CONTRIBUTION OF AROMATIC RESIDUE INTERACTIONS TO THE STABILITY OF MYOGLOBIN

V. ENHANCEMENT BY AROMATIC COMPOUNDS OF THE RATE OF HEAT DENATURATION

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ABSTRACT Aromatic compounds like chlorpromazine and benzoate and its homologs strongly enhance the rate of heat denaturation of myoglobin. The latter apparently exert their action by complexing with a single kind of site in the hemeprotein. Both charge-transfer and hydrophobic interactions are implicated in complex formation, most probably with the heme moiety.

INTRODUCTION

Aromatic compounds as diverse as benzene and chlorpromazine have an enormous and specific enhancing effect upon the rate of reaction of Zn^{++} with myoglobin and the rate of urea denaturation of the protein (1–4). Kinetic and spectroscopic evidence has been advanced in support of the interpretation that these compounds exert their effect by forming electron donor-acceptor (charge-transfer) complexes with the heme moiety of myoglobin, the aromatic compounds serving as donors. Charge-transfer is not the only force involved, however, Thus, there is a substantial contribution of hydrophobic interaction to the stability of the complex. These experiments have now been extended to include the heat denaturation of myoglobin, which exhibits the same exquisite sensitivity to aromatic compounds as the other reactions of myoglobin. Moreover, the same kinds of forces are held responsible for their interaction with a very limited portion of the hemeprotein.

METHODS

The sperm whale ferrimyoglobin was obtained from Mann Research Analytical Laboratories, New York. The chlorpromazine hydrochloride [2-chloro-10-(3-dimethylaminopropyl)-phenothiazine hydrochloride] was kindly supplied by Smith, Kline and French Laboratories, Phila-

delphia, Pa. The highest quality, commercially available methyl viologen [N,N'-dimethyl-(4,4'dipyridine)dichloride] and benzoic acid and its homologs were purified further by one or two recrystallizations and their melting points checked against literature values. Chlorpromazine was used at concentrations less than its critical micelle concentration (5); and methyl viologen is freely soluble up to a concentration greater than 0.1 M.

Heat denaturation experiments were made at 77°C on 0.05% myoglobin in 0.1 M sodium acetate buffer, pH 5.98. The ionic strength of reaction mixtures containing the aromatic salts was maintained at 0.1 *M* by downward adjustment of the acetate buffer concentration. The reaction mixture was prepared by mixing 1 ml of 1% myoglobin in water at room temperature with 19 ml of the desired medium at 85°C in such a fashion that the temperature dropped rapidly to 77°C and was maintained by a thermoregulated oil bath. During the course of denaturation, insolubilized protein settled out of solution. At programmed times, aliquots of the supernatant were diluted five-fold with 5 M NaCl at 0°C. After incubation for 1 hr at 0°C, the dilution mixtures were filtered through medium grade sintered glass and their Soret bands recorded with a Beckman DK-2 spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.) using the appropriate light path. The maximum wavelength (408 mμ), half-width, and shape of the band were the same within a small experimental error as those of the parallel control. Furthermore, the ratio of the absorbance at 408 mμ to that at 208 mμ (measured

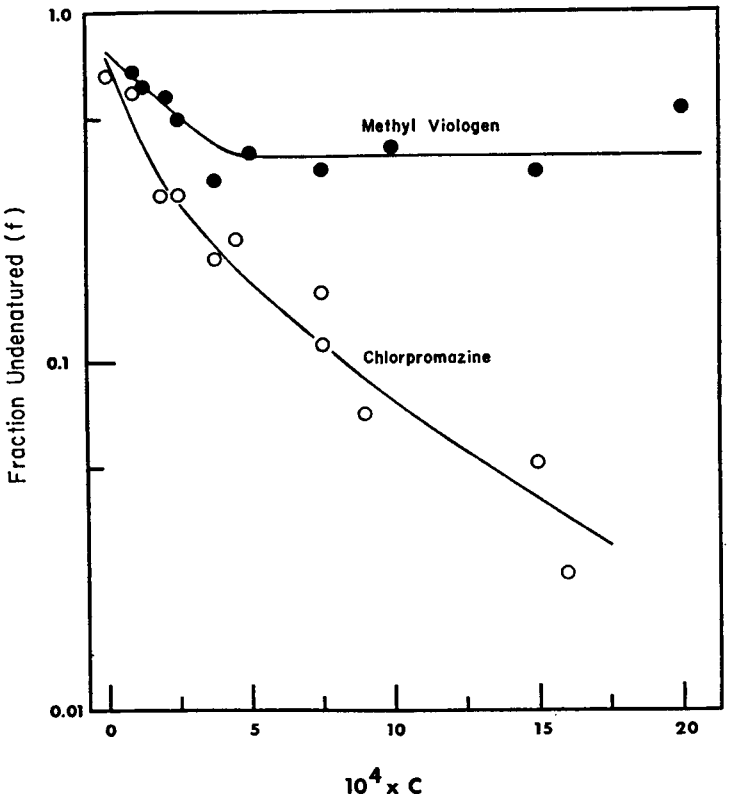

FIGURE 1 Comparison of the effect of chlorpromazine and methyl viologen upon the rate of heat denaturation of myoglobin.

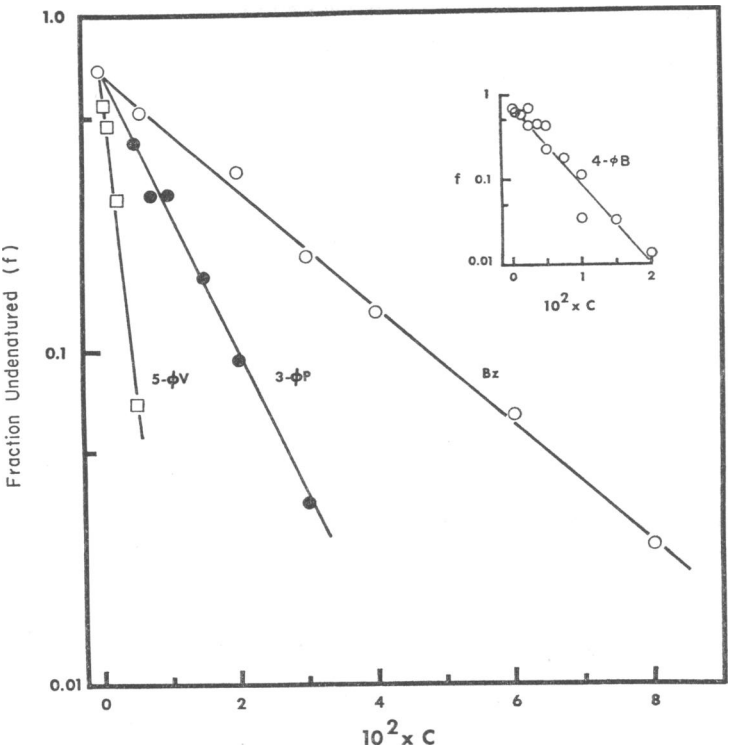

FIGURE 2 Comparison of the effect of benzoate and its homologs upon the rate of heat denaturation of myoglobin: Bz, benzoate; 3-φP, 3-phenylpropionate; 4-φB, 4-phenylbutyrate; 5-φV, 5-phenylvalerate. See text for phenylacetate.

only in the absence of aromatics) was the same as the control value. In light of these observations, the ratio of absorbance at 408 mμ to that of the control was taken as the instantaneous fraction of undenatured myoglobin.

Preliminary experiments in the absence of aromatic compound revealed a complex time-course of denaturation in which the logarithmic rate decreased progressively with time during early stages of reaction after which it remained essentially constant. Although denaturation fails to follow the simple rate law expected of a reaction which is of the first order in the protein concentration, the reaction is evidently not of an order higher than the first with respect to protein since the fraction undenatured (about 0.7) after 5 hr of heating was independent of protein concentration over the range, 0.013–0.1%. Accordingly, the standard condition of 5 hr at 77°C was adopted for comparative measurements on the effect of aromatic compounds upon the rate of heat denaturation. The results are displayed in Figs. 1 and 2 as semilogarithmic plots of the fraction undenatured, f, against the molar concentration, C, of aromatic compound in the reaction mixture.

This manner of displaying the results derives from a kinetic description which makes the following *assumptions*. (*a*) Native myoglobin in solution is heterogeneous with respect to heat denaturation and from this point of view can be considered as a mixture of components, N_1, $N_2 \cdots N_j$, which denature independently at different rates. Some justification for this assumption is found in the chromatographic heterogeneity of myoglobin (6). (*b*) In the

absence of aromatic compound, the rate-limiting step for the denaturation of a given component is the first-order reaction, $N_j \xrightarrow{k_{oj}} D_j$, in which case a semilogarithmic plot of f vs. time will be nonlinear; but f will be independent of the total protein concentration. (*c*) Binding of aromatic compound, A, to a single kind of site on the macromolecule causes enhancement of its rate of denaturation according to the kinetic scheme

$$N_j \xrightarrow{k_{oj}} D_j$$

$$N_j + A \overset{K_{1j}}{\rightleftharpoons} N_j A \xrightarrow{k_j} D_j A$$

$$N_j A + A \overset{K_{2j}}{\rightleftharpoons} N_j A_2 \xrightarrow{k_j} D_j A_2$$

$$\vdots$$

$$N_j A_{i-1} + A \overset{K_{ij}}{\rightleftharpoons} N_j A_i \xrightarrow{k_j} D_j A_i ,$$

in which the first-order rate constant $k_j > k_{oj}$; and the binding equilibria are established instantaneously with association constants, K_{ij} . For sufficiently large excess of A, f will vary with time, t, as

$$f = \sum^{j} \alpha_j \exp(-\beta_j t) \tag{1}$$

$$\beta_j = \frac{k_{oj} + k_j \sum^{i} C^i \prod^{i} K_{ij}}{1 + \sum^{i} C^i \prod^{i} K_{ij}}, \tag{2}$$

where α_j is the initial proportion of N_j . It follows that

$$\left(\frac{\partial \ln f}{\partial C}\right)_t = -\left\langle \frac{(k_j - k_{oj}) \sum^{i} iC^{i-1} \prod^{i} K_{ij}}{[1 + \sum^{i} C^i \prod^{i} K_{ij}]^2} \right\rangle t \tag{3}$$

where the factor $\langle\rangle$ is the value of $d\beta_j/dC$ averaged over all j with respect to $\alpha_j \exp(-\beta_j t)$. Equation 4 tells us that a semilogarithmic plot of f vs. C at constant t can be curvilinear with progressively decreasing slope. Moreover, for appropriate values of the several constants, the curve can show a rather rapid initial drop followed by a leveling off to a very small slope as $\beta_j \to k_j$, i.e., as the concentration of uncomplexed protein approaches zero. Within experimental error this behavior might give the appearance of plateauing. Both types of curves can be seen in Fig. 1; but such data alone cannot distinguish between our model and one in which the binding sites differ in that the several complexes denature with different k's. On the other hand, if all $K_{ij}C \ll 1$, equation 4 reduces to

$$\left(\frac{\partial \ln f}{\partial C}\right)_t = -\langle K_{1j}(k_j - k_{oj})\rangle t, \tag{4}$$

and if all $K_{ij}k_j$ have the same value, the average quantity $\langle\rangle$ is independent of C. In that case the semilogarithmic plot is linear as in Fig. 2; and such linearity is distinctive for binding to

a single kind of site. Since the factor $\Diamond$ is a function of t, the slope of the plot is disproportionate with t.

RESULTS

It is apparent from Figs. 1 and 2 that the rate of heat denaturation is strongly enhanced by the addition of aromatic compounds to the reaction mixture. In order to delineate the forces responsible for rate enhancement, two groups of compounds were examined. The first pair was chosen to explore the role of charge-transfer in the small molecule-macromolecule interaction. As shown in Fig. 1, the rate of denaturation is enhanced to a considerably greater extent by the electron donor, chlorpromazine, than the electron acceptor, methyl viologen, which exhibits saturation at quite low concentration.

The second group of compounds examined was an homologous series judged to have about the same electron-donating tendency but progressively increasing hydrophobicity. The series consists of benzoate and its homologs in which the straight carbon chain separating the phenyl ring from the carboxylate group becomes progressively longer on going from phenylacetate to 5-phenylvalerate. As illustrated in Fig. 2, the rate of heat denaturation is first-order with respect to these compounds.[1] The results for phenylacetate are not included in the figure because of its aberrant behavior at high concentrations. Whereas the data points define a straight line lying between those for benzoate and 3-phenylpropionate at concentrations below 0.02 M, at higher concentrations they depart markedly therefrom and describe a curve which bends ever downwards. This is suggestive of a chemical reaction whose product(s) interacts strongly with the protein. Indeed, after heating to 90°C, buffered solutions of 0.04 M phenylacetate containing no protein gave a positive and permanent color test for keto compounds with 2,4-dinitrophenylhydrazine (test mixture made alkaline with NaOH); and it is conceivable that myoglobin may catalyze the reaction. Clearly, rate data obtained at high concentrations are suspect; but we believe that those obtained at concentrations below 0.02 M are a true expression of the inherent rate-enhancement of phenylacetate.

Three conclusions can be drawn from these observations. (*a*) Benzoate and its homologs apparently exert their action by binding to a single kind of site in the hemeprotein; (*b*) Their ordering with respect to effectiveness in enhancing the rate of heat denaturation is benzoate < phenylacetate < 3-phenylpropionate < 4-phenylbutyrate < 5-phenylvalerate; and (*c*) Their effectiveness approximately

[1] In order to eliminate the possibility of a fortuitous first-order relationship somehow associated with the complex time-course of denaturation, experiments were also made on the effect of benzoate upon the fraction of protein remaining undenatured after 25 hr at 77°C. The fraction undenatured in the absence of benzoate was 0.45 as compared with 0.69 for the standard 5 hr of heating. The logarithm of the fraction undenatured decreased linearly with increasing benzoate concentration with a slope about twice that for the standard 5 hr. The disproportionality of the slope with time is consistent with the prediction of equation 4.

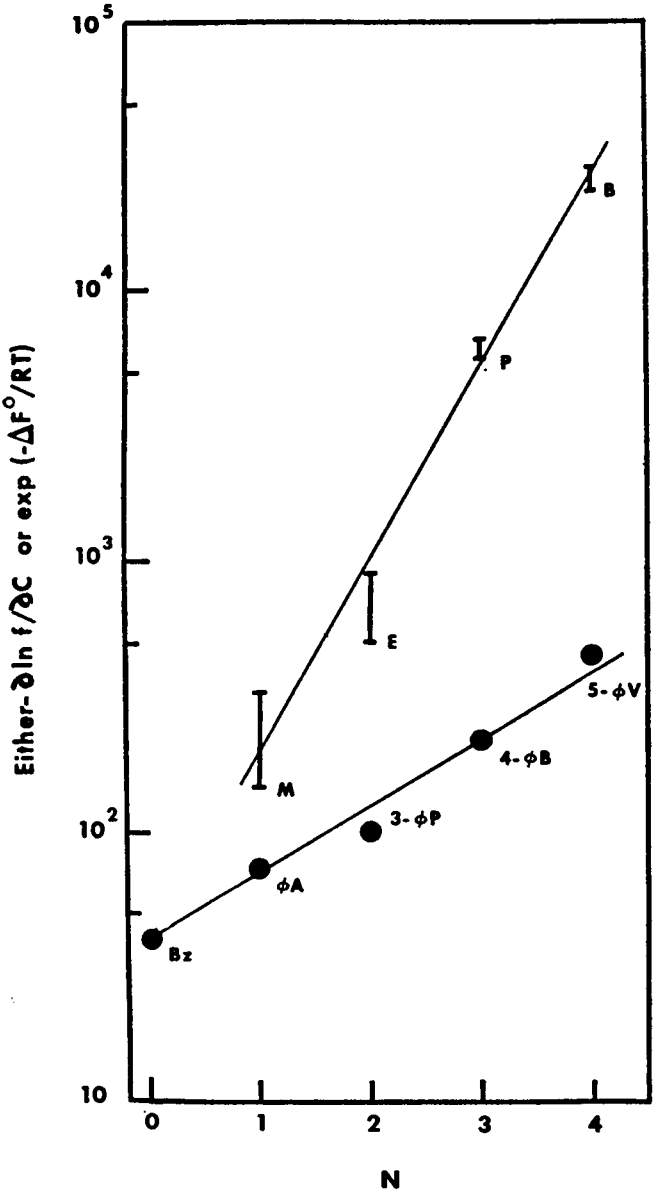

FIGURE 3 Semilogarithmic plots of $-\partial \ln f/\partial c$ liter mole^{-1}, for benzoate and its homologs (lower line) and the quantity, exp ($-\Delta F°/RT$), for aliphatic hydrocarbons against the number of carbon atoms, N, in the straight chain: M, methane; E, ethane; P, propane; B, butane; ϕA, phenylacetate; other symbols as in Fig. 2. The value of $-\partial \ln f/\partial c$ for ϕ A was derived from observations made at concentrations below about 0.02 M (see text). Values of $\Delta F°$ from Nemethy and Scheraga (7) were corrected to 77°C using values of $\Delta S°$ from same source.

doubles upon each addition of a methylene group to the carbon chain. The latter is very revealing, for we recall that a measure of the hydrophobicity of the lower, straight chain aliphatic hydrocarbons is given by exp $(-\Delta F^\circ/RT)$, where ΔF° is the standard free energy of transfer of a mole of hydrocarbon from aqueous solution to the liquid phase (or nonpolar solution); and that $-\Delta F^\circ$ increases linearly with chain length. Comparison of the functional dependencies of $-\partial \ln f/\partial C$ and exp $(-\Delta F^\circ/RT)$ upon the number of carbon atoms in the straight chain is made in Fig. 3. It is immediately apparent that there is a close relationship between the logarithmic rate of change of f with C for a given homolog and the hydrophobicity of its aliphatic carbon chain. These results provide strong support for increasing hydrophobicity as the major force responsible for the ordering of benzoate and its homologs with respect to their rate enhancement.

DISCUSSION

The effect of aromatic compounds on the rate of heat denaturation of myoglobin is virtually the same as described previously (1–4) for the reaction of Zn^{++} with myoglobin and the urea denaturation of the protein. The only important difference concerns the order of reaction with respect to added aromatic. For example, while urea denaturation is second order in the homologs of benzoate, heat denaturation is first order. An extensive body of evidence indicates that the complex formed at the lower temperature is fundamentally of the electron donor-acceptor type with an important contribution of hydrophobic interaction to its stability; and the same forces are implicated in the heat denaturation system. Thus, the strong electron donor, chlorpromazine, enhances the rate of heat denaturation much more so than the acceptor molecule, methyl viologen. Chlorpromazine is also a more effective rate-enhancer than the benzoate homologs which are, in turn, weaker donors. As for stabilization of the complex by hydrophobic interactions, the results on benzoate and its homologs speak for themselves. These findings generalize the effect of aromatics to include three different reactions of myoglobin each of which disrupts the native macromolecular conformation undoubtedly by different mechanisms. They must share, however, some common feature which is sensitive to the binding of aromatic compounds.

It has been demonstrated spectroscopically that chlorpromazine complexes with hemin and does so with the heme moiety of myoglobin (3). Hemin also forms molecular complexes with β-naphthoate and 3-indolebutyrate (4). Since hematoporphyrin likewise complexes with chlorpromazine and β-naphthoate as well as with iodobenzene and nitrobenzene (3, 4), it seems clear that in the case of hemin, complex formation is with the protoporphyrin ring system and/or its vinyl side chains rather than with the iron. Moreover, there is a nice correspondence between the relative strength of complexing of aromatic compounds with hemin and hematoporphyrin and their synergism with urea. Hematoporphyrin has been shown to be a

good electron acceptor (3); and the new visible absorption bands of the chlorpromazine-hemin and chlorpromazine-myoglobin complexes may be charge-transfer bands. Although these various measurements were made at 28°C and the standard enthalpy of formation of charge-transfer complexes is negative, extrapolation to 77°C seems justified since the value of the association constant for a typical π-complex would decrease only by a factor of about two over this temperature range (8). Accordingly, our previously proposed mechanism of action of aromatic compounds is retained for heat denaturation. We visualize aromatic donor molecules as forming charge-transfer complexes directly with the heme moiety of myoglobin, thereby relieving its π-bonding interactions with the two aromatic rings of phenylalanine residues, CD1 and 15H (9). This in turn labilizes the macromolecular conformation to the disrupting action of chemical and physical agents. Since the heme moiety resides in a nonpolar pocket of the protein (9), it is understandable that hydrophobic interactions make such a large contribution to the stability of the complex.

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