of $(Hr)_b$ with SCN^-, but not N_3^- ions, and probably the reduction of $(Hr)_b$ by $S_2O_4^{2-}$ ion. The reactivity of methemerythrin will thus depend on factors such as pH or the concentration of added large anions that control the amount of the base form. Finally, it should be emphasized that in any investigation of the reactions of these species, particularly at lowered temperatures, sufficient time should be allowed for equilibration of the acid and base forms to be achieved. This might require several hours at 4 °C.

Registry No. BF_4^-, 14874-70-5; PF_6^-, 16919-18-9; Ph_4B^-, 4358-26-3; SCN, 302-04-5; perchlorate, 14797-73-0; dithionite, 14844-07-6.

References

Araiso, T., & Yamazaki, I. (1978) *Biochemistry 17*, 942.

Boyd, W. C. (1965) *J. Biol. Chem. 240*, 4097.

Darnall, D. W., Garbett, K., & Klotz, I. M. (1968) *Biochem. Biophys. Res. Commun. 32*, 264.

Davis, L. A., Schejter, A., & Hess, G. P. (1974) *J. Biol. Chem. 249*, 2624.

Epstein, N., & Schejter, A. (1972) *FEBS Lett. 25*, 46.

Garbett, K., Darnall, D. W., & Klotz, I. M. (1971a) *Arch. Biochem. Biophys. 142*, 455.

Garbett, K., Darnall, D. W., & Klotz, I. M. (1971b) *Arch. Biochem. Biophys. 142*, 471.

Gorman, E. G., & Darnall, D. W. (1981) *Biochemistry 20*, 38.

Hendrickson, W. A. (1978) *Nav. Res. Rev. 31*, 1.

Iizuka, T., Ogawa, S., Inubushi, T., Yonezawa, T., & Morishima, I. (1976) *FEBS Lett. 64*, 156.

Job, D., Richard, J., & Dunford, H. B. (1977) *Arch. Biochem. Biophys. 179*, 95.

Keresztes-Nagy, S., & Klotz, I. M. (1965) *Biochemistry 4*, 919.

Kihara, H., Saigo, S., Iizuka, T., & Ishimura, Y. (1978) *Biochim. Biophys. Acta 533*, 112.

Klotz, I. M., Klotz, T. A., & Fiess, H. A. (1957) *Arch. Biochem. Biophys. 68*, 284.

Kurtz, D. M., Jr., Shriver, D. F., & Klotz, I. M. (1977) *Coord. Chem. Rev. 24*, 145.

Loehr, J. S., & Loehr, T. M. (1979) *Adv. Inorg. Biochem. 1*, 235.

Meloon, D. R., & Wilkins, R. G. (1976) *Biochemistry 15*, 1284.

Muhoberac, B. B., Wharton, D. C., Babcock, L. M., Harrington, P. C., & Wilkins, R. G. (1980) *Biochim. Biophys. Acta 626*, 337.

Olivas, E., deWaal, D. J. A., & Wilkins, R. G. (1979) *J. Inorg. Biochem. 11*, 205.

Stenkamp, R. E., & Jensen, L. H. (1979) *Adv. Inorg. Biochem. 1*, 219.

Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 126*, 457.

Wilkins, R. G., & Harrington, P. C. (1983) *Adv. Inorg. Biochem. 5*, 51.

Kinetic and Equilibrium Studies on the Role of the β-147 Histidine in the Root Effect and Cooperativity in Carp Hemoglobin†

Lawrence J. Parkhurst,* Dixie J. Goss, and Max F. Perutz

ABSTRACT: In fish possessing a swim bladder, the alkaline Bohr effect is much enhanced. At pH ≤6, oxygen binding becomes noncooperative and the oxygen affinity very low. This phenomenon is known as the Root effect. In mammalian hemoglobin a major fraction of the alkaline Bohr effect is contributed by His-HC3(146)β. Perutz proposed that the Root effect is a consequence mainly of the replacement of Cys-F9(93)β in mammalian hemoglobin by Ser in fish hemoglobin. Model building showed that this Ser would stabilize the salt bridges between His-HC3 and Asp- or Glu-FG1 in the T structure by the formation of two additional hydrogen bonds. If this theory is correct, then enzymatic cleavage of His-HC3β should inhibit the Root effect. We have prepared des-His-HC3(147)β carp hemoglobin and compared its ligand-binding equilibria and kinetics with those of native carp hemoglobin and have interpreted the results in terms of the allosteric model. Our results show that removal of His-HC3(147)β halves the alkaline Bohr effect as determined from $\partial \log P_{50}/\partial$pH, accelerates the binding of CO at acid pH, and diminishes the pH dependence of the CO on rate. In native carp hemoglobin at pH 7, recombination of CO after photolytic dissociation of only part of the CO is faster than after full dissociation. In des-His carp hemoglobin at pH 7, this acceleration is not observed. These results show that removal of His-HC3 inhibits all the phenomena associated with the Root effect. Our measurements indicate that this is due to a destabilization of the T structure by the equivalent of 3.1 kcal/mol of tetramer.

The effects of pH on the oxygen affinities of the bloods and hemoglobins of a number of fish have been examined (Green & Root, 1933; Root & Irving, 1941; Scholander & Van Dam, 1954; Rossi-Fanelli & Antonini, 1960). For the bony fishes possessing a swim bladder, the pH changes altered the O_2 pressures for half-saturation (Bohr effect) and the shapes of the O_2 equilibrium curves. At low pH, the binding curves often became heterogeneous and leveled off before complete saturation could be reached. Scholander & Van Dam (1954) reported that at low pH certain fish hemoglobins remained only partially saturated when the O_2 pressure was as high as 100 atm. Tan et al. (1973) have shown how the effects for carp hemoglobin can be interpreted within the framework of the allosteric model. At low pH (5.6 or 6 in the presence of 1 mM

† From the Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304, and the Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received December 9, 1982; revised manuscript received June 7, 1983.* We thank the National Institutes of Health (Grant HL 15, 284), the National Science Foundation (Grant PCM 8003655), the Research Council, the University of Nebraska, and the Burroughs-Wellcome Foundation (travel grant to L.J.P.) for support of this research.

0006-2960/83/0422-5401$01.50/0

IHP),[1] the Hb is locked in the low-affinity T conformation in both deoxy and liganded states. At pH 9, the Hb is in the R state in both deoxy and liganded forms. At intermediate pHs, an R–T equilibrium obtains. This behavior has become known as the Root effect. Noble et al. (1970) reported the first detailed kinetic studies on a fish hemoglobin, the pooled hemoglobins of *Cyprinus carpio*, the carp. They found that the oxygen dissociation constant was more affected by changes in pH than the association constant, whereas for CO, both association and dissociation rate constants varied with pH. Tan et al. (1972) found no significant kinetic differences between the two major hemoglobins of carp. The pronounced Bohr effect in a marine annelid is also due largely to changes in the O_2 dissociation rate constant (Wiechelman & Parkhurst, 1973).

What is the origin of the Root effect? It is really just a much enhanced alkaline Bohr effect. In human hemoglobin a major fraction of the alkaline Bohr effect is contributed by His-HC3(146)β. The imidazole of this C-terminal histidine is free in the R structure, but in the T structure its pK is raised by about 1 unit because it forms a salt bridge with Asp-FG1(94)β (Perutz et al., 1969; Kilmartin et al., 1973). Enzymatic cleavage of the histidine from the β subunit reduced the alkaline Bohr effect and lowered both the allosteric constant L and K_T, the oxygen dissociation constant of the T structure (Kilmartin & Wootton, 1970; Kilmartin et al., 1978). Carp hemoglobin contains fewer histidines than human hemoglobin and Val-1α, which contributes to the alkaline Bohr effect of human hemoglobin, is replaced by acetylated serine in carp hemoglobin. The five histidines of the carp α chain are in the same locations as in human α chain and should not give rise to an enhanced Bohr effect. Apart from the heme-linked histidines that contribute no Bohr protons, the only histidines present in the β chain of carp Hb are HC3(147)β and FB4(98)β (Grujic-Injac et al., 1980). The latter is far from any other ionizable group and is known not to contribute significantly to the Bohr effect in human hemoglobin (Perutz et al., 1980). It stands to reason, therefore, that His-HC3-(147)β is likely to be a major source of the Root effect. At a meeting on allostery held in Cambridge in August 1980, Perutz proposed that the Bohr effect contributed by His-HC3(147)β might be enhanced by the replacement of Cys-F9(93)β in human by Ser-F9(94)β in fish hemoglobin. This serine could stabilize the C-terminal salt bridges of His-HC3(147)β by a pair of hydrogen bonds: its OH could donate a bond to one of the carboxylate oxygens and accept a bond from the peptide NH of the histidine (see Figure 1). These bonds would stabilize the T structure and raise the pK of the imidazole in that structure; in consequence, the Bohr effect would be increased, and L and K_T would be raised, as is required for the production of a Root effect (Perutz & Brunori, 1982).

A test of that hypothesis has become possible by the recent discovery of procedures for making native subunits of carp Hb, allowing us to carry out a number of studies on modified and hybrid hemoglobins to explore the Root effect in more detail (Parkhurst & Goss, 1983). In this paper, we report oxygen equilibria as well as O_2 and CO kinetic studies that compared carp hemoglobins and des-His-147β carp hemoglobins and interpret the findings in terms of allosteric models. The interpretation of our results rests upon the assumption that the structures of carp and human hemoglobin are closely alike. The evidence for the assumption comes from the homologies of most of the residues at the heme contacts and at the $\alpha_1\beta_2$ subunit boundary. Of the residues in contact with the hemes in human hemoglobin listed by Fermi & Perutz (1981), 16 out of 20 are identical in the α chains and 14 out of 20 in the β chains. At the $\alpha_1\beta_2$ contact, which forms the switch between the deoxy and oxy structures, 27 out of 32 residues listed by Fermi & Perutz (1981) are identical in the two species. These homologous residues would be stereochemical misfits unless the tertiary and quaternary structures superposed with a standard deviation of less than 1 Å.

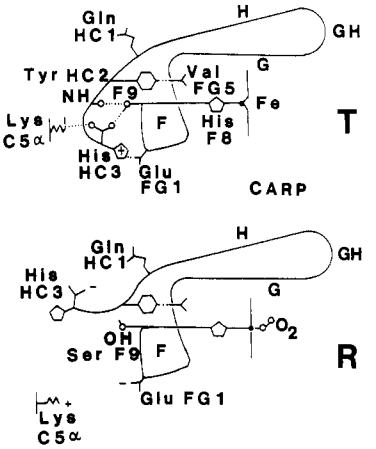


FIGURE 1: C-Terminal salt bridges showing the role of residues F9β and HC3β in the T and R structures of carp hemoglobin.

Materials and Methods

Materials. Sodium barbital buffer, pH 8.6, was freshly prepared from preweighed vials obtained from Sigma Chemical Co. (St. Louis, MO). Sephadex G-25, arginine, D-phenylalanine, dithiothreitol, glucose oxidase (from *Aspergillus niger*, type II), catalase (from bovine liver, purified powder), dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride], and Nessler's reagent (Sigma ammonia color reagent) were all obtained from Sigma Chemical Co. (St. Louis, MO). Carboxypeptidase B, type I, from porcine pancreas was supplied in solution (2.0 mg of protein/mL of solution), chromatographically purified, from Sigma Chemical Co. Histidase from *Pseudomonas fluorescens*, was also supplied by Sigma Chemical Co. Live carp (*Cyprinus carpio*) were obtained from a local supplier in Plattsmouth, NE.

Preparation of Des-His Carp β Chains and Reconstitution of Carp Hemoglobin. Carp (*C. carpio*) hemoglobin was prepared according to Tan et al. (1972). The preparation of isolated carp α and β chains is described in detail elsewhere (Goss & Parkhurst, 1983). The histidine was removed from the CO carp β chains by modification of the method of Kilmartin (1981). A total of 3–7 mL (35–100 μM, heme basis) of isolated carp β chains was equilibrated with 0.06 M barbital buffer, pH 8.6. To this solution were added 50–100 mg of D-phenylalanine and 5–10 μL of carboxypeptidase B in a small tube. The tube was sealed with a serum stopper. A needle was inserted for a vent, and a second needle was used to blow H_2O-equilibrated CO over the protein for 20 min. The two needles were removed from the serum stopper, and the solution was incubated at 37 °C for 18–24 h. An aliquot was removed for His analysis. Barbital was removed by gel filtration on Sephadex G-25. Arginine (150 mg) and dithiothreitol (1 mM final concentration) were added to the β chains. The protein was equilibrated with CO and stored at 4 °C before use. For

[1] Abbreviations: KP_i, potassium phosphate buffer; IHP, inositol hexaphosphate; +IHP, buffer contains 1 mM IHP; LFER, linear free-energy relation; des-His-Hb, carp hemoglobin obtained by removing both 147 histidines from the β-chains; Hb, hemoglobin; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

most kinetic and equilibrium measurements, the carboxypeptidase B was not removed from the sample, but arginine (20 mg/mL) was present to inhibit the enzyme. The des-His carp hemoglobin was prepared by mixing equal molar amounts of carp α chains and des-His β chains (assuming equal molar absorptivities at 420 nm for the carbonmonoxy form of the protein). All physical measurements on des-His-Hb were completed within 3 h of combining the α and β subunits. Disc gel electrophoresis showed that with Arg present as an inhibitor for the carboxypeptidase B, no detectable (<2%) cleavage of the α-chain Arg from the reconstituted hemoglobin occurred for incubation times up to 75 h at 20 °C in phosphate buffer. As a control, carp hemoglobin was reconstituted from carp β chains that had gone through the incubation procedure for removal of His but in the absence of carboxypeptidase B (see paragraph at end of paper regarding supplementary material).

Oxygen Equilibrium Measurements and Analyses. Oxygen equilibrium measurements on carp hemoglobin and on des-His carp hemoglobin were carried out in a thin-layer (0.13-mm path-length) oxygen equilibrium cell (Dolman & Gill, 1978). The concentration of the stock solution was 400–800 μM in heme. The hemoglobin was treated with dithiothreitol (1 mM) to prevent oxidation of the sulfhydryl groups, and an enzyme system (Asakura et al., 1972) was added to prevent methemoglobin formation. The pH of the sample was adjusted by adding 1 part of 1.0 M phosphate or 0.5 M Bis-Tris buffer at the desired pH to 3 parts of hemoglobin solution. Runs that showed significant met formation (>3%) or in which the thickness of the sample layer had changed were not used for further analysis. Spectra were recorded on a Cary 210 spectrophotometer interfaced to an Apple II microcomputer. Oxygen equilibria data were fit to an Adair model and a Monod, Wyman, and Changeux (MWC) allosteric model (Monod et al., 1965) by using a Fletcher–Powell (Fletcher & Powell, 1963) sum of squares minimization program. The fractional saturation function can be expressed as $Y = \mathrm{d}\ln P/\mathrm{d}\ln X$, where, for the Adair model, the binding polynomial is $1 + AX + BX^2 + CX^3 + DX^4$, where X is the oxygen concentration and $A = K_1$, $B = K_1K_2$, $C = K_1K_2K_3$, and $D = K_1K_2K_3K_4$. The K_i's are the association constants for

$$\mathrm{HbX}_{i-1} + \mathrm{X} \rightleftharpoons \mathrm{HbX}_i \qquad X = \mathrm{O}_2$$

For the MWC model, the binding polynomial P is $L(1 + cK_R^{-1}X)^4 + (1 + K_R^{-1}X)^4$, where $L = \mathrm{T/R}$ (for zero ligands bound), K_R is the oxygen dissociation equilibrium constant for the R state, and c is K_R/K_T.

Analytical Gel Filtration. Large-zone analytical gel filtration (Ackers, 1970, 1975) was used to determine the tetramer–dimer equilibrium constant for des-His carp hemoglobin. The sample (10–15 mL) was loaded onto a Sephadex G-100 column (0.6 × 50 cm) equilibrated with CO-saturated buffer–0.1 M potassium phosphate, pH 7.0. The column flow rate was 10 mL/h. The effluent volume was recorded with a Model 60 ISCO drop counter, and absorbance was measured by a Model UA-5 column monitor (ISCO, Lincoln, NE) with a dual-beam UV–vis optical unit. Carp hemoglobin (1%) and sperm whale myoglobin were applied to the column in small-zone runs as markers. The des-His hemoglobin was run in the large-zone mode at a concentration of 4 μM (heme basis).

Ligand Binding Kinetics.[2] Gas solutions were equilibrated as previously described (Steinmeier & Parkhurst, 1975). The des-His hemoglobin was sensitive to dithionite, so oxygen was removed from the solutions by addition of β-D-glucose, glucose oxidase, and catalase. Photolysis experiments were carried out with a Phase-R 2100 BXH dye laser with rhodamine 6G as the lasing dye. A Nova 2/10 minicomputer with 32K of memory was used for data acquisition (LaGow & Parkhurst, 1972). Association of oxygen and carbon monoxide and dissociation of oxygen were measured by laser photolysis. Oxygen association (k') was measured by observing the rapid phase of the reaction after photolysis of HbCO in the presence of O_2. The observing wavelength was 430 nm. Oxygen dissociation (k^*) was measured from the relaxation reaction at 420 nm as described elsewhere (Goss & Parkhurst, 1980). Oxygen was removed from the solution by addition of β-D-glucose, glucose oxidase, and catalase. CO recombination was then monitored at 425 nm. CO association was also measured in the stopped-flow apparatus by flash photolysis in which a photographic flash (Wabash type R-1140, equipped with a Sylvania R4330 electronic flash tube) was discharged 5 s after the flow-recombination reaction was over. For carp HbO_2 at pH 7, k^* was measured by flowing HbO_2 vs. a CO-saturated 0.5% dithionite solution.

Fitting Kinetic Data. The ligand-binding kinetic data were fit to one- or two-exponential decay models by a Fletcher–Powell (1963) sum of squares minimization program. The kinetics of overall oxygen dissociation (k) for carp hemoglobin (Noble et al., 1970) and of CO combination (l') by stopped-flow and recombination following photolysis were fit by allosteric models in which the R and T conformations were assumed to be in equilibrium at each stage of ligation. For carp hemoglobin, rate constants measured at pH 9 and 5.6 (or pH 6 in the presence of 1 mM IHP) were taken to represent, respectively, the appropriate rate constants for the R and T states. For the oxygen and CO kinetic experiments, expressions were derived for the concentrations of all species as a function of time in terms of the R- and T-state rate constants, L, c, and the fractional photolysis (F) (see Table II). The appropriate linear combinations of species were then fit to the observed kinetics. Choices for values of c are explained in Table II.

Results

The analyses for His and Tyr in the des-His β-chain preparations showed that at least 0.92 mol of His (but fewer than 0.03 mol of Tyr) was removed per mol of β chain. In one set of experiments, β chains from only the major carp hemoglobin (I) were used. When des-His β chains from carp Hb I were subjected to disc gel acrylamide electrophoresis, only a single band could be detected, placing an upper limit on residual unreacted β chains of 5%. A control experiment on the reconstitution procedure involved subjecting carp β chains to the incubation procedures required for the production of des-His β chains but in the absence of carboxypeptidase B. These chains were recombined with carp α chains, and an O_2 equilibrium curve was measured at pH 7.1. The Hill number (2.4) and P_{50} (13 mmHg) were within experimental error of the values obtained for native carp hemoglobin (2.42 and 14 mmHg, respectively). The value for $K_{T,D}$ for native carp HbCO (at 23 °C, 0.1 M potassium phosphate, pH 7) was 0.025 μM (Pham, 1981). Under the same conditions, $K_{T,D}$ for carp des-His-HbCO was 0.06 μM. Thus, we may neglect the presence of dimeric hemoglobin in any of the equilibrium and kinetic studies on des-His carp hemoglobin. The marked lowering of the Hill number (2.4 to 1.4) on removal of the β C-terminal His cannot be due to dissociation of the des-His

[2] The Roughton convention for rate constants is employed, where l and k refer respectively to rate constants for CO and O_2 binding, a primed rate constant denotes an association constant, and an unprimed constant denotes a dissociation constant.

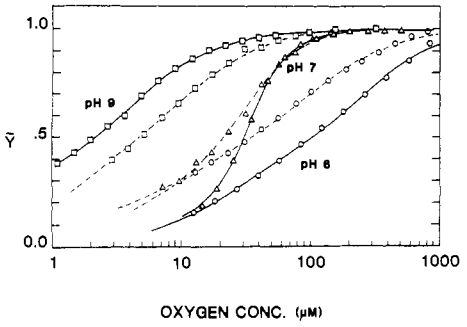


FIGURE 2: Oxygen equilibrium curves for carp and des-His carp hemoglobins. Solid lines are for carp Hb; dashed lines are for des-His-Hb. Squares denote pH near 9, triangles denote pH 7, and circles denote pH 6. The actual pH values were as follows: carp, pH 8.9; des-His, pH 8.8; carp, pH 7.00; des-His, pH 7.05; carp, pH 6.05 (+IHP); des-His, pH 6.2 (+IHP). $\bar{Y}$ is fractional saturation. The lines are the best fits to the data for the four-step Adair model.

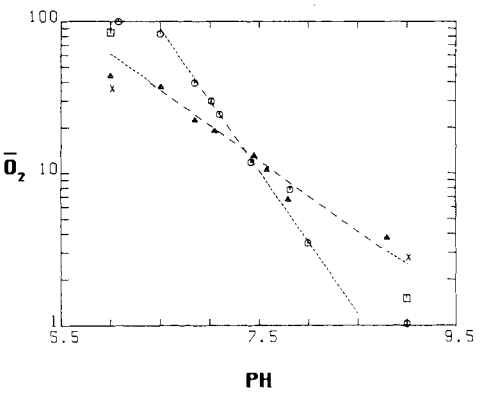


FIGURE 3: Plots of $\bar{O}_2$ (oxygen activity, micromolar, at half-saturation) vs. pH. The des-His data are triangles; the carp data are circles. The two X's and the two squares are respectively for des-His-Hb and carp Hb. They were obtained from the ratios of rate constants for oxygen dissociation (k^*) and association (k'). The long dashed lines denote the pH region over which a linear least-squares fit was made to the data to obtain n_H; the short dashed lines are extensions of these lines. For des-His-Hb, the linear pH interval was taken as pH 6.5–8.8; for carp, the interval was pH 6.85–7.4.

carp tetrameric HbO_2 to dimers.

Representative O_2 equilibrium curves are shown in Figure 2. The decrease in the steepness of the equilibrium curves for both hemoglobins at the pH extrema and the lesser sensitivity of the equilibria to pH of des-His carp compared to native carp hemoglobin are evident. Plots of log $\bar{O}_2$ vs. pH for both proteins are shown in Figure 3. $\bar{O}_2$ is the oxygen activity (μM) at half-saturation. The des-His-HbO_2 was usually unstable below pH 6.5. The two values for $\bar{O}_2$ marked by X's are for des-His hemoglobin and were obtained from laser photolysis results (k^*/k'). Analogous results for carp hemoglobin are shown as two squares on the figure. The value for k' was obtained from the rapid phase of the photolysis of HbCO in the presence of O_2 and that of k (k^*) from the slower eigenvalue that characterizes the relaxation process [see Goss & Parkhurst (1980)]. The constant k^* denotes the ligand-bound conformation or equilibrium distribution of conformations. The number of Bohr protons, n_H, per tetramer is obtained from −4 times the slope of log $\bar{O}_2$ vs. pH. We used the log $\bar{O}_2$ vs. pH plots over the pH interval 6.5–8.8 for des-His hemoglobin and 6.85–7.4 for carp hemoglobin. Beyond the pH intervals quoted above, decreased values of the Hill number were obtained, and these data were not used to obtain n_H. Values for the Adair constants and allosteric parameters for the most precise data, as well as the Bohr proton data, are

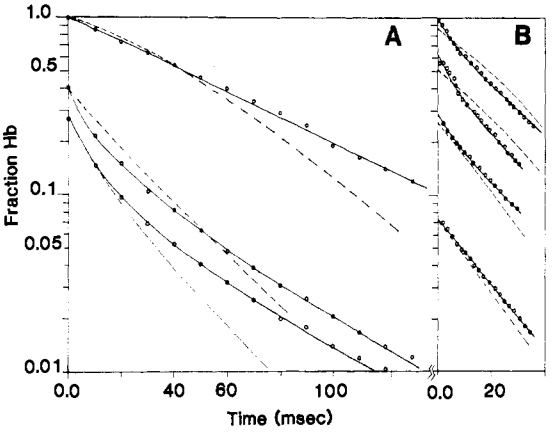


FIGURE 4: Plots of fraction unliganded hemoglobin vs. time for laser photolysis experiments on HbCO: (A) carp Hb; (B) des-His-Hb; pH 7 for both hemoglobins. The dashed lines are theoretical fits according to the allosteric model and represent the best fits according to the parameter values in Table II. The solid lines are for best fits to the data according to biexponential models. For carp Hb, c = 0.028; for des-His, c was taken as 0.28. For both proteins, the temperature was 20 °C, the buffer was 0.05 M KP_i, and the observing wavelength was 430 nm. Measurements were made in a 1-cm path-length cuvette. The protein concentration, heme basis, was 10 μM; (CO) = 81 μM.

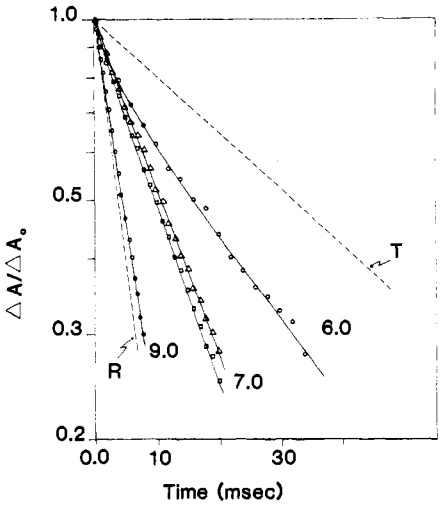


FIGURE 5: Time course of CO binding for des-His-Hb. Protein concentration was 5 μM in heme after the mixing in the stopped-flow apparatus; [CO] = 160 μM, T = 20 °C. The upper curve at pH 7 is for CO recombination following photolysis measured at the conclusion of the stopped-flow reaction. A variable small amount of rapid material was usually present in the des-His preparations. The solid lines are for best fits to the data according to biexponential models. The dashed lines are for carp Hb: (top curve) T is for pH 6 (+IHP); (bottom curve) R is for pH 9.

given in Table I. There were no significant differences in the Hill number or in the number of Bohr protons for either carp HbO_2 or des-His carp HbO_2 for 0.25 M KP_i vs. 0.13 M Bis-Tris buffer, pH 6.5–7.9.

In Figure 4 are shown results of laser-photolysis CO-recombination studies as a function of fractional photodissociation. For the carp HbCO at pH 7.0, the initial half-time for the recombination reaction decreases by a factor of 3.4 as the percent photodissociation decreases from 100% to 27%, and the reaction becomes increasingly biphasic. For des-His-HbCO, the initial rate was faster, but virtually no increase in recombination rate could be detected at pH 7.0. Also shown in Figure 4 are the best fitting theoretical fits according to the allosteric model, assuming equilibrium of R and T conformers at each stage of ligation.[3] In Figure 5 are shown the results

Table I: Properties of Carp and Des-His Carp Hemoglobins

	(A) Equilibrium Data carp Hb	des-His-Hb
n_{max} (Hill no.)	2.42 ± 0.09 (pH 7.1, 0.2 M KP_i)	1.4 ± 0.1 (pH 6.85, 0.2 M KP_i)
n_{max}	2.38 ± 0.10 (pH 6.7, 0.13 M Bis-Tris)	1.32 ± 0.1 (pH 6.6, 0.13 M Bis-Tris)
Adair constants	pH 6.85, 0.2 M KP_i	pH 6.71, 0.2 M KP_i
K_1 (μM^{-1})	0.056 ± 0.004	0.071 ± 0.02
K_2 (μM^{-1})	0.028 ± 0.003	0.004 ± 0.002
K_3 (μM^{-1})	0.0041 ± 0.001	0.015 ± 0.009
K_4 (μM^{-1})	0.079 ± 0.02	0.057 ± 0.03
allosteric fits[a]		
c	0.056 (pH 6.85–7.58, 0.2 M KP_i)	0.100 (pH 6.71–8.8, 0.2 M KP_i)
K_R^{-1} (μM^{-1})	0.196	0.11
L	3800 ± 750 (pH 6.85)	5180 ± 2000 (pH 6.71)
L	400 ± 75 (pH 7.20)	1030 ± 420 (pH 7.05)
L	3.05 ± 0.2 (pH 7.58)	983 ± 400 (pH 8.8)
n_H (Bohr protons)[b]	3.8 ± 0.1 (pH 6.85–7.4, 0.2 M KP_i)	1.9 ± 0.1 (pH 6.5–8.8, 0.2 M KP_i)
	3.6 ± 0.1 (pH 6.8–7.2, 0.13 M Bis-Tris)	1.8 ± 0.1 (pH 6.8–7.3, 0.13 M Bis-Tris)

(B) Kinetic Data carp Hb	des-His-Hb
$k'_R = 20\ \mu M^{-1}\ s^{-1}$ (pH 9)	$k'_{R'} = 21\ \mu M^{-1}\ s^{-1}$ (pH 9)
$k'_T = 8\ \mu M^{-1}\ s^{-1}$ [pH 6 (+IHP)]	$k'_{T'} = 11\ \mu M^{-1}\ s^{-1}$ [pH 6 (+IHP)]
k_T (=k^*) = 680 s^{-1} [pH 6 (+IHP)]	$k_{T'} = 380\ s^{-1}$ [pH 6 (+IHP)]
k_R (=k^*) = 30 s^{-1} (pH 9)	$k_{R'} = 56\ s^{-1}$ (pH 9)
$K_T = k_T/k_{T'} = 85\ \mu M$	$K_{T'} = 34.5\ \mu M$
$K_R = k_R/k_{R'} = 1.5\ \mu M$	$K_{R'} = 2.67\ \mu M$
$l'_T = 0.135\ \mu M^{-1}\ s^{-1}$ [pH 6 (+IHP)]	$l'_{T'} = 0.27\ \mu M^{-1}\ s^{-1}$ [pH 6 (+IHP)]
$l'_R = 1.2\ \mu M^{-1}\ s^{-1}$ (pH 9)	$l'_{R'} = 0.96\ \mu M^{-1}\ s^{-1}$ (pH 9)

[a] The allosteric fitting involved fitting multiple oxygen equilibrium curves assuming that c and K_R did not vary with pH. If K_R was allowed to vary with pH, the errors in L were often larger than than L. The fits were very poor when either K_R or c were forced to be identical for carp and des-His carp Hbs. The pH ranges for the curves are indicated. [b] A least-squares fit of the equilibrium data to a straight line was carried out to obtain the maximum value of n_H over the indicated pH ranges.

of CO combination from stopped-flow experiments in which deoxy-des-His-Hb flowed against CO at various pHs. At pH 7.0, the stopped-flow CO-combination rate was the same as for recombination following 100% photolysis. The latter was measured on the same hemoglobin in the stopped-flow apparatus within 5 s to 5 min of the CO-combination reaction. The sensitivity of the des-His carp hemoglobin to dithionite prevented our measuring the overall rate of O_2 dissociation by flowing HbO_2 vs. dithionite or measuring O_2 dissociation by O_2 pulse (Gibson, 1973). Rather, we measured the rate constant k^*, as defined above. For a hemoglobin in which $Hb(CO)_4$ and $Hb(O_2)_4$ are very largely in the R state, k^* is essentially identical with k_R. Since, for des-His-HbO_2, the

[3] Only L was varied in fitting the data shown in Figure 4A. The optimal L was essentially the same as for the L found for fitting the O_2 equilibrium data with $c = 0.0078$ (Table IIA). The mismatch shown in Figure 4A for 27% photolysis would be even greater were a smaller value of L used. A full kinetic analysis for fixed L would involve varying the four rate constants for $k_n(R \rightarrow T)$, $n = 0$–3, where n denotes the number of bound ligands. It appears, however, that the fitting in Figure 4A would be little improved by this effort for $L \sim 3800$.

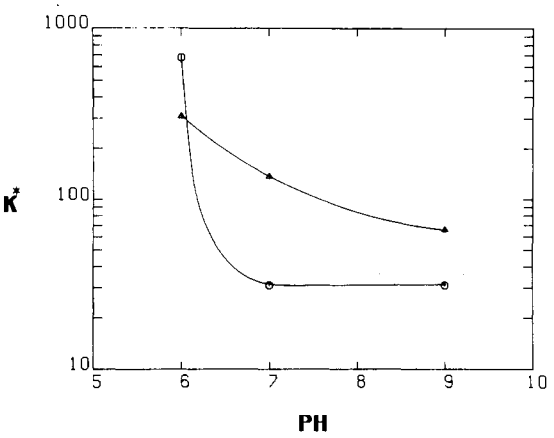


FIGURE 6: A plot of k^*, oxygen dissociation rate constant for the liganded form as a function of pH, 20 °C, for carp (circles) and des-His (triangles). Buffers: pH 9, 0.05 M borate; pH 7, 0.05 M KP_i; pH 6, 0.05 M KP_i + 1 mM IHP. The pH 7 point for carp was determined by stopped flow; the other points were determined by laser photolysis.

Hill number is quite low, we expect little difference between k and k^*. Figure 6 shows plots of k^* vs. pH for both carp hemoglobin and des-His carp hemoglobin. At pH ≥7, oxygen dissociation of des-His is faster than in the native hemoglobin, but it fails to show the steep acceleration below pH 7 that is characteristic of the native form.

Discussion

The high sensitivity of the equilibrium and kinetic properties of carp hemoglobin to pH and the decrease in that sensitivity following removal of the C-terminal His from the β subunits are evident in Figures 2 and 3. The observed decrease in n_H, the number of Bohr protons per tetramer, following removal of the β His-147, is 1.8 ± 0.2, and this decrease is essentially the same whether measured in phosphate or in Bis-Tris buffers (see Table I). The measured n_H is the difference in the number of heme-linked bound protons for the deoxy and oxy forms of the protein. Let $n_H = n(\text{deoxy}) - n(\text{oxy})$, and f_T and f_R denote mole fractions of T and R states, respectively. Then, for a Bohr effect that derives entirely from quaternary R–T changes, $n(\text{deoxy}) = n_T f_T(\text{deoxy}) + n_R f_R(\text{deoxy})$, and $n(\text{oxy}) = n_T f_T(\text{oxy}) + n_R f_R(\text{oxy})$. In terms of an allosteric model, $n(\text{deoxy}) = (n_T L + n_R)/(L + 1)$, and $n(\text{oxy}) = (n_T L c^4 + n_R)/(Lc^4 + 1)$. For carp hemoglobin at pH 7, the values for the allosteric parameters (Tables I and II) allow us to write $n_H = n_T - n_R$. For des-His, values of $c = 0.1$ and $L = 500$ allow us to write, to a good approximation, the same equality; only, for reasons given below, the T and R states for carp and des-His are not quite the same. Were they the same, we would expect $\Delta n_H = n_H(\text{carp}) - n_H(\text{des-His})$ to equal 2 only in the limit that the pK's for the histidines-147 were $\gg$7.5 for deoxy and $\ll$7.5 for oxy. If the pK of the His-147 were 7 for oxy and 8 for deoxy carp hemoglobin, then at pH 7.5 we would expect $\Delta n_H = 1.04$. If the pK's for the His in oxy and deoxy hemoglobin, respectively, were 6.5 and 8.5, then at pH 7.5, $\Delta n_H = 1.64$. Assume that the pK's for the His-147 in oxy and deoxy carp hemoglobin are respectively 7 and 8. If des-His carp hemoglobin were to have the same T and R conformations as carp hemoglobin and electrostatic interactions of His-147 with the heme-linked protons were negligible, then removal of the two His-147 residues should lower n_H (at pH 7.5) from 3.6 to 2.6. Table I shows that K_T is more affected by removal of the His residues than is K_R. Altogether, the behavior of the des-His T structure is shifted significantly toward that of the native R structure. We may express this by assigning to

Table II: Allosteric Model Fits for Kinetic Data

(A) Stopped-Flow Results

carp hemoglobin											des-His-Hb	
$c = 0.0078$[a]				$c = 0.0176$[b]		$c = 0.056$[c]		$c = 0.028$[d]				
pH	k(obsd) (s^{-1})	L	SSQ[e]	L	SSQ	L	SSQ	l'(obsd) (μM^{-1} s^{-1})	L	SSQ	pH	l'(obsd) (μM^{-1} s^{-1})
5.6	680							0.135			6 (+IHP)	0.27
6.5	100	1.1×10^6	0.036	6.4×10^4	0.044	1160	0.057	0.18	2.7×10^4	0.029		
7	50	3.4×10^3	0.049	550	0.051	37	0.052	0.22	3.8×10^3	0.046	7	0.43
7.5	35	14	0.012	5.6	0.012	1.4	0.011	0.60	6.2	0.038		
8	32	3.1	0.0023	1.3	0.0022	0.38	0.0021	0.93	1.1	0.012		
9	30							1.20			9	0.96

(B) Laser Photolysis[f]

carp HbCO[g] ($c = 0.028$, $L = 3800$)			des-His-Hb[h]							
			$c = 0.10$, $L = 30$, $l'_T = 0.135$[i]				$c = 0.1$, $L = 710$, $l'_T = 0.27$[j]		$c = 0.28$, $L = 46$, $l'_T = 0.27$	
F	fraction rapid	SSQ	F	l'(obsd)	l'(calcd)	SSQ	l'(calcd)	SSQ	l'(calcd)	SSQ
1.0	0	0.045	1.0	0.43	0.36	0.275	0.33	0.189	0.37	0.090
0.4	43 ± 4	0.046	0.58	0.49	0.59	0.036	0.40	0.135	0.44	0.055
0.27	65 ± 3	0.041	0.28	0.46	0.83	0.564	0.53	0.026	0.53	0.032
			0.07	0.51	0.91	0.568	0.62	0.067	0.59	0.034

[a] This value of c was obtained from the ratio of oxygen affinities at pH 5.6 and 9 (Noble et al., 1970). [b] This value of c was obtained from our kinetic data, Table IB. [c] This value for c was obtained from the equilibrium fitting, Table IA. [d] This value was obtained from ratios of the rate constants at pH 5.6 and pH 9 (Noble et al., 1970). [e] SSQ is the sum of squared residuals for 20 points equally spaced in time over three half-lives for the normalized observed data. [f] Observed and calculated curves are shown in Figure 4. The experimental conditions are in the caption for Figure 4. F is fraction photolysis. [g] For the data fitting, $l'_T = 0.135$ μM^{-1} s^{-1}, and $l'_R = 1.2$ μM^{-1} s^{-1}. The normalized observed data were fit well (SSQ = 0.0001) to the following one-parameter (ϕ) model: $\phi \exp(-l'_4[CO]t) + (1 - \phi) \exp(-l'[CO]t)$ where ϕ is the fraction of rapidly reacting material, $l' = 0.22$ μM^{-1} s^{-1}, the observed apparent rate constant at pH 7 from stopped-flow data, and $l'_4 = 1.15$ μM^{-1} $s^{-1} = (Lc^4 l'_T + l'_R)/(1 + Lc^3)$. [h] The CO association data were always slightly biphasic. For theoretical fitting, the rapid phase was ignored and only the slow phase, represented by a single exponential decay, was fit. In the fitting, $l'_R = 0.96$ μM^{-1} s^{-1}. The values for l'(calcd) are from the best one-exponential fits to each theoretical curve. Except for the first theoretical curve (SSQ = 0.136), all theoretical curves could be represented very well by single exponentials over three half-lives (sum of squares <0.01 and often <0.0001). The units for l' in the table are μM^{-1} s^{-1}. [i] This is the value for carp Hb at pH 5.6. [j] This is the observed value for des-His Hb at pH 6 (+IHP).

it a fraction of R structure that reduces the number of Bohr protons bound to deoxy-des-His at loci other than His-147β. If we assume that carp and des-His carp hemoglobins have the same R structure, but that the T of des-His is best described as having 30% R character and that n_H varies linearly with the fraction of R state in the deoxy form, the n_H(des-His) = (0.7)(2.6) = 1.8. Thus, removal of the His-147 residues produces a *larger* effect than would be expected from the contribution of the His in the native protein because its removal also produces a shift in the quaternary equilibria.

In fitting both the equilibrium and the kinetic data, we tested models that assumed that the T and R states for des-His carp hemoglobin and carp hemoglobin were the same (same parameter c) and that differences in the kinetics derived entirely from changes in L and models that allowed c to differ for the two proteins. Equilibrium data could not be fit at all well with c and K_R or just c the same for carp and des-His carp hemoglobins. Optimal fits of the laser photolysis results for both carp and des-His hemoglobins at pH 7 were obtained not only for c(des-His) approximately 10 times c for carp hemoglobin but also for L(des-His) approximately one-fourth to one-thirtieth that for carp Hb, indicating that the R–T conformational equilibrium is poised much more toward the R (or from a modified T, T′, to R′, a modified R) conformation for the des-His protein. Figure 4 and Table II, photolysis results, show that the allosteric model does not fit the kinetic results in detail but merely exhibits, for both proteins, the same qualitative dependence of the rates on fractional photolysis. Our model assumed that the conformational equilibria were established infinitely rapidly following ligation or loss of ligand. At 100% photolysis, the observed data for carp hemoglobin do not show the predicted acceleration after an initial lag phase, suggesting that T → R is slow for early stages of ligation. At 50% photolysis, the CO-recombination rate is too fast for the rapidly equilibrating model, suggesting that a larger fraction of $Hb(CO)_3$ and $Hb(CO)_2$ initially have the R structure than assumed in the model. For the CO association kinetics, the lack of fit may derive from conformational changes that are slow with respect to ligation and/or small departures of the $(TX_n)/(RX_n)$ equilibria from the values Lc^n required by the allosteric model. The allosteric model, with rapid conformational equilibria, provides a framework for interpreting the observed oxygen dissociation kinetics. Table II gives the observed (which are also the calculated) apparent first-order rate constants for the dissociation of oxygen from carp hemoglobin, along with the best fitting values of the allosteric parameter L for choices of c. Overall O_2 dissociation from carp HbO_2 can be fit reasonably well by the allosteric model. The CO-combination curves in Figure 5 show that the binding reaction is sensitive to changes in pH in both native and des-His carp hemoglobin but that removal of the β His-147 decreases the range of the variation. Figure 6 shows values for the apparent rate constant k^* for O_2 release from the ligand-bound form of carp and des-His carp hemoglobins. At pH 6 (+IHP) and 9, the values should correspond to k_T and k_R respectively. At pH 7, k^* should equal $(k_R + k_T Lc^4)/(1 + Lc^4)$. The calculated value for k^* is 30 s^{-1} for either pair ($L = 3400$, $c = 0.0078$; $L = 37$, $c = 0.56$) and is in excellent agreement with the data (31 s^{-1}). There is a reduced span for the ranges of k^* and l' for des-His-Hb values compared to carp hemoglobin data. In terms of an allosteric model, this reduction can be accommodated either by changing c (and L), so that the affinity of the T state for des-His is different from that for carp hemoglobin, or by fixing c to be the same for

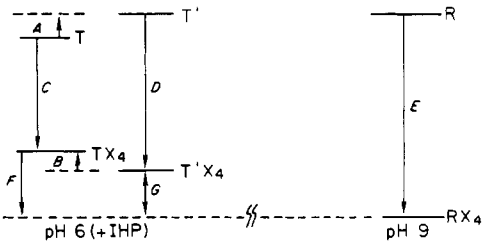
both proteins but varying L differently with pH for the two hemoglobins, so that values of L and Lc^4 nearer unity are required at all pHs, including pH 9 and 6, for des-His than for carp Hb. As for the CO kinetics, the latter procedure did not give good fits to the data; rather, it was necessary to make c for des-His much larger than that for carp Hb and assign the rate constants of des-His hemoglobin at pH 6 and 9 to states T′ and R′ rather than to an equilibrium mixture of T and R. For des-His-Hb, the reduction in the Hill number at pH 7, where cooperativity appears maximal, arises *both* from the larger value of c for des-His-Hb and from a smaller value of L compared to the values required for carp Hb.

According to a linear free-energy analysis (Szabo, 1978) of the Bohr effect, we can calculate "α", the fractional coordinate, for both carp and des-His carp hemoglobins if we know $\bar{O}_2$ values and k^* values for the pH extremes where cooperativity vanishes. The parameter α is calculated from $\log (k^*_T/k^*_R) = (1 - \alpha) \log (K_T/K_R)$, where the K's are O_2 equilibrium dissociation constants and α varies from 0 to 1. There is some variation in the reported values of $\bar{O}_2$ for carp Hb at low pH (Noble et al., 1970; Tan et al., 1972; Chien & Mayo, 1980; this work) owing to instability of the protein. If we use our values from Table I, α is 0.23 for carp Hb and 0.25 for des-His carp Hb, showing that the transition state for O_2 binding occurs early in the ligation process and at essentially the same stage for both proteins. For both hemoglobins, the Bohr effect derives mainly from changes in the O_2 dissociation rate constant. For carp Hb, data from Noble et al. (1970) for CO binding yield a value for α of 0.53, implying a later transition state for CO binding and about equal involvement of both ligand-association and -dissociation processes in the Bohr effect.

According to Perutz & Brunori (1982), the Root effect is due mainly to two additional hydrogen bonds that stabilize the C-terminal salt bridges of His-HC3(147)β with Glu-FG1(95)β and Lys-C5(40)α in the T structure. These are provided by Ser-F9(94)β, which donates a hydrogen bond to the carboxylate oxygen that is not bonded to Lys-C5(40)α and accepts a hydrogen bond from the peptide NH of His-HC3-(147)β (Figure 1). The inhibition of the Root effect, the reduction of the Bohr effect, and the lowering of L and K_T in des-His carp Hb at acid pH are all consistent with that theory. Acid pH strengthens the salt bridge between His-HC3(147)β and Glu-FG1(95)β in the T structure by giving the imidazole a positive charge; alkaline pH loosens it by discharging the imidazole. In the *human* R structure and, by implication, also in the R structure of carp hemoglobin, Lys-C5(40)α moves 13 Å away from His-HC3(147)β so that the salt bridge with the carboxylate of His-HC3(147)β cannot be made. However, we cannot exclude the possibility that in the presence of Ser-F9(94)β, the imidazole of His-HC3(147)β could form the salt bridge with Glu-FG1(95)β at acid pH also in the R structure, thus raising K_R. All these effects would disappear in des-His carp hemoglobin.

Studies of abnormal and chemically modified hemoglobins have shown that rupture of any bond that constrains the T structure also lowers K_T and L, even in the absence of any stereochemical changes detectable by X-ray analysis (Fermi & Perutz, 1981). Our results therefore do not imply that des-His carp Hb has a conformation different from that of native carp Hb, except of course at the C-terminus of the β chain, but merely that the T structure of des-His carp Hb is less constrained and therefore does not resist the changes in tertiary structure that accompany ligand binding as strongly as native carp Hb. Thermodynamically, this corresponds to a raising of the free energy of the T state.

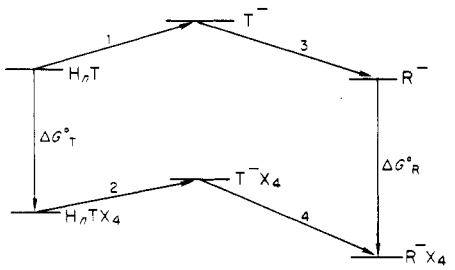
Chart I



One may ask to what extent the β-chain C-terminal His helps stabilize the T state in this model. Figure 1 shows that there is a straight, direct link running from Ser-F9(94)β via the proximal histidine to the heme iron. At pH 6, removal of His-147 and of its hydrogen bonds to Ser-94 may therefore loosen directly the constraints at the heme iron and raise its ligand affinity. At pH 9, carp hemoglobin has the R structure where the C-terminal salt bridge in the β chain does not exist. Several of our observations are consistent with these results obtained by model building. First, the fits of the oxygen equilibrium data to the allosteric model require that c, the ratio of the ligand affinities in the T and R states for des-His-Hb, be at least twice that for carp Hb. Second, the fitting of the des-His data for CO association by laser photolysis requires that c be on the order of 0.28, about 10 times that calculated for carp Hb on the basis of CO kinetic data (Table II, Figure 4). If α for binding of CO to des-His-Hb is also 0.53, then c would be 0.09, 3 times that for carp Hb. Third, the CO association rate constant, l', at pH 6 (+IHP) where the protein is noncooperative, is about twice that for carp Hb, because the T structure is less constrained than in carp hemoglobin. A similar conclusion is reached in comparisons of k^* at pH 6 (+IHP) for carp and des-His carp hemoglobins. According to a linear free-energy relation (LFER) analysis, a value of α for O_2 binding of 0.25 implies that the O_2 off rate constants (and hence, see above, the Bohr effect) should be particularly sensitive to structural changes in the *unliganded* forms of carp Hb at pH 6 and 9. We propose that the large difference between carp Hb and des-His Hb at acid pH arises from the fact that in carp hemoglobin His-147 can maintain a large tension at the heme irons, at least during the first quarter of Fe–O_2 bond formation. Removal of the His should lower the tension at the heme irons and decrease k^* (and hence K_T) toward R-state values. A tertiary change following ligation in the T state might facilitate loss of the proton on the imidazole (see below).

Chart I summarizes the free-energy changes involved. The various transitions pertaining to ligation, T → $T(O_2)_4$, etc., are the standard-state (1 M) free-energy changes per mole of tetramer for the reactions of protein with ligand in the various buffers. The species T and T′ are at pH 6 in 1 mM IHP; R and RX_4 are at pH 9 in 0.05 M borate. Whereas we know from the binding isotherms the energy changes for a given species, we are uncertain about the relative positions of the energy levels of the unliganded or liganded forms. At pH 6, the R state is not shown for carp. The unliganded R state would be located $RT \ln L$ above that of T, where L is probably $>10^6$. Similarly, at pH 9, the T state is not shown since it lies indeterminately higher than R. In terms of the Ser–His hydrogen-bonded model, T should lie below T′, the latter being reached by deprotonating the imidazolium moiety and any other changes in the protein that would arise from removal of His-147. The positioning of T′ above T by an energy A, however, leaves uncertain whether the free-energy change $\Delta G°$

Chart II



for $T(O_2)_4 \rightarrow T'(O_2)_4$ is positive, zero, or negative, since ln $K_{T'} <$ ln K_T. We can argue that $G°_{T}(O_2)_4 \leq G°_{T'}(O_2)_4$ because some of the T state constraints in carp are lacking in des-His hemoglobin.

Perutz et al. (1978) have shown that the difference spectrum between 287 and 293 nm of carp HbCN at pH 9 vs. pH 6 (+IHP) reflects differences between the liganded R and T states. In Chart I, if we neglect differences between liganded R-state carp Hb and des-His carp Hb, this corresponds to F. If such spectroscopic changes were linearly dependent on free-energy changes, then G/F is 0.5, the ratio we obtain from difference spectra for carp and des-His carp HbCN complexes.

We can rewrite Chart I and consider one of several possible cycles relating the T- and R-state ligation energetics. In Chart II, we let X represent a bound ligand and H_n the n Bohr protons. From measurements, we obtain $\Delta G°_T$, $\Delta G°_R$, and n. The difference $\Delta G°_T - \Delta G°_R$ is $1 - 2 + 3 - 4$ and is simply the overall heterotropic free-energy change, $\Delta G°_{12}$, measuring the total apparent free energy of interaction of ligand X and Bohr protons per mole of tetramer [see Wyman (1964)]. In our model, this is $-4RT \ln (K_R/K_T) = -4RT \ln c$. We might consider $\Delta G°_{12}$ as a measure of the net constraints to ligation (or a "barrier") associated with proton binding. We can then relate the $\Delta G°_{12}$ values for carp and des-His carp Hbs. Their difference measures the difference in net barrier heights for ligation for acid and alkaline oxygenation. For carp Hb, $\Delta G°_{12}$ is 9.4 kcal/mol of tetramer; for des-His carp Hb, it is 6.0 kcal/mol of tetramer. Alternatively, we might define the free energies of carp Hb at pH 6 (+IHP) and at pH 9 as characterizing T and R, respectively, and measure the thermodynamic fraction of R state (f_R) in terms of free energies:

$$f_{Ri} = (\Delta G°_T - \Delta G°_i)/(\Delta G°_T - \Delta G°_R)$$

With this functional (as opposed to structural) definition, for the des-His carp Hb at pH 6 (+IHP), $f_R = 0.22$; at pH 9, $f_R = 0.86$. From LFER analysis, we can use l' values in this functional sense [assuming the same α (0.53) for carp and for des-His carp Hb] to calculate

$$f_{Ri} = \log (l'_i/l'_{T(carp)})/\log (l'_{R(carp)}/l'_{T(carp)})$$

from which we obtain for des-His carp Hb, at pH 6 (+IHP), $f_R = 0.31$; and at pH 9, $f_R = 0.90$. These various measures suggest that around 0.5 of the energetics of the R–T change in carp might be ascribed to participation of the β His-147.

In the model proposed by Perutz for the Root effect, the β chain has a strong tendency to assume the tertiary t conformation at low pH. Recent studies (Parkhurst & Goss, 1983) on hybrid human–carp hemoglobins provide additional support for this proposal. In the hybrid α(human)β(carp), the CO–hemoglobin at pH 6 can be well described in terms of a "Koshland intermediate": a tertiary r conformation for the human α chains and a tertiary t conformation for the carp β chains. A similar mixed species for unliganded hemoglobins can be detected at pH 7 for this hybrid following laser photolysis. In this case, the β chains, although initially r state, revert to t state while the human α chains are still in the r state. In both cases, the β chains show a marked tendency to assume a t conformation independent of the conformation of the complementary α chain, perhaps because the imidazoles of His-HC(147)β form the salt bridges with Glu-FG1(95)β, as suggested.

Supplementary Material Available

A description of the isolation of carp β chains, the analyses for histidine, and the details of fitting the CO and oxygen kinetic data (4 pages). Ordering information is given on any current masthead page.

Registry No. His, 71-00-1.

References

Ackers, G. K. (1970) *Adv. Protein Chem. 24*, 343–446.

Ackers, G. K. (1975) in *The Proteins I* (Neurath, H., Hill, R. L., & Boeder, C.-L., Eds.) pp 1–94, Academic Press, New York.

Asakura, T., Tamura, M., & Shin, M. (1972) *J. Biol. Chem. 247*, 3693–3701.

Chien, J. C. W., & Mayo, K. H. (1980) *J. Biol. Chem. 255*, 9790–9799.

Dolman, D., & Gill, S. J. (1978) *Anal. Biochem. 87*, 127–134.

Fermi, J., & Perutz, M. F. (1981) *Hemoglobin and Myoglobin. Atlas of Molecular Structures in Biology*, Series 2, Clarendon Press, Oxford.

Fletcher, R., & Powell, M. J. D. (1963) *Comput. J. 6*, 163–168.

Gibson, Q. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1–4.

Goss, D. J., & Parkhurst, L. H. (1980) *J. Biochem. Biophys. Methods 3*, 315–322.

Goss, D. J., & Parkhurst, L. J. (1983) *Biochemistry* (in press).

Green, A. A., & Root, R. W. (1933) *Biol. Bull. (Woods Hole, Mass.) 64*, 383–404.

Grujic-Injač, B., Braunitzer, G., & Stangl, A. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1629–1639.

Kilmartin, J. V. (1981) *Methods Enzymol. 76*, 167–171.

Kilmartin, J. V., & Wootton, J. F. (1970) *Nature (London) 228*, 766–767.

Kilmartin, J. V., Breen, J. J. Robers, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246–1249.

Kilmartin, J. V., Imai, K., Jones, R. T., Faruqi, A. R., Fogg, A., & Baldwin, J. M. (1978) *Biochim. Biophys. Acta 534*, 15–25.

LaGow, J., & Parkhurst, L. J. (1972) *Biochemistry 11*, 4520–4525.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.

Noble, R. W., Parkhurst, L. J., & Gibson, Q. H. (1970) *J. Biol. Chem. 245*, 6628–6633.

Parkhurst, L. J., & Goss, D. J. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Ed.) Elsevier/North-Holland, New York.

Parkhurst, L. J., & Goss, D. J. (1983) *Biochemistry* (in press).

Perutz, M. F., & Brunori, M. (1982) *Nature (London) 299*, 421–426.

Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., & Kilmartin, J. V. (1969) *Nature (London) 222*, 1240–1243.

Perutz, M. F., Sanders, J. K. M., Chenery, D. H., Noble, R. W., Pennelly, R. R., Fung, L. W. M., Ho, C., Giannini, I., Porschke, D., & Winkler, H. (1978) *Biochemistry 17*, 3640–3652.

Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J. G., & Rollema, H. S. (1980) *J. Mol. Biol. 138*, 649–70.
Pham, T. T. (1981) Ph.D. Dissertation, University of Nebraska.
Root, R. W., & Irving, L. (1941) *Biol. Bull. (Woods Hole, Mass.) 81*, 307–323.
Rossi-Fanelli, A., & Antonini, E. (1960) *Nature (London) 186*, 896–897.
Scholander, P. F., & Van Dam, L. (1954) *Biol. Bull. (Woods Hole, Mass.) 107*, 247–259.
Steinmeier, R. C., & Parkhurst, L. J. (1975) *Biochemistry 14*, 1564–1572.
Szabo, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2108–2111.
Tan, A. L., DeYoung, A., & Noble, R. W. (1972) *J. Biol. Chem. 247*, 2493–2498.
Tan, A. L., Noble, R. W., & Gibson, Q. H. (1973) *J. Biol. Chem. 248*, 2880–2888.
Wiechelman, K. J., & Parkhurst, L. J. (1973) *Biochem. Biophys. Res. Commun. 52*, 1199–1205.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 241.

Interactions of Porphyrins with Nucleic Acids†

Robert F. Pasternack,* Esther J. Gibbs,*,‡ and Joseph J. Villafranca§

ABSTRACT: The interactions of nucleic acids with water-soluble porphyrins and metalloporphyrins have been investigated by stopped-flow and temperature-jump techniques. Both natural DNA (calf thymus) and synthetic homopolymers [poly(dG-dC) and poly(dA-dT)] have been employed. The porphyrins studied belong to the tetrakis(4-*N*-methylpyridyl)porphine (H_2TMpyP-4) series and can be divided into two groups: (i) those which have no axial ligands when bound to nucleic acids [e.g., Ni(II), Cu(II), and the nonmetallic derivatives] and (ii) those which maintain axial ligands upon binding [e.g., Mn(III), Fe(III), Co(III), and Zn(II) derivatives]. The reaction of both axially and nonaxially liganded porphyrins at AT sites is too rapid to be measured by the kinetic methods utilized, whereas at GC sites the interaction of the nonaxially liganded porphyrins is in the millisecond time range and can be monitored by both stopped-flow and temperature-jump techniques. These results corroborate previous static studies, utilizing visible spectroscopy and circular dichroism, which indicate that the formation of an intercalated complex occurs only at GC base pair sites with porphyrins which do not possess axial ligands. With all the porphyrins investigated, the complexes formed at AT sites are envisioned as being of an "external" type involving some degree of overlap between the porphyrin and the bases of the duplex. In relaxation experiments of poly(dG-dC) with H_2TMpyP-4, a large, reproducible effect is observed which can be analyzed as a single exponential. Rate constants for association and dissociation of the H_2TMpyP-4/poly(dG-dC) complex are 3.7×10^5 M^{-1} s^{-1} and 1.8 s^{-1}, respectively. Relaxation studies of mixtures of poly(dA-dT) and poly(dG-dC) with H_2TMpyP-4 indicate that the transfer of the porphyrin from one homopolymer to another occurs via a mechanism involving dissociation rather than direct transfer. With calf thymus DNA and H_2TMpyP-4, a multiphasic relaxation profile is observed. Both the amplitude and concentration dependencies of these kinetic effects indicate that the processes being observed involve the redistribution of porphyrin among the various sites on the polynucleotide. A comparison of the relaxation times obtained for this system with those obtained for mixtures of the synthetic homopolymers with H_2TMpyP-4 strongly suggests that, for the natural system, the porphyrin can move from site to site without first dissociating into the solvent medium.

Interactions of synthetic and natural nucleic acids with tetrakis(4-*N*-methylpyridyl)porphine (H_2TMpyP-4, Figure 1) and several of its metal derivatives have been studied in some detail (Fiel et al., 1979; Fiel & Munson, 1980; Pasternack et al., 1983). The combined evidence from thermodynamic, circular dichroic, and visible spectroscopic data indicates that the type of complex formed depends upon the composition of neighboring base pairs of the nucleic acids and the detailed structural features of the metalloporphyrin (Pasternack et al., 1983). It has been suggested that a truly intercalated species requires guanine/cytosine (GC) base pairs and porphyrin moieties which have no axial groups. Thus, H_2TMpyP-4, Cu^{II}TMpyP-4, and Ni^{II}TMpyP-4 all interact extensively with GC regions whereas Zn^{II}TMpyP-4, Mn^{III}TMpyP-4, Fe^{III}TMpyP-4, and Co^{III}TMpyP-4 do not. Also worth noting is that tetrakis(2-*N*-methylpyridyl)porphine (H_2TMpyP-2), for which the rotational barrier for the peripheral pyridyl groups is very large (Eaton & Eaton, 1975; Eaton et al., 1978), also does not interact with GC regions (Pasternack et al., 1983).

A fundamentally different type of complex is formed at adenine/thymine (AT) regions of nucleic acids. This latter type of interaction leads to an "externally" bound complex in which the porphyrin is located in a groove and/or is only partially intercalated. In either case, evidence exists for some degree of drug/nucleic acid base interaction in addition to the anticipated Coulombic attraction of the positive porphyrin periphery for the negative phosphate backbone. It may well be that upon formation of such an externally bound complex,

† From the Department of Chemistry, Swarthmore College, Swarthmore, Pennsylvania 19081. *Received February 1, 1983.* This research has been supported by a grant from the National Institutes of Health (GM 17574 to R.F.P.).
‡ Present address: Department of Chemistry, Goucher College, Towson, MD 21204.
§ J.J.V. is an Established Investigator of the American Heart Association. Permanent address: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

0006-2960/83/0422-5409$01.50/0