Relative Stabilities of the Two Quaternary Conformations of Human Fetal Hemoglobin†

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ABSTRACT: The pH dependence of several functional properties of human fetal and adult hemoglobins has been studied to determine the relative stabilities of the high and low affinity (R and T) quaternary conformations of the two proteins under different conditions. Fetal aquomethemoglobin undergoes changes in sulfhydryl reactivity, absorption spectrum, and circular dichroism in the presence of inositol hexaphosphate which are consistent with a transition from the R to T quaternary state, but only at pH values below 6.8. In adult hemoglobin this transition can be induced at pH values below 7.2. Even in the absence of phosphates, the ultraviolet (uv) circular dichroism spectrum of fetal aquomethemoglobin at low pH indicates the presence of some T conformation. The initial value for the second-order rate constant for combination of fetal deoxyhemoglobin with carbon monoxide is comparable to that for adult hemoglobin in the absence of organic phosphates and is not reduced by organic phosphates as much as that for the adult protein. The apparent first-order rate constant for dissociation of CO from fully liganded fetal hemoglobin, measured by replacement with NO, increases threefold in the absence of organic phosphates, and fourfold in the presence of organic phosphates, with decreasing pH; the midpoint of the pH dependent transition occurs around 6.8. A similar increase in the apparent first-order rate constant for O_2 dissociation, as measured by replacement with CO, can also be seen with decreasing pH. NO-hemoglobin F can be converted to the T state even when fully liganded simply by lowering the pH, as judged by uv circular dichroism, visible difference spectrum in the region of the α and β bands, and a dramatic increase in the rate of NO dissociation, measured by replacement with CO in the presence of dithionite. These results are all consistent with a model for fetal hemoglobin in which the organic phosphate site may be functionally weakened by replacement of a residue involved in ionic interactions with the negatively charged phosphate groups, but in which the low affinity T conformation is intrinsically more stable than that of adult hemoglobin. According to this model, the differences between fetal and adult hemoglobin can be accounted for primarily in terms of the relative stabilities of R and T conformations in each of the proteins, with differences in the intrinsic properties of the individual conformations contributing effects of only secondary importance.

Fetal respiration is dependent upon transfer of oxygen from the placental capillary bed to the fetal circulation. This transfer occurs under conditions where the oxygen tension is considerably lower than that encountered in the alveoli, and would seem to require a fetal hemoglobin with higher oxygen affinity than that of the mother's. Highly purified preparations of fetal hemoglobin F (Hb F),[1] however, have lower oxygen affinities than comparable preparations of adult hemoglobin A (Hb A), in apparent contradiction to the physiological observations (Tyuma and Shimizu, 1970). This paradox was resolved when it was discovered that hemoglobin F binds organic phosphates less effectively than hemoglobin A so that, within the erythrocyte, the adult protein acquires a lower affinity for oxygen than the fetal protein (Tyuma and Shimizu, 1970; Maurer et al., 1970).

Recent investigations in which we have participated have probed factors which determine the relative stabilities of the high and low affinity quaternary conformations of hemoglobin A under various conditions (Perutz et al., 1974a,b, 1976). We have extended these studies to fetal hemoglobin in an effort to determine the origin of its low oxygen affinity in the absence of organic phosphates. Our results indicate that the low affinity (T) quaternary conformation of fetal hemoglobin exhibits a strongly pH dependent stability, and may actually predominate over the high affinity (R) structure in liganded hemoglobin F at low pH under certain conditions.

In many of the previous studies on the quaternary conformational equilibrium in hemoglobin, strongly bound organic phosphates, such as inositol hexaphosphate (IHP), have been employed to induce a transition from the high to low affinity structure. Ambiguities have arisen in interpretation of some of these earlier experiments because of the possibility of induction of a totally new conformation, only superficially resembling the structure of unliganded hemoglobin, by the bound phosphate. The results presented here offer an opportunity to provide correlative evidence for the conclusion that IHP, or any analogous allosteric negative effector, stabilizes the low affinity T quaternary state, instead of generating an entirely new structure.

Materials and Methods

Hemoglobin A was prepared from samples of adult blood according to the procedure of Drabkin (1946). Hemoglobin F was prepared from cord blood hemolysates by chromatography on CM-Sephadex according to the procedure of Wind (1973). Purity of the isolated hemoglobin F was verified by the alkali denaturation procedure of Brinkman and Jonxis (1935).

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[1] Abbreviations used: Hb A and Hb F, hemoglobin A and F, respectively; IHP, inositol hexaphosphate; CM, carboxymethyl; PCMB, *p*-chloromercuribenzoic acid; CD, circular dichroism; uv, ultraviolet; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

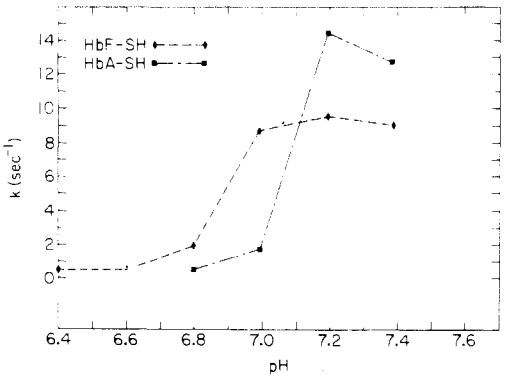

FIGURE 1: Apparent first-order rate constant for reaction of aquomethemoglobins A and F with *p*-chloromercuribenzoic acid (PCMB) as a function of pH. Hemoglobin concentrations, 5×10^{-5} M tetramer; PCMB concentration, 2×10^{-4} M. All reactions were carried out in 0.05 M Pipes buffers, containing 0.1 M NaCl.

Methemoglobin preparations were prepared by the procedure of Antonini et al. (1964). The reaction of the sulfhydryl group of cysteine F9(93)β with *p*-chloromercuribenzoic acid (PCMB) was measured with a Durrum D-110 stopped-flow spectrophotometer equipped with a xenon light source and a transient recorder (Biomation Assoc.) according to the procedure described by Perutz et al. (1974b). The rate of combination of deoxyhemoglobins A and F with carbon monoxide was measured according to the procedure of Gibson (1959); the rates of replacement of CO by NO and of O_2 by CO were determined by the methods of Salhany et al. (1975), McDonald and Noble (1972), and Olson et al. (1971); and the rate of NO replacement by CO in the presence of dithionite was measured according to the procedure of Moore and Gibson (1976), using the same apparatus. Kinetic constants were computed from exponential curves according to the method of Guggenheim (1926). Visible difference spectra of aquomethemoglobin F and NO-hemoglobin F were determined according to procedures of Perutz et al. (1974b, 1976). Uv circular dichroism measurements were carried out as described by Simon and Cantor (1969) and Perutz et al. (1974b).

Results

I. Sulfhydryl Reactivity of Methemoglobins A and F. Perutz et al. (1974b) have described the effect of inositol hexaphosphate (IHP) on the rate of reaction of cysteine F9(93)β with sulfhydryl reagents in methemoglobin A. High-spin ferric derivatives, such as aquomethemoglobin A and fluoromethemoglobin A, undergo a marked decrease in reactivity with decreasing pH, with a midpoint in the transition from high to low reactivity at pH 7.2. The apparent first-order rate constant for the reaction of aquomethemoglobins A and F with *p*-chloromercuribenzoic acid (PCMB) in the presence of IHP, determined under conditions of effectively constant PCMB concentration, as a function of pH is shown in Figure 1. Fetal aquomethemoglobin clearly undergoes a reduction in sulfhydryl reactivity which is comparable in magnitude to that observed for aquomethemoglobin A, but the apparent midpoint of the transition is shifted almost 0.4 pH unit to pH 6.8. The values obtained for the rate constants at pH values below 6.8 and above 7.2 are comparable to the values measured for deoxyhemoglobins A and F and oxyhemoglobins A and F, respectively, indicating that the conformations of the fetal and adult protein in the vicinity of the reactive sulfhydryl group

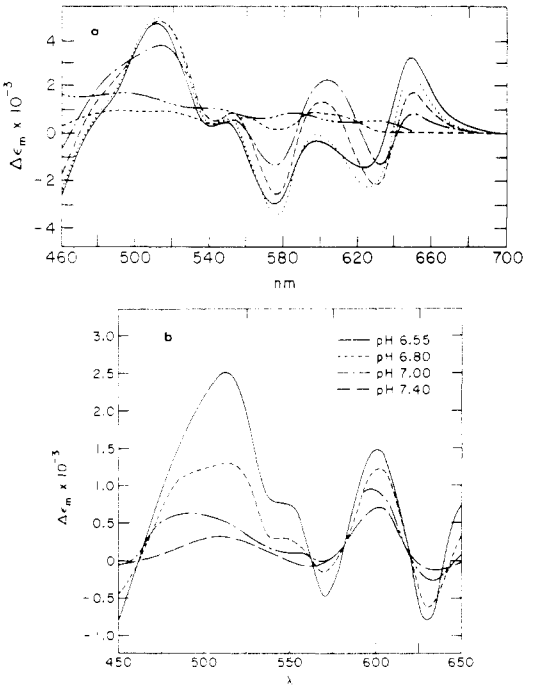

FIGURE 2: Effect of pH on the difference absorption spectrum of aquomethemoglobins A and F in the presence vs. in the absence of inositol hexaphosphate (IHP). (a) Aquomethemoglobin A, 4×10^{-5} M tetramer; 5×10^{-5} M IHP; 0.05 M Bistris buffers containing 0.1 M NaCl. (. . .) pH 5.5; (—) pH 6.0; (— —) pH 6.5 (— - —) pH 7.0; (— - - - —) pH 7.5; (- - -) pH 8.0. (b) Aquomethemoglobin F, 5×10^{-5} M tetramer; 2×10^{-4} M IHP; 0.05 M Bistris buffers containing 0.1 M NaCl. Data taken in part from Perutz et al. (1974c).

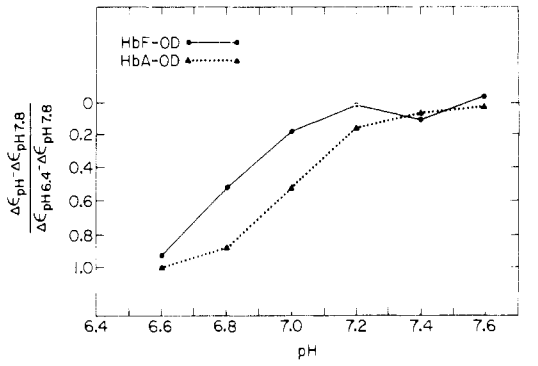

FIGURE 3: Effect of pH on the difference absorption spectra of aquomethemoglobins A and F in the presence vs. in the absence of IHP, expressed as fraction of maximum $\Delta\epsilon$ at 500, 575, and 625 nm, averaged for the three wavelengths.

are very similar under these conditions of acid and basic pH.

II. Difference Absorption Spectra of Methemoglobins A and F. Perutz et al. (1974b) have also reported on a difference absorption spectrum induced in aquomethemoglobin A upon binding of IHP and have interpreted the results (1974c) in terms of an organic phosphate-induced quaternary conformational transition in the protein. In Figure 2, part of the visible difference spectrum of aquomethemoglobin A in the presence vs. the absence of IHP at different pH values, as measured by Perutz, is compared with the difference spectrum of aquomethemoglobin F under identical conditions. Clearly, IHP produces extremely similar spectral changes in the two proteins, which are strongly pH dependent. In Figure 3, the pH dependences of these two sets of difference spectra are

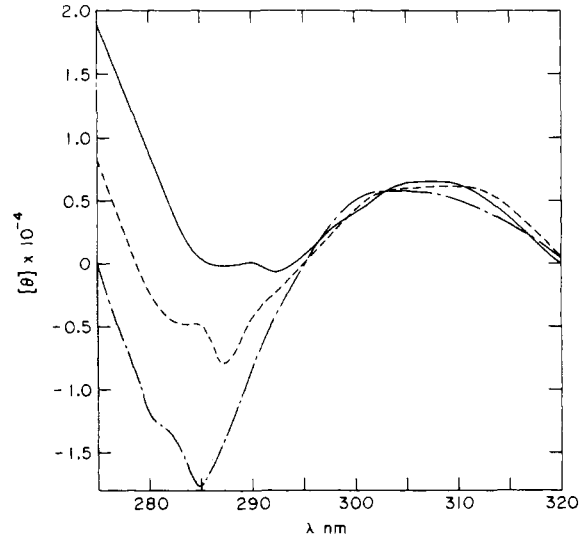

FIGURE 4: Circular dichroism of aquomethemoglobin F in the presence and absence of IHP. Hemoglobin concentration, 5×10^{-5} M tetramer; 0.05 M Bistris buffers containing 0.1 M NaCl. (—) pH 7.0 − IHP; (- - -) pH 6.0 − IHP; (— - —) pH 6.0 + 2×10^{-4} M IHP.

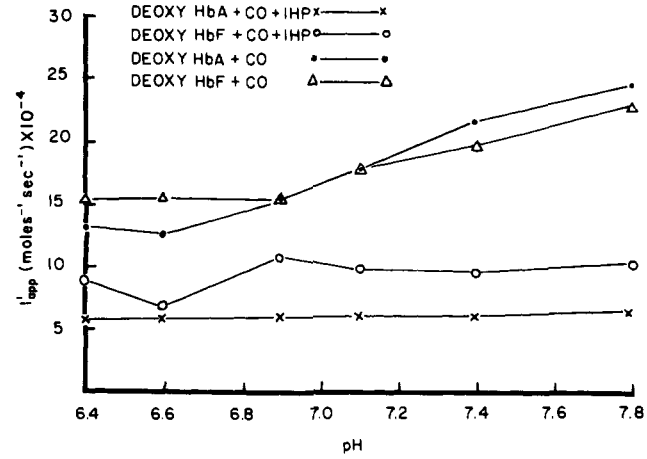

FIGURE 5: Effect of pH on apparent second-order rate constant for combination of CO with deoxyhemoglobins A and F in the presence and absence of IHP. Hemoglobin concentration, 2.5×10^{-5} M tetramer; CO concentration, 5×10^{-4} M; IHP concentration, 2×10^{-4} M; 0.05 M Bistris buffers containing 0.1 M NaCl. Reactions were monitored at 568 nm.

plotted, expressed as an average of the magnitude of the difference between the $\Delta\epsilon$ at any pH value and that measured at pH 7.8, normalized to the maximum difference produced, taken at several wavelengths where the $\Delta\epsilon$ values are largest. As in the case of the sulfhydryl reactivity measurements reported above, the development of the IHP-induced difference spectrum in fetal aquomethemoglobin occurs at more acid conditions than the transition observed for aquomethemoglobin A. Comparison of the pH dependences of the difference spectra in adult and fetal aquomethemoglobins with the pH dependences for sulfhydryl reactivity suggests that, within the limits of error of the two experimental techniques, the transition in aquomethemoglobin A, which occurs at approximately pH 7.2, occurs in aquomethemoglobin F as well, but about 0.4 pH unit lower, at approximately pH 6.8.

III. Ultraviolet Circular Dichroism of Methemoglobin F. A third measure of quaternary conformation discussed by Perutz et al. (1974a,b) is the ellipticity of a hemoglobin sample in the region around 287 nm. Strong negative ellipticity in this region is characteristic of the T structure, whereas weak positive ellipticity is associated with the R structure, regardless of the electronic state of the iron or the presence of ligands bound to the heme. In the presence of IHP, aquomethemoglobin F shows a marked negative ellipticity at pH 6.2 (Figure 4), further supporting the indications that the protein is indeed converted into the T state by the organic phosphate at low pH. Since this measure of quaternary conformation is unaffected directly either by iron spin state, or by pH, unlike the visible absorption spectrum or sulfhydryl reactivity which change with pH even when there is no quaternary structural transition, we examined aquomethemoglobin F CD spectra in the absence of IHP over the pH range from 7.4 to 6.2. The results, also shown in Figure 4, indicate that, even in the complete absence of organic phosphates, aquomethemoglobin F develops some negative ellipticity around 287 nm at low pH, indicative of a significantly populated fraction of molecules in the low affinity T state.

IV. Kinetics of CO Binding to Deoxyhemoglobins A and F. One measure of the functional properties of the low affinity T state of hemoglobin is the kinetics of its combination with ligands. The kinetics of binding of CO to deoxyhemoglobin exhibit a characteristic autocatalytic profile, as described by Gibson and Roughton (1956). The apparent second-order rate constant increases over the first half of the reaction, and then levels off. This rate constant is related to Z_1', the constant for the combination of the first CO molecule with deoxyhemoglobin. We have not undertaken a detailed examination of the autocatalytic nature of the CO-binding reaction for either fetal or adult deoxyhemoglobin in the absence or presence of IHP, but report only an average value of the second-order constant observed during the early phases of the combination, l_{app}'. The reaction measured under our conditions is in fact pseudo-first-order in deoxyhemoglobin since the free CO concentration does not change significantly over this limited extent of reaction. We have monitored the reaction profile at wavelengths where any heterogeneity in individual chain reactivity, as reported by Gray and Gibson (1971), would not be readily detectable. In Figure 5, we have reported the pH dependence of the apparent second-order constant for the early phases of the combination of CO with deoxyhemoglobins A and F in the presence and absence of IHP. In the absence of the organic phosphate, the combination constants for the two deoxyhemoglobins are very similar. Addition of IHP reduces both constants, although the reduction for hemoglobin A is greater than that for hemoglobin F. The two combination constants are quite pH independent in the presence of IHP. The reduced effect of IHP on fetal hemoglobin is completely consistent with the arguments of Arnone (1972, 1974) on the organic phosphate binding site of deoxyhemoglobin: the substitution of histidine β143 in hemoglobin A by serine in hemoglobin F eliminates a residue directly involved in organic phosphate binding. However, the intrinsically lower affinity of Hb F for ligands in the *absence* of organic phosphates is apparently not due to a slower combination rate.

V. Kinetics of Replacement of CO by NO in Hemoglobin F. Measurement of CO dissociation from fully liganded hemoglobin is most easily accomplished by replacement with NO: the rapidity of NO combination rates relative to CO dissociation rates ensures that the protein remains in the fully liganded state. Until recently, it was not considered that any fully liganded molecules could assume the low affinity structure. However, recent reports by Salhany et al. (1975) and by Perutz et al. (1976) have shown that NO-hemoglobin is indeed

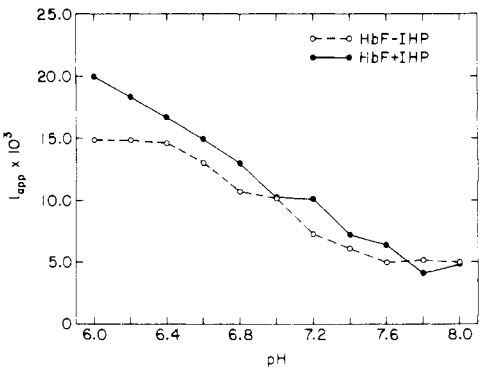

FIGURE 6: Effect of pH on apparent first-order rate constant for substitution of NO for CO in hemoglobin F in the presence and absence of IHP. Hemoglobin concentration, 2.5×10^{-5} M tetramer; NO concentration, 5×10^{-4} M; 0.05 M Bistris buffers containing 0.1 M NaCl. (- - -) minus IHP; (—) plus 2×10^{-4} M IHP. The reaction was monitored at 591 nm.

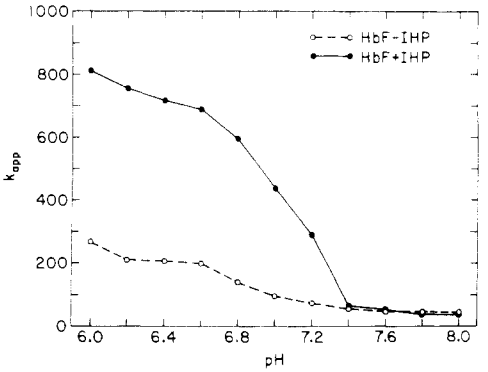

FIGURE 7: Effect of pH on apparent first-order rate constant for substitution of CO for oxygen in hemoglobin F in the presence and absence of IHP. Hemoglobin concentration, 2.5×10^{-5} M tetramer; CO concentration, 5×10^{-4} M; 0.05 M Bistris buffers containing 0.1 M NaCl. (- - -) minus IHP; (—) plus 2×10^{-4} M IHP. Reactions were monitored at 578 nm.

capable of assuming the T quaternary structure under certain conditions. Salhany et al. (1975) have also shown that the rate of CO dissociation from the low affinity quaternary structure, as measured in hemoglobin Kansas, is ninefold greater than that from the high affinity conformation. Dissociation of the first CO molecule from fully liganded hemoglobins other than this low affinity mutant appears to occur from the R state of the protein. If, during replacement of CO ligands by NO, a quaternary structural change were to take place, more than one CO molecule would appear to dissociate simultaneously from the protein, giving rise to an increase in the first-order dissociation rate constant.

Salhany et al. (1975) have interpreted such increases in the apparent dissociation constant of CO from fully liganded hemoglobin A in the presence of IHP in terms of this mechanism. It must be cautioned, however, that alterations in the intrinsic properties of the R state of CO-hemoglobin could also give rise to increases in the apparent dissociation constant.

We have reexamined the replacement of CO by NO in hemoglobin F, both in the absence and in the presence of IHP, as shown in Figure 6. At high pH values, the value of l_{app}, the apparent dissociation constant of CO from the fully liganded molecule, is consistent with the value of 1.75×10^{-3} s^{-1} reported by Salhany et al. (1975) for l_{app} for hemoglobin A. As the pH is lowered to 6.0, a threefold increase in l_{app} develops in the absence of IHP; in the presence of the organic phosphate, the increase is closer to fourfold. A qualitatively similar increase in l_{app} with decreasing pH in the absence of organic phosphates has been reported for hemoglobin F over a more limited pH range by McDonald and Noble (1972), but neither they nor Salhany et al. observed such marked pH dependent changes in l_{app} for phosphate-free hemoglobin A.

VI. Kinetics of Replacement of O_2 by CO in Hemoglobin F. A replacement procedure analogous to that used for measuring CO dissociation has also been employed for measuring O_2 dissociation. In this replacement reaction, however, dissociation of O_2 may not always be slower than combination with CO, especially if the protein undergoes a conformational transition from the R to the T state. Accordingly, we have examined this reaction only to provide correlative evidence for the pattern of increase seen in the CO–NO replacement reactions, and have not undertaken a complete kinetic analysis according to the procedures described by Olson et al. (1971). We have employed sodium dithionite as described by Gibson and Roughton (1955) to maintain the concentration of free oxygen as close to zero as possible, and have used buffers containing 0.05 M Bistris and 0.1 M NaCl to minimize nonspecific binding of organic phosphates. As can be seen in Figure 7, in the absence of IHP, the value of k_{app}, the apparent dissociation constant of O_2 from the fully liganded molecule, increases by about fourfold in hemoglobin F, as the pH is lowered from 8.0 to 6.0. This result is also in agreement with the findings of McDonald and Noble (1972) over a more limited pH range. Upon addition of IHP, particularly at the low pH values, between 10 and 20% methemoglobin was generated in the hemoglobin F solutions, as evidenced by a slow increase in the absorbance at 578 nm, following the initial rapid absorbance decrease. Solutions in the absence of IHP always contained less than 10% methemoglobin, as judged by this criterion. The value of k_{app} increases by more than 40-fold as the pH of these hemoglobin F solutions is lowered from 8.0 to 6.0 in the presence of IHP. The midpoint of the increase in k_{app} occurs at pH 6.8–6.9, both in the absence and in the presence of the organic phosphate.

TABLE I.

	j_{app}	
pH	Hemoglobin F	Hemoglobin A
6.0 (−IHP)	5.61×10^{-3}	1.36×10^{-3}
6.5	3.23×10^{-3}	9.50×10^{-4}
7.0	1.58×10^{-3}	2.76×10^{-4}
7.5	1.65×10^{-4}	5.89×10^{-5}
8.0	1.31×10^{-4}	3.80×10^{-5}
6.0 (+ 5×10^{-4} M IHP)	7.48×10^{-3}	6.78×10^{-3}
7.0 (+ IHP)	7.60×10^{-3}	7.49×10^{-3}

VII. Kinetics of Replacement of NO by CO in Hemoglobin F. Moore and Gibson (1976) have recently described a proton-stimulated increase in the first-order rate constant for dissociation of NO from hemoglobin A, as measured by replacement with CO in the presence of dithionite, and have interpreted their results in terms of a quaternary structural transition in the fully liganded protein. We have examined this reaction in hemoglobin F as well as hemoglobin A, both in the presence and absence of organic phosphates, from pH 8.0 to pH 6.0.

We have not undertaken a complete kinetic analysis of the reactions, but report here only the initial first-order rate con-

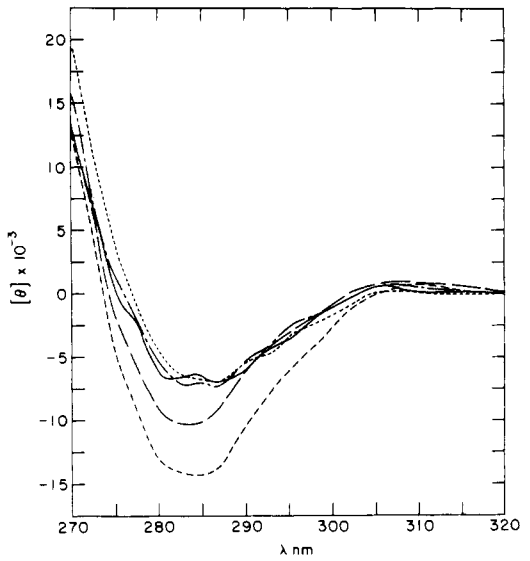

FIGURE 8: Circular dichroism of NO-hemoglobin F in the absence of organic phosphates as a function of pH. Hemoglobin concentration, 5×10^{-5} M tetramer; 0.05 M Bistris buffers containing 0.1 M NaCl. (- - -) pH 6.0; (— —) pH 6.5; (— - —) pH 7.0; (. . .) pH 7.5; (—) pH 8.0.

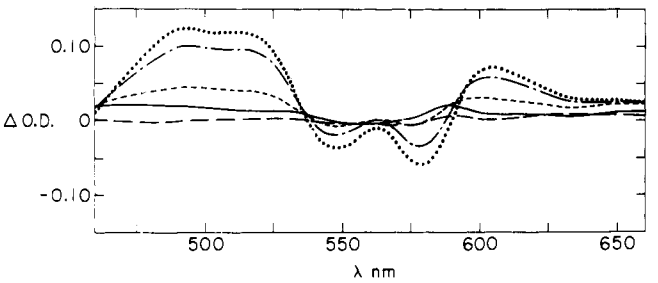

FIGURE 9: Effect of pH on the difference absorption spectrum of NO-hemoglobin F at various pH values vs. pH 8.0. Hemoglobin concentration, 1.13×10^{-4} M heme; 0.1 M Bistris buffers with no organic phosphates present. (. . .) pH 6.0; (— - —) pH 6.5; (- - -) pH 7.0; (—) pH 7.5; (— —) pH 8.0.

stant, j_{app}. However, it should be noted that the characteristic pattern of decelerating rates at progressively greater extents of replacement with CO described by Moore and Gibson was clearly apparent in our traces. The results, shown in Table I, indicate that the rate of dissociation of NO from hemoglobin F at pH 6.0 in the complete *absence* of organic phosphates is very similar to that observed for both hemoglobins A and F in the presence of IHP. Such comparison suggests the possibility that NO-hemoglobin F is virtually quantitatively converted from the R to the T conformation at low pH in the absence of organic phosphates. The pH dependence for the NO dissociation rate constant in the absence of organic phosphates is similar for hemoglobin A and F over the pH range we have studied $[(\partial \log j_{app})/(\partial \text{pH}) = -0.9]$, but the rate constant for hemoglobin F at each pH is greater than that for hemoglobin A. This could reflect intrinsically different values of j_R and j_T for hemoglobins F and A, although the identity of the values of j_{app} in the presence of IHP argues against such differences, at least for j_T. However, we recognize the limitations of using parameters determined in the presence of this tightly bound organic phosphate as quantitatively representative of the properties of the T state of the phosphate-free protein.

VIII. Spectral Properties of NO-Hemoglobin F. In view of the apparently similar effects of protons and organic phosphates on the replacement kinetics of CO by NO and on the NO dissociation rate in hemoglobin F, we undertook an examination of the visible difference spectrum and circular dichroism of NO-hemoglobin F to probe further the effect of pH on the quaternary structure of this protein. Figure 8 illustrates the circular dichroism of solutions of NO-hemoglobin F *in the complete absence of organic phosphates* at 0.5 pH unit intervals from 8.0 to 6.0. The appearance of pronounced negative ellipticity around 285 nm at pH values below 7.0 is identical with that observed by Salhany (1974) and Perutz et al. (1976) for NO-hemoglobin A plus IHP and by Perutz et al. (1974b) for aquomethemoglobin A plus IHP. In Figure 9, the visible spectra of NO-hemoglobin F in the absence of organic phosphates at various pH values vs. that at pH 8.0 in the region of the α and β bands are illustrated. These difference spectra are identical with those observed by Perutz et al. (1976) for NO-hemoglobin A plus IHP.

Discussion

The results we have reported here are all consistent with a model which accounts for the functional properties of hemoglobin primarily in terms of the relative stabilities of two quaternary conformational states (Hopfield et al., 1971). The application of a model of this type to fetal hemoglobin suggests that the low affinity or T conformation of this protein is more stable than that of hemoglobin A. This unusual stability becomes somewhat more pronounced in the presence of organic phosphates, but is still evident even in the absence of organic phosphates. The first line of evidence for this interpretation comes from the experiments with adult and fetal methemoglobins. Perutz et al. (1974a–c) have recently shown that it is possible to switch the quaternary conformation of certain derivatives of hemoglobin without altering the state of ligation as long as the two quaternary states have approximately equal free energies. Objections have been raised that the criteria employed by Perutz et al. for arguing their case for a quaternary structural transition in high-spin methemoglobins are not adequate since the T structure of deoxyhemoglobin dissociates into subunits far less readily than does aquomethemoglobin in the presence of IHP, and even some low spin derivatives undergo some changes in functional properties induced by organic phosphates (Hensley et al., 1975). These criticisms are based on the assumption that the T structure of all hemoglobin derivatives, regardless of the state of the heme, will possess a unique set of functional properties. Although such a conclusion would be justified by rigorous application of the two-state theory of Monod et al. (1965), the extensive evidence for specific tertiary structural features induced by changes in heme iron valency or ligation (Anderson, 1973, 1975; Perutz and Ten Eyck, 1971) suggests that a range of functional properties might well be observed in different derivatives in either of the two quaternary structures.

Allowing for the caveat that sulfhydryl reactivity and visible difference spectra alone do not constitute a proof of a quaternary structural change, we suggest that, below pH 6.8, fetal and adult aquomethemoglobins share functional properties which are consistent with a reduction in the free energy of the T quaternary conformation. This stabilization apparently persists in the adult protein until the pH is raised to above 7.2. It is possible, but unlikely, that the loss of the histidine at position H21(143)β could account for a 0.4 pH unit change in the pK of the organic phosphate binding site in fetal methemoglobin. A more reasonable assumption is that the replacement of the histidine results in a weakening of the ability of fetal hemoglobin to bind organic phosphates, and it is only

when the pH falls below 6.8 that the deoxy or T quaternary structure becomes sufficiently inherently stable in fetal hemoglobin that addition of organic phosphates can induce a quaternary transition in methemoglobin F. Presumably the extra set of positive charges along the dyad axis of the organic phosphate binding site in hemoglobin A adds sufficient strength to maintain adult methemoglobin in the T structure in the presence of IHP until the pH rises above 7.2.

A direct test of this proposed stabilization of the T state of methemoglobin F at low pH is provided by the CD results on fetal aquomethemoglobin in the absence of IHP. Although the protein is not quantitatively converted from R to T structure at low pH in the absence of organic phosphates, a significant contribution of molecules in the T state to the observed negative ellipticity is evident. This contrasts with the failure of phosphate-free solutions of aquomethemoglobin A to show any change in CD spectrum when the pH is lowered to 6.0.

The recent study by Chien and Snyder (1976) on the uv circular dichroism of oxy- and deoxy-Co-hemoglobin emphasizes the sensitivity of this spectral test for quaternary conformation, and its freedom from interference by alterations in the spin state of the heme, by the specific heme ligand bound, or even by the metal complexed to the porphyrin ring. We believe the CD results on fetal aquomethemoglobin offer strong support for our conclusion that the effects of IHP on sulfhydryl reactivity and visible absorption spectrum are also related to the quaternary state of the fetal protein. The fraction of aquomethemoglobin F molecules in the T state at pH 6 in the absence of phosphates is not sufficient to be detected by sulfhydryl reactivity since the β93-SH group reactivity falls with decreasing pH even in the absence of quaternary structural changes (Guidotti, 1967).

We have endeavored to demonstrate that the marked stabilization of the T state in aquomethemoglobin F at low pH persists in the ferrous protein as well and represents the primary contributing factor to the reduced affinity for oxygen. The support for this conclusion is based on correlation of the kinetic properties of hemoglobin F with those of hemoglobin A. Changes in such parameters as the initial rate of combination of the deoxygenated protein with ligands, or the initial rate of ligand dissociation, can be interpreted either in terms of alterations in the intrinsic properties of the high and low affinity conformations, or in terms of a shift in the allosteric constant, L, i.e., the ratio of R to T states. Our results on the rates of combination of deoxyhemoglobins A and F with CO suggest that the properties of the T states of these two proteins are quite similar in the absence of organic phosphates. The changes induced by IHP probably reflect perturbations in the T state induced by organic phosphate binding rather than any further shift of the R–T equilibrium in the direction of the T state and are consistent with the known alterations in the organic phosphate binding site in fetal hemoglobin.

The interpretation of the ligand dissociation kinetics is less unambiguous. It is conceivable that these pH-dependent increases in ligand dissociation rates could all reflect alterations in the properties of the R state. However, the weight of the experimental evidence, especially the data on NO-hemoglobin F, suggests that a significant part of pH dependence for all the ligands arises from the same fundamental pH dependence of the allosteric constant, L. The key feature of this interpretation is that the pH dependence is an intrinsic property of the protein, and not of the ligands which are bound. Thus, the failure of fully liganded oxy- or CO-hemoglobin F to undergo the transition from R to T state seen in NO-hemoglobin F would be explained by difference in the value of c for these different ligands, i.e., different ratios of the affinity of R and T states for different ligands. The studies by Moore and Gibson (1976) indicate that, at least for NO, c is quite pH independent. The pH dependence of L is in fact the major stereochemical basis of the Bohr effect. In this context, it is noteworthy that hemoglobins A and F have a very similar Bohr effect (Arczynska and Prod'hom, 1973) and, in the experiments in which the actual slope of the pH dependent changes in functional properties can be measured both for hemoglobins A and F, these slopes are virtually identical for the two proteins.

We would interpret the fourfold increases in the rates of O_2 and CO dissociation from hemoglobin F at low pH as arising primarily from a significant transition of the species, $Hb(O_2)_3$ and $Hb(CO)_3NO$, to the T state, leading to rapid loss of the remaining ligands. The marked increase in the rate of O_2 dissociation from fetal hemoglobin in the presence of IHP appears to be associated specifically with the presence of aquomethemoglobin in the reaction mix. Preliminary studies on O_2 dissociation rates, as measured by CO replacement, have been carried out on mixtures of oxy- and methemoglobin F in the absence of organic phosphates, and similar large increases have been observed (Law, L. E., and Simon, S. R., unpublished observations). We attribute these increases to a significant transition of the species, $FHb(O_2)_x(Fe^{3+})_y$ $(x + y = 4)$, from R to T state at low pH. Although we have not attempted to rule out conclusively the possibility that the O_2 and CO dissociation kinetics can be ascribed to pH dependent changes in the R state by further kinetic studies, we believe our results with the NO-protein are less subject to this potential ambiguity.

These findings, together with the recent studies by Tan et al. (1972, 1973) on carp hemoglobins, offer a means of verifying the interpretations of the allosteric effector role of IHP in experiments with hemoglobin A. In the studies of Perutz et al. (1974a,b, 1976) and Salhany et al. (1975), it has been argued that IHP neither contributes substantially itself to the T state-like properties nor forces the protein into an altogether new conformation which only superficially resembles the low affinity T quaternary structure. X-ray evidence (Arnone, 1974) supports these arguments, but verification by using an alternative allosteric effector has not been possible with hemoglobin A. Our studies indicate that, because of the intrinsically greater stability of the T state in hemoglobin F, protons can function as an alternative effector, producing the same changes in fetal hemoglobin that are seen in the adult protein only in the presence of strongly bound organic phosphates.

Direct tests of the relationship between the pH dependence of the quaternary structural transition and oxygen affinity in fetal hemoglobin have not yet been systematically undertaken. Shimizu and Bucci (1974) have reported low values of $p_{O_2(50\%)}$ and of n_H in analysis of oxygen equilibrium curves of fetal hemoglobin in the presence of benzenehexacarboxylate at low pH values. Such reductions in values of n_H are to be expected if the product of the values of L and c increases to the point that the T state persists to some extent even in fully liganded molecules. Studies in our laboratories on the oxygen affinity of hemoglobin F in the presence of physiological concentrations of 2,3-diphosphoglycerate as a function of pH should provide additional information on the physiological significance of the enhanced stability of the T quaternary conformation in this protein.

References

Anderson, N. L. (1973), *J. Mol. Biol. 79*, 495.
Anderson, N. L. (1975), *J. Mol. Biol. 94*, 33.

Antonini, E., Wyman, J., Brunori, M., Taylor, J. F., Rossi-Fanelli, A., and Caputo, A. (1964), *J. Biol. Chem. 239,* 907.
Arczynska, W., and Prod'hom, L. S. (1973), *Pediatr. Res. 7,* 126.
Arnone, A. (1972), *Nature (London) 237,* 146.
Arnone, A., and Perutz, M. F. (1974), *Nature (London) 249,* 5452.
Brinkman, R., and Jonxis, J. H. P. (1935), *J. Physiol. 85,* 117.
Chien, J. C. W., and Snyder, F. W. (1976), *J. Biol. Chem. 251,* 1670.
Drabkin, D. L. (1946), *J. Biol. Chem. 164,* 703.
Gibson, Q. H. (1959), *Prog. Biophys. Chem. 9,* 1.
Gibson, Q. H., and Roughton, F. J. W. (1955), *Proc. R. Soc. London, Ser. B 143,* 310.
Gibson, Q. H., and Roughton, F. J. W. (1956), *Proc. R. Soc. London, Ser. B 146,* 206.
Gray, R. D., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 7168.
Guidotti, G. (1967), *J. Biol. Chem. 242,* 3673.
Guggenheim, E. A. (1926), *Philos. Mag. 2,* 538.
Hensley, P., Edelstein, S. J., Wharton, D. C., and Gibson, Q. H. (1975), *J. Biol. Chem. 250,* 952.
Hopfield, J. J., Shulman, R. G., and Ogawa, S. (1971), *J. Mol. Biol. 61,* 425.
Maurer, H. S., Behrman, R. E., and Honig, G. R. (1970), *Nature (London) 277,* 388.
McDonald, M. J., and Noble, R. W. (1972), *J. Biol. Chem. 247,* 4282.
Monod, J., Wyman, J., and Changeux, J. P. (1965). *J. Mol. Biol. 12,* 88.
Moore, E. G., and Gibson, Q. H. (1976), *J. Biol. Chem. 251,* 2788.
Ogata, R. T., and McConnell, H. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 335.
Olson, J. S., Anderson, M. E., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 5919.
Perutz, M. F., Fersht, A. R., Simon, S. R., and Roberts, G. C. K. (1974b), *Biochemistry 13,* 2174.
Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., Ho, C., and Slade, E. F. (1974c), *Biochemistry 13,* 2187.
Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., and Simon, S. R. (1976), *Biochemistry 15,* 378.
Perutz, M. F., Ladner, J. E., Simon, S. R., and Ho, C. (1974a), *Biochemistry 13,* 2163.
Perutz, M. F., and Ten Eyck, L. F. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 295.
Salhany, J. M. (1974), *FEBS Lett. 49,* 84.
Salhany, J. M., Ogawa, S., and Shulman, R. G. (1975), *Biochemistry 14,* 2180.
Shimizu, K., and Bucci, E. (1974), *Biochemistry 13,* 809.
Simon, S. R., and Cantor, C. R. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63,* 205.
Tan, A. L., DeYoung, A., and Noble, R. W. (1972), *J. Biol. Chem. 247,* 2493.
Tan, A. L., Noble, R. W., and Gibson, Q. H. (1973), *J. Biol. Chem. 248,* 2880.
yuma, I, and Shimizu, K. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 1112.
Wind, M. (1973), Thesis, Department of Pharmacology, New York University School of Medicine.