A STRUCTURAL INTERPRETATION OF THE ACIDITY OF GROUPS ASSOCIATED WITH THE HEMES OF HEMOGLOBIN AND HEMOGLOBIN DERIVATIVES

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Since the discovery by Bohr and coworkers in 1904 of the effect of acidity on the oxygen equilibrium of hemoglobin, there have been a large number of investigations directed towards clarification of the physicochemical relationships of acid groups with the hemes in hemoglobin and its derivatives. It is the purpose of this paper to analyze modern quantitative data, including those for ferrihemoglobin (methemoglobin), in order to throw new light on the general problem, to establish the ionization constant of an acid group in ferrihemoglobin previously unrecognized, and to give a structural interpretation of shifts in the pK of groups caused by changing chemical environment.

Characterization of the Acid Groups by pK Values

In recent papers (1) Taylor and Taylor and Hastings have summarized results of a detailed investigation of the hemoglobin electrode potential in the pH range 5 to 9, obtained with borate and phosphate buffers of ionic strength 0.1 to 0.2. When the potentials measured for solutions containing equal quantities of horse ferrohemoglobin and ferrihemoglobin are plotted against pH, there is obtained a line with slope $\Delta E_0'/\Delta\text{pH}$ of zero at pH 5, curving smoothly to a line of slope -0.06 ($-2.30\ RT/F$) between pH 8 and 9, and the points are satisfactorily represented by the well known theoretical E.M.F.-pH equation with the assumption of an acid group of pK 6.65 for the oxidized form. No evidence

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was observed in the data Dr. Taylor has kindly put at our disposal for other pK values for either the ferro or the ferri form.

It is of interest to correlate this information about ionization constants of groups connected with the hemes with what is already known about the ionization constants of groups in ferrohemoglobin and ferrihemoglobin. Wyman's analysis (2) of the German and Wyman (3) differential acid-base titration data between ferrohemoglobin and oxyhemoglobin established the existence in ferrohemoglobin of two acid groups of pK 5.25 and 7.81 whose pK values are shifted upon oxygenation to 5.75 and 6.80. Haurowitz (4) showed the existence of an acid group in horse ferrihemoglobin of pK 8.2, a value which has been checked at ionic strength 0.10 for dog hemoglobin spectrophotometrically as 8.10 by Austin and Drabkin (5) and for cow hemoglobin magnetometrically as 8.07 by Coryell, Stitt, and Pauling (6).

There have thus been recognized four acid groups associated with the hemes of ferrohemoglobin or ferrihemoglobin with pK values in the range 5 to 9, each of which is of significance to the physical chemistry of hemoglobin. The electrode potential E_0' of a one-step system at half reduction is commonly expressed by the general equation

$$E_0' = E_0 + \frac{RT}{F}\left[\sum \ln (K_r + H^+) - \sum \ln (K_o + H^+)\right] \qquad (1)$$

where K_r is the acid constant of the ferro or reduced form, and K_o that of the ferri or oxidized form. The great majority of the acid groups of the protein parts of hemoglobin compounds has identical values of K_r and K_o and leads to no observable effect of pH on E_0'. If a value of K_o for one acid group accidentally agrees with a value of K_r for another, a similar cancelation of effects occurs. Now the decrease of magnetic susceptibility observed (6) in solutions more acid than pH 6 and the decrease in the dissociation constant of ferrihemoglobin fluoride in acid solutions noted by Lipmann (7) point to the existence of a previously unrecognized heme acid group in ferrihemoglobin with a relatively low pK value, whose ionization affects somewhat the magnetic properties of ferrihemoglobin and the affinity of the iron atom for fluoride ion. The existence of this acid group is made certain by the fact that the values of E'_0 of Taylor are constant between pH 5.0 and 6.0,

which requires that ferrihemoglobin possess an acid group with pK practically identical with that, 5.25, of the known (2) acid group of ferrohemoglobin.

We estimate that a difference in pK values of the two groups greater than 0.2 would have produced an observable effect in the electrode potential data in this pH range, and we assign to the new acid group the approximate pK value 5.3.

A similar cancelation seems to occur in the potentiometric study, within experimental error, between the pK of ferrihemoglobin at 8.1 and that of ferrohemoglobin at 7.81. The effect on E_0' of the ferrihemoglobin acid group with pK 6.65 (change of slope) would tend to mask the small effect (about 18 millivolts extended over 2 pH units) of the real difference in pK of these two acid groups.

It is interesting that oxyhemoglobin and carbonmonoxyhemoglobin have identical pK values, as shown by the work of Hartridge

TABLE I

Heme-Linked Acid Groups in pH Range 4.5 to 9

Hb^+	$pK_1 = 5.3$ *Mo*	$pK_2 = 6.65$ *Si, Mi, Po*	$pK_3 = 8.10$ *So, Mo*
Hb	$pK_1 = 5.25$ *Mi*		$pK_2 = 7.81$ *Si, Mi*
HbO_2	$pK_1 = 5.75$ *Mi, Po*	$pK_2 = 6.80$ *Si, Mi, Po*	
HbCO			

(8) on the oxyhemoglobin-carbonmonoxyhemoglobin equilibrium, which is independent of pH over the range from the point of acid destruction of oxyhemoglobin (about pH 6) to quite alkaline solutions. It will be of considerable importance to determine pK values of such groups associated with the hemes in other hemoglobin derivatives; the case discussed above suggests that only a relatively small number of different categories exist. Russell and Pauling (9) report an additional acid group with pK 9.5 in the covalent ferrihemoglobin-imidazole complex. It seems probable that this group is the imino group in the imidazole ring rather than another acid group of ferrihemoglobin.

The pK values of the known acid groups associated with the hemes of ferrihemoglobin (Hb^+), ferrohemoglobin (Hb), and oxyhemoglobin and carbonmonoxyhemoglobin (HbO_2 and HbCO) are collected in Table I. They are numbered in the order of increasing pK values, and are described by the symbols *S*, *M*, and

P, denoting spectrophotometrically, magnetometrically, and potentiometrically, respectively, and *o* or *i*, denoting operative or inoperative. Since each acid group has a definite effect in any physicochemical equilibrium involving the substance containing it, all are of the class *Po*, but *Pi* is used with brackets where cancelation occurs among these in the ferrohemoglobin-ferrihemoglobin electrode potential.

The two acid groups of ferrohemoglobin have been called the "oxy-labile" groups. Since the acidity of these groups has been shown to be affected by the oxidation to ferrihemoglobin, it is proposed that the more general name *heme-linked groups* be used for them instead. For convenience we designate the various heme-linked acid groups which give rise to pK_1, pK_2, and pK_3 as Groups I, II, and III respectively, and the forms of hemoglobin derivatives predominating in the pH ranges just before neutralization has proceeded half-way as forms I, II, and III. In the case of ferrihemoglobin, the form occurring in alkaline solution with all acid groups neutralized has been recognized as the hydroxide complex, HbOH (6).

Magnetometric Evidence for Value of pK_1 of Ferrihemoglobin

From the data reported in Table III of Coryell, Stitt, and Pauling (6) we have been able to check indirectly the value of pK_1 for ferrihemoglobin given in Table I. The magnetic data were treated assuming the value 5.30 for pK_1 and a calculation made of the effect of the acid form in lowering the observed susceptibility below the asymptotic value given in Fig. 1 of the Coryell, Stitt, and Pauling paper. Their figure has been amended and is presented here as Fig. 1. The broken portions of Curves A and B represent the asymptotic susceptibility which applies to the second form (Hb^+ II). The solid curve falling off in the acid range was calculated for the value 5.30 for pK_1 and the value $12{,}570 \times 10^{-6}$ c.g.s.u. for the molal paramagnetic susceptibility of the most acid form (corresponding to an increase in susceptibility on the loss of the proton of 1500×10^{-6}). The average deviation of the points in the acid range is 110×10^{-6}, comparable to deviations found in the other magnetic studies. This is accordingly a satisfactory treatment of the magnetic susceptibility data for ferrihemoglobin in the pH range from 5 to 12. We

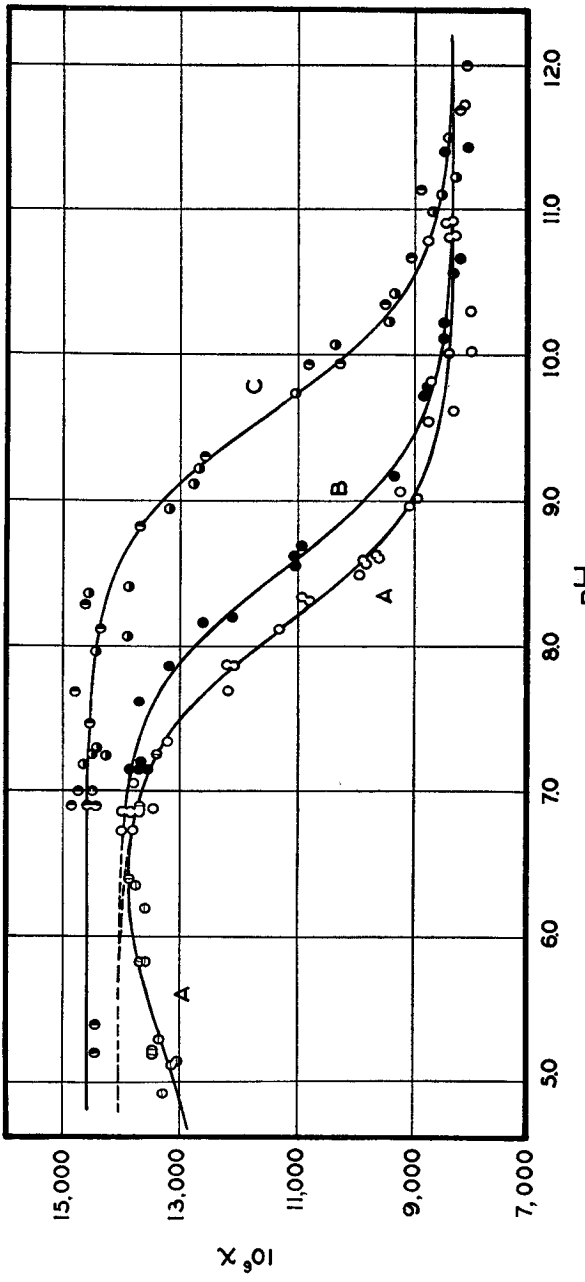


FIG. 1. Dependence of magnetic susceptibility of ferrihemoglobin solutions on pH. Curve A, ionic strength 0.20, $pK_1 = 5.30$, $pK_2 = 8.15$. Curve B, ionic strength 1.3, $pK_2 = 8.56$; Curve C, low and high ionic strength with added fluoride, apparent $pK_2 = 9.62$. Molal susceptibilities ($\times 10^{-6}$ c.g.s.u.) Hb^+I, 12,570; Hb^+II and Hb^+III, 14,070; HbF, 14,610; HbOH, 8340.

estimate that the magnetic determination of the value 5.30 for pK_1 is reliable to about 0.2 unit. The data show that ionization of the second acid group ($pK_2 = 6.65$) causes no change in magnetic properties.

The approximation of two points close to the fluoride Curve C in the acid range (pH $\sim$ 5.3) indicates either that the first acid group of ferrihemoglobin fluoride does not ionize in the range investigated or that it is magnetically inoperative. (The data of Lipmann (7) are not extensive enough for a quantitative treatment of this question.) The inflection at pH 9.6 on this curve is that for replacement of fluoride ion (bonded to the iron atom) by hydroxide ion in 0.34 M fluoride solution.

Structural Interpretation of Acidity of Heme-Linked Acid Groups

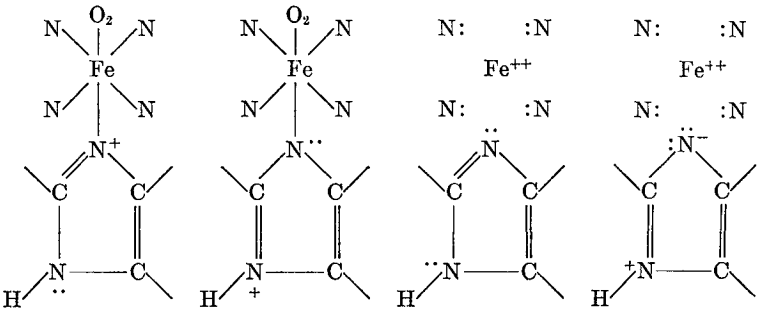
The reduction of ferrihemoglobin to give ferrohemoglobin is accompanied by the loss of one plus charge for the iron atom, and one acid group for the molecule in the range which has been explored (Table I). The formation of ferrihemoglobin fluoride involves bonding the fluoride ion to iron by an ionic bond (6), and the fluoride ion is displaced by the hydroxide ion in alkaline solution. Since reaction with fluoride gives rise to an apparent shift in pK_3 of ferrihemoglobin in accord with the physicochemical requirements of this equilibrium (6) (see Fig. 1), we conclude that the corresponding acid Group III is due to the ferric atom itself. The behavior of a positive ion as an acid group is a well known phenomenon, as for instance in the first step of hydrolysis of the free ferric ion.

It seems reasonable, furthermore, that there are two other acid groups associated with the hemes, for which changes in the bonding of the iron atom give rise to the various values observed for pK_1 and pK_2. Wyman (2) has already concluded from a consideration of the pK values themselves and of the heats of ionization (about 6500 calories per equivalent) of the group that both of these acid groups are due to imidazole groups of histidine residues contained in the globin part of the molecule. We present here structural explanations for the shifts from the values observed in ferrohemoglobin caused by complex formation (with oxygen or carbon monoxide) and by oxidation.

Let us first consider acid Group II. Since the iron atom is

held by essentially ionic bonds in ferrohemoglobin (10) and also in ferrihemoglobin (6), the chief effect of oxidation is to increase the positive formal charge on the iron atom by 1 unit. This is seen to have the effect of decreasing pK_2 by 1.16 units. Assuming that the electrostatic interaction of the iron atom and the proton of the acid group is solely responsible for this change in pK_2, we can estimate roughly the distance between acid Group II and the iron atom from comparison with some dipositive acids such as the salts of the alkyl diamines. The first and second pK values of propylenediamine and butylenediamine differ by 2.04 and 1.54 units, respectively (11). These values must be decreased by log 4 or 0.60 for the symmetry effect, which does not operate between the chemically unlike second and third acid groups of ferrihemoglobin. The observed decrease in pK_2 on oxidation of the iron atom lies between the corrected diamine differences (ΔpK) 1.44 and 0.94. The distance between the nitrogen atoms of the two diammonium salts, assuming extended configuration and the angles and distances given by Pauling (12), are 4.94 and 6.27 Å., respectively. We conclude that acid Group II of ferrohemoglobin and ferrihemoglobin is roughly 5 Å. from the iron atom, about the expected distance between an iron atom near one of the ring nitrogen atoms of a histidine residue and the second nitrogen atom of the ring.

On combination of ferrohemoglobin with oxygen or carbon monoxide, the bonds to the iron atom change from the essentially ionic to the essentially covalent type (10). In order to explain the shift in pK_2 caused by this change ($\Delta pK_2 = -1.01$ units),



we make the assumption that the iron atom is close to one of the histidine heterocyclic nitrogen atoms. Important structures contributing through resonance to the normal states of the two compounds are shown in the accompanying diagrams. Only the imidazole skeletons of the histidine residues and the 4 nitrogen atoms of the porphyrin group are shown.

The effect of resonance on acid strength may be discussed in the following way (12). If Structure A alone represented the normal state of oxyhemoglobin, the acidity of the NH group of the imidazole ring would be very low, since the structure $-\ddot{N}-H$ is characteristic of amines (such as dimethylamine) which are basic rather than acidic. If Structure B alone represented the normal state, the group would be rather strongly acidic; the group $=N^{+}-H$ in the pyridinium cation,[1] for example, has $pK_A = 5.1$. With resonance between Structures A and B, the group should be somewhat less acidic than the pyridinium cation. This is observed for imidazole derivatives of this type; thus the observed value (13) of pK for the N-methylimidazolium cation is 7.35. For the imidazolium ion itself the observed value of pK, 6.95, becomes 7.25 when corrected by the amount of log 2 to correct for the presence of 2 equivalent ionizable hydrogen atoms; and the imidazolium group in the histidine cation (14) has pK = 6.04, which becomes 6.34 on correction, the change from the imidazolium value being attributable to interaction with the charges in the ionized amino and carboxyl groups of the amino acid. Some variation in substituted imidazoles is to be expected also because of the difference in electronegativity of the attached groups. We would predict on the basis of our postulate about the structure of oxyhemoglobin that pK_2 for this substance should lie in this region, near 7; the observed value, 6.80, is in satisfactory agreement with this prediction.

A qualitative prediction of the effect on pK_2 of removing the oxygen molecule can be made, in the following way. Structures A and B for oxyhemoglobin are seen to be closely similar (they are equivalent in the imidazolium ion), and hence they contribute

[1] The Kekulé-like resonance in this substance is not expected to affect the acidity very greatly.

nearly equally to the normal state of the molecule. The decrease from large contribution of a structure of the type of Structure B (pyridinium ion) to a contribution of about 50 per cent is accompanied by an increase in pK by about 2 units. Now in ferrohemoglobin itself Structure B′ makes a still smaller contribution than 50 per cent, because this structure, with separated electric charges, is less stable than Structure A′, in which the nitrogen atoms have their normal covalence; hence it is predicted with certainty that *the change of bond type for the iron atom accompanying removal of the oxygen molecule must be accompanied by a decrease in the acidity of the attached imidazole group.* A quantitative prediction of the magnitude of the expected change in pK_2 from oxyhemoglobin to ferrohemoglobin cannot be made at present; but the observed change from 6.80 to 7.81 is reasonable, in the light of the above discussion.

In explaining the changes in pK_1 recorded in Table I we make use of the suggestion of Conant (15) that a second imidazole ring of a histidine residue lies near the opposite side of the porphyrin ring from the one responsible for acid Group II. We assume that the 3-nitrogen atom of this ring is restrained by the configuration of the hemoglobin molecule to a relatively unfavorable position for electrostatic coordination with the iron atom, so that a proton can be added, breaking the bond to the iron atom, at high enough acidity. These assumptions explain the occurrence of the low pK_1 value 5.25 in ferrohemoglobin for the imidazolium structure postulated for acid Group I. The coordination of the iron atom with an oxygen molecule on the same side of the porphyrin ring would be expected to prevent the interaction with the iron atom and thus to make the imidazolium group show more nearly the same pK value as in histidine itself (pK = 6.04).

On oxidation of the iron to the ferric form, the pK_1 value would at first sight be expected to be lowered, as is the pK_2 value; instead, no appreciable change is observed. If, however, after addition of a proton to the imidazole group a water molecule coordinates (through dipole attraction) with the iron atom in the ferric state more strongly than with it in the ferrous state, the corresponding extra stabilization of the acid form by the water molecule will tend to offset the expected decrease in pK_1 when the iron atom is oxidized. The cancelation of these effects seems to be complete.

It is noteworthy that physicochemical analysis offers the main method for studying the nature of the binding of the prosthetic group to the protein in hemoglobin. Without doubt, theories which stand the test of further experimental investigation of this substance will be of general value in the study of other heme pigments. The spectroscopic study of the acid groups of ferricytochrome C by Theorell and Åkesson (16) together with the as yet unpublished new magnetic studies carried out in these laboratories by Theorell illustrates this point.

We are indebted to Dr. J. F. Taylor of Harvard University for making the results of his experiments available to us before their publication in detail.

SUMMARY

The existence of an acid group interacting with the heme in ferrihemoglobin (methemoglobin) with the pK value 5.3 has been established from the consideration of electrode potential data and magnetic susceptibility data. The magnetic susceptibility of ferrihemoglobin over the pH range 5 to 12 has now been completely correlated with heme-linked acid group ionizations.

A table is presented giving the spectroscopic and magnetic characteristics of the three known heme-linked acid groups of ferrihemoglobin, the two of ferrohemoglobin, and the two of oxyhemoglobin and carbonmonoxyhemoglobin occurring in the pH range 4.5 to 9.5. An explanation is offered for the fact that only one effective pK value is observed in studies of the ferrohemoglobin-ferrihemoglobin electrode potential.

Structural interpretations of the acidity of the heme-linked acid groups in hemoglobin and the changes in acidity caused by oxygenation and oxidation are given. It is postulated that acid Group I is a histidine imidazolium ion in poor position for electrostatic coordination of the basic form with the iron atom, and that acid Group II is the imino group of a histidine residue (on the opposite side of the porphyrin ring from acid Group I) whose 3-nitrogen atom is strongly coordinated by either an essentially ionic or an essentially covalent bond with the iron atom. Acid Group III of ferrihemoglobin is the iron atom itself, which may add hydroxide ion, or a water molecule coordinated to the iron atom, which may lose a proton.

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