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Proton Magnetic Resonance Study of the Switch between the Two Quaternary Structures in High-Affinity Hemoglobins in the Deoxy State†

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ABSTRACT: High ligand affinity hemoglobins in the deoxy state have been studied by proton magnetic resonance to look for the switch between the two quaternary structures (T and R). In des-Arg-hemoglobin around pH 7, the paramagnetically shifted resonances are quite similar to those of normal deoxyhemoglobin, and the exchangeable proton peak at −14 ppm which is an indicator of the T quaternary structure is observed. At pH 9 the spectrum is quite different from that at pH 7 and can be regarded as that of the R structure. A similar spectral change from R to T is observe in Nes-des-Arg-hemoglobin when inositol hexaphosphate is added at pH 7. In the pH dependence of the nuclear magnetic resonance spectrum of des-Arg-hemoglobin there is an indication that the rate of interconversion between T and R can be as slow as 10^4 sec^{-1}.

The allosteric model of Monod *et al.* (1965) proposed the existence of two conformational states to explain cooperative oxygen binding in hemoglobin. The two states were attributed to the two distinct quaternary structures of deoxygenated and ligated hemoglobins elucidated by Perutz in X-ray studies (Perutz, 1970). There are two basic assumptions in the two-state model. One is that the two states or two structural forms have quite different functional properties, high and low ligand affinities, for example, and maintain their specific characteristics independent of the degree of ligation. The second is that the two structural forms are in equilibrium regardless of the degree of ligation. Since hemoglobin starts with one structural form (T) predominating in the deoxygenated state and finishes with the other form (R) predominating in the fully ligated state, there should be during the process of ligation a point where hemoglobin switches its structural form from one to the other and the two forms are relatively equally populated. The switch between the two forms without changing the state of ligation was demonstrated in nmr studies of valency hybrids in which two of the four subunits were in the ligated state (Ogawa and Shulman, 1972). Analyses of the functional properties of hemoglobin in terms of the two state model have also indicated that this switching should occur after two to three oxygen molecules are bound to normal hemoglobin (Edelstein, 1971; Hopfield *et al.*, 1971).

The switch between the two structural forms, or two quaternary structures, occurs when the difference of the free energy between the two becomes very small, and therefore it should be possible to observe the switch at various degrees of ligation in various hemoglobins in which the intersubunit interactions are modified by chemical methods or by mutations. Hemoglobin Kansas, a mutant hemoglobin with low oxygen affinity, has been shown to retain the T quaternary conformation even when fully liganded with carbon monoxide in the presence of inositol hexaphosphate (IHP)[1] (Ogawa *et al.*, 1972). On the other hand, in high-affinity hemoglobins with diminished cooperativity, the switch from T to R would be expected to occur at earlier stages of ligation. A preliminary study of these high-affinity hemoglobins by nuclear magnetic resonance (nmr) has been reported, showing that the switch can be induced by pH and phosphates (Ogawa *et al.*, 1973).

Material and Methods

Hemoglobin A was prepared from freshly drawn adult blood. Des-Arg-HC3(141)α-hemoglobin (des-Arg-Hb) was prepared by digestion of 6×10^{-3} M solutions of hemoglobin A with carboxypeptidase B (Worthington enzyme COB–diisopropyl fluorophosphate treated) in 0.05 M barbital buffer

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[1] Abbreviations used are: IHP, inositol hexaphosphate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Nes, *N*-ethylsuccinimide.

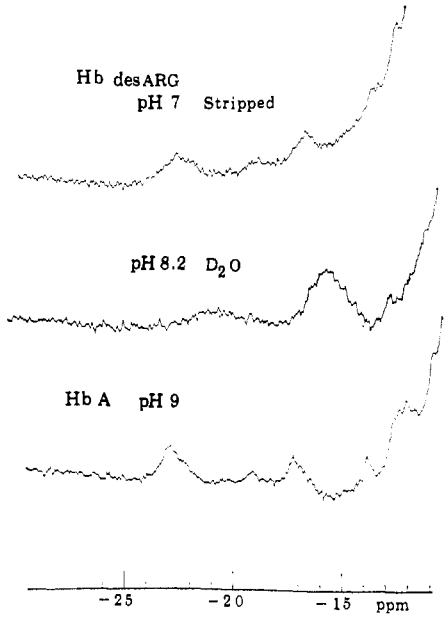

FIGURE 1: A comparison of 220-MHz nmr spectra of deoxy-des-Arg-Hb and deoxy-HbA at 20°: (a) des-Arg-Hb at pH 7 in Bis-Tris buffer in H_2O; (b) des-Arg-Hb at pH 8.2 in Tris buffer in D_2O; (c) HbA at pH 9 in borate buffer.

(pH 7.5) containing 0.1 M NaCl, using an enzyme:substrate ratio of 1:100. Digestion was allowed to proceed for 12 hr at 20°, and the extent of reaction was assayed by amino acid analysis of released arginine, as described by Kilmartin (Kilmartin and Hewitt, 1971). Separation of des-Arg-Hb from unreacted protein was accomplished by ion-exchange chromatography on a 2.5 × 30 cm column of Bio-Rex 70 using a gradient generated by 1000 ml each of 0.02 M potassium phosphate and 0.2 M potassium phosphate buffer (pH 6.5). Des-Tyr-His-Hb was prepared by digestion of hemoglobin A with carboxypeptidase A (Worthington enzyme COA–diisopropyl fluorophosphate treated) under the same conditions, except the buffer contained 0.3 M NaCl. Assay of reaction and separation of products were carried out as described above. Nes-des-Arg-Hb was prepared by reaction of des-Arg-Hb with *N*-ethylmaleimide (Pierce Chemicals) as described by Kilmartin, and separation of products as described above.

The samples for nmr experiments were prepared in the following way. An aliquot of 300 μl of hemoglobin solution, 10^{-2} M in heme, in Tris buffer was placed in a closed vessel fitted with a pH electrode. The solution was deoxygenated as much as possible by passing N_2 gas through the vessel. A small volume of 0.1 M dithionite solution anaerobically prepared in Tris buffer was added to the hemoglobin solution to ensure the full reduction of hemoglobin. The pH of the solution was measured with a Radiometer Model 26 pH meter. Adjustments of pH were made by mixing the Tris buffer solution with Tes solution. The sample was then transferred anaerobically to a N_2-filled nmr sample tube. A test in which a sample in an nmr tube was retransfered to the vessel showed that the pH value did not decrease more than 0.02 pH unit. The final buffer concentrations of the nmr samples were mostly 0.3 M Tris with varying amounts of Tes and 0.01 M dithionite. The final heme concentration was about 8×10^{-3} M. In a few experiments on des-Arg-Hb around pH 9, samples were prepared by simple deoxygenation by N_2 gas without any reducing reagents. There were no appreciable differences in the nmr spectra of any consequences to our interpretation between the two kinds of sample preparations, although in the latter preparation there were significant amounts of oxidized material and residual oxy form. In a few experiments, an enzymatic reduction system with ferredoxin, ferredoxin reductase, and TPNH was used to maintain the hemoglobin in the reduced state.

The proton magnetic resonance spectra were taken on Varian HR-220 and Varian HR-300 spectrometers. The peak positions were expressed in parts per million from 2,2-dimethyl-2-silapentanesulfonate.

Results

In Figure 1, parts of the 220-MHz nmr spectra of deoxy-HbA and deoxy-des-Arg-Hb in the low-field spectra region are shown. As previously reported (Shulman *et al.*, 1969; Davis *et al.*, 1971; Ogawa and Shulman, 1972), the broad peaks below −15 ppm all come from proton resonances shifted by paramagnetic interaction with the deoxy heme iron. In HbA the peaks at −23 and −17.5 ppm have been attributed to the protons coming from the β subunits and the α subunits, respectively. These peaks did not show any sensitivity to variation of pH between 7 and 9 in phosphate-free solutions of HbA, although the peak at −23 ppm arising from the β subunit showed the effect of phoshate binding at pH 7 by a slight movement of the peak position to −24 ppm. The peak at −17.5 ppm is a single peak and has an intensity of about six protons per tetramer. It is reasonable to assign this peak to a methyl resonance. The peak at −23 ppm has an intensity of about eight protons per tetramer and has a poorly resolved structure. It is more likely to be a superposition of a methyl and single proton resonance rather than a superposition of four discrete single proton resonances. The assignments of these peaks have not yet been established but heme methyls are likely candidates. However, it is possible that resonances of nearby methyl groups from the heme pocket in the globin are shifted to this spectral region by interaction with the paramagnetic heme iron. There are a few minor peaks around −19 and −20.5 ppm. The peak at −14 ppm in the spectrum of HbA at pH 9 (Figure 1c) is an exchangeable proton peak which is not observable in D_2O solution. This particular peak has been shown to be observable only in the T quaternary structure of hemoglobin (Ogawa *et al.*, 1972; Mayer *et al.*, 1974), regardless of the state of ligation. There are other exchangeable proton peaks, two of which at −13 and −12 ppm are also observed in oxyhemoglobin and are pH invariant in the physiological pH range.

In contrast to the insensitivity of the deoxy-HbA spectrum to the variation of pH, deoxy-des-Arg-Hb displays a strong pH dependence in its nmr spectrum. In Figure 1 the spectra at pH 7 (H_2O) and at pH 8.2 (D_2O) are shown. At pH 7 the spectrum of des-Arg-Hb (Figure 1a) is very similar to that of HbA, although the α-subunit peak at −17.5 ppm in HbA is slightly shifted to −16.8 ppm, indicating some minor structural perturbation induced by the removal of the C-terminal arginine. The exchangeable proton peak at −14 ppm is still observable, although the peak is somewhat broader than the corresponding peak in HbA. At high pH the spectrum undergoes a drastic change as shown in Figure 1b. In order to elucidate the details of this spectral change, a series of 300-MHz nmr spectra in H_2O have been taken at various pH values around pH 8 and are shown in Figure 2. This figure includes the upfield region which extends above the high-field region of normal diamagnetic protein spectra and contains some peaks shifted by paramagnetic interaction. These paramagnetically shifted peaks in the upfield region change their

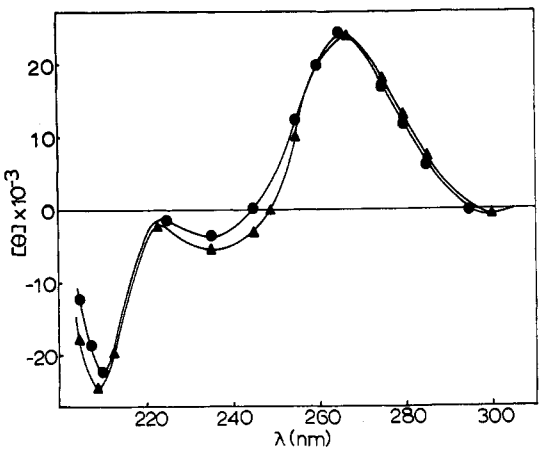

FIGURE 3: CD spectra of R17 RNA (●) and globin mRNA (▲) in 0.01 M Tris-HCl–0.04 M NaCl–4 × 10^{-3} M Mg^{2+} (pH 7.5) at 28°. The measurements were performed in triplicate. The error at 265 nm is ±2%.

of the intense positive band there is a small difference in particular at the high-temperature end. The red shift seems to be terminated at about 272 nm for globin mRNA, whereas for R17 RNA the peak is close to 274 nm. The CD melting curves of R17 RNA and globin mRNA in the presence of 4 × 10^{-3} M Mg^{2+} are shown in Figure 5. Considering the variation of [θ] at 266 nm (left ordinate) two distinct melting regions are apparent for both RNAs. In the case of globin mRNA one region lies below 42° having less of a slope than the one above 42°. For R17 RNA the breaking point is about 48°. Before the sharp breaking point the transitions occur with little change in [θ]. It can also be seen that in the melting region with the larger slopes R17 RNA melts at a higher temperature than globin mRNA. On the right ordinate of Figure 5 the shift of the peak ellipticity is plotted for both melting curves. In that case the difference between the melting behavior of both RNAs is even more pronounced. The shift

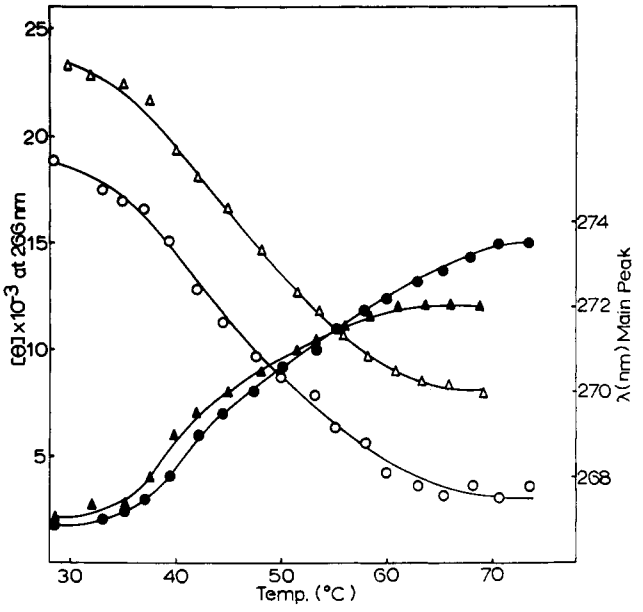

FIGURE 4: CD melting curves of R17 RNA and globin mRNA in 0.01 M Tris-HCl–0.01 M NaCl–0.001 M EDTA (pH 7.5). The melting studies were done in duplicate. Two parameters are plotted: on the left ordinate the peak residue ellipticity at 266 nm for R17 RNA (○), and globin mRNA (△); on the right ordinate the red shift of the peak of the intense positive band for R17 RNA (●) and globin mRNA (▲).

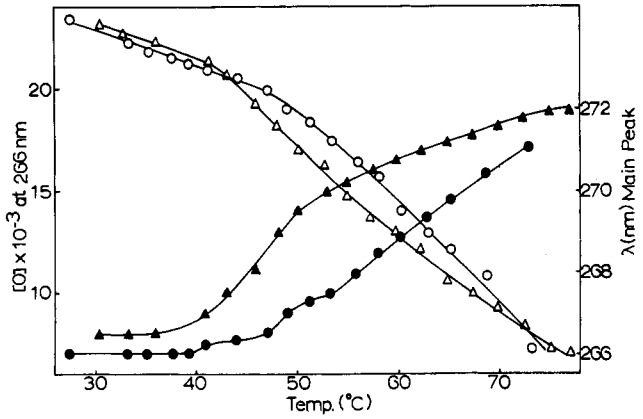

FIGURE 5: CD melting curves of R17 RNA and globin mRNA in 0.01 M Tris-HCl–0.04 M NaCl–4 × 10^{-3} M Mg^{2+} (pH 7.5). The melting studies were done in duplicate. Two parameters are plotted: on the left ordinate the peak residue ellipticity at 266 nm for R17 RNA (○) and globin mRNA (△); on the right ordinate the red shift of the peak of the intense positive band for R17 RNA (●) and globin mRNA (▲).

in wavelength of the band maximum consists of two distinguishable regions for globin mRNA. Between 40 and 50° a large change in the peak position is observed, whereas above 50° the slope of the curve is considerably smaller. Study of the peak shift curve of R17 RNA reveals two small cooperative shifts with transition midpoints of about 40 and 49°, followed by a larger one with a transition midpoint of greater than 65°. A similar observation has already been reported for R17 RNA in the presence of Mg^{2+} in a different buffer system. The corresponding transition values were 37 and 46° in 0.1 × SSC buffer (0.015 M NaCl–0.0015 M sodium citrate (pH 7.0)) (Phillips and Bobst, 1972). The original CD spectra can be restored for both RNAs. Even the 298-nm band can be obtained back if the RNAs are allowed to renature at 8–10° for about 12 hr.

Discussion

The analysis of the temperature–absorbance profiles in terms of compositions of double helices indicates (Table I) that without the addition of Mg^{2+} the percentage of bases in base-paired regions is similar for both mRNAs. In terms of thermal stability both seem to have similar overall melting properties. Both melting curves show discontinuities which slightly differ from one another. It is believed that the discontinuities represent separate populations of double helices. As to be expected, the secondary structure is to a certain extent different for both RNAs. The major difference, however, is found in the considerable large amount of nonbase paired adenylic acid residues for globin mRNA. About 7% more adenylic acids are free at 28° in the mammalian messenger than in the bacteriophage RNA. It is tempting to assume that this is the amount present as poly(A) in globin mRNA. Using polyacrylamide gels Gaskill and Kabat (1971) determined a molecular weight of 220,000 for rabbit mRNA. Thus, the free poly(A) stretch should be about 40–50 nucleotides long. Incidentally, this estimation would coincide with the value found for the adenylate rich sequence in mouse globin mRNA. In that case the adenylate sequence was determined to be about 50 nucleotides long based upon its migration relative to poly(A) standards (Morrison *et al.*, 1973).

It is well established that in an RNA chain the optical activity of single-stranded structures might be large, whereas the hypochromicity is small (Boedtker, 1967; Gratzer, 1966).

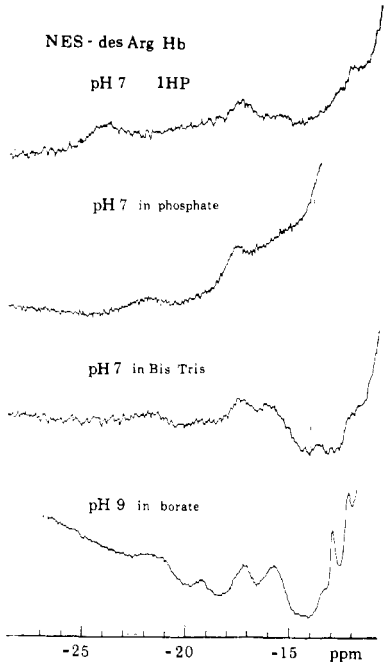

FIGURE 4: Spectral changes in 220-MHz nmr induced by phosphates in deoxy-Nes-des-Arg-Hb at 20°: (a) at pH 7 with IHP (5×10^{-3} M) in Bis-Tris buffer; (b) at pH 7 in 0.1 M phosphate buffer; (c) at pH 7 in 0.2 M Bis-Tris buffer; (d) at pH 9 in borate buffer.

1974). At pH 9 in borate buffer in H_2O, the spectrum of deoxy-Nes-des-Arg-Hb (Figure 4d) is similar to but not identical with the spectrum of deoxy-des-Arg-Hb at pH 9. The intensity around −16 ppm corresponding to the two superposed strong peaks in deoxy-des-Arg-Hb at high pH is clearly resolved into two peaks in Nes-des-Arg-Hb. These two peaks in Nes-des-Arg-Hb have intensities of about six protons per tetramer each and are most likely methyl resonances of α and β subunits. Since these two resonances are so well resolved, it should be possible to obtain some information about the relative affinities of the α and β chains in the R quaternary structure by monitoring the peaks as a function of saturation of Nes-des-Arg-Hb with ligands. The broad weak peak at −21.5 ppm (Figure 4d) appears to be a superposition of two resonances of roughly equal intensity. The two resonances can be more clearly resolved in a 300-MHz spectrum at 34°. In Bis-Tris buffer at pH 7, the spectrum of deoxy-Nes-des-Arg-Hb is essentially identical with that observed at pH 9 (Figure 4c). Addition of IHP (5×10^{-3} M) to the pH 7 Bis-Tris buffer transforms the spectrum of deoxy-Nes-des-Arg-Hb to that shown in Figure 4a. This spectrum is very similar to that of deoxy-HbA. In 0.1 M orthophosphate buffer, in the absence of organic phosphates, spectrum b in Figure 4 is obtained. The peak at −16 ppm is now considerably broader than in spectrum 4c or 4d, but the peak at −22 ppm remains weak and is detected at a position very close to that in spectrum 4c.

Similar nmr spectral changes have been observed for samples of other modified or mutant deoxyhemoglobins which are characterized by high oxygen affinity and little or no cooperativity. The spectrum at pH 7 of deoxy-des-TyrHis-Hb, prepared by digestion with carboxypeptidase A, is similar to that of deoxy-des-Arg-Hb at pH 7; at pH 9, the spectrum is not completely converted to a pattern like that of Figure 4d, but resembles more closely an intermediate spectrum similar to Figure 4d. The spectrum of deoxy-des-TyrHis-Arg-Hb, prepared by digestion sequentially with carboxypeptidases B and A, resembles that of Nes-des-Arg-Hb at pH 7 and 9.

Discussion

Nmr Spectra in T and R Quaternary Structures. The nmr peaks which have been paramagnetically shifted as described above can respond to structural changes around the heme which alter the electronic state of the heme or the geometrical arrangements of the protons relative to the heme. Therefore, regardless of the precise nature of these paramagnetic shifts, such peaks can be sensitive indicators of the local structural changes in the environment of the hemes. The spectrum of deoxy-des-Arg-Hb at low pH is very similar to that of normal deoxyhemoglobin (Figure 1): this similarity suggests that the structures around the hemes in the two proteins are very much alike, especially at the unmodified β subunits. Furthermore the exchangeable proton peak at −14 ppm, the presence of which has been shown to indicate T quaternary structure (Patel *et al.*, 1970; Ogawa *et al.*, 1972; Mayer *et al.*, 1974), is observable at low pH. Therefore it can be concluded that deoxy-des-Arg-Hb is in the T quaternary structure under these conditions. This conclusion is consistent with the crystallographic observation that human deoxy-des-Arg-Hb crystallizes isomorphously with deoxy-HbA (Perutz and TenEyck, 1971).

The spectrum of deoxy-des-Arg-Hb at pH 9 is very different from that of normal deoxyhemoglobin under the same conditions, or from that of des-Arg-Hb at low pH. We have concluded that this spectrum can be interpreted as arising from the R quaternary structure of deoxy-des-Arg-Hb on the basis of the following considerations: (a) similarity of the nmr spectral features to those observed in other hemoglobins known to retain the R quaternary structure in the absence of ligands; (b) other electronic spectral evidence which indicates that deoxy-des-Arg Hb assumes the R structure at high pH; (c) the concerted nature of the pH dependence of the spectral transition observed here; (d) functional properties of des-Arg-Hb at high pH; and (e) X-ray crystallographic evidence that, in contrast to the human protein, horse deoxy-des-Arg-Hb can be crystallized in a form isomorphous with that of normal oxyhemoglobin.

The differences between the high and low pH spectra of deoxy-des-Arg-Hb are comparable to, although not identical with, the spectral differences between deoxy-HbA and the isolated deoxy α and β chains (Ogawa *et al.*, 1971). The assignment of the high pH spectrum of deoxy-des-Arg-Hb to a spectrum characteristic of hemoglobin in the R quaternary structure is also consistent with previous nmr studies of valency hybrids (Ogawa and Shulman, 1972). In the nmr spectra of both of the deoxy-CN-met valency hybrids, $\alpha_2^{Fe^{3+}CN}\beta_2^{Fe^{2+}}$ and $\alpha_2^{Fe^{2+}}\beta_2^{Fe^{3+}CN}$, in the R structure, the peak intensity around −16 ppm was greater than that seen in the oxygenated hybrids. This extra paramagnetically shifted peak in the deoxygenated hybrids, superposed on the strong peaks of the cyanoferric subunits, coincides in peak position with the peak around −16 ppm in deoxy-des-Arg-Hb at pH 9. When the deoxy hybrid, $\alpha_2^{Fe^{3+}CN}\beta_2^{Fe^{2+}}$, was shifted to the T structure by addition of organic phosphates, the extra intensity at −16 ppm disappeared and a new peak at −22 ppm appeared. Similarly, when the hybrid, $\alpha_2^{Fe^{2+}}\beta_2^{Fe^{3+}CN}$, was shifted to the T structure, the peak at −16 ppm moved about 1 ppm downfield. The nmr spectrum characteristic of the R quaternary structure as described above, therefore, has strong resonances at −16 ppm and weak resonances around −20 ppm. The intensity of the peak at −16 ppm in deoxy-des-Arg-Hb at pH 9 is twice that of the peak at −17.5 ppm seen in the same protein at pH 7, and, on the basis of this correlation with the spectra of the valency hybrids in the T and

R states, represents the resonances arising from the α and β chains of the R quaternary structure of the protein.

The spectrum of deoxy-des-Arg-Hb at pH 9 is also similar to, but not as clearly resolved as, the spectrum of deoxy-Nes-des-Arg-Hb (*cf.* Figure 1b and Figure 4c,d). The extensive electronic spectral studies on Nes-des-Arg-Hb recently reported by Perutz *et al.* (1974) offer strong evidence that this derivative does assume the R structure in the absence of ligands throughout the pH range we have studied. The differences between the high pH spectrum of des-Arg-Hb and the spectrum of Nes-des-Arg-Hb could arise from small structural differences between the two derivatives. Alternatively, the R structure of deoxy-des-Arg-Hb at pH 9 might not be as stable as that of deoxy-Nes-des-Arg-Hb, and the high pH spectrum might reflect a partial mixture of states. These possibilities should be checked further. The spectral difference is certainly far less than the differences between the spectra of hemoglobins in the two different quaternary states, as can be seen by comparing Figures 1a and 4a with Figures 1b, 4c, and 4d. Since the functional properties of these two modified hemoglobins at high pH are very similar, as will be discussed below, the small spectral differences cannot indicate the presence of a structural change of functional consequence between the two proteins in the R quaternary structure.

We have described three paramagnetically shifted resonances and one slowly exchangeable proton resonance in deoxy-des-Arg-Hb, all of which undergo pH-dependent shifts or changes with the same midpoint, at pH 8.1. It is significant that these spectral changes arise from the heme environments of both α and β subunits. It might be argued that the identity of the pH profiles of the spectral shifts merely reflects similar but functionally independent transitions in local conformations of individual subunits induced by pH. However, similar spectral transitions can be induced in Nes-des-Arg-deoxyhemoglobin at a fixed pH by addition of organic phosphates. Therefore, it is reasonable to conclude that the nmr spectral change observed in deoxy-des-Arg-Hb around pH 8.1 represents an allosteric transition between the two quaternary structures which are in close equilibrium. This conclusion is consistent with the conclusion derived from CD spectral studies of deoxy-des-Arg-Hb (Radochay *et al.*, 1972). The pH dependence of the CD spectral transition also has a midpoint at pH 8; this spectral change has been established as an indicator of the quaternary T to R structural transition (Simon and Cantor, 1969). These concerted structural transitions at various degrees of ligation have now been demonstrated in several hemoglobin derivatives in which the difference in free energy of the T and R states is sufficiently small that interconversion between the two is possible by altering pH or organic phosphate concentration without changing the state of ligation of the hemes.

The crystallographic studies of Perutz and TenEyck (1971) and the complementary functional analyses of Kilmartin and Hewitt (1971) on the role of the C-terminal residues in stabilizing the deoxy or T quaternary structure of hemoglobin add further support to our interpretation of the high and low pH nmr spectra of deoxy-des-Arg-Hb. Although human deoxy-des-Arg-Hb crystallizes isomorphously with unmodified human deoxyhemoglobin, horse deoxy-des-Arg-Hb forms crystals isomorphous with those of unmodified horse oxyhemoglobin. Perutz and TenEyck account for this apparent contradiction by reasoning that the two quaternary conformations are of comparable stability in each derivative in solution, and the least soluble conformation crystallized out, *viz.*, human deoxy and horse oxy. Deoxy-Nes-des-Arg-Hb always crystallizes in forms which are very similar to those of unmodified oxyhemoglobin, suggesting that the oxy or R quaternary structure is significantly more stable than the deoxy structure in this derivative under all conditions. Kilmartin and Hewitt have noted that des-Arg-Hb is capable of binding oxygen cooperatively, with a Hill constant, n, of approximately 2 at pH 7. At pH 9, however, n has dropped to 1, and the affinity of the derivative for oxygen has increased to a value comparable to that of Nes-des-Arg-Hb. Unlike des-Arg-Hb, Nes-des-Arg-Hb never displays cooperative oxygen binding, even at pH 7, and has an affinity for oxygen which is insensitive to pH. Kilmartin and Hewitt interpret their results as indicating that deoxy-des-Arg-Hb undergoes a quaternary conformational change upon ligand binding at low pH, but retains the high-affinity R conformation in the absence of ligands at high pH. Nes-des-Arg-Hb retains the R conformation at all pH values, according to Kilmartin and Hewitt's functional analysis. Our conclusions derived from the nmr spectra of deoxy-des-Arg-Hb and deoxy-Nes-des-Arg-Hb are fully consistent with these results.

Rate of $R \rightleftarrows T$ Conversion. It is possible to obtain some information on the rate of interconversion between the R and T quaternary structures in deoxy-des-Arg-Hb from the pH-dependent nmr spectral changes. Peak A shifts continuously with increasing pH over a range of approximately 300 Hz. Similarly, the pH-induced shift in peak C, not including the increase in intensity, appears to be continuous over a range of 300 Hz. These shifts suggest that the rate of the conformational interconversion is faster than $2\pi \times 300/2$ sec^{-1}. Additional information about the interconversion rate could be obtained in principle from the changes in width of these peaks. However, the width of peak C cannot be measured over the full pH range because a new peak appears at almost the same resonance frequency at high pH; also, peak A appears to undergo some secondary broadening process at high pH. Therefore, we cannot put an upper limit on the rate of conformational interconversion from these resonances alone.

The movement of peak B is not unequivocally established. If peak B at higher pH values merely splits into the separate weak peaks at -20 ppm or progressively broadens, the peak movement does not provide any information on the upper limit of the interconversion rate. However, if the movement of peak B is truly discontinuous, from -23 to -16 ppm, as described earlier, the rate of interconversion is in the intermediate exchange region with respect to the separation ($2\pi \times 2000$ sec^{-1}) of the two positions (Gutowsky and Holm, 1956) and an approximate interconversion constant can be estimated. In a computer simulation of nmr spectra for two interconverting species with identical intrinsic $1/T_2$ values ($\pi \times 300$ sec^{-1}), separated by 2000 Hz, a shift in the resonance of one of the species, comparable to the observed small shift (300–400 Hz) of peak B from low pH to the midpoint of the titration, corresponds to a case where the interconversion rate at the midpoint is about 3×10^3 sec^{-1}. We intend to establish the precise nature of the shift of peak B by titrating Nes-des-Arg-Hb with organic phosphates. This procedure should generate a series of mixtures of the two quaternary conformations of the protein, analogous to the apparent equilibrium mixtures in deoxy-des-Arg-Hb at various pH values.

The interconversion rate may also be estimated from the behavior of the exchangeable proton peak at -14 ppm. This peak has been observed only in hemoglobins with low oxygen affinity, such as deoxy-HbA, HbM Iwate, and Hb Kansas, all which have been shown to assume the T conformation; it has not been observed in oxy-HbA, or in any other hemoglobin

derivatives which possess a quaternary conformation unlike that of the T state. Presumably in this latter class of derivatives, the rate of proton exchange between the protein and the solvent water is too rapid to give any intensity at a peak position sufficiently removed from the H_2O line to be measurable. On the basis of this identification of the T quaternary structure with a slow proton exchange rate and the R structure with a rapid rate, the peak width of the −14 ppm resonance may be used to estimate a lower limit of the lifetime of the T state in deoxy-des-Arg-Hb. This peak broadens progressively with increasing pH (Figure 2), indicating that the lifetime of the T structure is pH dependent. By comparing the −14-ppm peak with the −13-ppm peak, which is unaffected by quaternary state, we have calculated the interconversion rate from T structure to R structure to be 3–5 $\times$ 10^2 sec^{-1} at pH 7.6 and 6–15 $\times$ 10^2 at pH 7.7, at 25°. The corresponding rates for R to T structure are around 10^4 sec^{-1}.

The relatively slow interconversion rate between the two structures at equilibrium as indicated in the present study is far from agreement with the lower limit value of the rate (10^6 sec^{-1}) found in laser flash experiments on unmodified HbA by McCray (1972). We have attempted to verify a slow interconversion rate in deoxy-des-Arg-Hb by an independent procedure, in which the deoxy protein could be rapidly generated from the liganded protein and the relative concentrations of the two quaternary conformations estimated kinetically. In a preliminary experiment in which CO was fully photodissociated from a 3 $\times$ 10^{-4} M solution of des-Arg-Hb by flash photolysis, the subsequent CO recombination rate did not show marked pH dependence (Lamola and Ogawa, unpublished results). If the conversion rate were extremely fast, the two quaternary conformations should be in equilibrium within less than a millisecond after photodissociation, and the subsequent apparent CO binding rate would be expected to be a pH-dependent weighted average between 1 $\times$ 10^6 M^{-1} sec^{-1} for T (Hewitt and Gibson, 1973) and 5 $\times$ 10^6 M^{-1} sec^{-1} for R, depending upon the ratio of the two conformational states. Therefore the quaternary conformational interconversion rate for des-Arg-Hb could indeed be as slow as the present nmr study indicates. It is possible that the free energy of activation for the R–T switch in all hemoglobins could depend on the difference between the free energies of the initial and final states. According to this hypothesis, the R–T switch would be as slow as we have observed only in partially saturated intermediates of unmodified HbA, but not necessarily in the fully liganded or unliganded species.

Apparent pK Value of the R–T Transition in Deoxy-des-Arg-Hb. The titration of all the resonances described above with a pK of 8.1 (Figure 3), as well as the CD titration with a pK of 8, suggests that the C-terminal histidine of the β subunits (Kilmartin *et al.*, 1973) is the residue whose ionization plays a role in energetically stabilizing the T structure in deoxy-des-Arg-Hb. The measurements of Kilmartin *et al.* were performed on samples of unmodified deoxyhemoglobin A, which undergoes no conformational changes over the pH range in which the two C-terminal histidine residues titrate, whereas our studies were performed on derivatives which do undergo a quaternary conformational change. The consequences of this difference in experimental systems are seen in the difference in the shapes of the titration curves obtained by Kilmartin *et al.* and by us. In the absence of a quaternary structural change, the histidine residues display a normal titration curve, extending over approximately three pH units. The concerted changes we see in our nmr spectra display a steeper pH dependence, associated with a cooperative transition. This cooperative transition would be reflected in an apparent facilitation of the concerted titration of the two C-terminal histidines. The uncertainties in peak positions and intensities in our nmr experiments rule out quantitation of the extent of cooperative interactions by Hill plots, but an analysis of the circular dichroism spectral changes in this manner has indicated that the quaternary conformation-linked titration in deoxy-des-Arg-Hb has a value of n equal to 2.02.

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