Burton, K. (1956) *Biochem. J. 62*, 315–323.
Capony, F., & Rochefort, H. (1975) *Mol. Cell. Endocrinol. 3*, 233–251.
Carlstedt-Duke, J., Okret, S., Wrange, O., & Gustafsson, J. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4260–4264.
Evans, E., Baskevitch, P. P., & Rochefort, H. (1982) *Eur. J. Biochem. 128*, 185–191.
Garcia, M., Greene, G., Rochefort, H., & Jensen, E. V. (1982) *Endocrinology (Baltimore) 110*, 1355–1361.
Greene, G. L., Fitch, F. W., & Jensen, E. V. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 157–161.
Greene, G. L., Nolan, C., Engler, J. P., & Jensen, E. V. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5115–5119.
Jordan, V. C., Collins, M. M., Rowsby, L., & Prestwich, G. (1977) *J. Endocrinol. 75*, 305–316.
Layne, E. (1957) *Methods Enzymol. 3*, 444–454.
Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J., & Pratt, W. B. (1979)) *J. Biol. Chem. 254*, 11884–11890.
Mann, H. B., & Whitney, D. R. (1947) *Ann. Math. Stat. 18*, 52–54.
Moncharmont, B., Su, J. L., & Parik, I. (1982) *Biochemistry 21*, 6916–6921.
Nishigori, H., & Toft, D. (1980) *Biochemistry 19*, 77–83.
Rochefort, H., & Borgna, J.-L. (1981) *Nature (London) 292*, 257–259.
Rochefort, H., Borgna, J.-L., & Evans, E. (1983) *J. Steroid Biochem. 19*, 69–74.
Weichman, B. M., & Notides, A. C. (1977) *J. Biol. Chem. 252*, 8856–8861.
Westley, B., & Rochefort, H. (1980) *Cell (Cambridge, Mass.) 20*, 353–362.
Westphal, H. M., Moldenhauer, G., & Beato, M. (1982) *EMBO J. 1*, 1467–1471.
Yamamoto, K. R., & Alberts, B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2105–2109.

Oxygen Equilibrium Studies on Carp–Human Hybrid Hemoglobins†

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ABSTRACT: Hybrid hemoglobins have been made in which one chain is derived from human hemoglobin and the other from carp hemoglobin. Both hybrid hemoglobins show low cooperativity in oxygen binding. Hybrid I (α carp:β human) has a very small Bohr effect, whereas hybrid II (α human:β carp) has a Bohr effect nearly as large as that for human hemoglobin. Both hemoglobins have P_{50}'s more closely resembling carp hemoglobin than human hemoglobin in the region of pH 7, and for both hybrids, as for carp, cooperativity virtually disappears at acid and alkaline pHs. Since both hybrids are formed from chains derived from cooperative parent hemoglobins, it is difficult to account for the low cooperativity in terms only of the T-state salt bridges and the α_1–β_2 contacts involved in the R–T switch region. We suggest that the F9 Ser in the carp β-chain as well as α_1–β_1 interactions is important in controlling the allosteric transitions in these hybrids.

Oxygen binding equilibria have been extensively studied in both human and carp hemoglobins (Roughton & Lyster, 1969; Imai & Yonetani, 1975; Imai, 1973; Noble et al., 1973; Tan et al., 1973; Chien & Mayo, 1980; Parkhurst et al., 1983). In human hemoglobin, the cooperativity is virtually unchanged over the pH range 6–9, and the ligation has frequently been interpreted to first order in terms of R and T quaternary conformations (Shulman et al., 1975; Baldwin, 1975). Within this framework, the Bohr effect corresponds to a shift of the R–T equilibrium toward T as the pH is decreased from 9 to 6. In many fish hemoglobins, the pronounced Bohr effect is known as the "Root effect" (Root, 1931; Root & Irving, 1941, 1943). At pH 6 (+IHP),[1] carp hemoglobin, in both deoxy and liganded forms, is in the T state; at pH 9, both deoxy and liganded forms are in the R state (Tan et al., 1972, 1973). At these extremes of pH, the hemoglobin is noncooperative in oxygen or CO binding. At intermediate values of pH, an equilibrium exists between the R and T forms, and cooperativity is observed in ligand binding. The importance of the β-93 Ser in carp and its interaction with His-147β have recently been discussed (Parkhurst et al., 1983; Perutz & Brunori, 1982). We were interested in observing the ligation properties of hybrid hemoglobins made by combining complementary chains from these two cooperative parent hemoglobins. We report in this paper the oxygen equilibrium binding properties of the two human–carp hybrid hemoglobins and discuss the ligand equilibria in terms of allosteric models and substitutions at selected sites.

Materials and Methods

Protein Preparation. Human hemoglobin was prepared from whole blood and separated into α- and β-chains by the methods described by Geraci et al. (1969). The separated chains were stored frozen as small pellets under liquid nitrogen. Carp hemoglobin was prepared from red blood cells (also stored in liquid nitrogen) as described by Tan et al. (1972). The carp hemoglobin was then separated into α- and β-chains as described in the following paper (Goss & Parkhurst, 1984). For these preparations, the CO form of the protein was used to prepare carp hemoglobin chains. Polyacrylamide gel electrophoresis was carried out in slabs following Brewer &

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[1] Conventional three-letter codes are used for the amino acids: Ser, His, Glu, Asp, Val, Gln, Pro, Ala, Lys, Arg, Trp, and Thr refer respectively to the amino acids serine, histidine, glutamic acid, aspartic acid, valine, glutamine, proline, alanine, lysine, arginine, tryptophan, and threonine. Results referred to with +IHP are for experiments in which the buffer contained 1 mM inositol hexaphosphate (IHP).

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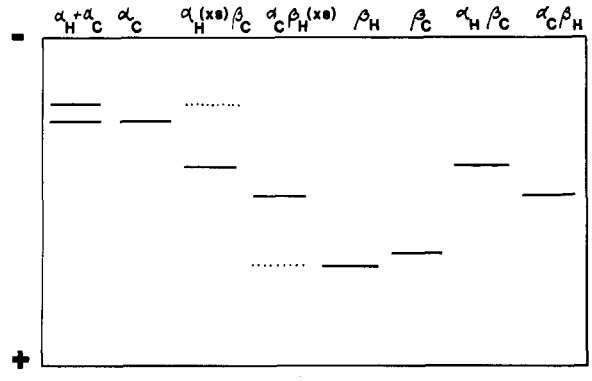

FIGURE 1: Polyacrylamide gel electrophoresis patterns. All protein samples were 200 μM in heme before application. The samples applied to the gel were, left to right, as follows: 15 μL each of carp α-chains and human α-chains; 20 μL of carp α-chains; 20 μL of human α-chains and 15 μL of carp β-chains; 15 μL of carp α-chains and 20 μL of human β-chains; 20 μL of human β-chains; 20 μL of carp β-chains; 15 μL each of human α-chains and carp β-chains; 15 μL each of carp α-chains and human β-chains. The carp β-chains were from carp Hb II. When unfractionated carp Hb is used, a more acidic β-chain is observed from Hb I.

Ashworth (1969). The patterns for the chains and hybrids are shown in Figure 1. The two hybrid hemoglobins were formed by mixing equal molar amounts of a given chain (as the CO form) with the complementary chain from the other species, with the assumption of equal molar absorptivities at 540 nm. The hybrid hemoglobins formed readily upon mixing the complementary chains, and polyacrylamide gel electrophoresis showed less than 5% uncombined free chains. The hybrid hemoglobins were prepared for oxygen equilibrium measurements by passing the hemoglobin over a Sephadex G-25 column equilibrated with 0.001 M potassium phosphate buffer, pH 7, and then by concentrating the hemoglobin in an Amicon concentrator (Model 3, Amicon Corp., Lexington, MA) with a PM10 membrane under carbon monoxide at 40 psi. The concentration of the resulting stock solution was 300–600 μM in heme. The hybrids were then treated with an enzyme system (Asakura et al., 1972) to prevent oxidation of the heme groups and with 1 mM dithiothreitol to prevent oxidation of the free sulfhydryl groups. The hemoglobin was then brought to the appropriate pH by addition of 1 part of 1 M phosphate buffer at the desired pH to 3 parts of hemoglobin stock solution. At pH 9, the protein solution was 0.1 M in borate; at pH 6, the solutions contained 1 mM IHP and are denoted pH 6 (+IHP). The pH values of the final solutions were determined with an Instrumentation Laboratory Inc. Model 245 pH meter and Ingold electrode (no. 14043). Runs that showed significant (>3%) met formation or changes in thickness of the sample layer during the run were not used for data analysis.

Oxygen Equilibrium Measurements. The fractional oxygen saturation of both hybrid hemoglobins, and both parents, was measured as a function of the partial pressure of oxygen in a specially designed oxygen equilibrium cell (Dolman & Gill, 1978). The cell employs a thin layer of hemoglobin trapped between a quartz window and a membrane (a silicone copolymer, General Electric, MEM 213, 1 mm) permeable to gaseous oxygen and argon. The chamber was first flushed with oxygen (Union Carbide, ultrahigh purity), until the hemoglobin was totally converted to the oxy form (HbO_2), and the absorbance was recorded. The concentration of oxygen in the chamber was decreased stepwise by successive additions of a known amount of argon (Union Carbide, prepurified grade) to the chamber through a calibrated valve. After the protein had reached equilibrium following each addition, the absorption spectrum was recorded on a Cary 210 spectrophotmeter linked to an Apple II microcomputer. At the end of a run, the cell was flushed with argon to obtain a spectrum of the fully deoxy form of the hemoglobin. See paragraph at end of paper regarding supplementary material.

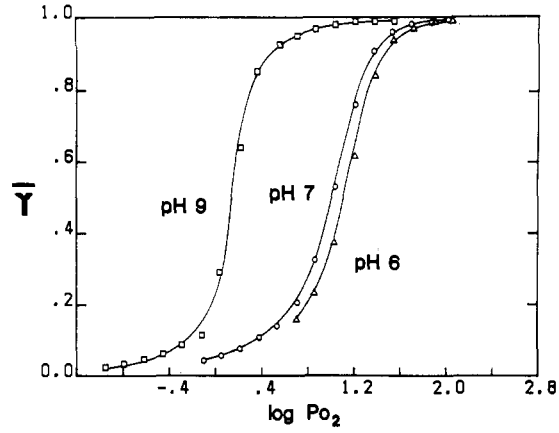

FIGURE 2: Oxygen equilibrium curves for human hemoglobin at pHs near 6 (+IHP), 7, and 9 at 20 °C. $\bar{Y}$ is fractional saturation; oxygen activities are in millimeters of Hg.

Data Processing. Let $\bar{Y}$ be the fractional saturation of the hemoglobin by oxygen. The $\bar{Y}$ data were fit to Adair and allosteric models. For the Adair model

$$\bar{Y} = N/(4D) \quad (1)$$

where

$$D = 1 + K_1X + K_1K_2X^2 + K_1K_2K_3X^3 + K_1K_2K_3K_4X^4$$

where the K_i's are association constants for $HbX_{i-1} + X \rightleftharpoons HbX_i$ ($X = O_2$), $N = X\,dD/dX$, and X = ligand activity. For the allosteric two-state model

$$\bar{Y} = N/(4D) \quad (2)$$

where

$$D = L(1 + cK_R^{-1}X)^4 + (1 + K_R^{-1}X)^4$$

$N = X\,dD/dX$, X = ligand activity, K_R and K_T are ligand dissociation equilibrium constants for the R and T states, respectively, $c = K_R/K_T$, and $L = (Hb)_T/(Hb)_R$ (Monod et al., 1965). For both (1) and (2), the Fletcher–Powell algorithm (Fletcher & Powell, 1963) was used for least-squares minimization. The Hill numbers (n^*) were obtained from

$$n^* = d \log [\bar{Y}/(1 - \bar{Y})]/d \log X \quad (3)$$

at $\bar{Y} = 0.5$ and $X = \bar{X}$, where $\bar{X}$ is the ligand activity for half-saturation. Values for the number of Bohr protons per tetramer, n_H, were obtained from the slopes of linear fits of the equilibrium data as a function of pH (see Figure 6):

$$n_H = -4(d \log \bar{X}/d\ pH) \quad (4)$$

Results and Discussion

Figures 2–5 show oxygen equilibrium curves for the two parent hemoglobins, human and carp, and the two hybrids, in 0.25 M phosphate buffer at 22 °C at pH 6 and 7. At pH 9, the solutions were 0.1 M in borate. Data derived from these and other curves are in Tables I and II. Figures 2 and 3 clearly differ, showing that for human Hb there is little change in the slope of the binding isotherm with pH whereas (Figure 3), for carp, the pH 7 curve is much steeper than the pH 6 and 9 curves. [Control experiments showed that the kinetic and equilibrium properties of carp hemoglobin reconstituted from isolated chains were identical with those of the native protein (Parkhurst et al., 1983).] The hybrid hemoglobin

Table I: Oxygen Equilibrium Data

hemoglobin	pH	n^*	$\bar{O}_2$ (μ)	n_H (pH range)[a]
carp	9.0	1.1 ± 0.1	1.2 ± 0.1	
	7.1	2.4 ± 0.09	24 ± 1	3.8 ± 0.1 (6.85-7.4)
	6 (+IHP)	0.9 ± 0.1	94 ± 4	
human	9.0	3.1 ± 0.1	2.4 ± 0.1	
	7.1	2.9 ± 0.1	17.2 ± 0.6	2.6 ± 0.2 (7.1-7.5)
	6 (+IHP)	2.7 ± 0.2	21.6 ± 0.5	
hybrid I (α carp:β human)	9.0	1.0 ± 0.1	6.1 ± 0.5	
	7.0	1.4 ± 0.1	32.4 ± 0.9	1.6 ± 0.1 (6.8-7.4)
	6 (+IHP)	1.1 ± 0.1	42 ± 2	
hybrid II (α human:β carp)	9.0	1.2 ± 0.1	5.3 ± 0.5	
	6.9	1.45 ± 0.09	46.5 ± 0.9	2.2 ± 0.1 (6.8-7.5)
	6 (+IHP)	1.08 ± 0.09	59 ± 3	

[a] pH range for n_H.

Table II: Adair Constants and Interaction Energies[a]

hemoglobin	pH	K_1	K_2	K_3	K_4	ΔG_I (kcal)	$\Delta G_{I,II}$ (kcal)
carp	6.85	0.056 ± 0.004	0.028 ± 0.003	0.0041 ± 0.0041	0.079 ± 0.02	1.81	2.64
human	7.5	0.059 ± 0.01	0.0031 ± 0.002	0.209 ± 1	0.15 ± 0.06	2.16	1.28
hybrid I	7.03	0.22 ± 0.02	0.023 ± 0.003	0.018 ± 0.002	0.025 ± 0.002	0.35	1.12
hybrid II	6.88	0.31 ± 0.06	0.13 ± 0.01	0.0041 ± 0.0003	0.029 ± 0.002	0.23	1.4

[a] Adair constants are in units of reciprocal micromolar. Interaction free energy, ΔG_I, is in kilocalories per mole of heme sites. Heterotropic interaction free energy, $\Delta G_{I,II}$, is in units of kilocalories per mole of proton binding sites.

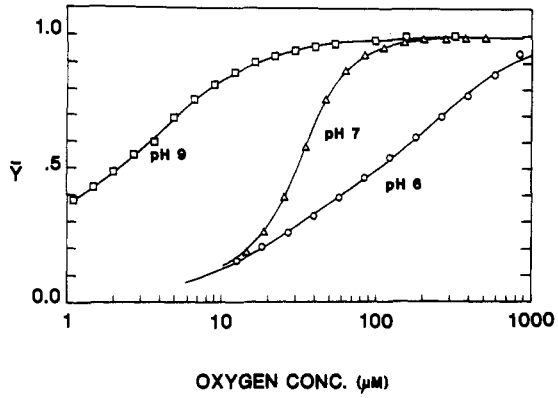

FIGURE 3: Oxygen equilibrium curves for carp hemoglobin at pHs near 6 (+IHP), 7, and 9 at 20 °C. $\bar{Y}$ is fractional saturation; oxygen activities are in micromolar. The actual pH values were 6.0, 6.95, and 8.85.

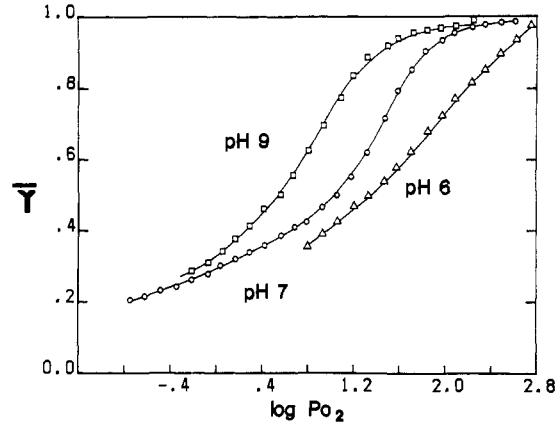

FIGURE 4: Oxygen equilibrium curves for hybrid I (α carp:β human) hemoglobin. $\bar{Y}$ is fractional saturation; oxygen activities are in millimeters of Hg. T = 20 °C.

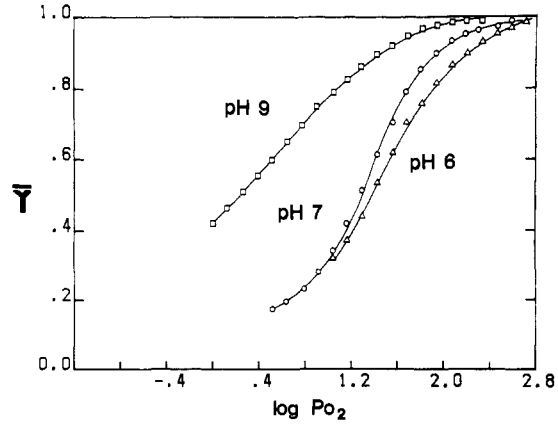

FIGURE 5: Oxygen equilibrium curves for hybrid II (α human:β carp) hemoglobin. $\bar{Y}$ is fractional saturation; oxygen activities are in millimeters of Hg. T = 20 °C.

curves (Figures 4 and 5) resemble the carp hemoglobin curves in having affinities more nearly those of carp than of human hemoglobin (see Figure 6) and in having Hill numbers (n^*) that vary with pH—in both cases approaching 1 at pH 6 (+IHP). For both hybrid hemoglobins (Table I) the Hill number is much reduced from that observed for the two parent hemoglobins. As will be shown in the following two papers, these parent–hybrid differences can be interpreted quite differently for the two hybrids. In terms of a two-state allosteric model, for the α-carp:β-human hybrid, hybrid I, there appear to be larger values of c (or a smaller difference in free energy changes for ligation to the R and T states) for oxygen binding than for the two parent hemoglobins. In the case of α-human:β-carp hemoglobin, hybrid II, however, a multiplicity of detectable intermediates is associated with a reduction in cooperative ligand binding.

Studies of the dimer–tetramer equilibria for the two hybrids from pH 6 to 9 and in various states of ligation show that K_{TD} never exceeds 2 μM. We may therefore disregard dissociation of the tetramer as a source of the low cooperativity shown by the hybrids, since our equilibrium measurements were made at heme concentrations in excess of 300 μM. The overall free energy of interaction (Wyman, 1964) is $\Delta G_I = RT \ln (16K_4/K_1)$, in terms of the K's in the above binding polynomial. These quantities are given in Table II for the hybrids. As expected from the Hill number data, the interaction energies

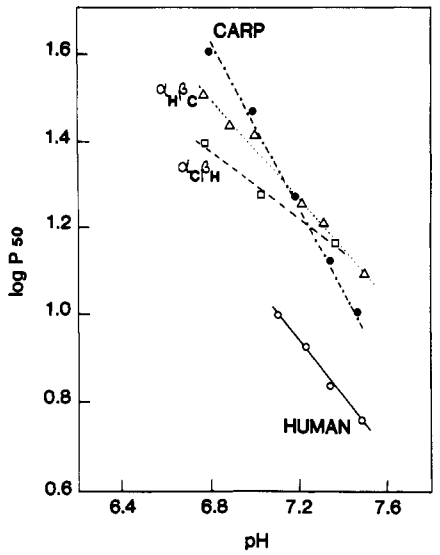

FIGURE 6: Plot of log of the half-saturation oxygen activity, $\bar{O}_2$, vs. pH to determine the number of Bohr protons for the four hemoglobins: (open circles) human Hb; (closed circles) carp Hb; (open squares) hybrid I (α carp:β human); (open triangles) hybrid II (α human:β carp).

are lower for the two hybrids than for either parent hemoglobin. The ligand kinetics for the two hybrids (see the following two papers) provide evidence for weak cooperativity at both pH 6 and 9, though again their behavior is more nearly that of carp—essentially T state at pH 6 (+IHP) and R state at pH 9. If we equate $\bar{O}_2$ with the median ligand activity, we may calculate the overall heterotropic interaction energy $\Delta G_{I,II} = -RT\Delta(\ln \bar{O}_2)$ for the pH 6–9 interval. For carp HbO_2, this is a measure of the difference in the free energy of protonation of the R and T states, and for the hybrids it is approximately so. In the case of the α-human:β-carp hybrid (Parkhurst & Goss, 1984), our present inability to determine the quaternary state of the liganded hybrid at pH 6 (+IHP) prevents our analyzing the $\Delta G_{I,II}$ quantities in terms of the individual chains and quaternary states. We may, however, consider the n_H values determined in the vicinity of pH 7, where cooperativity is maximal for carp Hb and the hybrids. The quantity n_H is the difference in the mean number of protons bound per tetramer by deoxy- and oxyhemoglobins. Suppose that n_H is not only additive but transferable for each chain—that is, we assume that the number of Bohr protons assigned to a given chain will not be affected by changes in ionization of the complementary chain over the interval pH 6.8–7.5 and that the oxy–deoxy conformations for a given chain are respectively the same in all proteins. Then

$$n_H(\text{human}) = n_H(\alpha\text{-human}) + n_H(\beta\text{-human}) = 2.6 \quad (5)$$

$$n_H(\text{carp}) = n_H(\alpha\text{-carp}) + n_H(\beta\text{-carp}) = 3.8 \quad (6)$$

$$n_H(\text{I}) = n_H(\alpha\text{-carp}) + n_H(\beta\text{-human}) = 1.6 \quad (7)$$

$$n_H(\text{II}) = n_H(\alpha\text{-human}) + n_H(\beta\text{-carp}) = 2.2 \quad (8)$$

We cannot solve for the n_H's, however, since the four equations above are linearly dependent. We can, however, check for consistency, since we have experimental values for the four quantities, n_H, and these are given to the right of the second equality in each line above. If we calculate $n_H(\text{II}) = (5) + (6) - (7)$, we obtain 4.8, compared to the observed 2.2. Similarly, $n_H(\text{I})$, calculated, is $(5) + (6) - (8) = 4.2$, compared to a measured value of 1.6. Or, we might attempt to calculate the difference in the number of Bohr protons for carp β-chain vs. human β-chain, which equals –0.4 from (8) – (5) but equals 2.2 from (6) – (7). Similar inconsistencies are obtained for the α-chains. At least one and perhaps both of the assumptions underlying the above two-term decomposition of n_H must be in error. The kinetic studies provide strong support for considering the ligand binding properties of a given chain, and presumably its conformation, to depend strongly on the complementary chain in the intact hemoglobin, contradicting the transferability hypothesis.

The two-state allosteric model gave somewhat disappointing results for both hybrids. For hybrid I, a family of three oxygen equilibrium curves, with a total of 56 points, measured at pHs 6.78, 7.03, and 7.36 was fit such that c and K_R were constrained to be the same for all three curves and only L was allowed to vary with pH. This constrained minimization was adopted after repeated attempts at fitting individual curves led to enormous errors in the three parameters L, c, and K_R. For the constrained minimization, $c = 0.18 \pm 0.08$ and $K_R = 6.7 \pm 2.2$ μM, but the response surface in the L direction was so flat that error estimates for L exceeded the value of that parameter. The overall fitting was quite good, however, with a value of 0.069 for the sum of squared residuals (SSQ). This value for c is larger than the value we have determined for human Hb (0.01) or for carp Hb (0.05) by the same fitting procedure. In terms of the allosteric model, the R and T states of this hybrid are rather similar, differing by only 1.0 kcal for the binding of 1 mol of oxygen. The value for K_R is much closer to that for carp (5.1 μM) than for human Hb (2.4 μM) and, as discussed in the following paper, is in excellent agreement with the value calculated from kinetic data.

For hybrid II, three curves, 50 data points total (pHs 6.88, 7.32, 7.52), were also fit as a family with rather similar results: $c = 0.16 \pm 0.09$, $K_R = 4.8 \pm 2.0$ μM, and the estimated standard deviation of L was 3–5 times L. In this case, however, the high value of c did not agree with the results of kinetic measurements (Parkhurst & Goss, 1984) that showed, for CO association, values typical of the R and T states for human Hb. We then carried out a grid search in c and K_R where the same values of c and K_R were used for all three curves and only L was allowed to vary for each curve. An excellent fit was obtained for $c = 0.092$ and $K_R = 2.9$ μM. The values for L at pHs 6.88, 7.32, and 7.52, respectively, were 8200 ± 600, 3760 ± 840, and 680 ± 90. The observed points for pH 7.32 are shown in Figure 7, along with the theoretical curve. In Parkhurst & Goss (1984), evidence is presented for an intermediate state denoted "S" in which the α-chain (human) has R state properties and the β-chain (carp) has T state properties. Recent papers have called attention to the importance of the replacement of the β-93 SH of mammalian hemoglobins by serine in fish hemoglobins (Perutz & Brunori, 1982) and the interaction of that serine with the β C-terminal His in stabilizing the T state (Parkhurst et al., 1983). This stabilization may account for the observation of the intermediate S state. We can treat hybrid II as a three-state allosteric problem with $c = K_R/K_T$, where the K's are equilibrium dissociation constants, $L = (\text{Hb})_T/(\text{Hb})_R$, and $M = (\text{Hb})_T/(\text{Hb})_S$. The data for pH 7.32 were fit according to a three-state model with $c = 0.092$ and $K_R = 2.9$ μM by using a two-dimensional grid search for L and M. The optimum values were $L = 4190$ and $M = 222$. The theoretical curve in Figure 7 is for these values. The fitting, though slightly better than that for the two-state model (SSQ = 0.0018 vs. 0.0025), did not represent a significant improvement in the fitting. It is reasonable, however, because of the kinetic evidence, to treat the data according to such a model. The fitting allows one to calculate concentrations of the three states as a function of fraction saturation, and these are shown in Figure

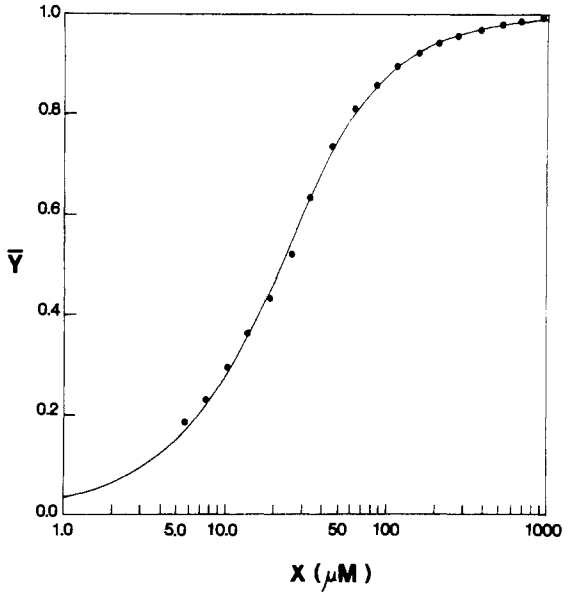

FIGURE 7: Oxygen equilibrium curve for hybrid II at pH 7.32. The fractional saturation, $\bar{Y}$, is plotted vs. oxygen activity, micromolar, on a logarithmic scale. The solid line is a fit to the data according to a three-state allosteric model; see text for details. On this scale, the fit according to a two-state model is indistinguishible from the three-state fit.

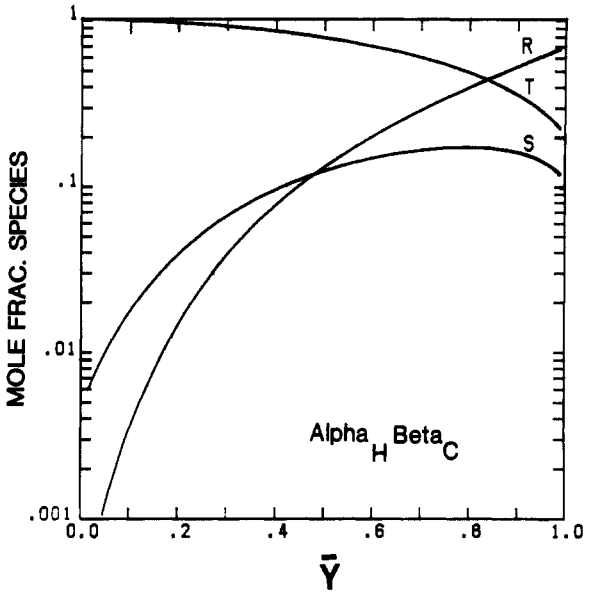

FIGURE 8: Plot, derived from the fitting in Figure 7, of the mole fractions of the three allosteric states R, S, and T for hybrid II as a function of fractional saturation with oxygen.

8. These equilibrium populations can then be considered in discussing the various kinetic experiments (Parkhurst & Goss, 1984). The occurrence of an intermediate S state with properties and conformations between those of R and T should lead to reductions in both the Hill number (n^*) and number of Bohr protons (n_H).

Recent discussions of hemoglobin function (Perutz, 1976; Chothia et al., 1976; Baldwin & Chothia, 1979) have attempted to account for the ligation properties in terms of a small number of residues. These sets of residues include (1) the Bohr proton sites, (2) residues involved in a change in surface area of the protein exposed to solvent in the R–T transition, (3) the T-state salt bridges, and (4) residues at "switch" and "flexible-joint" sites.

With regard to set 1, the present study provides little information except to emphasize that the magnitude of the Bohr effect is strongly dependent on the identity of both the α- and β-chains but is not simply additive. It is clear from studies of mutant Hbs that substitutions strongly affect n_H, though in the present studies, unlike many of the mutant Hb studies, each chain is derived from a parent Hb that is cooperative and has a sizeable Bohr effect. The above n_H analysis does show, however, that the number of Bohr protons cannot simply be assigned to a given chain without regard for conformation. With regard to set 2, hybrid II has two substitutions (relative to human Hb) at the sites listed in Figure 3 of Chothia et al. (1976): Glu-101β → Asp and Glu-43β → Val. Unless the masking is very different for the β-chain substitutions, hydrophobic considerations involving just these residues cannot account for the marked decrease in cooperativity in hybrid II compared to the parent hemoglobins. Similar considerations hold for hybrid I where there are three substitutions: Thr-38α → Gln, Pro-44α → Ala, and Val-96α → Ala. Examination of models of horse oxyhemoglobin and of human deoxyhemoglobin with regard to the above substitutions fails to suggest any large difference in masking in the hybrid compared to that in the cooperative parent hemoglobins. Without detailed structural data, the actual change in surface accessibility, of course, cannot be determined. Consider set 3, the T-state salt bridges. In human hemoglobin, the interactions are (a) α_1 Lys-40···β_2His-146, (b) α_1 Arg-92···β_2 Glu-43, (c) α_1 Arg-141···α_2 Lys-127, and (d) α_1 Arg-141···α_2 Asp-126. In carp Hb, the β-43 Glu is replaced by Ala; thus, carp Hb can have salt bridges a, c, and d. In the hybrids, the following salt bridges are possible: (hybrid I) a–d; (hybrid II) a, c, and d. In terms only of the salt bridges, we would expect hybrid I to have a greater tendency toward forming the T state than carp, yet it does not. It is, however, from pH 6 to 9 poised between T and R, apparently with a restricted conformational range (Goss & Parkhurst, 1984). Hybrid II should be identical with carp—in fact, liganded hybrid II at pH 6 (+IHP) is not T state but rather S state (Parkhurst & Goss, 1984). We will return to these points after discussing the residues in set 4.

Baldwin & Chothia (1979) in discussing mammalian hemoglobins have called attention to specific changes at the $\alpha_1\beta_2$ interface in the R–T transition and to two regions, referred to as switch and flexible-joint regions, respectively: α_1 C···β_2 FG and α_1 FG···β_2 C (2-fold symmetry-related interactions are not listed here). The residues that interlock somewhat differently in R and T to form a flexible joint are α Arg-92, Asp-94, and Pro-95 and β Trp-37 and Arg-40. These same residues occur in carp Hb, and hence, the same joint can occur in the hybrids. Regarding the switch region, the following interactions have been described: β_2 His-97 is between α_1 Thr-41 and α_1 Pro-44 in the T state and moves past the α_1 Thr-41 to a position between that of Thr-41 and α_1 Thr-38 in the R state. The movement of the β His past α Thr-41 is seen as the main evident barrier for the T ↔ R transition. A hydrogen bond between β_2 Asp-99 and the OH of α_1 Tyr-42 also occurs in the T state, but not in R. There are no substitutions in carp for the two β-chain residues in this region, but there are two substitutions in the α-chain: Thr-38 → Gln and Pro-44 → Ala. This switch region can therefore be the same in human Hb and in hybrid II, and as will be shown later (Parkhurst & Goss, 1984), similar R- and T-state properties for CO ligation can be observed at pH 7 for these two hemoglobins. At pH 6, the properties are different from those of human, presumably because of the F9 Ser and its interactions with the β His-147 (Parkhurst et al., 1983). On the other hand, the barrier and the neighboring interactions should be very similar in carp and in hybrid I if only the T-state salt

bridges and flexible-joint and switch regions were important. In fact, hybrid I has quite low cooperativity and cannot quite assume a carp T state at low pH and, surprisingly, cannot assume a normal human R state. This may point both to the importance of the β carp Ser for assumption of the T state in carp Hb and the importance of $\alpha_1\beta_1$ interactions for determining the extent of R-state properties. Baldwin & Chothia (1979) list 68 residues involved in the $\alpha_1\beta_1$ contact. Of these, in a comparison of human and carp Hbs, there are 20 substitutions in the α-chain and 20 in the β-chain. These substitutions may prevent carp β-chains from reacting as rapidly in the carp R state with CO as in hybrid II. As is reported elsewhere (Parkhurst & Goss, 1982; Pham, 1981), carp α-chains behave quite differently from human α-chains. They have a slow component with kinetics more nearly characteristic of T state than R and reminiscent of lamprey Hb, have differing aggregation in deoxy-Hb and HbCO forms. In subsequent papers, evidence will be presented in support of a model in which the carp α-chain appears to be relatively noncompliant—requiring, for T-state properties, a complementary chain with a strong tendency to assume the T state. The carp α-chains also appear to prevent the complementary chain from assuming full R-state properties. The β carp chain has such a strong tendency to assume the T state that it can force the α carp chain into this state. In the liganded form at low pH, presumably, the α human and β carp chains in hybrid II do not adequately mesh, and one finds the S state, where the carp β-chain has T-state and the human α-chain has R-state properties. Clearly, the properties of a given chain in hemoglobin depend strongly on the nature of its contacts with the complementary chain.

Supplementary Material Available

Absorbance spectrum for hybrid I (2 pages). Ordering information is given on any current masthead page.

Registry No. Oxygen, 7782-44-7.

References

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

Baldwin, J. M. (1975) *Prog. Biophys. Mol. Biol. 29*, 225–320.

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Brewer, J. M., & Ashworth, R. B. (1969) *J. Chem. Educ. 46*, 41–45.

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

Chothia, C., Wodak, S., & Janin, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3793–3797.

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

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

Geraci, G., Parkhurst, L. J., & Gibson, Q. H. (1969) *J. Biol. Chem. 244*, 4664–4667.

Goss, D. J., & Parkhurst, L. J. (1984) *Biochemistry* (second paper of three in this issue).

Imai, K. (1973) *Biochemistry 12*, 798–808.

Imai, K., & Yonetani, T. (1975) *J. Biol. Chem. 250*, 7093–7098.

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

Noble, R. W., Parkhurst, L. J., & Gibson, Q. H. (1973) *J. Biol. Chem. 245*, 6628–6633.

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

Parkhurst, L. J., & Goss, D. J. (1984) *Biochemistry* (third paper of three in this issue).

Parkhurst, L. J., Goss, D. J., & Perutz, M. F. (1983) *Biochemistry 22*, 5401–5409.

Perutz, M. F. (1976) *Br. Med. 32*, 195–208.

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

Pham, T. (1981) Ph.D. Dissertation, University of Nebraska.

Root, R. W. (1931) *Biol. Bull. (Woods Hole, Mass.) 61*, 427–456.

Root, R. W., & Irving, L. (1941) *Biol. Bull. (Woods Hole, Mass.) 81*, 307–323.

Root, R. W., & Irving, L. (1943) *Biol. Bull. (Woods Hole, Mass) 84*, 207–212.

Roughton, F. J. W., & Lyster, R. L. J. (1969) *Hvalradets Skr. 4*, 185–192.

Shulman, R. G., Hopfield, J. J., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325–420.

Tan, A. L., De Young, 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.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 223.