Contribution of Aromatic Residue Interactions to the Stability of Myoglobin*

John R. Cann

ABSTRACT: Benzene and other aromatics have a strong and specific enhancing effect upon the rate of reaction of Zn with myoglobin. Enhancement of the rate evidently involves formation of charge-transfer complexes between two aromatic molecules and two sites in the hemeprotein.

It is proposed that complex formation relieves π-bonding interactions between the two aromatic rings of the phenylalanine residues, CD1 and H14, and the heme. These results indicate that aromatic side chain interactions play a significant role in maintaining the structural integrity of myoglobin.

Previous experiments on the reversible reaction of Zn ions with sperm whale myoglobin (Cann, 1963, 1964a,b) have focused attention upon the structural complex involving the heme and adjacent portions of the protein moiety as the critical site of attack by Zn. Kendrew's 2-A model of myoglobin (Kendrew *et al.*, 1961; Kendrew, 1961, 1962; Stryer *et al.*, 1964) reveals that the iron of the heme is bonded to the imidazole group of the F8 histidyl residue. Except for its two propionic acid groups which are at the surface of the molecule and probably involved in salt linkages, the heme rests snugly in a pocket where it is surrounded almost entirely by nonpolar residues. There are at least 90 van der Waals contacts between the heme and neighboring atoms, and two aromatic rings (phenylalanine residues, CD1 and H14) are arranged parallel, or nearly so, to its pyrrole rings or vinyl groups. Kendrew points out that π-bonding interactions must be significant here.

Reaction of Zn with myoglobin causes major changes in the physical and chemical properties of the protein, the most characteristic spectral charge being a marked reduction in Soret-band intensity. Our previous studies have led to the conclusion that the *rate-controlling* step in suppression of the Soret band involves macromolecular conformational changes concomitant with rupture of the otherwise inaccessible Fe^{3+}–F8 imidazole linkage and occupancy of the imidazole group by Zn. Such a mechanism would almost certainly necessitate rupture of π-bonds between the aromatic ring side chains and the heme. In that event, the addition of aromatic hydrocarbons such as benzene or naphthalene to the reaction mixture should increase the rate of reaction. Such compounds might be expected to relieve the intramolecular π-bonding interactions, thereby decreasing the activation energy. Indeed, preliminary experiments (Figure 1) showed that saturation of the reaction mixture with benzene increases by about two orders of magnitude, the rate of suppression of Soret intensity. It has been found that other aromatic compounds are also effective. The detailed experiments reported below support the idea that they act by relieving the π-bonding interactions, evidently by forming charge-transfer complexes with two sites in the macromolecule. These results also indicate that aromatic side chain interactions play a significant role in maintaining the structural integrity of myoglobin.

Experimental Section

Solutions of Mann's sperm whale ferrimyoglobin were dialyzed against distilled water in the cold and lyophilized. The benzene, toluene, *m*-xylene, and isooctane were spectroquality; fluorobenzene was Matheson Coleman and Bell material, bp 84–85°; other chemicals were reagent grade. Rate measurements were made at 408 mμ in a thermostated Beckman DK-2 spectrophotometer fitted with the time drive accessory for recording absorbance *vs.* time. Except where noted measurements were made at 26 ± 0.01°. In a typical experiment a KCl solution of myoglobin (0.1% protein) and a $ZnAc_2$–HAc–KCl solution[1] were saturated with benzene by shaking with the organic phase in a thermostated bath. Saturation of the Zn solution with benzene did not change its pH. The reaction mixture (about 0.01% protein) was prepared by mixing the two saturated solutions in a stoppered cuvet containing benzene vapor. The pipets also contained benzene vapor. The reaction proceeded without significant change in pH, and the protein did not precipitate. Reliability of the experimental procedure was established by determining the relationship of absorbance of benzene at 237 mμ to its

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[1] Abbreviation used in this work: Ac, acetate.

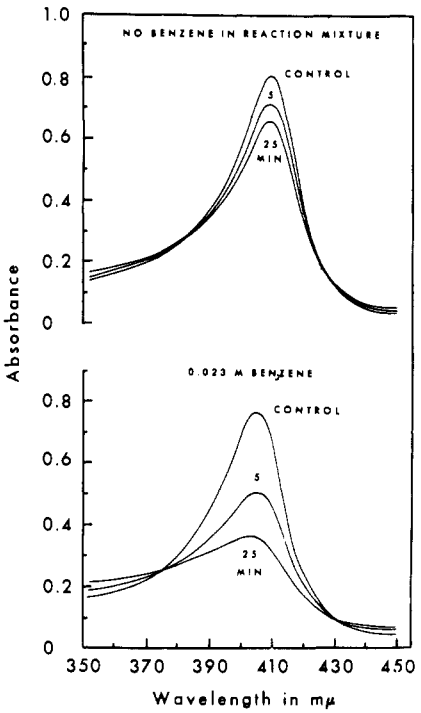

FIGURE 1: Enhancement of the rate of Zn-mediated suppression of the Soret band of ferrimyoglobin by addition of benzene to the reaction mixture. Solvent, 0.01 M KCl-9 × 10^{-3} M $ZnAc_2$, pH 6.4. Controls same as reaction mixtures except for substitution of NaAc for $ZnAc_2$.

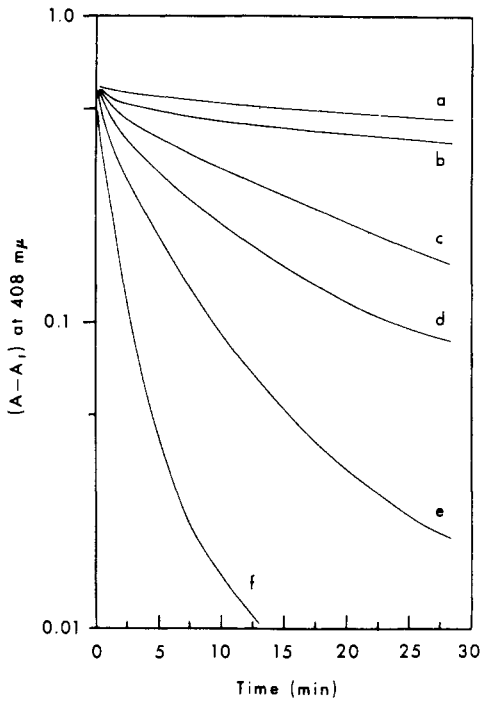

FIGURE 2: Effect of several different aromatic compounds on the time course of the Zn-mediated suppression of Soret absorption of myoglobin. Semilogarithmic plot of the difference between the instantaneous value of absorbance at 408 mμ and its value upon completion of reaction, $A - A_f$, *vs.* time in minutes: a and b, controls without aromatic compounds; c, 2.59 × 10^{-4} M naphthalene; d, 0.054 M benzyl alcohol; e, 0.018 M sodium benzoate; f, 0.023 M benzene. Solvents: a, 0.01 M KCl-9 × 10^{-3} M $ZnAc_2$–0.054 M ethanol, pH 6.33; b, 0.04 M KCl-9 × 10^{-3} M $ZnAc_2$, pH 6.30 (addition of 0.018 M sodium acetate or 0.2 M ethanol to this solvent increased the rate of reaction by at most 30%); c, same as b with 0.2 M ethanol; d, same as a except for the ethanol; e and f, same as b.

concentration in 0.04 M KCl. (For measurements at concentrations less than half-saturation, the usual precaution of flushing the cuvet with air containing benzene vapor was neither necessary nor desirable.) The solubility of benzene in water and its activity coefficient in KCl solution were taken from Alexander (1959) and McDevit and Long (1952), respectively. The absorbance obeyed Beers' law over the entire concentration range up to saturation, and the derived molar extinction coefficient agreed to within 4% with the literature value.

Previously (Cann, 1964a), semilogarithmic plots of absorbance *vs.* time were linear during early stages of reaction, so that initial logarithmic rate constants were obtainable. The myoglobin used in this study showed a somewhat different behavior, an initially rapid decrease in absorbance preceding the linear portion of the plot. This difference may be related to the heterogeneity of myoglobin in solution (Edmundson and Hirs, 1962). It is possible that the preparation used in this study contained a small amount of unusually reactive material. Accordingly, the slope of the linear portion was taken to be the initial rate for the bulk of the protein. Confidence in this procedure is afforded by the fact that it gives an activation energy in the absence of benzene which is in excellent agreement with the previously reported value.

Results

Representative measurements on the effect of aromatic compounds on the time course of the Zn-mediated suppression of Soret absorption of myoglobin are presented in Figure 2. It is strikingly apparent from these experiments that low concentrations of aromatic compounds can cause enormous enhancement of the rate of suppression. Thus, 0.023 M benzene increased the rate about 50-fold. Even 0.054 M benzyl alcohol, the least effective of the compounds studied, increased the rate by an order of magnitude. Nor are these merely irreversible denaturation effects. Myoglobin solutions, protein concentration 0.1%, can be saturated with benzene by shaking with the organic phase without denaturing the protein. (In contrast, the same concentration of Zn-treated myoglobin is partially precipitated from solution when shaken with benzene. However, no

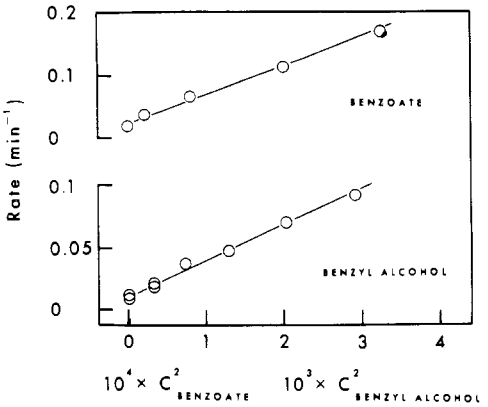

FIGURE 3: Order of the Zn-mediated suppression of Soret absorption with respect to rate-enhancing aromatic compounds: C symbolizes molar concentration of aromatic compound; solvents as in Figure 2.

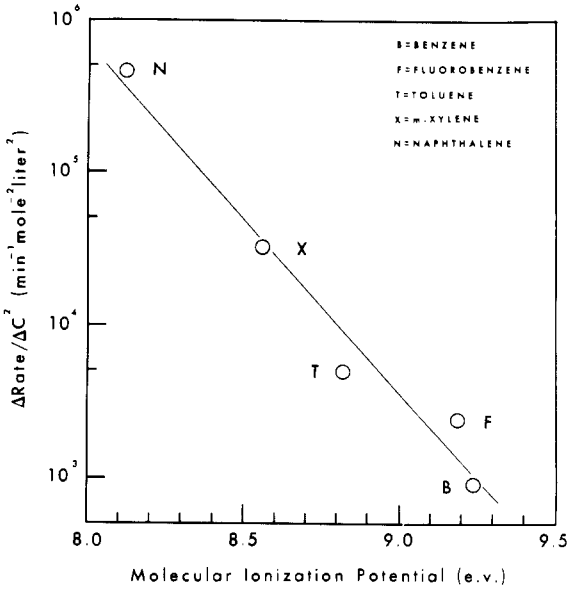

FIGURE 4: Correlation of effectiveness of aromatic compounds in enhancing the rate of Zn-mediated suppression of Soret absorption with molecular ionization potential, the latter values taken from Watanabe (1957) and Watanabe *et al.* (1962). Solvent, 0.04 M KCl–9 × 10^{-3} M $ZnAc_2$, pH 6.3. More rigorously, the logarithm of $\Delta rate/\Delta C^2$ is a linear function of $(I - B)^{-1}$, where I is the molecular ionization potential and B is a constant. However, for $B < 6$ ev, it can be approximated over the range of I values of interest with a function linear in I.

precipitate formed during the benzene-enhanced reaction of 0.01% myoglobin with Zn.) The resulting saturated solutions show only a slightly depressed Soret absorption (compare controls in Figure 1) which does not change on aging. When the benzene was evaporated from such a solution prior to addition of Zn with no great precaution to remove all of the benzene, the rate of suppression of the Soret band was only slightly greater than that of the control never exposed to benzene. Finally, as described previously (Cann, 1964a) for the Zn–myoglobin reaction in the absence of benzene or other aromatic, the benzene-enhanced reaction can also be reversed by sequestering the metal ion with ethylenediaminetetraacetate.

In contrast to aromatics compounds, isobutane at 1 atm partial pressure had no significant effect on the rate of reaction, and acetate anion and ethanol at concentrations comparable to their aromatic analogs had only slight effect. This indicates specificity for the aromatic ring. Furthermore, experiments on the dependence of rate upon concentration of aromatic compounds indicate that they exert their action on a very limited portion of the protein molecule. Measurements on benzene indicated a second- or at most third-order reaction, but uncertainties in the lower concentrations due to evaporative losses of material prevented a more definitive conclusion. Accordingly, measurements were also made on the two nonvolatile aromatics, benzoate anion and benzyl alcohol (Figure 3). The reaction is clearly second order with respect to these compounds, and we assume that the same is also true for benzene.

All the aromatic compounds which have been tested enhance the rate of the Zn–myoglobin reaction. Their effectiveness in doing so increases in the order *benzyl alcohol* < *benzoate* < *benzene* < *fluorobenzene* < *toluene* < *m-xylene* < *naphthalene*, which from benzene to naphthalene is also the order of decreasing molecular ionization potential of these compounds. Benzyl alcohol has a surprisingly low ranking, possibly due to hydrogen bonding of the alcohol in aqueous solution rather than to an intrinsically low effectiveness. Otherwise, the ordering is that to be expected if these molecules form charge-transfer complexes with groupings in myoglobin. Accordingly, the kinetic law was derived for a process in which the activated complex of the *rate-controlling* step is composed of a myoglobin molecule, a Zn ion, and 2 molecules of an aromatic compound, the latter being bound at separate sites by charge-transfer complexing. [It was shown previously (Cann, 1964a) that the *rate-controlling* step is first order in both myoglobin and Zn.] This treatment predicts that the logarithm of the change in rate of reaction with respect to the square of the concentration of aromatic, $\Delta rate/\Delta C^2$, should increase linearly with decreasing molecular ionization potential of the aromatic compounds. As shown in Figure 4, this is approximately true for our system and, together with the observed second-order reaction with respect to aromatics, constitutes strong presumptive evidence for charge-transfer complexing of the aromatic compounds at two sites in the myoglobin molecule. It cannot be inferred, however, that the small molecule is necessarily the electron donor since alternate hydrocarbons possess approximately constant molecular electronegativity, which is the sum of ionization potential and electron affinity. This

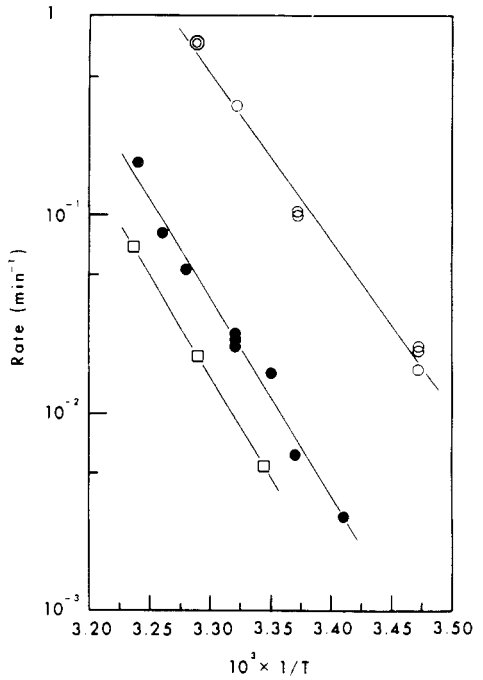

FIGURE 5: Arrhenius plot of logarithm of rate of reaction *vs.* reciprocal of the absolute temperature. □, 0.01 M KCl–9 × 10^{-3} M $ZnAc_2$, pH 6.33, E = 48 kcal $mole^{-1}$; ●, 0.04 M KCl–9 × 10^{-3} M $ZnAc_2$, pH 6.40, E = 46 ± 1.9 kcal $mole^{-1}$, from Cann (1964a); ○, 0.01 M KCl–9 × 10^{-3} M $ZnAc_2$–saturated with benzene at each temperature, pH 6.36, rates corrected for the small increase in solubility of benzene with increasing temperature, E = 39 ± 1.7 kcal. $mole^{-1}$. A second set of measurements on solutions saturated with benzene, using a slightly modified procedure, gave E = 38 ± 2.2 kcal $mole^{-1}$.

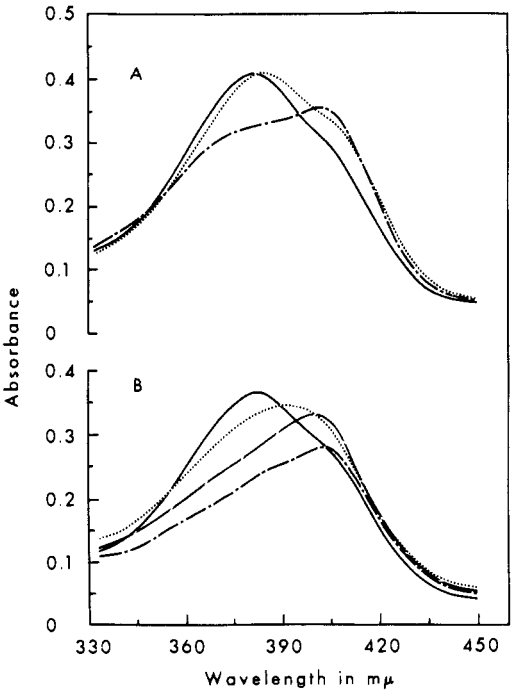

FIGURE 6: Spectrum of hemin. A, 6.6 × 10^{-6} M hemin in following solvents: ———, 100% acetone; ---------, 50% acetone–50% benzene or toluene; –·–·–·–, 50% acetone–50% fluorobenzene. B, 5.9 × 10^{-6} M: ———, 100%; ---------, 90% acetone–10% benzyl alcohol; – – – –, 70% acetone–30% benzyl alcohol; — — —, 50% acetone–50% benzyl alcohol. The spectrum in 50% acetone–50% isooctane is essentially the same as in 100% acetone except for a slightly lower maximum absorbance.

means that, although the ionization potential of naphthalene, for example, is less than that of benzene, its electron affinity is greater (Becker and Wentworth, 1963). As to the structure of the complexes, one would suppose that they are probably of the π,π-type (Brieglieb, 1961) involving ring systems in the macromolecule.

As might be expected, the benzene-enhanced rate of reaction has a somewhat lower temperature dependence than the rate in the absence of benzene (Figure 5). The difference in activation energy is −7 ± 2.6 kcal $mole^{-1}$. Other things remaining constant, the difference in entropy of activation is −16 ± 8.7 eu. It is satisfying to note that these values agree rather well with those for the enthalpy and entropy of transfer of 2 moles of benzene from aqueous solution to the solid state (−6 kcal $mole^{-1}$ and −22 eu, respectively).

Finally, it seemed pertinent to inquire whether benzene and other aromatic compounds interact with hemin in solution. The experiments presented in Figure 6 indicate that this is the case. The differences between the Soret band in acetone–aromatic mixtures and pure acetone can hardly be attributed entirely to nonspecific solvent effects.

Discussion

Quantitative description of the various forces and their cooperative action in determining and maintaining the conformation of macromolecules which, in turn, determines their biological activity is one of the most provocative of biophysical problems. Myoglobin and hemoglobin are ideal proteins for such studies since their three-dimensional structures have been largely established by crystallographic X-ray analyses, thereby providing the fundamental information required for a detailed description of the mechanisms of their reactions. Such experimental translation of geometrical structural details into physicochemical terms is one method for delineating conformation-determining forces. The experiments described above provide partial assessment of one such force.

These experiments show that aromatic compounds have a strong and specific enhancing effect upon the rate of reaction of Zn with myoglobin. Enhancement of the rate evidently involves formation of charge-transfer complexes between two aromatic molecules and two sites in the hemeprotein. We propose that complex formation disrupts π-bonding interactions be-

tween the two aromatic rings of the phenylalanine residues, CD1 and H14, and the heme. One can imagine several possible mechanisms whereby these interactions could be relieved. For example, the two aromatic molecules might interact directly with the heme at positions distal to the side chain rings. It is even conceivable that the side chain rings are displaced from the heme by insertion of the aromatic molecules between the rings and the heme. Alternatively, the aromatic molecules might interact not with the heme but rather with the side chain rings themselves. Of course, the possibility that they might exert their action by complexing with residues elsewhere in the protein molecule cannot be ignored, but likely loci are not obvious. In any event, our experiments indicate that aromatic residue interactions play a significant role in maintaining the structural integrity of myoglobin, evidently contributing at least 2 kcal $mole^{-1}$ to the free energy of activation, about 20 kcal $mole^{-1}$, of the Zn–myoglobin reaction. They also bring into sharper focus the cooperative action of the heme moiety and protein part of myoglobin in determining macromolecular structure.

Acknowledgment

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