Acid Denaturation of Carbonylhemoglobin. Protein Unfolding without Heme Detachment*

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ABSTRACT: When ferrihemoglobin (Hb^+) is acid denatured, migration of heme from its native site and protein unfolding occur simultaneously. With carbonylhemoglobin (COHb) new spectroscopic evidence confirms earlier suggestions that the heme remains attached to globin at the native site when anaerobically acid denatured. When COHb is denatured at pH 1.8 to 3.3, unfolding is established by uv difference spectra which contain typical tryptophan blue shift troughs and other peaks attributed to phenylalanine; also, in optical rotatory dispersion measurements the helix Cotton effect trough at 233 mμ is greatly diminished. Stop-flow kinetics at the tryptophan trough (284 mμ) are first order and the rate constants for unfolding of protein increase with decreasing pH, but appear to approach a limiting value; horse and human COHb give identical results. Other difference bands appear only when unfolding is induced at pH less than 3.0. At pH 3.0 to 3.3 the Soret Cotton effect (due to heme–protein interaction) *increases* and shifts to more negative rotations. Thus in this range unfolding occurs with the heme attached and optically active in a changed environment. At pH less than 3.0 the difference spectra show a heme trough at 269 mμ, deepening with decreasing pH and parallel with the disappearance of the Soret Cotton effect. Stop-flow kinetics at 269 and 421 mμ give second-order rate constants (heme dimerization) in good agreement, and also independent of species. Analysis of an anomalous pH dependence of these dimerization rates gives the pK of the heme-linked imidazole and an estimate of the association constant for CO-heme and imidazole. The species independence of the rate of unfolding of COHb is unique and is discussed in terms of additional or enhanced bonding between heme and globin other than their involving the iron–imidazole bond.

Investigations of the conformational transitions of hemoglobin have often been carried out on the ferric form of the protein because the ferrous form is extremely autoxidizable in the denatured state. More recently it has become apparent that there are subtle but important differences between the unfolding behavior of ferri- and ferrohemoglobins. One of these differences is in the stability of ferri- and ferrohemoglobins toward acid denaturation. COHb is more stable in acid than Hb^+ even in the presence of air (Zaiser and Steinhardt, 1951). A more recent study of COHb denaturation with oxygen strictly excluded has shown that the Soret band persists even though other measurements indicate that the protein is fully acid unfolded (Steinhardt *et al.*, 1966). This is in vivid contrast to the case for Hb^+ where the Soret band completely disappears in a three-stage process as the protein is denatured (Polet and Steinhardt, 1969; Allis and Steinhardt, 1969). However, anaerobic denaturation of COHb at a pH lower than that used by Steinhardt *et al.* (1966) in the earlier case resulted in the loss of much of the Soret band as if it had undergone oxidation. However, oxidation of the heme could not have played a part in this disappearance, because upon regeneration at pH above 4 no Hb^+ was formed (Geddes and Steinhardt, 1968). In the same study, the back-titration curve of denatured COHb was found to depend upon both the pH at which the denaturation took place and the length of time the protein had been denatured.

The present paper reports a systematic study of the sequence of events which occur when COHb is rapidly denatured at low pH values under strictly anaerobic conditions. This work has resolved the apparent contradictions raised by the earlier work. It has also made even more vivid the marked difference between the physicochemical behavior of carbonyl and ferri form of hemoglobins.

Experimental Procedure

Materials. Horse COHb was crystallized three times from the blood of a single animal using the method of Ferry and Green (1929) as described earlier (Steinhardt *et al.*, 1966). Human COHb was also crystallized three times from pooled human red blood cells as described previously (Steinhardt and Hiremath, 1967) using the procedure of Drabkin (1949). The preparations were stored frozen in 5% solutions. Two preparations from each species were used for this work. The molar extinction coefficients were: at 419 mμ, 185,000–190,000 except for one human preparation which was 174,000; at 538 mμ, 12,500–14,300; 569 mμ, 12,200–13,700. No measurable peaks at 500 or 630 mμ were present, indicating the absence of the ferri forms.

Methods. All experiments were carried out in the presence of 0.02 M chloride ion and HCl was used as the denaturing agent. Kinetic experiments were conducted at 25 ± 0.1° and difference spectra and optical rotatory dispersion measurements were made at room temperature (usually 23°).

Spectrophotometry. Absorption spectra and difference spectra were measured with a Cary Model 14 spectrophotometer. An expanded-scale slide-wire, 0–0.10-absorbance unit full scale, was used for the difference spectra. Denatured COHb

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[1] Abbreviations used are: COHb, carbonylhemoglobin; Hb^+, ferrihemoglobin.

solutions were prepared in a tonometer with a 0.50-mm path-length absorption cell attached, essentially as described previously (Steinhardt *et al.*, 1966); the tonometer was evacuated and refilled three times with a CO atmosphere before the acid and the protein solutions were mixed. No evidence of denatured ferrihemoglobin was found, *i.e.*, no band at 650 mμ, in any of the spectra reported. Protein concentrations for difference spectra were typically 0.43% at 240–300 mμ and 0.16% at 220–240 mμ. For direct spectra, 0.43% protein and path lengths of 0.1–0.5 mm were used.

Hemin chloride solutions were made as previously described (Polet and Steinhardt, 1969). At the final dilution CO-saturated water was used and 1 or 2 mg of sodium dithionite was added (per 100 ml of solution) to reduce the hemin to ferroheme and remove any trace of oxygen. A bright pink color characteristic of carbonylferroheme resulted, which was stable at neutral pH for at least 2 hr. The concentration of heme was 1.26×10^{-5} M; the spectra were measured in a 5.00-mm cell. The absorption coefficient of the Soret band at 407 mμ was 1.70×10^5 l. mole^{-1} cm^{-1}.

Spectropolarimetry. Optical rotatory dispersion over the far-ultraviolet and Soret regions was measured with a JASCO Model UV 5 spectropolarimeter. In many cases the solution used for the difference spectra was used for the optical rotatory dispersion measurements; these can be distinguished by the identical pH's given for the two measurements. In the cases where a different sample was prepared for the optical rotatory dispersion, they were prepared in precisely the manner described for the difference spectra. The path length of the cell used for all optical rotatory dispersion measurements was also 0.50 mm and the protein concentration was 0.43%.

Stop-Flow Kinetics. All kinetic measurements were made on a Durrum-Gibson stop-flow spectrophotometer. It was necessary to use much lower protein concentrations for these measurements than those referred to above because the cuvets available for this instrument (1.85 and 2.13 mm) are longer than used in the other measurements, and because a higher absorbance is desirable for difference spectroscopy and optical rotatory dispersion than for these kinetic measurements. Protein concentrations for kinetic measurements were 0.020–0.045%.

The solutions used for kinetics were freed of any vestige of oxygen before the measurements were made. A simple apparatus was constructed consisting of two 250-ml-round-bottom flasks joined through a sintered glass disk. On one of these flasks was a two-way pressure stopcock and on the other was a one-way pressure stopcock. The one-way stopcock was fitted with a serum cap and 50 ml of either acid solution or COHb solution was placed in that flask. In the other flask was placed a small magnetic stirring bar and a small amount of platinized asbestos. The apparatus was closed and the double stopcock was connected to a vacuum pump and a tank of 90% H_2–10% CO gas. All reagents had previously been saturated with this gas. The closed apparatus was alternatively evacuated and filled with the H_2–CO mixture a total of three times. Then the reagent solution was drawn into the other flask through the sintered disk and into contact with platinized asbestos and stirred for 5 min before being passed back through the sintered disk to the original flask. The solution was then withdrawn through the serum cap using the reservoir syringe of the Durrum-Gibson instrument. The syringes had been thoroughly greased and allowed to stand with CO inside for at least 0.5 hr. The flow system of the spectrophotometer was always flushed with CO-saturated water for about 2 hr before the experiment. An inert atmosphere (N_2) under continuous flow was provided to bathe the rear of the drive syringes on the instrument in early experiments. Later, Hamilton gas-tight syringes were used as drive syringes and this practice was no longer necessary. It was known from previous work with platinized asbestos as an oxygen scavenger that the hemoglobin adsorbed to the asbestos. The use of relatively large volumes of solution and small amounts of asbestos kept the concentration of protein to within 1% of its original value. Sodium dithionite was used as an oxygen scavenger in early experiments, but was discarded after it was noted that the denaturation rate of COHb was faster in the presence of dithionite than when traces of oxygen were present. The fact that the reaction products of dithionite react with the protein of hemoglobin has been reported previously by Benesch and coworkers (1964) as well as by other workers.

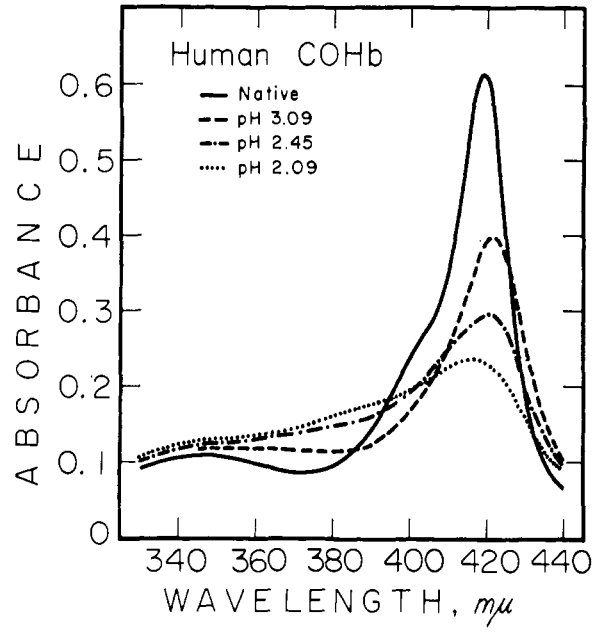

FIGURE 1: Spectra of the Soret region for human COHb, native and denatured at three pH values. All curves are measured at equilibrium. Protein concentration is 0.44%, optical path is 0.50 mm, chloride ion concentration is 0.02 M, and temperature is about 23°. pH of native COHb solution is 6.2.

Results

Soret Spectra. The spectrum of human COHb in the region of the Soret absorption band is shown in Figure 1. The corresponding spectrum of horse COHb is nearly identical. In the figure the native spectrum is compared with those at each of three denaturing pH's. Less of the Soret band is retained after denaturation at the lower pH's. These data confirm observations on horse COHb by Geddes and Steinhardt (1968) who found substantial decrease of the Soret at pH 2.40 and Steinhardt *et al.* (1966) who found about 80% retention of the Soret band at pH 3.37. The shift of the Soret

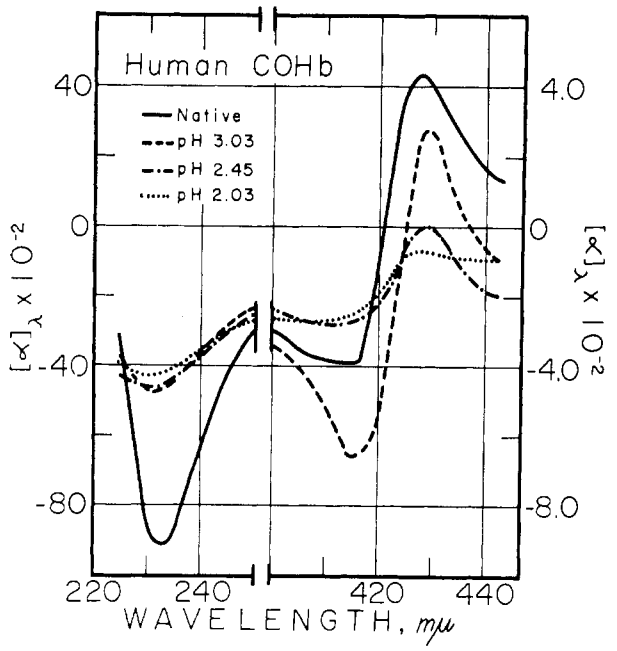

FIGURE 2: Optical rotatory dispersion of human COHb in the far-uv and Soret absorption regions for native protein and protein denatured at three pH values. Protein concentration is 0.42%, optical path is 0.50 mm, chloride ion concentration is 0.02 M, and temperature is about 23°.

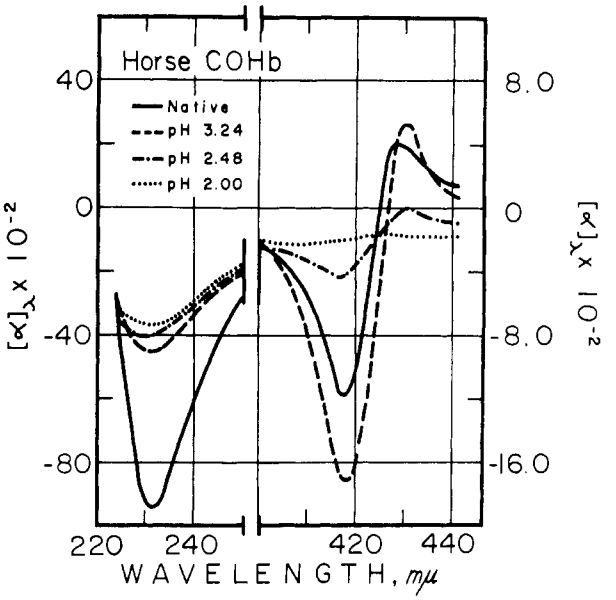

FIGURE 3: Optical rotatory dispersion of horse COHb in the far-ultraviolet and Soret absorption regions for native protein and protein denatured at three pH values. Protein concentration is 0.43%, optical path is 0.50 mm, chloride ion concentration is 0.02 M, and temperature is about 23°.

maximum from 419 mμ in native protein to 421 mμ for acidified protein (Figure 1) has been noted previously for horse COHb (Steinhardt *et al.*, 1966). This shift is immeasureably fast by stop-flow techniques at 25°.[2] The apparent shift of the Soret peak back to shorter wavelengths at the lowest pH is due solely to the decrease in the size of the Soret peak relative to the absorption at shorter wavelengths.

The peak at 421 mμ is not caused by free carbonylferroheme in solution; rather, it must be from acidification of the native protein. Free carbonyl-ferroheme has a Soret maximum at 407 mμ in aqueous solution at neutral pH. Its Soret band is stable as long as oxygen is excluded at neutral pH, but disappears rapidly at acid pH. A Soret peak at 408 mμ is obtained when COHb is denatured in 50% glacial acetic acid, but this peak disappears in a few minutes when the protein begins to precipitate.

Optical Rotatory Dispersion. It has been demonstrated previously (Steinhardt *et al.*, 1966) that COHb (horse) is completely denatured at pH below 3.4; this is confirmed in Figure 2 (human COHb) and Figure 3 (horse COHb) where the optical rotatory dispersion measurement shows the Cotton trough at 232 mμ has decreased to about half the size of the native band at all denaturing pH values indicating that essentially the same amount of structure has been destroyed in each case.

On the other hand, the Cotton effects at the Soret band (shown in the same figures) are not at all similar at the three denaturing pH's. This Cotton effect, due to an asymmetry in the interaction of the heme group with the protein, actually becomes larger at pH > 3 than in native protein (for an earlier observation, see Steinhardt *et al.*, 1966). However the enhanced Cotton effect decreases at lower pH and is nearly gone at pH about 2. The increase in size of the Cotton effect at pH above 3 is accompanied by a small shift to higher wavelength. Thus, while the protein structure is destroyed at pH between 3.0 and 3.4, the asymmetry of the heme interactions, while changed, is not destroyed but actually enhanced. Only at lower pH, where changes in helix content are no longer important, is the Soret Cotton effect, and presumably part of the specific heme–protein interaction, destroyed. The sole difference between the horse and human optical rotatory dispersion spectra is that the Soret Cotton effect of horse protein is larger and has a deeper trough.

Difference Spectra. Further evidence of a difference in the behavior of denatured COHb above and below pH 3.0 is shown in the uv difference spectra shown in Figure 4 (human) and Figure 5 (horse). In the aromatic absorption region (240–300 mμ) at pH 3.0 the difference spectra show two main features: a double trough at 284–285 and 291–292 mμ both typical of a tryptophan blue shift, and five peaks (or troughs) in the 240–270-mμ region due to phenylalanine perturbation (Herskovits and Sorenson, 1968; Polet and Steinhardt, 1968). These features indicate that the protein has unfolded and that tryptophan and phenylalanine which are shielded from water in the native structure have come into contact with the solvent in the denatured protein.

At the two lower pH values for each protein, another band appears which has a minimum at 269 mμ. We attribute this new band to a change undergone by the heme group since its appearance parallels the disappearance of the Soret Cotton effect in the optical rotatory dispersion spectra. Monomeric heme in the Fe(II) oxidation state is known to have an absorption band with maximum at 270 mμ when it is associated

[2] A similar red shift as the first stage of the acid denaturation of Hb^+ has been shown to be time dependent at 0° (Allis and Steinhardt, 1969).

with a nonaromatic polypeptide chain; this band is absent for heme in the Fe(III) state (Urry, 1967). Kinetic evidence for this assignment will be presented later. The difference spectra in the far-ultraviolet region show a trough (at 228 mμ) to which both protein and heme apparently contribute (Figure 4).

The foregoing data show that there are two distinctly different sets of results depending upon the pH at which the denaturation is carried out. Above pH 3.0 only protein unfolding occurs and the heme group retains its close association with its native binding site on the protein, although the environment of the heme has been modified. At pH below 3.0 protein unfolding is accompanied by loss of the Soret absorption and Cotton effect and a change in the absorption at 269 mμ. Polet and Steinhardt (1969) have shown with ferrihemoglobin that the heme separates from its native site on the protein during denaturation and subsequently dimerizes. Similar events may also be responsible for the observation in the present case, although lower pH values are required than with ferrihemoglobin.

Kinetics. The technique used by Polet and Steinhardt (1969) of following different events in the reaction sequence by observing changes at wavelengths at which each separate event predominates has been applied to the present work. Five wavelengths have been chosen from the spectral data (Figures 1, 4, and 5) at which kinetic measurements are made: four are in the aromatic absorption region, 250, 269, 284.5, and 291.5 mμ; the fifth is the Soret maximum, 421 mμ.[3]

At pH greater than 3.0 the data from all wavelengths give first-order kinetics with the same rate constant for a given pH. When the first-order rate constants are plotted logarithmically as a function of pH (Figure 6) the data are in fair agreement with the older denaturation data of Steinhardt *et al.* (1966) (filled circles). The latter results were obtained from measurements which did not depend on spectral properties, such as (a) pH-Stat measurements where the titration of imidazole groups not available in the native protein is followed, and (b) precipitation of denatured protein as a function of time. These older measurements were made on horse protein.

At pH below 3.0 the kinetic results are more complex. At 291.5 mμ two reactions are clearly distinguishable since they proceed with changes in absorbance which reverse sign; the first reaction is much faster than the second. From the kinetic trace, the first reaction appears to be 5–10 times faster than the second, varying somewhat with pH. The faster reaction when plotted by the method of Guggenheim (1926)[4] follows first-order kinetics. The slower reaction follows second order kinetics for the case where the initial concentrations of the reactants are equal.

At 284 mμ only the faster, first-order reaction is observed. This wavelength appears to be fortuitously isosbestic for the slower reaction (*i.e.*, no changes occur in heme absorption). Therefore an end value for the faster reaction can be measured

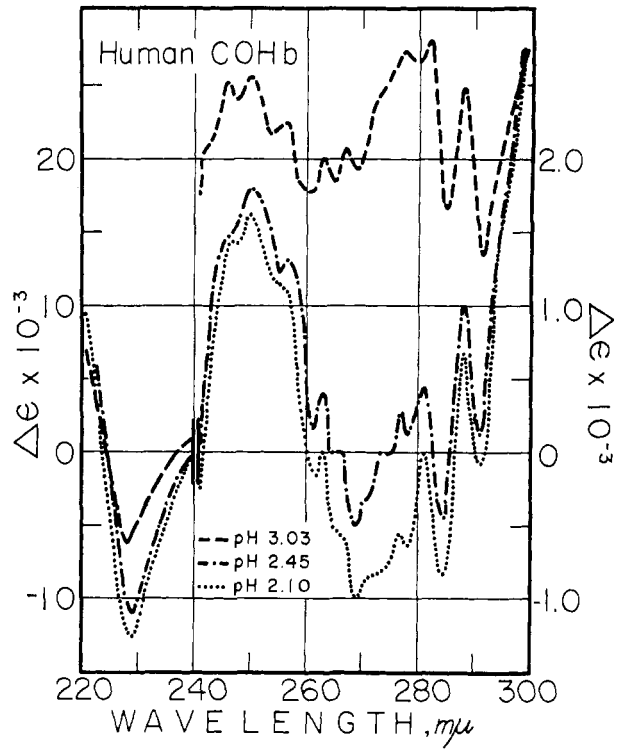

FIGURE 4: Human COHb difference spectra between native protein and protein denatured at three pH values. The spectra are measured in the far-ultraviolet and aromatic absorption region of the ultraviolet. Protein concentration is 0.16% for 200–240 mμ and 0.42% for 240–300 mμ. Optical path is 0.50 mm, chloride ion concentration is 0.02 M, and temperature is about 23°.

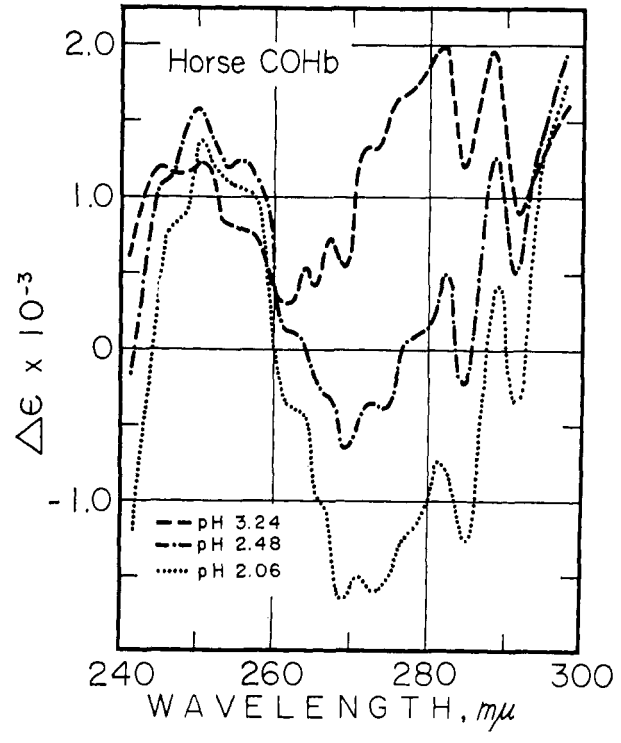

FIGURE 5: Horse COHb difference spectra between native protein and protein denatured at three pH values. The spectra are measured in the aromatic absorption region of the ultraviolet. Protein concentration is 0.43%, optical path is 0.50 mm, chloride ion concentration is 0.02 M, and temperature is about 23°.

[3] It should be borne in mind that the spectral band pass for the Gibson Durrum is not nearly as small as for the Cary. The band pass for the kinetic experiments in the ultraviolet is 3 to 4 mμ and the kinetic data are an average of the peaks or troughs that are so sharply defined in the difference spectra obtained with the Cary.

[4] The difference in absorbance over successive time intervals is plotted logarithmically as a function of time. Initial or final absorbance values are not required when this method is used.

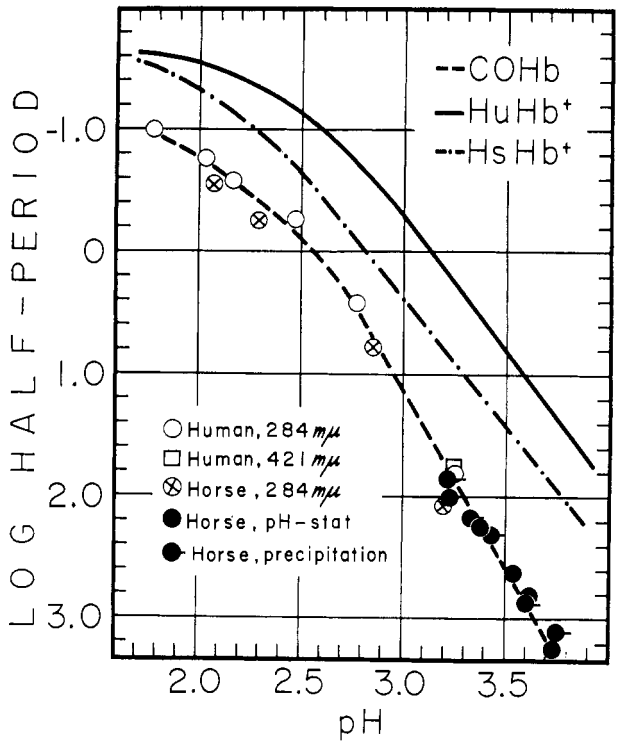

FIGURE 6: Dependence of the logarithm of the first-order rate of denaturation of COHb upon pH at 25.0° and anion concentration of 0.02 M. The half-time, $t_{1/2}$, is related to the first-order rate constant, k_1, by $t_{1/2} = (\ln 2)/k_1$. The open points are calculated from measurements at 284 mμ and the filled points are taken from Steinhardt *et al.* (1966). Similar curves for horse and human Hb^+ are included for comparison (Polet and Steinhardt, 1969).

and the normal method of calculating first-order rates can be used.[5] The first-order rate constants obtained are plotted in Figure 6. Measurements at several pH values for both horse and human protein are given, and fall on a line continuous with the data discussed above. It is clear that these first-order rates describe the denaturation of the protein since they are taken at a tryptophan difference band and are continuous with similar data measured by entirely different techniques.

An unexpected result of this analysis is that both horse and human COHb have the same kinetic pH profile. With the ferrihemoglobins, different profiles were found. The Hb^+ profile of the two species are included in Figure 6 for comparison. It should be noted that at the same pH COHb always denatures much more slowly than the Hb^+ of either species. At very low pH all the profiles appear to approach a limiting velocity of denaturation and the velocity for COHb continues to be lower than those for Hb^+ (see Discussion). The slope of the linear portion of the velocity profile is about three for the COHb data and about two with the ferrihemoglobins.

At 269 mμ the slower, second-order reaction predominates with an absorbance change about twice as large as that at the other ultraviolet wavelengths. Departure from second-order kinetics in the early part of the reaction appears to be due either to spectral interference of the faster reaction or the need for the faster reaction to provide the reactants for the slower reaction, or perhaps to both effects combined. The early departure from second-order kinetics disappears after a time approximating three half-periods of the first-order reaction (87% complete) at the same pH. The kinetic plots obtained are qualitatively similar to those illustrated previously (Figure 6, Allis and Steinhardt, 1969) at different wavelengths, and need not be repeated here. The fact that second-order rate behavior consistent with a dimerization process is observed at this wavelength lends strong support to the earlier assignment of this band to a reaction of the heme group.

The kinetics at 250 and 421 mμ are like those at 269 mμ, predominantly second order except for the departure noted in the early part of the reaction. The presence of the second-order reaction at 250 mμ is probably a reflection of the fact that about half of the phenylalanines of hemoglobin are in contact with the heme in the native structure (Perutz *et al.*, 1968), as well as the fact that there is probably spectral interference at this wavelength from the relatively broad heme band at 269 mμ. The Soret band (421 mμ) is, of course, principally a heme band and reflects the reactions of the heme. Its very large absorbance change under these conditions makes it particularly attractive for kinetic analysis.

The second-order rate constants were measured at 269 and 421 mμ at pH below 3.0, where the second-order reactions appear. Since these rate constants have been determined at two different wavelengths, they will appear to be a function of the strengths of the absorption bands at these wavelengths, unless they are calculated in terms of the conventional units of second-order rate constants (l. $mole^{-1}$ sec^{-1}) by the method described previously (Allis and Steinhardt, 1969) before direct comparison. The experimental rate constants, so calculated, are plotted as the apparent rate constants, k_{app}, in Figure 7. These rate constants are pH dependent in strong contrast to the case of the second-order reaction found during Hb^+ denaturation (Polet and Steinhardt, 1969), and are dealt with further in the Discussion. The data for horse and human proteins at both wavelengths give the same results. Below pH 2.1 the second-order reaction rate is pH independent but between pH 2.1 and 3.0 there is a strong dependence on pH. The line drawn with slope −2.0 is the best fit through the experimental points in the latter region. Thus, the apparent second-order rate constant is proportional to the square of the hydrogen ion concentration at pH between 2.1 and 3.0. The *maximum* dimerization rate of ferroheme in this system is slower by a factor of about 2.5 than that for ferriheme as calculated from the data of Polet and Steinhardt (1969).

Discussion

Reaction Mechanism. The presence of a second-order process with both reactants at the same concentration is consistent with only one molecular process in these solutions; that is the dimerization of heme after the unfolding of the protein. This interpretation is also consistent with the difference spectra and optical rotatory dispersion data discussed above, and is identical with the explanation given for the second order process in the denaturation of Hb^+ (Polet and Steinhardt, 1969). Two major differences between Hb^+ and the present case remain to be explained: (a) the absence of a second-order reaction when denaturation occurs at pH above

[5] The kinetic plots obtained are qualitatively similar to those illustrated by Polet and Steinhardt (1969) in their Figure 4; however, they were forced to use the Guggenheim method for their analysis which is not the case at 284 mμ in this work.

3.0, and (b) the pH dependence of the dimerization rate in the case of COHb but not with Hb^+. Both these differences arise from the fact that in COHb there is a covalent bond between the heme iron and the imidazole of histidine F8, its normal binding site. In Hb^+ this bond is very highly polar, essentially ionic. The data presented here show that when CO Hb is unfolded, the heme–imidazole bond is not disrupted unless the pH is brought below 3.0. The pH dependence in the region pH 2.1–3.0 is due to the fact that imidazole, a prototropic group which normally protonates at pH 6.5, is not free to receive protons until the hydrogen ion activity is high enough to compete with iron for the basic form of imidazole, *i.e.*, until there is an equilibrium between bound and unbound heme. Below pH 2.1, essentially all the heme is detached from its normal site and the pH dependence disappears.

Based on these facts, the mechanism for the denaturation of COHb appears to be

$$\text{COHb} + x\text{H}^+ \longrightarrow \text{COHbH}_x^+ \qquad (1a)$$

$$\text{COHbH}_x^+ \xrightarrow{k_1} \text{D-heme} \qquad (1b)$$

$$\text{D-heme} + \text{H}^+ \overset{K_{eq}}{\rightleftharpoons} \text{DH}^+\text{-heme} \qquad (2)$$

$$2\text{DH}^+\text{-heme} \xrightarrow{k_2} \text{dimerized heme–protein complex} \qquad (3)$$

where $COHbH_x^+$ is the protonated form of native COHb at the denaturing pH, D-heme refers to acid-unfolded COHb with heme still attached at its normal imidazole binding site, and DH^+-heme is acid-unfolded COHb with the imidazole of histidine F8 protonated and the heme bound to the protein at some other site. Equation 1a is the protonation of COHb and is much too fast to measure by stop-flow techniques. Equation 1b is the fast first-order acid-unfolding of the protein which affects tryptophan bands (and may be followed with their aid) and is pH dependent. Equation 3 is the slow concentration-dependent second-order reaction, the kinetics of which are followed principally at 269 and 421 mμ. Equation 2 is *not* observed kinetically, because the equilibrium must be established much more rapidly than the rate of the second-order reaction.

The reaction represented by eq 2 is interpreted as the breaking of the iron–imidazole bond accompanied by protonation of the imidazole. This is followed by displacement of the heme group to another binding site where it is attached to the protein by a mechanism other than through the iron–imidazole bond, possibly through hydrophobic contacts. In this latter state the heme is free to dimerize although the dimer must remain attached to the globin molecule, while in the state represented by D-heme, dimerization does not occur. The final product is a complex of dimerized heme and acid-unfolded protein.

A similar mechanism for heme dimerization after unfolding of Hb^+ is implied by the results of Polet and Steinhardt (1969). They observe no precipitation of heme after denaturation (an observation made by many workers) although dimerized heme is insoluble at acid pH. The spectrum observed by Polet and Steinhardt is the same as that of *soluble* dimerized heme, which may be observed at pH values well above 7. Polet and Steinhardt also observe only a single dimerization

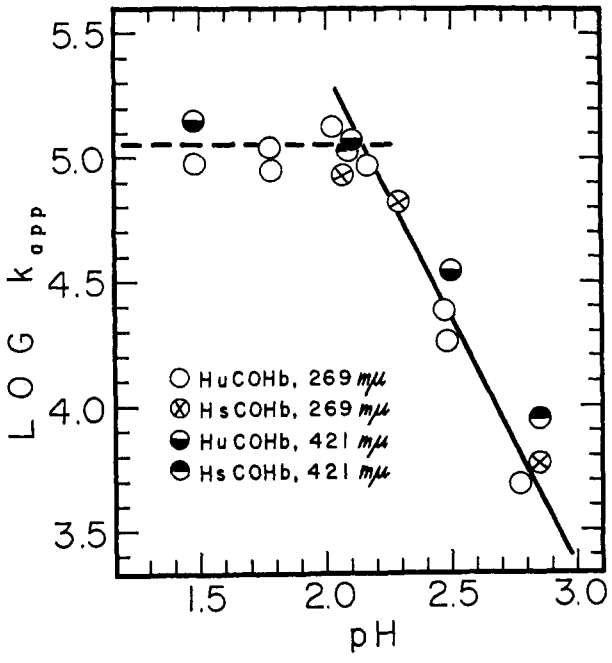

FIGURE 7: Dependence of the logarithm of the apparent second-order rate constant, k_{app}, upon pH at 25.0° and chloride ion concentration of 0.02 M. The solid line is drawn with slope −2 and the dashed line represents the average *maximum* rate constant.

rate constant, in contrast to the observation of Inada and Shibata (1962) who observed several successively slower rate constants when they allowed free hemin to dimerize without protein or other ligand present. This result was interpreted as the formation of higher homologs of the dimeric species until a large enough polymer was formed to precipitate. Since this result is not observed with unfolded Hb^+ present, the dimeric heme must be bound to the protein chain in some fashion that prohibits the formation of higher homologs of hemin polymer.

On the basis of the mechanism proposed, a rate law for the dimerization may be developed. This development is simplified by the fact that the unfolding of the protein (eq 1b) is much more rapid than the dimerization under the conditions of these experiments, and by the assumption that the equilibrium in eq 2 is rapid with respect to the dimerization, mentioned previously. The rate of appearance of dimerized products is given by

$$\text{rate} = \frac{\text{d[dimerized products]}}{dt} = k_2[\text{DH}^+\text{-heme}]^2$$

If the total amount of monomeric heme is T, then at any given time

$$T = [\text{D-heme}] + [\text{DH}^+\text{-heme}]$$

Now the quantity [D-heme] may be eliminated between this expression and that for K_{eq} (from eq 2). After solving the resulting expression for [DH^+-heme] and substituting into the above rate law, we obtain

$$\text{rate} = k_2\left(\frac{K_{eq}[\text{H}^+]T}{1 + K_{eq}[\text{H}^+]}\right)^2$$

This development predicts a second-order rate law will be followed at all pH values if the assumption that method of detection of the reaction does not distinguish between the two monomeric forms of heme, D-heme, and DH^+-heme, when compared with the dimerized form. This assumption appears to be valid for the spectroscopic wavelengths used in this study. The rate law also predicts an apparent second-order rate constant which is dependent on pH

$$k_{app} = \frac{k_2 K_{eq}^2 [H^+]^2}{(1 + K_{eq}[H^+])^2}$$

In the limit of low pH, k_{app} loses its dependence on pH and becomes equal to the true second-order rate constant, k_2. At high pH, $k_{app} = k_2 K_{eq}^2 [H^+]^2$ showing a second power dependence on the hydrogen ion concentration. These are the features of the data illustrated in Figure 7.

The lines drawn in Figure 7 represent the *asymptotic* values of the rate law. The scatter in the data is sufficient to preclude a more exact fitting of the data to the rate law except by complex iterative procedures. However, log k_2 can be evaluated from the low pH region as 5.05, and log $K_{eq} = 2.2 \pm 0.2$ can be calculated using this value and any point on the line of slope -2 from Figure 7.

Log K_a for the association of protons with the histidine imidazole in a polypeptide chain is about 6.5. Log K_a for the protonation of the imidazole tied to hemin has been shown to be 2.2 (protons and hemin have the same affinity for the heme-linked imidazole at this pH). Therefore, log K_a for the association of carbonylferroheme with globin at its normal imidazole binding site *moved from another binding site on the unfolded polypeptide chain* is calculated to be about 4.3. In comparison, the log K_a for imidazole binding to ferrihemoglobin (essentially a bisimidazole complex), a low-spin complex, is about 2.6 at neutral pH (Russel and Pauling, 1939). For the closest comparable ferro complex for which data are available, bispyridine–ferroprotoporphyrin kept in solution by cationic detergent, log K_a is 5.4 (Falk, 1964). That the carbonylheme–imidazole link in denatured COHb is more stable than the imidazole–ferrihemoglobin complex is not surprising since ferroheme complexes are generally more stable than even low-spin ferriheme complexes. While the higher stability of the bispyridine complex might be accounted for by the possibility that pyridine and imidazole may not have the same affinity for the heme iron, or by alternative hypotheses, it appears that the value obtained above for the strength of the bond from protein to heme in COHb is plausible.

Species Independence. The data of Figures 6 and 7 show that the reaction scheme represented by eq 1–3 is entirely species independent. It is not surprising that k_{app} is the same for both species since in each case it is dependent upon detachment of the heme by breaking an iron–imidazole bond followed by dimerization of the heme (albeit bound to unfolded protein). However, the fact that the unfolding of the protein, eq 1b (with rate constant k_1), is also the same for horse and human proteins deserves special note, especially since the denaturation rates for the ferrihemoglobins of horse and human are markedly different (Figure 6). This difference does not disappear when azide or cyanide are bound (Steinhardt *et al.*, 1963; Molday and Steinhardt, 1969), even though these ligands form low-spin complexes with Hb^+. Thus, we conclude that while the globins of different species differ in their primary structure and stability at acid pH, they are apparently brought to the same stability by the presence of ferroheme with low-spin bonds, *e.g.*, O_2Hb or COHb. COHb may represent the form of maximum stability for hemoglobin since species differences appear to be eliminated, and no other form of hemoglobin is known to be more stable (Steinhardt *et al.*, 1963).

The limiting factor in determining the rate of denaturation of COHb is *not* the breaking of the heme–imidazole bond which is negligible at pH greater than 3.0. Therefore another and weaker interaction between heme and apoprotein must be broken when levels of protonation at pH 3.0–3.5 are reached.

The conversion of the iron bonds in heme from high spin into low spin is accompanied by changes of a few tenths of an Ångstrom unit in the iron bond lengths (Hoard, 1966; Countryman *et al.*, 1969) and it appears likely that ferroheme will behave similarly. Changes in the iron-bond lengths are intimately related to larger conformational changes in the polypeptide surrounding the heme group and forming the bridges between the hemes in the hemoglobin tetramer (Banerjee *et al.*, 1969; Perutz *et al.*, 1968). Thus, the strong Fe–N bond in COHb results in conformational changes in the globin which may result in the formation of the second "bond" referred to above. This "bond" may simply be an increase in the hydrophobic contacts between the heme and surrounding protein, although other types of bonding are also possible. However, the presence of protoporphyrin confers a great deal of stability to globin (Sebring, 1969) by what must be essentially hydrophobic contacts. It is reasonable to expect that hydrophobic contacts may be further enhanced by the changes that take place in the heme bonds and the conformation of the protein during the formation of COHb. These changes coupled with the changed relationship of monomer units to one another in the tetramer (Perutz *et al.*, 1968) may explain the increased resistance of COHb to acid denaturation and the elimination of the species differences found in this study.

Application of Mechanisms to Previous Work. In light of the mechanism proposed in this paper several apparently contradictory results from previous work may be clarified. First, the conflict between only 20% loss of Soret absorption noted by Steinhardt *et al.* (1966) when COHb is denatured at pH 3.37 is reconciled with the very substantial loss of Soret absorption observed by Geddes and Steinhardt (1968) when pH 2.40 is used. No heme is detached from its native binding site in the former case while substantial amounts are detached in the latter. In the latter case when the pH of the denatured COHb solution was raised in order to regenerate native protein the authors expected that some ferrihemoglobin would be formed, since the disappearance of the Soret band might have been caused by oxidation. The proposed mechanism shows clearly that this need not be the case (as indeed demonstrated by their own results). The increased effectiveness of higher regeneration pH (demonstrated in the same paper) is due to increased reforming of the heme–imidazole bond with the decrease in competition of H^+ for the imidazole.

One difference between the two older works not yet resolved is the fact that Geddes and Steinhardt report the position of the Soret band for regenerated COHb to be 419 mμ

even though the lowest regeneration pH was 3.18. This is below pH 3.37 used by Steinhardt and coworkers in the denaturation experiment in which the Soret maximum was 421 mμ, as in the present work. Both pH values are sufficiently low for the protein to be totally acid unfolded at equilibrium.

The option suggested by Steinhardt *et al.* (1966) that the heme in COHb might be held in the protein as a clathrate, that is a caged but unbound molecule, can now be firmly rejected. If true, the heme would be released as soon as the protein unfolds, which we have shown is not the case.

The most difficult of the older data to understand was the effect of pH and length of time of denaturation on the back-titration curves of COHb (Figure 3, Geddes and Steinhardt, 1968). For protein denatured at pH 3 and back-titrated after 3 sec virtually no unmasking of prototropic groups was found, even though at least threed enaturation half-periods had passed,[6] and the back-titration was measured 0.2 sec after mixing of reagents. The authors presumed that remasking of unmasked groups had occurred in this very short time. It is now evident that no heme was detached from its native site in this experiment; more important 3 sec was very likely not long enough for the molecular domains of the individual polypeptide chains of the denatured protein to become intermingled in this relatively concentrated solution (0.236%). Therefore refolding about the heme held in place could be very rapid. In an experiment in which COHb was denatured at pH 2.73 for 3 sec before back-titration, some heme was undoubtedly detached from imidazole F8. However, the 3-sec exposure (13 half-periods at this pH) was still short enough to preclude a high degree of intertwining of the polypeptide chains. The intermediate back-titration curve obtained may reflect the fact that some molecules are free to re-fold quickly and others are not.

Experiments carried out with 30-min exposure and pH of either 3 or 2.7 yield similar results; both appear to show full titration of unmasked prototropic groups. The 30-min exposure to acid is easily sufficient for molecular chains to become entangled with one another. This process must play at least as important a role in slowing the refolding reaction as detachment of the heme from the imidazole, since at the higher pH virtually no heme is detached. At the lower pH, substantial amounts of heme have become detached and dimerized, with the heme bound to the protein in a position other than the native site. This process also must be reversed to regenerate the native protein. Nearly all the native protein can be recovered in these experiments; however, the recovery occurs on a time scale much longer than required for the titration experiments.

[6] These data are for 0.3 M Cl^- and the rates of denaturation of COHb and of dimerization of heme are not directly comparable to those in the present paper. The higher ionic strength greatly increases the rate of unfolding, and probably does so for heme dimerization also.

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