Carbon Monoxide Binding to Human Hemoglobin A_0[†]

Enrico Di Cera, Michael L. Doyle, Patrick R. Connelly, and Stanley J. Gill*
Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215
Received April 14, 1987; Revised Manuscript Received June 8, 1987

ABSTRACT: The carbon monoxide binding curve to human hemoglobin A_0 has been measured to high precision in experimental conditions of 600 μM heme, 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, 0.1 M NaCl, 10 mM inositol hexaphosphate, 1 mM disodium ethylenediaminetetraacetic acid, pH 6.94, and 25 °C. Comparison to the oxygen binding curve in the same experimental conditions demonstrates that the two curves are not parallel. This result invalidates Haldane's two laws for the partitioning between carbon monoxide and oxygen to human hemoglobin. The partition coefficient is found to be 263 ± 27 at high saturation, in agreement with previous studies, but is lowered substantially at low saturation. Although the oxygen and carbon monoxide binding curves are not parallel, both show the population of the triply ligated species to be negligible. The molecular mechanism underlying carbon monoxide binding to hemoglobin is consistent with the allosteric model [Di Cera, E., Robert, C. H., & Gill, S. J. (1987) *Biochemistry 26*, 4003–4008], which accounts for the negligible contribution of the triply ligated species in the oxygen binding reaction to hemoglobin [Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., & Robert, C. H. (1987) *Biochemistry 26*, 3995–4002]. The nature of the different binding properties of carbon monoxide stems largely from the lower partition coefficient of the T state (123 ± 34), relative to the R state (241 ± 19). These values closely resemble the partition coefficients for the isolated α and β chains, respectively, thus supporting the prediction of the model that ligation occurs first at the α chains in the T state and then at the β chains after the conformational transition to the R state.

It has been known since 1912 that carbon monoxide combines with very high affinity to human hemoglobin and dramatically affects the oxygen dissociation curve of whole blood (Douglas et al., 1912). Haldane was the first to elucidate the toxicity of this effect and proposed two famous laws regarding the binding of carbon monoxide and oxygen to human hemoglobin (Haldane & Smith, 1897). The first law says that when hemoglobin is exposed to a mixture of the two gases the ratio of carbon monoxide $\bar{Y}$ to oxygen $\bar{X}$ molecules bound to human hemoglobin is proportional to the ratio of the partial pressures of the two gases (y for CO; x for O_2) according to a constant factor m, the partition coefficient, that is

$$\frac{\bar{Y}}{\bar{X}} = m\frac{y}{x} \tag{1}$$

The second law says that the total saturation $\bar{T} = \bar{Y} + \bar{X}$ is given by

$$\bar{T} = \bar{Y} + \bar{X} = f(x + my) \tag{2}$$

and is a function of $x + my$ only. According to these laws the carbon monoxide binding curve simply parallels the oxygen binding curve, the displacement of the two curves being given by a constant factor, the partition coefficient m.

Wyman (1948) showed that either law is a consequence of the other over the domain of $\bar{T}$ in which it holds. The basic question arises as to the extent of this domain. Haldane believed that it extended over all values of $\bar{Y}$ and $\bar{X}$, and therefore that the entire CO dissociation curve would merely be the O_2 dissociation curve shifted to lower ligand partial pressures. Barcroft (1928) reported a slight, but distinct, difference of the two dissociation curves in whole blood, particularly at low saturations. Although the data available at that time were not accurate enough to prove this point, the observation was later confirmed in more accurate studies on whole blood (Roughton, 1970; Okada et al., 1976; Hlastala et al., 1976). These results are at variance with the prediction of Haldane's first and second laws.

However, a rigorous test of Haldane's laws is best obtained by examination of CO binding to the purified hemoglobin molecule. Preliminary work on the competition reaction between CO and O_2 to human hemoglobin has demonstrated the validity of Haldane's prediction (Wyman et al., 1982) in the domain where the macromolecule is fully saturated ($\bar{T} = 4$). A more recent investigation of the partitioning of CO and O_2 to the isolated α and β chains (Bishop & Gill, 1986) has revealed a significant difference in the partition coefficients, suggesting that a difference exists in the partition coefficients of the two chains in the assembled molecule.

Determination of CO binding curves to hemoglobin is also motivated by the recent observation that in the case of O_2 binding the triply ligated species makes virtually no contribution to the oxygenation process (Gill et al., 1987). This result has been interpreted within the framework of an allosteric model (Di Cera et al., 1987), which bears on the stereochemical mechanism of oxygenation proposed by Perutz (1970). A critical question arises as to the generality of the low population of triply ligated species. This observation has been made for O_2 binding to human hemoglobin over a variety of solution conditions (Gill et al., 1987; Doyle et al., 1987), suggesting that the mechanism that leads to the low population of triply ligated species is an intrinsic property of the molecule (Di Cera et al., 1987). If so, then one might observe a similar result for the ligand CO.

In this paper we report precision carbon monoxide binding curves to human hemoglobin A_0. The aim of the present study is twofold: examining the validity of Haldane's first and second laws over the entire domain of $\bar{T}$, from 0 to 4, and testing the molecular mechanism by which hemoglobin expresses a co-

[†] This work was supported by National Institutes of Health Grant HL22325.

0006-2960/87/0426-6494$01.50/0

operative behavior. The results are compared to oxygen binding under identical experimental conditions.

Theory

In this section we summarize the basic aspects of the thermodynamic linkage relationships employed in this study.

The phenomenological examination of the partition of CO and O_2 to hemoglobin is based upon the fundamental rules of the linkage theory (Wyman, 1948, 1964). For a nondissociating tetrameric macromolecule, the binding polynomial (Wyman, 1964) or, in most general terms, the binding partition function Ξ (Hill, 1985) can be expressed exactly in terms of four ligand binding sites. The binding partition function for oxygen binding to hemoglobin is then

$$\Xi(x) = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 \qquad (3)$$

where x is the oxygen partial pressure and β_i is the overall Adair constant (Adair, 1925) for the reaction $M + iX = MX_i$ (M denotes hemoglobin). The number of moles of O_2 bound per mole of hemoglobin is readily obtained from $\Xi(x)$ by differentiation as

$$\bar{X} = \frac{d \ln \Xi(x)}{d \ln x} \qquad (4)$$

By substituting x with y, the carbon monoxide partial pressure, and $\bar{X}$ with $\bar{Y}$, the number of moles of CO bound per mole of hemoglobin, we obtain the thermodynamic description of CO binding.

When both CO and O_2 are present in solution, hemoglobin can react with either ligand and the binding partition function is expanded along two "coordinates", corresponding to the chemical potentials of the two ligands. It is here that the fundamental principles of linkage thermodynamics (Wyman, 1948, 1964) come into play. The linkage between CO and O_2 to hemoglobin is particularly attractive since it is an *identical linkage*. Both ligands have the same binding site, and either ligand, once bound, excludes the binding of the other to the same site. One can depict the general reaction scheme as

$$M + iX + jY = MX_iY_j \qquad (5)$$

with a corresponding overall Adair constant β_{ij} ($\beta_{00} = 1$) for the species with i oxygens and j carbon monoxides bound. The basic feature of this reaction is that the sum $i + j$ cannot exceed 4, the total number of binding sites, for whatever values of i and j. Accordingly, the binding partition function becomes

$$\Xi(x,y) = \sum_{i=0}^{4} \sum_{j=0}^{4-i} \beta_{ij} x^i y^j \qquad (6)$$

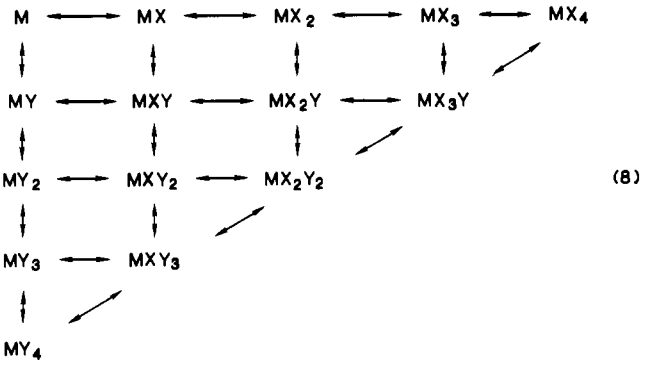
The functions $\bar{X}$ and $\bar{Y}$ are readily obtained by differentiation as

$$\bar{X} = \left(\frac{\partial \ln \Xi(x,y)}{\partial \ln x} \right)_{\ln y} \qquad (7a)$$

$$\bar{Y} = \left(\frac{\partial \ln \Xi(x,y)}{\partial \ln y} \right)_{\ln x} \qquad (7b)$$

From the relations above it is easy to verify that $\bar{X} + \bar{Y} \leq 4$.

The linkage scheme described in eq 6 can be diagrammed in the form of a triangle of species along the two x and y coordinates:



(8)

The critical step is the experimental determination of all these species by equilibrium measurements of CO and O_2 binding to hemoglobin.

Materials and Methods

Experimental Conditions. In the past, attempts in our laboratory to measure the CO binding curve of human hemoglobin by use of the thin-layer method (see below) have failed due to the extremely long equilibration time for removing CO from the sample solution. The strategy in the present study has been to find solution conditions that reduce this equilibration time to a practical level (5–25 min). In this connection one considers minimizing the CO affinity by lowering the pH to a region where the median partial pressure is maximum and employing effector molecules such as IHP.[1] Additionally, one can lower the hemoglobin concentration.

Experiments were therefore conducted under solution conditions of 0.1 M HEPES buffer, 0.1 M NaCl, 1 mM Na_2EDTA, 10 mM IHP, 25 °C, and pH 6.94. The HEPES buffer ($pK = 7.5$) was used to optimize pH control (Gill et al., 1987). The Na_2EDTA minimizes oxidation (Chu et al., 1984). The enzymic reducing system of Hayashi et al. (1973) was used in all cases to reduce formation of metHb. All components of the reductase system, the HEPES buffer, and IHP were products of Sigma. Human hemoglobin A_0 was prepared according to the method reported by Williams and Tsay (1973) and stored in liquid N_2.

The hemoglobin concentration for this study has been chosen on the basis of two criteria: (1) The concentration should be high enough to minimize the complicating effects of CO- and O_2-linked dissociation of the tetramers, yet (2) it should be low enough to enable removal of CO from the sample solution within a measurable time frame. A hemoglobin concentration of 600 μM heme was found to meet both criteria. It should be pointed out that in the absence of IHP a heme concentration of 600 μM does not meet the first criterion (Mills et al., 1976; Johnson & Ackers, 1977; Gill et al., 1987) nor the second. On the other hand, employment of IHP is known to stabilize the oxygenated tetrameric form of hemoglobin with respect to dimers (Chiancone et al., 1974; Imai, 1979). Moreover, the carboxy tetramer has been reported to dissociate even less than the oxy tetramer (Guidotti, 1967).

To establish that the ligand-induced dissociation of human hemoglobin under the experimental conditions of the present study was negligible, we have measured the O_2 binding curve over the range of 30 μM to 1.2 mM heme and the CO binding curve over the range 100–600 μM heme. Saturating amounts of IHP were observed to greatly reduce the ligand-linked

[1] Abbreviations: Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; IHP, inositol hexaphosphate.

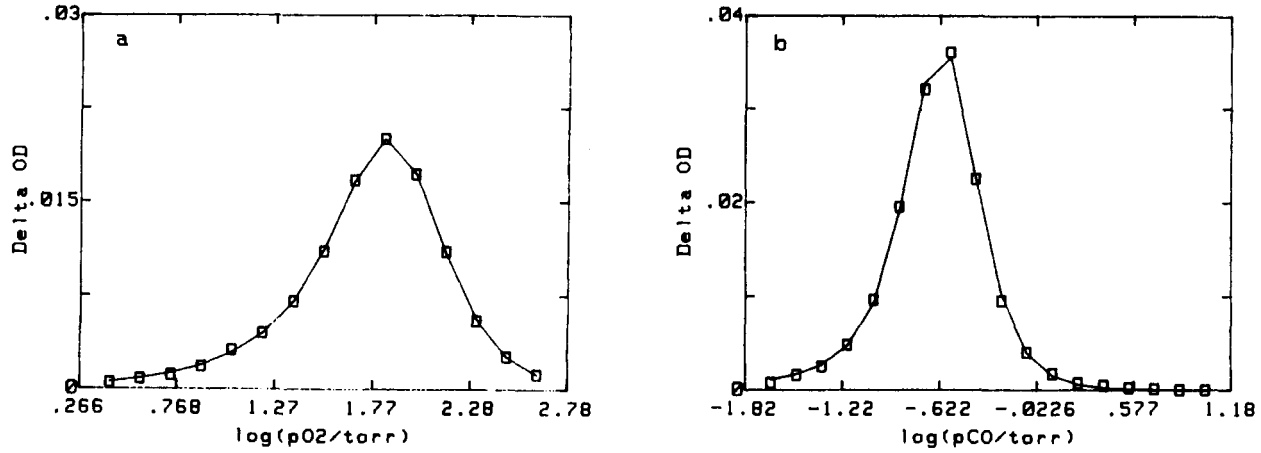


FIGURE 1: Optical density changes for stepwise changes in ligand partial pressure above a thin-layer (0.0025-cm) sample of Hb A_0. Experimental conditions are reported in the text. The changes are plotted versus the logarithm of ligand partial pressure. The solid line was drawn according to the best fit values of the β's reported in Table I, with eq 3 as the binding partition function. (a) O_2; (b) CO.

dissociation of hemoglobin into dimers, as judged by the constancy of the overall Adair equilibrium parameters. The effect of dimers was insignificant (under the experimental conditions described above) in the O_2 binding curve at heme concentrations as low as 300 μM and in the CO binding curve at heme concentrations as low as 100 μM.

Differential Binding Curve Measurement. An important concern in the acquisition of precise ligand binding curves is the ability to know the solution activity of the ligand at each point along the binding curve. This can be accomplished by equilibrating the sample solution with a well-defined ligand gas phase. The binding curves in this study were obtained with a thin-layer optical technique that makes use of this principle. The technique has been discussed in detail elsewhere[2] (Dolman & Gill, 1978; Gill et al., 1987). Dilutions of the ligand partial pressure were made with nitrogen gas, by means of a dilution valve connected to the gas-tight sample cell. The partial pressure p_i of the ligand after the ith stepwise dilution is calculated from

$$p_i = p_0 D^i \tag{9}$$

where p_0 is the starting partial pressure of the ligand and D is a constant dilution factor (typically about 0.6955 ± 0.0005). Experiments were typically initiated with CO at 2.499% of the atmospheric pressure and O_2 at atmospheric pressure. Changes in the fractional saturation of either CO or O_2 were measured as changes in optical density with a CARY 219 spectrophotometer at either 419 or 414 nm, respectively. The sample thickness was 0.0025 cm, leading to rapid equilibration with each new partial pressure of the gaseous ligand. The percentage of metHb was measured before and after each experiment by the ratio of absorbances at 578 and 500 nm in the spectrum of the fully oxygenated molecule (Kilmartin et al., 1978) to be less than 2%.

Data Analysis. At each dilution in the ligand partial pressure, the fractional saturation change is computed and multiplied times the total optical density change for fully saturating the molecule. The observed change in optical density ΔOD_i for the ith dilution step where the ligand partial pressure changes from p_{i-1} to p_i is described by

$$\Delta OD_i = \Delta OD_T(\theta_i - \theta_{i-1}) \tag{10}$$

where θ is the fractional ligand saturation of the macromolecule at a given step and ΔOD_T is a parameter representing the optical density change on taking the macromolecule from zero to complete saturation. The degree of saturation θ is simply equal to the number of ligand molecules bound per mole of hemoglobin, $\bar{X}$ for O_2 and $\bar{Y}$ for CO, divided by 4, the number of binding sites.

All binding parameters entering the function θ and ΔOD_T were estimated by least-squares optimization of the appropriate fitting equations to the data, with the Gauss–Newton algorithm as modified by Marquardt and others (Frazier & Suzuki, 1973). One standard deviation confidence intervals for all the parameters were determined by F tests (Magar, 1972). Hewlett-Packard 9816 and 9000/300 computers were employed for all analyses.

RESULTS

Although the thermodynamic treatment of the CO–O_2 linkage to hemoglobin is straightforward, nevertheless only two edges of the thermodynamic triangle have been explored so far in a quantitative way, namely, the top edge, representing the O_2 binding reaction (Imai, 1982; Chu et al., 1984; Gill et al., 1987), and the diagonal edge, representing the competition reaction of CO and O_2 in the fully saturated molecule (Wyman et al., 1982). Due to the very high affinity of CO for hemoglobin, no information has been available on the distribution of the remaining species of the thermodynamic triangle.[3]

O_2 and CO Binding Curves. The top horizontal edge of the triangle in eq 8 represents the O_2 binding curve of hemoglobin in the absence of CO, and the left vertical side represents the CO binding curve in the absence of O_2. Both of these binding curves were measured under identical solution conditions and are shown in Figure 1 as differential binding curves. The data on these curves represent the change in optical density for a change in ligand partial pressure. In the case of the O_2 binding curve, the hemoglobin moves from MX_4 to M, through the intermediates MX_3, MX_2, and MX (see eq 8). The analysis of the data yields the values for β_{10}, β_{20}, β_{30}, and β_{40}. Similarly the values of β_{01}, β_{02}, β_{03}, and β_{04} are determined from the CO binding curve.

The striking conclusion drawn from the best fit values of the Adair constants reported in Table I is that β_{30} and β_{03} are

[2] The particular application to the study of CO and O_2 identical linkage to hemocyanins has demonstrated the basic features of the method (Richey et al., 1985; Zolla et al., 1985).

[3] Stoichiometric titrations of Hb with CO have been reported (Anderson & Antonini, 1968), but in extremely low hemoglobin concentrations (below 1 μM heme) where the dissociation into dimers is severe.

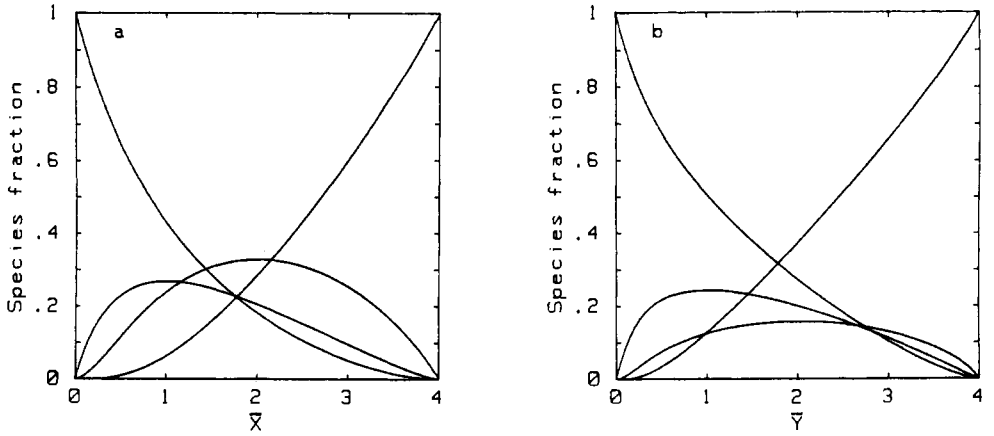


FIGURE 2: Species fractions of stoichiometric intermediates for the experimental conditions reported in the text as a function of the moles of ligand bound per mole of macromolecule, with the best fit values of the β's summarized in Table I. (a) O_2; (b) CO.

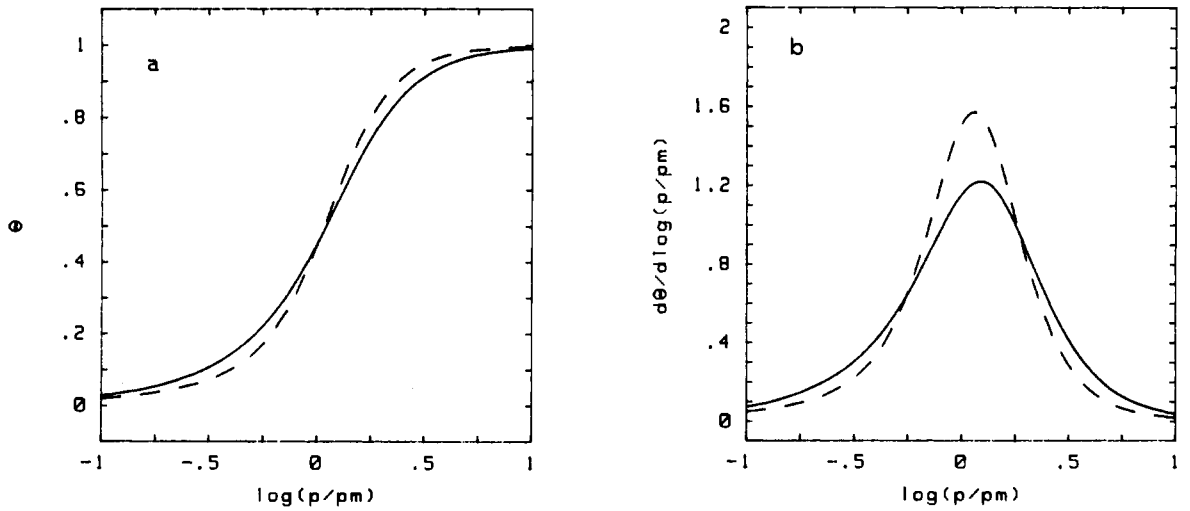


FIGURE 3: (a) Normalized binding curve for O_2 (continuous line) and CO (dotted line) to Hb A_0. The two curves were calculated from the best fit values of the β's reported in Table I, after scaling the ligand partial pressure by the p_{median} in both cases. The figure shows a different shape for the CO binding curve, particularly at low and high saturations. (b) Derivatives of the normalized binding curves for O_2 (continuous line) and CO (dotted line). The derivative of the binding curve is proportional to the cooperativity, since it provides a measure of the steepness of the binding curve. From these derivative curves the higher cooperativity for CO binding to Hb A_0 is evident. The maximum Hill coefficient for CO is 2.8, and 2.4 for O_2.

negligibly small. Consequently, the triply ligated species is negligible, regardless of the ligand. This result has been recently obtained with O_2 binding to hemoglobin in a variety of solution conditions (Gill et al., 1987; Doyle et al., 1987) and is found here also for CO. However, a glance at Figure 2 shows the distribution of the intermediate species of ligation is different for O_2 and CO. Namely, the doubly ligated species is more populated in the case of O_2. The immediate conclusion is that the shape of the binding curve is different for O_2 and CO, as shown in Figure 3a, with CO displaying a higher degree of cooperativity, as shown in Figure 3b. The maximum Hill coefficient is equal to 2.4 for O_2, in agreement with previous studies (Tyuma et al., 1973), and 2.8 for CO. A recent investigation of the ligation intermediates of Hb with CO (Perrella et al., 1986) has suggested a high degree of cooperativity for CO binding to Hb. However, the distribution of intermediates reported by these authors is not confirmed in the present study (see Figure 2b), possibly due to the presence of IHP in our experimental conditions.

The ratio of the median partial pressure for O_2 and CO (see Table I) gives the partition coefficient m_4 for the fully ligated molecule. The value obtained in these two experiments is 262, in agreement with the literature (Benesch et al., 1972, Wyman et al., 1982). With the values of the overall Adair constants for both O_2 and CO, one can calculate the values of $m_1 = 176$ and $m_2 = 153$, corresponding to the singly and doubly ligated molecule (see Table I), which are clearly lower than the value of m_4. Finally, one can plot the partition coefficient m of eq 1 as a function of hemoglobin saturation. In Figure 4 we show that the partition coefficient, as a consequence of the different shape of the O_2 and CO binding curves, is a function of the ligation stage, thus violating Haldane's first and second laws.

Table I: Overall Adair Constants for O_2 and CO Binding to Hemoglobin and Corresponding Confidence Intervals within One Standard Deviation (67%) Determined by F Testing

O_2 binding constants	CO binding constants
β_{10} = 0.015 (0.014–0.018) $Torr^{-1}$	β_{01} = 2.64 (2.47–2.79) $Torr^{-1}$
β_{20} = 0.00032 (0.00031–0.00035) $Torr^{-2}$	β_{02} = 7.49 (7.15–7.99) $Torr^{-2}$
β_{30} = 0 (1.5×10^{-8}) $Torr^{-3}$	β_{03} = 0 (2.7) $Torr^{-3}$
β_{40} = 4.9×10^{-8} [(4.4–5.1) × 10^{-8}] $Torr^{-4}$	β_{04} = 230 (211–237) $Torr^{-4}$
ΔOD_T = 0.1062 (0.1052–0.1071)	ΔOD_T = 0.1483 (0.1469–0.1499)
σ = 0.00015	σ = 0.00038
$m_1 = \beta_{01}/\beta_{10}$ = 176 (137–199) $m_4 = (\beta_{04}/\beta_{40})^{0.25}$ = 262 (254–271)	$m_2 = (\beta_{02}/\beta_{20})^{0.5}$ = 153 (143–160)

O_2–CO Competition Experiment. The diagonal edge of the triangle in eq 8 depicts the fully ligated hemoglobin in all

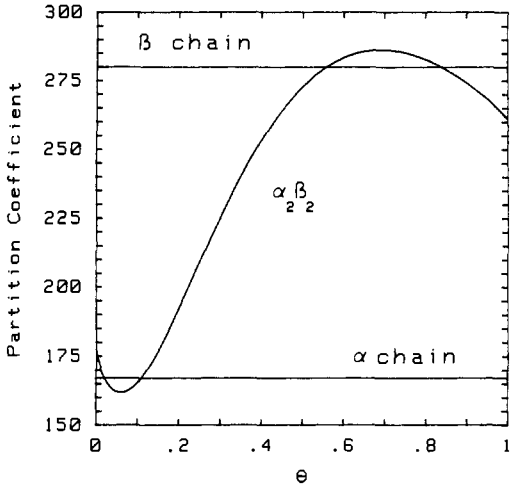


FIGURE 4: Partition coefficient for CO and O_2 binding to Hb A_0 plotted as a function of the fractional saturation, as described in eq 1. The theoretical line was drawn according to the best fit values of the β's reported in Table I. The two horizontal lines represent the partition coefficient of the β_4 tetramer (top) and of the α_2 dimer (bottom) as reported in the literature (Bishop & Gill, 1986). The figure shows that the partition coefficient is not constant throughout the saturation range, in contrast to the prediction drawn from Haldane's first and second laws (Haldane & Smith, 1897).

possible combinations of CO and O_2. The equilibria between the species can be explored by saturating the molecule with one ligand and measuring its replacement through a series of increasing relative activities of the other ligand (Wyman et al., 1982). Figure 5a shows the optical density changes at the CO maximum absorbance (419 nm) as observed upon incremental dilution of the CO with O_2. Evaluation of the data enables determination of the equilibrium constants β_{40}, β_{31}, β_{22}, β_{13}, and β_{04} (see Appendix). In agreement with previous studies (Wyman et al., 1982), the fully ligated species of hemoglobin is partitioned into all its possible combinations of CO and O_2 in a statistical fashion (i.e., with all the subunits being identical and independent in the replacement reaction of CO by O_2). The values of the pertinent Adair constants are reported in Table II.

Binding Curve at a Constant Ratio of O_2/CO. In this experiment one populates all the fully ligated species with a specified mixture of CO and O_2 gas partial pressures and then

Table II: Binding Constants for Competition and Constant Ratio Experiments and Corresponding Confidence Intervals within One Standard Deviation (67%) Determined by *F* Testing[a]

competition experiment	constant ratio experiment
C_1 = 1455 (1415–1503)	E_1 = 7.36 (7.02–8.00) $Torr^{-1}$
$C_2 = 4.2 \times 10^5$ [(4.0–4.5) × 10^5]	E_2 = 74.4 (71.1–81.2) $Torr^{-2}$
$C_3 = 7.7 \times 10^7$ [(7.2–8.1) × 10^7]	E_3 = 0 (25.3) $Torr^{-3}$
$C_4 = 4.7 \times 10^9$ [(4.3–5.1) × 10^9]	E_4 = 6035 (5431–6517) $Torr^{-4}$
ΔOD_T = 0.1564 (0.1549–0.1577)	ΔOD_T = 0.1134 (0.1121–0.1145)
σ = 0.00016	σ = 0.00045

[a] The relationships between these constants and the overall Adair constants are given in the Appendix.

dilutes the gas mixture with N_2 (Richey et al., 1985; Zolla et al., 1985). The hemoglobin system thus moves from the diagonal edge of the thermodynamic triangle in eq 8 to the unligated species M, passing through all the possible intermediates. Analysis of this experiment involves all the Adair constants in eq 6 (see Appendix). Results from this experiment confirm a negligible value of β_{30} and β_{03} and also indicate that β_{21} and β_{12} are negligible. Therefore, no triply ligated species, regardless of the combination with CO and O_2, make a significant contribution to the ligation process. As shown in Table II, the amount of fully ligated species in this experiment is in good agreement with that observed in the competition experiment, while the singly and doubly ligated species match the values found in the separate CO and O_2 binding curves.

Simultaneous Analysis of Binding Data. The four types of binding experiments described above provide a self-consistent test of the thermodynamic triangle for the identical linkage between CO and O_2. Therefore, one should be able to extract all the β's from a simultaneous fit of the four experiments. This is a critical test since all the parameters except one, β_{11}, are determined from the analysis of two different experiments (for example, β_{03} is determined in the CO binding curve and in the constant ratio experiment). The two parameters β_{40} and β_{04} are determined in three different experiments (the constant ratio and the competition experiments and either the CO or O_2 binding curve). Since the values of these common parameters must be the same in each of these experiments, the simultaneous fit is highly constrained, and any possible discrepancy among different experiments will be evident. The best fit values of the β's obtained in the simultaneous fit are summarized in Table III and are in ex-

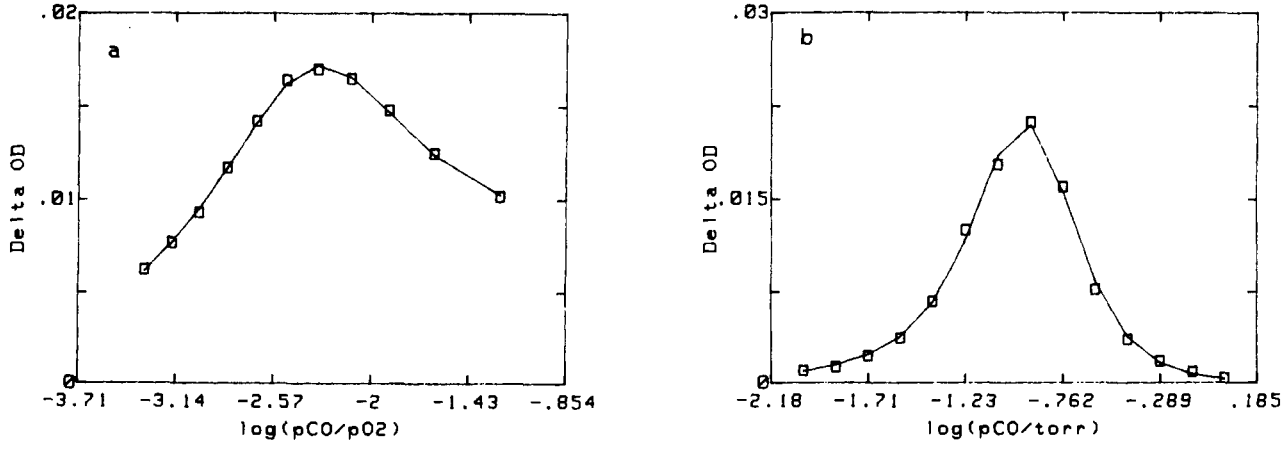


FIGURE 5: (a) Competition experiment: optical density changes for stepwise changes in CO partial pressure (starting at 2.499% of the atmospheric pressure) above a thin-layer (0.0025-cm) sample of Hb A_0. The reaction was followed as a change of CO saturation at 419 nm upon stepwise dilutions with pure O_2. The abscissa represents the logarithm of *z* (see Appendix), the ratio of CO and O_2 partial pressures at any given dilution step. The theoretical curve was drawn according to the best fit values of the *C*'s (see Appendix) reported in Table II. (b) Constant ratio experiment: optical density changes for stepwise changes in both CO and O_2 partial pressures above a thin-layer (0.0025-cm) sample of Hb A_0. The reaction was followed as a change of the total CO + O_2 saturation at 410 nm upon stepwise dilution with pure N_2. The optical density changes are plotted as a function of the logarithm of the CO partial pressure (see Appendix), and the theoretical line was drawn according to the best fit values of the *E*'s (see Appendix) reported in Table II.

Table III: Overall Adair Constants As Determined by a Simultaneous Fit of All Four Experiments Discussed in the Text[a]

$\beta_{10} = 0.016 \pm 0.002$ Torr^{-1}	$\beta_{04} = 231 \pm 15.4$ Torr^{-4}
$\beta_{20} = 0.00031 \pm 0.00003$ Torr^{-2}	$\beta_{31} = 7.1 \times 10^{-5} \pm 5.0 \times 10^{-6}$ Torr^{-4}
$\beta_{30} = 0 \pm 2.4 \times 10^{-8}$ Torr^{-3}	$\beta_{22} = 0.020 \pm 0.002$ Torr^{-4}
$\beta_{40} = 4.8 \times 10^{-8} \pm 3.8 \times 10^{-9}$ Torr^{-4}	$\beta_{13} = 3.80 \pm 0.28$ Torr^{-4}
$\beta_{01} = 2.72 \pm 0.48$ Torr^{-1}	$\beta_{21} = 0 \pm 6.0 \times 10^{-6}$ Torr^{-3}
$\beta_{02} = 7.23 \pm 0.61$ Torr^{-2}	$\beta_{12} = 0 \pm 0.002$ Torr^{-3}
$\beta_{03} = 0 \pm 4.1$ Torr^{-3}	$\beta_{11} = 0.12 \pm 0.02$ Torr^{-2}
$\sigma = 0.00029$	$\xi^b = p_0(O_2)/p_0(CO) = 315 \pm 9$
$m_1 = \beta_{01}/\beta_{10} = 170 \pm 37$	$m_2 = (\beta_{02}/\beta_{20})^{0.5} = 153 \pm 20$
$m_4 = (\beta_{04}/\beta_{40})^{0.25} = 263 \pm 27$	

[a] The errors on the parameters are twice the actual errors estimated in the fit. [b] In the constant ratio experiment (see Appendix).

cellent agreement with the values determined in the separate fits. The results obtained for a given set of experiments were found to be highly reproducible when in repeated experiments the same or different material preparation was used.

Discussion

For the particular solution conditions discussed above, namely, at high heme concentration with excess IHP present, we find that the shape of the binding curve of CO is different from that of O_2. The partition coefficient m in eq 1 is therefore a function of saturation and is shown in Figure 4. This observed dependence of m on ligation violates Haldane's first and second laws and supports the early observation of Barcroft (1928) and later by Roughton (1970) on whole blood.

Figure 4 shows that at low saturation the value of the partition coefficient is similar to that found for the α_2 dimer, while at high saturation it approaches the value found for the β_4 tetramer (Bishop & Gill, 1986). This is consistent with the possibility that ligation may occur first to the α chains and then to the β chains. However, alternative explanations might also be invoked. In fact, the unequivocal determination of the order of ligation strictly demands direct evidence of the binding properties of the two types of chains. However, for the experimental conditions discussed above, NMR data have indeed shown a preferential binding to the α chains at low saturations (Viggiano & Ho, 1979).

The population of triply CO-ligated species is found to be negligible, as in the case for O_2 (Gill et al., 1987). This supports the idea that a sharp conformational transition occurs at the third stage of ligation. We have recently proposed an allosteric model (Di Cera et al., 1987) that contains this feature and accounts for the negligible population of the triply ligated species. The ligation mechanism proposed by the model is based on the Perutz stereochemical mechanism (Perutz, 1970) and is substantiated by recent crystallographic studies (Baldwin & Chothia, 1979; Fermi et al., 1984; Brzozowski et al., 1984). The key element of this mechanism is blockage of the heme pocket of the β chain in the T state by the distal residue Val-67 (Perutz, 1970). The blockage does not leave enough room for binding either O_2 or CO.

Here we employ this model in the interpretation of the CO binding results. The model postulates the existence of two quaternary states in equilibrium, the T or low-affinity state and the R or high-affinity state. An extreme heterogeneity between the α and β chains in ligand binding affinity is assumed in the T state, while in the R state all the subunits are independent and have equal ligand affinity. Consequently, ligation proceeds first to the α chains in the T state and then, after the conformational transition, to the β chains in the R state. This model nicely handles the two main features of the CO binding reaction to hemoglobin, namely, the low population of triply ligated species and the change of the partition coefficient as ligation proceeds. In fact, if CO first binds at the α chains in the T state like O_2, then the partition coefficient at low saturations is expected to be similar to that found for the isolated α chains (α_2 dimer), as is observed here. At high saturation the molecule switches to the R state, and the β chains are ligated. Then the partition coefficient assumes a value similar to that found for the isolated β chains (β_4 tetramer), which are known to have an R-like structure (Arnone et al., 1982).

Table IV: Model Parameters As Determined by a Simultaneous Fit of All Four Experiments Discussed in the Text[a]

$\kappa_{T\alpha X} = 0.011 \pm 0.003$ Torr^{-1}	$L = 2.3 \times 10^{12} \pm 3.8 \times 10^{10}$
$\kappa_{RX} = 19.9 \pm 0.12$ Torr^{-1}	$\gamma_{XX} = 2.68 \pm 0.78$
$\kappa_{T\alpha Y} = 1.35 \pm 0.04$ Torr^{-1}	$\gamma_{YY} = 4.52 \pm 0.82$
$\kappa_{RY} = 4821 \pm 440$ Torr^{-1}	$\gamma_{XY} = 2.40 \pm 0.20$
$\sigma = 0.00044$	$\xi^b = p_0(O_2)/p_0(CO) = 310 \pm 7$
$m_T = \kappa_{T\alpha Y}/\kappa_{T\alpha X} = 123 \pm 34$	$m_R = \kappa_{RY}/\kappa_{RX} = 241 \pm 19$

[a] The errors on the parameters are twice the actual errors estimated in the fit. [b] In the constant ratio experiment (see Appendix).

The predictions of the model are also consistent with the result obtained in the competition experiment. As demonstrated in a previous study (Wyman et al., 1982), the distribution of the species in the diagonal edge of the thermodynamic triangle is statistical, since both CO and O_2 bind to the fully ligated species independently and noncooperatively. According to the model the fully ligated molecule is in the R state, where the subunits are independent and bind with the same affinity. The competition experiment demonstrates that the partition coefficients for the α and β chains in the R state are equal. Since the isolated α chains have a lower partition coefficient than the isolated β chains (Bishop & Gill, 1986) and the fully saturated Hb tetramer, the α_2 dimer must be in a different quaternary state. Crystallographic analysis might help to clarify this point. However, since the functional properties as determined by the partition coefficient of the α_2 dimer are identical with the binding properties at low saturation of the Hb tetramer, known to be in the T form, this suggests that a similar structural state exists for the α_2 dimer.

The binding partition function of the model for the identical linkage between CO and O_2 to hemoglobin may then be written as

$$\Xi(x,y) = L(1 + 2\kappa_{T\alpha X}x + 2\kappa_{T\alpha Y}y + \gamma_{XX}\kappa_{T\alpha X}^2x^2 + \gamma_{YY}\kappa_{T\alpha Y}^2y^2 + 2\gamma_{XY}\kappa_{T\alpha X}\kappa_{T\alpha X}xy) + (1 + \kappa_{RX}x + \kappa_{RY}y)^4 \quad (11)$$

where $\kappa_{T\alpha X}$, κ_{RX}, $\kappa_{T\alpha Y}$, and κ_{RY} are the affinity constants for O_2 and CO in the two quaternary states, L is the allosteric constant defined as [T]/[R] in the absence of ligands, and the γ's are the interaction constants (Di Cera et al., 1987) between the α chains in the T state when they are both ligated with O_2 (γ_{XX}) or CO (γ_{YY}) or in the mixed configuration (γ_{XY}). The lack of cooperativity and the feature of identical linkage are seen directly in the term to the fourth power for the R state.

In Figure 6 we report the result of the simultaneous fit of the four experiments to the model. The best fit values of the model parameters are summarized in Table IV. The extent and precision of the experimental data and the consistency of the model allow accurate resolution of all the parameters. The interaction constants γ's are significantly different from 1 (93% confidence as determined by F test), thus indicating the existence of nearest-neighbor interactions between the α chains in the T state (Di Cera et al., 1987). Interestingly, the extents of these interactions are similar for CO and O_2. As expected

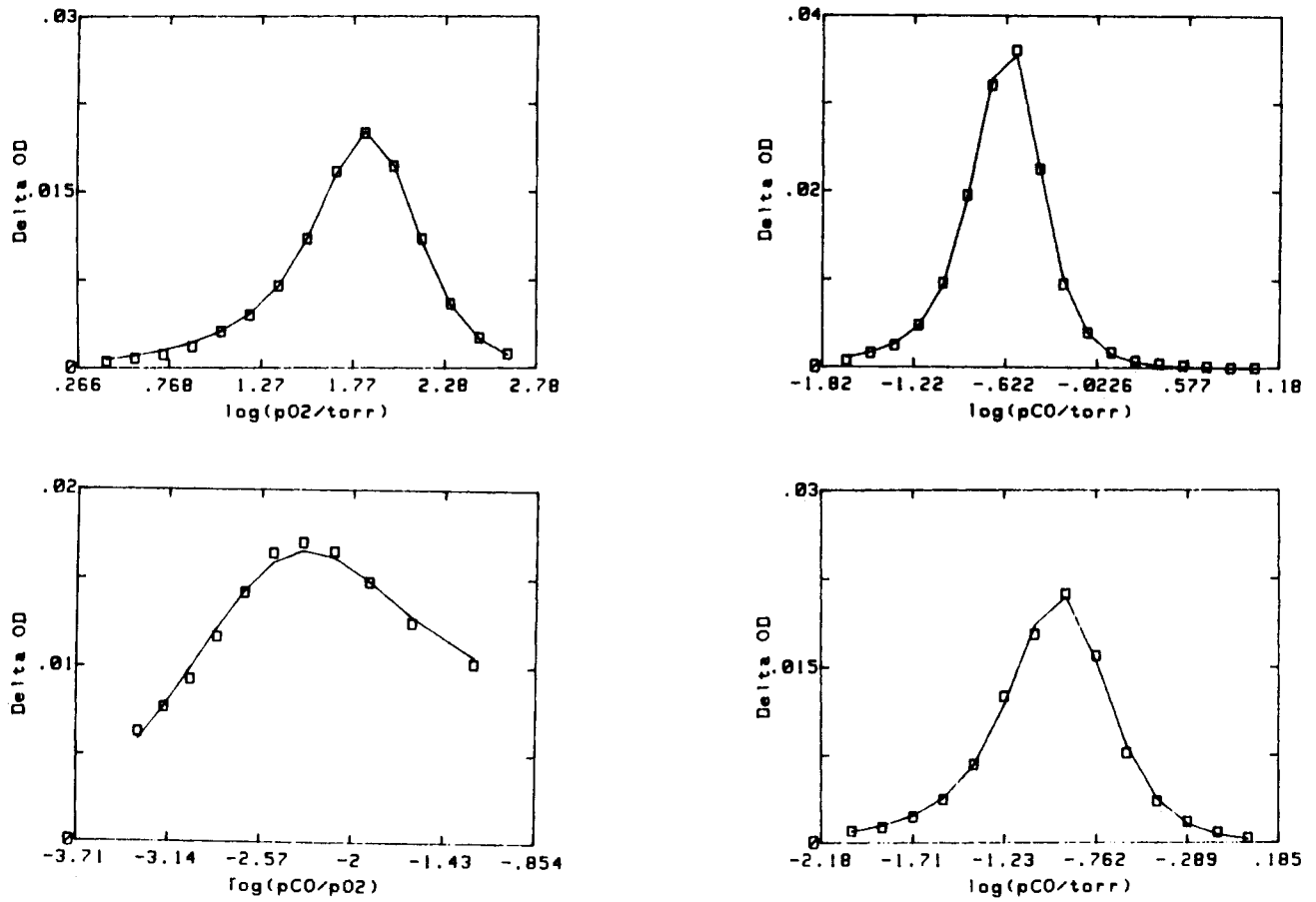


FIGURE 6: Result of the simultaneous fit of the four experiments [(top left) O_2 binding curve, (top right) CO binding curve, (bottom left) competition experiment, (bottom right) constant ratio experiment] to the allosteric model discussed in the text. The theoretical curves were drawn according to the best fit values of the model parameters reported in Table IV.

from the Adair analysis, the partition coefficient for the T state (123 ± 34) is determined from the fit to be substantially lower than that of the R state (241 ± 19) and accounts for the change shown in Figure 4.

What is the origin of the different cooperativity in binding CO and O_2 to hemoglobin? According to the model discussed here, the main difference found in binding these two ligands to hemoglobin stems from the change in the affinity when the molecule switches from the T to the R state. The ratio $\kappa_R/\kappa_{T\alpha}$ is lower for O_2 than for CO. Since in the R state all the structural constraints are released (Perutz, 1970; Baldwin & Chothia, 1979; Shaanan, 1983), the origin of the difference would be that the binding of CO to the T state has more steric hindrance than the binding of O_2, as indicated by structural studies (Heidner et al., 1976). Furthermore, it is not surprising to observe a different cooperativity between CO and O_2 binding, in view of the fact that the kinetic mechanism underlying the cooperative behavior of hemoglobin is different for these two ligands (Szabo, 1978; Moffat et al., 1979).

In conclusion, the results presented in this study demonstrate that the domain of validity of Haldane's two laws (Haldane & Smith, 1897) is limited to the fully saturated molecule ($\bar{T} = 4$), the partition coefficient m being a function of the degree of ligation. The mechanism of hemoglobin cooperativity, when explored under the two different coordinates provided by O_2 and CO binding, is consistent with an allosteric model recently proposed (Di Cera et al., 1987). The negligible population of the triply ligated species (Gill et al., 1987) is therefore the result of an intrinsic property of Hb A_0 in its reaction with ligands.

ACKNOWLEDGMENTS

We thank Dr. Bo Hedlund for providing portions of the hemoglobin used in this study.

APPENDIX

In the competition experiment we saturate the molecule with CO at 2.499% P_a (P_a = atmospheric pressure) and then dilute this gas with O_2 at atmospheric pressure. At each dilution step the CO partial pressure is

$$p_i(\mathrm{CO}) = p_0(\mathrm{CO})D^i = 0.02499P_aD^i \qquad \text{(A1)}$$

and the O_2 partial pressure is

$$p_i(\mathrm{O_2}) = p_0(\mathrm{O_2})(1 - D^i) = P_a(1 - D^i) \qquad \text{(A2)}$$

The binding partition function in this experiment can be written in terms of the fully ligated species MX_4, MX_3Y, MX_2Y_2, MXY_3, and MY_4. If one takes MX_4 as the reference species, the binding partition function is

$$\Xi(x,y) = \frac{\beta_{40}x^4 + \beta_{31}x^3y + \beta_{22}x^2y^2 + \beta_{13}xy^3 + \beta_{04}y^4}{\beta_{40}x^4} \qquad \text{(A3)}$$

Upon rearrangement eq A3 becomes

$$\Xi(z) = 1 + C_1z + C_2z^2 + C_3z^3 + C_4z^4 \qquad \text{(A4)}$$

where $C_1 = \beta_{31}/\beta_{40}$, $C_2 = \beta_{22}/\beta_{40}$, $C_3 = \beta_{13}/\beta_{40}$, $C_4 = \beta_{04}/\beta_{40}$, and $z = y/x$, the ratio of CO and O_2 partial pressures that can be calculated according to eq A1 and A2 at any given dilution step. The analysis of the competition experiment yields the C's as summarized in Table II.

In the constant O_2/CO binding curve we saturate the molecule with a gas mixture of O_2 and CO. The gas mixture

can be produced with high precision by starting with a 2.499% P_a of CO, followed by appropriate dilutions with pure O_2 in the thin-layer cell. After six dilution steps one calculates a ratio between O_2 and CO partial pressures of 313. At this point both gases are diluted with N_2, and the binding curve is monitored at 410 nm [where the absorbances of oxyHb and carbonmonoxyHb are equal] as changes in the CO + O_2 saturation ($\bar{T}/4$). In this experiment all the species of the thermodynamic triangle are present in solution. Since the ligation process is followed at the isosbestic point[4] for carbonmonoxyHb and oxyHb, the fitting equation is similar to eq 10, that is

$$\Delta OD_i = \Delta OD_T\{[\theta_i(CO) + \theta_i(O_2)] - [\theta_{i-1}(CO) + \theta_{i-1}(O_2)]\} \quad (A5)$$

The fractional saturations $\theta(CO)$ and $\theta(O_2)$ are obtained from eq 7a and 7b, and the sum $\theta(CO) + \theta(O_2)$ is given by

$$\theta(CO) + \theta(O_2) = \frac{1}{4}\frac{\sum_{i=0}^{4}\sum_{j=0}^{4-i}(i+j)\beta_{ij}x^iy^j}{\sum_{i=0}^{4}\sum_{j=0}^{4-i}\beta_{ij}x^iy^j} \quad (A6)$$

The initial ratio $\xi = x/y$ of 313 between O_2 and CO partial pressures remains unchanged during this experiment; therefore, one can write eq A6 as

$$\theta(CO) + \theta(O_2) = \frac{1}{4}\frac{d \ln (1 + E_1y + E_2y^2 + E_3y^3 + E_4y^4)}{d \ln y} \quad (A7)$$

where

$$E_1 = \beta_{10}\xi + \beta_{01} \qquad E_2 = \beta_{20}\xi^2 + \beta_{11}\xi + \beta_{02}$$
$$E_3 = \beta_{30}\xi^3 + \beta_{21}\xi^2 + \beta_{12}\xi + \beta_{03} \quad (A8)$$
$$E_4 = \beta_{40}\xi^4 + \beta_{31}\xi^3 + \beta_{22}\xi^2 + \beta_{13}\xi + \beta_{04}$$

The analysis of this experiment gives the E's as summarized in Table II. As one can see, the best fit value of E_3 is zero, thus demonstrating that all the triply ligated species must necessarily be negligible.

In the simultaneous fit of the four experiments one can express the C's and the E's as functions of the β's, since these constants are common to at least two experiments. Consequently, in the simultaneous fit one can resolve all the β's in a very precise way. The constant ξ was treated as a floating parameter in the simultaneous fit. The value recovered in the analysis (see Tables III and IV) is identical with the value of 313 calculated for six dilution steps.

Registry No. Hb A_0, 54651-57-9; CO, 630-08-0; O_2, 7782-44-7.

REFERENCES

Adair, G. S. (1925) *Proc. R. Soc. London, A 108*, 627–237.

Anderson, S. R., & Antonini, E. (1968) *J. Biol. Chem. 243*, 2918–2920.

Arnone, A., Briley, P. D., Rogers, P. H., & Hendrickson, W. A. (1982) in *Hemoglobin and Oxygen Binding*, Elsevier, New York.

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

Barcroft, J. (1928) in *The Respiratory Function of the Blood*, Cambridge University Press, Cambridge.

Benesch, R. E., Maeda, N., & Benesch, R. (1972) *Biochim. Biophys. Acta 257*, 178–182.

Bishop, G. A., & Gill, S. J. (1986) *Biopolymers 25*, 1381–1384.

Brzozowski, A., Derewenda, Z., Dodson, E., Dodson, G., Grabowski, M., Liddington, R., Skarzynski, T., & Vallely, D. (1984) *Nature (London) 307*, 74–76.

Chiancone, E., Anderson, N. M., Antonini, E., Bonaventura, J., Bonaventura, C., Brunori, M., & Spagnuolo, C. (1974) *J. Biol. Chem. 249*, 5689–5694.

Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry 23*, 604–617.

Di Cera, E., Robert, C. H., & Gill, S. J. (1987) *Biochemistry 26*, 4003–4008.

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

Douglas, C. G., Haldane, J. S., & Haldane, J. B. S. (1912) *J. Physiol. (London) 44*, 275–304.

Doyle, M. L., Di Cera, E., Robert, C. H., & Gill, S. J. (1987) *J. Mol. Biol.* (in press).

Fermi, G., Perutz, M. F., Shaanan, B., & Fourme, R. (1984) *J. Mol. Biol. 175*, 159–174.

Frazier, R. D. B., & Suzuki, E. (1973) in *Physical Principles and Techniques of Protein Chemistry*, Academic, New York.

Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., & Robert, C. H. (1987) *Biochemistry 26*, 3995–4002.

Guidotti, G. (1967) *J. Biol. Chem. 242*, 3685–3693.

Haldane, J. S., & Smith, J. L. (1897) *J. Physiol. (London) 22*, 231–258.

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

Heidner, E. J., Ladner, R. C., & Perutz, M. F. (1976) *J. Mol. Biol. 104*, 707–722.

Hill, T. L. (1985) in *Cooperativity Theory in Biochemistry*, Springer-Verlag, Berlin.

Hlastala, M. P., McKenna, H. P., Franada, R. L., & Detter, J. C. (1976) *J. Appl. Physiol. 41*, 893–899.

Imai, K. (1979) *J. Mol. Biol. 133*, 233–247.

Imai, K. (1982) in *Allosteric Effects in Haemoglobin*, Cambridge University Press, Cambridge.

Johnson, M. L., & Ackers, G. K. (1977) *Biophys. Chem. 7*, 77–80.

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

Magar, M. E. (1972) in *Data Analysis in Biochemistry and Biophysics*, Academic, New York.

Mills, F. C., Johnson, M. L., & Ackers, G. K. (1976) *Biochemistry 15*, 5350–5362.

Moffat, K., Deatherage, J. F., & Seybert, D. W. (1979) *Science (Washington, D.C.) 206*, 1035–1042.

Okada, Y., Tyuma, I., Ueda, Y., & Sugimoto, T. (1976) *Am. J. Physiol. 230*, 471–475.

Perrella, M., Sabbioneda, L., Samaja, M., & Rossi-Bernardi, L. (1986) *J. Biol. Chem. 261*, 8391–8396.

Perutz, M. F. (1970) *Nature (London) 228*, 726–739.

Richey, B., Decker, H., & Gill, S. J. (1985) *Biochemistry 24*, 109–117.

Roughton, F. J. W. (1970) *Ann. N.Y. Acad. Sci. 174*, 177–188.

Shaanan, B. (1983) *J. Mol. Biol. 171*, 31–59.

Szabo, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2108–2111.

Tyuma, I., Imai, K., & Shimizu, K. (1973) *Biochemistry 12*, 1491–1498.

[4] This is not an absolute requirement. The experiment can be followed at any other wavelength. The general fitting equation is then

$$\Delta OD_i = \Delta OD_T(CO)[\theta_i(CO) - \theta_{i-1}(CO)] + \Delta OD_T(O_2)[\theta_i(O_2) - \theta_{i-1}(O_2)]$$

since the different spectral contributions of carbonmonoxyHb and oxyHb must be taken into account. Clearly, measurement at the isosbestic point for CO and O_2 leads to $\Delta OD_T(CO) = \Delta OD_T(O_2)$ and then to eq A5.

Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673–3677.
Williams, R. C., & Tsay, K. (1973) *Anal. Biochem. 54*, 137–145.
Wyman, J. (1948) *Adv. Protein Chem. 4*, 407–531.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 233–286.
Wyman, J., Bishop, G. A., Richey, B., Spokane, R., & Gill, S. J. (1982) *Biopolymers 21*, 1735–1747.
Zolla, L., Brunori, M., Richey, B., & Gill, S. J. (1985) *Biophys. Chem. 22*, 271–280.

Chemical Modification of Arginine Residues in the Lactose Repressor†

Peggy A. Whitson and Kathleen S. Matthews*
Department of Biochemistry, Rice University, Houston, Texas 77251
Received November 3, 1986; Revised Manuscript Received May 20, 1987

ABSTRACT: The lactose repressor protein was chemically modified with 2,3-butanedione and phenylglyoxal. Arginine reaction was quantitated by either amino acid analysis or incorporation of ^{14}C-labeled phenylglyoxal. Inducer binding activity was unaffected by the modification of arginine residues, while both operator and nonspecific DNA binding activities were diminished, although to differing degrees. The correlation of the decrease in DNA binding activities with the modification of ~1–2 equiv of arginine per monomer suggests increased reactivity of a functionally essential residue(s). For both reagents, operator DNA binding activity was protected by the presence of calf thymus DNA, and the extent of reaction with phenylglyoxal was simultaneously diminished. This protection presumably results from steric restriction of reagent access to an arginine(s) that is (are) essential for DNA binding interactions. These experiments suggest that there is (are) an essential reactive arginine(s) critical for repressor binding to DNA.

Expression of the lactose metabolizing enzymes of *Escherichia coli* is regulated by a repressor protein. This protein binds with high affinity to the operator sequence in the DNA, physically blocking RNA polymerase transcription of these coordinately regulated enzymes (Miller & Reznikoff, 1980). In the presence of inducer molecules, the repressor undergoes a conformational change, which results in diminished affinity for operator DNA. The excess of nonspecific DNA binding sites in the *E. coli* genome can then compete effectively with the operator for repressor binding, and transcription of the mRNA for the *lac* enzymes can be initiated. The affinities of the *lac* repressor for various nonspecific and operator-containing DNAs have been measured under a variety of conditions (deHaseth et al., 1977; Record et al., 1977; Revzin & von Hippel, 1977; Barkley et al., 1981; Winter & von Hippel, 1981; Winter et al., 1981; Whitson & Matthews, 1986; Whitson et al., 1986).

The inability to produce X-ray-quality crystals of the repressor has led to the use of alternative methods in an attempt to delineate structure–function relationships of this protein. In concert with genetic mapping, chemical modification studies provide information regarding functional roles and local environments of individual amino acid residues (Means & Feeney, 1971; Glazer, 1976; Glazer et al., 1975). The roles of specific amino acid residues in the functioning of the lactose repressor have been examined by genetic methods (Müller-Hill et al., 1977; Pfahl et al., 1974; Coulondre & Miller, 1977; Miller, 1979; Miller et al., 1979) and chemical modification (Fanning, 1975; Hsieh & Matthews, 1981, 1985; O'Gorman & Matthews, 1977; Brown & Matthews, 1979; Alexander et al., 1977; Manly & Matthews, 1979; Whitson et al., 1984). The importance of basic residues in DNA binding activity can be deduced from the requirement for the protein to interact with the polyacidic nucleic acid. Basic groups on the surface of the protein must be available to participate in ion pairs with the phosphate groups of the DNA. Lysines-33, -108, and, to a lesser extent, -37 were found to be involved in DNA binding by chemical modification (Whitson et al., 1984), and genetic evidence indicated lysines critical for DNA binding at positions 2, 33, 84, and 290 (Pfahl et al., 1974; Coulondre & Miller, 1977; Miller, 1979; Miller et al., 1978, 1979; Schmitz et al., 1978; Miller & Schmeissner, 1979). Amino acid replacements resulting from missense mutations have indicated that arginines-22, -51, -168, and -303 influence DNA binding activity of the repressor (Miller & Schmeissner, 1979; Miller, 1979). Chemical modification of arginine residues has been employed to provide further insight into the participation of these residues in repressor function. We have modified the lactose repressor with 2,3-butanedione and phenylglyoxal, which specifically react with the guanidinium functional group of arginines, to correlate effects on binding activities with reaction of these residues.

Materials and Methods

Purification of Repressor. The lactose repressor was purified from *Escherichia coli* CSH 46 grown in a 100-L fermentor. Cells were stored frozen at −20 °C until use. Repressor was isolated by the method of Rosenberg et al. (1977) as modified by O'Gorman et al. (1980). The protein yield was approximately 1–4 mg/g of cells. Repressor concentrations were determined spectrophotometrically with an $A_{280}^{1mg/mL}$ = 0.6 (Huston et al., 1974). The purity of the repressor (>95%) was assessed by sodium dodecyl sulfate gel electrophoresis.

Assay of Repressor. Isopropyl β-D-thiogalactoside (IPTG)[1] binding activity was determined by the nitrocellulose filter and

† This work was supported by grants from the National Institutes of Health (GM-22441) and the Robert A. Welch Foundation (C-576).
* Author to whom correspondence should be addressed.

[1] Abbreviations: bp, base pair(s); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; PGO, phenylglyoxal; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-6502$01.50/0