of co-operative and non-co-operative binding are predominantly determined by the values of k_{int} and k_{int}^c .

Results and Discussion

The theory has been applied to the interaction of sodium *n*-dodecyl sulphate with three globular proteins, ribonuclease A, β -lactoglobulin and bovine serum albumin. The binding data for ribonuclease A and bovine serum albumin have been reported previously (Tipping *et al.*, 1974). The data for the binding of sodium *n*-dodecyl sulphate to β -lactoglobulin was obtained using a method identical with that previously described (Jones *et al.*, 1975). The molecular parameters for the proteins used in the calculations are given in Table 1. In order to calculate R_j from eqns. (11) and (12), it is necessary to know the specific volumes of the medium and the surfactant. These were taken to be $1 \text{ cm}^3 \cdot \text{g}^{-1}$ and $0.886 \text{ cm}^3 \cdot \text{g}^{-1}$ respectively (Reynolds & Tanford, 1970). Also required is the hydration parameter δ_1 , which for a native protein is usually assumed to have a value of $0.2 \text{ g} \cdot \text{g}^{-1}$. It is not clear what value should be used for this parameter for a surfactant-protein complex; at saturation, Reynolds & Tanford (1970) quote $0.9 \text{ g} \cdot \text{g}^{-1}$; as a compromise we take here a value of $0.5 \text{ g} \cdot \text{g}^{-1}$. The parameter δ_2 is given by the eqn.

$$\delta_2 = \frac{288.39}{M} j \quad (19)$$

where M is the molecular weight of the protein and 288.39 is the molecular weight of sodium *n*-dodecyl sulphate.

The total number of binding sites, n , for each protein was estimated from the previously reported

Table 1. Molecular parameters for globular proteins

Protein	Mol.wt.	$\bar{v}_2$ ($\text{cm}^3 \cdot \text{g}^{-1}$)*	$\bar{z}$ (pH)	$\delta_1 v_1^0$ ($\text{cm}^3 \cdot \text{g}^{-1}$)
Ribonuclease A	13683	0.728	+4† (7.3)	0.5
β -Lactoglobulin	35000	0.751	+20‡ (3.5)	0.5
Bovine serum albumin	66000	0.734	-9§ (7.0)	0.5

* Tanford (1961).

† Tanford & Hauenstein (1956).

‡ Cannon *et al.* (1942).

§ Tanford *et al.* (1955).

Table 2. *Binding parameters*Parameters used to fit plots of $\bar{v}$ against $\log[\text{sodium dodecyl sulphate}]$.

Protein	n	$k_{\text{int}} (\text{M}^{-1})$	$k_{\text{int}}^c (\text{M}^{-1})$	E/kT	x
Ribonuclease A	145	0.1	1.0×10^{-4}	10.0	0.086
β -Lactoglobulin	170	0.1	1.0×10^{-4}	9.0	0.110
Bovine serum albumin	360	0.1	1.0×10^{-4}	10.4	0.080

Fig. 2. Binding of sodium *n*-dodecyl sulphate to ribonuclease A ($\bar{v}$) as a function of the logarithm of the free surfactant concentration

○, Experimental points; pH 7.3; $I = 0.005$ mol/litre; 25°C ; protein concentration, 0.03% (Tipping *et al.*, 1974); solid line, theoretical curve calculated from $k_{\text{int}} = 0.1 \text{ M}^{-1}$, $k_{\text{int}}^c = 10^{-4} \text{ M}^{-1}$, $E/kT = 10.0$, $x = 0.086$ and $n = 145$; dotted lines, non-co-operative (a) and co-operative (b) contributions.

(Tipping *et al.*, 1974) Scatchard plots for ribonuclease A and bovine serum albumin. For β -lactoglobulin n was calculated from the saturation value of $1.4 \text{ g} \cdot \text{g}^{-1}$ (Pitt-Rivers & Impiombato, 1968). The values of n are given in the second column of Table 2. Figs. 2, 3 and 4 show the experimental values of $\bar{v}$ as a function of the logarithm of the free sodium *n*-dodecyl sulphate concentration, together with the theoretically calculated curves. The dotted curves show the resolution of the theoretical solid lines into the contributions from non-co-operative and co-operative binding. The adequacy of eqn. (16) to represent the co-operative hydrophobic free energy is confirmed by the large rate of increase of $\bar{v}$ with $\log[\text{sodium dodecyl sulphate}]$ for the co-operative contributions. The curves for both β -lactoglobulin and bovine serum albumin show pronounced kinks on the onset of co-operative binding. The experimental points are insufficiently precise to either

Fig. 3. Binding of sodium *n*-dodecyl sulphate to β -lactoglobulin ($\bar{v}$) as a function of the logarithm of the free surfactant concentration

○, Experimental points; pH 3.5 (sodium acetate buffer); $I = 0.01$ mol/litre; 25°C ; protein concentration, 0.06%; solid line, theoretical curve calculated from $k_{\text{int}} = 0.1 \text{ M}^{-1}$, $k_{\text{int}}^c = 10^{-4} \text{ M}^{-1}$, $E/kT = 9.0$, $x = 0.110$ and $n = 170$; dotted lines, non-co-operative (a) and co-operative (b) contributions; dashed lines, $n = 190$ (upper curve) and $n = 150$ (lower curve).

confirm or refute the existence of such a kink in these plots, although kinks occur in the Scatchard plots. The agreement between the theoretical line and the experimental points at low binding values for ribonuclease A is not good. However, the theory would not be expected to hold for low values of $\bar{v}$. In Fig. 3 the effect of a variation in n by ± 20 is illustrated. For the non-co-operative region, the effect of changing n by this amount is relatively small.

The effect of decreasing W_{el} by 10% is shown in Fig. 4. This has an almost negligible effect on the

Fig. 4. Binding of sodium *n*-dodecyl sulphate to bovine serum albumin ($\bar{v}$) as a function of the logarithm of the free surfactant concentration

○, Experimental points; pH 7.0; $I = 0.005$ mol/litre; 25°C ; protein concentration, 0.03% (Tipping *et al.*, 1974); solid line, theoretical curve calculated from $k_{\text{int}} = 0.1 \text{ M}^{-1}$, $k_{\text{int}}^c = 10^{-4} \text{ M}^{-1}$, $E/kT = 10.4$, $x = 0.080$ and $n = 360$; dotted lines, non-co-operative (a) and co-operative (b) contributions; dashed line was calculated with W_{e1} decreased by 10%.

Fig. 5. Affinity profile for the binding of sodium *n*-dodecyl sulphate to bovine serum albumin

Left-hand axis and solid line, jk_j against j (non-co-operative interactions); right-hand axis and broken line, jk_j^c against j (co-operative interactions).

values of $\bar{v}$ and hence changing the shape of the model of the protein-sodium dodecyl sulphate complexes from that of a sphere to an ellipsoid would not seriously alter the results of the calculations.

Four adjustable parameters are used in the fitting of a curve to the experimental points. However, to restrict the variability between the parameters for the different proteins, the values of k_{int} and k_{int}^c were kept constant. This procedure also increases the significance of the values of E/kT and x .

The initial choice of values of k_{int} and k_{int}^c was made on the basis of trial and error in fitting the ribonuclease A data. The initial choice of the magnitude of the hydrophobic energy parameter, E , was made from the consideration that the energy of hydrophobic bonding per CH_2 group is of the order of $6 \text{ kJ} \cdot \text{mol}^{-1}$ (Tanford, 1970). Hence, for the hydrophobic interaction between one CH_2 group of the *n*-dodecyl sulphate chain with a hydrophobic side chain of an amino acid residue, E/kT would have a value of approx. 2.5. Since there are 11 CH_2 groups and one CH_3 group in the surfactant chain, we might expect E/kT to have a value in the range 2.5–30. The value of E/kT which satisfactorily represented the non-co-operative region of the binding curve was found, and then the co-operative region was fitted by adjustment of the co-operativity parameter, x . Table 2 gives the parameters used for the theoretical curves of Figs. 2, 3 and 4. It is interesting that, despite the

large variation in the molecular weights, initial net charges and the number of binding sites for the proteins, the variation in the values of E/kT is consistent with the formation of approximately four hydrophobic interactions on binding of sodium *n*-dodecyl sulphate to these proteins. It should be stressed, however, that the energy of ion-pair formation (approx. $4 \text{ kJ} \cdot \text{mol}^{-1}$; Davies, 1962) is of a comparable magnitude to the formation of a CH_2 hydrophobic bond, and, in the initial stages of non-co-operative binding, E/kT could be made up of contributions from both ionic and hydrophobic interactions.

Finally we consider the variation of the calculated association constants for non-co-operative (k_j) and co-operative (k_j^c) binding as a function of the extent of binding. Fig. 5 shows the plots of jk_j and jk_j^c as a function of j for the interaction of sodium *n*-dodecyl sulphate with bovine serum albumin. These plots would be linear if the binding sites were completely independent (Klotz, 1946). The non-co-operative jk_j plot shows the characteristic effects of attenuating interactions. The latter occur as a consequence of the increase in electrostatic free energy of the complex as binding proceeds, which will have an inhibitory effect on further binding. In contrast, the co-operative jk_j^c plot passes through a maximum. The rise in jk_j^c at low values of j is characteristic of accentuating interactions, which must result in a co-operative process. However, competing against this process is the rise in the electrostatic free energy of the complex and the limitations imposed by a finite number of binding sites. The jk_j and jk_j^c plots for ribonuclease A and β -lactoglobulin are analogous.

References

- Adair, G. S. (1925) *J. Biol. Chem.* **63-64**, 529-545
- Burkhard, R. K. & Stolzenberg, G. E. (1972) *Biochemistry* **11**, 1672-1677
- Cannon, R. K., Palmer, A. H. & Kibrick, A. C. (1942) *J. Biol. Chem.* **142**, 803-822
- Davies, C. W. (1962) *Ion Association*, p. 128, Butterworths, London
- Hartley, G. S. (1936) *Aqueous Solutions of Paraffin-Chain Salts*, p. 32, Hermann, Paris
- Jones, M. N., Skinner, H. A. & Tipping, E. (1975) *Biochem. J.* **147**, 229-234
- Klotz, I. M. (1946) *Arch. Biochem.* **9**, 109-117
- Klotz, I. M. (1974) *Acc. Chem. Res.* **7**, 162-168
- Oakes, J. (1974) *J. Chem. Soc. Faraday Trans. I* **70**, 2200-2209
- Pitt-Rivers, R. & Impiombato, F. S. A. (1968) *Biochem. J.* **109**, 825-830
- Reynolds, J. A. & Tanford, C. (1970) *J. Biol. Chem.* **245**, 5161-5165
- Reynolds, J. A., Herbert, S., Polet, H. & Steinhardt, J. (1967) *Biochemistry* **6**, 937-947
- Reynolds, J. A., Herbert, S. & Steinhardt, J. (1968) *Biochemistry* **7**, 1357-1361
- Tanford, C. (1961) *Physical Chemistry of Macromolecules*, p. 358, Wiley, New York
- Tanford, C. (1970) *Adv. Protein Chem.* **24**, 2-93
- Tanford, C. & Hauenstein, J. D. (1956) *J. Am. Chem. Soc.* **78**, 5287-5291
- Tanford, C., Swanson, S. A. & Shore, W. S. (1955) *J. Am. Chem. Soc.* **77**, 6414-6421
- Tipping, E., Jones, M. N. & Skinner, H. A. (1974) *J. Chem. Soc. Faraday Trans. I* **70**, 1306-1315