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PH DEPENDENT VOLUME CHANGES ACCOMPANYING THE BINDING REACTIONS OF HUMAN AND PIGEON METHEMOGLOBINS

Gabriel B. OGUNMOLA
Department of Chemistry, University of Ibadan, Ibadan, Nigeria

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Dilatometric measurements of the volume changes accompanying the binding reactions of azide ion to human adult and pigeon methemoglobins as a function pH at 25°C demonstrate pH values of maximum volume change ($pH_{\Delta V max}$) which are different for the different hemoglobins. $pH_{\Delta V max}$ occurs at pH 6.7 for human methemoglobin A and at pH 7.7 for pigeon methemoglobin. The $pH_{\Delta V max}$ occurs near the characteristic pH (pH_{ch}) of maximum enthalpy of the same binding reaction. It is shown that the large pH variation in ΔV can arise if the configuration of charged groups on the surface of the molecule is different in methemoglobin and methemoglobin complex. When such a difference in configuration exists the addition of the same number of protons to methemoglobin and methemoglobin complex will give rise to different changes in the partial molar volume of the two species.

1. Introduction

The measurement of volume change ΔV, by dilatometry [1] affords a direct observation of the change in an extensive property during a chemical process and attempts to relate ΔV to known chemical changes are at the basis of most of the studies on volume changes in protein reactions [2,3]. In the previous work [4], we reported the ΔV accompanying the binding of ligand L, to methemoglobin ($HbOH_2$) and metmyoglobin ($MbOH_2$) as a function of pH using the dilatomeric measurement and the pressure dependence of the equilibrium constant for the reaction [4] $HbOH_2 + L \rightleftharpoons HbL + H_2O$. It was observed that the ΔV, accompanying the axide and cyanide binding reactions varied with pH and showed a maximum [4]. Similar pH profile of enthalpy of ligand binding has been observed [5]. If this behaviour is general to different methemoglobins in their binding reactions, it will be an indication of a common structural factor relating the pH characteristic of enthalpy of ligand binding to another obligatory thermodynamic constant, in this case the ΔV of the ligand binding to methemoglobin.

Of particular relevance to the work described in this paper is the concept of characteristic pH [5,6] of a hemoglobin (pH_{ch}). We define the characteristic pH of a hemoglobin as the pH at which the enthalpy of formation of a methemoglobin complex with a charged ligand passes through a maximum and we have shown that it is sensitive to changes in the amino acid composition. For a large number of hemoglobins, pH_{ch} is correlated with differences between the number of lysine and arginine residues and the number of glutamic acid and aspartic acid residues in the molecule [7]. These effects would arise if the charged amino acid on the surface of the molecule change to a new configuration in a concerts manner in the region of the characteristic pH. Such changes in the configuration of charged groups through changes in hydration structure might give rise to significant volume changes and accompanying compensating enthalpy and entropy changes which has been shown to be characteristic of the complex formation in methemoglobins. The correlation between the pH_{ch} and the relative composition of charged amino acids [7] implies that a change of a single amino acid involving a change in charge can affect the magnitude of a property of methemoglobin. We have therefore been led to suggest that even-though ΔV is a small obligatory quantity in the thermodynamic expression for the total Gibbs free energy change, since its value and pH profile runs parallel to important thermodynamic constants in term of the enthalpy and entropy of the binding reactions for dif-

ferent methemoglobins, the pH profile of the ΔV associated with ligand binding to methemoglobin might vary with species in a manner that, there would exist a pH of maximum ΔV which might be related to charged amino acid composition as does the pH_{ch}. We have therefore measured the ΔV accompanying the reaction of pigeon and human A methemoglobin with azide ions at 25°C using dilatometeric technique, in order to find out if ΔV – pH profile of the methemoglobin binding reaction would vary with species and with the charged amino acid composition.

2. Materials and method

Human and pigeon methemoglobins were prepared as previously described [8]. Phosphate and borate buffers $I = 0.5$ were used throughout. Concentrated hemoglobin solutions were prepared by pressure dialysis against appropriate buffer solutions and hemoglobin concentration was determined using $\epsilon^{540}_{HbCN} = 10.9$ mM. Dilalometric measurements were made as described in the previous report [4] at 25°C.

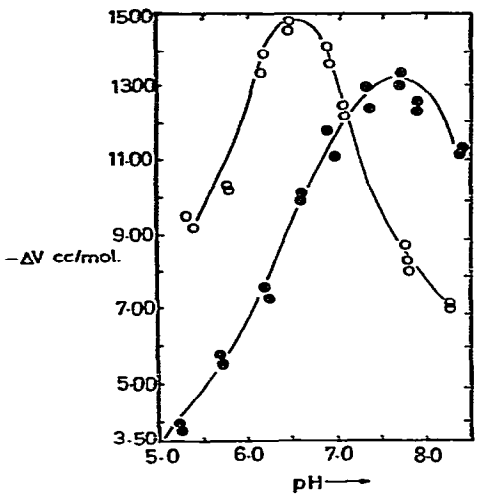

Fig. 1. Plot of ΔV against pH accompanying the reaction of azide ion with methemoglobins: o human adult methemoglobin A and • pigeon methemoglobin.

Table 1
Values of the volume change accompanying the binding of azide ion with human adult and pigeon methemoglobins

	$(-\Delta V)$ Volume change/mole Fe
	(a) human adult methemoglobin
5.30	9.32 ± 0.16
5.78	10.23 ± 0 08
6.27	13.62 ± 0.23
6.48	14.71 ± 0.14
6.92	14.37 ± 0.24
7.10	12.35 ± 0.15
7.76	9.03 ± 0.24
8.26	7.06 ± 0.07
	(b) pigeon methemoglobin
5.23	3.99 ± 0.08
5.68	5.72 ± 0.11
5.93	7.48 ± 0.16
6.60	9.83 ± 0.48
6.98	11.46 ± 0.34
7.36	12.72 ± 0.26
7.73	12.84 ± 0.48
7.96	12.48 ± 0.09
8.44	11.29 ± 0.10

3. Result and discussion

Table 1 gives the values of the ΔV accompanying the binding of azide ions to human adult and pigeon methemoglobins at 25°C and fig. 1 is the plot ΔV accompanying the azide binding reaction against pH for pigeon and methemoglobin A. For each methemoglobi there is a contraction in volume on the binding of ligar making the ΔV accompanying the ligand binding reaction negative. The volume change becomes more negative with pH and reaches a maximum at a $pH_{\Delta V max}$ close to the characteristic pH of the human adult and pigeon methemoglobins. Above this pH of maximum ΔV, the volume change becomes less negative with increase in pH. $pH_{\Delta V max}$ occurs at pH 6.7 fcr methemoglobin A and pH 7.7 for pigeon methemoglobin.

The result in fig. 1 shows that ΔV–pH profile is qualitatively similar in character to the ΔH – pH profi for the binding reaction of methemoglobin, there bein a pH of maximum ΔV, $pH_{\Delta V max}$, which varies from

one hemoglobin to another and the position of $pH_{\Delta V max}$ occurs in the pH region which is close to the pH_{ch}. It is therefore suggestive that the $pH_{\Delta V max}$ and pH_{ch} might have a common structural origin and might be related to the composition of charged groups on the protein.

What explanations could be given to the bell shaped variation of ΔV with pH? In order to relate ΔV to known chemical and structural changes in the methemoglobin molecule we consider the equilibrium binding reaction

$$Hb^+OH_2 + L^- \rightleftharpoons HbL + H_2O.$$

The overall equilbrium constant K_L for the above reaction of methemoglobin ($HbOH_2$) with a ligand, L^- is related to the volume change associated with the same binding reaction through the pressure dependence of the overall equilibrium constant according to the expression:

$$\partial \ln K/\partial p = -\Delta V/RT.$$

At constant temperature, the pH dependence of the equilibrium constant could be accounted for if the ligand binding process is assumed linked to the release of a proton and we can write the methemoglobin reaction as

$$Hb^+OH_2 + L^- \rightleftharpoons HbL + \psi H^+ H_2O,$$

where ψ is the number of hydrogen ion released at a particular pH and arises from the differences between the pK values of linked ionizable groups on Hb^+OH_2 and HbL. Wyman linkage expression [9] for the binding reaction and the linked ionized group could be written as

$$\partial \log K/\partial pH = \psi.$$

In order to apply the linkage equation to account for the pH profile of volume changes, we differentiate the Wyman linked equation expression with respect to pressure and obtain the expression:

$$\frac{\partial}{\partial p}\frac{(\partial \log K)}{\partial pH} = \frac{\partial \psi}{\partial p}, \quad \frac{\partial}{\partial pH}\frac{(\partial \log K)}{\partial p} = \frac{\partial \psi}{\partial p}.$$

Substituting the expression in equation relating log K_L to ΔV we have

$$\frac{\partial}{\partial pH}\frac{(-\Delta V)}{2.303\,RT} = \frac{\partial \psi}{\partial p}.$$

At constant pressure $\partial\psi/\partial p$ would be zero, which should make the expression

$$\frac{\partial}{\partial pH}\left(\frac{-\Delta V}{2.303\,RT}\right)$$

equal to zero.

From the slope of ΔV – pH curve in fig. 1, $\partial V/\partial pH$ is finite on both sides of $pH_{max\Delta V}$. On the acid side of $pH_{max\Delta V}$ ($\partial \Delta V/\partial pH$) is positive with a value of ~ 5 cm^3/pH unit. The slope $\partial \Delta V/\partial pH$ for human adult A and pigeon methemoglobin are similar on either side of $pH_{max\Delta V}$ and in order to account for the pH dependence volume change for the reaction of methemoglobin A and pigeon with azide ion the Wyman's relationship does not hold. Obviously some of our assumptions must be incorrect or some of the assumptions that are inherent in the Wyman relations may not be valid. There are several assumptions contained in Wyman's expression, the most significant of which is the fact that the ratio of activity coefficients of the various protein species remains constant. This arises largely out of the choice of standard state. Wyman chooses the standard state for reactant and product to be a solution in which the hemoglobin molecule is in the state where proton has been added to all possible ionizable groups. Any variation of the pH dependence of the chemical potential of either reactant or product apart from those arising from loss of proton could be buried in the activity coefficient term defined in terms of the chosen standard state.

Specifically we choose a new standard state of one molar protein solution with properties such that as the protein concentration and ionic strength are reduced to zero, the activity coefficient approaches unity assuming that the protein retains the same net charge and protein configuration under the experimental condition of ionic strength and pH. On this basis following the earlier procedures introduced by Beetlestone and co-workers [10] the Wyman relationship is modified thus

$$\frac{\partial \log K_L}{\partial pH} = \psi - \frac{1}{2.303\,RT}\frac{\partial}{\partial pH}(\mu^0_{Hb\,X_u} - \mu^0_{Hb_u}),$$

where $\mu^0_{HbX_u}$, $\mu^0_{Hb_u}$ are the chemical potential of the methemoglobin with bound ligand and without ligands respectively. In both methemoglobin and its complex the linked groups are in unionized form. Using this ex-

pression we can derive the volume change relationship by differentiating the above expression with respect to pressure and obtained the expression

$$\frac{\partial}{\partial \mathrm{pH}}\frac{\partial \log K_L}{\partial p} = \frac{\partial \psi}{\partial p} - \frac{1}{2.303\,RT}\frac{\partial}{\partial \mathrm{pH}}\left(\frac{\partial \mu^0_{\mathrm{HbX_u}}}{\partial p} - \frac{\partial \mu^0_{\mathrm{Hb_u}}}{\partial p}\right)$$

$$\frac{\partial}{\partial \mathrm{pH}}\left(\frac{-\Delta V}{2.303\,RT}\right)$$

$$= \frac{\partial \psi}{\partial p} - \frac{1}{2.303\,RT}\frac{\partial}{\partial \mathrm{pH}}\left(\frac{\partial \mu^0_{\mathrm{HbX_u}}}{\partial p} - \frac{\partial \mu^0_{\mathrm{Hb_u}}}{\partial p}\right),$$

$$\frac{\partial}{\partial \mathrm{pH}}\left(\frac{-\Delta V}{2.303\,RT}\right) = \frac{\partial \psi}{\partial p}$$

$$- \frac{1}{2.303\,RT}\frac{\partial}{\partial \mathrm{pH}}(\phi V_{\mathrm{HbX_u}} - \phi V_{\mathrm{Hb_u}}).$$

At constant pressure $\partial\psi/\partial p$ will be zero, the above expression then becomes

$$\frac{\partial}{\partial \mathrm{pH}}(-\Delta V) = \frac{\partial}{\partial \mathrm{pH}}(\phi V_{\mathrm{HbX_u}} - \phi V_{\mathrm{Hb_u}}) = \frac{\partial}{\partial \mathrm{pH}}(\Delta \phi V)_{\psi V}$$

where $\phi V_{\mathrm{HbX_u}}$ and $\phi V_{\mathrm{Hb_u}}$ are the partial molar volume of the methemoglobin complex and methemoglobin respectively with the linked groups unionized in both cases. $(\partial/\partial \mathrm{pH})(-\Delta V)$ would be positive if $\phi V_{\mathrm{HbX_u}} > \phi V_{\mathrm{Hb_u}}$ tending to suggest that below $\mathrm{pH}_{max\Delta V}$ the pH variation of partial molar volume of the liganded methemoglobin would be greater than that of aquomethemoglobin.

On the alkaline side of $\mathrm{pH}_{max\Delta V}$ both the liganded and the aquo methemoglobin would be present in the form in which the linked groups are in their ionized forms and the species making contribution to the chemical potential would be largely $\mathrm{HbX_i}$ and $\mathrm{Hb_i}$. The expression in previous equation would become

$$\frac{\partial(-\Delta V)}{\partial \mathrm{pH}} = \frac{\partial}{\partial \mathrm{pH}}\left(\frac{\partial \mu_{\mathrm{HbX_i}}}{\partial p} - \frac{\partial \mu_{\mathrm{Hb_i}}}{\partial p}\right) = \frac{\partial}{\partial \mathrm{pH}}(\Delta \phi V)_{\mathrm{i}}$$

The negative slope of ΔV – pH profile above the $\mathrm{pH}_{max\Delta V}$ could be accounted for if the pH dependence of the partial molal volume of the aquomethemoglobin with the linked groups in the ionized form is greater than the partial molar volume of the liganded complex. We could therefore suggest that the partial molar volum change of the liganded and aquomethemoglobin undergoes a reversal of molar volume changes on passing the $\mathrm{pH}_{max\Delta V}$ due to the ionization of the linked groups on the protein.

As the pH increases on the acid side of the $\mathrm{pH}_{\Delta V max}$ ionization of charged groups would increase and this process is accompanied by a contraction of about 5 cm^3 per unit pH. This could be explained if charged groups on the surface of the protein carry into the bulk solvent some of the "frozen" water of hydration on the protein molecule, or that the volume of water which is not diffusible to ions due to electrostatic effect of image charg in the low dielectric provided by the protein interior decreases with complex formation. The configurations of the methemoglobin and its complex at any pH is determined by orientation of charge groups resulting in a unique configuration where positively and negatively charged groups are occupying positions that define a unique hydration structure of the methemoglobin and its complex at each pH. The position of the $\mathrm{pH}_{\Delta V max}$ would therefore depend intimately on the relative number of positive and negatively charged amino acids which in turn is related to pH_{ch} and hence $\mathrm{pH}_{\Delta V max}$ ma therefore follow closely pH_{ch} for each hemoglobin.

Drude and Nernst [12] showed that if a charge Z is uniformly distributed on the surface of a sphere of radius a and immersed in a dielectric, the dielectric will undergo a volume change ΔV due to electrostriction given by the expression

$$\Delta V = -\beta\,(V \mathrm{d}D/\mathrm{d}V)\,e^2Z^2/2aD^2,$$

where β is the compressibility, D and V are the dielectric constant and volume of the dielectric respectively. From this expression as the pH increases when the net charge Z on the methemoglobin and its complex also increases the positive value of $\partial V/\partial \mathrm{pH}$ on the alkaline side of $\mathrm{pH}_{\Delta V max}$ could be explained if as the pH increases addition of ligands decrease the net charge of the complex and the partial molar volume of the methemoglobin complex with respect to the methemoglobin at the same pH. In other words addition of the same number of protons to methemoglobin and its complex will give rise to different changes in the partial molar volume of the two species. Changes of orientation of these charged groups and the accompanying changes in

hydration are intimately linked to the volume and enthalpy changes associated with the ligand binding process through changes in the environment of the heme resulting from spin state changes and protolysis of crucial groups most especially the distal group in the molecule, whose configuration would respond to changes in the spin state of the iron as well as the hydration structure of the molecule. Such a mechanism may account for the variable volume and enthalpy changes with different ligands as has been previously observed.

The physical and biological significance of the pH dependent volume changes characteristic of methemoglobins may lie in the correspondence between it and the pH dependent changes in enthalpy with characteristic compensation in entropy changes both quantities of which show similar pH profile with a pH of maximum value characteristic of each methemoglobin. pH dependent volume changes like the pH dependent enthalpy and compensating entropy changes may therefore be dependent on the same process which derives a substantial contribution from fluctuations in spin and protein configurational changes.

To the extent that the characteristic pH of a methemoglobin that arises from its pH dependent enthalpy of ligand binding is intimately linked with the charged amino acid composition of the hemoglobin that determined the sensitivity of a hemoglobin oxygen affinity to the binding of diphosphoglyceric acid (DPG) which meets the metabolic need of the animal species [13], it is not inconceivable that volume changes like enthalpy changes with similar profile and pH dependence that derives from similar structural and thermodynami basis would also be equally related to function.

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