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The uptake of protons by heme-linked ionizable groups on azide binding to methemoglobin

Kehinde Onwochei OKONJO and Fernando J. VEGA-CATALAN
Departments of Chemistry and Computer Science, University of Ibadan

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When azide ion reacts with methemoglobin in unbuffered solution the pH of the solution increases. This phenomenon is associated with increases in the pK values of heme-linked ionizable groups on the protein which give rise to an uptake of protons from solution.

We have determined as a function of pH the proton uptake, Δh^+, on azide binding to methemoglobin at 20 °C. Data for methemoglobins A (human), guinea pig and pigeon are fitted to a theoretical expression based on the electrostatic effect of three sets of heme-linked ionizable groups on the binding of the ligand. From these fits the pK values of heme-linked ionizable groups are obtained for liganded and unliganded methemoglobins. In unliganded methemoglobin pK_1, which is associated with carboxylic acid groups, ranges between 4.0 and 5.5 for the three methemoglobins; pK_2, which is associated with histidines and terminal amino groups, ranges from 6.2 to 6.7. In liganded methemoglobin pK_1 lies between 5.8 and 6.3 and pK_2 varies from 8.1 to 8.5.

The pH dependences of the apparent equilibrium constants for azide binding to the three methemoglobins at 20 °C are well accounted for with the pK values calculated from the variation of Δh^+ with pH.

The reaction of ligands with heme proteins is influenced to an appreciable extent by the state of ionization of groups on the protein [1 – 7]. This effect is reciprocal, and pK changes of ionizable groups on the protein may occur when ligands bind to the iron atoms of heme proteins [7 – 9]. The number and nature of such heme-linked ionizable groups have been determined in some ferric heme proteins from the pH dependence of the kinetics of ligand binding [1 – 7].

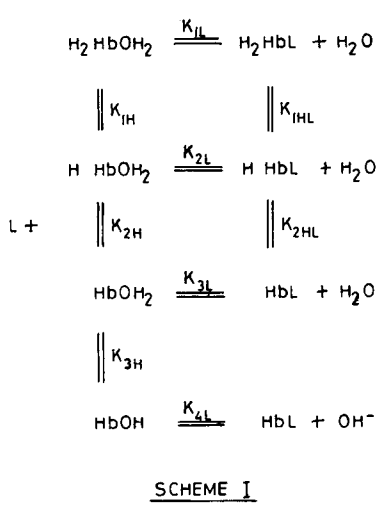
By studying the pH dependence of the apparent second-order complex-formation rate constant and of the first-order complex-dissociation rate constant of turnip peroxidases P_1 and P_7, Job and Ricard [7] demonstrated that the pK values of heme-linked ionizable groups on these proteins increase on ligand binding. A method such as this for determining the pK values of heme-linked ionizable groups of liganded and unliganded heme proteins can only be applied to methemoglobin with difficulty. We have shown that such pK values can be determined for unliganded methemoglobin with cyanide ion as ligand [6]. Unfortunately, however, the apparent dissociation rate constant of cyanide-methemoglobin complex is so small [6] that its determination is subject to a great deal of uncertainty. Thus the pK values of ionizable groups of liganded methemoglobin cannot be determined by this method with cyanide as the ligand. In contrast to the simple kinetic time course for cyanide binding [6], the complex kinetic time course for the binding of other ligands to methemoglobin precludes application of the method of Job and Ricard [7] to these ligands.

It is known that the binding of azide ion to methemoglobin in unbuffered solution gives rise to an uptake of protons from solution [10]. We have therefore determined the pH dependence of the proton uptake on azide binding to methemoglobin, in an attempt to measure the pK changes of heme-linked ionizable groups on methemoglobin. The data are fitted to a theoretical equation derived on the basis of a reaction scheme in which ligand binding is subject to the electrostatic influence of three sets of heme-linked ionizable groups [6]. We find that azide binding gives rise to increases in the pK values of heme-linked carboxylic, histidyl and terminal amino groups of methemoglobin.

Correspondence to K. O. Okonjo, Department of Chemistry, University of Ibadan, Ibadan, Nigeria

MATERIALS AND METHODS

To free it from small ions and organic phosphates, methemoglobin was passed slowly through a Dintzis column [11] into a jacketted (thermostatted) titration vessel, at the neck of which a Radiometer GK 2401 C combined electrode had been sealed. In order to eliminate CO_2 completely from the solution, nitrogen gas was passed through bubble bottles containing 0.4 M NaOH and distilled water and then over the methemoglobin solution which was stirred magnetically. After about 30 min the solution was brought to the desired pH by adding small quantities of either 0.1 M hydrochloric acid or 0.1 M NaOH. The pH was measured on a Radiometer PHM 4d pH meter. An aliquot was withdrawn from the titration vessel to determine the methemoglobin concentration, which was about 0.2 mM. An amount of 0.1 M NaN_3 solution, pretitrated to the same pH as the methemoglobin and calculated to give 90% reaction, was added to the titration vessel. The appropriate binding constant was assumed for the methemoglobin-azide complex at 20 °C [12]. On addition of azide, the pH of the solution increased and was brought back to the original pH with a known quantity of either 0.1 M or 0.01 M hydrochloric acid in an Agla syringe. The procedure was repeated at various pH values with fresh samples of methemoglobin. The result was calculated as moles of proton taken up per mole of azide reacted, after correction to 100% reaction. Values of proton uptake are subject to a standard



SCHEME I

error of ± 0.05. Each titration was done in the presence of 0.05 M NaCl.

RESULTS AND DISCUSSION

The reaction of methemoglobin with an anionic ligand may be formulated according to Scheme I in which three sets of heme-linked ionizable groups influence the binding of ligand. Scheme I is identical to the scheme in our previous report [6] except that it has been written to emphasize the displacement, by ligand, of the water molecule present at the sixth coordination position of the iron atoms of methemoglobin. Furthermore, the different liganded species are here written out explicitly to facilitate the inclusion of the ionization constants of liganded species. In Scheme I, K_{iH} ($i = 1$, 2, 3) are the ionization constants of heme-linked groups in unliganded methemoglobin, and they are identical to the K_i of our previous report [6]. The K_{iHL} ($i = 1$, 2) are the corresponding ionization constants of heme-linked groups in liganded methemoglobin. In terms of these parameters the proton uptake per mole of azide reacted, Δh^+, is given by:

$$\Delta h^+ = \frac{2[H^+]^2 + K_{1HL}[H^+]}{2\{[H^+]^2 + K_{1HL}[H^+] + K_{1HL}K_{2HL}\}} + \frac{K_{3H}}{K_{3H} + [H^+]} - \frac{3[H^+]^3 + 2K_{1H}[H^+]^2 + K_{1H}K_{2H}[H^+]}{3\{[H^+]^3 + K_{1H}[H^+]^2 + K_{1H}K_{2H}[H^+] + K_{1H}K_{2H}K_{3H}\}}. \quad (1)$$

In Scheme I and Eqn (1) it is assumed that azide is entirely in the anionic form. This assumption is justified under our experimental conditions (pH $\geqslant 6$) because pK_a for hydrazoic acid is 3.5 at 22 °C [13].

Fig. 1 shows the Δh^+ data for (A) human methemoglobin A [10], and the corresponding data for (B) guinea pig and (C) pigeon methemoglobins, respectively. The full lines in these figures are theoretical lines calculated from Eqn (1). It is seen that reasonably good fits are obtained. The fitting parameters are shown in Table 1. Details of the curve-fitting procedure have been described elsewhere [6, 14].

We recently presented arguments to show that the pK values reported in Table 1 cannot be assigned solely to individual ionizable groups close to the heme but to sets of ionizable amino acid residues with similar functional groups [6]. Members of each set may be anywhere in the protein, provided they are within the functional electrostatic domain of the

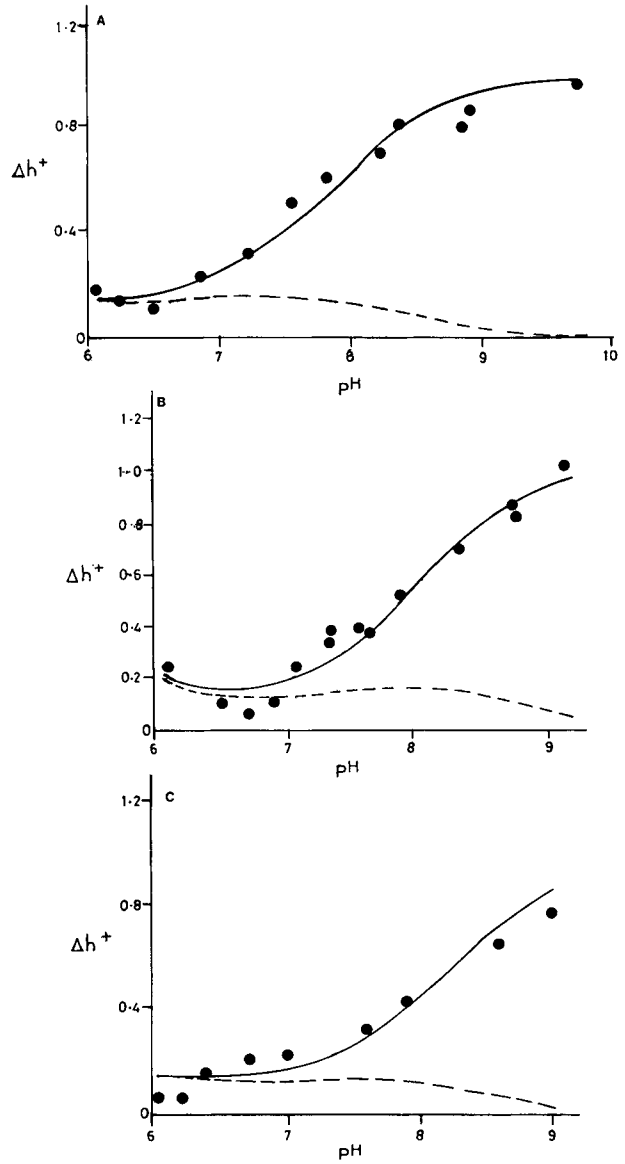


Fig. 1. *Reaction of methemoglobins with azide ion.* Molar ratio of proton uptake/azide reacted, Δh^+, as a function of pH at 20 °C, ionic strength 0.05 M (NaCl). (A) Human A (data of Anusiem et al. [10]); (B) guinea pig; (C) pigeon. The full lines through the data points are theoretical lines calculated with Eqn (1) of the text on the basis of Scheme I. The fitting parameters are shown in Table 1. The broken lines are theoretical lines calculated on the assumption that hydroxide ion is not displaced from alkaline methemoglobin by azide ion. All Δh^+ values are subject to a standard error of ± 0.05

Table 1. *Fitting parameters used to calculate the theoretical lines in Figs 1 and 2 according to Eqns (1) and (2) of the text, respectively (cf. Scheme I)*

Parameter	Value for methemoglobin		
	A	guinea pig	pigeon
pK_{1H}	5.22	3.98	5.52
pK_{2H}	6.23	6.70	6.74
pK_{3H}	8.00	8.18	8.32
pK_{1HL}	5.86	6.25	6.32
pK_{2HL}	8.15	8.48	8.37
$10^{-5}K_{1L}M^{-1}$	5.21	6.9	2.26
$10^{-5}K_{2L}M^{-1}$	5.0	1.9	2.25
$10^{-5}(K_{3L}+K_{4L})M^{-1}$	3.13	1.0	1.20

protein. We assigned pK_{1H} to carboxylic acid groups, pK_{2H} to histidines and terminal amino groups, and pK_{3H} to the acid-alkaline transition of unliganded methemoglobin [6]. pK_{1HL} and pK_{2HL} are the corresponding values for carboxylic acid groups and for histidines and terminal amino groups of liganded methemoglobin, respectively.

A comparison of pK_{1H} and pK_{1HL} for the three methemoglobins shows that the pK of heme-linked carboxylic groups increases on azide binding. Similarly pK_{2HL} is greater than pK_{2H}, indicating that the pK values of histidines and terminal amino groups also increase on azide binding. Thus when the positive charges on the iron atoms of methemoglobin are neutralized by azide ion the pK values of heme-linked ionizable groups increase. These increases in pK, and the consequent uptake of protons from solution, arise because the potential field sensed by the electrostatically heme-linked ionizable groups on the protein becomes more negative when the positive charges on the iron atoms are neutralized.

Job and Ricard [7] demonstrated that the pK values of ionizable groups in turnip peroxidases increase on ligand binding. However, these authors did not directly demonstrate that there is an uptake of protons on ligand binding to these proteins. On the other hand, while it was demonstrated that there is an uptake of protons on azide binding to methemoglobin [10], there was no indication of the number of groups involved or of their pK values. In this report we have been able to demonstrate directly the uptake of protons and have, in addition, calculated the pK values of the groups responsible for proton uptake on ligand binding.

We now want to address ourselves to the question of whether hydroxide ion can be displaced from alkaline methemoglobin by other ligands. This question is important because in a previous report [6] we found, in agreement with Job et al. [5], that the rate of replacement of hydroxide ion by cyanide ion was negligibly small and concluded that cyanide ion does not displace hydroxide ion from alkaline methemoglobin to any measurable extent. Moreover, Job and Ricard [7], in their kinetic models for fluoride and cyanide binding to turnip peroxidases P_1 and P_7, do not take into consideration the possibility of replacement of hydroxide ion from alkaline peroxidase.

If azide ion does not displace hydroxide ion from alkaline methemoglobin, then the last ligand binding step of Scheme I, namely,

$$L + HbOH \overset{K_{4L}}{\rightleftharpoons} HbL + OH^-$$

would be eliminated, and the expression for Δh^+ would be Eqn (1), but without the term $K_{3H}/(K_{3H} + [H^+])$. This modified expression for Δh^+ is shown plotted (with the pK values reported in Table 1) as broken lines in Fig. 1. It is clear that the fit to the data is very poor. Attempts to fit the modified expression to the data with other chemically reasonable pK values also failed. We are therefore led to conclude that azide ion does displace hydroxide ion from alkaline methemoglobin. Since cyanide ion has a higher binding constant than azide ion [12, 15], it is very likely that cyanide ion also displaces hydroxide ion. Our previous data [6], however, indicate that the rate of this displacement is several orders of magnitude slower than the rate of displacement of water from acid methemoglobin. The data in Fig. 1 indicate that hydroxide ion, displaced from alkaline methemoglobin, contributes to the uptake of protons from solution (compare the full and broken theoretical lines of Fig. 1).

The pK_{iH} values reported in Table 1 are similar to, though not identical with, those reported before [6]. The differences

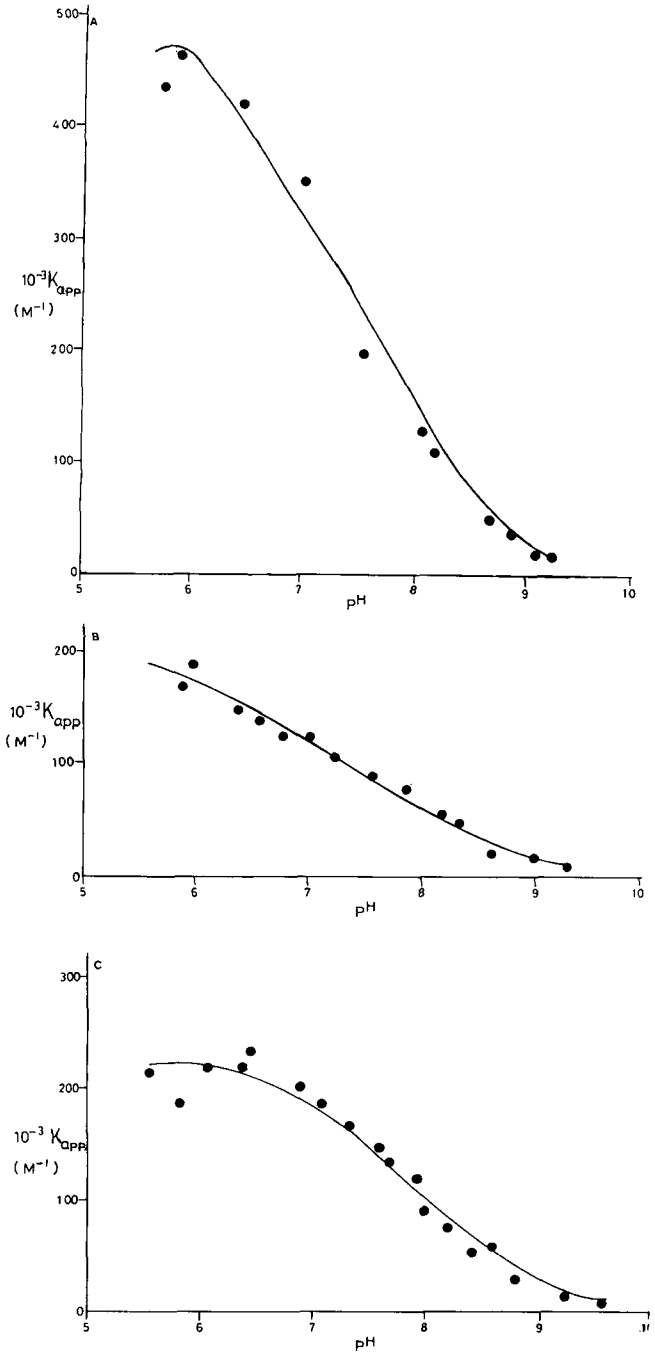


Fig. 2. *Reaction of methemoglobins with azide ion.* Apparent binding constants, K_{app}, as a function of pH at 20°C (data of Anusiem et al. [12]). (A) human A; (B) guinea pig; (C) pigeon. The lines through the experimental points are theoretical lines calculated with Eqn (2) using the pK_{iH} values calculated from Fig. 1. The binding constants, K_{jL} ($j = 1, 2, 3$ and 4), used to fit the data are shown in Table 1

may be attributed to the fact that in the present study azide ion is the ligand, whereas cyanide was employed in the previous study. A similar ligand-dependent effect on the pK values of ionizable groups was observed previously for fluoride and cyanide binding to turnip peroxidases [7]. On the other hand, it may be that the nonidentity of pK_{iH} values [6] (and this work) may have arisen because of the different methemoglobin concentrations used in the two sets of experiments: 2 μM heme [6] and 0.2 mM heme (this work).

The apparent equilibrium constants for azide binding to methemoglobins A, guinea pig and pigeon, determined under the conditions of [6], have been reported in the literature as a function of pH [12, 15]. The apparent binding constant, K_{app}, is related to the binding constants K_{jL} ($j = 1, 2, 3,4$) of Scheme I by the equation:

$$K_{app} = \frac{K_{1L}[H^+]^3 + K_{1H}K_{2L}[H^+]^2 + K_{1H}K_{2H}(K_{3L} + K_{4L})[H^+]}{[H^+]^3 + K_{1H}[H^+]^2 + K_{1H}K_{2H}[H^+] + K_{1H}K_{2H}K_{3H}} \quad (2)$$

Using the pK_{iH} values in Table 1, we have fitted the literature values of K_{app} to Eqn (2). The fits are shown in Fig. 2 and the K_{jL} values are reported in Table 1.

The good fits obtained indicate that the pK_{iH} values of Table 1 are satisfactory. The K_{jL} values ($j = 1, 2, 3$) are of the same order of magnitude, in marked contrast to the corresponding second-order rate constants [6]. It is likely that K_{4L} is much less than the other K_{jL}. Our method of analysis does not, however, allow for a separation of K_{3L} and K_{4L}, and we cannot therefore make a definite conclusion about the relative magnitude of K_{4L}.

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