Myles, G. M., Van Houten, B., & Sancar, A. (1987) *Nucleic Acids Res. 15*, 1227-1243.
Orren, D. K., & Sancar, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5237-5241.
Patterson, M., & Chu, G. (1989) *Mol. Cell. Biol. 9*, 5105-5112.
Rycyna, R. E., & Alderfer, J. L. (1985) *Nucleic Acids Res. 16*, 5949-5963.
Sancar, A., & Rupp, W. D. (1983) *Cell 33*, 249-260.
Sancar, A., & Sancar, G. B. (1984) *J. Mol. Biol. 172*, 223-227.
Sancar, A., & Sancar, G. B. (1988) *Annu. Rev. Biochem. 57*, 29-67.
Sancar, A., Franklin, K. A., & Sancar, G. B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7379-7401.
Sancar, G. B. (1990) *Mutat. Res.* (in press).
Sancar, G. B., & Smith, F. W. (1989) *Mol. Cell. Biol. 9*, 4767-4776.
Sancar, G. B., Smith, F. W., & Heelis, P. F. (1987) *J. Biol. Chem. 262*, 15457-15465.
Sibghat-Ullah, Husain, I., Carlton, W., & Sancar, A. (1989) *Nucleic Acids Res. 17*, 4471-4484.
Thomas, D. C., Levy, M., & Sancar, A. (1985) *J. Biol. Chem. 260*, 9875-9883.
Valerie, K., De Riel, J. K., & Henderson, E. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7656-7660.
Van Houten, B., Gamper, H., Hearst, J. E., & Sancar, A. (1988) *J. Biol. Chem. 263*, 16553-16560.
Wood, R. D. (1989) *Biochemistry 28*, 8287-8292.
Wood, R. D., Robins, P., & Lindahl, T. (1988) *Cell 53*, 97-106.
Zwetsloot, J. C. M., Vermeulen, W., Hoeijmakers, J. H. J., Yasui, A., Eker, A. P. M., & Bootsma, D. (1985) *Mutat. Res. 146*, 71-77.
Zwetsloot, J. C. M., Hoeijmakers, J. H. J., Vermeulen, W., Eker, A. P. M., & Bootsma, D. (1986a) *Mutat. Res. 165*, 109-115.
Zwetsloot, J. C. M., Barbeiro, A. P., Vermeulen, W., Arthur, H. M., Hoeijmakers, J. H. J., & Backendorf, C. (1986b) *Mutat. Res. 166*, 89-98.

Kinetics and Thermodynamics of Oxygen and Carbon Monoxide Binding to the T-State Hemoglobin of *Urechis caupo*†

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ABSTRACT: The tetrameric hemoglobin from *Urechis caupo* is nearly ideal for studying ligation to the T-state. Our previous EXAFS study had shown that the Fe is displaced 0.35 Å from the mean plane of the porphyrin in the HbCO derivative. We have carried out detailed kinetic studies of oxygen and CO ligation as a function of temperature in order to characterize both the kinetics and thermodynamics of ligation in this hemoglobin. The entropy change associated with ligation essentially corresponds to simple immobilization of the ligand and is virtually the same as that we have determined for leghemoglobin, an extreme R-state-type hemoglobin. The low ligand affinities thus derive from small enthalpies of ligation, which can be correlated with the large out of plane displacement of the Fe. Only oxygen pulse measurements revealed kinetic evidence for cooperative oxygen binding, but a direct measurement of oxygen binding gave a Hill number of 1.3. An allosteric analysis gave $L = 2.6$ and $c = 0.048$ (oxygen) and $c = 0.77$ (CO). The higher affinity state in this weakly cooperative hemoglobin is denoted T*, and it is for this state that thermodynamic quantities have been determined. The small differences between T and T* in CO binding were nevertheless sufficient to allow us to measure by flash photolysis the rate of the T* → T conformational change in terms of an allosteric model. The half-time for this transition was calculated to be 8-14 ms at 20 °C.

The hemoglobin of the echiuroid *Urechis caupo* is a tetramer of molecular weight 57 600 for which oxygen binding has been reported to be noncooperative and of low affinity (Garey & Riggs, 1984; Sima, 1979) or weakly cooperative (Mangum et al., 1983) and which we have characterized as a T-state hemoglobin (Schreiber & Parkhurst, 1984) with an unusually large displacement of the Fe out of the mean plane of the heme in the HbCO form (Chance et al., 1986). Garey and Riggs (1984) have recently reported that there is one predominant form of the tetramer consisting of nearly identical subunits. A recent X-ray crystallographic study at 5-Å resolution reported that the tetramer has unusual subunit contacts, giving rise to an "inside-out" quaternary structure with the G/H helices located on the outer surface (Kolatkar et al., 1988). We wished to extend our previous kinetic studies (Sima, 1979) to obtain the thermodynamics of oxygen and CO ligation in order to characterize a naturally occurring T-state hemoglobin. Oxygen and CO ligand binding kinetic experiments were carried out as a function of temperature to allow calculation of $\Delta H°$ and $\Delta S°$. We report here the oxygen and CO association and dissociation rate constants, equilibrium constants, and the activation energies and the standard-state changes in free energy, enthalpy, and entropy of ligation. In the course of these studies, we found from both kinetic and equilibrium experiments that the protein showed weak cooperativity. We were not only able to characterize this behavior in terms of

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the allosteric model but also able to measure the rate of the conformational change between the two low-affinity states, T* and T.

Materials and Methods

Preparation of Hb. Specimens of *Urechis caupo* were obtained from Sea Life Supply, Sand City, CA. Animals were bled by dorsal incision, the coelomic fluid (50 mL) was centrifuged at 4000*g* at 4 °C for 10 min, and the packed cells were washed two times with 1% NaCl with centrifugation after each washing. Lysing was achieved by subjecting the cells to three freeze–thaw cycles in 0.10 M KP_i (20-mL total volume) at pH 6.0. Cell debris was removed by centrifugation at 27000*g* for 30 min. The solution was diluted to 40 mL with 0.10 M KP_i, 0.10 g of celite was added with stirring, and the suspension was then centrifuged at 27000*g* for 30 min. The Hb was stored frozen, as droplets, in liquid nitrogen. Kinetic studies run on freshly prepared Hb showed functionality identical with that of the liquid nitrogen stored protein. In one study, the hemoglobin was fractionated on a 2.2 cm × 12 cm. column of CMC-50 Sephadex (Pharmacia) equilibrated with 0.02 M potassium phosphate, pH 6. The minor band eluted under these conditions; then, the major component was isolated by elution with 0.05 M Tris-HCl, pH 8.5.

Instrumentation and Data Analysis. The stopped-flow apparatus has been described elsewhere (Parkhurst et al., 1980). Data were acquired from both rapid flow and laser photolysis initiated reactions by a Computerscope Model ISC-16 (R.C. Electronics, Santa Barbara, CA) in an IBM PC-XT microcomputer. The data (typically 1000 points per run) were then digitally filtered with third-order polynomial smoothing (Savitzky & Golay, 1964) and least-squares fit to both one- and two-exponential models. For none of the reactions were the biexponential fits significantly better than for a single-exponential fit. For CO dissociation, a small stopped-flow apparatus was built into the Cary 210 spectrophotometer, with the cuvet situated in a thermostated brass block having three optical ports. The cuvet assembly was modified to include flow solution reservoirs, which were thermostated in the block along with the reaction chamber. A thermistor was embedded immediately adjacent to the reservoirs to monitor temperature. A high-energy photographic flash tube (Wabash Electroflash, type R-1140, Wabash Corp., Brooklyn, NY) was situated in the Cary at the third optical port, perpendicular to the monitoring beam, and was used for photolysis to drive the CO dissociation reactions to completion after approximately three half-times. The Cary was interfaced to an Apple II+ microcomputer for storage of Δ absorbance vs time data. For laser photolysis, a Phase-R dye laser (New Durham, NH), tube Model DL-18Y, was used with Coumarin 540A dye (Exciton Chemical Co., Inc., Dayton, OH). The cuvet was situated in a thermostated brass block with four optical ports and had a thermistor placed just above the path of the monitoring beam. It was convenient to use the regular stopped-flow instrument for the quantum yield studies, since the HbCO and MbCO[1] solutions could be alternately placed in the cell, and the closed system ensured that no gas exchange occurred during the course of the experiment. An argon ion laser (Model 75, Lexel, Palo Alto, CA) was used in the quantum yield studies. The beam was dispersed by a lens onto one end of an optical fiber bundle, which terminated in a rectangular array directly over the stopped-flow cuvet and perpendicular to the observing beam. The photolysis source was thus distributed evenly over the sample. A piece of heavy cardboard between the laser and the lens served adequately as a shutter for the slow relaxation reactions. The first-order relaxation curves were acquired by the Computerscope ISC-16 and treated as described above.

Individual Rate Constants. The gasses CO, O_2, and Ar were from Union Carbide, Linde Division (Chicago, IL), and NO was from Matheson (East Rutherford, NJ). All gases were CP grade. Sephadex, microperoxidase, horse heart myoglobin, and catalase were from Sigma (St. Louis, MO); dithionite (Manox brand) was from Holdman and Hardman, Miles Platting, Manchester, U.K. All buffers were 0.1 M KP_i at pH 7.0 unless otherwise noted.

(*1*) *Oxygen On* (k'). Stock HbO_2 was treated with an enzyme reducing system (Hayashi, 1973), diluted into 3 mL of 0.1 M KP_i, pH 7.0, to 20 μM in heme, and placed in a cuvet situated in a brass block thermostated at 20.0 °C. The HbO_2 was photolyzed directly and efficiently (in the absence of CO) with a dye-laser flash, and the reaction was followed at 430 nm. Free oxygen concentration was typically 253 μM. All gas concentrations were calculated from solubility tables (Hodgman, 1960). Nine reaction curves were recorded, least-squares fit to a one-exponential model, and averaged to obtain the reported value of k' at 20 °C. For all rate constants, the data fit the single-exponential model well but could not be satisfactorily fit to a two-exponential model, since negative rate constants or very large parameter errors were then found. The HbO_2 sample was prepared in like fashion for the activation energy determination, except that saturated CO was diluted into the buffer to final concentrations of 90 and 418 μM, to increase the quantum yield of photodissociation. The protein concentration was 7.5 μM. Saturated CO was prepared by bubbling water-saturated CO through buffer for 10 min. The reaction was run at seven temperatures from 7 to 30 °C and fit as for k' at 20 °C. The data were least-squares fit to an Arrhenius plot, and the value of the activation energy was obtained from the slope of the regression line. The errors in the rate constants and activation energies were obtained by standard statistical methods (Draper & Smith, 1981) with respect to the regression line. These errors in rate constants were somewhat larger than error estimates derived from the covariance matrix for fitting individual experiments.

(*2*) *Oxygen Off* (k). HbO_2 was prepared as for k' with a final concentration of free oxygen of 12–125 μM and a protein concentration of 15 μM, before mixing. This solution was rapidly flowed against a 0.05% dithionite solution in argon-saturated buffer and followed at approximately 411 nm, the metdeoxy isosbestic point, which was determined in the stopped-flow apparatus immediately prior to each experiment with met-Hb in place of HbO_2. The variable initial oxygen concentrations gave a range of partially saturated intermediates. The temperature was varied from 9 to 25 °C for determination of the activation energy, and the data were fit as for k', with the k reported at 20 °C taken from the regression line.

(*3*) *CO On* (l'). Initially, the dye laser was used for photolysis to determine a value for l' because the dead time (≈2 μs) allowed any small amount of a rapidly reacting component to be detected. Because a small but consistent difference was noted between the values obtained for l' by this method and the values obtained by flow, subsequent photolysis experiments were carried out on the stopped-flow apparatus with the photographic flash situated directly over the cuvet. The same sample at constant temperature could then be subjected to both

[1] Abbreviations: MbCO, carboxymyoglobin; Mb, deoxymyoglobin; BSA, bovine serum albumin.

rapid mixing and flash photolysis for a precise comparison, and the closed system ensured constant ligand concentration. In addition to photolysis vs flow comparisons, l' was determined as a function of CO concentration, fraction saturation, pH, and heme concentration. Solutions for all experiments were prepared similarly: the Hb solutions of 5–7.5 μM were prepared by dilution of stock HbO_2 into argon-saturated buffer and addition of a few crystals of dithionite to remove traces of oxygen. CO solutions were prepared by diluting CO-saturated buffer with argon-saturated buffer to the desired concentration and adding dithionite. The reactions were followed at 430 nm, thermostated at 20 °C, and the values for l' determined by averaging the fits of four replicate curves. The final value of l' reported at 20 °C was obtained by stopped flow with a final CO concentration of 93 μM. Following each flow experiment, the bound CO was photolyzed, and the data were fit to obtain a corresponding value for l'_{flash}. The effect on l' of variations in fraction saturation following photolysis was determined at two CO concentrations, 134 and 475 μM, and the Hb solution contained the enzyme reducing system and 1 mM DTT. In these experiments, the HbCO was photolyzed in the stopped-flow cuvet, and variations in fraction saturation were achieved by varying the distance of the photographic flash unit from the cuvet. Fractional photolysis varied from 0.08 to 1. Comparisons of l' from flash photolysis and from flow at pH 7 and 9 were made for two CO concentrations, 86 and 518 μM. The buffer for the Hb solution was 0.2 M KP_i (before mixing) for pH 7.0 and 0.2 M borate for pH 9.0 and contained 22 000 units/10 mL catalase and 1 mM DTT. The CO solutions were prepared as described above except in distilled water rather than in buffer. An identical CO solution was then used for both pHs for a precise comparison. The effect of heme concentration was determined by stopped flow with a final CO concentration of 451 μM and heme concentrations of 2 and 40 μM. The Hb solution contained 22 000 units/10 mL catalase and 1 mM DTT. The reaction using 40 μM Hb was followed at 557 nm. The syringe containing the CO remained unchanged for both heme concentrations. The photographic flash was used to obtain the value of the activation energy for l', with a heme concentration of 5 μM and [CO] = 329 μM. The temperature was varied from 8.0 to 34.0 °C. Reactions were run at seven temperatures, and each curve was fit to obtain a value for l'. The data were least-squares fit to an Arrhenius plot as for k'. The activation energy for l' was also determined from flash photolysis experiments employing the dye laser (11 temperatures, 8–20 °C) with 100 μM CO and from stopped-flow measurements (10 temperatures, 12–36 °C), with 45 μM CO after mixing.

(*4*) *CO Off* (l). Both NO replacement and competitive binding by microperoxidase were used to obtain a value for l. The HbCO solution for the first method was prepared by blowing H_2O-saturated Ar over stock HbO_2 in a tonometer for 30 min then diluting the protein into deoxygenated buffer containing 30 μM CO, to a final concentration of Hb of 15 μM. One crystal of dithionite was added to the solution, since traces of oxygen produce side reactions with NO, and great care was taken to eliminate oxygen from all buffers and lines. The NO solution was prepared by bubbling water-saturated NO into deoxygenated buffer for 5 min, the NO first having been passed through 2 M KOH. A small thermostated stopped-flow unit was built into a Cary 210 spectrophotometer. The dual-beam monitoring allowed the reaction to be followed to long times with no evidence of base-line drift. The monitoring wavelength was 423 nm. After approximately three half-times, residual HbCO was photolyzed to completion by a photographic flash situated in the Cary. The absorbance data were collected by an Apple II^+ computer interfaced to the Cary and processed by least-squares fitting as for k. For the activation energy value, 17 curves were collected from 14 to 30 °C and fit to an Arrhenius plot. The reported value of l and the associated error are from the regression line at 20.0 °C. Microperoxidase was used in a single-beam stopped flow, thermostated at 20.0 °C, (a) for verification of the NO value and (b) in order to search for evidence of cooperativity in l. Microperoxidase was prepared by dissolving the powder in 0.5 mL of water, deoxygenating the solution by blowing H_2O-saturated Ar over it for 30 min, and diluting to 25 μM (from the weight of the lyophilized powder) with deoxygenated 0.1 M KP_i, pH 7.0 buffer. Just prior to use, 1% (by volume) of a 1% (w/v) solution of dithionite was added to the microperoxidase. The HbCO solution was prepared as for the NO experiment, but with final concentrations before mixing of 5 μM for both Hb and CO, and the buffer contained 1 mM DTT. The reaction was monitored at 435 nm. Microperoxidase binds CO over 350-fold more tightly than does *Urechis* Hb and, under these conditions, 1800-fold faster, allowing us to neglect the rebinding of CO to Hb during the dissociation reaction. Sharma's (1975) studies showed that concentrations of microperoxidase 1–10-fold over that of myoglobin all gave the same l for that reaction. Since l' for *Urechis* Hb is 1 order of magnitude slower than l' for Mb and the affinity of *Urechis* for CO is 5-fold less than that for Mb, a microperoxidase excess of 1–10-fold should be more than adequate for *Urechis* HbCO. Because microperoxidase binds the CO as it is released from HbCO and there is no replacement molecule for those vacated sites, an "overall" l is measured. To try to differentiate this overall l (and that for the liganded conformation, determined by NO replacement) from a more rapid "T-state" rate, the reaction was photolyzed to 60–90% completion very soon after mixing, creating distributions of intermediates weighted toward $Hb(CO)_1$, and values of l for these distributions were obtained.

Determination of M. The HbO_2/HbCO partition coefficient M is the equilibrium constant for the reaction $HbO_2 + CO \rightleftharpoons HbCO + O_2$. It can be calculated from the rate constants, since $M = K_D/L_D = kl'/k'l$, or determined directly from $M = [HbCO]/[HbO_2] \times [O_2]/[CO]$, all concentrations at equilibrium. Standard spectra for HbO_2 and HbCO were established from 400 to 500 nm in the following manner. To obtain the standard HbCO spectrum, a cyanomet solution of the protein was prepared by adding a 3-fold molar excess of $K_3Fe(CN)_6$ and KCN to a stock oxygen solution, incubating for 20 min at room temperature, and passing the mixture over a G-25 column equilibrated at pH 8. The Hb^+CN^- was then diluted into CO-saturated 0.1 M KP_i, pH 7.0, to approximately 50 μM and the cyanomet spectrum recorded from 600 to 400 nm on a Cary spectrophotometer. Crystals of dithionite were added, converting the protein to HbCO, and that spectrum was recorded. The HbO_2 spectrum was obtained similarly, by diluting stock HbO_2 into O_2-saturated buffer, recording the spectrum from 600 to 400 nm, and then adding KNO_2 and KCN to convert the protein to its cyanomet derivative. The concentrations of the HbCO and HbO_2 samples were scaled to a common concentration via their respective cyanomet spectra, the ratio of their absorbances at 541 nm being used to obtain the scale factor. A mixture was then made from stock HbO_2 that was 5 μM in heme, with the total concentrations of O_2 and CO equal to 1189 and 45 μM, respectively, and the absorbance was recorded over the same wavelengths.

The HbO_2 solution and the mixture contained the enzyme reducing system and approximately 0.5% (w/v) BSA for added stability. For all three spectra (HbCO, HbO_2, mixture), the absorbance at each wavelength was computer collected and stored. A least-squares fit of 40 points (from 401 to 440 nm) from each of the three spectra gave the optimal value for the $HbCO/HbO_2$ ratio of the mixture. For $\Delta H°_M$, the temperature was varied from 14 to 25 °C, and the absorbance of the mixture was recorded from 400 to 500 nm at each temperature, and fit as above for the ratio of the two species. Because σ ($[O_2]/[CO]$) remained essentially constant in this closed system, $M \propto [HbCO]/[HbO_2]$ (=ρ), and ln ρ vs $1/T$ was fit in a van't Hoff plot to give the standard enthalpy change for the partition constant, and thus a direct determination of the difference of $\Delta H°$ for CO and O_2 ligation. The reported value of M was determined from the value of ρ at 20 °C on the regression line and from the known value of σ. The regression line variance gave the error in ρ, and the error in σ was an average value obtained from a series of previous experiments.

Quantum Yield Determination. The quantum yield, Φ, of *Urechis* HbCO was determined relative to that of whale MbCO. In the presence of CO and O_2, equilibria are described by

$$HbO_2 + CO \underset{k'}{\overset{k}{\rightleftharpoons}} Hb + CO + O_2 \underset{l}{\overset{l'}{\rightleftharpoons}} HbCO + O_2 \qquad (1)$$

Following a perturbation, for CO and O_2 in large excess over total heme, equilibrium is reestablished after a brief transient phase with the relaxation constant

$$R = \frac{l'[CO]k + k'[O_2]l}{k'[O_2] + l'[CO]} \qquad (2)$$

If high-intensity light is absorbed by the sample, the apparent rate constant for one of these reactions, l, will be altered, because CO is preferentially photolyzed by light and the equilibrium will shift to the left (Noble et al., 1967). Considering only the effect of the light

$$-d[HbCO]/dt = SI°A\Phi = SI°\epsilon b\Phi_{HbCO}[HbCO] \qquad (3)$$

where S includes geometrical factors, conversion factors, and the cross-sectional area of the photolyzing light, $I°$ is the intensity of the incident light, A is absorbance (assumed to be small in the direction of photolysis), ϵ is the molar absorptivity of HbCO, and b is the path length (2 mm). Then, in the light

$$R_{light} = \frac{l'[CO]k + k'[O_2](l + SI°\epsilon b\Phi_{HbCO})}{k'[O_2] + l'[CO]} \qquad (4)$$

and D, the difference in relaxation constants in the light and in the dark, is

$$D = R_{light} - R_{dark} = \frac{k'[O_2]SI°\epsilon b\Phi_{HbCO}}{k'[O_2] + l'[CO]} \qquad (5)$$

With conditions such that $k'[O_2] \gg l'[CO]$

$$D_{Hb} = SI°\epsilon b\Phi_{HbCO} \qquad (6)$$

Similarly, D for Mb is $SI°\epsilon b\Phi_{MbCO}$, and if S, $I°$, and b are held constant

$$\frac{D_{Hb}}{D_{Mb}} = \frac{\epsilon_{HbCO}\Phi_{HbCO}}{\epsilon_{MbCO}\Phi_{MbCO}} \qquad (7)$$

We therefore determined D_{Hb}, D_{Mb}, and the ratio $\epsilon_{HbCO}/\epsilon_{MbCO}$ for the lines emitted by the argon ion laser. For determination of R_{light} and R_{dark}, the Hb solution was prepared by diluting stock HbO_2 into 9 mL of air-equilibrated 0.1 M KP_i buffer, pH 7.0, plus 1 mL of CO-saturated buffer to final concentrations of 9 μM Hb, 95 μM CO, and 220 μM O_2, yielding $l'[CO]/k'[O_2] = 0.012$. MbO_2 was generated from met-Mb with the enzyme reducing system (Hayashi, 1973) and diluted into 5 mL of air-equilibrated buffer, 4 mL of argon-saturated buffer, and 1 mL of CO-saturated buffer to final concentrations of 9 μM total Mb, 95 μM CO, and 123 μM O_2. In this case, $l'[CO]/k'[O_2] = 0.028$. The cell was thermostated at 20.0 °C. The Hb solution was put into the cell with the shutter in place. Opening and closing the shutter then gave relaxation processes for which R_{light} and R_{dark}, respectively, were calculated. These reactions were followed at 426.6 nm. This procedure was immediately repeated with the Mb solution, the reaction being observed at 430 nm. The sequence was repeated at 11 and 25 °C. Two final curves of 1000 data points each were acquired for each relaxation, least-squares fit to a one-exponential model, and averaged. For multiline illumination, the quantity $\epsilon_{Hb}/\epsilon_{Mb}$ is replaced by

$$\Sigma(I_{\lambda i}/I°)\epsilon_{HbCO_{\lambda i}}/\Sigma(I_{\lambda i}/I°)\epsilon_{MbCO_{\lambda i}}$$

where $I_{\lambda i}/I°$ is the fraction of the total intensity of one of the five lines emitted by the argon ion laser running in the multiline mode. To determine $I_{\lambda i}/I°$, each of the five lines was isolated with a prism monochromator and its power measured to give the fraction of each band's contribution to $I°$. The values of ϵ_{HbCO} and ϵ_{MbCO} at each of the five wavelengths were derived from the associated cyanomet spectra as described for the determination of M, after the molar absorptivity of *Urechis* HbCN at 541 nm was established first to be 11.0 ± 0.2 by the pyridine hemochromogen method (Porra & Jones, 1963).

Oxygen Pulse. The method of oxygen pulse designed by Gibson (1973) was used to search for a change in k with a change in the fractional oxygen saturation. Solutions containing various concentrations of oxygen are flowed against a solution of deoxy-Hb and $S_2O_4^{2-}$. The oxygen rapidly binds to the Hb, to varying degrees of saturation, and the subsequent dissociation rates are then measured. The Hb solution was prepared by diluting stock HbO_2 into argon-saturated buffer to a concentration of 15 μM. The buffer contained 0.05% (w/v) dithionite and 1 mM DTT for stability. The initial oxygen solution was air-equilibrated buffer (121 μM in O_2 after mixing), which was successively diluted seven times to a final oxygen concentration of approximately 1 μM after mixing. The reaction was run at 22 °C, followed at 430 nm, and the total experimental time was 40 min. Either one or two reaction curves were obtained at each oxygen concentration and well fit by least squares to a one-exponential model. When two curves were collected, the first-order rate constants were averaged.

Demonstration of Deoxy Intermediate. The rate constant obtained by flowing HbO_2 against CO + $S_2O_4^{2-}$ is often used to obtain an "R-state" value for k, because CO binds very rapidly to each heme as O_2 is released. Because of the unusual combination in *Urechis* of a fast k and a slow l', it was possible to generate deoxy-Hb as an intermediate species with this reaction. In the simple model

$$HbO_2 \xrightarrow{k_1} Hb \xrightarrow{k_2} HbCO$$

if $k_1 = k_2$ and if $\epsilon_{HbO_2} = \epsilon_{HbCO}$, the theoretical maximum absorbance change (and maximum [Hb]) will occur at time $t^* = 1/k$ (where $k = k_1 = k_2$). The HbO_2 solution was prepared by diluting stock HbO_2 with the enzyme reducing system into air-equilibrated buffer to achieve a concentration of 20 μM. The CO solution was 441 μM in CO after mixing and contained 0.7 mg/mL of dithionite. The reaction was thermostated at 13 °C; at this temperature, with 441 μM CO, $k_1 = k_2 = 18$ s^{-1} and t^* is predicted to be 56 ms. The observing

Table I: Oxygen and CO Rate Constants, Activation Energies, and Calculated Equilibrium Constants for *Urechis* Hb[a]

	assoc constant (M^{-1} s^{-1})	activation energy (kcal/mol)	dissoc constant (s^{-1})	activation energy (kcal/mol)	K_d (μM)
O_2	2.10 (± 0.03) × 10^6	8.36 ± 0.23	37.5 ± 0.4	17.81 ± 0.24	17.8 ± 0.3
CO_{flow}	5.50 (± 0.02) × 10^4	8.59 ± 0.09	0.0098 ± 0.0001	20.25 ± 0.42	0.178 ± 0.002
CO_{flash}	6.62 (± 0.02) × 10^4	8.17 ± 0.07	0.0098 ± 0.0001	20.25 ± 0.42	0.148 ± 0.002

[a] 20.0 °C; pH 7.0; 0.1 M KP_i.

wavelength was approximately 431 nm, the HbO_2/HbCO isosbestic point. Multiple reactions were run to establish the reproducibility of the reaction curves. The computer-collected values of ΔAbs vs time are plotted along with the theoretically predicted curve for this model in Figure 1.

Rate of a Conformational Change. Because of a small (6–10%) but consistent difference in l' obtained from flash photolysis and flow, we investigated this difference as a function of CO concentration. Finding that the apparent l' determined by flow was independent of CO concentration, our interest focused on the change in $l^{*\prime}$ from flash as CO was varied (the asterisk denotes a more quickly reacting form of the protein). The information from the CO concentration dependence would allow calculation of an approximate value for the rate constant (k_i) for the conformational change from Hb*, the unliganded T* species, to Hb, the unliganded T-state species. The experiment was carried out in the stopped-flow apparatus. The protein solution was prepared by diluting stock HbO_2, containing the enzyme reducing system, into argon-saturated buffer which was 1 mM in DTT and adding a few crystals of dithionite. The Hb concentration after mixing was 5 μM. The initial CO solution was prepared by bubbling H_2O-saturated CO through buffer for 10 min and adding a small amount of dithionite, giving a calculated CO concentration of 491 μM after mixing. Sequential dilutions were made with argon-saturated buffer to a final CO concentration of 69 μM after mixing. The reactions were at 20.0 °C and were followed at 430 nm. For each dilution, the solutions were flowed together, and the value for the rate of that reaction was obtained by a least-squares fit of the smoothed data to a one-exponential model. By use of $l'_{flow} = 5.5 \times 10^4$ M^{-1} s^{-1}, the CO concentration was calculated. The CO was then photolyzed by a photographic flash situated immediately over the cuvet; the association reaction was recorded and fit as that for flow to obtain the value for l'_{flash}. For each concentration of CO, the photolysis data were fit very well by a one-exponential model. Because there *was* a change in l'_{flash} with CO concentration, the data were fit to an allosteric model in two ways, the distinction stemming from the assumptions that were made in each case. With the fitted rate constants, a normalized data vector was generated for each of the five experimental curves. To these points, 1% random, uncorrected noise was added. (The individual curves were fit to a normalized σ fit of 0.75%, but separate statistical studies suggest that this error somewhat underestimates the variance in sets of successive curves.) For each of the two proposed models, an analytical solution was derived for the response function, and a two-dimensional grid search was conducted in k_i and l'_T. For each set of (k_i, l'_T) values, a total sum of squares was obtained from the variance between the experimental family of curves and the corresponding theoretical family of curves. The optimal values for k_i and l'_T were determined from the minimum of the total variance. An F test for lack of fit was made, and an F contour at the 95% confidence level was constructed for each of the two models.

Results and Discussion

A minor *Urechis* hemoglobin had previously been found in our laboratory (Sima, 1979) and elsewhere (Mangum et al., 1983), and Garey and Riggs (1984) have reported multiple hemoglobins. In the latter study, there appeared to be two phenotypes, one with hemoglobins F-II and F-III (component F-III is the minor Hb, unstable with regard to oxidation) and the other with components F-I, F-II, and F-III. Both F-I and F-II appear to have at least five electrophoretic components, but both F-I and F-II have the same amino acid composition, and it was considered that they may differ only by slight posttranslational modifications. In our preparations, only two components were typically found, one of which was F-III and comprised no more than 9% of the total hemoglobin. Studies of CO and oxygen binding for this minor hemoglobin showed that, at the wavelengths employed for the kinetics reported here, that component would have contributed less than 3% to the total signal change. The detailed kinetics reported here were for a mixture containing F-III, but some studies of CO and oxygen binding were carried out on the purified major fraction and were compared with those on the mixture. For CO binding, both the mixture and the major band showed very homogeneous kinetics, with the pure component reacting about 4% faster than the mixture. Band F-III reacted at about 70% of the rate of the major band. After having passed twice over the CMC-50 column, however, the major component was indistinguishable in kinetics from the mixture. In oxygen binding kinetics, the rate of the mixture was 14% slower than that for the major fraction that had passed once over the column but only 8% slower than the rate of the major fraction that had passed twice over the column. Furthermore, the kinetics observed for the mixture were *not* the weighted sum of the kinetics for F-III and for the major component. This led us to conclude that the column preparation was slightly altering the behavior of the major component. Since we found that fraction F-III makes an insignificant (<3%) contribution to the observed kinetics, we regard the measurements reported here on the mixture to reflect quite accurately the properties of the major *Urechis* hemoglobin in our preparations.

Tables I–III show the values for O_2 and CO rate constants, activation energies, equilibrium constants, calculated thermodynamic values, and the O_2/CO partition coefficient. The

Table II: Calculated Thermodynamic Values for *Urechis* Hb for the Association of O_2 and CO[a]

	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
O_2	−6.36 ± 0.01	−9.45 ± 0.33	−10.53 ± 1.13
CO_{flow}	−9.05 ± 0.01	−11.7 ± 0.4	−8.9 ± 1.5
CO_{flash}	−9.16 ± 0.01	−12.1 ± 0.4	−10.3 ± 1.5

[a] 20.0 °C; pH 7.0; 0.1 M KP_i. [b] Standard state = 1 M. To convert to a standard state of 1 atm. for oxygen add respectively 3.83 kcal/mol, −3.36 kcal/mol, and −24.5 eu to $\Delta G°$, $\Delta H°$, and $\Delta S°$ and for CO add respectively 4.00 kcal/mol, −3.10 kcal/mol, and −24.2 eu to the tabulated values. To obtain values for unitary free energy and entropy changes, add respectively −2.3 kcal/mol and 8 eu to the tabulated values. Values for $\Delta H°$ and $\Delta S°$ for O_2 and CO dissolved in H_2O were obtained by fitting solubility data (Hodgman, 1960) from 2 to 30 °C, correcting for the vapor pressure of H_2O.

Table III: Partition Coefficient and Associated Enthalpy Change

	M (20.0 °C)	$\Delta H°_M$ (kcal/mol)
calculated	100.00 ± 2	−1.7 ± 0.5
direct measurement	97.0 ± 2.5	−2.0 ± 0.6

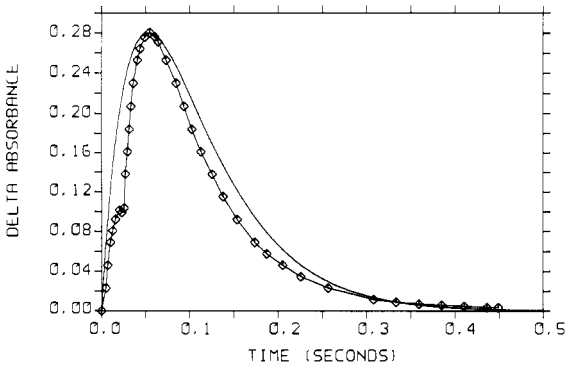


FIGURE 1: Generation of the deoxy intermediate formed as oxygen dissociates from HbO_2 in the presence of CO. The experimental curve is shown by (◇). The smooth line is the theoretically predicted curve from the simple model $HbO_2 \rightarrow Hb \rightarrow HbCO$ for observation at the HbO_2–HbCO isosbestic, with $k_1 = k_2 = 18\ s^{-1}$, $T = 13$ °C, for which t^* is calculated to be 56 ms.

direct determination of the O_2/CO partition coefficient, M, determined via ρ and σ as described earlier, gave a value for M of 97.0 ± 2.5 at 20 °C. M calculated from the measured rate constants is 100 ± 2. The ΔH°_M calculated from the activation energies of the rate constants has a large uncertainty due to considerable error propagation but does predict a slightly larger enthalpy change for the binding of CO (see Table III). The values for the quantum yield of *Urechis* HbCO relative to that of MbCO are as follows: 0.40 at 25.3 °C, 0.88 at 20 °C, and 0.96 at 10.9 °C. A significant change in Φ_{HbCO} occurred with a change in temperature, and the slope of that change predicts a Φ relative to MbCO above unity below 10.9 °C. The value for l' was the same (within 3%) for heme concentrations of 2 and 40 μM, suggesting either no significant protein aggregation changes in this range or no linkage of CO affinity to aggregation in this concentration region. Previous work (Sima, 1979) showed no sizable change from 1 to 100 μM in heme. Garey and Riggs (1984) concluded from oxygen equilibrium measurements that no change in the state of aggregation is associated with oxygenation.

Figure 1 shows the generation of the deoxy intermediate as oxygen dissociates and before CO binds. The predicted t^* (56 ms), the reaction time at the maximum concentration of the intermediate, is virtually identical to the observed t^*. (The inflection in the initial part of the experimental curve is wavelength dependent and is due to reduction of a small amount of met-Hb always present in the HbO_2 preparations. The absorbance change for met-Hb → Hb is large and in the opposite direction to that of the reaction of interest.) Even in the presence of the enzyme reducing system, a small amount (~2%) of the iron is in the ferric form. In keeping with our simple model [$\Delta Abs_t = Abs_t - Abs_\infty = (\epsilon_{Hb} - \epsilon_{HbO_2})b(HbO_2)_0\ kte^{-kt}$], the wavelength for observation was constrained to be the HbO_2/HbCO isosbestic point, therefore, this interference could not be optically eliminated. With our experimental conditions, the interfering reaction was over in 50 ms and most likely accounts for the departure from prediction in the oxygen dissociation part of the curve. The model is oversimplified in that the individual k and l' reactions are treated as single heme processes. In reality, CO binds to some tetramers that still have O_2 bound. Thus, the actual CO binding is probably described by a value of l' for the simple model that is slightly larger than that obtained from stopped flow, which was used to devise the experiment.

The reaction curves for all kinetics experiments were monorather than multiphasic, indicating negligible subunit heterogeneity. These conclusions are complemented by the structural work of Garey and Riggs (1984), who identified three chromatographic fractions, F-I–F-III. The major fractions F-I and F-II are tetramers whose amino acid compositions are very similar and may differ only in a few post-translational modifications. Garey and Riggs found that the subunits of F-I are nearly identical. In contrast, the composition of minor fraction F-III was found to be markedly different from those of F-I and F-II, in that it was brownish on columns, apparently unstable, and easily oxidized. Although we saw no functional differences that might arise from F-III, we did find instability that could be attributable to this component and observed that the addition of the enzyme reducing system, DTT, and BSA markedly improved the stability of our preparation. We have concluded that functionally, for the ferrous reactions in this study, *Urechis* Hb is composed of homogeneous tetramers with equivalent subunits.

The collective kinetic data together with an independent equilibrium determination (Larsen et al., 1988) support the conclusion that *Urechis* hemoglobin is weakly cooperative and, overall, of low affinity. Data from the oxygen equilibrium measurement give a Hill number of 1.30 ± 0.01 and a K_D (or $\bar{X}$, oxygen concentration at half-saturation) of 19.60 ± 0.16 μM at 21.7 °C. Garey and Riggs reported a K_D of 21.4 μM at 20 °C and a Hill number of 1, and Mangum et al. (1983) reported a K_D of 19.8 μM for intact coelomic cells at 20 °C and a Hill number of 1.11. The oxygen dissociation and the CO association reactions both yielded evidence of cooperativity. The two methods used to obtain the rate constant for CO dissociation often show differences that reflect CO cooperativity. The rate constant from NO replacement should be the slower T^* rate constant (l^*) whereas that obtained with microperoxidase is "l overall" and should be less than l^*. When the HbCO was photolyzed to 40–10% saturation in the presence of microperoxidase, there was ample time for conformational relaxation, and the CO dissociation kinetics should have reflected the increased fraction of T-state conformers. For none of the experiments, however, were there consistent differences among the various values of l (all within 6%).

For k, the different states of saturation produced by varying the initial oxygen concentration resulted in no significant differences (within 1%) in the values for the dissociation constant. The oxygen pulse experiment, however, showed a 3–4-fold increase in k as the initial oxygen concentration was changed from 121 to 10 μM. From $[O_2] = 120\ \mu M$ to $[O_2] = 30\ \mu M$, k remained relatively constant near 50 s^{-1} (at 22 °C) and then rapidly became faster and remained near 150 s^{-1} for initial O_2 concentrations of ≤10 μM.

Two studies of CO ligation gave further evidence of functionally different states: (1) Binding was studied as a function of fractional photolysis at low and at high CO. At a low CO concentration (94 μM), full photolysis consistently produced a value for l' 7–10% slower than the value obtained from partial photolysis (10–15%). At a high CO concentration (475 μM), the value for l'_{flash} appeared to be independent of the percent of photolysis. Low concentrations of free CO allow time for the conformational change (Hb* → Hb) following full photolysis with subsequent binding at the slower T-state rate, whereas partial photolysis leaves the protein largely in the T* state, resulting in the faster recombination. At high CO concentration, the rate of the conformational change cannot compete effectively with the rapid ligand binding and the rate appears independent of the percent of photolysis. The difference in the value of l' that results from varying [CO], with full photolysis, is depicted in Figure 3. (2) Different rates were obtained for l' by flow and by flash photolysis on the same

Scheme I

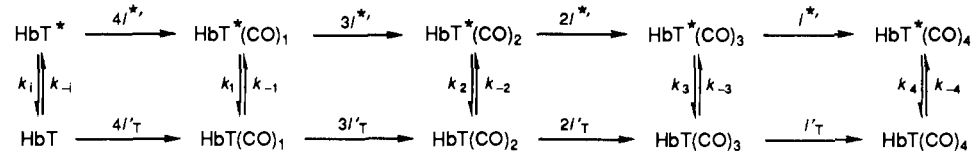


Table IV: Values for CO Association Obtained by Flash Photolysis as a Function of CO Concentration[a]

[CO] (μM)	l' (M^{-1} s^{-1})	l'(stopped flow)/l'(photolysis)
372	5.89 ± 10^4	0.93
192	5.90 ± 10^4	0.93
139	5.71 ● 10^4	0.96
98	5.63 ± 10^4	0.98
69	5.58 ± 10^4	0.99

[a] 20.0 °C; pH 7.0; 5 μM heme; complete photolysis.

sample, but the apparent l' by flow was independent of [CO]. Table IV shows l'_{flash} as a function of CO concentration.

Our oxygen kinetic data corrected to 21.7 °C via energies of activation give an apparent K_D of 19.6 ± 0.5 μM. The combined oxygen studies lead to the conclusion that this constant represents the higher affinity state of the protein. The very low concentration of oxygen required to produce the T-state rate for oxygen dissociation in the oxygen pulse experiment implies that the T* to T switch occurs at a low level of ligation. In fact, the allosteric crossover point is less than 1. Therefore, the measured rate for k is largely a rate for the high-affinity conformation. The oxygen association constant, k', obtained from the relaxation experiment in the presence of CO (Parkhurst, 1979) is likewise for the T* state, with the CO binding and maintaining the T* conformation. It will be shown that the rate of the conformational change is slow compared to the binding of oxygen, so even in the absence of CO, the protein would not have had time to change from T* to T before the oxygen recombined. There is excellent agreement between this kinetically determined value for K_{T*} and the value obtained from our equilibrium measurement. Having confidence that K_{T*} was established and knowing n_H and $\bar{X}$, we proceeded to examine allosteric mapping to determine values for the allosteric parameters L and c.

Figure 2 shows an allosteric mapping from an "experimental space" (n_H, $\bar{X}/K_{T*}$) to $1/c$, L or "allosteric space". Only points above the line in panel I give real and/or positive values of L and c. We show the mapping in panel II for the locus of allowed points with $n_H = 1.3$ and $\bar{X}/K_{T*} < 3$. It is clear that if an allosteric model is to fit the data, L must be small, since, in fact, $\bar{X}$ from equilibrium measurements and K_{T*} differ only by experimental error. The Adair constant K_1, however, provides, in addition to $\bar{X}$ and n_H ($K_1 = 0.08$), a third relationship among K_{T*}, L, and c, allowing us to establish a point on the curve in panel II that best describes the O_2 binding in terms of the allosteric model. That point corresponds to $L = 2.61$ and $c = 0.048$. Having an allosteric description of the O_2 equilibrium properties, we then sought to explore the oxygen and CO kinetics in terms of the model. For CO binding, L must also equal 2.61, but c is expected to differ. The data for l' from flash photolysis as a function of CO concentration were fit to an allosteric model for two cases, as described under Materials and Methods. For both cases, the only fitted parameters were k_i and l'_T, and the model shown in Scheme I was used. A different assumpion was made in each case, concerning the equilibration between the T* and T species after the first ligand is bound. For case I, complete equilibration was assumed for $Hb(CO)_1$ through $Hb(CO)_4$, so that the rates represented by k (±1) through k (±4) are occurring much

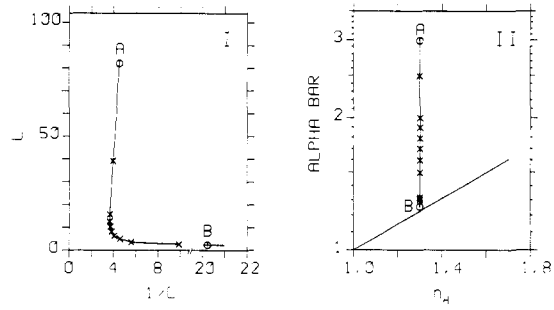


FIGURE 2: Allosteric mapping of oxygen equilibria for *Urechis* Hb. Panel I shows a mapping in allosteric parameter space of the points from A → B in the experimental n_H, $\bar{\alpha}$ space. In panel II, n_H is the Hill number, and $\bar{\alpha} = \bar{X}/\bar{X}_{T*}$, where $\bar{X}$ is the half-saturation oxygen concentration and $\bar{X}_{T_l} = k_{T*}/k'_{T*}$, where k_{T*} and k'_{T*} are respectively apparent oxygen dissociation and association rate constants for the T*-state. In panel II, only points in the region above the upward sloping line beginning at (1, 1) give rise to real, positive values for L and c.

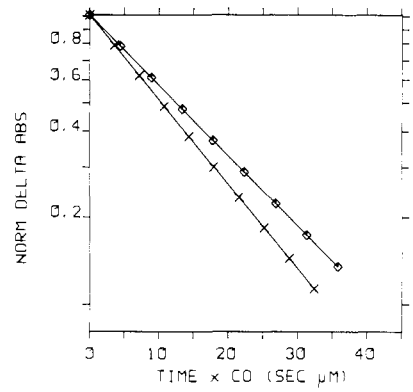


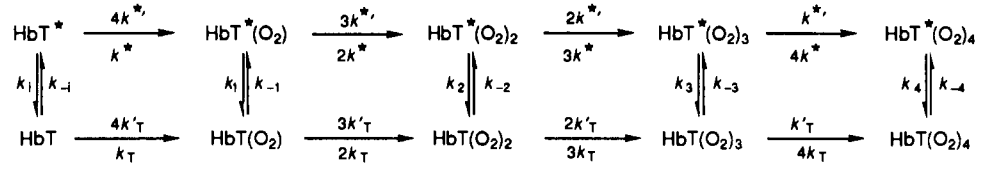
FIGURE 3: A semilogarithmic plot of $\Delta Abs(t)/\Delta Abs(0)$ vs a scaled time from a flash photolysis study of CO recombination. Results at 491 μM (CO) are depicted by (×) and those at 69 μM in CO by (◇). Successive dilution of CO produced a family of curves between the two limits shown, for which there was a monotonic increase in the absolute value of the slope with increase in [CO]. If there were no change in the apparent second-order rate constant with [CO], all curves would be superimposed.

faster than any of the ligation processes. For case II, we assumed no equilibration; k (±1) through k (±4) = 0. The value of L, determined from the oxygen studies, was used to assign a value to k_{-i} for each k_i, since $k_{-i} = k_i/L$. The value for $l^{*\prime}$ was set at 6.62×10^4 M^{-1} s^{-1}, which was the rate constant determined at high CO concentration and partial photolysis. For case I, the problem was simplified by using the fact that after the first ligation the kinetic model collapses to a single line and for the nth ligation step, $n > 1$, the apparent rate constant in the sequential scheme is

$$(4 - n + 1)[(l^{*\prime} + Lc^{n-1}l'_T)/(1 + Lc^{n-1})]$$

Since for most hemoglobins it is the CO association constant that most significantly affects CO affinity (Schreiber & Parkhurst, 1984) and since CO dissociation with microperoxidase gave no evidence for enhancement of l, we assumed that $c = l'_T/l^{*\prime}$. For both cases, closed-form analytical solutions were readily obtained for concentrations from Laplace transformation of the rate constant matrix and application of

Scheme II



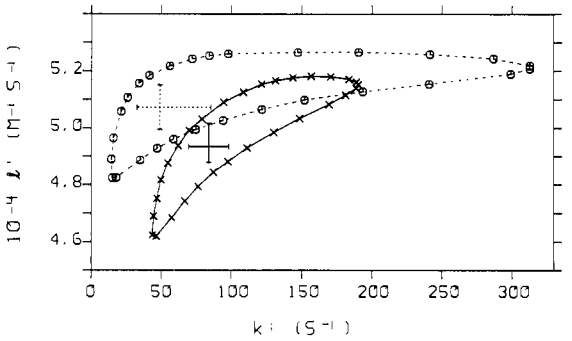


FIGURE 4: F contours at the 95% confidence level for the two-parameter (k_i, l'_T) fit of the CO association kinetic data to an allosteric model. The broken line represents the contour for case I, which assumes rapid conformational equilibration between the intermediates; the smooth line represents the contour for case II, which assumes no conformational equilibration between the partially liganded intermediates. The perpendicular lines inside each contour intersect at the optimal parameter values and show the limits of a contour at the 68% confidence level.

the Bromwich (1916) integral to the appropriate matrices (Goertzel & Tralli, 1960). Appropriate response functions were generated for data fitting.

The grid search in k_i and l'_T was then conducted. The optimal parameter values were, for case I, $k_i = 50\ s^{-1}$ and $l'_T = 5.075 \times 10^{-4}\ M^{-1}\ s^{-1}$; for case II, $k_i = 85\ s^{-1}$ and $l'_T = 4.935 \times 10^{-4}\ M^{-1}\ s^{-1}$. Neither model could be rejected on the basis of the F test applied to test for lack of fit. F contours at the 95% confidence level were found for each model and are displayed in Figure 4 together with the parameter limits shown by lines parallel to the axes for a 68% level of confidence. The allosteric parameter $c = l'_T/l^{*\prime}$ was then known for each model and alllowed calculation of the associated unique coordinates of n_H and $\bar{\alpha} = (\bar{X}/\bar{X}_{T^*})$. For case I, $n_H = 1.013$ and $\bar{\alpha} = 1.17$ ($c = 0.768$). For case II, $n_H = 1.015$ and $\bar{\alpha} = 1.186$ ($c = 0.750$). From $\bar{\alpha}$ and K_{T^*}, the half-saturation concentration, $\bar{X}$, for CO can be obtained: for case I, $\bar{X} = 0.173\ \mu M$; for case II, $\bar{X} = 0.176\ \mu M$. We conclude that the correct model probably lies somewhere in between the two extreme cases of I and II, that the two conformations of the protein bind CO at rates that are distinct but relatively close in value (within 25%), and that the rate of the conformational change between the two states is slow, with a half-time in the range of 8–14 ms. For this range of values for k_i, CO ligation and k_i are in competition at high CO concentrations and complete pohtolysis, but at low CO concentrations (69 μM), ligation is 3–4-fold slower than k_i, and binding occurs essentially to the low-affinity conformation.

Adequate information is now available to examine the oxygen kinetics in terms of the allosteric model. Specifically, it was of interest to know if the results of the oxygen pulse experiment could be explained in terms of the model, in accord with the values of all of the rate constants and the allosteric parameters previously determined. A simulation was done, with the model shown in Scheme II. The rate of the conformational change from Hb* to Hb is ligand independent and so could be applied to this model for oxygen dissociation. In contrast to CO ligation, the value of c for oxygen binding results primarily from differences in the off constant between the two states (Schreiber & Parkhurst, 1984). (We had previously found that the observed k' was independent of the percent photolysis, and the low value for k_i assured that the measured k' could be assigned to the T^* state.) We therefore assigned the value of 766 s^{-1} to k for the T-state, on the basis of $c = 0.048$ and $k^* = 37.46\ s^{-1}$, as previously determined. The values of k_1 to k_4 are equal to Lc^n, where n = the number of bound ligands. The values of the associated rate constants were unknown in the model, but all of the T* → T conformational changes were assigned the k_i value of 85 s^{-1}, constraining the opposing reaction to satisfy the value of the associated K_n. Our interest was in knowing whether the model could accurately predict the observed rates of oxygen dissociation at high $[O_2]$, with nearly complete saturation of the protein, and at very low $[O_2]$, with ligation proceeding essentially only to the first step. Therefore, the simulation was done for $[O_2] = 10$ and 130 μM. Numerical integration was used to obtain values for all concentrations as a function of time, leading directly to a value for the response function. Under these conditions, the simulated values for the rates for oxygen dissociation at the two concentrations were within 10% of the values observed experimentally, and we concluded that both the O_2 and the CO kinetics could be very satisfactorily explained in terms of an allosteric model.

At pH 9.0, l' is consistently slightly lower (2–7%) than at pH 7.0, a result opposite of expectation based on the behavior of hemoglobins which, unlike *Urechis* (Sima, 1979), have a significant alkaline Bohr effect. At low CO concentration, l'_{flash} from total photolysis and from flow agree at pH 7.0, whereas these values show a difference of 4.5% at pH 9. Analogous to mammalian hemoglobins, this difference suggests an even slower rate of change from Hb* to Hb at pH 9.0 than at pH 7. Extensive experiments, however, were not done at pH 9.0, and the data were not fit as for pH 7.0.

Our initial objective in this research was to obtain highly precise values for rate constants from which equilibrium constants and thermodynamic quantities could be obtained for a naturally occurring T-state hemoglobin which might serve as a general model for the T-state. Previous work (Sima, 1979) in our laboratory had given little evidence for cooperativity in ligand binding. Improvements in data collection, analysis, and flow–flash instrumentation allowed us to detect evidence for slight cooperativity in the binding of CO. Very careful equilibrium studies combined with oxygen pulse measurements showed that oxygen binding was also cooperative and that allosteric parameters could be obtained. Were there a significant Bohr effect in this protein, the variations of the rate constants, equilibrium constants, and derived thermodynamic quantities with temperature should have been corrected for the small pH changes of the buffers with temperature (Wyman, 1949), but that correction was not necessary for *Urechis* Hb. The question now is to what extent can we correlate our measurements with those of a "T" state? For CO binding, there was never any detectable acceleration in CO dissociation with fractional ligation, and the differences between flow and flash kinetics suggest that, in terms of an allosteric model, the low- and high-affinity forms differ by only 23% ($c = 0.77$).

The low value for L (2.61) means that determinations of $l^{*\prime}$ at high CO and less than complete photolysis can be assigned to the higher affinity state. The activation energies reported in Table I for flow and flash photolysis are virtually identical, as expected. The CO binding parameters thus are calculated for the higher affinity state, which we denote as T* since it is one of low ligand affinity and appears from all of our measurements to be but slightly different from T. For oxygen binding, the Hill number of 1.3 assures that we are dealing with low cooperativity, and further analysis and modeling of the oxygen pulse experiments shows that the crossover between T and T* is between zero and one bound ligand. Here, the only rate constant that contains any information on the T state is that for oxygen dissociation at low saturation (<20%) as measured by oxygen pulse. For that reason, the kinetic and thermodynamic quantities reported also can be assigned to the T* state for oxygen ligation. Separate nanosecond geminate studies show that for CO and oxygen the geminate recombination is slow (Parkhurst and Gibson, unpublished observation), consistent with the high quantum yield and assignment of the initial photolysis state as a T state. Our EXAFS (Chance et al., 1986) studies have shown that the Fe–out of plane distance for the HbCO form is unusually large (0.35 Å). Associated with this large displacement are the low values for $\Delta G°$ and $\Delta H°$ for the binding of both oxygen and CO. In contrast, the values for $\Delta S°$ are nearly the same as those we have measured for leghemoglobin (unpublished results), a hemoglobin with a large heme cavity and one with extreme R-state properties for the binding of both CO and oxygen. For this latter hemoglobin, we showed that the entropy changes were consistent with a simple model in which the ligand was merely immobilized at the binding site with little alteration in the protein or solvent. Such appears to be the case for *Urechis* Hb as well, for which the low affinity derives from a low heat of formation for the Fe–CO and Fe–oxygen bonds which in turn can be correlated with the large displacement of the Fe from the center of the porphyrin. These enthalpy changes are, for CO and oxygen, respectively 4.34 and 3.95 kcal/mol smaller than those for ligation to leg-Hb for which EXAFS studies (Chance et al., 1986) have shown the Fe to be in the plane of the heme for LbCO. We cannot carry the thermodynamic analysis further at this time since we lack information on the Fe–out of plane distance for *Urechis* deoxy-Hb. A very detailed calorimetric study of CO binding to hemoglobin I of trout showed that the enthalpy of ligation to the T-state was positive (Barisas & Gill, 1979) and that the reaction was driven by a very large positive change in entropy. The enthalpy change for ligation to the R state was negative, however. Trout HbI shows high cooperativity in CO binding and clearly undergoes large conformational changes associated with ligation. It is now clear that both entropy and enthalpy changes need to be considered in discussions of R- and T-state properties. *Urechis* Hb appears to be an ideal system for isolating the energetics of ligation in a T-state.

References

Barisas, B. G., & Gill, S. J. (1979) *Biophys. Chem. 9*, 235–244.

Bromwich, T. J. I'a. (1916) *Proc. London Math. Soc. 15*, 401.

Chance, M. R., Parkhurst, L. J., Powers, L. S., & Chance, B. (1986) *J. Biol. Chem. 261*, 5689–5692.

Draper, N., & Smith, H. (1981) *Applied Regression Analysis*, 2nd ed., pp 8–30, Wiley, New York.

Garey, J., & Riggs, A. (1984) *Arch. Biochem. Biophys. 228*, 320–331.

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

Goertzel, G., & Tralli, N. (1960) *Some Mathematical Methods of Physics*, pp 38–49, McGraw-Hill, New York.

Hayashi, A., Suzuki, T., & Shin, M. (1973) *Biochim. Biophys. Acta 310*, 309–316.

Hodgman, C. D., Ed. (1960) *Handbook of Chemistry and Physics*, 41st ed., pp 1706–1707, Chemical Rubber Co., Cleveland, OH.

Kolatkar, P. R., Meador, W. E., Stanfield, R. L., & Hackert, M. L. (1988) *J. Biol. Chem. 263*, 3462–3465.

Larsen, T., Mueser, T., & Parkhurst, L. J. (1988) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 2*, A325.

Mangum, C. P., Terwilliger, R. C., Terwilliger, N. B., & Hall, R. (1983) *Comp. Biochem. Physiol. 76A*, 253–257.

Noble, R. W., Brunori, M., Wyman, J., & Antonini, E. (1967) *Biochemistry 6*, 1216–1222.

Parkhurst, L. J. (1979) *Annu. Rev. Phys. Chem. 30*, 503–546.

Parkhurst, L. J., Sima, P., & Goss, D. J. (1980) *Biochemistry 19*, 2688–2692.

Porra, R. J., & Jones, O. T. G. (1963) *Biochem. J. 87*, 181–185.

Savitzky, A., & Golay, M. J. E. (1964) *Anal. Chem. 36*, 1627.

Schreiber, J., & Parkhurst, L. J. (1984) *Comp. Biochem. Physiol. 78A*, 129–135.

Sharma, V. S., Ranney, H. M., Geibel, J. F., & Traylor, T. G. (1975) *Biochem. Biophys. Res. Commun. 66*, 1301–1306.

Sima, P. (1979) Ph.D. Dissertation, University of Nebraska.

Wyman, J. (1948) in *Advances in Protein Chemistry* (Anson, M. L., & Edsall, J. T., Eds.) pp 458–467, Academic, New York.