THE COOPERATIVE INTERACTION OF ANILINE WITH METHEMOGLOBIN*

John J. Mieyal and Linda S. Freeman
Department of Pharmacology, Northwestern University
Medical and Dental Schools, Chicago, Illinois 60611

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SUMMARY

The interaction of aniline with human methemoglobin was studied by UV spectroscopy. The observed spectral changes reflected conversion of high-spin aquomethemoglobin to the low-spin aniline complex and indicated that aniline bound cooperatively; Hill coefficient n = 2.2. This high cooperativity is unusual, since most ligand-methemoglobin interactions show n ≃ 1. Apparently spin-state change is not sufficient for cooperativity, because imidazole (which also forms a low-spin complex) bound non-cooperatively under the same conditions.

More attention has been afforded to ligand interactions with ferrohemoglobin than with ferrihemoglobin, probably because the former functions in O_2 and CO_2 transport in vivo, and no function has been ascribed to methemoglobin. Nevertheless, since binding to the former is cooperative whereas binding to the latter has generally been judged non-cooperative (1), studies of interactions with both forms are important in elucidating the basis for cooperativity. Moreover, there is a controversy currently regarding whether or not certain ligand-methemoglobin interactions are cooperative (2,3). In the present study we directly compared the binding of imidazole and aniline to methemoglobin and found that imidazole binds non-cooperatively (as reported previously (4)), whereas aniline binding displays high cooperativity, Hill coefficient n = 2.2.

RESULTS

Figure 1 shows that the Soret band of the UV/visible spectrum of human methemo globin in the presence of aniline has undergone a bathochromic shift and the bands associated with the high-spin form of methemoglobin (∿500 nm and ∿630 nm) have been

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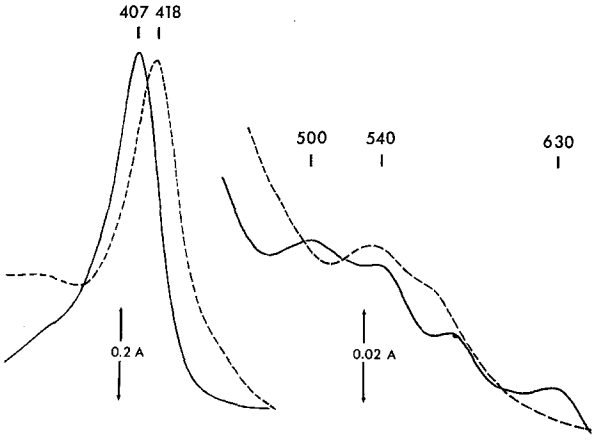

Fig. 1 UV/Visible Spectra of Aquomethemoglobin and Anilino-methemoglobin.

(———) Aquomethemoglobin, ∿1 μM in 20 mM K phosphate, pH 6.8, 38°.
(-----) Anilinomethemoglobin: neat aniline liquid and a solution of methemoglobin were equilibrated to achieve a resultant solution 0.3 M aniline, ∿1 μM methemoglobin in 20 mM K phosphate, pH 6.8, 38°.

Crystalline human hemoglobin (Sigma) was dissolved, treated with $K_3Fe(CN)_6$ to convert it totally to methemoglobin and then separated from excess oxidant and other small molecules by Sephadex G-25 chromatography. Hemoglobin concentrations were determined according to Van Kampen and Zijlstra (5). Spin states of methemoglobin complexes were approximated from the positions of the Soret bands in their UV spectra using fluoromethemoglobin and cyanomethemoglobin as the standards of pure high-spin and pure low-spin complexes, respectively (6).

diminished, while the absorbance at ∿540 nm (β band indicative of low-spin ferric iron) has increased. These changes are all consistent with the transformation of aquomethemoglobin, predominantly high-spin (∿81%), to anilinomethemoglobin which appears to be predominantly low-spin (∿84%). (See legend to Fig. 1).

When increasing amounts of aniline were combined with methemoglobin, the magnitude of the resultant difference spectrum (λ_{min} = 405 nm, λ_{max} = 425 nm; see Legend, Fig. 2) was increased. (This difference spectrum is analogous to the effect of aniline on the spectrum of ferricytochrome P450 (7)). The relationship between the absorbance changes at equilibrium and the aniline concentration (Figure 2, lower curve) is clearly sigmoidal in shape, indicating cooperative binding. To test the validity of this spectral procedure we studied imidazole under the same conditions, because it induces similar changes in the methemoglobin spectrum, but it has been reported to bind non-cooperatively (4). The low-spin

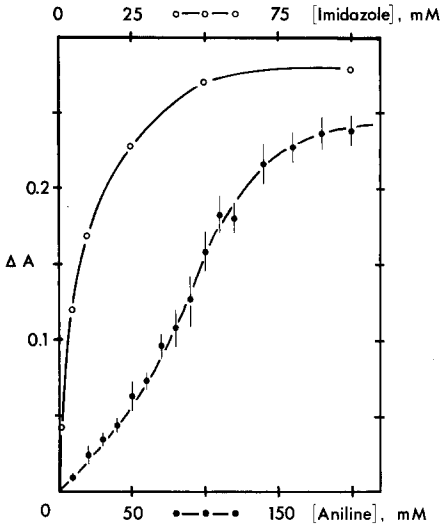

Fig. 2 Dependence of ΔA of Methemoglobin Spectra on [Aniline] (-•-•-) or [Imidazole] (-o-o-).

ΔA values (obtained as described below) were plotted against ligand concentration. Each point for the aniline curve represents the average of at least five separate determinations and the vertical bars represent the mean ± standard deviation of the mean. Each point for the imidazole curve represents the average of two separate determinations.

Mixing cuvettes (Pyrocell) were used in an Aminco DW-2 spectrophotometer operating in the split beam and baseline correction modes. One ml of 2 μM methemoglobin in 20 mM K phosphate, pH 6.8 was added to one chamber of both the sample and reference cuvettes. One ml of ligand solution of appropriate concentration also in buffer was added to the other chamber. A flat baseline was set after the solutic had equilibrated to 38°. The sample cuvette was inverted and the resultant differer spectrum (sample minus reference) was recorded between 350 and 450 nm as a function of time until no further change was observable. For aniline, λ_{min} = 405 nm, λ_{max} = 425 nm; for imidazole, 410 nm and 435 nm, respectively.

nature of the imidazole-methemoglobin complex has been determined directly by magnetic susceptibility measurements (8). The typical rectangular hyperbola (Figure 2, upper curve) demonstrates that imidazole binds non-cooperatively with a dissociation constant ≃ 8 mM. The qualitative and quantitative agreement of these data with those previously reported (i.e. for human methemoglobin-imidazole at 27° and pH 7, K_d ≃ 10 mM (9)) validates the experimental procedure and thereby confirms the cooperativity of the aniline binding.

The Hill plot for the aniline data (Fig. 3) showed maximum cooperativity at the midsection of the curve (n = 2.2), and the slopes at the extremes reverted to

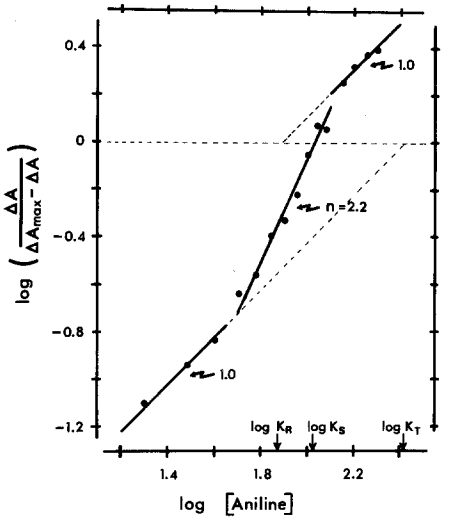

Fig. 3 Hill plot of Aniline-Methemoglobin Interaction Data.

ΔA_{max} was obtained by replotting the reciprocals of the ΔA values from the terminal protion of Fig. 2(i.e. ΔA values for [Aniline] = 0.12 to 0.30 M) versus the reciprocals of the corresponding aniline concentrations. Extrapolation of the linear portion of that curve to 1/[Aniline] = 0, yielded $1/\Delta A_{max}$. The ΔA_{max} value is an internally consistent measure of the change in absorbance associated with total aniline-methemoglobin complex formation, i.e. fraction bound = 1.0. ΔA is then the measure of intermediate amounts of association such that $\frac{\Delta A}{\Delta A_{max} - \Delta A}$ is equivalent to $\frac{y}{1 - y}$ in the common symbolism of the Hill plot.

n = 1. This is the typical form of the complete Hill plot and allows calculation of the various parameters which describe the cooperativity according to the Two-State Theory (10, 11). Thus, cooperativity is assumed to relate to a substrate-induced conformational transition from a "tense" low affinity form (T) to a "relaxed" high affinity form (R). The apparent overall dissociation constant (K_S), the dissociation constants for the two forms (K_T and K_R) and the ratio (L) of the initial concentrations of the T and R forms can be estimated directly from the Hill plot (11), if it is assumed that binding of ligand to the α and β chains is equivalent. In this case K_S = 105 mM, K_T = 260 mM, K_R = 75 mM, L = T/R $\simeq$ 4, and c = K_R/K_T = 0.29.

DISCUSSION

The Two-State Theory appears to be the chief current model for hemoglobin

cooperativity (10,11), and efforts have been made to provide physical evidence for the proposed conformational transition. From xray and spectroscopic data, Perutz et al. (12,13) concluded that aquomethemoglobin exists predominantly in the oxyhemoglobin conformation equated to R. It should follow that ligand interactions with methemoglobin display very little cooperativity. This conclusion has generally been confirmed, but Hill coefficients as high as n = 1.55 and 1.76 have been reported (2,14). In the present study, imidazole and aniline were compared directly; imidazole bound non-cooperatively as predicted, but aniline displayed high cooperativity. However, the values for L ($\sim$4) and c (0.29) are in conflict with the basic Monod-Wyman-Changeux model wherein high cooperativity is predicted only for large L and small c values[2]; e.g. for O_2 binding to deoxyferrohemoglobin (where n $\simeq$ 2.8), L $\simeq$ 9000, c $\simeq$ 0.014 (10). This conflict may in part reflect non-equivalence of aniline binding to the α and β chains (11), but it seems unlikely that correction for such non-equivalence, if observed, would alter the calculated value of L by several orders of magnitude. Hence the high cooperativity observed here suggests that a relatively small excess of a conformational isomer may be compatible with cooperative ligand binding; indeed the smallness of the conformational equilibrium may provide an explanation for the inconsistency in reports concerning cooperative binding to methemoglobin (2,3). Nevertheless, it remains to be demonstrated that a specific conformational transition does occur in each case where cooperative binding to methemoglobin has been reported, and that the stoichiometry is 4 ligands/tetramer, as assumed.

Banerjee et al. (2) recently suggested that a unifying principle governing hemoglobin cooperativity (regardless of oxidation state) may be the high- to low-spin state transition that occurs concomitantly with binding of ligands which show cooperativity. It is recognized that conformational alterations accompany spin

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[2]If the values L = 4 and c = 0.29 are used in a computer program for the basic Monod-Wyman-Changeux model, a Hill plot is generated which differs markedly from the one we observed, having a maximum slope of n = 1.22. We thank Professor Brian Hoffman, Department of Chemistry, Northwestern University for this calculation and for valuable discussions.

state changes (15); but the spin state change alone cannot be the sufficient correlate of cooperativity in the present case, because imidazole causes the same change in spin state as does aniline, but binds non-cooperatively.

In the other studies (16-18), we found that aniline displays typical Michaelis-Menten substrate kinetics (K_M = 8 mM) for the O_2- and NADPH-requiring reaction in which human hemoglobin catalyzes hydroxylation of aniline. The finding that aniline binds weakly and cooperatively with ferrihemoglobin suggests that the catalytically important aniline-hemoglobin complex involves another form of hemoglobin. This conclusion was supported by the observation that interaction of aniline with oxy-ferrohemoglobin is non-cooperative and shows a half-maximal response at 8 mM aniline (18); i.e. identical to K_M for the overall reaction (17). Besides pertaining to the chemical properties of human hemoglobin, these studies may serve as a model for interpretation of mechanisms of cytochrome P450-catalyzed hydroxylation reactions (19).

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