Analogous Effect of Protons and Inositol Hexaphosphate on the Alteration of Structure of Nitrosyl Fetal Human Hemoglobin†

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ABSTRACT: We have determined the low temperature EPR spectra and room temperature ligand dissociation rate constants of human NO-hemoglobins F and A as a function of pH and inositol hexaphosphate levels in order to assess the contribution of a quaternary structural equilibrium in the two proteins to their spectral and functional properties. Our results are consistent with an increased stability of a ligated low affinity structure in the fetal protein; the functional properties of this structure appear to be essentially the same in the two hemoglobins, even though its stability relative to a high affinity conformation is different. The pH dependence of the NO dissociation constant in both adult and fetal hemoglobin can be assigned primarily to the pH-dependent equilibria of high and low affinity forms as monitored by EPR.

The cooperative equilibrium between tetrameric hemoglobin and gaseous ligands is generally thought to be a reflection of the existence of at least two conformational states of the protein, one of which has high affinity. Several studies have attempted to account for the properties of the protein, primarily in terms of two extreme conformation states, in a fashion analogous to that originally proposed by Monod et al. (1965; Perutz, 1970, 1972; Hopfield et al., 1971; Szabo & Karplus, 1972). Other studies suggest that a description of the function of hemoglobin that is based on only two conformation states is an oversimplification (Huestis & Raftery, 1973; Imai & Yonetani, 1975; Ogata & McConnell, 1972; Peisach, 1975). Because human hemoglobin A is so highly cooperative in its ligand binding properties, direct tests of the functional characteristics of these two states have generally involved comparison of fully ligated and unligated protein.

Recently, reports have appeared in which a conformational transition from a high affinity conformation to a low affinity conformation has been effected without changing the state of ligation of the molecule (Ogawa & Shulman, 1972; Perutz et al., 1974a-c). Many of these studies have involved unligated or partially ligated mutant or chemically modified hemoglobins, in which a low affinity conformation is much less stable than in hemoglobin A in the absence of organic phosphates. The low affinity conformation could then be stabilized by addition of a strongly bound phosphate derivative, typically inositol hexaphosphate.

The symmetrically related model, in which a ligated derivative could be induced to rest in a low affinity form, has been much less amenable to study. Fully ligated hemoglobin Kansas, however, can be induced to assume a low affinity conformation but should be studied only at high concentrations (Gibson et al., 1973) because of its tendency to dissociate into dimers. Recently, Salhany et al. (1975) and Perutz et al. (1976) have shown that the fully ligated nitrosyl derivative of hemoglobin A can be made to undergo a transition to a low affinity form in the presence of IHP.[1] We have reported that hemoglobin F, human fetal hemoglobin, can be induced to undergo a similar structural transition by lowering the pH *even in the absence of organic phosphates* (Wind et al., 1976). In addition, Moore & Gibson (1976) have analyzed the kinetics of NO dissociation from hemoglobin A as a function of pH in the absence of organic phosphate and have shown that a marked increase in the first-order rate constant for dissociation of ligand with decreasing pH is consistent with a change in the relative concentrations of the high and low affinity forms in the fully ligated protein. However, their interpretations were based on fitting their kinetic data to a model of pH-dependent equilibrium of quaternary conformations, rather than direct measurement of the conformational change by independent experimental means.

As a consequence of these findings, it was of interest to ascertain whether the alteration of quaternary structure of nitrosyl hemoglobin induced by organic phosphates and monitored by spectral changes was the same or different from the putative conformational change effected by lowering pH and detected by kinetic measurements. Therefore, we examined the relationship between the NO dissociation rate constant and the low temperature EPR spectrum of NO-hemoglobins A and F to clarify the relationship between these two proteins in terms of their relative stabilities of low affinity conformations and to substantiate the interpretation that the increasing ligand-dissociation rate with decreasing pH is truly a reflection of an altered quaternary structural equilibrium. Our results suggest that the pH dependence of the NO dissociation rate and the

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[1] Abbreviations used: IHP, inositol hexaphosphate; EPR, electron paramagnetic resonance; SHF, superhyperfine; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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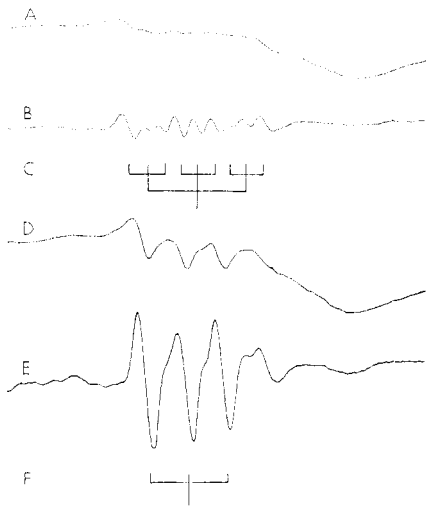

FIGURE 1: First derivative EPR spectra near g_{mid} taken at 1.6 K of nitrosyl hemoglobin F studied at (A) pH 7.4 and (D) pH 6.0. In the spectrum of the sample prepared at the lower pH, a three-line superhyperfine pattern can be resolved with splitting of 17–18 G. The spectrum of the sample prepared at pH 7.4 suggests higher multiplicity in the superhyperfine pattern. B and E show the spectrum taken using the third harmonic procedure of the same samples as A and D, respectively. A nine-line superhyperfine pattern is well resolved in B. In C, an analysis of the superhyperfine pattern of B into a triplet of triplets is given with splittings of about 7 and 23 G. In F a similar analysis of the triplet pattern of E is given with a splitting of 17 G. Note the presence of a residual nine-line component in spectra D and E.

functional differences between hemoglobins F and A arise primarily from a shift in this conformational equilibrium.

Materials and Methods

Hemoglobins A and F were isolated from lysates of washed erythrocytes from samples of adult and cord blood, respectively, by column chromatography on CM-Sephadex. The purity of the hemoglobin F preparation was shown to be greater than 95% by the alkali denaturation test of Brinkman & Jonxis (1935). Kinetics of NO dissociation by replacement with CO in the presence of sodium dithionite were determined by a procedure essentially identical to that described by Moore & Gibson (1976), employing a Durrum D110 stopped-flow spectrophotometer with a Biomation 802 transient recorder.

EPR spectra were studied under two conditions, the first at pumped helium temperature and the second at temperatures near liquid nitrogen. The pumped helium experiments were employed in order to confirm the existence of two types of EPR ascribable to nitrosyl heme, one with a nine-line and the other with a three-line superhyperfine (SHF) pattern, which are observed for both hemoglobin F as well as for hemoglobin A at high and low pH values, respectively.

EPR spectra were recorded at 1.6 K on a superheterodyne spectrometer first described by Feher (1957), using cavities described by Berzofsky et al. (1971). Spectra were run at a modulation amplitude of 1–2 G and at power settings where the EPR spectrum was not saturated. Magnetic fields were recorded simultaneously with the EPR spectrum using a digital Hall probe that had been calibrated against the proton resonance of water. Fresh samples of nitrosyl hemoglobin were prepared for each spectral determination by first converting the hemoglobin to the carbonmonoxy form with CO replacing O_2. Then, the sample was treated with NO in the absence of O_2. Excess NO was removed with purified nitrogen and the nitrosyl hemoglobin (5×10^{-4} M) was transferred to the EPR cavity and immediately frozen in liquid nitrogen. Before freezing, an aliquot of the protein was removed in order to verify that NO-ligation was complete by taking the optical spectrum on a Cary 14 spectrophotometer. Another aliquot was removed for pH measurement under anaerobic conditions.

The second study, near liquid nitrogen temperatures, 120 K, included a more extensive analysis of the pH dependence of the line shape of the SHF pattern at g_{mid} and was carried out with a Varian E4 instrument. Here, nitrosyl hemoglobin samples were introduced into quartz EPR tubes which were immersed into liquid nitrogen. Spectra were studied at a microwave frequency of 9.115 GHz and at a power of 10 mW. The modulation amplitude (~10 G) was carefully checked for each sample to ensure hyperfine structure was not broadened. The intensities of the spectra at $g_{max} = 2.08$ and $g_{min} = 1.96$ were always close for samples at the same protein concentration but at different pH values, and were further adjusted to correct for any mismatch by making small changes (<10%) in the receiver gain.

Fresh samples of nitrosyl hemoglobin were prepared for each determination by initially deoxygenating a sample of 4×10^{-3} M oxyhemoglobin in the appropriate buffer by repeated flushing with nitrogen, then introducing 1 mg/mL sodium dithionite anaerobically to ensure complete deoxygenation of the protein and reduction of methemoglobin impurities. Finally, NO was introduced into the vessel. The excess dithionite is oxidized by the excess NO, which was removed, along with any N_2O formed, by evacuation and flushing with nitrogen. The entire elapsed time from initial exposure of the protein to NO to freezing of the EPR tube was never more than 5 min. Completion of conversion to the NO derivative was established by direct anaerobic transfer of the samples into an optical cuvette and measurement of the visible absorption spectrum on a Cary 17 spectrophotometer.

Results

Figure 1 illustrates the first derivative EPR spectrum of nitrosyl hemoglobin F taken at 1.6 K and at high (A) and low (D) pH values. The values of g_{max}, g_{mid}, and g_{min} obtained from such spectra at 1.6 K, as reported in Table I, are identical with those measured from the spectra obtained at 120 K. As can be seen, the spectra are typical of nitrosyl heme complexes whose features consist of three resolved apparent g values and a superhyperfine pattern in the g_{mid} region (Chien, 1969; Dickinson & Chien, 1971). At low pH this pattern consists of three lines with splitting of 17–18 G.[2] At high pH there is some indication for higher multiplicity that cannot be resolved by direct EPR but can be resolved using a third harmonic technique (Chevion et al., 1977b).

Figure 1 also compares the EPR spectra of the same samples recorded by the third harmonic procedure (B and E) introduced by Chevion et al. (1977b). The resolution of fine structural details at the sacrifice of the more prominent features of

[2] It should be pointed out that EPR of nitrosyl heme proteins have often been observed with a strong three-line SHF pattern but the spectral line shape different from those observed here (Rein et al., 1972; Trittelvitz et al., 1975; Kon, 1968, 1975). This pattern appears as "spikes" and instead of lying at the apparent g_{mid} is observed at the apparent g_{min} end of the spectrum. This spectrum can be duplicated by denaturing nitrosyl hemoglobin with various chaotropic agents or (Kon, 1968) by lowering the pH so that the hemoglobin molecule is grossly denatured. If care is not taken so that O_2 is introduced into a hemoglobin sample already containing excess NO, the pH is lowered and the spectrum with a superhyperfine pattern at g_{min} is obtained. Moreover, in our own studies if we remove our samples of nitrosyl hemoglobin after spectra are recorded, expose them to temperatures above 60 °C, and reexamine the EPR, a spectrum with a similar SHF pattern at g_{min} is obtained.

TABLE I: Magnetic Parameters for NO Derivatives of Hemoglobin A and Hemoglobin F.

	pH	IHP content	App g values			Multiplicity of SHF pattern at g_{mid}
Hemoglobin A	6.4	−	2.078	2.007	1.964	9
	6.0	+	2.074	2.004	1.962	3, 9
	7.0	−	2.080	2.007	1.968	9
	7.0	+	2.076	2.004	1.974	3, 9
Hemoglobin F	6.0	−	2.075	2.008	1.962	3, 9
	6.35	+	2.076	2.008	1.961	3, 9
	7.0	−	2.078	2.005	1.960	9
	7.6	+	2.075	2.006	1.962	9

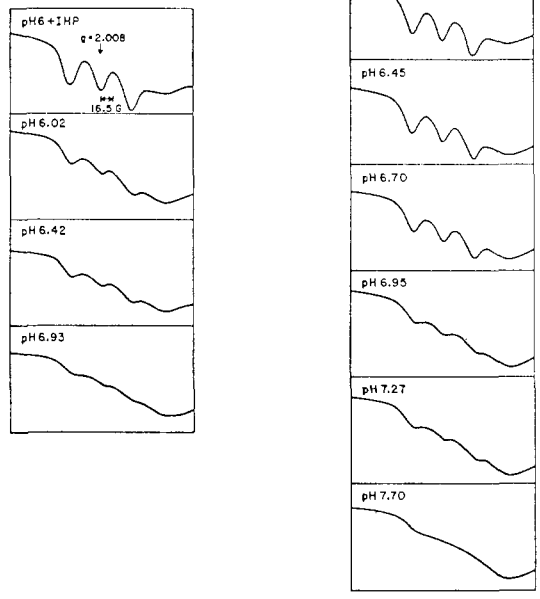

FIGURE 2: EPR spectra of NO-hemoglobins F and A in the region from $g = 2.07$ to $g = 1.95$, determined on the Varian E-4 spectrometer as described in Materials and Methods. The superhyperfine pattern of three lines, with splittings of 16.5 G, is apparent in the low pH samples and in the samples with 5×10^{-3} M IHP. (Left) NO-hemoglobin A; (right) NO-hemoglobin F.

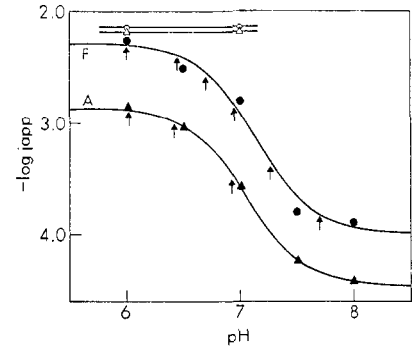

FIGURE 3: The apparent first-order rate constant, j_{app}, for dissociation of NO from fully ligated hemoglobins A and F as a function of pH and IHP, determined as described in Materials and Methods. (●) Hemoglobin F without IHP; (▲) hemoglobin A without IHP; (○) hemoglobin F plus 5×10^{-4} M IHP; (△) hemoglobin A plus 5×10^{-4} M IHP. The computer-drawn solid lines represent least-squares fits to a titration curve. The values of j_{app} for hemoglobin F are consistently higher than those for hemoglobin A at all pH values in the absence of IHP. The pH values at which EPR spectra have been determined (Figure 2) have been noted with vertical arrows.

g_{max} and g_{min} is characteristic of these spectra and is fully explained in the discussion of the method by Chevion et al. (1977b). In B, the third harmonic spectrum clearly shows an SHF pattern consisting of nine lines that can be analyzed as a triplet of triplets with splittings of 6.5–7 and 23–24 G. This SHF pattern indicates an interaction of the unpaired electron, originating in the NO, with two inequivalent nitrogen nuclei located along the normal to the heme plane, one from the NO and the other from the proximal imidazole (Griffith, 1956).

At low pH or in the presence of IHP, one observes with nitrosyl hemoglobin F an EPR spectrum with a three-line superhyperfine pattern similar to the one observed at low pH in the absence of phosphate (Figure 1D). Using the third harmonic procedure (Figure 1E), one observes a residual nine-line SHF component in the spectrum whose magnitude is about half of that which is observed either at high pH or in the absence of IHP. Thus, about half of the hemes in tetrameric nitrosyl hemoglobin F exist in a form exhibiting a nine-line SHF. This retention of a nine-line component in the three-line spectrum is analogous to the EPR reported by Chevion et al. (1977a) both for hemoglobin A in the presence of organic phosphates and for hemoglobin Kansas at neutral pH.

In Figure 2, the superhyperfine component of the EPR spectrum of hemoglobin A is illustrated at several values of pH, along with the spectrum obtained at pH 6.0 in the presence of 5×10^{-4} M IHP. (A spectrum obtained at pH 7.0 in the presence of 5×10^{-4} M IHP was indistinguishable from that shown at the lower pH and is not illustrated). A similar series of EPR spectra of NO-hemoglobin F is shown in the same figure. The intensities of these spectra at g_{max} and g_{min} are all nearly equal for both hemoglobin A and hemoglobin F so that comparison of the intensities of the hyperfine structure is possible within this frame of reference. Several features are clearly apparent from visual inspection of the spectra. The intensities of the SHF structure in NO-hemoglobin A and NO-hemoglobin F spectra in the presence of IHP are virtually identical. Moreover, the spectrum of NO-hemoglobin F in the complete absence of phosphates at pH 6 is also virtually identical with those obtained for both hemoglobins in the presence of IHP. At pH 6, the spectrum of phosphate-free NO-hemoglobin A has a much smaller three-line component than that obtained for hemoglobin F under the same conditions and is quantitatively comparable to the NO-hemoglobin F spectra at pH 6.70 and pH 6.95.

In order to make a comparison of the EPR for various forms of nitrosyl hemoglobin with functional properties of these molecules, in Figure 3 we have illustrated the pH dependence of the apparent first-order constants for dissociation of NO from fully ligated hemoglobins A and F. The open symbols

represent determinations made in the presence of 5×10^{-4} M IHP; the solid points represent determinations made only in the presence of 0.05 M Bistris buffers. The solid lines are least-squares fits of the data points for organic phosphate free samples to a cooperative titration curve. For the NO-hemoglobin A data the n value was determined to be 1.7 from the fit. This value was used as a constant in the fitting of the NO-hemoglobin F data as these were of poorer quality. The apparent pK for both fits was determined to be 7.1. These curves are not meant to reflect any attempt to fit the observed pH dependence to any specific mechanism; however, the results of these fits are consistent with the assumption that the pH dependence of the increase in dissociation rate is identical for the two proteins. We have marked those pH values along the solid lines at which we have determined EPR spectra. Visual examination of the intensities of the SHF structure in these various spectra shows that at any one pH for which the line shape of an NO-hemoglobin F spectrum is approximately equivalent to that for hemoglobin A at a different pH, the apparent first-order rate constant for dissociation of NO from hemoglobin F agrees with that for hemoglobin A.

In order to make this comparison somewhat more quantitative, we have attempted to estimate the intensity of the three-line component in each of the ESR spectra by a graphical integration procedure. This procedure must be regarded as only approximate, as the shape of the true baseline between g_{max} and g_{min} is not readily determined in the spectra with large SHF splittings. Bearing this uncertainty in mind, we have illustrated these intensities, normalized to the spectrum with the largest SHF component, as a function of pH, along with normalized values of the apparent dissociation constant of NO from hemoglobins A and F in Figure 4. We have employed the same reference for the maximum values of the SHF conponent and the NO dissociation constant; this reference is hemoglobin A + IHP. As a minimum limit for the value of j_{app}, we have employed the value obtained for hemoglobin A at pH 8.0. There is considerable uncertainty in the use of this value as the lower limit, but an error of ±100% in this lower limit introduces less than 1% error in the normalized values of j_{app} which can be related to the normalized SHF intensities. It is clear from Figure 4 that the normalized SHF intensities and NO dissociation rate constants are highly correlated, although not identical, for both hemoglobins.

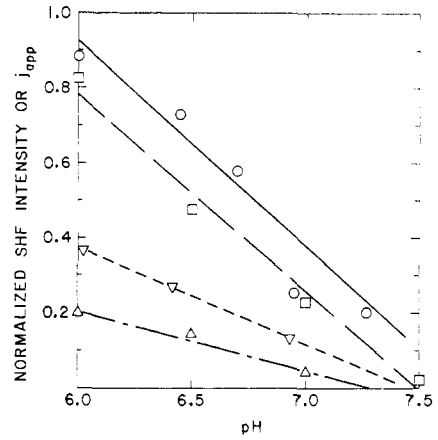

FIGURE 4: Fractional change in three-line SHF component and NO dissociation rate constant for fetal and adult hemoglobins as functions of pH. The intensities of the SHF component in the spectra were determined by weighing the excised peaks. All values of SHF intensity and of j_{app} have been normalized to the values of these parameters obtained with hemoglobin A + IHP. (○) Normalized SHF intensity, hemoglobin F; (□) normalized values of j_{app}, hemoglobin F; (▽) normalized SHF intensity, hemoglobin A; (△) normalized values of j_{app}, hemoglobin A.

Discussion

The EPR spectrum of nitrosyl hemoglobin has been interpreted in terms of the spectra of the individual subunits, and may be adequately described as a simple sum of the component spectra when the protein is in a high affinity quaternary conformation (cf. Reisberg et al., 1976). Upon conversion to a low affinity conformation, the spectrum can be interpreted best in terms of another summation of component spectra, with half the hemes contributing a marked three line superhyperfine structure. The major features of the EPR spectra of nitrosyl hemoglobin A and hemoglobin F are virtually identical (Table I), either in the presence or the absence of IHP, suggesting that the substitution of γ chains for the β chains of hemoglobin A does not change the electron distribution at the heme site significantly. Yet the functional properties of these proteins in the absence of organic phosphates are clearly different at any one pH. We believe the evidence presented here is consistent with the assignment of the origins of these differences to alterations in quaternary structural equilibria.

For the nitrosyl derivatives of hemoglobin Kansas and hemoglobin A, there is a strong correlation between the presence of a three-line SHF pattern in the EPR spectrum and ligand affinity (Chevion et al., 1977a). That is, whenever a three-line pattern is observed at g_{mid} the NO-ligated protein is in a low affinity form while the nine-line pattern is representative of a high affinity form. The addition of IHP to both HbA and HbF radically alters the EPR so that the SHF pattern changes from nine to three lines and, at the same time, the ligand dissociation rate increases. A similar change in the EPR can be effected by lowering the pH even in the absence of organic phosphates, where, once again, a three-line SHF pattern can be observed. A major difference between nitrosyl hemoglobin F and hemoglobin A, however, is that the three-line spectrum observed for the former in the absence of organic phosphate persists to a higher pH than it does for the latter. In this study we have attempted to relate this difference in intensity of the three-line component specifically to a difference in the relative proportions of high- and low-affinity quaternary conformations.

If the EPR spectra obtained in the presence of IHP at pH 6 or 7 are taken as representative of complete conversion to a low affinity form of hemoglobin, it would appear that in the absence of organic phosphates, at pH 6 this form predominates far more in NO-hemoglobin F than it does in NO-hemoglobin A. In fact, the similarity of the NO dissociation rate constants obtained for hemoglobin F at pH 6 in the presence or the absence of IHP, as well as the similarity in the EPR spectra, suggests that the ligated fetal protein is virtually completely converted to a low affinity form by 10^{-6} M H^+ alone. This structural interpretation is in agreement with the results we have previously reported on the visible absorption spectrum and UV circular dichroism of ligated hemoglobin F at low pH in the absence of organic phosphates (Wind et al., 1976).

In contrast to the EPR data, a structural interpretation of the NO dissociation kinetics is not readily obtained. Moore & Gibson (1976) have related the first-order constant for the early phases of the replacement of NO by CO in the presence of sodium dithionite to a pH dependent quaternary structural equilibrium between high and low affinity forms of the protein. We have seen increases in apparent NO dissociation rate in hemoglobin F with similar pH dependence, which we have also interpreted in terms of a quaternary conformational transition. Our interpretation of the consistently higher value of the NO dissociation constant for hemoglobin F than for hemoglobin

A at any one pH has been in terms of an intrinsically higher value of the stability of a low affinity form of the molecule in the fetal protein. The EPR results reported here provide an opportunity to verify these interpretations.

The significance of our results lies in the observation that the pH dependence of a functional property, the NO dissociation rate, appears to agree quantitatively with the pH dependence of a structural probe, the EPR spectrum, for both hemoglobin A and hemoglobin F. A comparison of the relationship between the NO dissociation constants for the two hemoglobins with the relationship between the EPR spectra suggests that the low affinity states of the two nitrosyl proteins probably have very similar kinetic and spectral properties both in the presence and absence of phosphates. Moreover, the pH dependence of the quaternary structural equilibrium is very similar for hemoglobins A and F, as judged by the independent criteria of NO dissociation kinetics and EPR spectra. In this context, it is significant that hemoglobins A and F have been reported to have very similar alkaline Bohr effects (Mann & Romney, 1968). The one difference between the two proteins which emerges as a result of this comparison is the actual ratio of affinity states at any one pH. Hemoglobin F apparently has an intrinsically higher value of this ratio, or L as it is designated in the formulation of Monod et al. (1965) but the contribution to stabilization of the low affinity state by protons is identical for both adult and fetal proteins.

A rigorous test of this conclusion requires analysis like that presented in Figure 4. If the *only* pH dependent property of NO-hemoglobin is the ratio of high affinity to low affinity states, and if the ESR spectra and the NO-dissociation rates are both true measures of this quaternary conformational ratio, then the fractional change in the functional property of NO-dissociation rate should precisely agree with the fractional change in the spectral measure of conformation. The allosteric model of Monod et al. (1965) was employed by Moore & Gibson (1976) with conclusions of this type, although no direct measures of conformational change were undertaken by these workers. Our own results show that the pH dependence of the fractional change in the SHF component of the ESR spectra and the pH dependence of the fractional change in the NO dissociation constant are highly correlated both for hemoglobin F and for hemoglobin A, although the values do not precisely agree at any one pH. Rather than attempting to overinterpret the data in this figure, we must draw attention to the sources of error which limit the sensitivity of this graphical test. The uncertainty of accurately determining the intensity of the SHF component in our ESR spectra has already been discussed. This uncertainty has the smallest effect on the results when the low affinity conformation, which gives rise to the three-line component, predominates. The NO dissociation rate data, on the other hand, indicate that the value of j_{app} undergoes an increase of approximately two orders of magnitude as the conformation changes from a high affinity state to a low affinity state. The presence of a small fraction of low affinity conformation in a solution in which the high affinity state predominates is reflected in marked changes in j_{app}, but when the low affinity conformation predominates, the value of j_{app} is not very sensitive to changes in the fraction of high affinity structure. Thus, our spectral measure of quaternary conformation tends to be most accurate under the conditions in which our measure of functional properties, the kinetic constant, is least sensitive. We believe that the strong correlation between these two measures supports our conclusion that the intensity of the SHF component of the ESR spectrum of NO-hemoglobin is proportional to the fraction of molecules with a rapid NO-dissociation rate.

Our results differ somewhat in interpretation from those of Hille et al. (1977), who suggest that differences in the normalized intensity of the three-line SHF component seen in a mixture of NO- and deoxyhemoglobin A at pH 7 and in fully liganded NO-hemoglobin A + IHP reflect differences in the conformation of a low affinity state. We would suggest that some of this difference in SHF intensity may arise from the fact that, in the 32% saturated NO-hemoglobin A solution prepared as Hille et al. describe, almost one-fourth of the nitrosyl hemes are found in the species $Hb(NO)_3$ and $Hb(NO)_4$, which are likely to be predominantly in a high affinity quaternary conformation, and another 44% are found in the species $Hb(NO)_2$, which may contain a mixture of high and low affinity states. Some indication of contribution of high and low affinity states to these partially saturated preparations might be obtained by a double mixing experiment in which the NO dissociation rate was measured immediately after ligation. However, the difficulties of obtaining such data reliably from dissociation measurements in the presence of dithionite but in the absence of CO are already well documented (Moore & Gibson, 1976). Certainly, the fashion in which we have prepared our NO-hemoglobin solutions would ensure that the slow development of the three-line SHF component observed by Hille et al. would have reached completion in all our samples. The question of the functional properties of molecules in which the three-line component has not yet reached its final intensity cannot be addressed by any of our results. We conclude only that in NO-hemoglobin solutions in which the three-line SHF component has reached its final intensity, that intensity is directly proportional to the fraction of molecules in the low affinity conformation. This proportionality holds regardless of whether the variant used is hemoglobin A or hemoglobin F, and regardless of whether the SHF component is generated by pH or by IHP.

The data at this level of resolution are not adequate to permit development of a model for stabilization of a low affinity state by protons. However, our results do provide support for the analysis by Moore & Gibson (1976), as well as our earlier studies on hemoglobin F, and may provide an independent check of the pK changes associated with the quaternary structural transitions which are presumed to give rise to the Bohr effect.

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Spin-Label Studies of the Sulfhydryl Environment in Bovine Plasma Albumin. 1. The N-F Transition and Acid Expansion†

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ABSTRACT: The environment of the sulfhydryl group in plasma albumin was previously characterized by employing spin-labels of varying chain lengths (Hull, H. H., Chang, R., & Kaplan, L. J. (1975) *Biochim. Biophys. Acta 400,* 132). It was established that the sulfhydryl is in a crevice approximately 10 Å deep but this crevice was not identified further. We now report the results of titrating albumin through the acidic conformational transitions while monitoring the electron-spin resonance of the bound nitroxide. With short spin-labels a general change is observed as the pH is lowered but the N-F transition is not discernible. However, with a spin label previously shown to project to the lip of the crevice a clear N-F transition as well as the subsequent acid expansion are observed. These results indicate that the sulfhydryl is in the crevice, formed by the domains of albumin, which opens during the N-F transition. Further results indicate that bound fatty acids do not influence the integrity of the sulfhydryl environment at neutral pH.

Bovine plasma albumin is known to undergo several pH dependent conformational transitions. On the acidic side of the isoionic point BPA[1] undergoes the reversible structural isomerization known as the N-F transition (Aoki & Foster, 1956) and the so-called acid expansion (Yang & Foster, 1954). A large number of studies have been undertaken to elucidate the nature and details of these transitions (see Foster (1960) for a review of the early literature and Peters (1975) for a recent review). In general, these and subsequent studies (Sogami & Foster, 1968) have revealed that, as the pH is lowered, the protein undergoes a fairly cooperative conformational change (N-F transition) that involves a slight expansion of the molecule resulting from a separation of intramolecular domains and the opening of a crevice. Further decrease of pH causes the protein to undergo a more complete expansion with a substantial increase in flexibility of the molecular structure.

BPA contains 17 disulfide bonds and (in the mercaptalbumin fraction) a single reactive sulfhydryl group located relatively close to the amino-terminal end of the polypeptide chain (King & Spencer, 1972; Brown, 1975). While the sulfhydryl has long been implicated in important physiological functions (King, 1961; Eagle et al., 1960; Putnam, 1965), recent in vitro evidence has demonstrated the specific catalytic role the sulfhydryl plays in the formation of a disulfide interchanged isomer (Nikkel & Foster, 1971; Stroupe & Foster, 1973). This isomer is also formed in vivo and is an intermediate in the catabolism of albumin (Wallevik, 1976). Additional isomers are formed when the sulfhydryl is oxidized to oxidation states higher than the well-known mixed disulfides (Janatova et al., 1968).

Due to the importance of the sulfhydryl group, it appeared to us that a specific study of the environment of the sulfhydryl residue and the conformational changes that take place in the

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[1] Abbreviations used: BPA, bovine plasma albumin; N, the normal conformer of bovine plasma albumin existing in the isoionic pH range; F, the conformer existing in the pH 3.5-4.0 range; ESR, electron spin resonance; MSL I, II, III, IV, and V, the maleimide spin labels whose structures are shown in Figure 1.

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