Evidence for Nonequivalent Binding Sites in Human Methemoglobin*

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SUMMARY

A careful analysis of the isotherm for the binding of imidazole to the iron of human methemoglobin A reveals that the equilibrium cannot be described with a single constant. The results are fit minimally by assuming two classes of independent and nonequivalent sites.

On the basis of nuclear magnetic resonance spectra, Davis, Charache, and Ho (1) have recently suggested that the heme groups of the α and β methemoglobin chains are not equivalent. Gibson, Parkhurst, and Geraci (2) have reported biphasic kinetics for the reaction of methemoglobin with a variety of iron-coordinating ligands. These results were also explained assuming nonequivalent heme groups. In contrast, Ansieus, Beetlestone, and Irvine (3); Beetlestone and Irvine (4); and Beetlestone, Epega, and Irvine (5) have reported hyperbolic curves for the binding to methemoglobin of all the ligands they have investigated. This latter observation suggests that the four heme iron bind ligands with identical, intrinsic affinities. There is, therefore, an apparent contradiction between the thermodynamic results, and the kinetic and nuclear magnetic resonance observations. Because of the large difference in time scales, it is possible that the binding sites may appear equivalent by one technique, but not by another. On the other hand, if the difference in binding energies is small, nonequivalent binding would be difficult to detect.

To test for this latter possibility, we undertook a careful analysis of the methemoglobin binding curves. We have found that the binding of imidazole to human methemoglobin A is best interpreted on the basis of two nonequivalent binding sites.

METHODS

The preparation of human methemoglobin A has been described previously (6). Although methemoglobin concentration is most often determined spectrally, we have found that the protein absorbance deviates from Beer's Law. We, therefore, determined the concentration of ligand-binding sites by cyanide titration. Varying amounts of a potassium cyanide solution, prepared on the day of use and previously calibrated with AgNO₃ (7), were added with a syringe microburette (Micrometric Instruments, Cleveland, Ohio) to 10-ml volumetric flasks, each containing a fixed amount of methemoglobin in 0.01 M sodium borate, pH 8.66. All volumetric glassware had been previously standardized with water. The solutions were diluted to the mark and equilibrated for 2 hours at 10°C, and the absorbances were measured at 533 nm with a Cary model 16 spectrophotometer in which the samples were also kept at 10°C. The results of one titration are presented in Fig. 1. Each point is derived from a separate protein sample. When the protein concentration is near \(10^{-4} \text{M} \) and under the conditions described here, the binding of cyanide is almost stoichiometric; a slight deviation from linearity is always observed near the equivalence point. The two straight lines of the titration curves are fit by linear regression analysis, and the equivalence point is calculated by solving the two resulting equations. The experimental reproducibility of this procedure is 0.1%, and the estimated accuracy is 0.3%.

Ligand-binding studies were performed at concentrations of \(4 \times 10^{-4} \text{M} \) or greater, since we have found that the protein dissociates below this concentration. Our routine procedure, called here the multiple sample procedure, was to fill individual volumetric flasks with a fixed amount of methemoglobin and with ligand solution as described above. All solutions were in pH 7.20 (determined at 24°C), 0.050 M Tris (cacodylate). Protein and ligand were equilibrated for 1 to 2 hours at 22.0 ± 0.1°C; the absorbance of each sample was measured at the same temperature and at a suitable wave length.

To improve the experimental reproducibility, we have also used a single sample procedure. A solution of methemoglobin (35 to 50 ml) in 0.20 M Tris (cacodylate), pH 7.20, was introduced into a constant temperature flask located outside the spectrophotometer. The solution maintained at 22.0°C was pumped through a flow cell (Precision Cells, Inc., New York) located inside the cell compartment. The spectrophotometer output was recorded with a paper tape punch (Diatoc Corporation, Dayton, Ohio) activated every 3 to 4 seconds. After addition of ligand to the solution outside the spectrophotometer, the system was equilibrated; the pump was stopped; and the absorbance was continuously read to obtain approximately 100 data points. The absorbance readings stored on the tape were then averaged with the use of a Wang 372 (Wang Laboratories, Inc., Tewksbury, Massachusetts) equipped with teletype. By
6. FIG. 1. Cyanide titration of methemoglobin. The procedure has been described under "Methods." Each point represents an individual sample. The absorbance values are arbitrary and have been normalized to correct for small differences in flask volumes.

This relatively crude, time-averaging technique, instrument noise level was reduced to approximately 0.02% of the absorbance reading. The entire binding curve was determined with one protein sample, repeating the above procedure for each ligand addition. Since the protein was progressively diluted, the absorbance observed after each addition was accordingly corrected.

Imidazole (grade III) and Tris were used as purchased from Sigma. Other reagents were purchased as analytical grade and were used with no further purification. All experiments were performed in glassware rinsed with alcoholic KOH to remove traces of detergent. Volumetric glassware was treated with cleaning solution after contact with protein and stored in EDTA solutions for at least 1 day before use. Water double distilled from quartz was used throughout the experiments.

The equilibria of imidazole and thiocyanate were studied since both these ligands have been reported to bind hyperbolically and with relatively low affinities to methemoglobin (3-5). Our preliminary experiments verified these observations. The results of a thiocyanate experiment, in which the multiple sample procedure is used, are plotted in a linear form of the Langmuir isotherm (Fig. 2). The data appear to fit the linear relationship extremely well, suggesting that the four heme sites bind ligand with identical affinities.

To verify the thiocyanate binding assay, and also to look for spectral evidence for nonequivalent sites, we investigated the behavior of two apparent isosbestic points at 477.5 mp and 412.4 mp (uncorrected). The results are presented in Fig. 3. Within a standard deviation of 0.1%, and in one case up to approximately 90% saturation, the absorbancies at these wave lengths remained constant. The presence of at least two isosbestic points suggests only two light-absorbing species. Therefore, within experimental error, the spectrophotometric assay
globin A is described, within experimental error, by the model isosbestic points in the thiocyanate reaction. This may not be the derived with the assumption that both binding sites exhibit the same absorbance change on saturation. This is consistent with the observation of two isosbestic points in the thiocyanate reaction.

We conclude that the binding of imidazole to human methemoglobin A is described, within experimental error, by the model

\[ A = A_0 + \Delta A_f \left\{ K(L) \over 1 + K(L) \right\} \]  

(1)

where \( A \), \( A_0 \), and \( \Delta A_f \) represent the protein absorbance at the concentration of free ligand \( (L) \), the absorbance of the free protein, and the total absorbance change associated with saturation. Since the imidazole equilibrium is characterized by such a low affinity constant, the free ligand concentration is effectively equal to the total ligand concentration, and no correction for bound imidazole was required. In the analysis the three constants \( A_0 \), \( \Delta A_f \), and \( K \) were taken as adjustable parameters. The second binding equation tested describes a model with two pairs of independent, but nonequivalent, sites.

\[ A = A_0 + \Delta A_f \left\{ K_1(L) \over 1 + K_1(L) \right\} + \left\{ K_2(L) \over 1 + K_2(L) \right\} \]  

(2)

So as not to prejudice the comparison of Equations 1 and 2, only three of these four constants, \( \Delta A_f \), \( K_1 \), and \( K_2 \), were treated as adjustable parameters.

The observed absorbances were fit to both Equations 1 and 2. The computed constants were then used to obtain theoretical binding curves, and the data were recalculated as the ratio of calculated to observed absorbance. The results of one experiment are presented in Fig. 4. The same experimental absorbance changes can be fit to both Equations 1 and 2 with a standard deviation of approximately 0.27. Clearly, at this level of reproducibility the two binding models cannot be distinguished. A further statistical analysis revealed that with data of this quality the nonequivalent site model could be differentiated only if the affinities of the two hypothetical classes of sites differed by more than 800 cal per mole.

In order to distinguish a smaller energy difference, better data were required. We, therefore, introduced two important, procedural modifications. A single protein sample was used to reduce the experimental manipulations, and the spectrophotometer output was time-averaged to reduce the noise level. Data obtained with this single sample procedure (see "Methods") were analyzed as described above, and the results are presented in Fig. 5. Although the hyperbolic model (Equation 1) still fits with a standard deviation of approximately 0.27%, the results show clearly that this mechanism is not operative. In contrast, the nonequivalent sites model (Equation 2) yields a better fit with a significantly better standard deviation. Equation 2 was derived with the assumption that both binding sites exhibit the same absorbance change on saturation. This may not be the case, but a small difference cannot be unambiguously established with these data. This is consistent with the observation of two isosbestic points in the thiocyanate reaction.

We conclude that the binding of imidazole to human methemoglobin A is described, within experimental error, by the model...
when a painstakingly high level of reproducibility is achieved. The thermodynamic results are, thus, consistent with the kinetic and nuclear magnetic resonance results, which were explained on the assumption of nonequivalent heme groups in the \( \alpha \) and \( \beta \) chains (1, 2).

The small, observed energy difference, 600 cal per mole, cannot be interpreted as being due to small differences in the chemistry of the sites themselves. The binding of a ligand to met-hemoglobin is, in reality, the substitution of that ligand for the water molecule already attached to the sixth coordination position of the iron. The binding reaction at one heme iron should be written as

\[
\text{Fe}_6\text{H}_2\text{O} + L \rightleftharpoons \text{Fe}_6\text{L} + \text{H}_2\text{O} \quad ; \quad K = \frac{(\text{Fe}_6\text{L})(\text{H}_2\text{O})}{(\text{Fe}_6\text{H}_2\text{O})(L)} \quad (3)
\]

The corresponding partial equations are

\[
\text{Fe}_6 + \text{H}_2\text{O} \rightleftharpoons \text{Fe}_6\text{H}_2\text{O} \quad ; \quad K_{\text{H}_2\text{O}} = \frac{(\text{Fe}_6\text{H}_2\text{O})}{(\text{Fe}_6)(\text{H}_2\text{O})} \quad (4)
\]

\[
\text{Fe}_6 + L \rightleftharpoons \text{Fe}_6\text{L} \quad ; \quad K_L = \frac{(\text{Fe}_6\text{L})}{(\text{Fe}_6)(L)}
\]

Therefore,

\[
K = \frac{K_L}{K_{\text{H}_2\text{O}}} \quad (5)
\]

If we postulate two substitution processes characterized by \( K \) and \( K' \), then the following ratio exists.

\[
\frac{K}{K'} = \frac{K_L}{K'_{\text{H}_2\text{O}}} \cdot \frac{K'_{\text{H}_2\text{O}}}{K_{\text{H}_2\text{O}}} \quad (6)
\]

Hence, the difference between the two binding constants, \( K \) and \( K' \), depends on the differences of the related partial constants, and may be small or large, depending on the degree of compensation. This is analogous to the substitution of carbon monoxide for oxygen on reduced hemoglobin. This substitution reaction yields a hyperbolic curve even though the formation of the oxygen and carbon monoxide complexes from deoxyhemoglobin is sigmoidal.

An extrapolation of these results, obtained with methemoglobin, to a discussion of the oxygenation of deoxyhemoglobin must be considered with great caution. However, the observation of nonequivalence in a thermodynamic process involving methemoglobin opens the possibility of an analogous nonequivalence in the oxygenation process. Any thermodynamic model which does not consider such a possibility, therefore, may be incomplete.

REFERENCES
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