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Significance of the Initial Fast Reaction in the Acid Denaturation of Ferrihemoglobins*

John W. Allis and Jacinto Steinhardt

ABSTRACT: Acid denaturation measurements of both horse and human ferrihemoglobins (Hb^+) at 2.5° show that the initial red shift of the Soret band at low pH is slowed to a rate measurable by stop-flow techniques. Parallel changes occur in the visible absorption bands of Hb^+; the bands at 500 mμ and 630 mμ disappear and a new band appears at 525 mμ (horse Hb^+) or 531 mμ (human Hb^+). These changes are suppressed by the presence of formate ion only, among several common anions tried.

These spectral events, which occur only at pH <3, although acid denaturation occurs at much higher pH, are interpreted as changes in the environment of the heme group which affect the iron orbitals coordinating with the ligands. The changes are probably due to the effect of the high-charge density acquired by the protein at low pH values prior to heme separation; contrary to an earlier report, the initial fast reaction is probably *not* important to the over all acid denaturation scheme of Hb^+ previously described; it is a manifestation of an alternative pathway, superimposed on the basic mechanism. In contrast to the result at 25°, the pH–denaturation rate profiles for the two species at low temperature do not approach the same limiting rate; the difference corresponds to the known difference in the final products at high and low temperatures at least with horse ferrihemoglobin.

The acid denaturation of both horse and human ferrihemoglobin (Hb^+), at low ionic strength, followed by changes in their Soret absorption bands, has been previously reported as a three-step sequential process (Polet and Steinhardt, 1969). The first step (A → B) is a very fast reaction (half-life at 25° <10 msec) manifested by a red shift of the Soret band, from 405 to 408 mμ for horse Hb^+ and to 410 mμ for human Hb^+. The second step (B → C) is characterized by the disappearance of the red-shifted Soret band and the simultaneous rise of a new band at 397.5 mμ; it has been shown that this change is caused by the expulsion of the heme from the protein, and the simultaneous or nearly simultaneous collapse of the protein's tertiary structure. The final step (C → D), the disappearance of the band at 397.5 mμ, and the appearance of a broad band at 370 mμ, is caused by the dimerization of the free heme. The three steps are seen as distinct processes at pH values below about 2.4. At higher pH values the second and third steps cannot be separated because the second, the rate of which is pH dependent, becomes rate limiting.

When the acidifying agent is formate buffer instead of HCl, the sequence is essentially the same except that the initial red shift, A → B, is not present (Polet and Steinhardt, 1969). With formate, at protein concentrations high enough so that the second-order reaction C → D is so fast that the rate measured is that of the first-order reaction B → C alone, identical kinetic results have been obtained at the Soret band and at 630 mμ (Zaiser and Steinhardt, 1953).

In this paper the original intent was to extend the earlier spectroscopic studies to temperatures as low as 2.5° and to pH ranges down to 1.8 in order to examine the kinetics of the initial very fast reaction (A → B) and to carry out measurements in the visible (490–670 mμ) as well as in the Soret region (350–425 mμ). It became immediately clear that the first very rapid reaction (A → B) is time dependent rather than instantaneous, and that drastic changes in the visible spectral region occur which can be correlated with coincident changes of the Soret spectrum. The results are reported here, together with interpretations which throw light on the interaction of heme and globin and on the stabilization of hemoglobin by its heme linkages.

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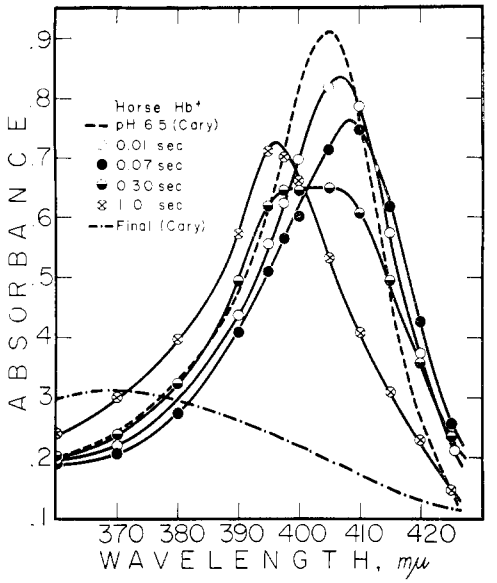

FIGURE 1: Decrease of Soret absorption band of horse Hb^+ during acid denaturation. Red shift (A → B) is complete in 0.07 sec. Protein concentration is 0.0047%, pH is 2.12, chloride ion concentration is 0.02 M, temperature is 2.5°, and optical path is 1.85 cm.

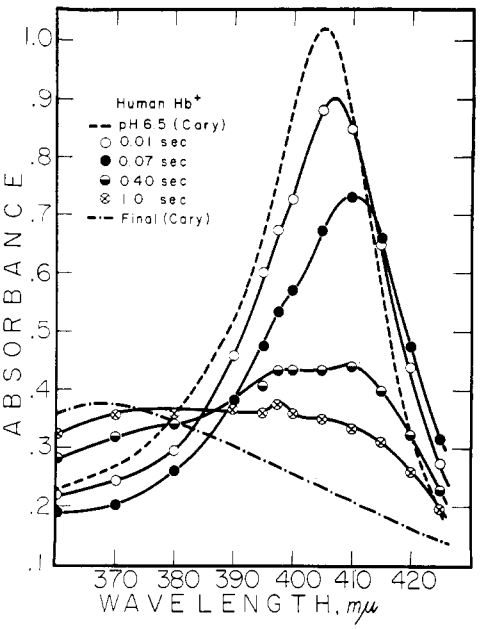

FIGURE 2: Decrease of Soret absorption band of human Hb^+ during acid denaturation. Red shift (A → B) is complete in 0.07 sec. Protein concentration is 0.046%, pH is 2.08, chloride ion concentration is 0.02 M, temperature is 2.5°, and optical path is 0.213 cm.

Experimental Procedure

Materials

Horse COHb was prepared from blood of a single animal by the method of Ferry and Green (1929) as described previously (Steinhardt *et al.*, 1966). Human COHb was prepared from pooled cells as described in an earlier work (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 afterward dialyzed against phosphate buffer (pH 6.8) followed by distilled water. The extinction coefficients at 405 mμ were 165–170 × 10^3 l./mole cm for human Hb^+ and 170–175 × 10^3 l./mole cm for horse Hb^+.

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

Methods

The ionic strength of all experiments was maintained at 0.02 unless otherwise noted. Hemin chloride solutions were made by the method of Inada and Shibata (1962) as previously described (Polet and Steinhardt, 1969).

Stop-Flow Measurements. A Durrum-Gibson stopped-flow spectrophotometer was used in the kinetics experiments as previously described (Polet and Steinhardt, 1969). For the Soret region, a 2-cm cell was used with Hb^+ concentrations of 0.005% (3 × 10^{-6} heme equiv if the average monomer molecular weight = 16,700); a 2-mm cell was used with 0.05% Hb^+. For the visible region, the 2-cm cell was always used and the Hb^+ concentration was 0.1% for the time-dependent spectra. Some kinetic measurements in the visible region were made at 0.005% in order to make comparisons with kinetics in the Soret region. Most of the measurements were made at 2.5 ± 0.2° with others at 8, 12, 16, 20, and 25°. The temperature at the drive syringes was continuously monitored.

Spectrophotometry. Static absorption spectra were measured with a Cary Model 14 spectrophotometer. For solutions of hemin chloride of concentration 1.56 × 10^{-6} M, a 5.0-cm cell was used. The normal slide wire (0–1.0 absorbance unit) was used for Soret region measurements and an expanded-scale slide wire (0–0.1 absorbance unit) was used for visible region measurements.

Results

Figures 1 and 2, the spectra at various times after initiation of the reaction, constructed as described by Polet and Steinhardt (1969), show that at low temperatures the event A → B which causes the red shift, although very fast, can be followed through at least part of its course. At 2.5° the reaction is complete at 60–70 msec after mixing and at 8.0° is complete in 30–40 msec.

This temperature dependence demonstrates that the red shift is due to a chemical reaction involving an activation energy and is not merely the result of protonation of some of the prototropic groups of the protein. The extent of the red shift at the lower temperatures is the same as at 25°. Measurements at pH 2.5 show the red shift to occur at the same rate and to the same extent as at pH 2.1. However, since the red shift is not seen at pH 3.0 it must attain its full extent over a very short pH range. Thus a highly cooperative effect must be involved. Such a cooperative effect might be the result of a conformation change in the apoprotein brought about by the reaction of a single heme if the conformation change promotes the same reaction at the other hemes.[1]

[1] The work of Perutz *et al.* (1968) identifies the amino acid side chains in contact with the heme group as well as the "bridges" that join the environments of the neighboring hemes.

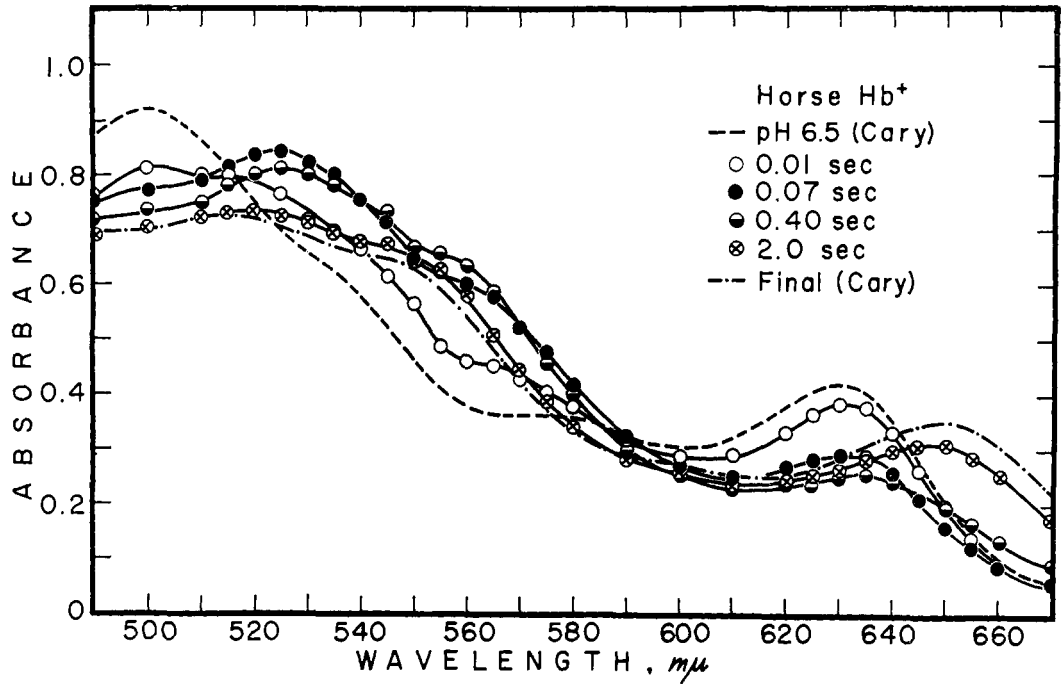

FIGURE 3: Spectral changes in the visible spectrum of horse Hb^+ during acid denaturation. The appearance of transient bands is complete in 0.07 sec. Protein concentration is 0.096%, pH is 2.11, chloride ion concentration is 0.02 M, temperature is 2.5°, and optical path is 1.85 cm.

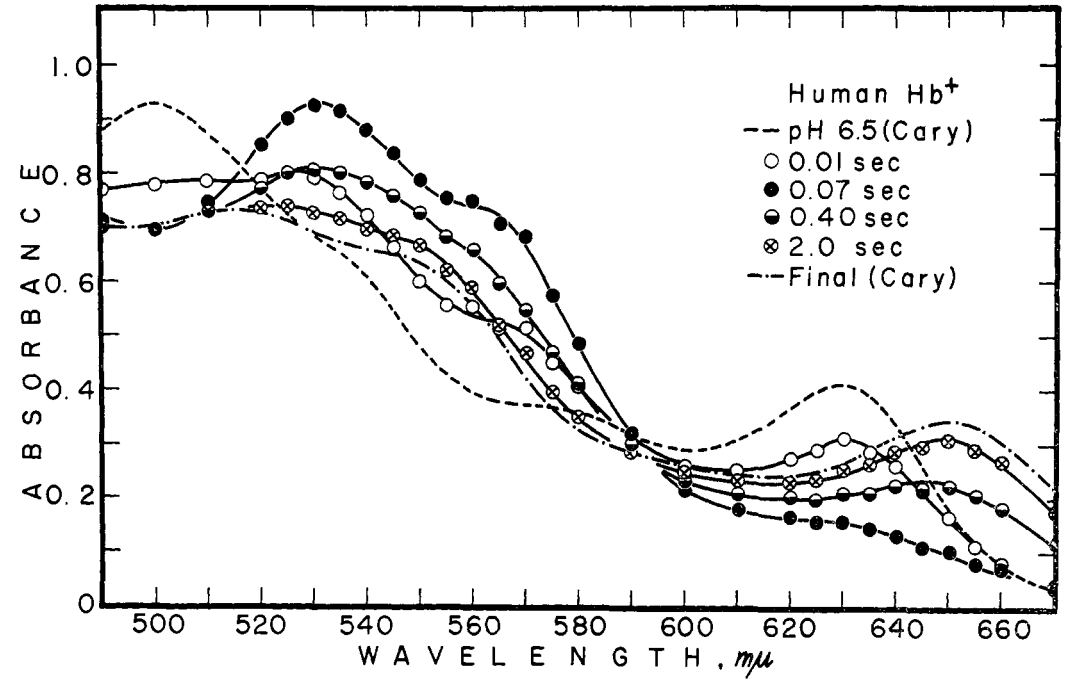

FIGURE 4: Spectral changes in the visible spectrum of human Hb^+ during acid denaturation. The appearance of transient bands is complete in 0.07 sec. Protein concentration is 0.102%, pH is 2.10, chloride ion concentration is 0.02 M, temperature is 2.5°, and optical path is 1.85 cm.

The reaction A → B also affects a similar sequence of spectra in the visible region, shown in Figures 3 and 4. Here the native proteins of both species have the familiar bands at 500 and 630 mμ, while the two denatured proteins have a band at 515 mμ with a shoulder at 545 mμ, and another band at 650 mμ. As the proteins of both species unfold, the peaks at 500 and 630 mμ decrease very rapidly while an increase over the 515–590-mμ region occurs. These changes are complete in 60–70 msec and develop new transient peaks at 525 mμ (horse Hb^+) or 531 mμ (human Hb^+). Thereafter, the spectra change monotonically until the denatured state is reached.

Since the disappearance of the native peaks and the appear-

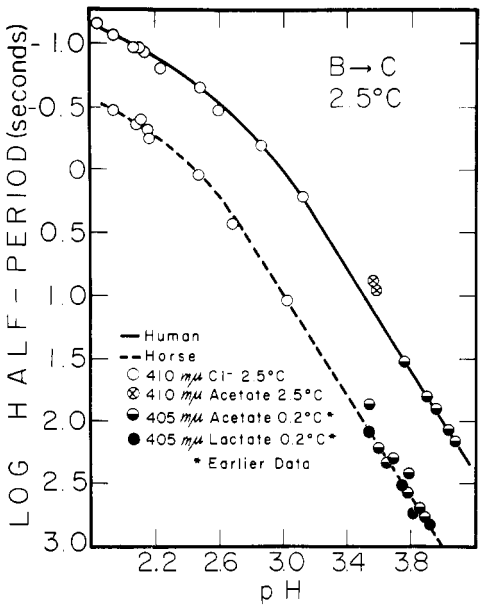

FIGURE 5: Dependence of the rate of decrease of the Soret absorption band upon pH at 2.5°. Ionic strength is 0.02. The data at 405 mμ and 0.2° are taken from Steinhardt *et al.* (1963) (horse Hb^+) and from Molday and Steinhardt (1969) (human Hb^+).

ance of the new peaks in the visible region occur in the same short time span as the Soret red shift, it may be assumed that the same molecular event is responsible for all changes. This view is supported by kinetic measurements at several wavelengths at which these rapid changes are most readily measured. Table I gives the half-period of the first-order reaction for each species at three wavelengths in the visible and one in the Soret region. These data were obtained by the Guggenheim (1926) method because a consecutive but much slower reaction precluded the estimation of final values. The kinetics of the reaction A → B are measured at 405 mμ because its spectral change is large at this wavelength (see Figures 1 and 2) although the reaction B → C contributes somewhat to the apparent rate. The Soret band decreases even as the red shift (A → B) is taking place, indicating that the consecutive reaction B → C is taking place and removing B, though at a much slower rate than B is formed, *i.e.*, the maximum amount of B

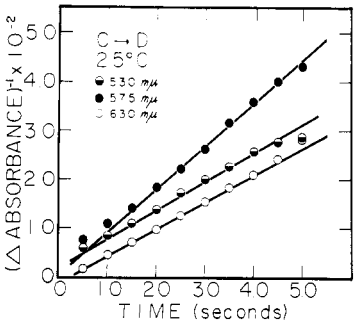

FIGURE 6: Second-order kinetic plots for the change in absorbance at 530, 575, and 650 mμ for human Hb^+ in 0.02 M Cl^-, pH 2.27, and at 2.5°. The ordinate of the 650-mμ curve has been decreased by 0.5 × 10^2 for this plot. Protein concentration is 0.0097%. Δ absorbance is the difference in absorbance at time, *t*, and a 1-min end value.

TABLE I: Kinetic Constants at Several Wavelengths for Initial Fast Reaction, A → B, at 2.5°.

Protein	Wavelength (mμ)	Concn (%)	pH	$t_{1/2}$ (msec)
$HsHb^+$	405	0.00471	2.15	11
$HsHb^+$	500	0.0956	2.11	9
$HsHb^+$	575	0.0956	2.11	12
$HsHb^+$	630	0.0916	2.53	10
$HsHb^+$	630	0.0940	2.10	15
$HsHb^+$	630	0.0956	2.12	14
$HuHb^+$	405	0.00472	2.08	11
$HuHb^+$	500	0.1018	2.11	14
$HuHb^+$	530	0.00970	2.27	13
$HuHb^+$	575	0.00970	2.27	12
$HuHb^+$	575	0.1018	2.09	10
$HuHb^+$	630	0.1018	2.10	13

never has a chance to accumulate. As a result, the calculated half-periods for A → B at 405 mμ may be shorter than they should be. The scatter in the other data in Table I is due to limitations of the instrument; it can only be said that any difference in rate between the two species must be small.

That these rapid spectral changes are due to changes in the heme-protein relationship is further supported by their absence in aqueous spectra of hemin chloride. It is known that monomeric heme rapidly dimerizes under conditions of low pH, high ionic strength, or high concentration with the disappearance of its band at 397 mμ and simultaneous appearance of the broad band at 370 (Inada and Shibata, 1962; H. Polet and J. Steinhardt, unpublished data).[2] The visible spectrum of monomeric hemin closely resembles that of native Hb^+. When the solution is acidified, causing the hemin to dimerize quickly, the visible spectrum changes until it resembles that of denatured Hb^+. The rapid intermediate changes in the visible spectrum are not observed with the aqueous hemin system.

The slower changes in the visible spectra (at 650, 575, and 530 or 525 mμ) have been linked to the reactions B → C and C → D of the Soret region by studying the kinetics. With human Hb^+ denatured at pH 2.26, the heme separation–unfolding reaction (B → C) is over in about 1.1 sec (seven half-periods, see Figure 5) and at concentrations below 0.01% the dimerization reaction is significant for 5–10 sec. Analysis of the data from visible wavelengths under these conditions show that the second-order rate law is obeyed (Figure 6). The rate constants agree closely with those obtained at 397.5 mμ (Table II), previously shown to be due to the dimerization of heme. The data at 650 mμ follow second-order kinetics from the first point while data at the remaining wavelengths do not follow second-order kinetics until the reaction B → C is over. At higher pH similar results are obtained but the reaction B → C takes longer, and the second-order plots for 530 and 575 mμ are of poorer quality.

[2] With human Hb^+, however, the 370-mμ band changes at a *faster* rate than the other bands in the Soret region. The origin of this discrepancy is not yet understood (see also Steinhardt and Hiremath, 1967).

TABLE II: Comparison of Second-Order Rate Constants (C → D) from Soret Band and Visible Region at 2.5°.

Protein	Wavelength (mμ)	Concn (%)	pH	Second-Order k_2 (l./mole sec)
$HsHb^+$	397.5	0.00471	2.08	1.6×10^5
$HsHb^+$	525	0.00471	2.08	
$HsHb^-$	575	0.00471	2.08	
$HsHb^+$	650	0.00471	2.08	1.6×10^5
$HuHb^+$	397.5	0.00472	2.08	1.6×10^5
$HuHb^+$	530	0.00970	2.27	2.6×10^5
$HuHb^+$	575	0.00970	2.27	1.9×10^5
$HuHb^+$	650	0.00970	2.27	2.4×10^5

The kinetics of horse Hb^+ denaturation under similar conditions and at pH 2.08 show good second-order behavior at 650 mμ, but not at 525 and 575 mμ. It can be seen from Figure 5 that horse Hb^+ at pH 2.08 denatures (B → C) more slowly than human Hb^+ at pH 2.26 by a factor of about 3. These data are interpreted to mean that the rise of the band at 650 mμ depends solely upon the appearance of dimerized heme (C → D), but that at the other two wavelengths in the visible, both reactions B → C and C → D contribute time-dependent spectral components. Therefore at the visible bands it is possible to detect the heme dimerization, C → D, only when the protein denaturation, B → C, is over relatively quickly and does not interfere.

Similar experiments with both proteins at about 0.1%, show kinetic behavior intermediate between first and second order. Thus, this concentration, although much higher, is not high enough for the first-order reaction to completely obscure the following second-order reaction; the rates of the two reactions must be approximately equal. The similarity in the rate of the two reactions at 0.05% can be seen in Figure 2. Since ϵ_{500} is much lower than ϵ_{405} it was necessary to use the high concentration to construct the spectra in Figures 3 and 4, and the curves therefore reflect the fact that B → C and C → D are proceeding at similar rates. The low concentration kinetic data were obtained by very high magnification of small changes on the oscilloscope screen.

Obviously the second-order plots in Figure 6 do not have identical slopes; the reason is that second-order rate constants are dependent upon the units of concentration used. In the plots absorbance is the variable proportional to concentration, and the different absorbance changes at the three wavelengths masquerade as different concentrations. The units of k from the slopes of the lines in Figure 6 are ΔA^{-1} sec^{-1} and must be transformed to l. $mole^{-1}$ sec^{-1} for meaningful comparisons. This has been accomplished by applying $\Delta A/\Delta C = l\Delta\epsilon_\lambda$, where l is the light path length in centimeters and $\Delta\epsilon_\lambda$ is taken as the difference in absorption coefficient between the end of the fast reaction A → B and the final state; that is, the reaction A → B is complete in 0.07 sec (Figures 3 and 4) and spectral changes from this point on have been taken as indicative of the slower reactions B → C and C → D.

When denaturation is carried out in formate buffers at 25° the Soret red shift is suppressed and the 630-mμ band decreases at the same rate as the Soret band. Kinetic results at 2.5° and pH 2.5 at the Soret band, 500, 525, and 630 mμ show that the Soret red shift does not appear, that the kinetics at 500 and 630 mμ agree closely with those at the Soret band and 525 mμ is an isosbestic point; none of the more rapid spectral changes found when HCl is used appear when formate buffer is used as the denaturant. A series of kinetic measurements at 630 mμ with several common reagents[3] including phosphate buffer (pH 2.34), hydrobromic acid (pH 2.59), chloroacetic acid (pH 2.30), acetate buffer (pH 2.54), and formaldehyde–HCl (pH 2.16) show that formate, already known to have unique stabilizing effects on ferrihemoglobin (Zaiser and Steinhardt, 1954), is the only buffer anion that has this effect. Since formate stabilizes Hb^+ against acid denaturation, it is obvious that the partial reaction (in its presence) A → C is considerably slower than the partial reaction B → C which must predominate when formate is not present.

TABLE III: Apparent Activation Energies for Ferrihemoglobin Denaturation, B → C.

Protein	pH	Temp Range (°C)	E^* (cal/mole)
$HsHb^+$	2.10	11–25	15,000
$HsHb^+$	2.10	2.5–11	7,000
$HuHb^+$	2.10	13–25	17,000
$HuHb^+$	2.10	2.5–13	2,400

Rate data obtained for the first-order kinetics in HCl at 410 mμ at 2.5° for both proteins are shown in Figure 5 as a function of pH. The new data are demonstrably continuous with that obtained earlier at 0.2° in acetate and lactate buffers at pH 3.3 (Steinhardt *et al.*, 1963; Molday and Steinhardt, 1969). The corresponding data at 25° (Figure 5, Polet and Steinhardt, 1969) are significantly different. First the separation between the profiles for the two species has increased from 0.3 pH unit to 0.5 pH unit, and second, the profiles for the two species do not appear to approach identical limiting velocity of denaturation at low pH as they do at 25°, from which Polet and Steinhardt (1969) drew important conclusions as to reaction mechanism. It appears that the two species approach separate limiting values at low temperature and that the earlier conclusions require reevaluation.

A further difference between horse and human Hb^+ is demonstrated by the Arrhenius plot of denaturation rate data (reaction B → C) at several temperatures, shown in Figure 7. Above 15°, both proteins show a high apparent activation energy, E^*, of about the same magnitude; however, at temperatures below about 12°, E^* becomes much smaller and a difference appears between the two species. The values of E^* are presented in Table III and show that at low temperatures the activation energy for horse is three times as great as for human, although both are quite small.

[3] Reagents giving rise to marked shifts in the spectral bands of Hb^+, such as azide and cyanide, were excluded from this study since they do not display bands at 500 or 630 mμ.

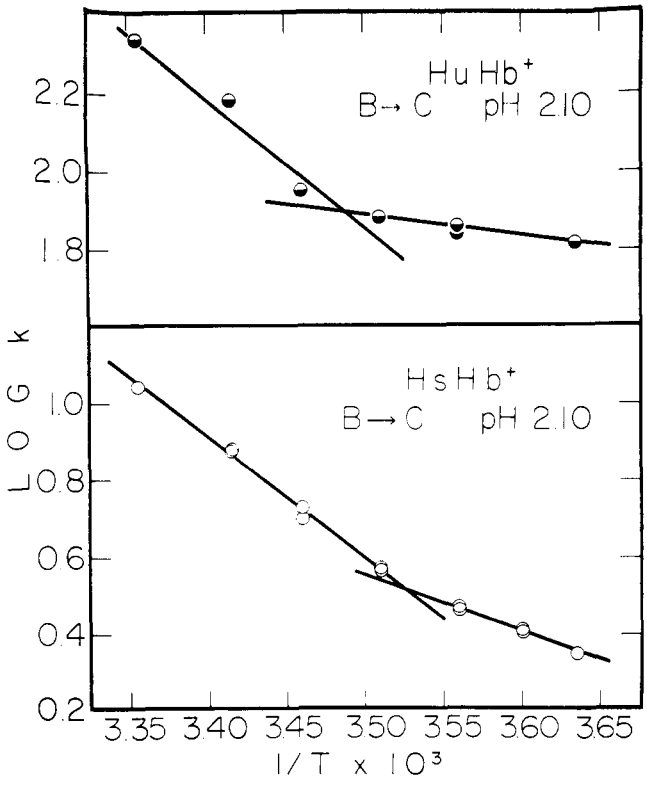

FIGURE 7: Arrhenius plot of the first-order denaturation rate constants (B → C) for horse and human ferrihemoglobins. Chloride ion concentration is 0.02 M. First-order rate constants are measured at 410 mμ.

Beychock and Steinhardt (1960) found a difference between acid-denatured horse ferrihemoglobin when formed at high and low temperatures. When measured at 0°, the hemoglobin denatured at 0° has a higher intrinsic viscosity than has hemoglobin denatured at 25°. If the viscosity measurement is carried out at 25°, where it was postulated that rapid interconversion occurs, the difference disappears. The results presented here support the conclusion of a difference in the product formed at 0 and 25° in both horse and human hemoglobin. Further characterization of this difference by means of their optical properties is to be published elsewhere.

Discussion

All the band shifts incident to the fast reaction, A → B, correspond to the changes observed by Schoffa (1964) when Hb^+ complexes become more "covalent" in nature: the Soret band shifts to the red to a maximum of 420 mμ, the band in the 500-mμ region shifts toward 540 mμ, and the band in the 630-mμ region shifts to longer wavelengths and vanishes in the case of the Hb^+ complexes which have the fewest and least polar Fe–N bonds. The Soret shift parallels in linear fashion the change in magnetic susceptibility (Figure 2, Schoffa, 1964); thus, the size of the red shift seen corresponds to a reduction of the susceptibility from 5.7 to about 4 BM for horse Hb^+ and perhaps slightly less for human Hb^+. This would indicate the transient existence of the iron in an intermediate or resonating spin complex, such as is known to exist with fluoride at 0° and thiocyanate at other temperatures (Brill and Williams, 1961).

Metmyoglobin is known to contain a water molecule at the sixth coordination site of the heme iron (Stryer *et al.*, 1964) and Hb^+ also must contain the same ligand since a proton is dissociated at the same pH in both proteins. It has been pointed out (Hoard, 1966) that the iron atom is displaced from the plane of the heme system in high-spin Hb^+, on the side closer to the proximal histidine (F8). Therefore the iron-to-water bond distance is quite long, and the water may be only a dielectric filler (Hoard *et al.*, 1965) at low pH and the FeIII may be in either a five or six coordination state.

The spectral changes accompanying the reaction A → B indicate that a transient state exists as a prelude to heme separation in which there is stronger ligand bonding at position 6 than provided by the water molecule. Following protonation at pH <3 (where the apoprotein acquires a high positive charge, most of its exposed prototropic groups having been protonated), the tertiary structure of the molecule, and in particular the heme pocket, is under great electrostatic strain. This may allow the distal histidine (E7) to approach the sixth coordination position of the iron more closely than is possible in the native structure. This histidine nitrogen would provide the stronger ligand field necessary to effect the orbital rearrangement,[4] giving rise to the observed spectral shifts. It would be necessary for the iron-bound histidine to be still unprotonated when the observed shift occurs. This presents no difficulty since an H_8O^+ ion would not be expected to penetrate the hydrophobic heme pocket until it collapses. Nor would proton transfer into the heme pocket be likely since the associated water molecule is protected from contact with the solvent by the pocket structure. This water would have to be moved aside, however, in order to allow the approach of the distal histidine to the iron coordination site.

When the initially unprotonated iron-bound histidine is protonated, the slower unfolding reaction occurs, which turns the heme completely out of the pocket. The unfolding reaction probably also competes with the approach of the distal histidine to the heme iron. The Soret band data at pH <3 are consistent with the latter interpretation, since the earliest curves are lower than the native Soret peak, and continue to decrease even as the Soret red shift takes place (see Figures 1 and 2). The interpretation given on an earlier page of the suppression of the red shift by formate ion is consistent with a substantial amount of competition when formate is absent.

Alternatively the spectral changes may result from changes in the donor–acceptor interactions between the π electrons of the porphyrin ring system, including its vinyl groups, and the protein environment. When the heme pocket structure starts to collapse the porphyrin ring may be brought into closer contact with the environment, as is believed to be the case when the ferro protein binds oxygen (Caughey, 1967). In either case, it appears that the reaction A → B is not a necessary precursor to the unfolding reaction. There are two pathways to reach state C (free monomeric heme) with the one involving A → B becoming important only when the charge density on the protein is high, and then only because it is so much faster than even the unfolding reaction A → C. Thus no Soret red shift is observed at denaturing pH values above 3.0 and the pH *vs.* rate profiles (Figure 5; and Figure 5, Polet and Steinhardt,

[4] A recent communication by Countryman *et al.* (1969) has established that the bisimidazole complex of hematin is a low-spin complex.

1969) are linear and continuous through the region where the red shift appears. Although Polet and Steinhardt (1969) did not provide for competition and a reaction path which could bypass B at pH >3.0, their model actually requires such an assumption to account for the results at pH >3.0. With this modification the present results are entirely in accord with the interpretation of the successive steps in the acid denaturation process set forth in the Polet and Steinhardt paper.

Neither of the suggestions so far accounts for the fact that the λ shifts of the Soret and 500-mμ bands are not the same in horse and human proteins. One difference between the proteins is that the horse protein contains three more carboxylic acids (or acid amides) in the β chain than does human (Smith, 1967); however, if this difference in composition were responsible then the position of the shifted peaks would be expected to be a function of the net charge. The absence of dependence upon pH has shown that this is not the case, at least in the range below pH 3.

Another species difference is found when the amino acid side chains in contact with the heme in horse hemoglobin (Perutz *et al.*, 1968) are compared with their counterparts in human hemoglobin (Braunitzer *et al.*, 1961). Three substitutions are present, all in the β chain. The residue valine (62), serine (70), and asparagine (97) in horse are replaced by alanine, alanine, and histidine, respectively, in human. The replacement of asparagine (97) by histidine changes the distribution of π electron charges about the heme in the β chains.

Formate buffers appear to suppress the fast initial reaction. It has been known for some time that formate (alone among the simple fatty acid anions) stabilizes Hb^+ against acid denaturation, much as an azide and cyanide do, although somewhat less effectively (Zaiser and Steinhardt, 1954; Steinhardt *et al.*, 1963). Although no spectral shifts occur, such as those found with cyanide and azide, there is a diminution in the absorption coefficients throughout the spectrum. Acetate affects the absorption spectrum in the same manner as formate but does not stabilize Hb^+, and denaturation at pH 2.54 shows that the rapid changes in the visible spectrum associated with the reaction A → B are also not affected. These rapid changes are also not affected by any of the other buffers tried. It is therefore concluded that the properties of Hb^+ denaturation by formate are unique; it is not uncommon for a one carbon compound to be markedly different in its properties from its higher homologs.

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