Comparisons of the Kinetic Stability of Normal and Sickle Cell Human Hemoglobins at Extremes of pH†

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ABSTRACT: Since differences (if any) between the rates of unfolding of normal human hemoglobin (HuA) and sickel cell hemoglobin (HuS) at extremes of pH would be attributable to a single codon substitution, they have been sought in both liganded and unliganded forms of both proteins. The strong destabilizing effects of long-chain alcohols have also been compared in the two proteins. The S apoprotein (globin) is unfolded about 50% faster than globin A, but the differences between the liganded forms of HbS and HbA are much smaller (Hu^+ and O_2Hu) or absent (COHu). Octanol enhances the rate of denaturation of the liganded forms of both A and S although to a smaller extent than with horse hemoglobin. The relations of octanol concentration to unfolding velocity and equilibrium are also different from the same effects with horse hemoglobin. There is no octanol effect at all on COHu. Contrary to earlier reports, the rates of denaturation of Hu^+S and Hu^+A in strongly alkaline solutions do not differ. The unliganded forms (Hu^0A and Hu^0S) show slightly larger differences than the liganded forms, but analysis of the results is complicated by (a) the existence of two deoxy forms of both proteins, which differ in rate of denaturation by about tenfold, (b) the strong stabilizing effects of trace quantities of diphosphoglycerate, inorganic phosphate, and Tris buffers, and (c) by the apparently spontaneous transformation of "unstable" Hu^0S to "stable" Hu^0S in storage. Hu^0S is less susceptible to enhancement of its acid denaturation rate by octanol; the effects of the latter are largely counteracted by 0.001 M phosphate, although not by Tris. The absence of more marked differences in stability between A and S indicate that the $\beta 6$ position plays only a small role in preserving the integrity of the native conformation. The presence of two deoxy forms which differ radically in stability suggests that more than a single T conformer may exist.

The hemoglobins of various mammalian species (*e.g.*, human and horse) are characterized by large numbers of amino acid sequence differences (about 60/dimer in the case of the example given). One result of these differences is that the pH profiles for both rates and equilibria of acid denaturation of their respective ferrihemoglobins differ substantially (Steinhardt and Hiremath, 1967; Molday and Steinhardt, 1969), *i.e.*, human ferrihemoglobin is denatured about five times faster than horse ferrihemoglobin at any pH and ionic strength. Furthermore, the equilibrium governing the transition in human ferrihemoglobin from fully folded (native) to the fully acid-unfolded forms is spread over twice as great a range of pH values as the corresponding range for horse.

Because of the large number of sequence differences in the two species it is difficult to relate particular differences, at specific sites, to the differences in either kinetic or equilibrium stability. Certain other pairs of species have fewer sequence differences in their respective hemoglobins, *e.g.*, human and rhesus monkey. However, the existence of human mutant hemoglobins provides a number of more extreme cases of limited well-defined differences, most often differences in a single side chain only. We have therefore studied the unfolding of the most plentiful of these known mutants, sickle cell hemoglobin (HbS)[1] in both unliganded and variously liganded forms, for comparison with normal human hemoglobin A (HbA). Use has also been made of very low concentrations of long-chain alcohols because of their effect on susceptibility to denaturation by acid, as recently reported (Cassatt and Steinhardt, 1971b, and unpublished data). Since this effect has hitherto been reported only for horse hemoglobin, it has been necessary to measure it with both unliganded and with various liganded forms of normal human hemoglobin also.

In the early course of this work, it became clear that the deoxyhemoglobins of both S and A prepared from various oxyhemoglobin stocks, belonged to two different populations with respect to their kinetic stability. Although we have not learned the structural basis of these differences knowledge of their existence has important consequences. Thus these differences are also reported here.

Experimental Section

Materials

Hemoglobin A. COHuA was crystallized three times from pooled human whole blood as described previously (Steinhardt and Hiremath, 1967) following Drabkin (1949). The COHuA was stored frozen in 6% aqueous solution and upon thawing it was passed through a 0.22 μ Millipore filter.

O_2HuA was prepared by one of two methods: (A) passing O_2 over 10 ml of a stirred solution of COHu in a 250-ml side-arm erlenmeyer flask. A 100-W incandescent light with reflector accelerated the photodissociation of COHu in an ice bath. The oxygenation, monitored spectrophotometrically, was usually complete in less than 1 hr. (B) The washed erythrocytes of

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[1] Abbreviations used are: HuA, normal human hemoglobins; HuS, sickle-cell hemoglobin; Hu^+, ferrihemoglobin; HuCN, ferrihemoglobin ferricyanide; O_2Hu, oxyhemoglobin; COHu, carbonylhemoglobin; Hu^0, deoxyhemoglobin; Hb, any form of human ferrohemoglobin; Hs, any form of horse ferrohemoglobin.

homozygous normal (AA) donors were lysed with water and toluene, and the lysates were dialyzed. The materials made by both methods gave identical results.

Hu^0A was prepared from 0.2% O_2Hu, containing no more than 10^{-6} M diphosphoglycerate, in a tonometer having a ground joint attached to a small bulb with a curved neck. The tonometer was evacuated to 20-mm pressure and flushed with N_2 a total of six times (with waits between each evacuation) to remove oxygen. The protein solution was then mixed with a slight stoichiometric excess of dry $Na_2S_2O_4$ previously placed in the attached bulb. It was then withdrawn with a syringe through a serum cap. The visible spectrum of the Hu^0 preparation showed strong absorption bands at 555 and 430 nm even after several hours.

Hu^+A was prepared by oxidation of COHu with two equivalents of $K_3Fe(CN)_6$ followed by dialysis against 0.2 M potassium phosphate at pH 6.8, and then against distilled water.

Hemoglobin S. HuS from homozygous (SS) donors was either crystallized as O_2Hu one to three times and then dialyzed free of phosphate, as above, or was obtained from blood cell lysates; and dialyzed against water, or first against 0.5 M NaCl and then water. When heterozygous (AS) donors were used the protein was prepared by fractionation of once-crystallized COHu or O_2Hu on DEAE-Sephadex (Tris buffers) by the method of Dozy *et al.* (1968) slightly modified by McGrath (1972). The effluent from the column was concentrated approximately 10-fold, first in a Dow hollow-fiber ultrafilter HfU-1 and then in an Amicon ultrafiltration cell equipped with a UM-2 membrane. Electrophoresis of the concentrate on Millipore cellulose acetate strips gave a single band corresponding to the less anodic band in the original mixture.

The globins of both S and A were prepared from Hu^+ by the method of Rossi-Fanelli *et al.* (1958) as described by Sebring and Steinhardt (1970).

The unliganded and liganded forms of the HuS were prepared as with HuA. In general HuS preparations were used soon after preparation (a few days to three weeks) and were therefore sometimes kept at 2° as O_2Hu rather than as COHu.

Other substances were used without further purification. Octanol (bp 195.8, 197.0°) was obtained from Mann Research Laboratories lot H1705; KCl, reagent grade, from Baker lot 33227; glycine, reagent grade, from Fisher lot 714050; chloroacetic acid, "Certified" chemical, from Fisher lot 722878. All ClAcH solutions were periodically tested with $AgNO_3$ and were not used if a white precipitate formed.

Methods

The unfolding of the various hemoglobins and their derivatives has been measured in two ways: (a) the least equivocal determines, by means of a pH-Stat, the rate of unmasking of basic groups which are inaccessible to solvents in the native state (Steinhardt *et al.*, 1966; Sebring and Steinhardt, 1970); (b) the second, which is faster and more convenient, follows the process of unfolding by measuring the decrease of absorbance at the Soret band maximum of each form (except COHb which was observed at 284 nm). It has been shown that the latter method is valid with horse Hb^+ (Polet and Steinhardt, 1969) and with CoHb at 284 nm (Allis and Steinhardt, 1970), but a searching analysis of its validity (freedom from interference by successive or parallel reactions) has not been made with either oxy- or deoxyhemoglobins. With Hb^+ and COHb the method is completely valid at pH values above about 3.0; at lower pH values, such as some of those used in the present investigation, the validity is limited to the first-order main portion of the reaction, after one or more very fast reactions (caused in part by wavelength shifts of the maximum) which occur much more rapidly at the beginning of the reaction (Polet and Steinhardt, 1969). The main portions of the reactions reported here are first order, within experimental error, and are completely consistent with measurements made at higher pH, where the validity is not in question. The conclusions drawn in the present paper thus apply only to the "main portion" of the reaction, and might differ from the conclusions which would follow from comparison of the respective initial fast reactions especially when the latter are measured at other wavelengths. The latter are now under investigation.

Measurements of the apparent equilibrium constant for the conversion of Hu^+ to denatured Hu^+ were made with 0.1% solutions of protein, with a Cary 14 spectrophotometer at the Soret maximum 406 nm after the lapse of 7–10 half-periods (Steinhardt and Hiremath, 1967). The absorbance of native Hu^+ was measured at pH 6.25 and that of completely denatured Hu^+ at pH 3.33. Measurements of pH were made with a Radiometer pH meter 25 calibrated with NBS buffer solutions. Ionic strength was maintained at either 0.02 or 0.025 as stated, with acetate, chloroacetate, or HCl–KCl mixtures (at low pH).

The time-dependent absorbance changes of 0.1% hemoglobin solutions rapidly mixed with acid buffers were measured in a 2-mm cell in a Durrum-Gibson stopped-flow spectrophotometer equipped with a Durrum D-131 logarithmic amplifier, and in most of the work with unliganded hemoglobins, with a Type 802 Biomation transient recorder. The Soret maxima were used for monitoring absorbance changes of Hu^+, HuCN, O_2Hu, and Hu^0. With COHu, a wavelength of 284 nm was used, since it is isosbestic for a second-order reaction which complicates the kinetics at other wavelengths, including the Soret maximum (Allis and Steinhardt, 1970). Oxyhemoglobin solutions were denatured at 2° to minimize acid-catalyzed ferrihemoglobin formation during the denaturation reaction which is relatively faster than oxidation. The other experiments were carried out at 25.0°. Alkali denaturation of ferrihemoglobins A and S was monitored at 580 nm under experimental conditions identical with those described by Beetlestone and Irvine (1962), using identical concentrations of A and S proteins separated from the mixed hemoglobins of a single AS individual on the same DEAE column.

The denaturation of the apoproteins (globins) was followed with a Radiometer pH-Stat, at an ionic strength of 0.02 with 0.5% protein solutions, as described by Sebring and Steinhardt (1970).

Results

Description of the results is divided into (A) comparison of the rates of acid denaturation of the apoproteins (globins) of the A and S hemoglobins; (B) the comparative susceptibility of liganded forms of A and S hemoglobins; (C) their comparative susceptibility to enhancement of denaturation rate by very low concentrations of octanol (1.6×10^{-3} M); (D) further information about the effects of octanol on human hemoglobins; (E) comparisons of the denaturation of A and S hemoglobins in alkaline solutions; and (F) comparisons of the susceptibility of the corresponding unliganded (deoxy) forms to acid denaturation.

Differences in the Apoproteins. Figure 1 shows that the globin of HbS is denatured about 50% faster than that of HbA

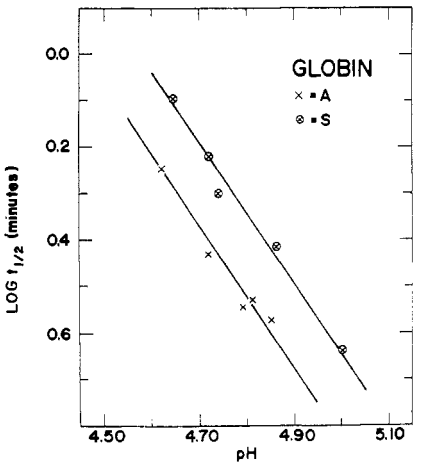

FIGURE 1: Dependence of the half-period of acid denaturation of normal human and sickle cell globins at 25° on pH at 0.02 ionic strength.

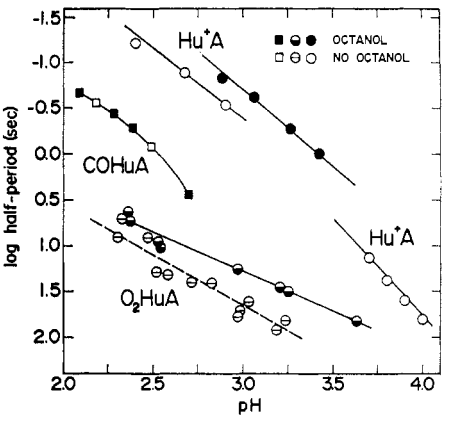

FIGURE 3: The effects of octanol (1.6×10^{-3} M) on the acid-denaturation rates of Hu^+A, O_2HuA and COHuA (crystallized preparations) at 0.02 ionic strength. The data for O_2Hu were taken at 2°, the other data at 25°. They have been displaced downward by 0.6 unit to avoid overlapping.

when compared by pH-Stat monitoring of the unmasking of inaccessible histidines (Sebring and Steinhardt, 1970). The dependence on pH is the same with both proteins. The two globins were obtained from a single AS individual, separated chromatographically on the same DEAE column, after a single crystallization, but data obtained with unchromatographed crystallized hemoglobin A are in close agreement with the data shown here for the chromatographed A protein.

Kinetics of Denaturation of Liganded Forms. The differences in stability of globins A and S almost disappear when a liganded prosthetic group is present. There are no large differences in the rates of denaturation of liganded forms of hemoglobins A and S when measured by the rates of decay of Soret absorbance. This is made clear in Figure 2 in which sets of data are shown for ferrihemoglobins A and S, ferrihemoglobin cyanides A and S; and oxyhemoglobins A and S. In the case of HuCN and the data for Hu^+ at higher pH the lines represent data obtained earlier with crystallized human hemoglobin A (Steinhardt and Hiremath, 1967; Molday and Steinhardt, 1969; Cassatt and Steinhardt, 1971a). Most of the results shown in Figure 2 were obtained with protein separated from AS mixtures by chromatography (Dozy *et al.*, 1968), but, it is clear, the results obtained with chromatographed A are very similar to those previously obtained with crystallized material from normal homozygous donors. There are only very small, but consistent, differences between the data for the various A and S proteins. Data for COHb for A and S (from a single AS individual) at pH 2.47, not shown in Figure 2, also show no significant difference.

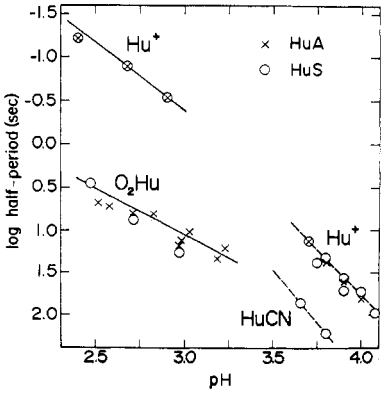

FIGURE 2: Comparison of the kinetic stabilities of various liganded forms of HuA and HuS (ferrihemoglobin, ferrihemoglobin cyanide, and oxyhemoglobin) as a function of pH. The broken lines through the data for HuCN (S) and for Hu^+ (S) represent data for HuCN (A) and Hu^+ (A) (see text). The data for O_2Hu were taken at 2°, the other data at 25°; ionic strength, 0.02.

The effects of octanol on the acid denaturation rates of Hu^+A, O_2HuA, and COHuA, made from crystallized protein, are shown in Figure 3. The enhancing effect of octanol is substantial in the cases of Hu^+A and O_2Hu but with COHuA there is no effect. The rate of denaturation is increased only two- to fourfold by 1.5×10^{-3} M octanol, as compared with a factor of 8 observed with horse Hb^+ (Cassatt and Steinhardt, 1971b). Experiments with $Hu^+(S)$, not shown on the figure, show that with S the effects of octanol are very similar in magnitude to those shown in Figure 3. The rates with Hu^+A shown in the figure are slightly slower than the rates for Hu^+S shown in Table I.

The results with O_2Hu are noteworthy in two respects: (a) their relatively low dependence on pH, and (b) the fact that the data obtained with octanol present appear to converge with the results without octanol as the pH falls, *i.e.*, the pH profiles are not parallel. The lower pH dependence appears to be well-established although the data scatter more than with the other forms.

Dependence of Velocity and Equilibria on Concentration of Octanol. Figure 4 shows that the rate constant of unfolding of Hu^+A at pH 3.0 is about 1.4 sec^{-1} in the absence of octanol and rises *linearly* with the octanol concentration to over 3.5

TABLE I: Effects of Octanol (1.6×10^{-3} M) on the Acid Unfolding of Hu^+S Compared to the Effect of 1.5×10^{-3} M Octanol on Hu^+S at 0.02 Ionic Strength.

	Half-period (sec)		
	Hu^+S		Hu^+A
pH	No Octanol	Octanol	No Octanol (Interpol)
3.75	24	6.3	10.9
3.90	53	17	36
4.08	95	46	89

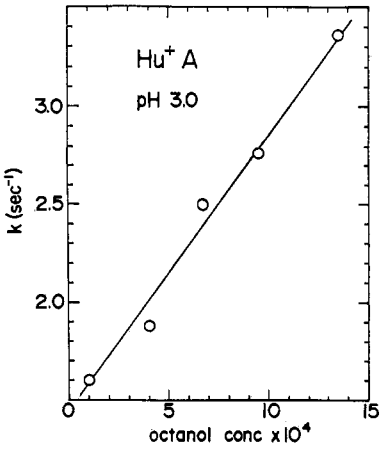

FIGURE 4: Dependence of acid-denaturation rate of Hu^+A on octanol concentration (moles/liter) at pH 3.0, ionic strength 0.02, at 25°.

sec^{-1} at 1.5×10^{-3} M. With horse hemoglobin the effect is not only larger, but quadratic with respect to concentration rather than linear (Cassatt and Steinhardt, 1971b). The effect on Hu^+S (J. C. Cassatt and J. Steinhardt, unpublished) is at least as large as the effect on Hu^+A.

The pH-dependent equilibrium constant for denaturation, K_D, is defined as the ratio of the concentration of denatured protein to that of native protein at a given pH. The pH dependence of log K_D for normal Hu^+, determined spectrophotometrically, is shown in Figure 5. Between pH 4.0 and 4.6 the slope of the pH profile of log K_D in the presence of octanol (3.3) is the same as in its absence but the data are shifted 0.1 unit toward higher pH. This contrasts with the corresponding results with horse Hs^+ for which the slope is 5 in the absence of octanol and 3 in the presence of octanol, *i.e.*, the unfolding reaction with the human is more highly concerted than with the horse protein (Cassatt and Steinhardt, 1971b). Hu^+ is less stable than Hs^+ with respect to acid denaturation both in the presence and absence of octanol (Cassat and Steinhardt, 1971b, and unpublished data), but the difference is smaller when alcohol is absent.

Equilibrium data have not been obtained with Hu^+S.

Unfolding at Alkaline pH. Figure 6 shows the kinetic behavior of the ferrihemoglobin of both A and S at alkaline pH (10.50). The two sets of data are as close as in the experiments at acid pH. This finding contradicts an earlier report (Beetlestone and Irvine, 1962) of a large difference in rates under these conditions. pH profiles of the two oxyhemoglobins at high pH lead to the same conclusion (Figure 7).

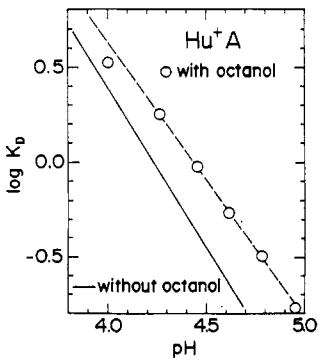

FIGURE 5: Variation with pH of the equilibrium constant for unfolding of Hu^+A by acid. Ionic strength 0.02, 25°, octanol concentration 1.5×10^{-3} M.

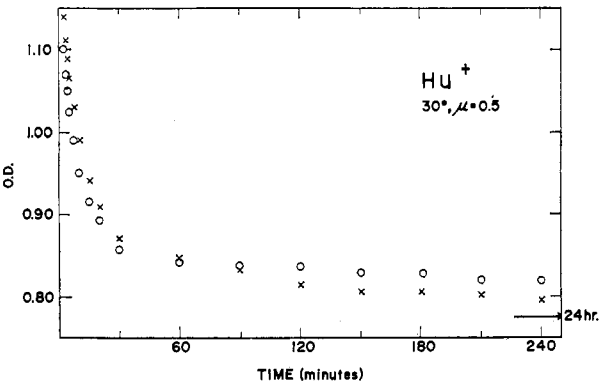

FIGURE 6: Unfolding of Hu^+ at pH 10.50 (carbonate) at 30°, at 0.5 ionic strength: circles, Hu^+A; crosses, Hu^+S.

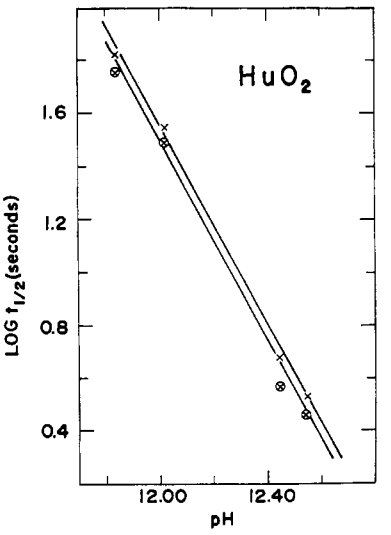

FIGURE 7: pH profiles for unfolding of O_2Hu A and O_2Hu S in 0.02 M NaOH–NaCl at 25°: circles, O_2Hu A; crosses, O_2Hu S.

Kinetics of Denaturation of Unliganded (Deoxy) A and S. Figure 8 shows that certain peculiarities, unique to the deoxy form, must be taken into account before the pH–rate profile of unliganded A and S hemoglobins are compared. The deoxy forms of both A and S may be characterized by either high or low kinetic stability (rates of denaturation) depending on the history of the preparation. Thus curves 1 and 3 represent the deoxy forms of "unstable" crystallized preparations, and curves 4, 5 and 6 present data obtained with the "stable" form of the deoxyhemoglobins. Note that the unstable S material (curve 3) was spontaneously transformed to the stable form (curve 4) during a storage period of three weeks, and that *very slightly* less stable material was obtained from the "stripped" uncrystallized lysates of erythrocytes from SS donors[2] (curve 5). Thus the much greater instability shown in 1 and 3 is not due to stripping of phosphates. Merely passing the unstable A preparation through a DEAE-Sephadex column equilibrated with Tris buffer (pH 8.1), and then dialyzing, converts the unstable preparation to the more stable form; (curve 7); in fact the mere addition of 10^{-4} M Tris suffices to produce this effect. With A *lysates*, however, stripping paradoxically *enhances* the stability significantly.

Although solutions of crystallized S changed from the

[2] "Stripping" refers to the removal of all phosphates (and other ions) by passage through a mixed bed resin (Bio-Rad T) after dialysis.

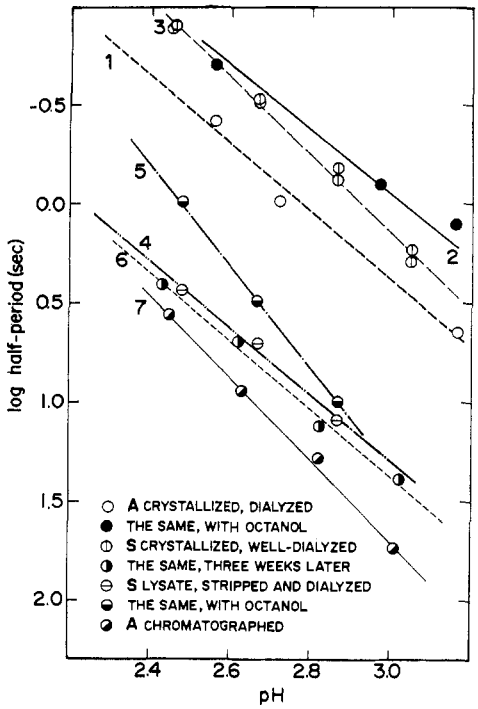

FIGURE 8: Examples of the pH profiles for the logarithm of the acid-denaturation rates of deoxyhemoglobins A and S, including the effects of 1.5×10^{-3} M octanol, and differences between preparations. Numbered curves (see text): ionic strength, 0.025; temperature, 25°.

unstable to the stable forms in less than three weeks (when stored as O_2Hu), unstable A material remained unstable during periods of storage as COHu for over 6 months at 2°. During that period traces of Tris always sufficed to change it to the stable form.

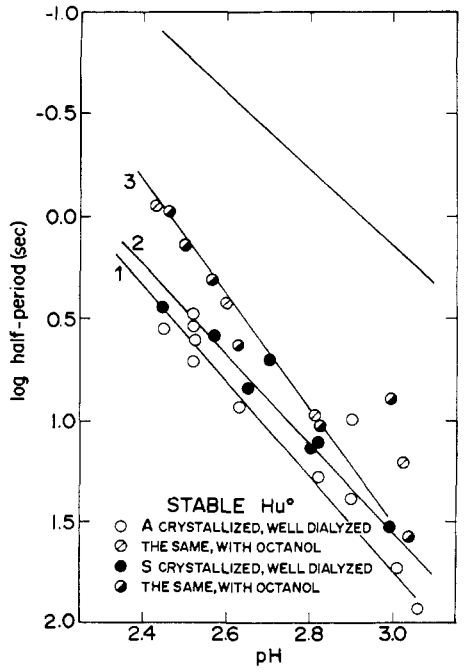

FIGURE 9: pH profiles for "stable" deoxyhemoglobins A and S with and without 1.5×10^{-3} M octanol: ionic strength, 0.025; temperature, 25°. The line at the top of the figure represents "unstable" deoxy S.

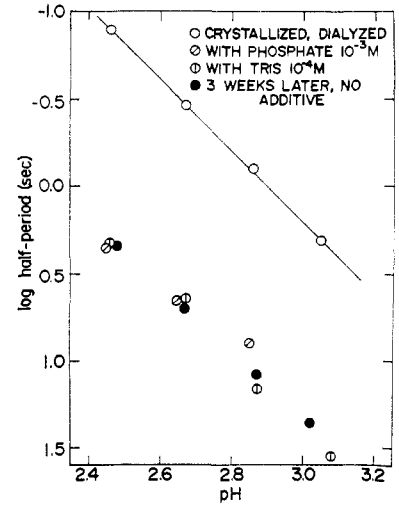

FIGURE 10: pH profiles at 25° of an initially unstable crystallized preparation of deoxyhemoglobin S stabilized by 10^{-3} M inorganic phosphate or by 10^{-4} M Tris, or by storage for less than 3 weeks.

Figure 8 also shows that there are large octanol effects on the unstable preparations, and smaller on the stable preparations.

Clearly, fortuitous (and incorrect) conclusions would be reached if unstable preparations of A were to be compared with stable preparations of S as in our preliminary work (Jones *et al.*, 1973). Data for stable preparations are compared in Figure 9. It appears that stable S is denatured about 40% faster than stable A (almost as much faster as the respective globins). The effect of octanol on A denaturation is larger (2.5 times) than on S denaturation (1.4–2.0 times, depending on pH). These two differences combine to make the kinetic data *with octanol* for both A and S hemoglobin appear to be superimposable. The important conclusions are that (a) differences in denaturation rate of A and S in the deoxy form are little if any larger than with the liganded forms,[3] and no larger than with the globins; and (b) that the effects of octanol are somewhat smaller with the S protein.

In Figure 10 some of the effects of the stabilizing additives (phosphate, Tris) on an initially unstable crystallized, well-dialyzed S preparation of deoxy S are summarized. Traces of both inorganic phosphate as well as of diphosphoglycerate (not shown) and Tris reduce the high denaturation rates of the unstable S by a factor of about 10, to the value which characterizes the same preparation after three weeks of storage as O_2Hu. Phosphate, but not Tris, counteracts the enhancing effect of long-chain alcohols on the denaturation rate; Tris acts only to reduce the rates of the unstable preparations and does not offset the effect of the alcohols.[4]

[3] Probably the most conclusive demonstration that there are only small differences in kinetic stability between Hu^0A and Hu^0S is afforded by data obtained with A and S separated from the erythrocytes of a single AS (heterozygous) individual on the same DEAE-cellulose column, adjusted to the same concentration. Such data are indistinguishable from the data shown in Figure 9. Furthermore Hu^0A and Hu^0S, if subjected to acid pH *before* fractionation, yield excellent first-order kinetics, without evidence of curvature or break until the reaction is at least 90% completed.

[4] Tris in very low concentration (2×10^{-3}) also completely inhibits gelling of concentrated Hu^0S solutions under conditions at which controls (at the same pH, ionic strength, and temperature) always gel. (D. Carroll and J. Steinhardt, unpublished data. See also Freedman *et al.*, 1972.)

Discussion

The following observations described in this paper require interpretation in terms of structure or conformation. (1) The apoprotein of S is substantially more rapidly denatured at low pH than the apoprotein of A; but (2) the various liganded forms of the respective hemoglobins differ to a much smaller degree in their rates of denaturation; (3) there are differences in their susceptibilities to enhancement of denaturation rate by octanol; and (4) the unliganded A and S do not differ in resistance to denaturation by acid by much more than do any of the liganded forms, but (5) there appear to be two forms of the deoxy hemoglobins which differ very greatly in stability.

When pH profiles of two first-order reactions (log half-period against pH) are given by two straight lines it is impossible to distinguish between differences in energies of activation, and differences in the pK's involved in determining the levels of rate-limiting ionic components, or triggering acid–base equilibria. However, unless the pK's involved are part of the prosthetic group, it seems more plausible to attribute the differences observed to changes in the free energy of activation of unfolding.

Since globin S (apohemoglobin S) is acid-denatured significantly faster than globin A at 25°, it is reasonable to conclude that this reaction has a lower activation free energy (by about 240 cal) than the A unfolding reaction, and that therefore somewhat larger percentage differences would have been found at lower temperatures. Combination with the prosthetic group, whether liganded or not, in every case reduces or obliterates the distinction. It has been shown elsewhere that the stabilization of horse globin by protoporphyrin IX (iron free) is almost as great as that bestowed by ferriheme (Sebring and Steinhardt, 1970); it therefore appears that hemoglobin is stabilized mainly sterically by the prosthetic group which prevents collapse of the heme cavity, *i.e.*, the tertiary structure does not depend entirely on iron–imidazole interactions, because it is constrained in the presence of heme to produce a "heme pocket." This stabilizing effect is so large that the small differences in the activation energy for the unfolding of *isolated* A and S globins does not effectively come into play. The limiting rate is set by the rate of heme ejection (Polet and Steinhardt, 1969) which apparently occurs at about the same rate in both S and A hemoglobins. It is this "main portion" of the reaction process (see Methods) which is given spectrophotometrically on the time scales employed in the present investigation.

The octanol effects demonstrated with all the normal human forms except COHu are nevertheless smaller and less concerted than those demonstrated with horse hemoglobin. They are still smaller with deoxy S than with deoxy A but this difference does not seem to affect the liganded forms. Cassatt and Steinhardt (1971a) have suggested that the effect of octanol is exerted in the heme pocket, and their later data (unpublished data) have served to support this hypothesis. Thus, for example, Stryer (1965) has shown that the dye 8-anilino-1-naphthalenesulfonate fluoresces strongly when adsorbed on apohemoglobin, from which it can be displaced and quenched by heme. Since traces of long-chain alcohols also quench the fluorescence of 8-anilino-1-naphthalenesulfonate–globin solutions, it would appear that octanol like heme, displaces 8-anilino-1-naphthalenesulfonate from the heme pocket. While differences between high-spin forms such as Hu^+ and low-spin forms such as COHu might be expected, it is difficult to explain why the effect of octanol on two low-spin forms, O_2Hu and COHu, should differ so greatly.[5] The replacement of Glu-6 by Val-6, which distinguishes HuS from HuA, is sufficiently outside the heme pocket to lead one to expect no differences in the effect of octanol in terms of the hypothesis of Cassatt and Steinhardt. The small effect found may signify that the substitution Glu-6 → Val-6, has small indirect conformational consequences beyond its immediate vicinity.

Denaturation at alkaline pH is more difficult to measure with precision than at acid pH, partly because most of the buffer ions available have charges greater than 1, and changes in ionic strength, as well as pH, occur as unmasked groups become accessible and react with the solvent. In addition, with Hu^+ the spectral changes produced must be distinguished from those due to removal of a proton from the hydrated iron, and from effects due to the ionization of tyrosine. Proteins are attacked by high pH in several ways, *e.g.*, reaction of disulfide groups when any are present. Complex kinetics result. The results presented in Figures 6 and 7 are therefore not analyzed further. The results nevertheless show that HuS and HuA behave almost identically when they unfold at high pH.

The results with deoxy S and A (Figures 8 to 10) should be the most revealing since it is only in the deoxy form that the gross manifestations of the sickle cell mutation appear. However, the present investigation has found only slightly larger differences in the kinetic behavior of unliganded A and S than in the liganded forms. The most unexpected outcome of the present work has been the discovery of two forms of deoxyhemoglobin in both HuS and HuA, which differ approximately tenfold in rate of unfolding. This phenomenon, together with its accompanying modification by phosphate, diphosphoglycerate, Tris, and octanol may, on further investigation, lead to new information about the architecture of the hemoglobin molecule. Such investigations are in progress.

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[5] Since the rates of unfolding of COHu were determined at a different wavelength (284 nm), the possibility is raised that a different reaction is measured than when measurements are made at 419 nm. This possibility was excluded by Allis and Steinhardt (1970); in addition measurements at 421 nm, the Soret maximum of acidified COHu, give the same pH profile that was obtained at 284 nm.

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Further Studies of the Sulfhydryl-Catalyzed Isomerization of Bovine Mercaptalbumin†

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ABSTRACT: As shown previously, bovine mercaptalbumin (N), when incubated at low ionic strength above pH 7.5, isomerizes to a new component, A, having a more positive electrophoretic mobility than N near pH 5. The reaction is sulfhydryl catalyzed. In the present study, a simple gel electrophoresis method was devised which separates A from N in a continuous pH 4.2 buffer system. Utilizing this method, the reaction was found to be faster than previously reported; the half-time at pH 8.6 and 23° in 0.01 M KCl is about 75 min. The reaction was demonstrated to be intramolecular, *i.e.*, the sulfhydryl group of the protein can catalyze reaction only within the same protein molecule. The equilibrium constant in 0.01 M KCl is near zero at pH 7.5 and rises to 0.58 at pH 8.9. KCl exerts a stabilizing effect on N and the lability of charcoal defatted bovine mercaptalbumin at low ionic strength was confirmed; in particular, prolonged (*ca.* 1 week) dialysis against water was found to result in substantial (*ca.* 10%) isomerization and denaturation of N. Evidence is presented that N and A differ covalently. Both have the same isoionic pH and acid end point. However, A binds more protons than N in the pH range of the N–F transition and fewer protons than N in the pH range of the neutral transition. A Scatchard plot of proton binding to the carboxylates of A shows no evidence for the N–F transition with all 98 carboxylates titrating with one intrinsic p*K*. The histidines of A also exhibit ideal titration behavior. Further investigations into the structural differences between N and A utilizing intrinsic fluorescence, ultraviolet difference spectroscopy, gel filtration, and susceptibility to proteolysis all show A to have a more open conformation than N at neutral pH. However, optical rotatory dispersion and circular dichroic measurements indicate N and A to have similar helix content. These results are interpreted in terms of the domain model for N.

The primary sequence of a protein is thought to contain all the information necessary to direct the folding of the polypeptide chain into a unique conformation which is stabilized by a unique set of disulfide bonds. However, Foster *et al.* (1965) considered the possibility that there might be several disulfide pairings which co-exist and give rise to the microheterogeneity observed in a population of albumin molecules. Evidence in support of this postulate was offered by Sogami and Foster (1968) and Sogami *et al.* (1969) who demonstrated that the heterogeneity of relatively homogeneous preparations of bovine plasma albumin was dramatically increased by a sulfhydryl-catalyzed "alkaline aging" reaction. The increase in heterogeneity was thought to reflect a randomization of the native set of disulfide bonds. Hagenmaier and Foster (1971) and Nikkel and Foster (1971) concluded that the aging reaction results in the formation of a new component, termed A,[1] having a positive electrophoretic mobility at pH 4.84, the isoelectric pH of native bovine mercaptalbumin. Nikkel and Foster (1971) further demonstrated the formation of A to be a function of pH and by reincubating isolated A they demonstrated the reaction to be reversible. Alkylation of the free sulfhydryl of bovine mercaptalbumin completely abolished the formation of A; however, the addition of low molecular weight thiols to the blocked sample catalyzed the formation of A. Peptide mapping studies by Nikkel and Foster suggested that the location of the free sulfhydryl did not change upon isomerization, showing the role of the free sulfhydryl group to be purely catalytic. If the reaction is a disulfide interchange it must involve at least two disulfide bonds.

This study utilized a more convenient technique of assaying for A to study the kinetics and equilibria of the reaction in more detail. A comparison of some of the physical properties of A with those of native bovine mercaptalbumin was undertaken. Since the isomerization is a function of pH and since separation of the two isomers is based on a difference in charge, particular attention was directed to a comparison of the hydrogen ion binding properties of A and the native protein.

Materials and Methods

Materials. Charcoal defatted bovine mercaptalbumin was prepared by the method of Hagenmaier and Foster (1971); all preparations had a sulfhydryl content greater than 0.9 mol/mol of protein. Other proteins utilized were crystalline bovine plasma albumin lots D71207 and G71812 from Armour Pharmaceutical Corporation, and porcine trypsin lot S-01-3

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[1] Abbreviations used are: N, the native form of bovine mercaptalbumin; A, the isomer of bovine mercaptalbumin formed by alkaline aging.