

976. *Reactivity Differences between Hæmoglobins. Part III.¹ The Effect of Ionic Strength and Multiple Charge Changes on the Free-energy and Enthalpy of Ionization of Methæmoglobins.*

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The effect of ionic strength on the linear relationship between the entropy and enthalpy of ionization of a methæmoglobin to its alkaline form is calculated using Tanford and Kirkwood's theory of electrostatic interactions in globular proteins. It is shown that when the ionic strength increases from zero to 0.05 the slope of the $T\Delta S$ versus ΔH plot decreases by about 10%. The conditions under which a linear $T\Delta S/\Delta H$ relationship will exist for a series of hæmoglobins differing by many charges are examined.

IN Part II of this series,¹ Kirkwood's theory² of electrostatic interactions in globular proteins was applied to the ionization of a methæmoglobin to its alkaline form. Expressions were derived for the difference in free-energies, enthalpies, and entropies of ionization at zero ionic strength of two methæmoglobins which differ from each other by changes in charged amino-acids at known positions in the molecule. It was shown that a linear plot of $T\Delta S^0$ versus ΔH^0 (the values of $T\Delta S$ and ΔH at zero ionic strength) is expected for a series of hæmoglobins in which the differences between the members consist only of changes in charges at the same site in the molecule. The equations derived in Part II are now extended to allow comparison with experimental data obtained at finite ionic strength, as well as to include hæmoglobins which differ from one another by charge changes at many sites in the molecule. The model used for the calculations in Part II and also in this Paper is that suggested by Kirkwood.² The protein is considered to be an impenetrable sphere of radius b and dielectric constant D_i . Point charges e_1, e_2, \dots, e_m characterize the average charge distribution of the molecule, r_{kl} is the distance between charges e_k and e_l , r_k and r_l are the distances of e_k and e_l from the centre of the sphere, and θ_{kl} is the angle between r_k and r_l . For reasons given in Part II the four iron atoms of methæmoglobin are assumed to have identical free-energies of ionization, and the calculations below are for one iron atom in the environment of the whole hæmoglobin molecule.

The Free-energy, Enthalpy, and Entropy of Ionization of Methæmoglobins A, S, and C at Finite Ionic Strength.—The nomenclature of the hæmoglobins was explained in detail in Part I. Hæmoglobin S differs from N-hæmoglobin A in having a valine instead of a glutamic acid residue in the sixth position from the terminal amino-acid residue of each of the β -polypeptide chains. In hæmoglobin C, the same glutamic acid is replaced by lysine in each β -polypeptide.

If ΔG_A^0 and ΔG_S^0 are the free-energies of ionization of methæmoglobins A and S at zero ionic strength, and the difference in free energies originates entirely from different electrostatic interactions, then

$$\Delta G_A^0 - \Delta G_S^0 = e\bar{G}_{P-(A)}^0 - e\bar{G}_{P-(S)}^0 - e\bar{G}_{PH(A)}^0 + e\bar{G}_{PH(S)}^0 \quad (1)$$

where $e\bar{G}_{P-(A)}^0$, $e\bar{G}_{PH(A)}^0$, $e\bar{G}_{P-(S)}^0$, and $e\bar{G}_{PH(S)}^0$ are the electrostatic contributions to the partial molal free energies of the alkaline and acid forms of methæmoglobins A and S. For any protein X the electrostatic contribution to the partial molal free energy expressed in cal. mole⁻¹ (see Tanford and Kirkwood³) is

$$e\bar{G}_X^0 = \frac{N}{4.2 \times 10^7} \left\{ \frac{1}{2} \sum_{k=1}^m \sum_{l=1}^m \frac{e_k e_l}{D r_{kl}} + \frac{1}{2} \sum_{n=0}^{\infty} \frac{(D_i - D)(n+1)A_n}{D_i b^{2n+1} [(n+1)D + nD_i]} \right\} \quad (2)$$

¹ Part II, Beetlestone and Irvine, *Proc. Roy. Soc.*, 1964, *A*, 277, 414.

² Kirkwood, *J. Chem. Phys.*, 1934, **2**, 351.

³ Tanford and Kirkwood, *J. Amer. Chem. Soc.*, 1957, **79**, 5333.

where $A_n = \sum_{k=1}^m \sum_{l=1}^m e_k e_l r_k^n r_l^n P_n(\cos \theta_{kl})$, N is Avogadro's number, D is the dielectric constant of the medium, and $P_n(\cos \theta_{kl})$ are the ordinary Legendre polynomials. Substitutions of equation (2) in equation (1), and use of the approximations and simplifications outlined in Part II, yields the equation

$$\Delta G_A^0 - \Delta G_S^0 = \frac{2N}{4.2 \times 10^7} \frac{e_q e_f}{r_{fq} D_E} \text{ cal. mole}^{-1} \quad (3)$$

where e_q is the charge which differentiates haemoglobins A and S, e_f the charge on the iron atom, r_{fq} the distance between e_f and e_q , and D_E the effective dielectric constant as defined by Kirkwood and Westheimer⁴ which is assumed to be constant for haemoglobins A, S, and C. An identical expression holds for $\Delta G_S^0 - \Delta G_C^0$, where ΔG_C^0 is the free-energy of ionization of methaemoglobin C at zero ionic strength.

At finite ionic strength an extra term must be added to equation (3). This term is a complicated function of the ionic strength, the position of the charges e_f and e_q , and the dielectric constants of the solvent and protein (see equation 5 of Tanford and Kirkwood³). However, at low ionic strength ($I < 0.01$) simplification of the term is possible,³ and the expression for the differences in free-energies of ionization of methaemoglobins A, S, and C at finite ionic strength becomes

$$\Delta G_A - \Delta G_S = \Delta G_S - \Delta G_C = \frac{2N e_f e_q}{4.2 \times 10^7} \left\{ \frac{1}{r_{fq} D_E} - \frac{\kappa}{D(1 + \kappa a)} \right\} \quad (4)$$

where $\kappa = \left(\frac{8\pi N e^2}{1000 D k T} \right)^{\frac{1}{2}} \sqrt{I}$, e being the electronic charge, k Boltzmann's constant, and T the absolute temperature. a is the distance of closest approach of the protein and a small ion.

In haemoglobins A, S, and C, $r_{fq} \simeq 30 \text{ \AA} \simeq a$. Equation (4) may therefore be written as

$$\Delta(\Delta G)_{AS} = \frac{2N e_f e_q}{4.2 \times 10^7 r_{fq}} \left\{ \frac{1}{D_E} - \frac{\kappa a}{D(1 + \kappa a)} \right\} \quad (5)$$

For reasons to be outlined in Part IV, comparison with experimental data is to be made at $I = 0.05$, and we assume equation (5) is approximately valid for this ionic strength. Then, substitution for $a = 30 \text{ \AA}$ and $\sqrt{I} = 0.224$ gives

$$\Delta(\Delta G^{0.05})_{AS} = P_{AS} \left[\frac{1}{D_E} - \frac{338}{D\{(DT)^{\frac{1}{2}} + 338\}} \right] \quad (6)$$

where

$$P_{AS} = \frac{2N e_f e_q}{4.2 \times 10^7 r_{fq}}$$

Hence,
$$\Delta(\Delta S^{0.05})_{AS} = \frac{\partial}{\partial T} \{-\Delta(\Delta G^{0.05})\} = P_{AS} \left\{ -\frac{\partial}{\partial T} \left(\frac{1}{D_E} \right) - 338Q \right\} \quad (7)$$

where

$$Q = \frac{D + T(\partial D/\partial T)}{2D(DT)^{\frac{1}{2}}\{(DT)^{\frac{1}{2}} + 338\}^2} + \frac{1}{D^2\{(DT)^{\frac{1}{2}} + 338\}} \frac{\partial D}{\partial T}$$

Therefore,
$$m_{0.05} \equiv \frac{\Delta(T\Delta S^{0.05})}{\Delta(\Delta H^{0.05})} = \frac{-T\partial(1/D_E)/\partial T - 338TQ}{\frac{1}{D_E} - \frac{338}{D\{(DT)^{\frac{1}{2}} + 338\}} - T\frac{\partial}{\partial T}\left(\frac{1}{D_E}\right) - 338TQ} \quad (8)$$

The comparable expression for $I = 0$ is given by equation (11) of Part II, *viz.*,

$$m_0 \equiv \frac{\Delta(T\Delta S^0)}{\Delta(\Delta H^0)} = T \frac{\partial \left(\frac{1}{D_E} \right)}{\partial T} \left/ \left\{ \frac{1}{D_E} - T \frac{\partial \left(\frac{1}{D_E} \right)}{\partial T} \right\} \right. \quad (9)$$

m_0 has replaced the symbol m used in Part II in order to emphasize that it is the slope of the $T\Delta S$ against ΔH plot at $I = 0$.

We are now in a position to ascertain the relative magnitudes of the slopes of the $T\Delta S$ against ΔH plots at $I = 0$ and $I = 0.05$. The Table gives the calculated values of m_0 and $m_{0.05}$ for different values of θ_{kl} , corresponding to different distances between the charges e_f and e_q . The method of calculating D_E and $\partial(1/D_E)/\partial T$ has already been outlined in Part II and the values appropriate to each value of θ_{kl} are also recorded in the Table. It is seen that except for the case where the charges e_f and e_q are very close, m_0 is expected to be about 10% greater than $m_{0.05}$. For $\theta = 90^\circ$ corresponding to $m_0 = 1.14$ (the experimental slope of $T\Delta S^0$ versus ΔH^0 for methæmoglobins A, S, and C⁵), m is expected to be 1.06.

Calculated values of m_0 and $m_{0.05}$.

θ_{kl}	D_E	$10^4 \partial(1/D_E)/\partial T$	m_0	$m_{0.05}$	r (Å)
180°	92	3.88	1.11	1.02	57
90	60	4.73	1.14	1.06	48
45	32.6	6.09	1.21	1.13	20
25	15.8	6.85	1.46	1.35	11.5
8	4.1	4.6	-1.25	-1.75	3.6

Electrostatic Contribution to the Differences in Free-energies and Enthalpies of Ionization of Methæmoglobins Differing by Many Charges.—Consider two hæmoglobins, X and Y, which differ by n charges $e_1, e_2 \dots e_1 \dots e_n$ which are at distances $r_1, r_2 \dots r_1 \dots r_n$, respectively, from the iron atom. If the difference between the free-energy of ionization of methæmoglobin X and of methæmoglobin Y is entirely electrostatic in origin, the corresponding equation to equation (3) is

$$\Delta(\Delta G^0)_{XY} = \sum_{i=1}^{i=n} \frac{N}{4.2 \times 10^7} \frac{e_i e_f}{r_{ij} D_{E(Y)}} \quad (10)$$

$D_{E(Y)}$ is the effective dielectric constant appropriate for the charges e_i and e_f , and it will vary according to the relative positions of these charges. Thus, in general, where we have two hæmoglobins which differ by many charges a simple expression analogous to equation (9) cannot be derived. However, under certain conditions, simplification of equation (10) is possible, as consideration of two extreme cases will indicate. Consider first the case of two hæmoglobins which differ by a charge as far as possible from the iron atom. In this case m_0 (see Table) is 1.11. The corresponding value of m_0 for a pair of hæmoglobins differing by a charge in such position that $\theta = 25^\circ$ is 1.46. At the same time the value of D_E changes from 92 to 16. Thus D_E changes by a factor of approximately 6 while m_0 changes by only 40%. The corresponding values of $m_{0.05}$ are 1.02 and 1.35. Assuming that charge changes can occur with equal probability anywhere on the surface of the protein, then $25^\circ < \theta < 180^\circ$ corresponds to 95% of all possible charge changes. Hence, for 95% of all possible charge changes both m_0 and $m_{0.05}$ remain constant to within $\pm 20\%$. The concept of an average m for 95% of all possible charge changes is therefore a useful one, even though the individual values of D_E and $\partial(1/D_E)/\partial T$ vary considerably. Corresponding to this average m , \bar{m} , we define an average effective dielectric constant \bar{D}_E . Equation (10) may now be rewritten

$$\Delta(\Delta G^0)_{XY} = P_{XY}/\bar{D}_E$$

⁵ Beetlestone and Irvine, *Proc. Roy. Soc.*, 1964, A, 277, 401.

where P_{XY} is a constant for the two haemoglobins and is independent of temperature. \bar{m}_0 , the slope of the $T\Delta S^0$ against ΔH^0 plot is then given by

$$\bar{m}_0 \equiv -T \frac{\partial}{\partial T} \left(\frac{1}{\bar{D}_E} \right) / \left\{ \frac{1}{\bar{D}_E} - T \frac{\partial}{\partial T} \left(\frac{1}{\bar{D}_E} \right) \right\}$$

The equation for $\bar{m}_{0.05}$ is the same as equation (8) with \bar{D}_E replacing D_E . For any series of methaemoglobins, for which electrostatic interactions are the sole origin of the differences in ΔH and $T\Delta S$, $\bar{m}_{0.05}$ will be constant only if in the series all charge differences are such that for each of them $25^\circ < \theta < 180^\circ$, since for $\theta < 25^\circ$ the concept of an average D_E for the purpose of calculating m is no longer valid, as the Table shows. Conversely, if for any series of methaemoglobins a plot of $T\Delta S$ versus ΔH of ionization is linear, and the points for methaemoglobins A, S, and C (where it has been established that the differences in $T\Delta S$ and ΔH are solely electrostatic in origin) fall on the line, we may conclude that (i) the differences between the values of ΔH and $T\Delta S$ of ionization are entirely electrostatic in origin, and (ii) the charge changes which differentiate the haemoglobins do not occur at positions such that $\theta < 25^\circ$; that is to say, they do not occur closer than 10 Å to the iron atoms. A change of only one charge for which $\theta < 25^\circ$ would cause a large deviation from the $T\Delta S$ against ΔH line because at this small distance the value of D_E is also small, and hence the electrostatic interaction is large.

We have shown above that for $25^\circ < \theta < 180^\circ$ the value of m_0 varies only between 1.11 and 1.46 and that of $m_{0.05}$ between 1.06 and 1.35. This has led us to introduce the concept of an average m (\bar{m}), and, correspondingly, an average dielectric constant (\bar{D}_E). The question now arises as to what values should be assigned to \bar{m} and \bar{D}_E at $I = 0$ and $= 0.05$, respectively. The problem is essentially one of finding the relative weighting to be given to each value of m and of D_E between $\theta = 25^\circ$ and $\theta = 180^\circ$. This requires calculation of the relative number of charged sites for each θ , assuming that charge changes can occur with equal probability anywhere on the surface of the molecule. Simple calculation along these lines show that the average slope, \bar{m} , is given by the equation

$$\bar{m} = \int_{0.139\pi}^{\pi} m \sin \theta \, d\theta / \int_{0.139\pi}^{\pi} \sin \theta \, d\theta$$

\bar{D}_E is given by the analogous equation with D_E replacing m . Graphical evaluation of the integral shows that \bar{m}_0 is 1.16 and $\bar{m}_{0.05}$ is 1.08.

The relative values of m_0 , $m_{0.05}$, \bar{m}_0 , and $\bar{m}_{0.05}$ calculated above will be compared with experimentally determined values in Part IV.

This work was supported in part by a grant from the United States Public Health Service.

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[Received, January 13th, 1964.]