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Correlation between Quaternary Structure and Ligand Dissociation Kinetics for Fully Liganded Hemoglobin†

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ABSTRACT: The quaternary structures of fully liganded adult hemoglobin and hemoglobin Kansas ($\alpha_2\beta_2$ 102 Asn → Thr) bound by carbon monoxide or nitric oxide were spectroscopically characterized using high-resolution nuclear magnetic resonance (NMR) and ultraviolet circular dichroism (CD). The spectral markers used for the quaternary transition were the line in the NMR spectrum in H_2O −14 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate and the negative peak at 285 nm in the ultraviolet CD spectrum. In the nitrosyl derivatives, these two structural markers were compared with the electron paramagnetic resonance (EPR) spectrum at room temperature for the purpose of correlating structural changes in the protein with changes at the heme. The kinetic properties of these hemoglobins were investigated by measuring the rate of CO dissociation by replacement with NO. The results indicated that fully saturated adult nitrosyl hemoglobin can be switched from the high ligand affinity (or R) quaternary structure to the low ligand affinity (or T) quaternary structure by the addition of inositol hexaphosphate (IHP). The fully saturated carbon monoxide form of adult hemoglobin is in the R state in the absence or presence of IHP. In stripped (phosphate-free) hemoglobin Kansas, simply replacing CO by NO produced a switch in the quaternary structure from the R state (for the CO form) to the T state (for the NO form). It was previously demonstrated that the addition of IHP to the carbon monoxide form of hemoglobin Kansas caused the quaternary structure to switch from R to T (Ogawa, S., Mayer, A., and Shulman, R. G. (1972), *Biochem. Biophys. Res. Commun. 49,* 1485-1491). Two types of room temperature EPR spectra were found which could be correlated with these changes in the quaternary structure of the protein. Stripped adult nitrosyl hemoglobin, which is in the R state according to the NMR and CD measurements, showed a symmetric room temperature EPR spectrum. Stripped nitrosyl hemoglobin Kansas and adult nitrosyl hemoglobin +IHP showed strongly anisotropic room temperature EPR spectra. These anisotropic room temperature EPR spectra were assigned to the T state based on the NMR and CD results. The kinetic properties followed these changes in the quaternary structure. The structural results shown above indicate that the CO replacement reaction by NO *will not always* be a replacement reaction within a single quaternary structure. This was found to be the case kinetically. Stripped adult hemoglobin is in the R state when both CO and NO are the heme ligands. The kinetics showed a slow, wavelength independent rate of CO dissociation. Both adult hemoglobin + IHP and stripped hemoglobin Kansas start in the R state with CO as the ligand and end in the T state with NO as the ligand. At pH 7.0, and appropriate protein concentrations, both of these conditions presented cooperative CO dissociation kinetics reflecting the switch in the quaternary structure during the reaction. Hemoglobin Kansas + IHP is in the T state with both CO and NO as heme ligands at appropriate solution conditions. Under these circumstances, the rate of CO dissociation was not cooperative and was an order of magnitude faster than the value in the R state. These results are discussed in terms of the two-state model of Monod et al. (Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88-118).

The possibility that *fully* liganded hemoglobin ordinarily in the high affinity R structure can be switched to the low affinity or T quaternary structure is a consequence of the model of Monod et al. (1965). This switch was first accomplished in the deoxygenated mixed valency hybrids, where hemoglobin could be maintained in solution with two ligands per tetramer (Ogawa and Shulman, 1971). In this way the energies of the R and T manifolds were closer to equality than they were with zero ligands, where the T state dominated, or with four ligands, where the R state dominated. With near equality there was enough energy in the preferential binding of organic phosphates like IHP[1] to the T

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[1] Abbreviations used are: bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; IHP, inositol hexaphosphate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Nes-des-Arg hemoglobin, hemoglobin allowed to react with *N*-ethylmaleimide and carboxypeptidase B; NMR, high-resolution nuclear magnetic resonance; EPR, electron paramagnetic resonance; CD, circular dichroism; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; DPG, 2,3-diphosphoglycerate; Tris, tris(hydroxymethyl)aminomethane. The phrase "stripped hemoglobin" is used in this paper to refer to organic phosphate free hemoglobin.

state, to switch from R → T. This switch was followed by changes in the NMR[1] spectra, and subsequently shown to be accompanied by a large change in ligand affinities (Cassoly et al., 1971). Other NMR lines have since been shown to be markers of the quaternary state. The reader is referred to a recent summary of these results (Shulman et al., 1973). In this paper we use the NMR line at −14 ppm downfield from DSS in H_2O (Patel et al., 1970; Ogawa et al., 1972; Mayer et al., 1973) as a spectral marker of the quaternary structure. Ogawa et al. (1972) showed that the mutant hemoglobin Kansas ($\alpha_2\beta_2$ 102 Asn → Thr) when fully liganded with CO possessed the −14-ppm NMR line only when IHP was present suggesting that IHP switched the quaternary structure of this fully liganded hemoglobin to the T form. Mayer et al. (1973) demonstrated that the low ligand affinity mutant hemoglobin Iwate (α_2 87 His → Tyr β_2) whose crystal structure was the T form, even when liganded (Greer, 1971) also possessed the −14 ppm line even in the absence of phosphate.

The second spectral indicator of quaternary structure used in this paper is the negative peak in the ultraviolet CD spectrum at 285 nm observed upon deoxygenation (Simon and Cantor, 1969). It was suggested that this transition in the CD spectrum indicated a change in quaternary structure. Recently, Perutz et al. (1974a,c) have shown that addition of IHP to certain deoxyhemoglobins known to be in the R state induces this same negative CD peak, consistent with the change in quaternary structure to T. Similar findings were observed by these authors for aquomethemoglobin. These CD results were shown to be a consequence of the different environments of the aromatic amino acids at the $\alpha_1\beta_2$ contact region, in particular tryptophan C3(37)β. This change in the CD spectrum is independent of the degree of ligation at the hemes, and seems to be a direct quaternary marker, just like the presence of the −14 ppm line.

There have been many investigations of the EPR spectrum of nitrosyl hemoglobin and related model compounds (Kon and Kataoka, 1969; Shiga et al., 1969; Trittelvitz et al., 1972; Rein et al., 1972; Henry and Banerjee, 1973). Generally two types of EPR spectra have been found at room temperature. One for the fully saturated hemoglobin is largely symmetric. The other spectrum, present when DPG or IHP was added or when $(\alpha(NO)\beta(deoxy))_2$ hybrids were studied, was asymmetric. The symmetric spectrum was the simple sum of the spectra for the isolated chains. The asymmetric spectrum was not. In the present paper we correlate these EPR spectra with quaternary structure determined using NMR and CD.

From preliminary kinetic studies, Salhany et al. (1974a) suggested that nitrosyl hemoglobin bound by IHP was in the T structure, as was stripped nitrosyl hemoglobin Kansas. The studies presented in this paper support this suggestion by using NMR, CD, and EPR as spectral probes of the hemoglobin structure. The definition and correlation of these markers for the change in quaternary structure provide the opportunity to more clearly study the kinetic consequences of this structural change in the fully liganded molecule. Communications related to the present paper have appeared (Salhany et al., 1974a,b; Salhany, 1974).

Materials and Methods

Preparation of Materials. All gases came from Matheson (East Rutherford, N.J.). The NO gas was washed through a 1 *M* KOH solution. The gas was flushed into the hemoglobin samples appropriately prepared so as to be free of oxygen. The other gases used in this study (i.e., CO and N_2) were also rendered oxygen free. IHP was obtained from Sigma Chemical Co. (St. Louis, Mo.) as a sodium salt.

Hemoglobin concentrations were monitored spectrophotometrically using a Cary 17 recording spectrophotometer and extinction coefficients from Antonini and Brunori (1971). The extinction coefficient for nitrosyl hemoglobin was 131 mM^{-1} cm^{-1} (419 nm). Conversion to cyanide was used to monitor the level of methemoglobin present (Van Kampen and Zijlstra, 1961).

Human hemoglobin A was freshly prepared and stripped of organic phosphates in a manner similar to that described elsewhere (Salhany et al., 1972). Hemoglobin Kansas was prepared according to the method described by Bonaventura and Riggs (1968) using blood from Mr. David Boyce. All organic and inorganic phosphates were removed. Nes-des-Arg hemoglobin[1] was prepared according to the method of Kilmartin and Hewitt (1971).

High-Resolution NMR. The concentrations of the protein used in these studies were between 5 and 8 m*M* in heme with sample volumes of about 0.5 ml. The NMR spectra were obtained using a Varian HR 220 spectrometer equipped with a Fabritek 1062 signal-averaging computer or a Bruker 270 spectrometer. An rf field about 20 dB above the optimum for ethylbenzene was used, with a sweep rate of about 50 Hz/sec. The temperature in the sample zone was regulated to ±1° and determined from the relative shifts of the resonances of ethylene glycol. Chemical shifts are expressed in parts per million relative to the internal standard DSS,[1] and the shifts to low field are assigned negative values.

Ultraviolet Circular Dichroism. Hemoglobin samples were prepared as already described. The spectra were recorded on a Cary 61 spectrometer in 1-cm cells which were capped and flushed with N_2 before the samples were added.

Electron Paramagnetic Resonance. The EPR experiments were performed on a Varian x-band EPR instrument. Flat quartz cuvets with an inner diameter of 0.3 mm were used to hold the samples. About 1 ml of concentrated hemoglobin solutions was placed in the deoxygenated EPR cuvets. The spectra were obtained with a field modulation of 0.3 G.

Stopped-Flow Apparatus. A Durrum Model D-110 stopped-flow spectrophotometer with a 2-mm cuvet was used and all studies reported here were made with a slit width of 0.15 mm. The stopped-flow output was fed directly to a Hewlett-Packard Model 7004B xy recorder equipped with a calibrated Model 17172A time base unit. The Biomation Model 802 recorder was used to collect data with faster time constants. The stopped-flow monochromator was calibrated using several mercury lines. Before each series of experiments the stopped-flow spectrophotometer was very carefully cleaned.

Results

Stripped adult nitrosyl hemoglobin with and without IHP was prepared and NMR spectra were investigated in the region between −11 and −15 ppm downfield from DSS in H_2O. The stripped protein fully saturated with NO showed peaks at about −12 and −13 ppm (Figure 1a), but no peak at −14 ppm. Upon the addition of IHP and keeping the pH 7.0, a well-resolved resonance appeared near −14 ppm (Figure 1b). This peak is similar to those previously seen in deoxyhemoglobin (Patel et al., 1970) and other low affinity hemoglobins (Ogawa et al., 1972; Shulman et al., 1973),

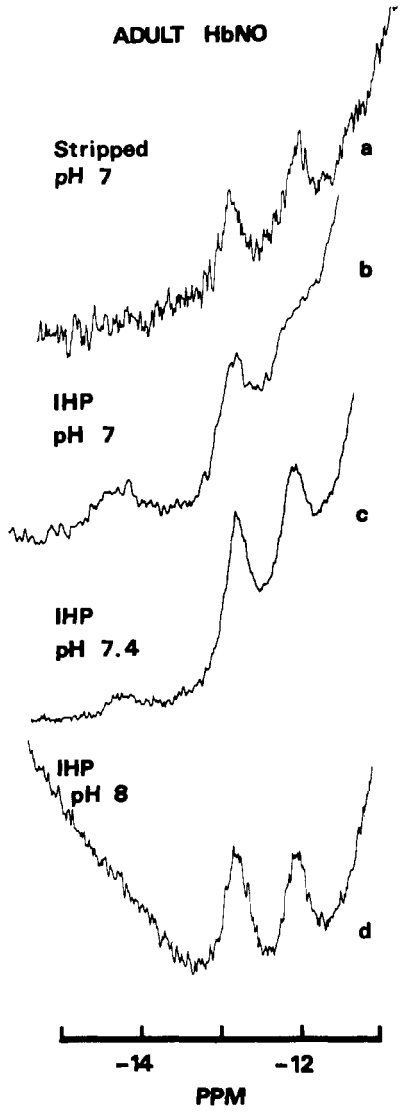

FIGURE 1: High-resolution nuclear magnetic resonance (220 MHz) spectra for adult nitrosyl hemoglobin in H_2O downfield from DSS: (a) stripped HbNO (pH 7.0) in 0.2 *M* bis-tris at 16°; concentration of heme, 5 m*M*; (b) HbNO + IHP (pH 7.0) in 0.2 *M* bis-tris at 18°; concentrations, heme, 6 m*M* and IHP, 5 m*M*; (c) HbNO + IHP (pH 7.4) in 0.1 *M* phosphate at 22°; concentrations, heme, 7 m*M*, and IHP, 5 m*M*; (d) HbNO + IHP (pH 8.0) in 0.1 *M* phosphate at 24°; concentrations, heme, 7 m*M*, and IHP, 5 m*M*.

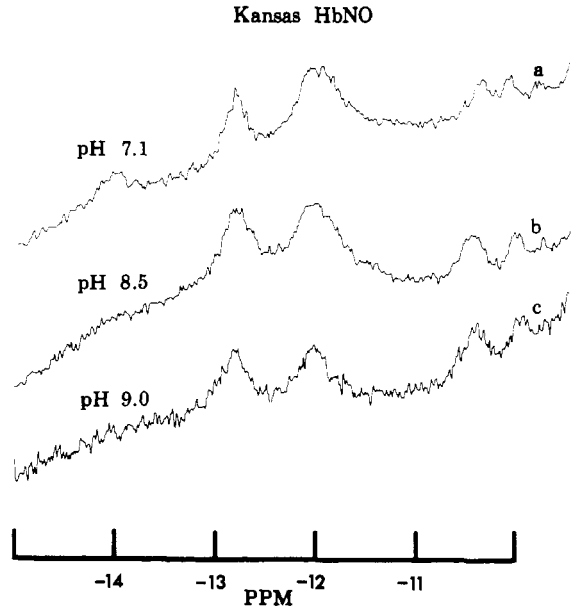

FIGURE 2: High-resolution nuclear magnetic resonance (270 MHz) spectra for stripped nitrosyl hemoglobin Kansas in H_2O downfield from DSS: (a) pH 7.1 in 0.2 *M* bis-tris, 20°; (b) pH 8.5 in 0.2 *M* Tris, 20°; and (c) pH 9.0 in 0.2 *M* Tris, 20°. The concentrations of all samples are about 8 m*M* in heme.

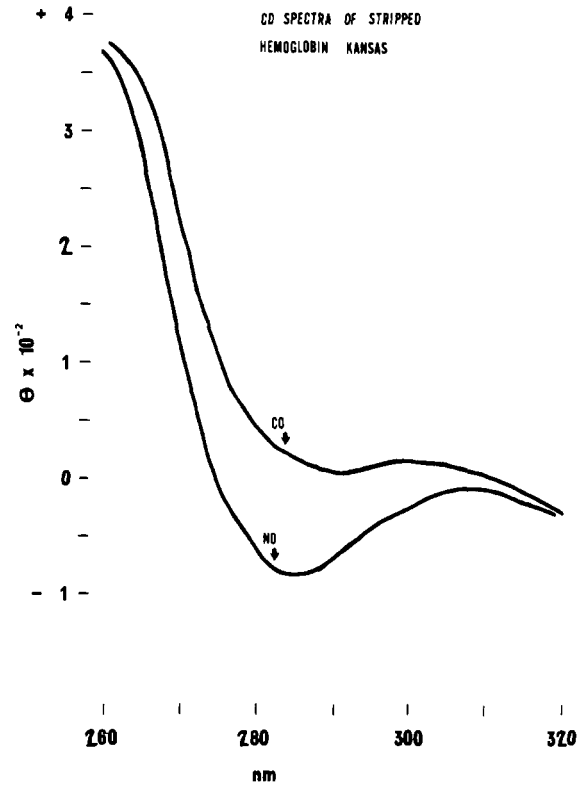

FIGURE 3: Ultraviolet CD spectra of stripped hemoglobin Kansas in the CO (top) and NO (bottom) forms in 0.2 *M* bis-tris (pH 7.0), 25°. The concentration of heme in both cases was 50 μM. The spectra are presented as ellipticity in degrees.

while peaks at this position are not observed in high affinity forms. The other NMR spectra in Figure 1 show that as the pH is raised to 8.0, the −14 ppm line disappeared, even in the presence of IHP. At pH 7.4 an intermediate intensity in the −14 ppm line was observed. This can be estimated by comparing the intensity of the −14 ppm line to the −13 ppm line which is unaffected by IHP.

Figure 2 shows the NMR results for stripped nitrosyl hemoglobin Kansas. The −14 ppm line was observed for nitrosyl hemoglobin Kansas in the *absence* of phosphate and over a *larger* pH range than in adult hemoglobin + IHP. As the pH rises above 8.0 (20°) the −14 ppm line becomes broader and is absent at pH 9.0 (20°). Thus, the estimated p*K* for the T → R switch in stripped nitrosyl hemoglobin Kansas is considerably higher (~8.5) than that for adult nitrosyl hemoglobin + IHP (~7.4). There was no −14 ppm NMR line for the nitrosyl form of Nes-des-Arg hemoglobin + IHP at neutral pH.

In a previous paper Salhany (1974) showed that the addition of IHP to adult nitrosyl hemoglobin produced a negative peak in the ultraviolet CD at 285 nm similar to that seen in other hemoglobins in the T state (Simon and Cantor, 1969; Perutz et al., 1974a–c). This negative CD peak was not present in stripped adult nitrosyl hemoglobin or in carbon monoxide adult hemoglobin + IHP similar to other

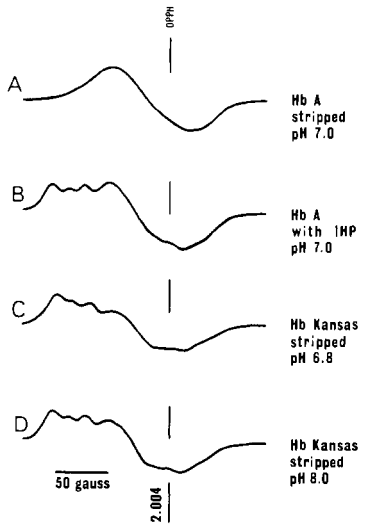

FIGURE 4: Room temperature electron paramagnetic resonance spectra of adult nitrosyl hemoglobin without and with IHP at pH 7.0 and stripped nitrosyl hemoglobin Kansas at pH 6.8 and 8.0. The concentrations used are: (A) stripped HbA − heme, 5.5 m*M*; (B) HbA + IHP − heme, 5.5 m*M*; IHP, 4 m*M*; (C) Hb Kansas − pH 6.8; heme, 4.45 m*M*; (D) Kansas − pH 8.0; heme, 1.78 m*M*. The adult hemoglobin was in 0.2 *M* bis-tris buffer and hemoglobin Kansas was in 0.1 *M* phosphate buffer. The spectra are not normalized to the same integrated intensity; DPPH, 1,1-diphenyl-2-picrylhydrazyl stable radical.

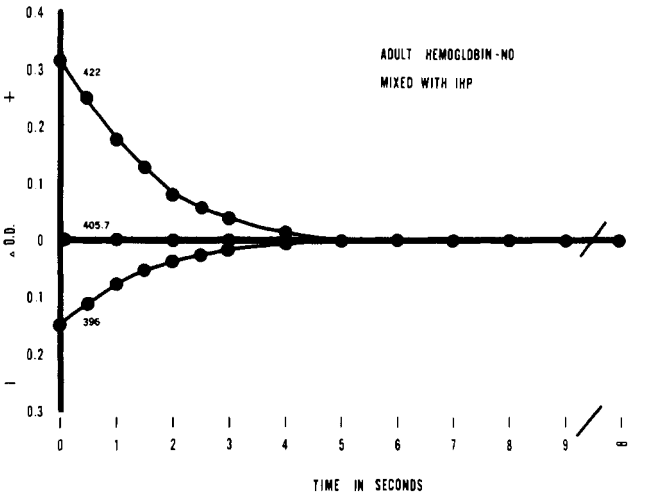

FIGURE 5: The time course of the spectral change observed upon mixing fully saturated adult nitrosyl hemoglobin with IHP in the presence of N_2. Concentrations after mix: heme, 70 μM; IHP, 3 m*M*; 0.2 *M* bis-tris (pH 7.0) + 0.1 *M* NaCl at 24°.

R state hemoglobins (Perutz et al., 1974a-c). These results correlate very well with the observation of the −14 ppm NMR line. In order to extend this correlation, the ultraviolet CD spectra of the stripped CO and NO forms of hemoglobin Kansas were measured (Figure 3). At pH 7.0, CO hemoglobin Kansas showed no negative peaks indicating an R state type spectrum. In contrast the nitrosyl form exhibited a negative peak with a minimum near 285 nm like adult nitrosyl hemoglobin + IHP (see Figure 2 of the paper by Salhany, 1974).

Stripped adult nitrosyl hemoglobin (R state) showed a nearly symmetric EPR spectrum (Figure 4A), whereas nitrosyl hemoglobin in the T state (adult nitrosyl hemoglobin plus IHP or stripped nitrosyl hemoglobin Kansas) showed an asymmetric EPR spectrum (Figure 4B-D). These T state EPR spectra are very similar to those found previously upon addition of organic phosphates to adult nitrosyl hemoglobin (Trittelvitz et al., 1972; Rein et al., 1972). The observation of a similar EPR spectrum in stripped nitrosyl hemoglobin Kansas indicates that the protein structural change, and not IHP per se, is the cause of this EPR spectrum at room temperature.

Optical Spectral Changes in Nitrosyl Adult Hemoglobin. Stripped hemoglobin A in 0.2 *M* bis-tris at pH 7.0 fully saturated with CO was mixed in the stopped flow with a 6 m*M* IHP solution in the same buffer saturated with CO. There was no detectable effect of IHP kinetically on the spectrum of CO hemoglobin A. This result agrees with Perutz et al. (1974c) who also found no significant effect of IHP on the static spectrum of CO-bound hemoglobin. When stripped adult nitrosyl hemoglobin in 0.2 *M* bis-tris at pH 7.0 + 0.1 *M* NaCl was mixed with 6 m*M* IHP in the same buffer (in N_2), a large and relatively slow spectral change was observed (Figure 5). At 422 and 396 nm a decrease and increase in absorbance were observed, respectively. This spectral change was isosbestic at 405.7 nm and the reaction came to a good base line. At the conditions specified above, this reaction was apparently first order with a rate of about 0.65 sec^{-1}.

The extinction change for nitrosyl hemoglobin A upon mixing with IHP at various wavelengths is shown in Figure 6. The entire spectrum showed a large decrease in both the Soret and visible regions (closed circles). When stripped Nes-des-Arg nitrosyl hemoglobin was mixed with IHP, no significant spectral change was seen (closed triangles). The kinetic difference spectrum for adult hemoglobin was confirmed in static experiments under similar conditions.

The kinetics of the extra spectral change were studied as a function of pH at a constant concentration of IHP after mixing (3 m*M*) and at roughly the same protein concentration after mixing (50 μM). There was a significant pH dependence in the magnitude (Figure 7A) and in the initial rate (Figure 7B) for this extra spectral change. The apparent p*K* value for the spectral change was found to be ~7.4 in 0.1 *M* phosphate buffer while the initial rates measured from the same data changed most rapidly between pH 6 and 7. The fact that both the extra spectral change and the rate were pH dependent suggested that protons were involved in this process.

The same extra spectral change was also observed upon jumping the pH of adult nitrosyl hemoglobin in 0.1 *M* phosphate from pH 8 to a final value of 6.7 (Figure 6A). Although the rate was similar, the magnitude of the extinction change was considerably smaller. In the absence of all phosphates, including inorganic phosphate, jumping the pH of adult nitrosyl hemoglobin over the same pH range as above produced no significant spectral change.

When fully saturated nitrosyl hemoglobin A in 0.2 *M* bis-tris at pH 7.0, no salt, was mixed with 6 m*M* IHP in the same buffer, the typical negative or positive decays at 422 and 396 nm, respectively, were initially observed followed by another much slower decay with an opposite sign (Figure 8). However, at 405.7 nm a good isosbestic was maintained throughout. This isosbesty suggests that at low salt concentration we are observing the appearance and partial disappearance of the same *spectral* species. In 0.1 *M* NaCl or 0.1 *M* inorganic phosphate, this unusual effect is not seen.

Earlier experiments (Salhany et al., 1974b) had shown that the slow spectral change (~0.65 sec^{-1}, pH 7.0, 0.2 *M* bis-tris + 0.1 *M* NaCl) was a change within the liganded T state after it is formed. This was demonstrated by showing

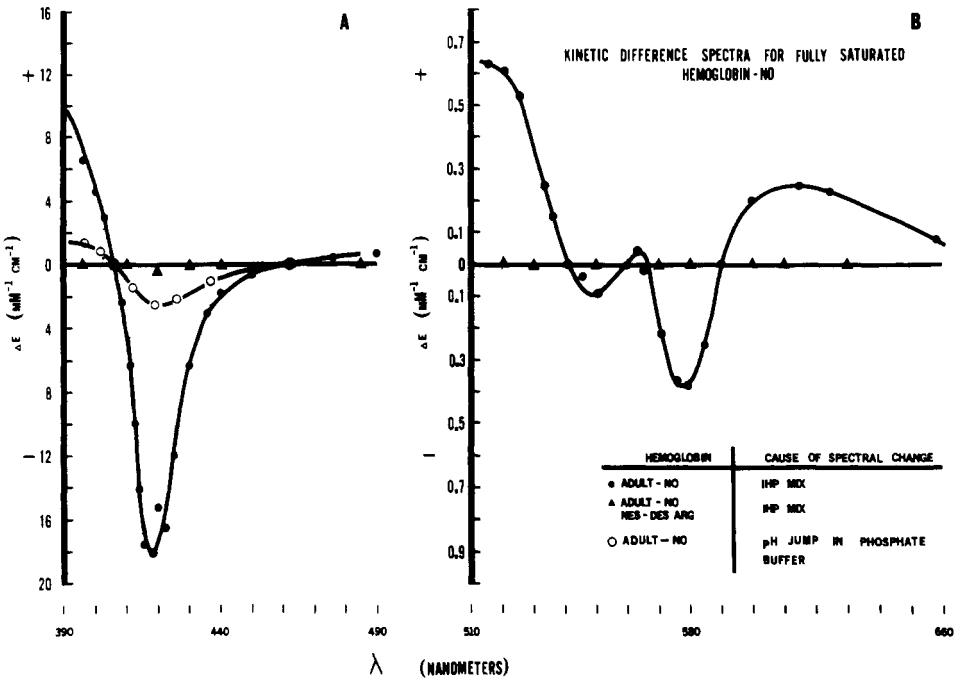

FIGURE 6: The kinetic difference spectrum for fully saturated adult nitrosyl hemoglobin. (A) Soret region. Closed circles are for adult nitrosyl hemoglobin mixed with 6 m*M* IHP in 0.2 *M* bis-tris (pH 7.0) + 0.1 *M* NaCl, 23°. Closed triangles are for Nes-des-Arg nitrosyl hemoglobin under the same conditions. The open circles are the spectral change which results when the pH of adult nitrosyl hemoglobin in 0.1 *M* phosphate is jumped in the stopped flow from 8.0 to a final value of 6.7. All extinctions were calculated by measuring the total absorbance change from the beginning to the end of the reaction, as in Figure 5, and dividing that value by the concentration of total heme after mix and the path length (2 mm). (B) Visible region. Symbols and calculations are the same as A above.

that the same spectral change seen in Figure 5 could also be obtained by mixing adult deoxyhemoglobin + IHP with NO saturated buffer (see Figure 2B of Salhany et al., 1974b). In that experiment NO binding occurs during the mixing time of the stopped flow. When phosphate free hemoglobin was investigated, no spectral changes were observed. When deoxyhemoglobin Kansas (plus or minus IHP) was mixed with NO, the extra spectral change was observed and showed the same wavelength dependence as adult hemoglobin + IHP (Figure 5). It was concluded by Salhany et al. (1974b) that this extra spectral change did not have the change in quaternary structure (defined by the NMR and CD markers) as the rate-determining step, since in their experiments it made no difference whether or not hemoglobin started in R or T.

In order to obtain further information about the structural basis of this extra spectral change, partial NO binding to deoxyhemoglobin in the presence of IHP was studied. In these experiments, deoxyhemoglobin + IHP was prepared and anaerobically transferred to the stopped flow which was also rendered oxygen free. The buffer solution containing less than equivalent amounts of NO was prepared by sucking a known volume of NO saturated buffer into the syringe which contained N_2 saturated buffer. In order to check the actual amount of NO bound to hemoglobin, the reacted material was flushed out of the stopped flow and into a 1-cm spectrophotometric cell containing a few crystals of dithionite. The cell was quickly put into the spectrophotometer and the visible spectrum was measured. The reaction in the stopped flow was studied at several wavelengths in the Soret region on the same sample and the ratio of the absorbance change at the various wavelengths was compared. When the ratio of the absorbance change for this slow spectral change was taken at 430 nm and two other wavelengths (422 or 396 nm) the value obtained was the same (within experimental uncertainties) for partial and full NO saturation (Table I).

The absence of sodium chloride in the medium also affects the behavior of the extra spectral change within T when the starting material is deoxyhemoglobin + IHP. Figure 9 shows that when deoxyhemoglobin + IHP is mixed with NO saturated buffer, the typical initial, slow spectral change is seen and this is followed by a slower, reverse process. Once again the isosbestic is maintained suggesting that we are observing the appearance and disappearance of the same *spectral* species. The magnitude and rate of this reverse phase were larger and faster than observed in Figure 8 when stripped, fully saturated nitrosyl hemoglobin was the starting material. Once again, this reversal is inhibited when the same experiment is performed in 0.1 *M* NaCl or 0.1 *M* inorganic phosphate. It should be noted that the NMR measurements made at infinite time indicated the existence of the T state both at low ionic strength, where there is a reverse color change, and at higher ionic strength, where there is no reversal of this extra spectral change. Furthermore, it does not appear that these reversals in the initial extra spectral change have to do with IHP per se because very similar spectral changes are seen for phosphate-free hemoglobin Kansas.

Replacement Reaction of CO by NO. The CO replacement by NO (performed by mixing CO saturated hemoglobin solutions with NO saturated buffer) for stripped adult hemoglobin was homogeneous and showed good isosbestics at 412.8 and 429 nm (see Figure 1 of Salhany et al., 1974b). The kinetic difference spectrum in the Soret region in the absence of phosphate at pH 6.35 is shown by the starred symbols of Figure 10. When IHP was added to this system all kinetic isosbestics were lost (Figure 10). The points near $\Delta\epsilon = 0$ in this figure occur because the beginning and end of the time course have about the same ab-

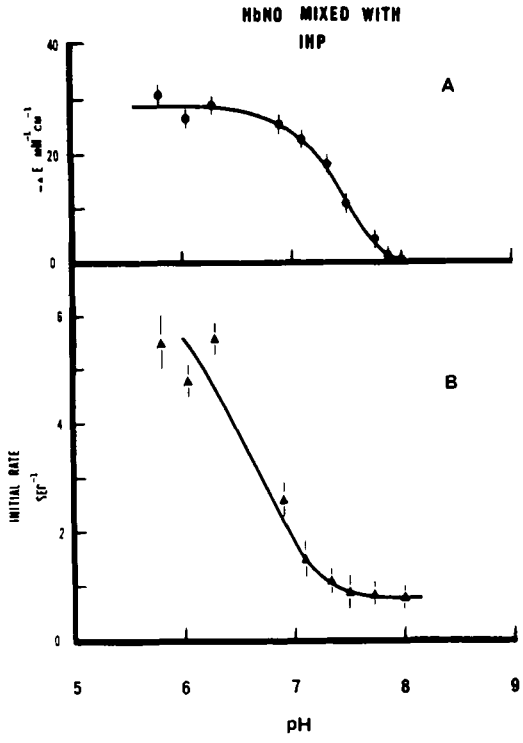

FIGURE 7: The pH dependence of the reaction of adult nitrosyl hemoglobin with IHP. (A) The change in extinction at 422 nm when adult nitrosyl hemoglobin is mixed with IHP at various pH values. Extinctions were calculated as described in Figure 6; wavelength, 422 nm; slits, 0.15 mm. (B) The initial rate of the spectral change in A. These reactions were studied in 0.1 *M* phosphate buffer. The concentration of IHP was 3 m*M* after mix. Ascorbate was present at 0.05 m*M* after mix and the reactions were performed in the presence of free NO; temperature, 24°

Table I: Ratio of Kinetic Absorbance Change at Soret Wavelengths for Different Degrees of NO Saturation.

	Fraction of HbNO	
Ratio of Absorbances	0.06	1.00
430 nm/396 nm	1.10	1.15
422 nm/430 nm	1.70	1.75

sorbance, while in the middle of the reaction the absorbance deviates from zero. The difference between the two difference spectra (stripped minus IHP) shown in Figure 10 (lower curve) was large with a minimum at 419 nm. Two zero crossing points are present, one at 405.7 nm and the other at 462 nm. When the replacement reaction was studied on Nes-des-Arg hemoglobin (Kilmartin and Hewitt, 1971) in the presence of IHP, the spectral heterogeneity was almost completely inhibited as shown in Figure 11 in agreement with the results of Figure 6.

In order to make true measurements of the CO off rate, it was necessary to study the reaction at wavelengths isosbestic for the extra spectral change. Measurements were made on stripped hemoglobin A with and without IHP as well as on Nes-des-Arg hemoglobin with IHP at 405.7 nm. This wavelength was chosen because it is an isosbestic wavelength for the extra spectral change in nitrosyl hemoglobin and it has a relatively large change in extinction when NO replaces CO (Figure 10). The results of these measurements are shown in Figure 12, where the normalized time courses are plotted semilogarithmically. When

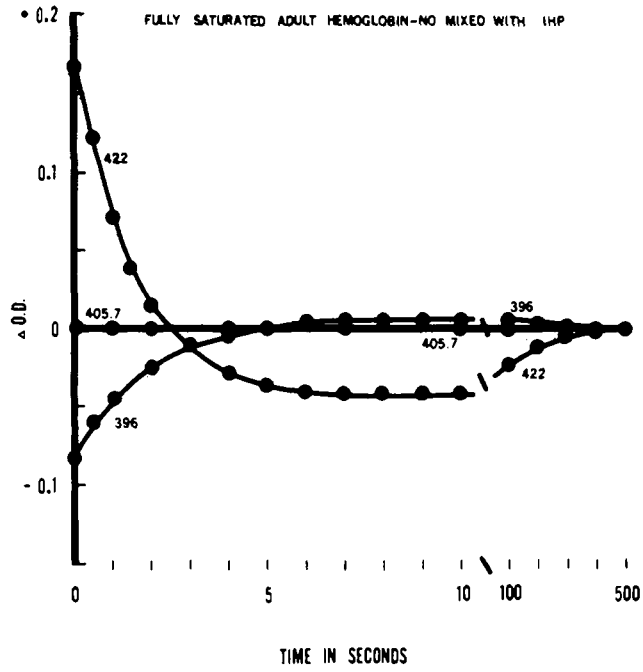

FIGURE 8: The spectral changes observed upon mixing stripped adult nitrosyl hemoglobin with IHP in the absence of salt. The concentrations of heme and IHP after mix were 64 μM and 3 m*M*, respectively, in 0.2 *M* bis-tris (pH 7.0), no salt, 24°. Note that both wavelengths overshoot their final values. Note further that λ 405.7 nm remains isosbestic indicating that the spectral overshoots seen are caused by the appearance and disappearance of the same *spectral* species.

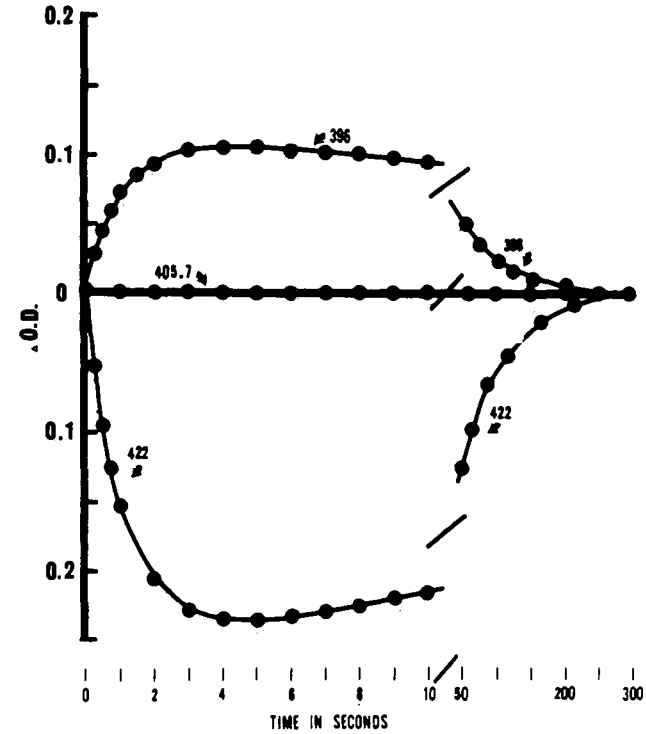

FIGURE 9: The spectral changes observed upon mixing deoxyhemoglobin + IHP with NO saturated buffer in low salt. The concentrations of heme and IHP after mix were 70 μM and 3 m*M*, respectively, in 0.2 *M* bis-tris (pH 7.0), no salt, 24°. The time courses are normalized to the final absorbance value. Note that at λ 405.7 nm, the isosbestic is maintained indicating that the same spectral species is being observed. In these deoxy experiments at low ionic strength, this color change completely disappears and does so at a more rapid rate. The amount of the reverse phase never exceeded the amount of the initial phase. The reader should compare this figure with Figure 2B in the paper by Salhany et al. (1974b) where the same reaction is studied in 0.1 *M* NaCl.

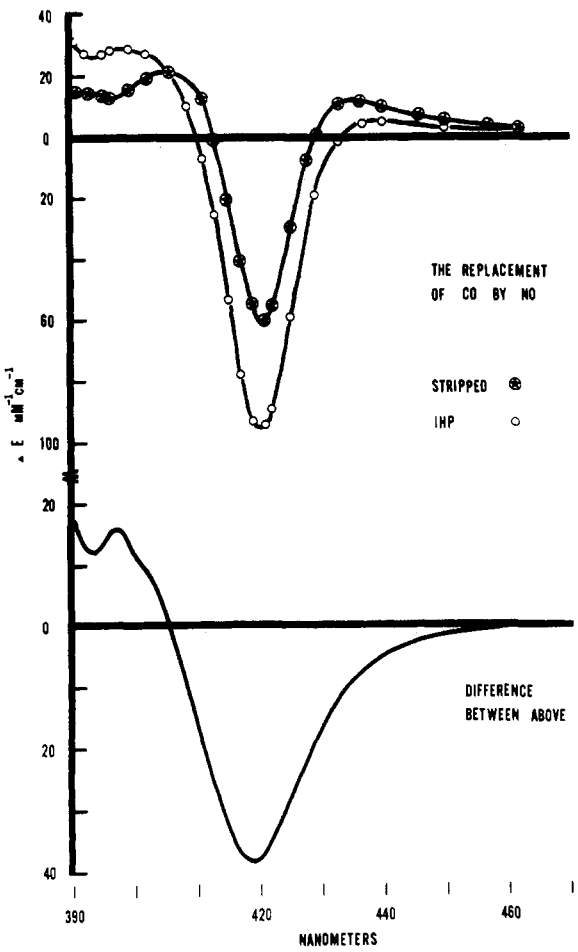

FIGURE 10: The replacement reaction of CO by NO for adult hemoglobin stripped and in the presence of IHP at various wavelengths in the Soret region. The upper portion of the figure shows the difference spectra for the replacement reaction of the stripped protein (circles with stars in the center) and for the exact same protein solution in the presence of 3 m*M* IHP after mix. These reactions were studied in 0.2 *M* bis-tris buffer (pH 6.35) + 0.1 *M* NaCl + 1 m*M* ascorbate (before mix in the NO buffer); 24°. It should be noted that in the presence of IHP there were no true isosbestic wavelengths. The pseudoisosbestic points in the spectrum with IHP occurred because the absorbance began and ended at the same value but deviated in between. The lower curve of this figure shows the calculated difference between the two experimental spectra in the upper portion of this figure. The extinctions were calculated based on total heme after mix in a 2 mm cell.

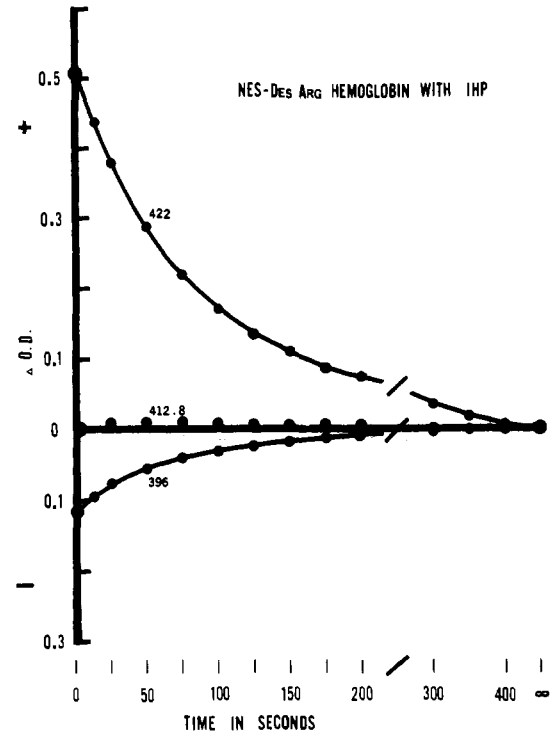

FIGURE 11: The CO replacement reaction by NO for adult Nes-des-Arg hemoglobin in the presence of IHP. The concentrations of reagents after mix were: heme, 50 μM; IHP, 3 m*M* in 0.2 *M* bis-tris (pH 7.0) + 0.1 *M* NaCl at 23°.

stripped hemoglobin A was studied (line 1), slow first-order time courses were observed. When hemoglobin A with IHP was investigated, autocatalytic time courses were seen (line 3). Nes-des-Arg hemoglobin + IHP (line 2) showed no autocatalytic behavior but did show a slightly faster rate. The values of the rate constants determined at the midpoint of the reaction are given in the legend to Figure 12. The initial portion of the time course for hemoglobin A + IHP showed the same apparent rate as that for stripped hemoglobin A. This indicates that the off constants of the first CO molecules which dissociate are unaffected by IHP.

The CO replacement reaction for stripped hemoglobin Kansas was studied in 0.2 *M* bis-tris + 0.1 *M* NaCl at pH 7.0 at 412.8 nm which is an isosbestic wavelength for the CO–NO reaction in stripped adult hemoglobin. The results are shown in Figure 13A. As can be seen, this wavelength was not isosbestic at pH 7.0 and no isosbestics could be found. The extra spectral change in nitrosyl hemoglobin Kansas with or without IHP was found to be the same as in adult hemoglobin + IHP. When hemoglobin Kansas was prepared at exactly the same protein concentration at pH 7.6 a large diminution of the extra spectral change was noted.

To measure the true CO off rate for hemoglobin Kansas, observations must be made at the wavelengths isosbestic for the extra spectral change. The results of these measurements are shown in Figure 13B. At pH 7.0 stripped hemoglobin Kansas showed an autocatalytic time course for the rate of CO dissociation. This result was similar to hemoglobin A + IHP at pH 7.0 in that the initial rate was about 0.007 sec^{-1} whereas the rate at about the halfway point was 0.026 sec^{-1} (see legend to Figure 12). At pH 7.6, the rate of CO dissociation was only moderately faster than stripped adult hemoglobin at pH 7.0.

It has already been mentioned that hemoglobin Kansas bound by CO + IHP was in the T state according to the NMR spectrum (Ogawa et al., 1972). A relatively concentrated solution of this mutant protein was prepared with IHP. The CO off rate was measured in the visible portion of the spectrum at an isosbestic wavelength for the extra spectral change (590.2 nm). The results are shown in Figure 13B. The time course was largely first order and the value of the CO off rate was nine times greater than the CO off rate for stripped hemoglobin A.

Discussion

Structural Properties. All of the physical studies presented above can be explained by the hypothesis that fully liganded nitrosyl hemoglobin can take two different quaternary structures. This interpretation rests on an understanding that the markers used indicate changes in the quaternary structure of hemoglobin. Correlations of the −14 ppm[2] NMR line and the negative peak in the ultraviolet

[2] This peak is tentatively assigned to the imidazole NH proton of α 122 histidine (S. Ogawa and D. J. Patel, manuscript in preparation).

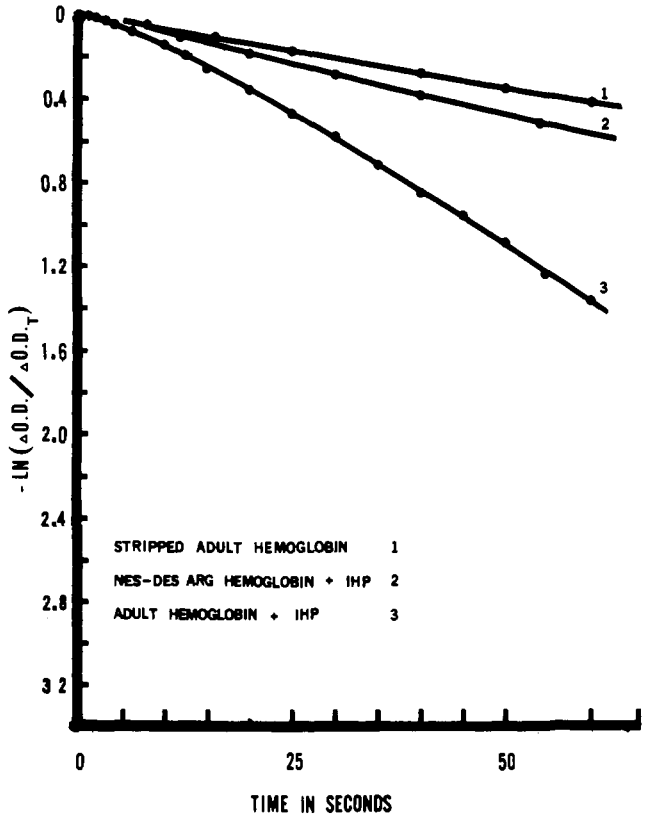

FIGURE 12: The normalized time courses for the CO replacement reaction by NO at an isosbestic wavelength (405.7 nm): (line 1) stripped adult hemoglobin, 42 μM heme, 23°; (line 2) Nes-des-Arg hemoglobin + IHP, 50 μM heme, 3 mM IHP, 24°; (line 3) adult hemoglobin + IHP, 42 μM heme, 3 mM IHP, 24°. All of the above concentrations of heme and IHP are given as after mix values. The buffer system in all of the above was 0.2 M bis-tris (pH 7.0) + 0.1 M NaCl. The rate constants were: (1) 0.007; (2) 0.009; and (3) 0.029 sec^{-1} at the half-way point.

CD with quaternary structure have been presented previously (Shulman et al., 1973; Perutz et al., 1974a,c; Salhany, 1974). The important point about both the NMR and CD markers is that their presence or absence depends on the quaternary structure and not on the degree of ligation or the type of heme ligand per se.

The observation of the −14 ppm line in adult nitrosyl hemoglobin + IHP and stripped nitrosyl hemoglobin Kansas suggests that these proteins are in the deoxy (or T) quaternary structure even when fully liganded with NO. The absence of the −14 ppm line in stripped adult nitrosyl hemoglobin and in nitrosyl Nes-des-Arg hemoglobin + IHP suggests that these molecules are in the oxy (or R) quaternary structure. The −14 ppm line showed a pH dependence in adult nitrosyl hemoglobin + IHP (Figure 1).

The T state in adult hemoglobin is stabilized by both protons and IHP binding. Both are less effective at higher pH values and this presumably accounts for the switch to the R state of adult nitrosyl hemoglobin plus IHP with a rise in pH. It is possible to switch stripped nitrosyl hemoglobin Kansas to the R state by raising the pH above 8 at room temperature. The apparent pK for the T → R transition of stripped nitrosyl hemoglobin Kansas is much higher ($\simeq$8.5; see Figure 2) than the apparent pK for the same transition in adult nitrosyl hemoglobin + IHP (~7.4; see Figure 1). Making the reasonable assumption, in light of the available information, that protons are equally effective in stabilizing the T states of adult hemoglobin and hemoglobin Kansas, we can conclude from these different switching points that in stripped nitrosyl hemoglobin Kansas the T state is more stable, *relative to R*, than is the T state of adult nitrosyl hemoglobin plus IHP.

The observation of a large negative CD peak centered around 285 nm for adult nitrosyl hemoglobin + IHP but

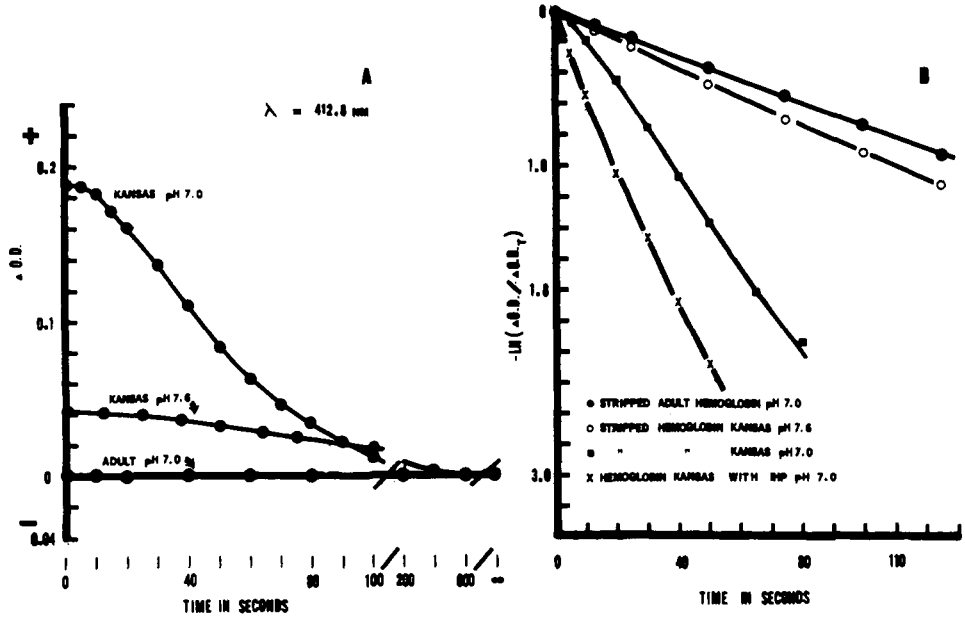

FIGURE 13: The CO replacement reaction by NO for hemoglobin Kansas. (A) Time course of the spectral changes for stripped hemoglobin Kansas at pH 7.0 and 7.6 at the HbCO-HbNO isosbestic point for adult hemoglobin (412.8). The concentration of heme for hemoglobin Kansas at each pH was 106 μM after mix. The reaction was studied in 0.2 M bis-tris + 0.1 M NaCl at 24°. (B) Normalized time courses for the CO replacement reaction at the isosbestic wavelengths for the extra spectral change in Figures 6 and 10. The conditions for stripped hemoglobin Kansas at pH 7.0 and 7.6 are as in A above. The conditions for stripped A and Kansas with IHP are: HbA, 42 μM heme, 23°; and Kansas, 363 μM heme; 3 mM IHP, 23°. The IHP and Hb Kansas were preincubated before the reaction and there was no sodium chloride present in this case since Edelstein has shown that the tetramer-dimer equilibrium constant is significantly affected by salt in the CO form of this mutant hemoglobin in the presence of IHP. The rate constants were: 0.007 (stripped HbA); 0.010 (stripped Hb Kansas, pH 7.6); 0.026 (stripped Hb Kansas, pH 7.0); and 0.062 sec^{-1} (Hb Kansas + IHP).

Table II: Correlation of the −14 ppm NMR Line with the Negative CD Peak at 285 nm.

pH ~ 7	−14 ppm NMR Line	Negative CD Peak at 285 nm
Stripped adult hemoglobin		
CO	−[a]	−[d]
NO	−[c]	−[j]
O_2	−[b]	−[g]
Aquomet	−[b]	−[i]
Deoxy	+[b]	+[g]
Adult hemoglobin + IHP		
CO	−[a]	−[j]
NO	+[c]	+[j]
Aquomet	+[e]	+[i]
Stripped hemoglobin Kansas		
CO	−[a]	−[c]
NO	+[c]	+[c]
Deoxy	+[a]	+[d]
Hemoglobin Kansas + IHP		
CO	+[a]	+[d]
NO	+[d]	+[d]
Nes-des-Arg hemoglobin		
Stripped deoxy	−[f]	−[h]
Deoxy + IHP	+[f]	+[h]
NO + IHP	−[c]	−[d]

[a] Ogawa et al. (1972). [b] Patel et al. (1970). [c] This paper. [d] Predicted. [e] S. Ogawa, unpublished observations. [f] Ogawa et al. (1974). [g] Simon and Cantor (1969). [h] Perutz et al. (1974c). [i] Perutz et al. (1974a). [j] Salhany (1974).

not for stripped adult nitrosyl hemoglobin or adult carbon monoxide hemoglobin + IHP (Salhany, 1974) offers independent structural evidence that the addition of IHP to the nitrosyl form of the protein induces the R → T transition in agreement with the above NMR results. Also in excellent agreement with the NMR results is the finding that stripped nitrosyl hemoglobin Kansas possesses a negative peak in the CD at 285 nm, whereas the CO form does not (Figure 3). At the concentrations of hemoglobin Kansas used in these CD measurements (50 μM), the R form of Kansas is known to be largely dimer using Edelstein's value for the stripped CO form of $K_{4,2} \simeq 412$ μM (Professor S. Edelstein, personal communication). The present experiments allow us to predict that *stripped nitrosyl* hemoglobin Kansas will be considerably more associated into tetramers compared with stripped carbon monoxide hemoglobin Kansas. A summary of the correlation between the NMR and CD results is given in Table II.

The EPR spectrum of nitrosyl hemoglobin probes the state of the unpaired electron of the NO molecule which is complexed at the sixth coordination position of the iron atom. The present EPR results and their correlation with the NMR and CD findings suggest that one type of EPR spectrum corresponds to the R state (stripped adult) and one type to the T state (adult + IHP, and Kansas pH 6.8 and 8.0) (Figure 4). The R state at room temperature is characterized by an almost completely symmetric first derivative spectrum centered at about $g \simeq 2.014$ in agreement with other results (Shiga et al., 1969; Henry and Banerjee, 1973). The T state presents a grossly asymmetric first derivative spectrum. Such an anisotropic EPR spectrum has been observed by other investigators when DPG or IHP was added to adult nitrosyl hemoglobin (Trittelvitz et al., 1972; Rein et al., 1972). However, the assignment of such a spectrum to the fully liganded T state was not explicit. One interesting and important result of Figure 4 is that stripped nitrosyl hemoglobin Kansas shows a T state EPR spectrum similar to that observed in adult hemoglobin + IHP. These findings show that this anisotropic room temperature spectrum does *not* depend on the presence of organic phosphates per se, but does depend upon the quaternary structure.

The NMR, CD, and EPR results presented here suggest that the structures of adult nitrosyl hemoglobin + IHP and stripped nitrosyl hemoglobin Kansas are quite different than the structure of stripped adult nitrosyl hemoglobin. Although this interpretation in structural terms is supported by the spectral evidence, it would be desirable to have some stereochemical evidence. How closely does the unliganded deoxy quaternary structure resemble the "liganded deoxy quaternary structure" we have claimed to isolate? Briehl and Salhany (1975) have recently shown that fully liganded, nitrosyl sickle cell hemoglobin + IHP forms gels and that these gels are birefringent under the polarizing microscope, just like deoxy sickle cell hemoglobin. This suggests a stereochemical similarity between the T form of nitrosyl hemoglobin + IHP and deoxyhemoglobin.

Extra Spectral Change in Nitrosyl Hemoglobin. The extra spectral change in nitrosyl hemoglobin described above occurs only when nitrosyl hemoglobin is in the T structure. This conclusion can be supported as follows. When NO binds to deoxyhemoglobin + IHP or deoxyhemoglobin Kansas the quaternary structure starts and finishes in T (as defined by the NMR and CD markers), yet this extra spectral change occurs (see Figure 2B of Salhany et al., 1974b). The extra spectral change cannot be due to any structural modification directly induced by the IHP binding to nitrosyl hemoglobin, because it is observable in hemoglobin Kansas without IHP (Figure 13A). When adult nitrosyl hemoglobin is in the R structure (i.e. complete absence of *all* phosphates), this extra spectral change does not occur between pH 6.8 and 8.5. Some additional support for the hypothesis that the extra spectral change occurs within T is obtained from partial NO binding to deoxyhemoglobin A with IHP shown in Table I. At low NO coverage one expects even more strongly that the molecules start and finish in T, yet the fractional extra spectral change is the same as in fully liganded nitrosyl hemoglobin. Furthermore, since this spectral change occurs at about the same rate when the system starts and ends in T (deoxy adult hemoglobin + IHP mixed with NO) as it does immediately after the R → T transition (stripped adult nitrosyl hemoglobin mixed with IHP), it would *not* seem reasonable to assume that this slow spectral change has the R → T transition as its *rate determining step* (Salhany et al., 1974b). Cassoly (1974) has independently observed the same slow spectral change when IHP is mixed with adult nitrosyl hemoglobin. He concluded that the slow spectral change was linked to the quaternary switch. He suggested from the slow rate of the extra spectral change that the final state was probably *not* the T state of the allosteric model. However, it is clear from the studies presented here and elsewhere (Salhany et al., 1974b) that the extra spectral change is some phenomenon within the T state and that the *quaternary structure* of this fully liganded molecule is very similar to the unliganded T state.

Recently Sugita (1975) has studied the optical spectrum of the NO hybrids and has shown that the spectral change which we (Salhany et al., 1974a,b) and Cassoly (1974) observe in nitrosyl hemoglobin comes almost exclusively from

the α^{NO} chain of the tetramer (he has also shown that the spectral change in deoxyhemoglobin which has been used as an indicator of the R → T transition also comes from the α chain which explains the results of Cassoly and Gibson (1972) on the cyanomet hybrids). In other words, when hemoglobin takes the low affinity quaternary form, as defined by the typical, large reduction in the rate of CO binding, the optical spectrum of the α subunits changes much more than that of the β subunits. The predominant differences in affinity seem to arise from the change in quaternary structure and not from these localized effects at the heme per se since one heme shows the localized effects while the other largely does not, yet both change their ligand affinities (Antonini et al., 1966; Cassoly and Gibson, 1972; Gibson and Parkhurst, 1968).

In summary, we see first that the extra spectral change is very localized, occurring almost exclusively at the α hemes. This degree of localization is quite different from the widespread quaternary changes probed by our NMR and CD spectral markers of the quaternary structure. Second, we point out that there is in fact a definite lack of correlation between these spectral changes, in nitrosyl and deoxyhemoglobin, and functional changes since the β heme affinities change strongly going from R → T yet their absorption spectra do not change appreciably.

Replacement Reaction of CO and NO in Hemoglobin. Measurement of the CO off rate by replacement with NO in the absence of IHP is straightforward. The time course of the normalized optical change is a wavelength independent simple first-order process. Therefore, there are only two optical species involved, CO and NO hemoglobin, both in the R state. However, when the T structure appears during the replacement reaction (adult hemoglobin + IHP or stripped hemoglobin Kansas), the time course of the reaction becomes complicated because of the extra spectral change of the NO heme in the T state. Since there are three significant optical species present, the measurement of the CO off rate must be done at the wavelengths where the extra spectral change in the NO heme is zero (Figures 6 and 10).

When there is a switch from R to T during the replacement reaction the time course of the reaction shows acceleration at isosbestic wavelengths for the extra spectral change, depending on the switch point. A large scale plot of the initial part of Figure 12 shows that the CO off rate from hemoglobin A with IHP starts with the R rate and as can be seen it increases nearly fourfold (Figure 12). Since the initial rate is almost identical with the rate in R, in spite of the presence of IHP, it suggests that the effect of IHP binding on the CO off rate in R is negligible. According to the NMR results, Nes-des-Arg hemoglobin is in the R state throughout the CO → NO reaction. We suggest that the slightly faster CO off rate for Nes-des-Arg hemoglobin is due to an alteration in the affinity within R due to the chemical modifications. Recent measurements (J. M. Salhany, unpublished results) show a similar increase in the CO off rate for stripped Nes hemoglobin consistent with the slightly lowered ligand affinity in the R state as reported by Kilmartin et al. (1975) for Nes hemoglobin.

In hemoglobin Kansas with IHP, the CO off rate is nine times faster than the rate observed for stripped adult hemoglobin in R (Figure 13B). In this case there is no quaternary switch during the reaction since the hemoglobin molecule begins and ends in T. Preliminary measurements (J. M. Salhany, unpublished results) of the NO off rate in R (i.e. phosphate-free HbNO → HbCO) and in T (i.e. phosphate-free hemoglobin Kansas, NO → deoxy and A + IHP,NO → deoxy) also show about a factor of 20 faster T off rate compared with R. Hemoglobin Kansas at pH 7.0, without phosphates (Figure 13B), has an initial CO off rate characteristic of the R state and the rate accelerates as the molecules switch to T.

In recent ultracentrifergation experiments, S. Edelstein (personal communication) has shown that IHP reduces the tetramer-dimer equilibrium constant ($K_{4,2}$) of CO Kansas by about a factor of 30 under the solution conditions of Figure 13B. This eliminates dimer contributions in the case where IHP is present. However, in stripped CO Kansas there is about 72% dimer present initially. It appears reasonable to assume that the T state in stripped nitrosyl hemoglobin Kansas is much more associated into tetramers than the R state when CO is the ligand. Thus, during the CO replacement reaction the molecule must shift its value of $K_{4,2}$ as the nitrosyl T state is formed. Such a shift to the tetrameric state is known to have rapid kinetics relative to the rate of CO dissociation in either R or T (Kellett and Gutfreund, 1970). The tetramer-dimer process should therefore be in rapid equilibrium throughout the reaction and would not become the *rate-determining step* for this particular reaction. The degree to which dimers become a problem for stripped hemoglobin Kansas will depend on the protein concentration used. This probably explains why Gibson et al. (1973) did not observe an autocatalytic CO off rate for stripped hemoglobin Kansas at 20 μM (a factor of 5 below that used here). The value of the CO off rate for stripped nitrosyl hemoglobin Kansas in their study was about a factor of 2 faster than stripped adult hemoglobin. Another point to consider is the slower, nonaccelerating CO off rate for stripped Kansas at pH 7.6. This may be explained if the *switch point* from one quaternary structure to the other is pH dependent and occurs later in the course of the reaction at pH 7.6 compared with pH 7.0. These particular kinetic measurements are extremely sensitive to the switch point.

The results of this paper can be explained using the two-state model of Monod et al. (1965) as discussed by Hopfield et al. (1971). Only two quaternary structures could be identified by these varied physical probes (Table II). When the probes indicated that the structures were constant throughout the reaction, the kinetics were *not* autocatalytic. When an R to T transition was indicated, the reactions were autocatalytic, provided the switch point was early enough (see Hopfield et al., 1971). Since all of this behavior occurred while the hemoglobin molecule was fully liganded, a Koshland type model (Koshland et al., 1966), in which affinities depend on the number of ligands bound, would seem to be ruled out. However, the type of ligand clearly has an influence on the molecule. The affect is, by definition, on the value of c.

Acknowledgments

We wish to express our thanks to Ms. C. L. Castillo for her excellent technical assistance. We also wish to thank Professor J. J. Hopfield for many helpful discussions and Professors S. Edelstein and Y. Sugita for informing us of their experimental results referred to in this paper. One of us (J.M.S.) wishes to thank Professors Robert Haselkorn and Paul Sigler for their support during his combined program of study at the University of Chicago and Bell Laboratories from which this work was taken.

Added in Proof

Elsewhere in this paper we predicted that stripped nitrosyl hemoglobin Kansas will be relatively more associated into tetramers than the CO form of the protein since the latter is in R while the former is in T. Recent ultracentrifugation measurements by S. Edelstein (personal communication) support this prediction. Replacement of CO by NO in stripped hemoglobin Kansas causes a large reduction in the tetramer–dimer equilibrium constant similar to what was observed when IHP was added to the CO form of the protein.

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