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A New Structure in the Vicinity of HisHC3[146]β of Pigeon Haemoglobin Induced by Inositol Hexakisphosphate

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Pigeon haemoglobin lacks an observable organic phosphate-induced additional Bohr effect, whereas the closely related chicken haemoglobin exhibits an additional Bohr effect. Moreover, the addition of one mole of inositol hexakisphosphate (inositol-P_6) to one mole of pigeon haemoglobin tetramers increases the partial pressure by 21.6 mmHg for 50% saturation of haemoglobin with O_2; the corresponding increase for chicken haemoglobin is 37.3 mmHg. Here, it is demonstrated that these differences arise because the salt bridge normally formed in haemoglobins between HisHC3[146]β and AspFG1[94]β is not formed in inositol-P_6 bound pigeon haemoglobin, so that HisHC3[146]β comes sufficiently close to the CysF9[93]β sulfhydryl group to interact with it electrostatically and so to influence its reactivity directly.

INTRODUCTION

Pigeon haemoglobin and the major haemoglobin of chicken have closely similar amino acid sequences [1-3]. It would, therefore, be reasonable to expect their functional properties to be closely similar. However, at pH of 7 the effect of inositol hexakisphosphate (inositol-P_6) in reducing the O_2 affinity of chicken haemoglobin is 1.7 times greater than its effect in reducing the O_2 affinity of pigeon haemoglobin [4]. Furthermore, chicken haemoglobin exhibits an organic phosphate-induced additional Bohr effect [5], whereas pigeon haemoglobin exhibits no observable additional Bohr effect [6]. The effects of organic phosphates are rather intriguing in view of the fact that the organic phosphate binding groups and the Bohr effect groups are identical in both haemoglobins [1-3].

Organic phosphates are known to produce an additional Bohr effect in haemoglobins from various animal species [5,6]. In view of the apparent inability of 2,3-bisphosphoglycerate (2,3-DPG) to produce an additional Bohr effect in pigeon haemoglobin [6] and the relative weak effect of inositol-P_6 on its O_2 affinity, it seemed reasonable here to investigate the effect of inositol-P_6 on the kinetics of the reaction between pigeon haemoglobin and 5, 5′-dithiobis (2-nitrobenzoate) [DTNB]. Inositol-P_6 normally reduces the reactivity of the CysF9[93]β sulfhydryl group of haemoglobin [7-9], including that of chicken [10].

The CysF9[93]β sulfhydryl group of haemoglobin has been employed as a monitor of tertiary and quaternary structure change over the past 30 years [7,11-19]. The nature and number of the ionizable amino acid residues in the neighborhood of this sulfhydryl group can be obtained from quantitative analysis of the pH-dependence profile of k_{app}, the apparent second order rate constant for the reaction of the sulfhydryl group with a sulfhydryl reagent [8,9,20-22]. Among several such sulfhydryl reagents [23-25] the one that is most sensitive to the environment of the sulfhydryl group is DTNB [20]. With DTNB as the reagent, the pK_a of the ionizable organic phosphate-binding groups has been determined [8,9,20-22], the groups which are responsible for half of the alkaline Bohr effect in haemoglobin [26].

From kinetic studies regarding the reaction of DTNB with haemoglobins possessing multiple reac-

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tive sulfhydryl groups [21,22], it is observed that, for the kinetic phase attributable to the CysF9[93]β sulfhydryl group, k_{app} has a simple pH-dependence profile resembling the titration curve of a diprotic acid. Such profiles have been quantitatively analyzed on the basis of the effect of the salt bridge formed between HisHC3 [146]β and AspFG1 [94]β on the reactivity of the CysF9[93]β sulfhydryl group of various haemoglobin derivatives [8,9,20-22]. Formation of this salt bridge is known to lower the reactivity of the CysF9[93]β sulfhydryl group of haemoglobin in the T quaternary structure [7,27]. The discovery of an R state Bohr effect involving HisHC3[146]β [28] proves that such a salt bridge is formed in R state haemoglobin.

A pH-dependence study of the kinetics of the reaction of DTNB with the aquomet, oxy and carbonmonoxy derivatives of pigeon haemoglobin has been carried out in the presence of inositol-P_6, a condition under which has been carried out it is known that the HisHC3/AspFG1 salt bridge is strengthened in human haemoglobin. For the kinetic phase attributable to the CysF9[93]β sulfhydryl of each derivative it was fully expected that a pH-dependence profile for k_{app} be obtained resembling the titration curve of a diprotic acid. Rather, a dramatic change in the profile of each derivative was observed. Instead of resembling the titration curve of a diprotic acid, each profile is bowl-shaped. This result implies that the HisHC3/AspFG1 salt bridge is not formed in pigeon haemoglobin in the presence of inositol-P_6. Based on this assumption the lack of an organic phosphate-induced additional Bohr effect in pigeon haemoglobin [6] and the relatively weak effect of inositol-P_6 on the O_2 affinity of pigeon compared to chicken haemoglobin [4] can be explained.

MATERIAL AND METHODS

The collection of blood samples and haemoglobin preparation is the same as [22]. The experimental procedure is also similar except that 10 μM of inositol-P_6 (sodium salt) was added to the haemoglobin solution. Transmittance readings from a Zeiss PMQ II spectrophotometer were recorded as a function of time on Philips PM 8261 Xt chart recorder. The reaction was allowed to proceed to completion. Each experiment was repeated at least twice. After converting transmittance readings to absorbance, the data were analyzed with a 1990 update of DISCRETE, a computer programme for analysis of multiple exponential signals [29,30]. The analyses gave two kinetic phases, fast and slow. The pseudo-first order rate constant for each phase, k_{obs}, was converted to k_{app}, the apparent second order rate constant, by dividing k_{obs} by the DTNB concentration. The standard error in the determination of k_{obs} was about 10% for the fast phase and about 20% for the slow phase.

RESULTS AND DISCUSSION

Nature of pH-Dependence Profiles

Fast Kinetic Phase

The reaction of DTNB with stripped pigeon haemoglobin displays biphasic kinetics [22]. For the fast phase of pigeon carbonmonoxyhaemoglobin, the variation of the apparent second order rate constant (k_{app}) with pH resembles the titration curve of a monoprotic acid [22]. This result is highly significant and will be discussed later in this report. For the fast phase of the reaction of the aquomet and oxy derivatives, the variation of k_{app} with pH resembles the titration curve of a diprotic acid. Such a profile is typical of the CysF9[93]β sulfhydryl group when its reactivity is affected by the salt bridge formed between HisHC3[143]β and AspFG1[94]β [8,9,20-22]. The formation of this salt bridge is known to hinder access to the sulfhydryl [7,27].

It is known that inositol-P_6 strengthens the HisHC3/AspFG1 salt bridge. In human haemoglobins, A and S, the pK_a of this histidine residue is increased by one pK_a unit when the organic phosphate is present [9]. Furthermore, the rate of the DTNB reaction is slowed down by about one order of magnitude [8,9]. Human and pigeon haemoglobins differ in a number of residues involved in organic phosphate binding [31,32], i.e. HisHC3[143]β and AspFG1[94]β of human haemoglobin are replaced by arginine and histidine residues, respectively [3]. Therefore, the negatively charged inositol-P_6 is expected:

i) To produce a drastic reduction in the reactivity of CysF9[93]β,

ii) To increase the pK_a of HisHC3[146]β by at least one pK_a unit.

Also obtaining a pH-dependence profile for k_{app} resembling the titration curve of a diprotic acid is expected. Nevertheless, none of the expected results were surprisingly obtained. Figure 1 shows the effect of inositol-P_6 on the pH-dependence profile of k_{app} for the aquomet, oxy and carbonmonoxy derivatives of pigeon haemoglobin. Each profile is bowl-shaped. Such a result has not been obtained previously for CysF9[93]β sulfhydryl group of any haemoglobin, with or without inositol-P_6 [8,9,13,20-22]. For comparison, the theoretical best-fit lines to the data for stripped pigeon haemoglobin [22] are included in Figure 1. It is noteworthy that the profile for each organic phosphate-induced derivative is strikingly different from the corresponding profile obtained for stripped haemoglobin.

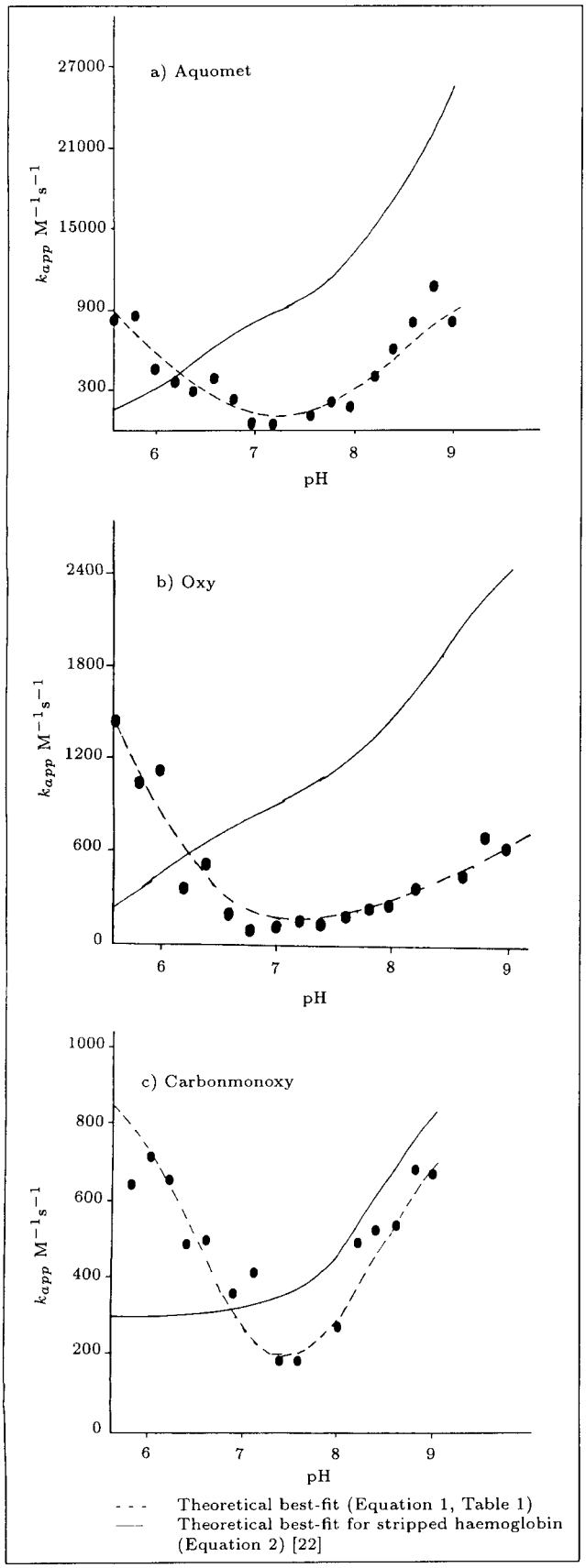


Figure 1. k_{app} versus pH for the reaction of DTNB with the CysF9[93]β of pigeon haemoglobin in the presence of inositol-P_6. I = 50 mM, Temperature = 20°C.

Furthermore, below pH of 6.2 (Figure 1a: aquomet; and Figure 1b: oxy) or 6.8 (Figure 1c: carbonmonoxy) the values of k_{app} for the stripped derivatives are generally lower than those for the corresponding organic phosphate-bound derivatives. These results are contrary to what would be expected if inositol-P_6 strengthened the HisHC3/AspFG1 salt bridge in pigeon haemoglobin [8,9]. Above these pH values, k_{app} is higher for stripped haemoglobin. However, the difference in k_{app}, with and without inositol-P_6, has a maximum value of only about 3-fold. The corresponding value for the closely similar chicken haemoglobin is 15-fold [10]. This relatively mild effect of inositol-P_6 on k_{app} of pigeon haemoglobin compared to chicken is reminiscent of its milder effect in reducing the O_2 affinity of pigeon compared to chicken [4].

Slow Kinetic Phase

For stripped pigeon haemoglobin the slow kinetic phase, which is attributable to CysB5[23]β, has a bowl-shaped k_{app} versus pH profile [22]. Figure 2 shows that this profile is retained in the presence of inositol-P_6. However, the organic phosphate, especially in the low pH range noticeably reduces values of k_{app}. For the carbonmonoxy derivative, k_{app} values are noticeably different above pH of 6.8, with or without inositol-P_6 (Figure 2c).

Quantitative Analysis of pH-Dependence Profile

Profiles like those reported in Figures 1 and 2 have been previously analyzed [22] with the following equation:

$$k_{app} = k_1 \frac{[H^+]}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]}, \qquad (1)$$

where k_1 is the limiting apparent second-order rate constant at low pH for the DTNB reaction when the sulfhydryl reactivity is linked to the ionization of a neighboring cationic group, with ionization constant K_1. The first fractional term is the fractional population of cationic form of this group; k_2 is the limiting apparent second-order rate constant at high pH when the sulfhydryl reactivity is linked to the ionization of the reacting sulfhydryl group, with ionization constant K_2. The second fractional term is the fraction of the thiol anion form of the reacting sulfhydryl.

Equation 1 was previously employed to analyze the bowl-shaped profiles obtained for the reaction of the CysB5[23]β sulfhydryl group of stripped pigeon haemoglobin [22]. Since the data in Figures 1 and 2 show that the pH-dependence profiles for the CysF9[93]β and the CysB5[23]β reactions of organic phosphate-bound pigeon haemoglobin are bowl-shaped, both sets of data have been fitted with Equation 1. The best-fit parameters are reported in

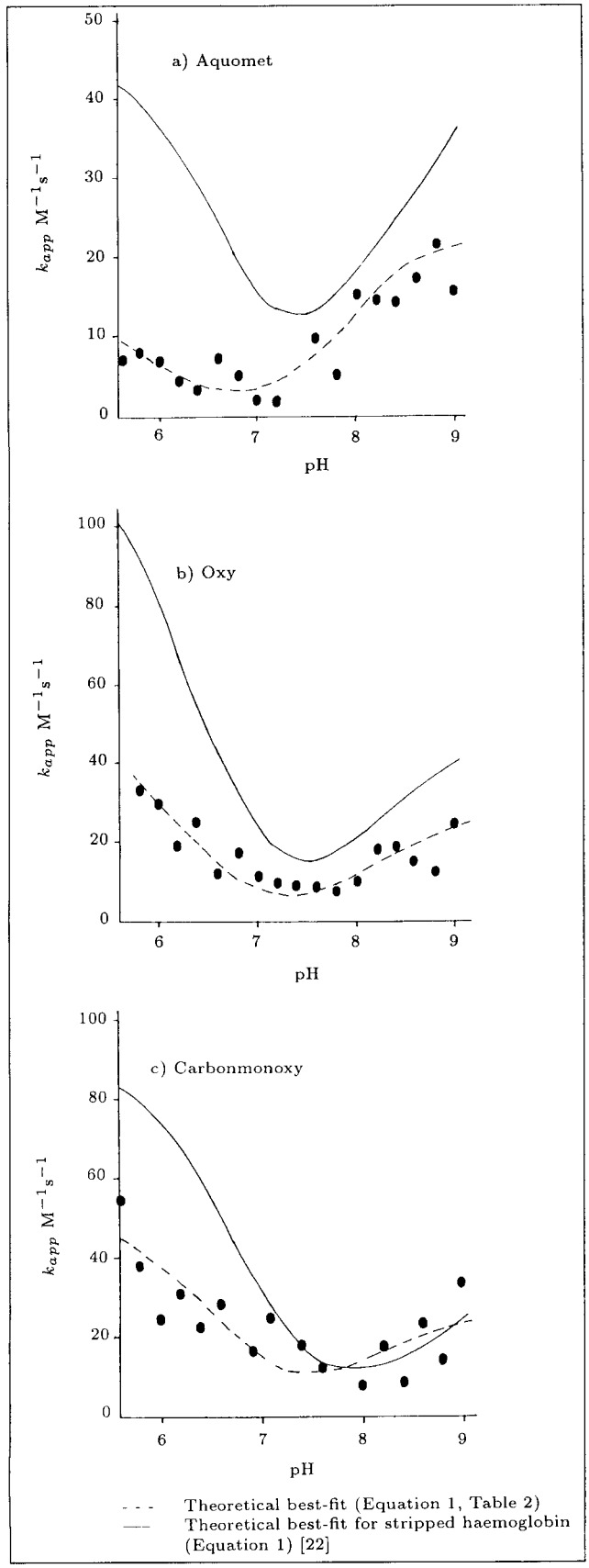


Figure 2. k_{app} versus pH for the reaction of DTNB with the CysB5[23]β of pigeon haemoglobin in the presence of inositol-P_6. I = 50 mM, Temperature = 20°C.

Table 1. Reaction of DTNB with the CysF9[93]β sulfhydryl group of pigeon haemoglobin derivatives in the presence of inositol-P_6. Best-fit parameters were employed to fit the experimental data in Figure 1 (compare with Equation 1).

Derivative	pK_1	pK_2	k_1 $M^{-1}s^{-1}$	k_2 $M^{-1}s^{-1}$
Aquomet	6.1	8.5	1000	1200
Oxy	5.7	8.1	2500	650
Carbonmonoxy	6.5	8.3	950	820

Table 1. For the fast phase (Figure 1) the mean value of pK_1 is 6.1 ± 0.3 and that of pK_2 is 8.3 ± 0.1. For the slow phase (Figure 2) the mean values of pK_1 and pK_2 are 6.2 ± 0.2 and 8.03 ± 0.03 respectively.

Theoretically, it is assumed that by using Equation 1 a bowl-shaped pH-dependence profile results if there is an ionizable cationic residue sufficiently close to the reacting sulfhydryl to interact with it electrostatically and to influence its reactivity directly. For the CysB5[23]β sulfhydryl of pigeon haemoglobin, the cationic group meeting this requirement is HisG19[117]β [22]. In principle, a bowl-shaped pH-dependence of k_{app} can also arise from a neutral group, close to the reacting sulfhydryl, ionizing to the anionic form with increasing pH. However, the pK_1 value of about 6 obtained for the required ionizable group (Table 1) is too high for aspartic and glutamic acid residues in haemoglobin. Additionally, such a close anionic group would make it impossible for cysteine residue to ionize into the anionic form, which reacts with DTNB [33,34].

Structural Implications of Bowl-Shaped pH-Dependence Profiles

Fast Kinetic Phase

The bowl-shaped pH-dependence profiles obtained form the reaction of DTNB with the CysF9[93]β sulfhydryl group of pigeon aquomet-, oxy- and carbonmonoxyhaemoglobin in the presence of inositol-P_6 (Figure 1) suggest that the salt bridge formed by HisHC3[146]β and AspFG1[94]β is not observed in these derivatives under the assumed conditions. In this regard, it is particularly noteworthy that k_{app} is higher for the inositol-P_6 bound than for stripped haemoglobin at pH≤6.2 (aquomet and oxy) and pH≤6.8 (carbonmonoxy) (see Figure 1). Such a result is not typical of the CysF9[93]β sulfhydryl reacting under the influence of HisHC3/AspFG1 salt bridge [7-10]. The results in Figure 1 suggest that in pigeon haemoglobin, inositol-P_6, rather than strengthening the HisHC3/AspFG1 salt bridge, causes the histidine residue to move away from the aspartate residue to come within a close distance to the CysF9[93]β residue. Consequently, the histidine interacts electrostatically

with the cysteine and influences its reactivity in such a manner that a bowl-shaped pH-dependence profile is obtained for k_{app} (see Figure 1). It is noted that the values of pK_1 obtained (Table 1) are those to be expected for a histidine [8,9,20-22]. An examination of the 3-D structure of pigeon haemoglobin shows that there is no other histidine sufficiently close to CysF9[93]β to act as the required cationic group.

Another support for the idea that the HisHC3/AspFG1 salt bridge is not formed in inositol-P_6 bound pigeon haemoglobin is provided by a comparison of the mean pK_1 value for the aquomet and oxy derivatives, 5.9 $\pm$ 0.2 (Table 1), with that for the corresponding stripped derivatives, 6.2 $\pm$ 0.2 [22, Table 1]. (On account of its monophasic profile [22] a pK_1 value cannot be calculated for the carbonmonoxy derivative of stripped pigeon haemoglobin). It is seen that, with or without inositol-P_6, the value of pK_1 remains about the same. By contrast, pK_1 values for human A and S, for which it is known that the salt bridge is strengthened by inositol-P_6, are increased by one pK unit in the presence of inositol-P_6 [9]. If the HisHC3/AspFG1 salt bridge was present in inositol-P_6 bound pigeon haemoglobin, a mean pK_1 value of at least 7.2 (6.2 for stripped plus 1) rather that 5.5 would have been obtained. Further support for the above mentioned idea is provided by a comparison of the second order rate constants k_1 and k_2 (Equation 1 and Table 1). In cases where the pH-dependence profile of k_{app} resembles the titration curve of a diprotic acid (i.e., the salt bridge is present), a comparison of k_1 values with the corresponding k_2 values shows that $k_1 < k_2$ (see Tables I and II of [20], Table 1 of [22], Table 1 of [8], Tables 1 and 2 of [9] and Table 1 of [10]). Constant k_1 is less than k_2 because, when the HisHC3/AspFG1 salt bridge is formed, TyrHC2[145]β sterically hinders the approach of a sulfhydryl reagent the relationship to the CysF9[93]β residue [27]. Table 1 shows that the relationship for inositol-P_6 bound pigeon haemoglobin is $k_1 \geq k_2$ for the reaction of CysF9[93]β. This is obtained previously for the CysB5[23]β sulfhydryl group of stripped pigeon haemoglobin [22, Table II], for which the 3-D structure shows that there is a neighboring histidine residue, HisG19[117]β. It is concluded that, in inositol-P_6 bound pigeon haemoglobin, HisHC3[146]β comes sufficiently close to CysF9[93]β to produce effects on its reactivity similar to those produced by HisG19[117]β on the reactivity of CysB5[23]β. This explains why bowl-shaped pH-dependence profiles are obtained for k_{app} of CysF9[93]β in the presence of inositol-P_6 (Figure 1).

Table 2. Reaction of DTNB with the CysB5[23]β sulfhydryl group of pigeon haemoglobin derivatives in the presence of inositol-P_6. Best-fit parameters were employed to fit the experimental data in Figure 2 (compare with Equation 1).

Derivative	pK_1	pK_2	k_1 $M^{-1}s^{-1}$	$k_2 M^{-1}s^{-1}$
Aquomet	6.0	8.0	12	25
Oxy	6.1	8.1	55	25
Carbonmonoxy	6.5	8.0	50	25

Slow Kinetic Phase

The slow kinetic phase for the reaction of stripped pigeon haemoglobin with DTNB has been previously assigned to the CysB5[23]β sulfhydryl [22]. According to the x-ray crystallographic data for human haemoglobin, the region of the β chain in which the CysB5[23]β residue appears does not undergo any significant structural change in O_2 binding to deoxyhaemoglobin [35]. An examination of the data in Figure 2 shows that in the presence of inositol-P_6 the reactivity of CysB5[23]β pigeon haemoglobin is noticeably lowered compared to stripped haemoglobin, especially at low pH. At pH of 6, for example, the extent of lowered reactivity is about 6-fold for aquomet haemoglobin and about 3-fold for oxyhaemoglobin and carbonmonoxyhaemoglobin. These changes are considered to be significant indicators of structural alterations induced by inositol-P_6 in the B5[23]β region. It is also significant that, for organic phosphate-bound pigeon haemoglobin, the reactivity of the aquomet derivative is about 4-fold lower than those of the oxy and carbonmonoxy derivatives at pH of 6. This represents an enhancement of the 2-fold lower reactivity of the aquomet derivative found in stripped haemoglobin [22], leading to the conclusion that in pigeon haemoglobin, the B5[23]β region undergoes a significant structural change on binding of inositol-P_6. It was found previously that in chicken haemoglobin, this region undergoes a significant structural alteration on change of haem ligand from O_2 to CO [10].

Implications of Bowl-Shaped Profiles for the Physiological Function of Pigeon Haemoglobin

The effects of organic phosphates on the O_2 affinities and Bohr effects of pigeon and chicken haemoglobins are different [4-6] despite their close similarity in primary structure [1-3]. Now, explanations for these differences in terms of the results are provided in Figure 1.

Bohr Effect

For the aquomet derivative of stripped pigeon haemoglobin it was found that HisHC3[146]β forms a salt bridge with AspFG1[94]β [7,22], demonstrating that aquomethaemoglobin at acid pH has the quaternary T conformation in the presence of inositol-P_6, that is, the same structure as deoxyhaemoglobin. The bowl-shaped profile for inositol-P_6 bound pigeon

aquomethaemoglobin (Figure 1a) strongly suggests that HisHC3[146]β of pigeon does not form a salt bridge with AspFG1[94]β in the presence of inositol-P_6. Since aquomethaemoglobin with inositol-P_6 has the same quaternary structure as deoxyhaemoglobin [7], it is assumed that HisHC3[146]β does not form a salt bridge with AspFG1[94]β in pigeon deoxyhaemoglobin when an organic phosphate is present.

Formation of the HisHC3/AspFG1 salt bridge raises the pK_a of the histidine in T-state deoxyhaemoglobin. In R-state oxyhaemoglobin this salt bridge is not as strong as in the T-state. Consequently, when deoxyhaemoglobin is oxygenated the pK_a of HisHC3[146]β (and also of ValNA1[1]α and HisH5[122]α) decreases, giving rise to the ejection of protons into the solution. This is the alkaline Bohr effect. Now the effect of organic phosphates on the alkaline Bohr effect of pigeon haemoglobins should be investigated in which it is known that the salt bridge is formed.

The addition of an organic phosphate to stripped haemoglobin increases the alkaline Bohr effect [5,6]. This is the so-called organic phosphate-induced additional Bohr effect. The organic phosphate binding groups in pigeon haemoglobin are ValNA1[1]β, HisNA2[2]β, LysEF6[82]β and ArgH21[143]β. The additional Bohr effect arises because, when deoxyhaemoglobin binds organic phosphate, those organic phosphate-binding groups with relatively low pK_a have their pK_a raised. Upon oxygenation, the binding of organic phosphate is weakened and the pK_a of these groups are lowered. This gives rise to the ejection of proton into the solution, i.e., the additional Bohr effect.

Bailey et al. [6] and Brygier et al. [5] have precisely determined the Bohr effects of various animal haemoglobins in the absence and presence of organic phosphate. For the animal haemoglobins employed in these studies, the Bohr effect in the presence of organic phosphate is higher than the value for the stripped haemoglobin (Figure 3 of [6] and Figures 5 and 6 of [5]). The only exception is pigeon haemoglobin, which apparently has no additional Bohr effect. Indeed, between pH of 6.5 and 7.6, the Bohr effect of pigeon haemoglobin in the presence of 2,3-DPG is slightly lower than that for stripped pigeon haemoglobin [6, Figure 3b].

Absence of an additional Bohr effect in pigeon is readily explained if one assumes that the HisHC3/AspFG1 salt bridge is not formed in pigeon deoxyhaemoglobin in the presence of 2,3-DPG. HisHC3[146]β is responsible for about half of the alkaline Bohr effect of haemoglobin [26]. Now, the additional Bohr effect is ignored temporarily. If the HisHC3/AspFG1 salt bridge is indeed not formed in 2,3-DPG bound pigeon deoxyhaemoglobin, as been proposed previously, an alkaline Bohr effect about half of normal would be resulted. Now, considering the organic phosphate-induced additional Bohr effect, a Bohr effect for 2,3-DPG bound pigeon haemoglobin is resulted that is not higher than that of stripped haemoglobin [6, Figure 3b].

Since pigeon and chicken haemoglobins have very similar amino acid sequences and compositions, it would be interesting to compare the Bohr effect data for both haemoglobins [5,6]. The authors kinetic data for chicken haemoglobin indicate that in the presence of inositol-P_6 the HisHC3/AspFG1 salt bridge is formed in the aquomet derivative [10]. Consequently, it is possible to assume that it would be formed in inositol-P_6 bound chicken deoxyhaemoglobin. Therefore, the Bohr effect of chicken haemoglobin in the presence of organic phosphate is expected to be higher than that of stripped haemoglobin. An additional Bohr effect is indeed present in chicken haemoglobin (see Figure 5 of [5]). By contrast, inositol-P_6 bound pigeon haemoglobin (for which the present data (Figure 1) strongly indicates that the salt bridge is not formed) appears not to have an additional Bohr effect groups and the organic phosphate binding groups are identical in chicken and pigeon haemoglobins.

Oxygen Affinity

P_{50} is the O_2 partial pressure required for 50% saturation of haemoglobin with O_2. At pH of 7 log P_{50} for stripped chicken haemoglobin is 0.055; in the presence of equimolar concentration of inositol-P_6, log P_{50} increases to 1.585 [4]. This represents an increase in log P_{50} of 1.530. Under the same experimental conditions, log P_{50} of stripped and inositol-P_6 bound pigeon haemoglobin are 0.67 and 1.42, respectively [4]. This represents an increase of 0.75 in log P_{50}, that is, half of the increase seen with chicken. This result would be reasonable only if it is assumed that in the presence of inositol-P_6, the T quaternary structure of pigeon deoxyhaemoglobin is not stabilized by a HisHC3/AspFG1 salt bridge, whereas that of chicken is.

Nature of k_{app} Versus pH Profile of Stripped Pigeon Carbonmonoxy Haemoglobin

The pH-dependence profiles of k_{app} for the fast phase of stripped pigeon aquomet- and oxyhaemoglobin resemble the titration curve of a diprotic acid (Figure 3 of [22]). Such profiles imply that the HisHC3/AspFG1 salt bridge is formed in these derivatives [8,9,20-22]. In contrast to the biphasic profiles for the aquomet and oxy derivatives, the profile for stripped pigeon carbonmonoxyhaemoglobin is monophasic, resembling the titration curve of a monophasic acid. Whereas k_{app} shows a very strong dependence for the aquomet and

oxy derivatives (Figure 3 of [22]); for the carbonmonoxy derivative it shows hardly any variation between pH of 5.8 and 7 (Figure 4 of [22]). How could this latter profile arise?

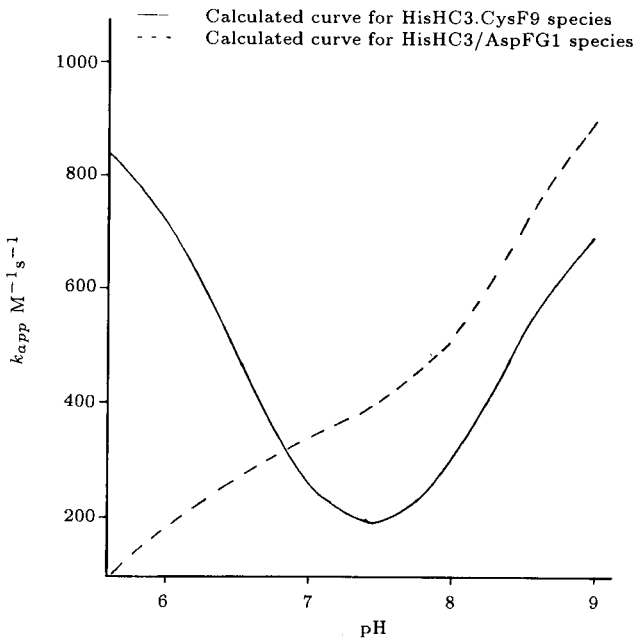
As seen in Figure 1, inositol-P_6 binding to pigeon haemoglobin induces a bowl-shaped pH dependence of k_{app}. Stripped aquomet- and oxyhaemoglobin, on the other hand, have profiles resembling the titration curve of a diprotic acid. It should be noted that the monophasic profile of stripped carbonmonoxyhaemoglobin may have arisen as a result of the presence in the solution of equilibrium mixture of HisHC3/AspFG1 salt bridge-forming species (cf. Full lines in Figures 1a and 1b) and a species in which HisHC3 is close to the CysF9 sulfhydryl and the salt bridge is not formed (broken lines in Figure 1). These species are referred to as the HisHC3/AspFG1 and HisHC3...CysF9 species, respectively. (Unless otherwise specified, all references to the HisHC3/AspFG1 and HisHC3...CysF9 species are the carbonmonoxy derivatives.) In order to calculate the proportions of these species in stripped carbonmonoxyhaemoglobin it is necessary, first, to construct the theoretical pH dependence profile of k_{app} for the HisHC3/AspFG1 species. One can then use a combination of this curve and the HisHC3...CysF9 curve that fits the stripped carbonmonoxyhaemoglobin (Figure 4 of [22]).

In calculating the theoretical pH dependence profile of k_{app} for the HisHC3/AspFG1 species the following equation is employed [20]:

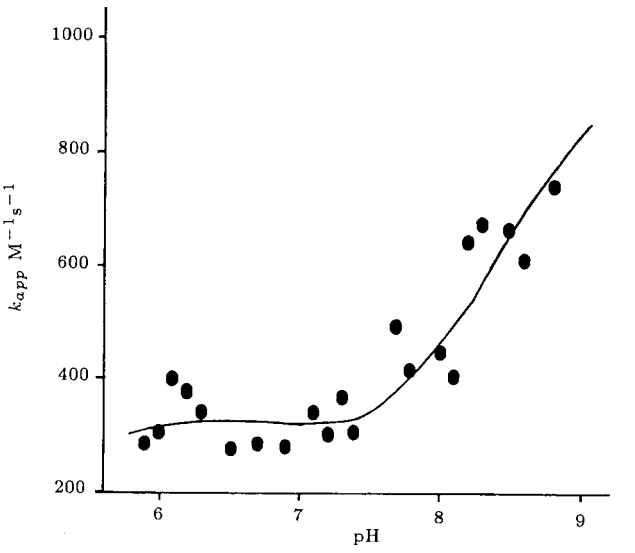
$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]}. \qquad (2)$$

With the exception of K_1, the parameters appearing in this equation are the same as those in Equation 1 and they are similarly defined. K_1 is the ionization constant of HisHC3[146]β which forms a salt bridge with AspFG1[94]β, thus restricting access to CysF9[93]β. The first fractional term of Equation 2 is the fraction of the neutral form of the histidine. For the construction of the theoretical pH-dependence profile of k_{app} for the HisHC3/AspFG1 species (by Equation 2) the following parameters were employed: $k_1 \approx 350\ M^{-1}s^{-1}, k_2 \approx 700\ M^{-1}s^{-1}, pK_1 = 6.0,\ pK_2 = 8.5$. (For details of how these parameters were estimated, see Appendix.) This theoretical curve is shown in Figure 3a (broken lines) together with the theoretical curve for the species HisHC3...CysF9 (full line; same as broken line in Figure 1c). In Figure 3b a theoretical k_{app} versus pH curve is shown for carbonmonoxyhaemoglobin containing an equilibrium mixture of the HisHC3/AspFG1 and HisHC3...CysF9 species.

This curve is the weighted mean of the HisHC3/AspFG1 and HisHC3...CysF9 curve (Figure 3a), calculated using the following equation:



a) Theoretical curve for the HisHC3.CysF9 and HisHC3/AspFG1 species.



b) Calculated weighted mean curve for HisHC3.CysF9 and HisHC3/AspFG1 species (Equation 3). This curve gives a good fit for the experimental data.

Figure 3. k_{app} versus pH for the reaction of DTNB with the CysF9[93]β of stripped pigeon carbonmonoxyhaemoglobin.

$$k_{app}(\text{mean}) = \alpha x k_{app}(\text{HisHC3...CysF9}) + (1-\alpha) x k_{app}(\text{HisHC3/AspFG1}). \qquad (3)$$

In Equation 3, α, which is a weighting factor, was adjusted until a good fit of the calculated curve to

the experimental data for the stripped pigeon carbonmonoxyhaemoglobin (see Figure 4 of [22]) was obtained. The best value of α that gave a good fit to the carbonmonoxyhaemoglobin data (Figure 3b) was 0.25. It is concluded that the monophasic nature of the k_{app} versus pH profile of pigeon carbonmonoxyhaemoglobin data (Figure 4 of [22]) arises because in solution there is an equilibrium mixture of two species: HisHC3...CysF9 (25%) and HisHC3/AspFG1 (75%). In view of the rather arbitrary nature of the choice of k_1 employed in calculating the HisHC3/AspFG1 profile (Figure 3a, broken line) (see Appendix), the numbers 25% and 75% should be regarded as approximations. This does not, however, detract from the conclusion that the monophasic nature of the stripped pigeon carbonmonoxyhaemoglobin profile arises because of the existence of two species (HisHC3...CysF9 and HisHC3/AspFG1) in solution.

It is likely that in the aquomet and oxy derivatives of stripped pigeon haemoglobin, the HisHC3...CysF9 species exist in equilibrium with the HisHC3/AspFG1 species, albeit in too small proportion to give a monophasic pH-dependence profile, such as observed for the carbonmonoxy derivative (Figure 4 of [22]). It is, therefore, possible that under experimental conditions that enhance the proportion of the HisHC3...CysF9 species, a monophasic pH-dependence profile could be obtained for the aquomet and oxy derivatives. All the experiments reported so far were carried out at an ionic strength of 50 mM. When a study was carried out on the stripped pigeon oxyhaemoglobin at an ionic strength of 200 mM (Figure 4), a monophasic pH-dependence profile was obtained for the biphasic profile obtained at an ionic strength of 50 mM. However, a similar study on aquomethaemoglobin (data not shown) showed that the biphasic profile obtained at 50 mM (Figure 3 of [22]) was retained at 200 mM.

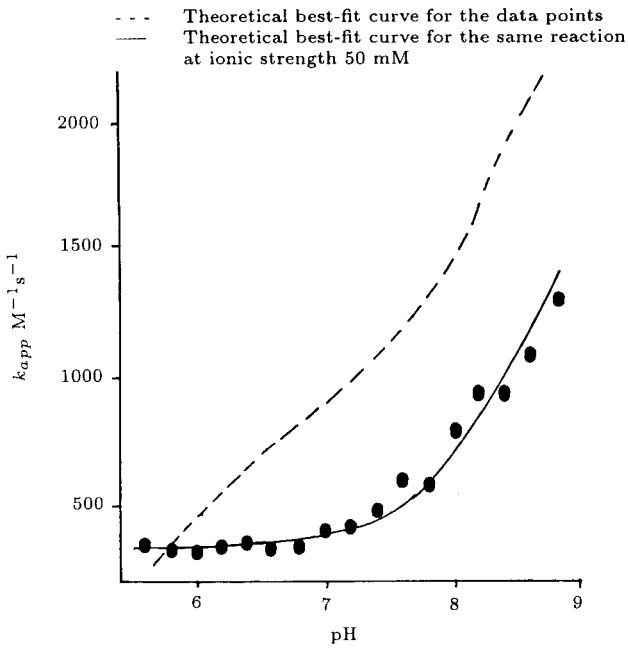


Figure 4. k_{app} versus pH for the reaction of DTNB with the CysF9[93]β of pigeon oxyhaemoglobin at ionic strength 200 mM. The data was fitted with the equation $k_{app} = k_2\{K_2/(K_2 + [H^+])\}$. $k_2 = 1600$ $M^{-1}s^{-1}$; $pK_2 = 8.5$ and a base line of $k_1 = 330$ $M^{-1}s^{-1}$.

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APPENDIX

Here, estimation of parameters employed in calculating the theoretical pH-dependence profile of the HisHC3/AspFG1 species of pigeon carbonmonoxyhaemoglobin is presented (cf. Figure 3a).

None of the parameters k_1, k_2, K_1 and K_2 of Equation 2 are known for the HisHC3/AspFG1 species. Therefore, they are estimated in the following manner: From the analyses of previous data with Equation 2, that is the analyses in which the effect of the salt bridge was taken into account, it is known that pK_1 values are closely similar for the oxy and carbonmonoxy derivatives of a haemoglobin (Table I of [8,9,21]). Therefore, for the HisHC3/AspFG1 species, the pK_1 value of 6.0 calculated for pigeon oxyhaemoglobin (Table 1 of [22]) was assumed. The pK_2 values in Table 1 are for the HisHC3...CysF9 species of the aquomet, oxy and carbonmonoxy derivatives. A comparison of these pK_2 values with those of the stripped aquomet, oxy and carbonmonoxy derivatives (Table I of [22]) shows that they are close. The mean pK_2 from both sets of data is 8.4 ± 0.1. It is therefore reasonable to expect pK_2 for HisHC3/AspFG1 species to have about the same value. Therefore, the pK_2 of 8.5 obtained for stripped carbonmonoxyhaemoglobin (Table I of [22]) was assumed for the HisHC3/AspFG1 species.

Now, the k_2 values in Table 1 are compared with those in Table I of [22]. For aquomethaemoglobin k_2 is about 2 times greater than k_2 for its HisHC3...CysF9 species. The corresponding relationship for oxyhaemoglobin is 2.8 times. In contrast, the k_2 values for carbonmonoxyhaemoglobin and its HisHC3...CysF9 are similar, 700 and 820 $M^{-1}s^{-1}$, respectively (compare Table 1 with Table I of [22]. Consequently, a k_2 of 700 $M^{-1}s^{-1}$ was estimated for the HisHC3/AspFG1 species. Since $k_1 = 1/2.k_2$ for stripped pigeon oxyhaemoglobin (Table I of [22]) $k_1 \approx 1/2.700$ $M^{-1}s^{-1}$, that is, 350 $M^{-1}s^{-1}$ was assumed as an estimate of k_1 for HisHC3/AspFG1 species.