Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970), *Biochim. Biophys. Acta 200*, 189.
Morell, S. A., Ayers, V. E., Greenwalt, T. J., and Hoffman, P. (1964), *J. Biol. Chem. 239*, 2696.
Nagel, R. L., Ranny, H. M., and Kucinskis, L. L. (1966), *Biochemistry 5*, 1934.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Perutz, M. F., and Lehmann, H. (1968), *Nature (London) 219*, 902.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219*, 131.
Riggs, A. (1961), *J. Biol. Chem. 236*, 1948.
Shiga, T., Shiga, K., and Kuroda, M. (1971), *Anal. Biochem. 44*, 291.
Taylor, J. F., Antonini, E., Brunori, M., and Wyman, J. (1966), *J. Biol. Chem. 241*, 241.
Tyuma, I., Shimizu, K., and Imai, K. (1971), *Biochem. Biophys. Res. Commun. 43*, 423.
Ueda, Y., Shiga, T., and Tyuma, I. (1970), *Biochim. Biophys. Acta 207*, 18.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.

Effects of Anions and Ligands on the Tertiary Structure around Ligand Binding Site in Human Adult Hemoglobin†

Ted R. Lindstrom‡ and Chien Ho*

ABSTRACT: We have studied the tertiary structure of the heme pockets of human adult carbon monoxide hemoglobin and oxyhemoglobin by investigating the ring-current shifted proton resonances in the 250-MHz nmr spectra. We have found that the conformation of the E11 valine residues in α and β chains relative to the heme plane is quite dependent on the nature of the anions and the pD of the solution as well as on the nature of the ligands. The E11 valines are located on the distal histidine side of the heme plane and are believed to play a vital role in the cooperative oxygenation of hemoglobin. We have attempted to correlate the structural transitions manifested by the differences in the ring-current shifts with the known functional effects produced by the different buffer systems. We have suggested a possible relationship between the conformation of the E11 valine methyl groups and the ligand affinity and have proposed a structural mechanism for the effects of anion binding on tertiary structure of the heme pockets in hemoglobin.

The nuclear magnetic resonance (nmr)[1] spectrum of human carbon monoxide hemoglobin A (HbCO A) is known to contain ring-current shifted proton resonances that arise from local magnetic fields produced by the delocalized π electrons in the heme groups (Ho *et al.*, 1970; Shulman *et al.*, 1970; Wüthrich *et al.*, 1972; Lindstrom *et al.*, 1972). In a previous report we have shown that the resonances at 6.58 and 5.86 ppm upfield from HDO can be assigned to the γ_1 and γ_2 methyl groups of the β67 (E11) valines, respectively (Lindstrom *et al.*, 1972). The other resonances have been assigned according to whether they are due to α or β chain residues. There is reasonably good evidence that the resonance at 6.48 ppm upfield from HDO is the γ_1 methyl of the α63 (E11) valine (Lindstrom *et al.*, 1972). The E11 valine residues are located on the distal histidine side of the heme plane, situated next to the ligand binding site, and are believed to play a vital role in the stereochemical mechanism for the cooperative oxygenation of hemoglobin (Perutz, 1970). Since the magnitude of the ring-current shifts is extremely sensitive to the geometrical relationship between the affected protons and the heme plane, these resonances are very sensitive to changes in the tertiary structure of the heme pockets. Our assignments of the E11 valine methyls allow us to probe the tertiary structure of the heme pockets with unprecedented sensitivity.

We have determined that the tertiary structure of the heme pockets is quite dependent on the nature of the supporting electrolyte and also on the nature of the ligand. The type of buffer, concentrations of added ions, and the pH of the solution all affect the tertiary structure of the heme pocket in a specific manner. We have attempted to relate these tertiary structural changes to the known functional effects of the perturbing conditions. In some cases, such as variations in pH and the use of phosphates, the tertiary structural changes may be correlated with changes in ligand affinities in hemoglobin.

† From the Department of Biophysics and Microbiology and the Department of Biochemistry, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. *Received July 27, 1972.* Supported by research grants from the National Science Foundation (GB-25053), the National Institutes of Health (HL-10383, HL-15165, and RR-00292), and the American Heart Association, Inc.

‡ Present address: Thomas J. Watson Research Center, International Business Machines Corporation, Yorktown Heights, N. Y. 10598.

* To whom communications and reprint requests should be addressed at the Department of Biophysics and Microbiology, University of Pittsburgh.

[1] Abbreviations used are: nmr, nuclear magnetic resonance; Hb A, human adult hemoglobin; HbCO, carbon monoxide hemoglobin; HbO_2, oxyhemoglobin; ppm, parts per million; DGP, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Tris, tris(hydroxymethyl)-aminomethane; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; mes, 2-(*N*-morpholino)ethanesulfonic acid.

Experimental Section

Materials

HbCO A and HbO_2 A were prepared from whole blood obtained from the local blood bank following the method of Drabkin (1946) with the cells being lysed with distilled water. The hemoglobin was stripped of phosphate by chromatography on a Sephadex G-25 column (2.5 × 40 cm) using 0.05 M Tris-HCl–0.1 M NaCl at pH 7.45 (Berman *et al.*, 1971). D_2O solutions of the hemoglobin were prepared by repeated dilution with D_2O (Merck, Sharp and Dohme) and concentration by ultrafiltration through an Amicon UM-20E membrane. 2,3-Diphosphoglycerate (DPG) was purhcased from Calbiochem as the pentacyclohexylammonium salt, converted to the acid form with Bio-Rad AG 50W-X8 cation exchange resins and titrated to pD 7 with NaOH. Inositol hexaphosphate (IHP) was purchased from Sigma as the sodium salt, dissolved in D_2O, and titrated to pH 7.0 with HCl. The 2-(*N*-morpholino)ethanesulfonic acid (Mes) was purchased from Sigma, dissolved in D_2O, and titrated to pH 7 with HCl. The tris(hydroxymethyl)aminomethane (Tris) was purchased from Sigma, dissolved in D_2O, and titrated to pD 7.0 with HCl. The 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (bis-tris) was purchased from Aldrich, dissolved in D_2O, and titrated to pH 7 with HCl. Concentrated stock solutions of buffers and salts in D_2O were prepared by repeated lyophilyzing and subsequent dissolution in D_2O. The stock solutions were added to hemoglobin D_2O solution to obtain the desired concentration of buffer or salt. Hemoglobin concentrations of the solutions used in the nmr studies were approximately 13% (2 mM tetramer). The concentrations of the hemoglobin solutions were determined spectrophotometrically with a Cary 14 spectrophotometer using $\epsilon_{540\ nm}$ 13.4 × 10^3 for HbCO A (Antonini, 1965). In cases where high salt concentration and low buffer concentration were desired, the dilution and ultrafiltration process was made with the desired deuterated buffer. The pD of the solution was determined by adding 0.4 pH unit to the value obtained from a Radiometer Model 26 pH meter equipped with a Beckman 39030 combination electrode (Glasoe and Long, 1960).

Method

Nmr spectra were obtained on the MPC-HF 250-MHz nmr superconducting spectrometer (Dadok *et al.*, 1970). Signal-to-noise ratios were improved by signal averaging with a Northern Scientific NS-544 digital memory oscilloscope. Chemical shifts are measured relative to the proton resonance of the residual water, HDO, and at the probe temperature of the spectrometer, 31°, the HDO signal is 4.84 ppm downfield from the customary reference, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). All chemical shifts reported are in ppm upfield from HDO and are accurate to ±0.02 ppm.

Results

In Figure 1, we show the nmr spectra of HbCO A in the region 5–7 ppm upfield from HDO. This spectral region is upfield from the resonances of the aliphatic protons in the protein and a portion of this aliphatic resonance causes the base line to rise on the left side of our spectra. Figure 1 indicates the effects of pD variation in 0.2 M potassium phosphate. At pD 7.0, there are ring-current shifted resonances at 5.18, 5.42, 5.86, 6.48, and 6.58 ppm upfield from HDO. As the pD is raised from 7 to 10.8, there is a progressive shift of the γ_1-methyl resonance of βE11 valine at 6.58 ppm to lower field.

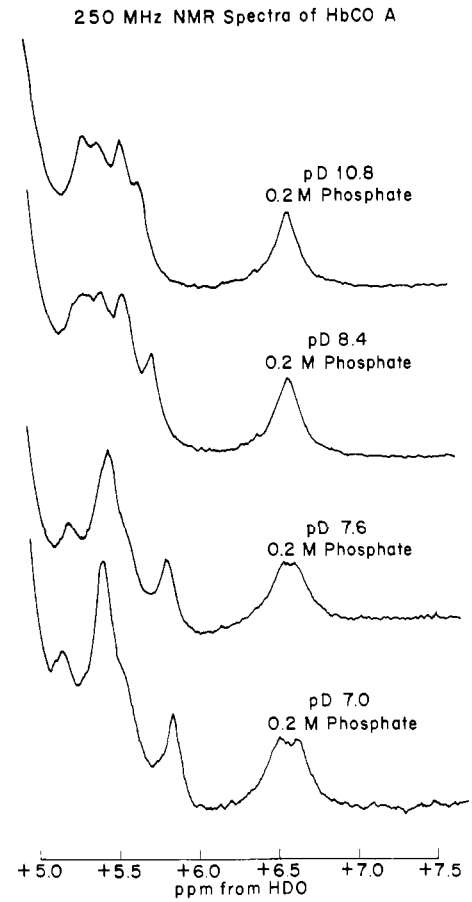

FIGURE 1: Effects of pD on the ring-current shifted proton resonances of HbCO A in 0.2 M potassium phosphate buffer at 31°.

At pD 8.4 and above, the resonances at 6.48 and 6.58 ppm have coalesced into a line at 6.48 ppm. With increasing pD, the βE11 γ_2-methyl line at 5.86 ppm also shifts to lower field going to 5.77 ppm at pD 7.6, 5.68 ppm at pD 8.4, and to 5.60 ppm at pD 10.8.

If the organic phosphates are removed by stripping with a Sephadex G-25 column, and bis-tris-HCl buffer is used, the spectra shown in Figure 2 are observed. At pD 7.1, there are resonances at 5.24, 5.38, 5.55, 5.78, and 6.51 ppm. Note that the γ_1-methyl resonances of α- and βE11 valines that appear at 6.48 and 6.58 ppm in phosphate appear as one resonance at 6.51 ppm when the phosphate is removed. As the pD is raised from 7.1 to 8.6 in bis-tris-HCl buffer, the βE11 γ_2 resonance at 5.78 shifts to lower field, appearing at 5.72 ppm at pD 7.55 and at 5.67 ppm at pD 8.6.

Figure 3 shows the results of adding DPG to stripped HbCO A in bis-tris-HCl. The spectra are essentially the same as for the 0.2 M phosphate solutions. At pD 7.0 in 0.1 M bis-tris-HCl and 0.004 M DPG, the ring-current shifted resonances appear at 5.13, 5.40, 5.81, 6.48, and 6.59 ppm. Again the peak at 5.55 ppm appears as a shoulder of the peak at 5.40 ppm. As the pD is increased, the βE11 γ_1-methyl peak at 6.59 ppm shifts to 6.54 ppm at pD 7.6 and to 6.50 ppm at pD 8.5. The βE11 γ_2-methyl resonance at 5.81 ppm shifts to 5.76 ppm at pD 7.6 and to 5.66 ppm at pD 8.5. If 0.1 M potassium phosphate or 0.004 M inositol hexaphosphate is used with stripped HbCO A in 0.1 M bis-tris-HCl, the spectrum of the ring-current shifts at pD 7.0 is similar to the spectra in 0.2 M phosphate or 0.004 M DPG shown in Figures 1 and 3. However, if only

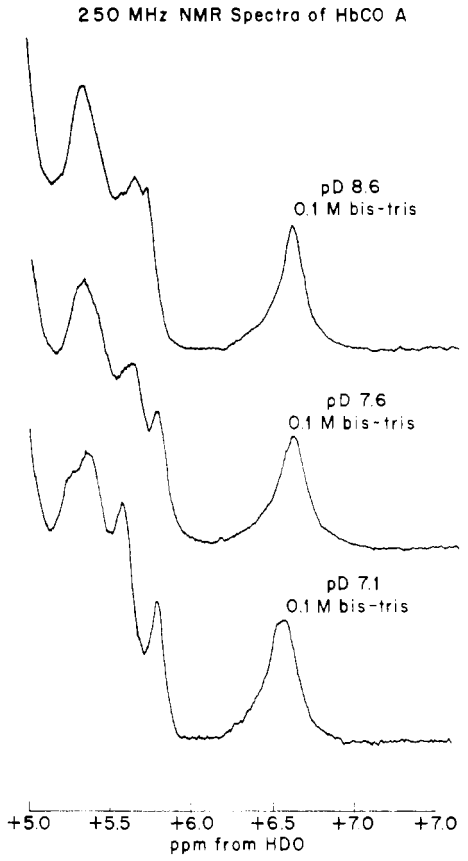

FIGURE 2: Effects of pD on the ring-current shifted proton resonances of HbCO A in 0.1 M bis-tris buffer at 31°.

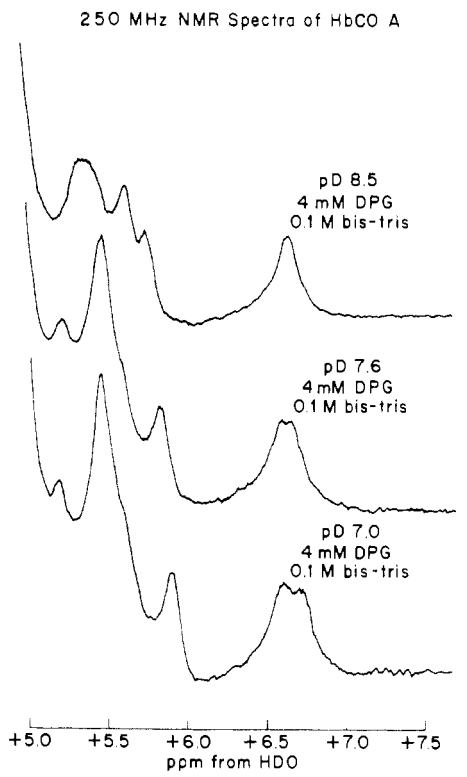

FIGURE 3: Effects of 2,3-diphosphoglycerate and pD on the ring-current shifted proton resonances of HbCO A in 0.1 M bis-tris buffer at 31°.

0.01 M potassium phosphate is used, the spectrum is similar to that of stripped HbCO A in bis-tris-HCl alone.

In Figure 4, we show the spectra of HbCO A solutions with a variety of buffers. In 0.2 M Tris-HCl buffer at pD 7.2, the resonances appear at 5.25, 5.43, 5.57, 5.79, and 6.53 ppm. In 2 M KCl plus 0.1 M bis-tris-HCl at pD 7.1, the resonances appear at 5.05, 5.33, 5.61, 6.33, and 6.44 ppm. This effect of sulfate is also produced by the use of a sulfonic acid buffer. In 0.25 M Mes at pD 6.6, the resonances appear at 5.28, 5.42, 5.57, 5.84, 6.42, and 6.52.

In Figure 5, we have compared the ring-current shifted resonances of oxy- and carbon monoxide hemoglobin. As already mentioned, the solution of HbCO A in 0.2 M (as well as in 0.1 M) phosphate at pD 7.0 has ring-current shifted resonances at 5.18, 5.42, 5.86, 6.48, and 6.58 ppm upfield from HDO. Oxyhemoglobin in 0.1 M phosphate at pD 7.3 has ring-current shifted resonances at 5.28 and 5.68 ppm and a broad resonance at 7.12 ppm. In similar solutions of HbO_2 at pD 8.2, the resonances appear at 5.38 and 5.59 ppm and the resonance at 7.10 is less broad.

Discussion

The results of our previous studies of these ring-current shifted resonances of HbCO A, in which assignments of the α- and βE11 valine methyls were made (Lindstrom *et al.*, 1972), allow us to make some important conclusions concerning the effects of ions and ligands on the structure of liganded hemoglobins. These effects are most readily observable in the relative line positions of the resonances we have assigned to the γ_1 methyls of the α- and βE11 valines. In this respect, there appear to be two forms of HbCO A. In one form the γ_1 methyls of the α- and βE11 valines have the same chemical shift. In the other form, the γ_1 methyls of the βE11 valine are approximately 0.1 ppm upfield from that of the αE11 methyl resonance. The former structure is produced in solutions of phosphate-free hemoglobin buffered by bis-Tris or Tris buffers. When DPG or IHP is present in amounts approximating the molar concentration of hemoglobin tetramer, the tertiary structure of the heme pocket is changed and the resulting nmr spectra show the γ_1-methyl resonances separated by 0.1 ppm. Inorganic phosphate and sulfate also produce this effect when present in quantities 100 times greater than the hemoglobin. Sulfonic acid buffers also shift the tertiary structure toward the form having the separated methyl resonances. These effects are not restricted to the E11 valine methyl resonances, but alter the line position of all the ring-current shifted resonances in the region 5–7 ppm upfield from HDO (Figures 1–4). The greatest effects seem to be exerted on the resonances assigned to the β heme pocket and the most noticeable perturbations are observed for the γ_1 and γ_2 methyls of the βE11 valine.

Increasing the pD of the phosphate or DPG solutions appears to change the tertiary structure of the heme pockets as if the phosphate were removed. We have also observed that the effects of sulfate and chloride ions are lost as the pD increases above 7.3. We feel that the increased pD causes these effects by deprotonating the anion binding sites and eliminating the hemoglobin–anion interaction. But this is not the only effect of increased pD. In bis-tris-HCl buffer, where there are no structural perturbations caused by anion binding, increasing the pD shifts the βE11 valine γ_2-methyl resonance to lower field. One may infer that changes in the deuterium ion concentration alter the tertiary structure of the heme pockets through a variety of mechanisms.

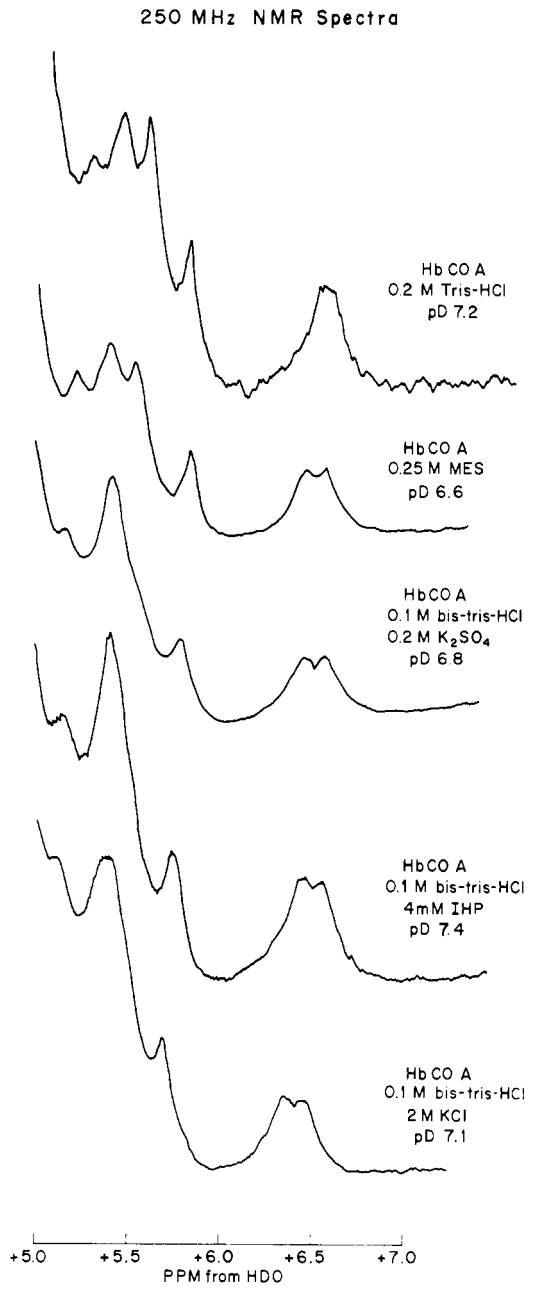

FIGURE 4: Effects of sulfate, inositol hexaphosphate, and KCl on the ring-current shifted proton resonances of HbCO A at 31°.

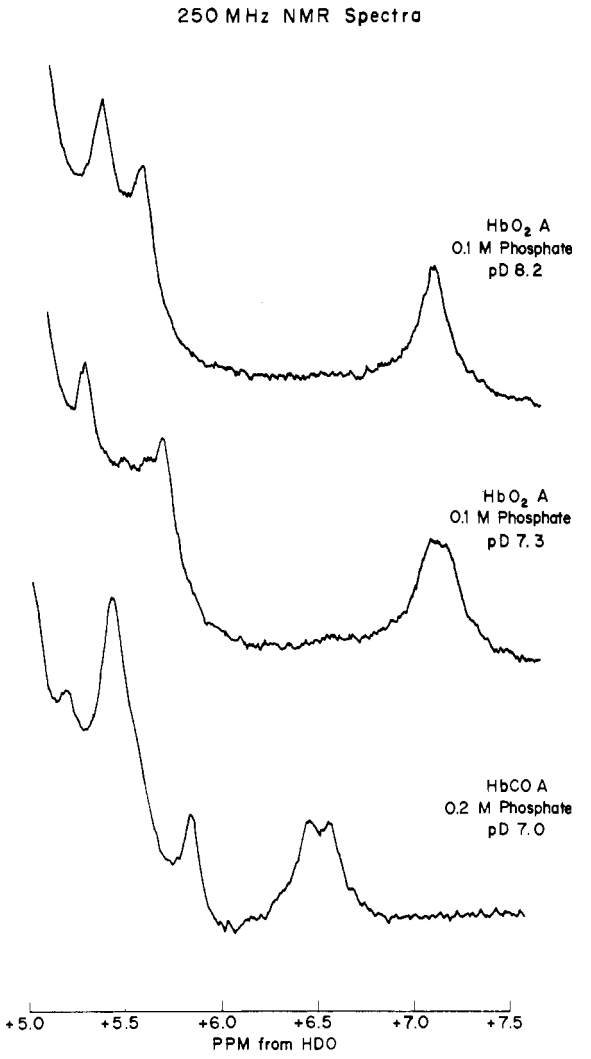

FIGURE 5: Effects of oxygen and carbon monoxide on the ring-current shifted proton resonances in Hb A in phosphate buffer at 31°.

Clearly, we are observing a dramatic change in the conformation of the heme pocket. The ring-current shifted resonances monitor directly the geometric relationship between the heme group and the amino acid side chains in the heme pockets. According to the concept of the ring-current shift (Johnson and Bovey, 1958), the closer a residue is positioned to the center of the heme group, *i.e.*, the iron atom, the greater result and ring-current shift. The calculations made by Shulman *et al.* (1970) indicate that a movement of 1 Å toward the iron atom will cause a change in the ring-current shift of up to 1 ppm depending on the direction of the movement. The changes in the ring-current shifted resonances that we observe in HbCO A under different buffering conditions indicate that the γ_1 and γ_2 methyls of the βE11 valines move closer to the iron atoms in the presence of certain anions.

The similarity of the effect produced by the anions suggests that a common binding site is involved. The number of such sites per tetramer is unknown as is the relationship between the heme pocket and the binding site. The literature on DPG binding would suggest anywhere from none to three binding sites per tetramer of HbCO A depending on the pH and ionic strength (Garby *et al.*, 1969; Tyuma *et al.*, 1971; Riggs, 1971; Chanutin and Hermann, 1969; Benesch *et al.*, 1969). Low pH and low ionic strength favor the binding of DPG to liganded hemoglobin. Information on IHP binding is more consistent. IHP binds to HbCO A and methemoglobin A in a ratio of one per tetramer (Janig *et al.*, 1971; Gray and Gibson, 1971; Tyuma *et al.*, 1971). Perutz *et al.* (1972)[2] suggested that the IHP–methemoglobin A binding site is similar to the site of DPG–deoxyHb A binding proposed by Bunn and Briehl (1970) and recently by Arnone (1972). In deoxyHb A, DPG binds to the N-terminal amino groups of the β chains, the β143 (H 21) histidines and the ϵ-amino group of one of the β82 lysines (EF6) (Arnone, 1972). In a crystallographic study of the DPG binding site in deoxyhemoglobin A, Arnone points out the presence of an anion bound between the N-

[2] Perutz, M. F., Ferst, A. R., Simon, S. R., Beetlestone, J. G., and Slade, E. F. (1972), unpublished data.

terminal amino groups of the β chains and the β82 lysine ε-amino groups. We suggest that DPG and IHP bind between the β chains of HbCO A as they do in deoxyHb A and that the inorganic phosphate and sulfate bind between the β N-terminal amino group and the β82 lysine ε-amino group, perhaps two per tetramer. We know from nmr studies of HbCO Hiroshima (β146 His→Asp) (Perutz *et al.*, 1971) that the HC3 (β146) histidine is not involved in the variation in ring-current shifted resonances with pD since the ring-current shifted spectra for HbCO A and HbCO Hiroshima in phosphate buffer at various pD values between 6 and 9 are identical (Lindstrom, Hamilton, and Ho, 1972[3]). We propose that the tertiary structural changes indicated by the changes in ring-current shifted spectra upon anion binding are caused by interactions between the anions and the β N-terminal amino groups and the β82 lysine ε-amino groups. The β82 lysine is in the EF corner and linking it to the N terminal could slide both the E and F helices with respect to the heme group. This would account for the movement of the βE11 valines upon the addition of DPG, IHP, phosphate, and sulfate.

We wish to point out the apparent relationship between ligand affinity and the presence of anions. Stripped Hb A in bis-tris or Tris buffer has a high oxygen affinity (Benesch *et al.*, 1969; Tyuma *et al.*, 1971). Addition of chloride lowers the affinity (Benesch *et al.*, 1969), addition of phosphate further decreases the oxygen affinity (Benesch *et al.*, 1969), and DPG and IHP have a still greater effect in lowering the oxygen affinity (Benesch *et al.*, 1969; Tyuma *et al.*, 1971). There appears to be a direct relationship between the ability of an anion to shift the βE11 valine closer to the iron atom and its ability to lower the oxygen affinity. We assume that the relative amount of anion needed to alter the tertiary structure is derived from the relative strength of the binding interaction. IHP binds most strongly to HbCO A followed by DPG, phosphate, sulfate, and chloride. The effects of the binding, once sufficient quantities of anion are present, seem to shift the βE11 valine methyls toward the ligand binding site. The movement of the βE11 valine methyls toward the iron atom could weaken the iron–ligand bond and reduce the observable ligand affinity. We would like to suggest that these structural changes in the heme pocket of the liganded form of Hb A contribute to the observed changes in ligand affinity.

DPG and IHP bind between the β chains and influence the equilibrium between the R and T quaternary structures in deoxyhemoglobin (Perutz, 1970, 1972; Perutz *et al.*, 1972;[3] Ogata and McConnell, 1972a,b; Ogawa and Shulman, 1971; Cassoly *et al.*, 1971; Hopfield *et al.*, 1971). We believe that DPG and IHP and inorganic phosphate and sulfate also affect the tertiary structure of the chains within the R quaternary structure. In the absence of the anions, the subunits in HbCO A take up the r tertiary structure. When DPG, IHP, phosphate, or sulfate is added, the tertiary structure of the subunits is altered to an r′ form which has a higher ligand dissociation constant than the r form. The presence of more than one r tertiary structure within the R quaternary structure emphasizes the flexible nature of the hemoglobin tetramer in solution and may indicate that a simple two-state model is insufficient in describing the process of cooperative ligand binding in hemoglobin. Indeed, caution must be taken when mechanisms are proposed that are based on oxygenation data obtained in phosphate or sulfonate buffers.

We would like to make a brief comment on the differences between the structures of the HbCO A and HbO_2 A heme pockets. According to the ring-current shifted resonances in HbO_2, a residue, possibly the E11 valine, is positioned much closer to the iron atom than in the case of HbCO A. This arrangement may contribute to the greater dissociation constant when oxygen is the ligand. It may also indicate that oxygen and carbon monoxide bind to the iron atom in a different fashion. Perhaps the oxygen molecule binds so that its long axis is parallel or at an angle to the heme plane and the carbon monoxide binds with its long axis perpendicular to the heme plane. The geometry would allow the E helix to lie closer to the heme plane in HbO_2 as the ring-current shifts indicate. It is well known that the binding constant for carbon monoxide is 200-fold greater than that of oxygen (Roughton *et al.*, 1955; Joels and Pugh, 1958) and perhaps the nearness of the methyls in the oxygenated form contributes to this great decrease in oxygen affinity relative to carbon monoxide affinity. Our results indicate that the tertiary structure of the heme pockets in hemoglobin is very sensitive to the nature of the ligands and suggest that the functional differences of various hemoglobin derivatives may be related, in part, to their differences in the environment of the ligand binding sites.

The results of this nmr study lead to some interesting conclusions. Inorganic phosphate, DPG, IHP, chloride, sulfate, and sulfonate buffers all bind to HbCO A at neutral pD and all affect the tertiary structure of the heme pockets in a characteristic manner. The changes in the line positions of the ring-current shifted resonances indicate that conditions which lower the ligand affinity also move the βE11 valine methyls closer to the ligand binding site. We suggest that the conformation of the E11 valines may contribute to the strength of the heme–ligand interaction and may account, in part, for the effects these anions produce on the ligand affinity.

Acknowledgment

We wish to extend our appreciation to Miss Dolores A. Bertoli for her excellent technical assistance.

Added in Proof

Recent kinetic studies by McDonald and Noble (1972) have demonstrated that the ligand dissociation rate is reduced within the R form of Hb A when the pH is increased from 6 to 9. This confirms our suggestion that we have observed a structural change within the R form that could alter the ligand dissociation properties.

References

Antonini, E. (1965), *Physiol. Rev. 45*, 123.
Arnone, A. (1972), *Nature (London), New Biol. 237*, 146.
Benesch, R., Benesch, R. E., and Yu, C. (1969), *Biochemistry 8*, 2567.
Berman, M., Benesch, R., and Benesch, R. E. (1971), *Arch. Biochem. Biophys. 145*, 236.
Bunn, H. F., and Briehl, R. W. (1970), *J. Clin. Invest. 49*, 1088.
Cassoly, R., Gibson, Q. H., Ogawa, S., and Shulman, R. G. (1971), *Biochem. Biophys. Res. Commun. 44*, 1015.
Chanutin, A., and Hermann, E. (1969), *Arch. Biochem. Biophys. 131*, 180.
Dadok, J., Sprecher, R. F., Bothner-by, A. A., and Link, T. (1970), Abstracts, 11th Experimental Nuclear Magnetic Resonance Conference, Pittsburgh, Pa.
Drabkin, D. L. (1946), *J. Biol. Chem. 158*, 703.

[3] Unpublished data.

Garby, L., Gerber, G., and de Verdier, C. H. (1969), *Eur. J. Biochem. 10*, 110.
Glasoe, P. D., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Gray, R. D., and Gibson, Q. H. (1971), *J. Biol. Chem. 246*, 7168.
Ho, C., Davis, D. G., Mock, B. H., Lindstrom, T. R., and Charache, S. (1970), *Biochem. Biophys. Res. Commun. 38*, 779.
Hopfield, J. J., Shulman, R. G., and Ogawa, S. (1971), *J. Mol. Biol. 61*, 425.
Janig, G. R., Ruckpaul, K., and Jung, F. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 173.
Joels, N., and Pugh, L. G. (1958), *J. Physiol. 142*, 63.
Johnson, C. E., and Bovey, F. A. (1958), *J. Chem. Phys. 29*, 1012.
Lindstrom, T. R., Noren, I. B. E., Charache, S., Lehmann, H., and Ho, C. (1972), *Biochemistry 11*, 1677.
McDonald, M. J., and Noble, R. W. (1972), *J. Biol. Chem. 247*, 4282.
Ogata, R. T., and McConnell, H. M. (1972a), *Proc. Nat. Acad. Sci. U. S. 69*, 335.
Ogata, R. T., and McConnell, H. M. (1972b), *Biochemistry 11*, 4792.
Ogawa, S., and Shulman, R. G. (1971), *Biochem. Biophys. Res. Commun. 42*, 9.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Perutz, M. F. (1972), *Nature (London) 237*, 495.
Perutz, M. F., Pulsinelli, P. D., Ten Eyck, L., Kilmartin, J. V., Shibota, S., Iuchi, J., Miyaji, T., and Hamilton, H. B. (1971), *Nature (London), New Biol. 232*, 147.
Riggs, A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2062.
Roughton, F. J. W., Otis, A. B., and Lyster, R. L. J. (1955), *Proc. Roy. Soc. (London), Ser. B 144*, 29.
Shulman, R. G., Wüthrich, K., Yamane, T., Patel, D. J., and Blumberg, W. E. (1970), *J. Mol. Biol. 53*, 143.
Tyuma, I., Imai, K., and Shimizu, K. (1971), *Biochem. Biophys. Res. Commun. 44*, 682.
Wüthrich, K., Keller, R. M., Brunori, M., Giacometti, G., Huber, R., and Formanek, H. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21*, 63.

The Nucleic Acid–Hydroxylapatite Interaction. I. Stabilization of Native Double-Stranded Deoxyribonucleic Acid by Hydroxylapatite†

Harold G. Martinson‡

ABSTRACT: The denaturation temperature (T_{mi}) of DNA can be markedly elevated by adsorption of the DNA to hydroxylapatite. This increase is always correlated with factors which stabilize the DNA–hydroxylapatite absorption interaction. For example, the hydroxylapatite T_{mi} *increases* linearly with *decreases* in the logarithm of the phosphate buffer concentration. Other factors which affect T_{mi} are changes of the hydroxylapatite, in the cation of the buffer or in the adsorption mode of the DNA. It is concluded that hydroxylapatite stabilizes the DNA double helix by interfering with the unwinding process required by denaturation.

Hydroxylapatite is widely used in the analysis of nucleic acids (see Kohne and Britten, 1971, and Bernardi, 1971). However, during the course of the present author's work which involved the analysis of RNA–DNA hybridization mixtures, the hydroxylapatite reacted in what at first appeared to be an unpredictable manner as experimental conditions were varied and different nucleic acids were used. This prompted a detailed analysis of the nucleic acid–hydroxylapatite interaction. It was found that the system is considerably more complex than is generally recognized. In this paper, the first of a series, the effect of hydroxylapatite on the thermal stability of ds-DNA is explored.

An increase in the thermal stability of ds-DNA when adsorbed to hydroxylapatite was first observed by Miyazawa and Thomas (1965). They ascribed the effect to an increase in the ionic strength of the buffer near the hydroxylapatite surface. However, the data presented below show that it is the physical immobilization in the adsorbed state which stabilizes the DNA and that electrostatic effects are not significantly involved. Thus as the concentration of phosphate is decreased the DNA becomes more firmly bound to the hydroxylapatite and the thermal stability of the DNA increases despite the reduction in ionic strength of the buffer.

Materials and Methods

Hydroxylapatite. Most of the results were obtained with the dried material "DNA-grade HTP" which was kindly supplied by Bio-Rad, Richmond, Calif. A few experiments were done

† From the Department of Biology, University of Lethbridge, Lethbridge, Alberta, Canada, and from the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received June 12, 1972.* This investigation was supported by Grant A-4926 from the National Research Council of Canada and by U. S. Public Health Service Research Grant AI 00634 from the National Institute of Allergy and Infectious Diseases.

‡ Current address: Department of Biology, University of Lethbridge, Lethbridge, Alberta, Canada.