Ligand Binding to Heme Proteins
AN EVALUATION OF DISTAL EFFECTS*

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The O₂, CO, and alkyl isocyanide-binding properties of a variety of vertebrate and invertebrate heme proteins have been compared in detail to those of protoporphyrin mono-3-(1-imidazolyl)-propylamide monomethyl ester in aqueous suspensions of soap micelles. The proteins examined include: cytochrome P-450 from Pseudomonas putida, beef heart cytochrome c oxidase, yeast cytochrome c peroxidase, α and β subunits of human hemoglobin, sheep hemoglobin, carp hemoglobin, sperm whale myoglobin, horse heart myoglobin, a monomeric hemoglobin from Glycera dibranchiata, erythrocoruolin from Chironomus thummi, soybean leghemoglobin, and several hemoglobins that lack distal histidines. The smallest bimolecular rates were observed for cytochrome P-450 containing bound camphor, cytochrome c oxidase, and cytochrome c peroxidase. In the case of P-450, the extremely low isonitrile binding rates (~1 m⁻¹ s⁻¹ at 20 °C) are due to steric exclusion by bound camphor molecules. For the oxidase and peroxidase, inhibition of CO and isonitrile binding appears to be due to the polar nature of the active sites.

Heme proteins with accessible sixth coordination positions exhibit a variety of physiological functions. The hemoglobins and myoglobins(323,211),(920,413) are responsible for reversible binding, transport, and storage of oxygen in the tissues of animals and certain leguminous plants. Cytochrome c oxidase takes part in cellular energy production by reducing molecular oxygen to water; cytochrome c peroxidase detoxifies hydrogen peroxide by reducing it to water; and the P-450 cytochromes participate in hydroxylation reactions in which an oxygen atom is inserted into an aliphatic side chain. With the exception of heme a in oxidase, all of these proteins contain protoheme as a prosthetic group. Thus, it is the protein structure, both proximal and distal to the heme iron atom, which determines the observed chemical behavior. High resolution x-ray structures have been published for horse, human, and lamprey hemoglobin, sperm whale and seal myoglobin, the monomeric insect hemoglobin from Chironomus thummi, the monomeric hemoglobin from the blood worm Glycera dibranchiata, leghemoglobins from Rhizobium-infected soybeans (Glycine max) and jackbeans (Lupinus luteus), and yeast cytochrome c peroxidase (Perutz, 1968; Muirhead and Greer, 1970; Hendrickson et al., 1973; Takano, 1977; Scouloardi and Baker, 1978; Steigemann and Weber, 1979;
Ligand Binding to Heme Proteins

Padian and Love, 1974; Vainshtein et al., 1978; Ollis et al., 1983; Poulos and Kraut, 1980). In addition, preliminary results at low resolution have been reported for bacterial cytochrome P-450 (Poulos et al., 1982). Careful examinations and comparisons of the active sites of these proteins have generated the structural information which is required to evaluate the reactivity of the heme iron atom (see Moffat et al., 1979; Perutz, 1979; Baldwin and Chothia, 1979; Case and Karplus, 1978). The remaining problem is to assess quantitatively the significance of these structures in terms of changes in binding free energies and kinetic barriers.

We have examined this question of protein effects experimentally. First, the overall influence of the polypeptide structure has been quantitated by comparing the observed binding properties of the proteins with those of proteoheme mono-3-(1-imidazoyl)-propylamide monomethyl ester dissolved in soap micelles. This model compound was chosen because it is pentacoordinate, stable, and exhibits spectral properties analogous to those of R state hemoglobin (Traylor et al., 1979). In addition, Traylor and co-workers have also shown that this model heme exhibits CO and O2 rate and equilibrium constants which are nearly identical to those exhibited by R state hemoglobin (Traylor et al., 1979; Traylor, 1981). The micellar solvent system was chosen to take into account partitioning of the apolar ligands between the aqueous solvent and the hydrocarbon interior of the protein molecules (see Olson et al., 1983). Second, we have attempted to discriminate between proximal and distal effects by using a homologous series of alkyl isocyanides. Since the bond strengths are the same for each member of this series, any differences in the free energies of binding are most likely to be due to distal protein steric effects. This approach is analogous to that taken originally by St. George and Pauling (1951) and later by Ainsworth et al. (1979), Reisberg and Olson (1980a, 1980b, 1980c), and Olson et al. (1982) in previous work with myoglobin and hemoglobin. The analysis presented here is more rigorous and, by using the model heme data as reference points, allows direct comparisons with results for O2 and CO binding. Third, by examining the distal effects for a series of proteins with differing structures in the sixth coordination region, the origin of some of the steric interactions can be assigned tentatively to specific amino acids.

A large number of isonitrile studies have already been published. Rate and equilibrium data for the pentacoordinate model heme dissolved in benzene and various soap micelles have been reported by Olson et al. (1983). In addition to the work with human hemoglobin (Talbot et al., 1971; Brunori et al., 1972; Reisberg and Olson, 1980a, 1980b, 1980c), systematic kinetic studies have been carried out for sheep hemoglobin (Ainsworth et al., 1960), sperm whale and horse heart myoglobin, and soybean leghemoglobin (Stetzkowski et al., 1979). Other less extensive data have been reported for the mononemic hemoglobins from Chironomus thummi (Blanck et al., 1972) and Aplysia limacina (Amiconi et al., 1972). In addition to further analysis and interpretation of this published work, we have carried out new isonitrile binding studies with the following reduced heme proteins: carp (Cyprinus carpio) hemoglobin at high and low pH, the monomeric hemoglobin II from Glycera dibranchiata, yeast cytochrome c peroxidase, cytochrome P-450 from Pseudomonas putida, and beef heart cytochrome c oxidase. Summaries of both the previously measured rates and those which are reported here for the first time are presented in Tables I through XII in the Miniprint.

RESULTS

An Overview of Association Rate Behavior—Schematic diagrams of the active sites of some of the heme proteins are shown in Fig. 1. A summary of the association rate constants measured for these compounds is presented in Fig. 2. As one would expect from the lack of distal restrictions, the largest bimolecular rates are observed for ligand binding to the proteoheme-imidazole complex (Table 1, Miniprint). In benzene the observed rate constant for isonitrile binding is 2 × 104 M⁻¹ s⁻¹, regardless of the structure of the alkyl side chain. In myristyl trimethylammonium bromide micelles, there is a linear increase in the logarithm of the association rate constant with increasing size of the ligand molecule which is readily interpreted in terms of preferential partitioning of the larger ligands into the micellar phase (i.e., a "hydrophobic" effect, Olson et al., 1983). In the case of the proteins, a marked decrease in the association rate constant is observed in going from CO to methyl isocyanide; then, depending on the sample examined, more modest fluctuations with increasing size and substitution are found.

The most striking feature in Fig. 2 is the wide separation between the isonitrile rates observed for the reversible oxy-

![Fig. 1. Active site structures of the model heme, hemoglobin, and myoglobin, cytochrome P-450, and cytochrome c peroxidase.](http://www.jbc.org/)

METHODS AND MATERIALS

1 Portions of this paper (including "Methods and Materials," Tables I-XII, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20814. Request Document No. 83 M-1604, cite the authors, and include a check or money order for $10.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Ligand Binding to Heme Proteins

Fig. 2. Dependence of the model heme and protein association rate constants on ligand length at pH 7, 20°C. The logarithm of the rate constant in M⁻¹ s⁻¹ units is plotted versus the number of atoms in the ligand molecule. Ligand abbreviations: M, methyl; E, ethyl; nP, n-propyl; nB, n-butyl; aA, n-amyl; and nH, n-hexyl isocyanide. Heme compound abbreviations: H, protopheme mono-3-(1-imidazoyl)-propylamide monomethyl ester in benzene (L); and in myristyl trimethylammonium bromide micelles (O, soap); P-450, cytochrome P-450 (A) in the presence (+CAM) and absence (-CAM) of camphor; Mb, sperm whale myoglobin (B); CCP, cytochrome c peroxidase (O); CTXO, cytochrome c oxidase (O); Lb, soybean leghemoglobin; Hb(R) and Hb(T), the high and low affinity states of human hemoglobin; CTT, hemoglobin for Chromonas thun- 
mitt; GLY, hemoglobin for Glycera dibranchiata; and CARP, hemoglobin from Cyprinus carpio. The data for the hemoglobins are not shown specifically; the arrows indicate the approximate positions of the rate constants for the intermediate sized isonitriles.

gen-binding pigments (k' ~ 10⁴ to 10⁶ M⁻¹ s⁻¹; Tables IV-XII, Miniprint) and those observed for cytochrome c oxidase and peroxidase (k' = 1 M⁻¹ s⁻¹; Tables II and III, Miniprint). Bacterial P-450cam seems to straddle these classifications. In the absence of bound camphor the observed rates are rapid, 1-2 × 10¹⁴ M⁻¹ s⁻¹, and roughly independent of ligand size. However, when the substrate is bound to the reduced enzyme, the observed bimolecular rates decrease by a factor of 10². Presumably the rigid camphor molecule is bound close enough to the iron atom to allow oxygen extraction at the 5' end of the equilibrium constant (see Fig. 1). Thus, ligand molecules containing 3 or more atoms are sterically excluded from replacing the iron atom. The enormous kinetic free energy barriers (~8 kcal/mol) suggest that camphor must be displaced before binding can occur (Peterson et al., 1971). Even the approach of CO appears to be sterically hindered since its rate of binding decreases 100-fold in the presence of bound substrate (Peter-

The slow rates of isonitrile binding to cytochrome c oxidase are less readily interpreted. In the oxidized enzyme, a Cu atom is located 3.7-3.9 Å away from the heme a₃ iron atom, presumably on the distal side (Powers et al., 1981). In the fully reduced state the Cu atom appears to move to at least 4 Å away from the iron atom, but probably not much farther since it is thought to stabilize and donate electrons to reduced oxygen intermediates. The total length of the Fe₃C = C₃N—CH₃ complex in a linear geometry is about 5 Å so that steric hindrance by the Cu atom or one of its ligands could easily explain the extremely slow rates of isonitrile binding.

The active site of cytochrome c peroxidase is shown in the lower right drawing in Fig. 1. Unlike the sixth coordination positions in hemoglobin and myoglobin, the distal region in the peroxidase is quite polar. In the ferric enzyme there is a tightly bound water molecule which is hydrogen bonded to a distal histidine and a positively charged arginine residue (Poulos and Krout, 1980). There is no reason to believe that this structure is altered when the enzyme is reduced in the absence of ligands. Thus, ligands must disrupt this hydrogen-bonding system in order to interact with the heme iron atom. Molecules containing oxygen atoms, particularly H₂O₂, appear to be able to do this readily, presumably because they can reform the hydrogen bonds either in the transition state or when bound to the iron atom (see Table III, Miniprint). On the other hand, isonitrile binding is severely restricted. This cannot be a purely size effect since ethyl peroxide is bound only 4 times less rapidly than hydrogen peroxide, whereas ethyl isocyanide is bound 10⁶ times more slowly (Table III, Miniprint). Thus, the polar or ionic character of the active site in cytochrome c peroxidase appears to inhibit the alkyl isocyanides from reaching the heme iron atom. Similar mechan-

Quantitative Analysis for the Oxygen-binding Heme Proteins—In contrast to the results for the cytochromes, the differences between the binding properties of the model heme and those of the oxygen-binding proteins are smaller and in some cases more subtle. Most of the proteins in this category exhibit an apolar active site structure which is either identical or very similar to that shown in the upper right-hand diagram in Fig. 1. A more systematic and quantitative analysis is required to interpret the differences between these proteins and to discriminate between the relative importance of proximal and distal effects.

Ligand binding involves a minimum of three distinct processes and can be considered in terms of Equation 1 which is adapted from the earlier work of Frauenfelder's group (Austin et al., 1975) and Reisberg and Olson (1980b).

\[ P + X \rightleftharpoons K_1 \rightarrow C \rightleftharpoons K_2 \rightarrow B \rightleftharpoons K_3 \rightarrow PX \]

Contributing factors: 1. Solvent or hydropho-

P and X represent the unbound protein and ligand molecules, respectively. State C represents ligand dissolved in outer hydrocarbon regions of the protein molecule; state B, a ligand molecule in the sixth coordination position with the correct orientation but not yet bound to the heme iron atom; and PX, the final iron-ligand complex.

Under almost all experimental conditions, the observed

\(^2\) It is also conceivable that the large dipole moment of the isonitrile group (3.85 D, see Olson et al., 1983) causes direct and specific interactions with the guanidine group in the active site of cytochrome c peroxidase. Weak binding to this positively charged residue would slow down the rate of approach of the ligand to the iron atom and, at the same time, reduce the rate of ligand diffusion out of the protein. This idea provides an explanation of the extremely low rates of methyl, ethyl, and n-propyl isocyanide dissociation from cytochrome c peroxidase (0.002 to 0.02 s⁻¹; Table III, Miniprint). However, once the isonitriles are bound, no favorable dipole interactions are possible between the alkyl side chains and the polar residues of the protein. This contrasts with the situation for ligands containing electronegative oxygen atoms. In this case hydrogen bonding or ionic interaction with the bound ligand is possible. At present, it is not possible to determine experimentally the relative contributions of these factors to the observed rate and equilibrium constants.
Ligand Binding to Heme Proteins

association equilibrium constant is given by the product of the individual constants: $K_{obs} = K_K K_s$ (a more general and rigorous discussion is given in the Miniprint, section B). $K_s$ represents the extent of ligand partitioning between the aqueous solvent phase and the more hydrophobic protein matrix. $K_s$ represents the equilibrium constant for taking the ligand molecule from the outer regions of the protein up to the sixth coordination position. As indicated by items 2 and 3 in Equation 1, at least two factors affect the magnitude of this parameter. First, there is the unfavorable "cage" effect of sequestering the ligand molecule in the small volume adjacent to the heme iron atom compared to the total volume of the protein. Second, specific protein residues distal to the heme iron atom can sterically hinder both the approach and final position of the ligand molecule in state $B$. $K_s$ is a reflection of the intrinsic strength of the iron-ligand bond and can be attenuated by any protein proximal effects which limit the reactivity of the heme iron atom.

Effects 1, 2, and 4 in this mechanism are general and should be observed with both the model heme and the proteins. Association equilibrium constants for the model heme are shown in Fig. 3. In benzene, the affinities of the pantocoordinate protoheme derivative are roughly the same for CO and all of the isonitriles ($K_{CO} = 2-3 \times 10^9$ M$^{-1}$, see Table 1, Miniprint). Thus, the cage effects and intrinsic bond strengths of these ligands are identical. When the heme-imidazole complex is dissolved in a soap micelle, solvent partitioning and surfactant behavior must also be considered. These effects are negligible in the case of CO but pronounced for the alkyl isocyanides. As shown in Fig. 3, the affinity constant for methyl isocyanide binding to the model heme decreases 20-fold in going from benzene to myristyl trimethylammonium bromide micelles. Olson et al. (1983) have argued that this decrease is due to stabilization of the polar isocyanato group in the aqueous phase and in the outer hydrated regions of the micelle rather than to destabilization of the heme-imidazole complex. The linear increase in going from methyl to n-hexyl isocyanide is due to preferential partitioning of the ligands from the aqueous solvent into the micelles and is directly proportional to the surface area of the alkyl side chain. The observed partitioning constant is equivalent to the $K_s$ term defined for the proteins in Equation 1 (see Olson et al., 1983).

Equilibrium constants for CO and isonitrile binding to sperm whale myoglobin are also presented in Fig. 3. As indicated by the undulating pattern, more complex behavior is observed due to the influence of proximal and distal protein structures. The magnitude of these combined effects can be computed directly by subtracting the free energy change observed for the model heme in micelles from that observed for myoglobin.

$$\Delta G_{ PROT} = RT \ln \left( \frac{K_{Prot}}{K_{obs}} \right)$$

The partition constants ($K_s$), the cage effect, and the intrinsic bond strengths for the model protoheme in the soap should be similar to the corresponding parameters and effects for the protein. Consequently, $\Delta G_{ PROT}$ can be considered a direct measure of amino acid interactions at the sixth coordination site and proximal restraints at the fifth coordination position.

In Equation 2, the model heme in a micelle is defined as the standard state to which the proteins are compared. In most cases differences between the hydrocarbon nature of the micelle and the interior regions of the proteins are due to rigid structures near the heme iron atom which sterically prevent ligand binding. As discussed in the preceding section, a polar active site can also inhibit binding with or without specific interactions. In our interpretations, this is also defined as a decrease in $K_s$ and attributed to distal effects (Equation 1).

Proximal effects are considered in the same way. $K_s$ for the model heme is a reflection of the flexibility and bonding characteristics of the alkylated imidazole. As indicated in Fig. 1, the proximal histidine in the $O_2$-binding pigments is attached to the F-helix of the protein structure. If this helix is rigidly fixed preventing movement of the proximal imidazole, the reactivity of the iron atom toward ligands will be significantly decreased. This idea was first proposed by Perutz (1970) to explain the low affinity of deoxyhemoglobin and has been expanded and elaborated by Gelin and Karplus (1977) and Warshel (1977) to take into account later crystallographic data. It is also possible the protein structure can enhance the reactivity of the heme by exhibiting greater proximal flexibility or by forcing the iron atom into the plane of the porphyrin ring. Steinem and Weber (1979) have suggested that this situation occurs in Chironomus hemoglobin and accounts for its high rate of reaction with carbon monoxide (see Table X, Miniprint).

Discrimination between proximal and distal effects can only be achieved by examining the dependence of $\Delta G_{ PROT}$ on the size and shape of the ligand molecules. As shown by the model heme results in benzene (Fig. 3, open squares), the alkyl isonitriles form a homologous series. In the absence of evidence to the contrary, protein proximal effects are assumed to apply equally to all of these compounds. The model heme results in benzene also suggest that, for equilibrium binding, CO can be considered a diatomic member of this series (Fig. 3). Differences between the $\Delta G_{ PROT}$ values for CO binding and those for isonitrile binding are interpreted in terms of differences in the $K_s$ term in Equation 1.

Mammalian Hemoglobins and Myoglobins—$\Delta G_{ PROT}$ values for the high ($R$) and low ($T$) affinity states of human hemoglobin and for sperm whale myoglobin are shown in Fig. 4. Estimation of the relative error in the $\Delta G_{ PROT}$ values can be obtained from the results for sperm whale myoglobin in Table VII of the Miniprint. For six of the isonitriles, independent measurements from 3 different laboratories are presented. The average deviation for the individual rate constants is ±10–30% so that the relative error in the equilibrium constants

![Fig. 3. Dependence of the observed association equilibrium constants for the model heme and sperm whale myoglobin on ligand length at pH 7, 20 °C. Abbreviations are given in Fig. 2. The ordinate represents the logarithm of the observed equilibrium constant in units of M$^{-1}$.](http://www.jbc.org/}

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is ±20–60%. Taking the higher estimate, the maximum error when comparing ΔG^prot values for the different proteins is about ±0.3 kcal/mol. This is also a reasonable error estimate when comparing the results for different ligands. The parameters for human hemoglobin represent averages of the properties of the α and β subunits. Although the β chain rates for the larger isonitriles are 10- to 50-fold greater than the corresponding α chain rates, the equilibrium constants are quite similar, and the individual ΔG^prot values differ by <0.4 kcal/mol (see Table IV, Miniprint). A detailed discussion of chain differences for human hemoglobin has already been presented by Reisberg and Olson (1980b, 1980c).

For CO binding to R state hemoglobin, ΔG^prot = 0 kcal/mol which implies no protein effects either on the proximal or distal side of the heme (see also Traylor, 1981). A 2–3 kcal/mol increase is observed when methyl isocyanide is bound; thus, placement of a third ligand atom in the active site is sterically hindered. No additional increase in ΔG^prot is observed when a fourth atom (ethyl isocyanide) is added. This is followed by a linear increase in ΔG^prot (~−0.8 kcal/mol per CH_2) in going from ethyl to n-butyl isocyanide, and finally the values level off as the side chain length is expanded beyond 4 alkyl carbon atoms. As shown in the right-hand panel in Fig. 4, there is also a linear decrease in ΔG^prot with increasing α substitution on the ethyl side chain.

Almost identical dependences on ligand size and shape are observed for sperm whale myoglobin and the R and T states of hemoglobin at pH 7, 20 °C. The protein effects were calculated using Equation 2. The abbreviations were taken from Fig. 2 and: iP, isopropyl; tB, tert-butyl isocyanide.

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FIG. 4. Dependence of ΔG^prot on ligand size and shape for sperm whale myoglobin and the R and T states of hemoglobin at pH 7, 20 °C. The protein effects were calculated using Equation 2. The abbreviations were taken from Fig. 2 and: iP, isopropyl; tB, tert-butyl isocyanide.

of distal steric restrictions shown in Fig. 5. In the original diagram of Reisberg and Olson (1980a), the unhindered region in the immediate vicinity of the heme iron was thought to accommodate up to 4 ligand atoms without significant steric hindrance. The results with the model heme compound show directly that bound methyl isocyanide is sterically hindered whereas bound carbon monoxide is not. This restriction is indicated by the dark band in Fig. 5. Diatomic molecules can easily fit into the sixth coordination position, but methyl isocyanide requires the displacement of an active site residue at a cost of 2–3 kcal/mol. Once this residue is shifted, the fourth atom in ethyl isocyanide experiences no additional hindrance to binding. Surrounding the area occupied by ethyl isocyanide is a larger region of restrictive protein structure into which the fifth and sixth atoms of n-propyl, isopropyl, n-butyl, and tert-butyl isocyanide extend (Fig. 5, hatched area). Still farther from the heme is a more fluid region into which the terminal carbon atoms of n-amyl and n-hexyl isocyanide protrude.

Carp, Glycera, and Soybean Hemoglobins—The generality of the active site diagram in Fig. 5 was tested by measuring the ligand-binding properties of a more diverse set of proteins. Carp hemoglobin was examined both because it is representative of the oxygen transport pigments of bony fishes and because it exhibits a marked Root Effect (see Root and Irving, 1941). At low pH, this hemoglobin behaves as if it were fixed in the T or low affinity state and exhibits little or no cooperativity (Noble et al., 1970; Tan et al., 1973). At high pH, the protein is fixed in the R or high affinity state, and again no cooperativity is observed. Under both conditions, marked differences between the rate constants of the α and β subunits are observed; however, the equilibrium constants for the two chains are similar at each pH (Table VI, Miniprint).

The reactions of carp hemoglobin with alkyl isocyanates at pH 6 and 9 have been analyzed empirically by fitting the observed time courses in terms of two independent components. In the absence of procedures for isolating and characterizing the α and β subunits of this protein, the two kinetically distinct species are designated only as fast and slow. This analysis assumes no cooperativity. If subunit interaction does occur, an analysis similar to that employed by Reisberg and Olson (1980c) would be required. Such a rigorous study did not seem justified, and as described by Reisberg and Olson...
with alkyl isocyanides are displayed in Fig. 6. Like R state the longer, straight chained alkyl isocyanides. However, sub-

In the immediate vicinity of the heme group, the high and low pH forms of carp hemoglobin appear to exhibit distal interactions which are similar to each other and to those of the R and T states of human hemoglobin. There is a 2 kcal/mol increase in going from CO to methyl isocyanide and little or no change in going from methyl to ethyl isocyanide. This is expected since the amino acid residues at the active site of carp hemoglobin are the same as those in human hemoglobin and sperm whale myoglobin (Fig. 1). Farther away from the active site these similarities break down. In carp hemoglobin there is a substantial resistance to the binding of long chain dibranchiata, was investigated for three reasons. First, the x-ray structure of this invertebrate protein has been solved; and second, the distal histidine is replaced by leucine; and third, and CO binding to this protein. The results of our studies show that the apparent contribution of the distal steric interactions can be obtained from the simpler two exponential fitting procedure. The mean values of \( \Delta G_{\text{rot}} \) for the two subunits of carp hemoglobin are displayed in Fig. 6.

![Dependence of \( \Delta G_{\text{rot}} \) on ligand size and shape for carp hemoglobin at high and low pH. Glycera hemoglobin, and leghemoglobin at 20 °C. \( \Delta G_{\text{rot}} \) was calculated using Equation 2, and the abbreviations are given in Figs. 2 and 4. The values for carp hemoglobin represent averages of the parameters for the fast and slow components. The data for Lb and Glycera were obtained at pH 7.](http://www.jbc.org/content/1983/7/14224/F6)

The monomeric hemoglobin from the marine worm, Glyceridae, was investigated for three reasons. First, the x-ray structure of this invertebrate protein has been solved; second, the distal histidine is replaced by leucine; and third, Parkhurst et al. (1980) reported extremely high rates of \( \text{O}_2 \) and CO binding to this protein. The results of our studies with alkyl isocyanides are displayed in Fig. 6. Like R state human hemoglobin, the active site of the Glycera protein appears to accommodate carbon monoxide without any steric hindrance (i.e. \( \Delta G_{\text{rot}} = 0 \) kcal/mol) but does restrict the binding of methyl isocyanide. In contrast to the other proteins, there is very little further hindrance to the binding of the longer, straight chained alkyl isocyanides. However, substituting on the \( \alpha \) carbon of ethyl isocyanide cause marked increases in \( \Delta G_{\text{rot}} \) (Fig. 6, right-hand panel) which suggest rigid structures adjacent to the sixth coordination position.

As shown in Fig. 6, the isonitrile binding data of Stetzkowsky et al. (1979) confirm this idea of an open active site. The values of \( \Delta G_{\text{rot}} \) for CO through ethyl isocyanide are equal to each other and less than 0 kcal/mol. This suggests a sixth coordination position which can accommodate up to 4 atoms without any difficulty. The negative values of \( \Delta G_{\text{rot}} \) suggest proximal effects which enhance the reactivity of the iron atom. Small increases in \( \Delta G_{\text{rot}} \) in going from ethyl to \( \text{n-butyl} \) and \( \text{tert-butyl} \) isocyanide indicate that some steric restrictions do exist. However, in general, leghemoglobin exhibits behavior more analogous to the model heme dissolved in soap micelles than to that of myoglobin or hemoglobin. In another sense, Stetzkowsky et al.’s results for leghemoglobin add further support to the legitimacy of using the model heme in soap as a standard state for evaluating distal effects.

Protein Kinetic Free Energy Barriers—Unlike the situation at equilibrium, the kinetic free energy barriers for each of the effects listed in Equation 1 do not contribute in a strictly linear fashion to the barrier height computed from the observed association rate constant, \( k' \). For rapid mixing experiments at room temperature, the expression for the observed bimolecular rate is

\[
k' = \frac{k_{h}k_{b}}{k_{-h}k_{-b} + k_{h}k_{b} + k_{-h}k_{-b}}
\]

where the \( k \) values with a positive subscript represent the forward or association rates in Equation 1 and those with negative subscripts represent the reverse or dissociation rates. Similar expressions have been derived by Austin et al. (1975), Reisberg and Olson (1980b), and Morris and Gibson (1980) and assume very small and steady state levels of states C and B during the overall reaction. The deviation from a linear combination of free energy barriers is a result of the denominator terms. A rigorous analysis of the protein contributions to the observed free energy barriers requires proof that the linear mechenism in Equation 1 is correct and then the assignment of values to all six rate constants in Equation 3. This is beyond the scope of our current work.

A more empirical approach has been adopted by again defining the model heme in soap micelles as a standard sterically unhindered state. The apparent contribution of the protein to the overall kinetic barrier is computed by analogy with the equilibrium situation,

\[
\Delta G_{\text{rot}} = RT \ln \left( \frac{k_{\text{obs}}}{k_{\text{calc}}} \right).
\]
and β subunits of human hemoglobin, and leghemoglobin are shown in Fig. 7.

Although the analysis is empirical, an examination of the dependence of $\Delta G_{HOT}$ on ligand size and shape leads to a number of interesting conclusions. First, the protein portions of the α and β subunits of hemoglobin appear to exhibit little resistance to CO diffusion up to the iron atom; the observed molecular rates are almost identical to those of the model heme (viz. $\Delta G_{HOT} = 0$ kcal/mol). The simplest interpretation of this result is that for both heme compounds, diffusion up to and away from the active site is much greater than the rate of bond formation (i.e. in Equation 3; $k_p, k_{zp} \gg k_s$). Doster et al. (1982) came to a similar conclusion for sperm whale myoglobin based on more direct measurements and suggested that at room temperature and low viscosity $k' = K_K_h$. Under these conditions, even if diffusion through the protein is slower than through the micellar or aqueous phases, it will exert no effect on the observed association rate constant. $\Delta G_{HOT}$ value of $+1.1$ kcal/mol for CO binding to myoglobin is most readily interpreted as a decrease in $k_s$ due to proximal effects.

Second, for all of the proteins there is a linear increase in $\Delta G_{HOT}$ ($-1$ kcal/mol per CH$_3$) in going from CO to n-propyl isocyanide. A similar increase is observed with increasing substitution (Fig. 7, right panel). These results must reflect decreases in the rates of ligand movement through the protein matrix (i.e. $k_s$ in Equation 3). This is particularly true in the case of leghemoglobin since for methyl and ethyl isocyanide there is no restriction to equilibrium binding and yet $\Delta G_{HOT}$ increases dramatically. Third, the greatest variation in behavior among the proteins is observed for the binding of the longer n-series isonitriles. As shown in Fig. 7, there is no change in $\Delta G_{HOT}$ in going from n-propyl to n-hexyl isocyanide for both human β chains and leghemoglobin. This is consistent with the idea in Fig. 5 of a rigid ring of protein structure surrounded by a more mobile area (see also Reisberg and Olson, 1980b). For the longer ligands only a portion of the molecule needs to move through the more restricted protein structure the remaining part stays in the outer layers. Myoglobin and human α chains appear to exhibit more viscous outer regions, and $\Delta G_{HOT}$ continues to rise, although less markedly, with increasing alkyl chain length.

**Discussion**

The importance of distal amino acids in determining the specificity and reactivity of heme proteins is obvious from a comparison of the structures in Fig. 1 with the observed rates in Fig. 2. The $\Delta G_{HOT}$ values for isonitrile binding to cytochrome c peroxidase are $9$–$10$ kcal/mol whereas those for R state hemoglobin and myoglobin are $3$–$4$ kcal/mol (see Table III, Miniprint). The polar Trp, His, and Arg residues in the peroxidase serve both to restrict the accessibility of the sixth coordination position to the isonitriles and to stabilize the anionic reduction intermediates of bound hydrogen peroxide. Presumably a similar mechanism applies to cytochrome oxidase where a Cu atom is near the distal side of heme a$_5$. In the case of bacterial P-450$_{202}$, the reactivity of the heme iron atom is dramatically influenced by the presence of substrate. Bound camphor effectively prevents the binding of any ligand molecule containing more than 2 atoms. The active sites in the hemoglobins and myoglobin are much more flexible, but even for these proteins there is a definite bias in favor of diatomic ligands.

**Steric Restrictions by the Distal Histidine**—The crystallographic structure of deoxyhemoglobin requires that the distal histidine and the γ$_1$ methyl group of Val E11 be displaced when even the small diatomic gases are bound (Perutz, 1970 and Fermi, 1975). Even in liganded hemoglobin, CO and cyanide appear to be bound in “strained off-axis" configurations due to interactions with these diatomic molecules (Moffat et al., 1979). The question is how much free energy is required to move the protein residues out of the way. If the active site were fluid and analogous to the situation in a soap micelle, then little net free energy would be required at room temperature. If the distal structures are extremely rigid, partial denaturation of the protein may be required. Under these conditions, a significant portion of the free energy released by bond formation would be consumed internally, with a marked decrease in the apparent affinity. The results in Figs. 3, 4, and 6 and Tables III–XII in the Miniprint represent our attempt to quantitate these distal steric effects systematically.

For all of the high affinity heme proteins, the $\Delta G_{HOT}$ values for CO binding are ≤$0$ kcal/mol. The observed reactivities are equivalent to those of pentacoordinate protoheme in a soap micelle. Thus, movements of the distal histidine and Val E11 to accommodate carbon monoxide appear to be energetically insignificant in these proteins, and the off-axis configuration may not be as energetically unfavorable as was originally thought. In contrast, the binding of methyl isocyanide is restricted significantly. Differences between the $\Delta G_{HOT}$ values for methyl isocyanide and CO binding are presented in Table 1, column 3. All of the vertebrate proteins exhibit a 2–3 kcal/mol difference which is a quantitative measure of the specificity of their active sites for diatomic molecules.

The origin of this restriction is not immediately clear. The alkyl carbon atom of methyl isocyanide could interact unfavorably with either the distal imidazole or the γ$_1$ methyl group of Val E11. The only direct structural information comes from the recent high resolution proton NMR studies of Mims et al. (1983). These workers measured the ring current shifts of the γ$_1$ methyl protons of Val E11 in sperm whale myoglobin and in the α and β chains of human hemoglobin. These shifts are extremely sensitive to the position of the methyl group with respect to the center of the porphyrin and, along with the proton shifts of a series of bound isonitriles, have allowed the construction of a rough geometrical diagram describing the
we examined the ligand-binding properties of the monomeric His hemoglobin from Glycera dibranchiata, since in this protein is replaced by Leu. The results are ambiguous. A large rise in $\Delta G_{\text{prot}}$ is observed in going from CO to methyl isocyanide but then no further increases occur for the n-series of ligands (Table I and Fig. 6). Thus, restriction of the sixth coordination position to two ligand atoms can be achieved without the presence of an imidazole and would appear to be a purely steric hindrance phenomenon. Similar inhibitions of isonitrile binding have been observed for Aplysia myoglobin and the monomeric hemoglobin from Chironomus, both of which contain altered distal structures (Tables X and XII, Miniprint). Unfortunately, the smallest alkyl ligand examined with these proteins was ethyl isocyamid.

The last column in Table I presents values of $\delta(\Delta G_{\text{prot}})$ in going from methyl to ethyl isocyanide. The results are rather surprising. In all cases, there is little or no change ($\pm 0.4$ kcal/mol) in steric hindrance when the fourth ligand atom is added. It would appear that once the E7 residue is displaced, the second alkyl carbon atom can fit easily into the active site.

Hydrogen Bonding to the Distal Histidine—A logical extension of the analysis prescribed by Equations 1 and 2 is to compute $\Delta G_{\text{prot}}$ values for oxygen binding and compare them with those for CO binding. Values of $\delta(\Delta G_{\text{prot}})$ in going from CO to O$_2$ for a wide variety of heme proteins are presented in Table I, second column. The absolute values and original rate and equilibrium constants are presented in Tables I–XII of the Miniprint and for the most part were not measured by us. Again O$_2$ binding to the model heme in soap suspensions is taken as the standard unhindered process. In this case, the observed association equilibrium constant is markedly lower than that for either CO or alkyl isocyanide binding (Table I, Miniprint). This result points out the major difficulty in interpreting the oxygen results. Since the intrinsic strengths of the iron-ligand bonds are markedly different, proximal protein effects may not be expressed equally on the observed equilibrium constants for O$_2$ and CO binding. However, even with this proviso, the results in Table I for oxygen binding are quite compelling. For each protein with a normal distal structure (viz. the residues depicted in Fig. 1), bound oxygen is stabilized with respect to carbon monoxide by $-1$ to $-2$ kcal/mol.

The ability of these heme proteins to discriminate between the gaseous ligands has been recognized for a long time and is most often discussed in terms of $M$ values, the partition constant for the displacement of O$_2$ by CO (Tucker et al., 1978; Collman et al., 1976). Two possible explanations of the $\delta(\Delta G_{\text{prot}})$ values in Table I have been proposed. First, because a linear Fe=C=O geometry is required, bound carbon monoxide could be destabilized by steric hindrance whereas bound oxygen would not be affected since a bent Fe-O=O geometry is allowed (Hoffman et al., 1977; Moffat et al., 1979; Baldwin, 1980). Collman (1977) originally suggested that the distal histidine is the residue which interacts unfavorably with bound carbon monoxide. Second, oxygen could be stabilized preferentially by hydrogen bond formation with the distal histidine (Pauling, 1964; see also Phillips and Schoenborn, 1981 and references cited therein). We feel that the latter explanation is correct and supported by most of the available experimental data.

Phillips and Schoenborn (1981) have used neutron diffraction to demonstrate directly the existence of a hydrogen bond between the distal histidine and bound oxygen in sperm whale myoglobin. Shaanan (1982) has reported that hydrogen bonding also occurs in the active sites of both the $\alpha$ and $\beta$ subunits in oxyhemoglobin. He compared the geometries of bound oxygen and the His E7 in both hemoglobin and myoglobin and concluded that the hydrogen bond in myoglobin is probably stronger. This structural conclusion is in agreement with the experimental results in Table I if it is assumed that

### Table I

Changes in the protein contribution to the binding free energy in going from O$_2$ to CO to methyl and ethyl isocyanide

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\Delta G_{\text{prot}, \text{O}_2}$</th>
<th>$\Delta G_{\text{prot, MNC}}$</th>
<th>$\Delta G_{\text{prot, ENC}}$</th>
<th>$\Delta G_{\text{prot, ENC-MNC}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Normal distal structures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Human hemo-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>globin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) $\alpha$ chains</td>
<td>$-0.83$</td>
<td>$+2.60$</td>
<td>$-0.57$</td>
<td></td>
</tr>
<tr>
<td>b) $\beta$ chains</td>
<td>$-0.91$</td>
<td>$+2.89$</td>
<td>$-0.06$</td>
<td></td>
</tr>
<tr>
<td>c) $\alpha$ (T)</td>
<td>$-1.41$</td>
<td>$+2.79$</td>
<td>$-0.03$</td>
<td></td>
</tr>
<tr>
<td>d) $\beta$ (T)</td>
<td>$-0.70$</td>
<td>$+2.15$</td>
<td>$-0.40$</td>
<td></td>
</tr>
<tr>
<td>2. Sheep Hb, type A (R state, pH 9)</td>
<td>$-0.85$</td>
<td>$+3.44$</td>
<td>$-0.11$</td>
<td></td>
</tr>
<tr>
<td>3. Sperma whale myoglobin</td>
<td>$-2.01$</td>
<td>$+2.16$</td>
<td>$-0.49$</td>
<td></td>
</tr>
<tr>
<td>4. Horse heart myoglobin</td>
<td>$-2.02$</td>
<td>$+2.11$</td>
<td>$-0.68$</td>
<td></td>
</tr>
<tr>
<td>5. Carp hemoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) pH 9.0, fast</td>
<td>$-0.91$</td>
<td>$+1.98$</td>
<td>$+0.46$</td>
<td></td>
</tr>
<tr>
<td>b) pH 9.0, slow</td>
<td>$-0.91$</td>
<td>$+1.91$</td>
<td>$+0.21$</td>
<td></td>
</tr>
<tr>
<td>c) pH 6.0, fast</td>
<td>$-1.14$</td>
<td>$+1.94$</td>
<td>$-0.16$</td>
<td></td>
</tr>
<tr>
<td>d) pH 6.0, slow</td>
<td>$-1.14$</td>
<td>$+2.28$</td>
<td>$-0.01$</td>
<td></td>
</tr>
<tr>
<td>B. Modified distal structures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. $\alpha$ chain, opossum Hb, R state (Gln E7, Ile E7)</td>
<td>$-0.84$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Elephant Mb (Gln E7)</td>
<td>$-1.27$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Aplysia Mb (no His E7)</td>
<td>$-1.23$</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4. $\beta$-Zurich (Arg E7)</td>
<td>$+0.29$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Glycera Hb (Leu E7)</td>
<td>$+1.25$</td>
<td>$+1.90$</td>
<td>$-0.45$</td>
<td></td>
</tr>
<tr>
<td>6. Chironomus Hb (Ile E7, displaced His E7)</td>
<td>$-0.39$</td>
<td></td>
<td></td>
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<tr>
<td>7. Leghemoglobin (flexible His E7)</td>
<td>$-1.86$</td>
<td>$-0.04$</td>
<td>$-0.02$</td>
<td></td>
</tr>
<tr>
<td>8. Ascaris, perienteric Hb (unknown changes)</td>
<td>$-5.94$</td>
<td></td>
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<td></td>
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</tbody>
</table>

* MNC, methyl isocyanide; ENC, ethyl isocyanide.
Oxygenated Intermediates of Catechol Dioxygenase

Terence A. Walsh, David P. Ballou, Ruth Mayer and Lawrence Oz Jr.

Materials and Methods

Catechol 1,2-dioxygenase was purified from Escherichia coli (O24) following the procedure of Fujie et al. (1975) with some modifications. An extinction value of 3.50 cm⁻¹ at 460 nm was used to determine the concentration of enzyme solutions. All experiments were performed in 0.1 M potassium phosphate buffer, pH 7.5 at 20°C.

Catechol and 4-methylcatechol were obtained from Aldrich Chemical Co. and purified by sublimation. 4-Fluorocatechol was synthesized from 4-acetamidocatechol (Corse and Leghoo, 1971).

Oxygen-labeling studies with catechol 1,2-dioxygenase were performed in the following manner: 460 nm was introduced into one compartment of a gas-tight modified vacuum line. A reaction flask was charged with a catalytic amount of immobilized enzyme, thoroughly degassed, and then filled with 0.5 atm. Four ml of a 4-methylcatechol solution (0.5 mg substrate in phosphate buffer, pH 7.5) was added to the photolabeled enzyme. After the addition of the enzyme and the flash, the flask was cooled at 0°C and 460 nm was introduced into the flask and condensed with liquid N2. The excised flash was then allowed to warm to room temperature and the reaction mixture until the reaction was complete.

The enzyme was then removed by ultrafiltration through an Amicon PM-10 membrane. The labeled product was obtained as follows: The solution was diluted to pH 7.5 with ICE, saturated with NaCl, and extracted with 1% ml of ethyl acetate. The ethyl acetate extract was dried over MgSO4 and the extract removed. The product thus obtained was subjected to mass spectral analysis by the analytical method at the Cornell Mass Spectrometry Laboratory. All experiments were performed under anaerobic conditions with dissimilar concentrations and wash-up procedures. These gave the doubly-labeled 3-Fluorocatechol releasing acid was observed at m/z 195 corresponding to the molecule ion, and that for the doubly-labeled 3-Fluorocatechol releasing acid was observed at m/z 145 corresponding to a loss of HF from the molecule ion. No loss of label was detected in either case.

Optical spectra and stopped-flow experiments were carried out by the procedures as described by Walsh and Ballou (1983) (Probing membrane).

A single turnover event was monitored by titrating the enzyme anatoxically with a sub-saturating amount of the appropriate substrate (typically 60-70% of the enzyme concentration) and rapidly mixing this with a solution of enzyme (the stopped-flow experiment). Because of the relatively low ES values for all the four-methylcatechol substrates used in Table I, there was minimal free substrate present in the enzyme solutions after illumination. The variable concentrations in the experiments were in excess of those of the substrate and were more than two fold greater than the ES value. This eliminates the problems associated with the interpretation of multiple turnover events and greatly simplifies the analysis.

Analysis of the kinetic data to determine the extinction of the two unknown intermediates is based on the method of Dolby et al. (1976) and we have given a detailed description of this process here to illustrate the effectiveness of the technique and to point out some possible pitfalls.

The relationship between the stopped-flow apparatus at 20 μl intervals over the wavelength range 320 - 750 nm. Inspection of these kinetic records revealed three phases in the reaction, especially evident in the region 460 - 660 nm, as the absorption changes due to each phase were observed (e.g., Fig. 1). It is essential to impact the data analyzed at several wavelengths as both the relative properties and the direction of absorption changes varies with wavelength. For example, the apparent rate of each process may be distorted by proceeding or following processes involving a large change in absorbance, or reaction rates involving these kinetic processes can be substantially apparent. Sharp or even monophasic in certain ranges of the spectrum. However, such regions are useful in that the reaction phase forming the major change in absorbance can be determined more accurately, as there is little interference from the other stages in the reaction. Thus, at 700 nm, A, predominates. At 460 nm, K, and A, are not affected by K, while at 460 nm the absorbance changes due to A, K, are more easily seen (see Figure 4). Thus, accurate values for all these rates constants can be estimated, either by direct semi-logarithmic analysis or by subtraction of sequential exponential decays in the case of multicomponent systems. This analysis, performed at different concentrations of oxygen, will also effectively demonstrate which reactions are second order with respect to oxygen.

Having determined the rates of the processes involved in the reaction, the spectra of the two intermediates can be determined. The spectra of the free enzyme and the enzyme-substrate complex as well as the appropriate ES values for each substrate are known from static spectral titrations. The absolute concentrations of each of the species during the course of the reaction can be calculated by computerized numerical integration techniques. At any given time point along the course of the reaction, the sum of these concentrations terms, multiplied by the appropriate extinction values, for the wavelength, most equal the observed absorbance. Thus, one has a large number of simultaneous equations at any given wavelength with only two unknowns, the extinction values for the two intermediates. These can be calculated by a least squares method. Computer simulations of reaction-time courses were performed using a Norn 2 minicomputer (Data General) employing a FOURIER-based fourth order Runge-Kutta method (Broyer and DePrince, 1969). The following scheme was used:

\[ A \rightarrow B \rightarrow C \rightarrow D \rightarrow E \rightarrow A \]

Where A is free enzyme, B is substrate, C is the enzyme-substrate complex, D is intermediate I, E is intermediate II and F is free product. The upper addition step, k1, is treated as pseudo-first order at any given oxygen concentration. The simulations were performed at several appropriate wavelengths to ascertain that with a given set of rate constants the fit is good and free for incertes where the proportions and direction of absorption change are widely different. Minor adjustments in the rate constants for a particular step are unnecessarily necessary (less than the uncertainty of the original determination by semi-logarithmic analysis) to obtain good fits for all wavelengths with a single set of rate constants. The data at all wavelengths is then fitted to give extinction values of the intermediates at 30 μsec intervals from 350 to 750 nm. One point should be stressed here. A rapid change in absorbance followed by a slower change does not necessarily imply that the rate constant governing the first process is faster than that of the second. (More and Petrasso, 1981).

In the simple example:

\[ A \rightarrow B \rightarrow C \text{ (a)} \]

Where the extinction of B, A, is unknown and two apparent rates can be determined from the biphasic spectroscopic changes due to the formation and decay of B, two equally appropriate solutions can be derived with the faster observed process occurring either first or second, i.e., the observed changes for the two spectrophotometric processes are inextricable and the extinction value of B is obscured to compensate. In practice, one of the two solutions to the equations can usually be eliminated as being unlikely or impossible (a negative value for example). In cases where k1 and k2 are not well separated (less than a factor of 2), it may be difficult to determine the true solution. We have tested our data by interchanging k1 and k2 in our kinetic scheme and solving for the extinctions of intermediates I and II. In the cases using 4-methylcatechol and 4-fluorocatechol as substrate, the ratios of k1/k2 are relatively large (1.2 and 3.1 respectively). For the solution where k2 is greater than k1, the extinction values of each of the intermediates decrease exponentially with the values for k2. In the smaller apparent rate constant, the resulting spectrum of intermediate II for the two substrates are very dissimilar with comparable high or low extinction rates and a fixed value of 4000 cm⁻¹·11 per 800-700 nm range that are 75% larger than those shown in Fig. 4. However, if the rate of the reaction is increased above those for the other substrates, and hence we have assigned the faster process as the one occurring first. This is a chemical approach to sorting out a difficult kinetic problem.
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Perutz, M. F. (1970) Nature (Lond.) 228, 726-739
Perutz, M. F. (1979) Annu. Rev. Biochem. 48, 327-386
Reisberg, P. I., and Olson, J. S. (1980a) J. Biol. Chem. 255, 4144-4150
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Reisberg, P. I., and Olson, J. S. (1980c) J. Biol. Chem. 255, 4159-4169
St. George, R. C. C., and Pauling, L. (1951) Science (Wash. D. C.) 114, 629-634
Additional references are found on p. 14219.
Ligand Binding to Heme Proteins: A Case of Haptic Effects

by Martha F. Wood, Arturo Foerster, John S. Olson, Forrest Peterson, and Robert W. Mohle

A. METHODS AND MATERIALS

All of the experiments were performed with a 310-nm, 0.1-mm, Photoreactor lamp and a 310-nm, 0.1-mm, Photoreactor lamp. The data were obtained from a Varian spectrophotometer and a Cary 110 spectrophotometer. The protein concentrations were determined by the method of Bradford (1976). The data were analyzed by the least squares method.

B. RESULTS AND DISCUSSION

The least squares line is represented by

\[ Y = a + bx \]

where \( a \) and \( b \) are determined by the least squares method.

C. CONCLUSIONS

The results of the experiments presented in this paper demonstrate that the least squares method is a powerful tool for analyzing data. The method is simple to use and provides accurate results. It is recommended for use in future studies.

References


Table I. Rates of ligand binding to pyruvate dehydrogenase complex. Present work was carried out in rat pyruvate dehydrogenase complex. The rates of ligand binding were measured at pH 7.4. The incubation time was 15 min. The assay was performed in the presence of 50 mM Tris-HCl buffer, pH 7.4, and 10 mM MgCl₂. The data are expressed as the mean ± S.E. of triplicate determinations.

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<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>1. Gluon</td>
<td>0.145 ± 0.013</td>
<td>0.145 ± 0.013</td>
<td>0.145 ± 0.013</td>
<td>0.145 ± 0.013</td>
</tr>
<tr>
<td>2. Heme protein</td>
<td>0.145 ± 0.013</td>
<td>0.145 ± 0.013</td>
<td>0.145 ± 0.013</td>
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</tr>
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</table>

Table II. Rates of ligand binding to pyruvate dehydrogenase complex. Present work was carried out in rat pyruvate dehydrogenase complex. The rates of ligand binding were measured at pH 7.4. The incubation time was 15 min. The assay was performed in the presence of 50 mM Tris-HCl buffer, pH 7.4, and 10 mM MgCl₂. The data are expressed as the mean ± S.E. of triplicate determinations.

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Table III. Rates of ligand binding to pyruvate dehydrogenase complex. Present work was carried out in rat pyruvate dehydrogenase complex. The rates of ligand binding were measured at pH 7.4. The incubation time was 15 min. The assay was performed in the presence of 50 mM Tris-HCl buffer, pH 7.4, and 10 mM MgCl₂. The data are expressed as the mean ± S.E. of triplicate determinations.

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<tr>
<th>Ligand</th>
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<td>2. Heme protein</td>
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Table IV. Rates of ligand binding to pyruvate dehydrogenase complex. Present work was carried out in rat pyruvate dehydrogenase complex. The rates of ligand binding were measured at pH 7.4. The incubation time was 15 min. The assay was performed in the presence of 50 mM Tris-HCl buffer, pH 7.4, and 10 mM MgCl₂. The data are expressed as the mean ± S.E. of triplicate determinations.

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<td>2. Heme protein</td>
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Table V. Rates of ligand binding to pyruvate dehydrogenase complex. Present work was carried out in rat pyruvate dehydrogenase complex. The rates of ligand binding were measured at pH 7.4. The incubation time was 15 min. The assay was performed in the presence of 50 mM Tris-HCl buffer, pH 7.4, and 10 mM MgCl₂. The data are expressed as the mean ± S.E. of triplicate determinations.

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</table>
Ligand Binding to Heme Proteins

Table V. Rates of Ligand Binding to type A haemoglobin molecules at pH 6.0, 37°C, 20% CHO. The rates were estimated as the rate constant for the formation of the initial complex (Kcal/mol + M, or in the case of a 1:1 reaction, for the formation of a 1:1 complex).

<table>
<thead>
<tr>
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<th>k on</th>
<th>k off</th>
<th>1/kt</th>
<th>1/kon</th>
<th>1/koff</th>
<th>1/kon.koff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>10.5</td>
<td>0.16</td>
<td>63.3</td>
<td>6.00</td>
<td>0.016</td>
<td>3.80</td>
</tr>
<tr>
<td>NO</td>
<td>1.0</td>
<td>0.002</td>
<td>500.0</td>
<td>0.002</td>
<td>500.0</td>
<td>1.00</td>
</tr>
<tr>
<td>CO</td>
<td>0.001</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
</tr>
<tr>
<td>HbO2</td>
<td>1.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table VI. Rate and equilibrium constants for ligand binding to type A heme proteins at pH 6.0, 37°C in the presence of 10% DMSO.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>k on</th>
<th>k off</th>
<th>1/kt</th>
<th>1/kon</th>
<th>1/koff</th>
<th>1/kon.koff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>10.5</td>
<td>0.16</td>
<td>63.3</td>
<td>6.00</td>
<td>0.016</td>
<td>3.80</td>
</tr>
<tr>
<td>NO</td>
<td>1.0</td>
<td>0.002</td>
<td>500.0</td>
<td>0.002</td>
<td>500.0</td>
<td>1.00</td>
</tr>
<tr>
<td>CO</td>
<td>0.001</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
</tr>
<tr>
<td>HbO2</td>
<td>1.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table VII. Rate and equilibrium constants for ligand binding to type A heme proteins at pH 6.0, 37°C in the presence of 20% DMSO.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>k on</th>
<th>k off</th>
<th>1/kt</th>
<th>1/kon</th>
<th>1/koff</th>
<th>1/kon.koff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>10.5</td>
<td>0.16</td>
<td>63.3</td>
<td>6.00</td>
<td>0.016</td>
<td>3.80</td>
</tr>
<tr>
<td>NO</td>
<td>1.0</td>
<td>0.002</td>
<td>500.0</td>
<td>0.002</td>
<td>500.0</td>
<td>1.00</td>
</tr>
<tr>
<td>CO</td>
<td>0.001</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
</tr>
<tr>
<td>HbO2</td>
<td>1.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
<td>1000.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Carbon monoxide rates were taken from data by Ozerov, O.K. (1999) Angew. Chem. Int. Ed. 38, 2038-2041. The oxygen rates were estimated as the rate constant for the formation of the initial complex (Kcal/mol + M, or in the case of a 1:1 reaction, for the formation of a 1:1 complex).
Ligand binding to heme proteins. An evaluation of distal effects.
M P Mims, A G Porras, J S Olson, R W Noble and J A Peterson


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