Sequential Stages in the Acid Denaturation of Horse and Human Ferrihemoglobins*

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ABSTRACT: The rates of acid denaturation for human and horse ferrihemoglobin followed by the disappearance of the Soret absorption band with the Gibson stop-flow apparatus have been extended to pH 1.8 and denaturation half-periods down to 25 msec. The results are essentially a linear extrapolation of the log velocity–pH profiles which prevail at higher pH values, maintaining a constant velocity difference between the species down to pH about 2.5. At lower pH the denaturation velocity for both human and horse proteins levels off to the same rate; the differences in stability of the two proteins at higher pH values may be due to the known differences in their content of carboxylic acids. The fast measurements at low pH make it possible to distinguish between several steps in the spectral changes: A → B, a small exceedingly fast reaction, accompanied by a red shift of the Soret band; B → C, expulsion of the heme from the protein (blue shift of the Soret band); C → D, the dimerization reaction of the free heme (disappearance of the Soret band). The same kinetic analysis of other spectral changes at λ 230 mμ, and measurements of the optical rotatory dispersion at this wavelength, lead to a demonstration that the unfolding of the protein (loss of helix) and the expulsion of the heme occur at the same rate over a very wide pH range above about 2.2. Thus, at pH below about 3, rates of changes in ε of the Soret band do not suffice to measure unfolding, because these changes are due to a complex series of events, one of which (dimerization of the heme) occurs *outside* the protein.

We have previously reported that human Hb^+ is denatured by acid about five times faster than horse Hb^+ over a wide range of pH (Steinhardt and Hiremath, 1967). In spite of this difference, with both proteins the rate dependence with respect to pH, temperature, nature of buffer, and complexing of ligands is the same; *e.g.*, the pH profile is merely shifted so that the data for human Hb^+ fall at higher pH values than the data for horse Hb^+ (Figure 2, Steinhardt and Hiremath, 1967).

Another species difference in the pH dependence of the equilibria is shown in Figure 3 of the same paper. The equilibrium covers a wider range of pH with the human protein. Also the denaturation rate is clearly affected more than the regeneration rate so that there is a shift in the half-denaturation pH. As the figure shows, there is a temperature of maximum stability which is not far from 20° in the case of both proteins; it is slightly higher in the case of horse Hb^+ than in the case of human Hb^+.

The proteins of the two species differ, however, in two other more striking respects: (a) the kinetics of the regeneration of native protein; and (b) the fact that with human Hb^+, it can be easily demonstrated that the disappearance of the Soret band at 406 mμ is a separate process from the appearance of the "denatured" band at 370 mμ (Steinhardt and Hiremath, 1967). In earlier papers from this laboratory it has been shown that with horse Hb^+ these two processes run exactly parallel and masquerade as a single molecular event.

Since the pH profile of the logarithm of denaturation velocity is a straight line of about the same slope with both species, we cannot tell whether the differences in rate or pH represent shifts along the pH axis (changes in the pK of trigger groups), or shifts in the vertical direction (changes in the rate of some time-dependent process); the latter would signify that unfolding in the human protein requires a lower activation energy than unfolding in the horse protein. The same question could be raised within a single species for the stabilizing effect of such ligands as cyanide.

If the shift in the pH profile is due to a difference in pK's, the linear region for both proteins must eventually terminate and the rates level off at a lower pH. If the curves should level off at the same rate for both proteins, differences in pK with no differences in activation energy might be indicated. If the rates should level off at different levels in the two species, activation energy differences are also involved. Study of the rates at low pH (below 3.2) requires stop-flow measurements because of the great speeds of the reactions. The results in this paper were obtained with the Gibson stop-flow apparatus and are based on spectra only. At pH values above 3 the unfolding kinetics and equilibria (unmasking of prototropic groups, notably imidazole) have been shown to be closely similar to the spectral kinetics (Steinhardt and Zaiser, 1953; Zaiser and Steinhardt,

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1954; Beychok and Steinhardt, 1959; E. Sebring and J. Steinhardt, unpublished data).

In this paper the pH range for the spectroscopic measurements is extended down to 1.8, by using stop-flow measurements in the region 360–420 mμ, to obtain half-periods as low as 25 msec. It has been possible to measure the absorption spectrum of the denaturing protein within this region, by repeatedly performing kinetic experiments at slightly different wavelengths to obtain optical densities at each such wavelength as a function of time, and thus for particular times. These time series have greatly increased our insight into the relation of Soret absorption to the role of the prosthetic group in stabilizing the native conformation. It has also been possible to correlate the rates of changes in Soret absorption with those at 230 mμ, and with the rates of changes in optical rotatory dispersion at 233 mμ measured in a flow-through system with a rapid mixing chamber.

Experimental Procedure

Materials

Horse COHb was prepared from blood from a single animal by the method of Ferry and Green (1929) as described in our earlier work (Steinhardt *et al.*, 1966). Human COHb was prepared as described previously (Steinhardt and Hiremath, 1967) using the procedure of Drabkin (1949). Both proteins were stored frozen in 5% solutions. The preparations were oxidized to Hb^+ in 2% solutions with 1.5 equiv of $K_3Fe(CN)_6$ (Steinhardt and Hiremath, 1967) and afterwards dialyzed against KCl followed by distilled water. The extinction coefficients at λ 406 mμ were for human Hb^+ 140–145 $\times 10^3$ and for horse Hb^+ 170–180 $\times 10^3$.

Hemin chloride from Nutritional Biochemicals was used. All other chemicals were reagent grade.

Methods

All experiments were carried out at 25°. The ionic strength was kept at 0.02 unless otherwise noted. Solutions of hemin chloride were made as described by Inada and Shibata (1962): hemin was dissolved in a small amount of 0.1 N KOH; water was added to make the concentration approximately 1×10^{-3} M. This stock solution was used for dilution with various solvents.

Stop-Flow Measurements. The stop-flow experiments were done in a Durrum-Gibson stopped-flow spectrophotometer equipped with a Tektronix storage oscilloscope and a Polaroid camera. Equal volumes of two solutions (protein, and acid or buffer) are mixed in a mixing chamber before entering the optical cell. The reading at the oscilloscope is triggered by the stop syringe at the same time or shortly before the flow of the two solutions is stopped. For Hb^+ concentrations of 0.005% (3×10^{-6} M, if M = 16,700) a 2-cm path-length cell was used; for concentrations of 0.05% a 2-mm cell was used. Oscilloscope traces were obtained over periods varying from 0.2 to 50 sec. The earliest reading was taken 10–15 msec after mixing. The oscilloscope reading is linear in transmission. From these data the optical densities were calculated and used for kinetic analysis. For small changes in transmission at λ 230 mμ the oscilloscope scale was expanded 5- or 10-fold. Repeated measurements at the same pH at a number of wavelengths allowed construction of time-dependent optical density curves of the Soret region; transmissions at particular times were then read from these traces, and absorption spectra for these times were then constructed. This procedure was repeated for several different times after mixing.

Spectropolarimetry. The optical rotatory dispersion was measured with a JASCO Model UV 5 spectropolarimeter at a constant wavelength, λ 223 mμ, in a flow-through system. A four-jet mixing chamber was attached at the entrance of a 1-mm path-length cell. Protein and acid solutions were delivered from two 50-ml syringes by a synchronous motor for 40 sec with a speed of 1 ml of each solution in 1 sec. The volume from the entrance of the mixing chamber to the middle of the window of the cell holder was 0.35 ml (0.3 ml to the lower end of the window and 0.4 ml to the upper end). The image of the slit is about as high as the window. Measurements were thus made at an average time of 0.175 sec after mixing. Only the last 10–20 sec of the reading represent a reading of the true rotation because of the slow response of the instrument.

Chromatography. Heme was separated from globin in a 25-mm diameter column packed with 30 g of Sephadex G-25 for approximately 2×10^{-7} mole of protein. The concentrations of the Hb^+ and of the hemin chloride solutions were approximately 3.5×10^{-5} M. The samples were poured on the column immediately after dilution with acid. The separation was followed by comparing the absorption bands of hemin at λ 398 mμ and those of globin plus hemin at λ 275 mμ.

Spectrophotometry. Absorption spectra were measured with a Cary Model 14 spectrophotometer. The concentration of hemin chloride in water (pH 6.8) was 1.23×10^{-6} M; the concentration of either hemin chloride or Hb^+ in other solvents was approximately 3.5×10^{-6} M. For difference spectra the solution was 2.15×10^{-5} M. A 2-cm path-length cell was used between 300 and 240 mμ, and a 0.5-cm cell below 240 mμ.

Results

Determination of the first-order denaturation rates of human and horse ferrihemoglobin at λ 405 mμ over a wide range gave the results shown in Figure 1. The new faster rates are demonstrably continuous with the data previously obtained in the region above pH 3.2, with both formate buffers and HCl solutions. At pH above 2.4 the rates in formate buffer are slower than those in HCl at the same pH for both hemoglobins. This difference corresponds to the "stabilization" effect of formate previously found at higher pH. Obviously at lower pH values a different situation prevails. The rates in HCl are a continuation of the rates of spectroscopic changes in acetate buffer and of the unfolding rates determined by titration of masked histidines in HCl. However at pH below 2.5 the kinetic analysis no longer showed simple first-order reactions beyond a brief initial

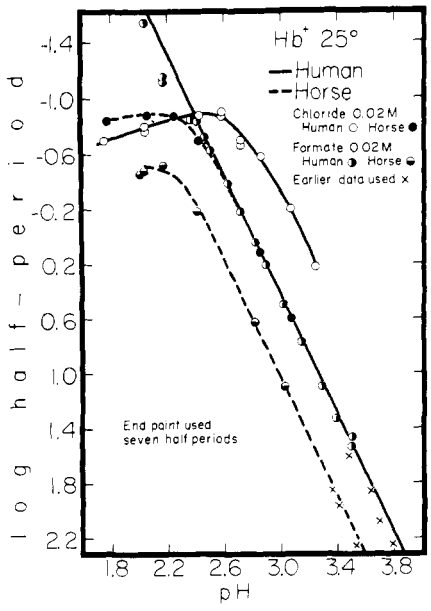


FIGURE 1: Dependence of the rate of the decrease of the Soret absorption band upon pH. Measured at λ 405 mμ at 25°. The end point used for the calculations is the optical density after seven half-periods. × are data from Figure 2, Steinhardt and Hiremath (1967).

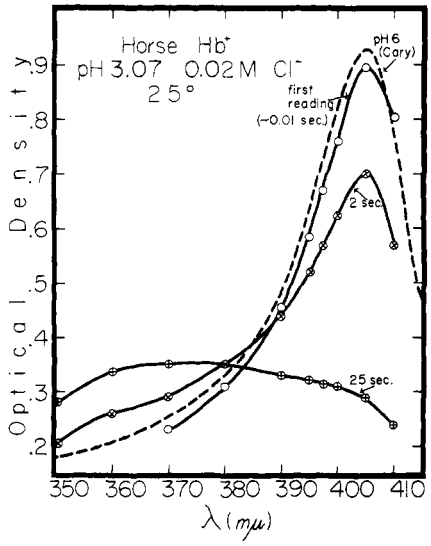
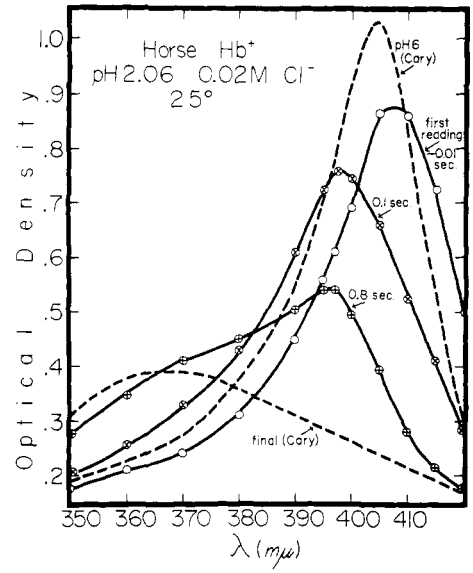


FIGURE 2: Decrease of the Soret absorption band during acid denaturation. Concentration 3 × 10^{-6} M; path length 2 cm.

period. The simple pH–rate profile thus becomes meaningless at low pH. It appeared likely that more than one reaction takes place during denaturation.

This conclusion is made quite clear in Figures 2 and 3 which show a sequence of spectra in the Soret region constructed from the kinetic traces for very short fixed time intervals at a number of different wavelengths. Figure 2 shows an example at pH 3, a pH which is high enough for the decrease of the optical density at λ 405 mμ to give a simple first-order reaction for six to



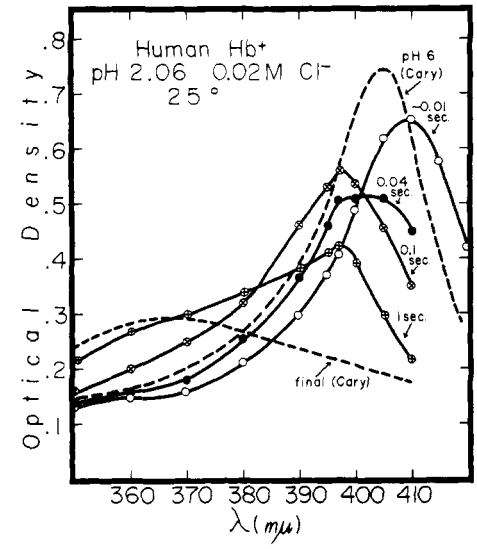


FIGURE 3: Decrease of the Soret absorption band during acid denaturation. Concentration 3 × 10^{-6} M; path length 2 cm.

seven half-periods. Figure 3a,b shows clearly that at low pH (both species) more than one reaction occurs: a first reaction A → B is indicated by a small red shift to λ 408 (horse) and to λ 410 mμ (human Hb^+). The second reaction B → C is the decrease of the extinction coefficient ε of this band and the building up of an absorption band with a maximum at 397–398 mμ (both species). It is evident that this new band increases as the 408-mμ band decreases: ε diminishes after a shift in λ_{max}. The third reaction, C → D, represents the slow decrease of the band at 398 mμ.

A kinetic analysis of each of these reactions, relatively unaffected by the others, can be made by working at different wavelengths. Reaction A → B is already complete at the first possible reading between 10 and 20 msec. Kinetic analysis of the reaction B → C, measured as a decrease of the optical density at λ 410 mμ,

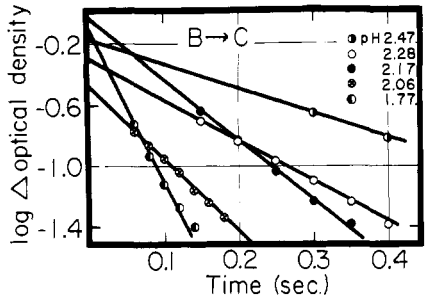


FIGURE 4: Guggenheim plots of the kinetics of the decrease in optical density at λ 410 mμ for $HsHb^+$ in 0.02 M Cl^- at 25°. The time interval for the ΔOD is close to one half-period. The data for pH 2.47 follow a straight line by 0.9 sec, covering almost five half-periods.

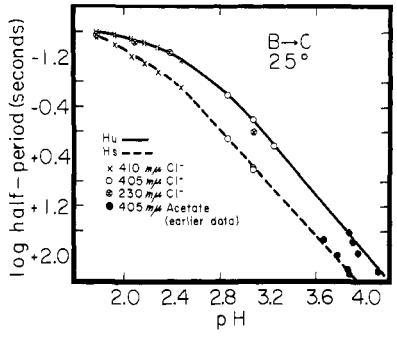


FIGURE 5: Dependence of the rate of the decrease of the Soret absorption band upon pH. Ionic strength = 0.02. The acetate data are taken from Figure 4 of Steinhardt and Hiremath (1967).

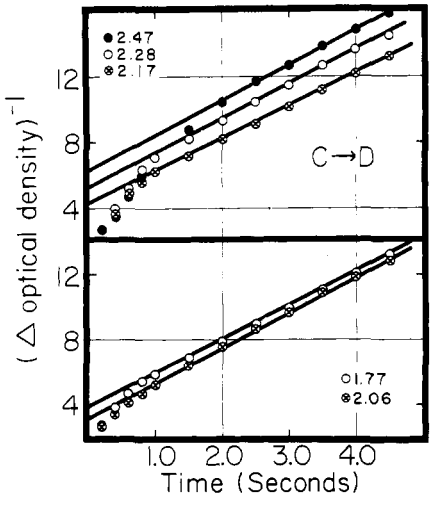


FIGURE 6: Second-order kinetic plots of the decrease of the optical density at λ 397.5 mμ for $HsHb^+$ in 0.02 M Cl^- at 25°. Concentration 3×10^{-6} M. ΔOD is the difference in optical density at time × and a 2-min end value.

showed first-order reactions for both species over the whole pH range for three to five half-periods (Figure 4). The rate is pH and species dependent (Figures 4 and 5). Reaction C → D, the disappearance of the band at 398 mμ (measured at 398), is shown to be a second-order reaction (both species) independent of pH in the pH range measured (pH 1.8–2.5) (Figures 6 and 7).

Figure 6 shows that the second-order reaction 2C → D is obeyed only after six to ten half-periods of the B → C reaction are over. This is to be expected, since both reactions cause density changes in opposite directions at λ 398 mμ. On the other hand, the decrease of the band at 398 mμ causes only very small density changes at λ 410 mμ during the first 0.5–1 sec, and it does not interfere with the beginning of the first-order reactions B → C. However a final end value of the optical density at λ 410 mμ cannot be used in the kinetic analysis because of further slow changes due to the 2C → D reaction. Kinetic analysis was therefore carried out by the Guggenheim (1926) method, which does not require that the end value be known experimentally. In Figure 5 the rates so calculated are shown to be a continuation of the kinetic data obtained at 405 mμ with normal first-order reactions at pH above 2.5. If we compare data of Figure 1—obtained at λ 405 mμ with an end value of seven half-periods—with data of Figure 5—obtained at λ 410 mμ, using the Guggenheim method and thus independent of an end value—it is obvious that the true rates of denaturation (defined as B → C) at low pH are faster than those shown in Figure 1, and that in HCl the curves level off at about the same rate *for the two species*.

The increase of the broad absorption band centered at λ 365 mμ, characteristic of denatured hemoglobins, could not be kinetically analyzed. Both reactions B → C and 2C → D cause an obvious increase in optical density which cannot easily be separated. It is worth mentioning that free spontaneously dimerized hemin chloride at pH 6, to be discussed later, shows this same absorption band with an ε value of 48×10^3 as compared with 60×10^3 for denatured Hb^+ at low pH.

Experiments in formate buffer show essentially the same reactions as those in dilute HCl with the exception

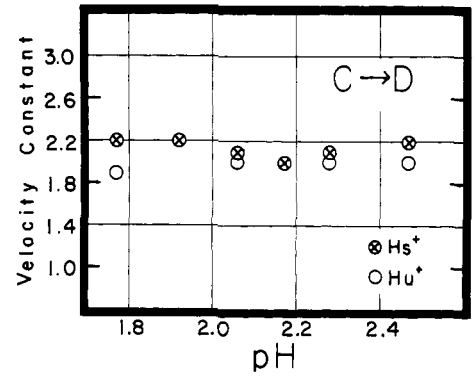


FIGURE 7: Dependence of the velocity constants of the second-order reaction upon pH. Concentration 3×10^{-6} M in 0.02 M Cl^-.

of the first step A → B: the small red shift observed in HCl at about pH 2.0 with both species is not present in formate. The further spectral changes are the same as in HCl. The true denaturation rate in formate buffer, calculated from optical density changes at λ 410 mμ independent of the end value, increase as compared with the rates given in Figure 1 in the same way as they do in HCl. The results obtained at pH about 2 were unexpected: while the rates for both proteins in HCl level off at low pH, the rates for formate buffer do not level off. With horse Hb^+ the pH profile of the log half-periods continues with the same slope to pH 2; with human Hb^+ the slope becomes even steeper. At pH 2.03 the denaturation rate in formate is *faster* than in HCl. The half-period in formate buffer is 24 msec, as compared with 32 msec for HCl at the same pH; whereas at the higher pH values formate has a stabilizing effect. Evidently in formate and at low pH a different reaction takes place. Formate buffers of ionic strength 0.02 and pH 2.03 are almost 1.5 M on formic acid. At this high concentration other reactions may well take over. We have been able to show that in high concentrations of acetic acid (50%), brought to pH 2.1 with acetate ion, the disappearance of the 405-mμ absorption band is immeasurably fast. In less than 20 msec the band is completely shifted to 398 mμ, the absorption band of monomeric hemin. It may well be that in 1.5 M formic acid the same reaction prevails. Because of this complication we did not pursue further kinetic studies in formate buffer solutions at low pH.

The first-order reaction is, as it should be, independent of the concentration of Hb^+, when 0.005% solutions are compared with 0.05% solutions. The second-order reaction shows the expected strong concentration dependence: with a 0.05% solution the rate of the second-order reaction has increased so much that the band at 398 mμ can no longer be observed.

Denaturation half-periods were also determined in the far-ultraviolet regions using spectral changes at λ 230 mμ. Figure 8 shows an acid difference spectrum of human Hb^+. The $\Delta\epsilon$ of 18,000 represents a change of 15% of the total absorption at this wavelength. For kinetic analysis again the Guggenheim method was used, to be independent of the end value. The reaction follows first-order kinetics for three to four half-periods, covering about 75–80% of the total optical density change (before becoming slower than first-order). The half periods for human Hb^+ are included in Figure 5. They evidently follow the same pH profile as the spectral changes at λ 405 and 410 mμ.

No measurements were made at 290 mμ since here the large absorption changes of the Soret region obviously overlap the protein "unfolding" difference spectrum (Wetlaufer, 1962).

Measurements of optical rotatory dispersion were used as another method to follow the unfolding of the protein directly. As described before we could not obtain time-dependent changes of the negative rotation at 233 mμ directly because of the slow response of the instrument. But in flow-through measurements we could determine the percentage of the total decrease of the negative rotation at the trough of the α-helix Cotton effect reached at a particular fixed time, the time the solution needs to flow from the mixing chamber to the cuvet. These measurements cannot give results of high precision. The time of the measurement lies between 0.15 and 0.20 sec, considering the lower and the upper end of the cell window.

At pH 2.24, where the denaturation half-period is 0.100 sec, the negative rotation at 233 mμ has reached a decrease of 74% (average of six experiments between 69 and 81%; see Table I) of its total change at this pH. The calculated value, using the half-period of the B → C reaction of 0.100 sec, is 70% at 0.175 sec after mixing.

At pH 2.33 the decrease found experimentally is 64% (average of six experiments between 59 and 68%); the calculated value at 0.175 sec using the determined half-period of the B → C reaction of 0.125 sec is 62%. Two

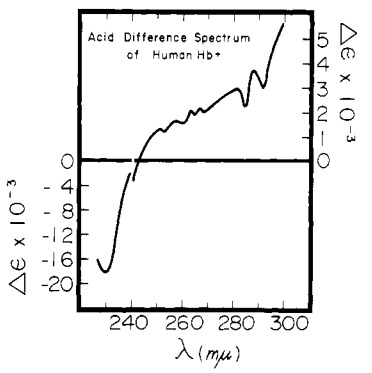


FIGURE 8: Acid difference spectrum of $HuHb^+$ in 0.02 M Cl^-. Sample cell: pH 2.3; reference cell: pH 6.7. (The "molar" extinction coefficients (M = 16,700) are 32,000 at 275 mμ and 140,000 at 230 mμ.)

TABLE I: Denaturation of Horse Ferrihemoglobin Determined by Optical Rotatory Dispersion 0.175 sec after Mixing of Protein with Acid.

pH	Hb Concn (%)	% of the Total Change of α at λ 233 mμ at the Same pH	% of the B → C Reaction Calcd with the Determd Half-Period
2.33	0.12	65	62
		65	
	0.06	63	
		63	
		68	
		59	
2.24	0.12	69	70
		69	
	0.06	76	
		81	
		78	
		76	

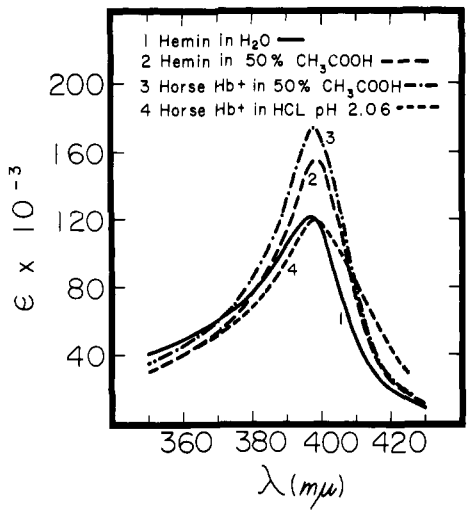


FIGURE 9: Absorption spectra of hemin chloride and $HsHb^+$ in different solvents. Curve 4 is taken from Figure 3a.

different concentrations of horse Hb^+ used for these experiments (0.06 and 0.12%) showed no difference.

Despite the large limit of error in these experiments the results show good agreement with the denaturation rates determined spectroscopically as the decrease of the optical density at λ 410 or 230 mμ.

In Figure 9 four spectra are compared: (1) the spectra of hemin chloride in water; (2) of hemin chloride in 50% glacial acetic acid–50% 0.01 N HCl; (3) horse Hb^+ in the same solvent; and (4) horse Hb^+ in HCl (pH 2.06), 0.1 sec after mixing of Hb^+ and acid. The last curve is taken from stop-flow measurements (see Figure 3a). The ϵ value at maximum absorption of free hemin in the acetic acid mixture is 155×10^3, and of horse Hb^+ in the same solvent 175×10^3. The two spectra are completely superimposable if their ϵ_{max} values are made equal. The two other spectra are very similar in shape. Curves 2 and 3 are stable for 1–2 hr. Lower concentrations of either acetic acid and HCl reduce the stability of both free hemin and denatured Hb^+. The absorption curve has a different shape if the solvent is 100% acetic acid.

It has not been possible to obtain kinetic data on the denaturation of Hb^+ in the mixture of 50% acetic acid with 0.01 N HCl, because the shift of the absorption band of native Hb^+ at 405 mμ to the band at 398 mμ is too fast. It is complete in less than 20 msec.

Separation of free monomeric heme from denatured globin in the acetic acid–HCl mixture has been achieved on a Sephadex column. The elution volume for hemin was exactly the same, whether hemoglobin or free hemin was placed on the column. A separation of denatured globin from heme in HCl without the acetic acid could not be obtained. This result was expected, since the free hemin dimerizes and polymerizes very rapidly in the absence of strong acetic acid, and can therefore not be completely separated from the globin by gel filtration. Nevertheless, the first fractions contained much more globin and much less hemin than would correspond to a 1:1 molar ratio.

Discussion

Three different steps may be distinguished during the acid denaturation of horse and human Hb^+ at low pH. At higher pH the existence of a great disparity in their rates collapses all the phenomena due to the three of them into an apparent single step, described more fully below. We cannot yet give any explanation of the first step: the small red shift in the Soret band to 410 mμ, complete in less than 20 msec. This shift does not occur in formate buffer. Although fast, it is not instantaneous, as shall be shown elsewhere; it is greatly slowed at temperatures just above 0°. The second reaction, the decrease of the absorption band at λ 410 mμ, and the buildup of a new band with the maximum at λ 398 mμ, must be the expulsion of the heme from the apoprotein, and the slower bimolecular reaction C → D the dimerization of the free heme. At pH values above about 2.7 the second first-order step is decisively rate limiting. These conclusions rest on the following: (1) the bimolecular reaction C → D occurs with the same rate with horse and with human Hb^+, and is independent of pH. Since it is postulated to occur outside the protein the nature of the apoprotein cannot affect it. (2) Inada and Shibata (1962) have shown that free hemin at pH 6.8 in very dilute aqueous solutions has its absorption maximum at λ 398 mμ. The relatively slow decrease of this band follows second-order kinetics. Inada and Shibata concluded that the decrease must be caused by dimerization of the free hemin. (3) It has been shown that the absorption bands of hemin under the following four conditions are in good agreement with one another (Figure 9): (1) hemin chloride at pH 6–7 in very dilute solutions, (2) hemin chloride in 50% acetic acid plus 0.01 N HCl, (3) horse Hb^+ in the same solvent as 2, and (4) horse Hb^+ at 0.1 sec after mixing with HCl (pH of the mixture is 2.06) (Figure 3a). Acetic acid (50%) was chosen as a solvent in which dimerization of hemin occurs exceedingly slowly; the absorption in this solvent has a maximum at 398 mμ, which decreases at a negligible rate. Curves 2 and 3 are fully superimposable, if the values at the maximum are normalized; curve 1 is broader at the low-wavelength side, indicating the start of dimerization; and curve 4 is broader on both sides: the heme is not completely expelled from the protein, which broadens the band at λ 410 mμ, and again the initial stages of dimerization cause a higher density at 365 mμ. (4) Free heme can be separated on Sephadex from denatured globin in the acetic acid–HCl mixture. It is known from the work of Rossi-Fanelli and Antonini (1960), and Banerjee (1962), that free heme is in equilibrium with hemoglobin. Fronticelli and Bucci (1963) showed this equilibrium is strongly pH dependent. The elution volume of the heme was found to be exactly the same, whether free hemin chloride or ferrihemoglobin is put on the column. The heme must be in the same state in both solutions. This separation is not possible with HCl solutions without acetic acid, because of the fast dimerization and polymerization of the heme.

The second reaction, the expulsion of the heme, is an obvious continuation of the over-all kinetic process at higher pH values in which no separate steps during

denaturation could be discerned. With both hemoglobin species this second reaction continues the pH–rate profile in HCl as well as in formate buffer. Since at higher pH dimerization is more rapid than heme separation or "ejection," the change in ϵ appears to occur at 405 rather than at 398 mμ.

The third reaction is concentration dependent. Its rate is independent of pH and species. It is obvious then that this reaction, following the B $\rightarrow$ C reaction, can only be observed if it is of equal or slower rate than the preceding one. In high concentrations of Hb^+ the dimerization rate is so fast that even at low pH, where B $\rightarrow$ C is fast, it cannot be observed at all. At low concentrations, and at higher pH, the B $\rightarrow$ C reaction, being strongly pH dependent, becomes the slower rate-limiting step, and the dimerization reaction again becomes invisible. Most kinetic studies on hemoglobin have been done at both higher concentrations and at higher pH so that the decrease of the Soret absorption is observed as a single reaction without change in λ. The experiments described in this paper at low pH and at low hemoglobin concentration ($\sim 3 \times 10^{-6}$ M) separated the reactions, which although "invisible" must also occur at higher pH in the same sequence. This conclusion does not include the first small red shift, which does not occur at higher pH.

Figure 5 shows that the denaturation rates for both proteins level off at low pH with the same velocity. The difference in rate for the two species over the whole pH range must therefore be explained as a shift of the curves along the pH axis, rather than as a shift along the log velocity axis.

Horse Hb^+ has three more carboxylic acids in the β chain than human Hb^+ (Perutz, 1965; Braunitzer *et al.*, 1961)[1] which causes the isoionic pH to be shifted slightly to more acid pH. In work from this laboratory (Beychok and Steinhardt, 1959; Steinhardt *et al.*, 1962; Steinhardt and Hiremath, 1967) it has been established that human hemoglobin has four more masked histidines (one per chain) than horse hemoglobin. Tanford and Nozaki (1966) found the same *differences* in calculating the titration data of Antonini *et al.* (1965), even though the number of masked groups identified as histidines by Tanford is considerably lower than those found by Steinhardt. Since both proteins have the same number of histidine residues, these results would indicate a higher positive charge for horse hemoglobin than for human hemoglobin at a given acid pH, which would partly compensate for the higher number of carboxylic acids in the horse species. If the net positive charge of horse Hb^+ at a given pH is lower than the net charge for human hemoglobin at the same pH, the unfolding would procede at a slower rate in the former.

The kinetic results obtained from the absorption change at λ 230 mμ (Figure 8) are in very good agreement with the B $\rightarrow$ C reaction: the expulsion of the heme from the apoprotein (Figure 5). A first-order reaction is followed for three to four half-periods. But again, a slower small change in optical density following this reaction indicates more complicated spectral changes. The free heme absorbs at this wavelength with an ϵ of $\sim$25,000. This value decreases with dimerization to $\sim$22,000. The slower small optical density changes may therefore be caused by the heme dimerization. But about 75–80% of the large $\Delta\epsilon$ of 18,000 must be due to changes in the environment of the aromatic amino acids and perhaps absorption changes of the peptide group due to loss of α helix. This "blue shift" of absorption bands at $\lambda \sim 280$ and at 230 mμ with unfolding of the protein is well established for many proteins without a prosthetic group (Wetlaufer, 1962; Glazer and Smith, 1961), and has been attributed to the bringing in contact with solvent of buried aromatic side chains after unfolding. The explusion of the heme group however will cause the same spectral changes if the prosthetic group was in close contact with aromatic amino acids. The recent X-ray analysis of Perutz *et al.* (1968) indeed shows 3 Phe and 1 Tyr from the α chain to be in close contact with the heme group; and 5 Phe from the α chain.

The difference spectra at 280 mμ show blue shifts due to Tyr and Trp, besides those due to Phe. Since only 0.5 Tyr/chain and no Trp is in contact with the heme group, most of the difference spectrum at $\lambda \sim 290$ mμ must be due to unfolding of the apoprotein. This will also cause a negative $\Delta\epsilon$ at $\lambda \sim 230$ mμ. These conclusions account for the fact that both the expulsion of the heme and the unfolding of the globin contribute to the $\Delta\epsilon$ at λ 230 mμ. Quantitative comparisons, however, are not possible, especially since the large absorption differences of the Soret region overlap the "unfolding" difference spectrum at $\lambda \sim 290$ mμ (Figure 8).

Our kinetic analysis of the spectral change at 230 mμ shows a simple first-order reaction for three to four half-periods, with the same rate as the reaction B $\rightarrow$ C, the expulsion of the heme. If both reactions contribute to this change, both must occur with about the same velocity.

Additional evidence that the unfolding occurs with the same rate as the expulsion of the heme is given by measurements of optical rotatory dispersion. Kinetic data could not be obtained, but the rotation at λ 233 mμ, the trough of the helical Cotton effect, could be measured with a flow-through cell at one fixed time after mixing. The percentage denatured is in good agreement with the calculated amount using the half-period determined in the stop-flow experiments for the expulsion of the heme (Table I).

We conclude that unfolding of the globin occurs with the same velocity as the expulsion of the heme over a wide pH range (2.2–4.0). It is not yet clear which of the two reactions is the initial one, or whether both reactions occur parallel and indistinguishable from one another, two facets of a single molecular event. The pH dependence and the known importance of heme in stabilizing globin suggest that an electrostatic repulsive force at low pH overcomes the affinity of heme to the contact points in the heme pocket, and that unfolding immediately ensues as the heme loses contact. It remains

[1] The newest data of Smith (1964, 1967) do not distinguish between the carboxylic acids and their amides, and thus leave some uncertainty as to the contents of aspartic and glutamic acids in horse hemoglobin.

to explain fully why the rates of heme ejection and probably of unfolding level off at identical rates in the two species at low pH. Experiments that measure unfolding directly, *e.g.*, further work on changes in Cotton effect, or in fluorescence quenching, must be resorted to, and are in progress.

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The Reductive Conversion of N-Terminal Pyroglutamyl into Prolyl Residues in Polypeptides and Proteins*

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ABSTRACT: The pyrrolidone ring of an N-terminal pyroglutamyl residue in a polypeptide or protein may be converted, by reduction with diborane in tetrahydrofuran or tetramethylurea, into a pyrrolidine ring. Thus, the originally nonbasic N-terminal residue is identified by its conversion into proline. Although the reduction shows only limited selectivity (carboxyl and peptide groups are partially reduced), yields of proline up to 46% have been obtained in the reduction of an octapeptide analog of gastrin. The generation of N-terminal proline by reduction of native or performic acid oxidized bovine γ-globulin is also observed. Diborane reduction offers a rapid and facile means of demonstrating the presence of an N-terminal residue of pyroglutamic acid and of initiating sequential analysis of the polypeptide chain.

A sizeable number of natural polypeptides and proteins are now known to possess N-terminal pyroglutamic acid (pyrrolidonecarboxylic acid) residues. The lack of basicity of the pyrrolidone nitrogen prevents the formation of derivatives which might be useful for N-terminal identification or for sequential cleavage of the polypeptide chain. Since a search for nucleophilic agents which could attack the pyrrolidone carbonyl selectively proved fruitless,[1] alternative approaches to the selective chemical modification of the cyclic lactam were explored.

We have found that diborane, which has already been

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[1] L. A. Cohen, unpublished data.