Vol. 82, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1978 Pages 634-640

THE INTERACTION OF BENZENE WITH HUMAN HEMOGLOBIN AS STUDIED BY ^{1}H FOURIER TRANSFORM NMR SPECTROSCOPY*

R.F. Novak, M. Dershwitz† and F.C. Novak
Department of Pharmacology, Northwestern University
Medical and Dental Schools, Chicago, Illinois 60611

Received March 24,1978

SUMMARY

The longitudinal relaxation rate, T_1^{-1}, of benzene in aqueous medium increased from 0.065 sec^{-1} in the total absence of hemoprotein to 2.38 sec^{-1} at 128 μM methemoglobin. Formation of cyanomethemoglobin decreased the relaxation rate of benzene from 2.38 sec^{-1} to 1.6 sec^{-1}, while fluoromethemoglobin enhanced the relaxation rate to 5.4 sec^{-1}. Variable temperature studies confirmed that exchange between free benzene and methemoglobin-complexed benzene occurred in the rapid exchange region of the temperature profile. Virtually no change in the relaxation rate of the methyl protons of a non-interacting internal reference, tetramethylammonium phosphate, was observed over the methemoglobin concentration range employed.

^{1}H nuclear magnetic resonance longitudinal relaxation rate measurements, T_1^{-1}, represent a sensitive method for the investigation of molecular interactions involving paramagnetic centers. In particular, NMR longitudinal relaxation rate measurements can be used to observe differential relaxation rate changes associated with the separate sites of a molecule interacting with or binding in proximity to a paramagnetic center. Such data should allow evaluation of the nature of the interaction or binding (e.g. direct coordination), accessibility to the binding site, distances of approach to the paramagnetic center, molecular orientation, and potentially, the deduction of the structural details of the binding cavity in both a structural and dynamic sense. We previously reported ^{1}H relaxation rate studies on the interaction of 2,6 dimethylaniline with the hemoproteins myoglobin, hemoglobin and partially purified mammalian cytochrome P450 (1,2). More recently we reported the initial results

*Supported in part by grant C77-14 from the Chicago and Illinois Heart Association to RFN and National Institutes of Health Training Grant GM-07263 to the Department of Pharmacology.

†National Institutes of Health Predoctoral Fellow.

0006-291X/78/0822-0634$01.00/0

of a comparative study on the interaction of aniline and imidazole with human methemoglobin using ^1H relaxation rate changes (2,3). It was suggested from results obtained in the above studies (1-3) that aniline and 2,6 dimethylaniline, in contrast to the ligand imidazole, may be capable of continued interaction with human hemoglobin in the vicinity of the heme even in the presence of directly-coordinating ligands such as cyanide or fluoride (1,2). These results suggest that attractive forces other than those directly associated with the positively charged iron atom may be involved in the interaction or binding of these molecules to methemoglobin and that a basic site on the molecule and/or direct coordination with the heme-iron center may not be absolute requirements for "ligand-methemoglobin" interactions. Studies have therefore been initiated on the interaction of pyridine (4) and the aromatic non-nitrogenous analog, benzene, with human hemoglobin in an effort to provide further information on "ligand-methemoglobin" interactions. We present here data implicating benzene in specific complex formation with human hemoglobin and demonstrate that this interaction occurs in proximity to the paramagnetic heme-iron atom.

METHODS

Human hemoglobin (Type IV, twice recrystallized, Sigma) was prepared as previously described (1). The concentration of hemoglobin in solution was determined according to the method of Van Kampen and Zijlstra (5). ^1H NMR spectra were obtained using a Varian CFT-20 Fourier Transform nuclear magnetic resonance spectrometer operating at 80 MHz and internally locked on the deuterium signal of the ^2H_2O solvent. T_1 relaxation time measurements were made using the standard inversion-recovery sequence, 180-τ-90, where τ represents the delay time in seconds.

Benzene (Fisher) was dissolved in 100% ^2H_2O (Aldrich) to give a stock solution of 37 mM. NMR samples were prepared using 300 µl of the benzene stock solution, methemoglobin, KPi buffer, pH_{obs} = 7.5 and ^2H_2O (100%) to give the required concentration of methemoglobin in 0.02 M KPi in a total volume of 400 µl. A teflon vortex plug was inserted into the 5 mm NMR tube to reduce benzene evaporation from the solution at magnet temperature ($\sim$ 29°C).

RESULTS

Figure 1 shows a typical inversion-recovery sequence obtained for benzene, δ = 7.43 ppm from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in ^2H_2O

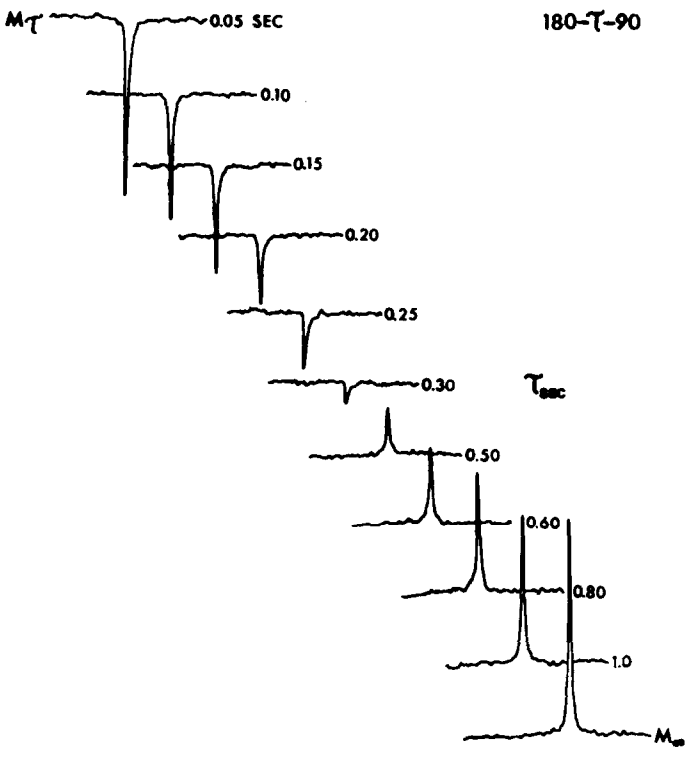

Figure 1

Inversion-recovery sequence, 180-τ-90, obtained for 28 mM benzene in aqueous medium which was 128 μM in methemoglobin, 0.02 M in phosphate buffer at pH 7.5, (T = 29°C). The delay time τ (sec) is given to the right of each partially relaxed Fourier Transform NMR spectrum. Here M_τ refers to the signal amplitude, positive or negative, obtained using the respective delay time τ and M_∞ represents the maximal signal amplitude obtained from a fully relaxed system (i.e. τ equals five times the T_1 value). The plot of $\ln(M_\infty - M_\tau)$ versus the delay time, τ, yields a straight line with the slope equal to T_1^{-1}.

Each spectrum in the above figure required 5 acquisitions and contained 8192 data points over a sweep width of 650 Hz. The 90° pulse width was 20 μsec and the residual HDO solvent peak was partially suppressed using ^1H homonuclear decoupling.

(100%), 0.02 M in KPi buffer at pH_{obs} = 7.5 in the presence of 128 μM methemoglobin. The series of partially relaxed Fourier Transform spectra allows calculation of the longitudinal relaxation rate, T_1^{-1}, of the benzene protons. A plot of the natural log of $(M_\infty - M_\tau)$ versus the delay time τ (sec) (see Figure 1 for definition of terms) yields a straight line with the slope equal to the relaxation rate T_1^{-1}.

Since hemoglobin is a protein of approximately 60,000 molecular weight,

it becomes necessary to assess those contributions to the relaxation rate changes which directly reflect interactions which occur at or in proximity to the paramagnetic center versus those which may result from interaction with the diamagnetic apoprotein. Methemoglobin exists predominately in the high-spin form ($\sim$ 85%) under ambient conditions (29°C, 0.02 M KPi, pH 7.5) and may be converted to either 100% high-spin form (S = 5/2) in the presence of fluoride ion or to the 100% low spin (S = 1/2) form by the presence of cyanide ion (6). Thus the addition of fluoride, which causes conversion of methemoglobin to 100% high-spin form, would be predicted to increase the relaxation rate, while the addition of cyanide would be expected to decrease the relaxation rate through either the displacement of benzene or conversion of the hemoprotein to the low-spin form. These changes were observed as shown in Figure 3. Fluoride, in addition to converting hemoglobin to the 100% high-spin form also increases the electron spin relaxation time from 1×10^{-10} to 9.1×10^{-10} (7,8) thereby effectively enhancing the paramagnetic effect. Thus, the relaxation rate changes of benzene in the presence of various concentrations of fluoromethemoglobin are substantially greater than simply predicted on the basis of an additional 15% conversion of the hemoprotein to the high-spin form. While the addition of cyanide decreases the relaxation rate appreciably, it does not reduce it to a value comparable to T_1^{-1} for the blank (0.0654 sec^{-1}). It thus appears that either a continued interaction between benzene and cyanomethemoglobin occurs or that benzene-apoprotein interactions contribute to the observed relaxation rate change (see Discussion). No change in the T_1^{-1} of the internal standard $(CH_3)_4N^+$ occurred over the hemoglobin concentration studied, in agreement with previously obtained results (1,2). Variable temperature studies confirmed that the exchange between the free and the methemoglobin-complexed benzene occurred in the fast exchange region of the NMR time scale as shown in Figure 3 (9,10). Hence, all data are consistent with the interpretation that specific complex formation between benzene and methemoglobin occurs. In addition, there may be a sizeable contribution to the observed relaxation rate change from benzene-apoprotein interactions.

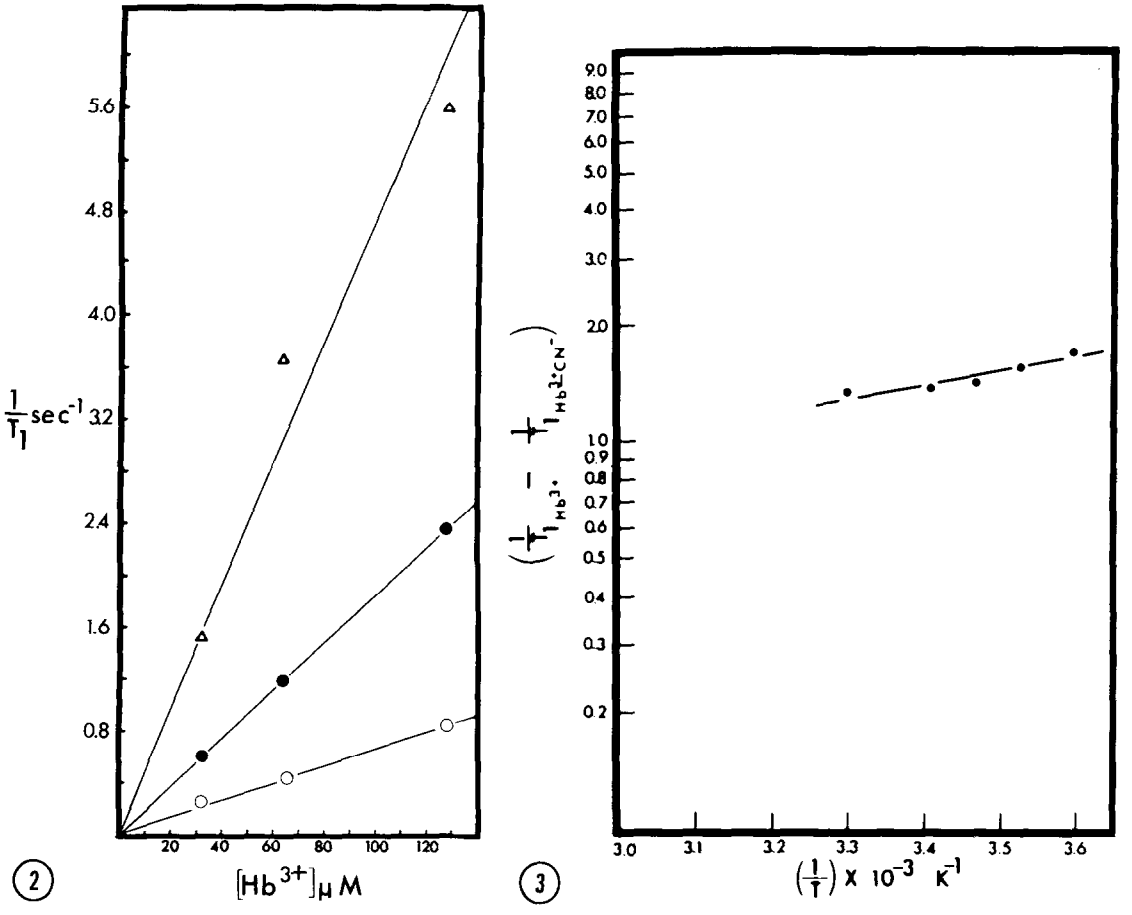

Figure 2

The change in relaxation rate T_1^{-1} of benzene in the presence of methemoglobin (●-●-●), cyanomethemoglobin (o-o-o) and fluoromethemoglobin (Δ-Δ-Δ). Each data point represents a minimum of three measurements and the standard error was less than 5% for each data point.

Cyanomethemoglobin was formed by the addition of 10 μl of 1.0 M KCN in ^2H_2O pH_{obs} = 7.5, to the methemoglobin-benzene solution to yield a solution 25 mM in KCN. Fluoromethemoglobin was formed by the addition of 50 μl of 1.0 M NaF in ^2H_2O, pH_{obs} = 7.5, to the methemoglobin-benzene solution to give a final fluoride concentration of 125 mM. The concentrations of KCN and NaF employed in the investigation caused complete conversion of methemoglobin to the respective cyano- or fluoro-derivative as determined from the absorbance wavelength of the Soret band of hemoglobin in the UV-Vis spectrum.

Figure 3

Changes in the relaxation rate, $T_1^{-1}{}_{Hb^{3+}} - T_1^{-1}{}_{Hb^{3+}\text{-}CN^-}$ over the temperature range 5-30°C. Here the difference $T_1^{-1}{}_{Hb^{3+}} - T_1^{-1}{}_{Hb^{3+}\text{-}CN^-}$ was used to approximate the paramagnetic contribution to the observed relaxation rate over the temperature range indicated. The results suggest that exchange occurs in the rapid exchange region of the temperature profile (9,10). Each temperature was measured with a special NMR thermometer inserted directly into the probe.

DISCUSSION

The investigation of substrate-hemoprotein interactions using T_1^{-1} relaxation rate changes offers a distinct advantage in that relaxation rates are extremely sensitive to intermolecular interactions and in particular to those involving active sites which contain paramagnetic metal ions. Since relaxation rate changes associated with molecular interactions at paramagnetic centers may be used to calculate distances between metal ion centers and various portions of a molecule (11,12) they provide a very powerful method for studying substrate-active site interactions in both a qualitative and quantitative fashion. Thus the observation of differential relaxation rate changes for various distinct sites of a molecule provides not only a qualitative appraisal of molecular orientation but with additional information such as the dissociation constant, K_D, for the complex and the correlation time, τ_c, offers an opportunity for quantative deduction of distances of separation (11,12).

We have reported here results which implicate benzene in specific complexation with human methemoglobin and have shown that changes in the spin state (or ligation) of the heme-iron atom directly affect the relaxation rate of benzene in the presence of methemoglobin.[1] We have further shown that two distinct contributions to the enhanced relaxation rate exist, a paramagnetic contribution which arises from the paramagnetic heme-iron atom and a potentially sizeable diamagnetic contribution from the apoprotein (e.g. $\sim$ 0.82 sec $^{-1}$at 128 μM methemoglobin) using the cyanomethemoglobin results in order to obtain approximations to the diamagnetic contributions.

Whether the potential diamagnetic protein contribution to the relaxation

[1]The addition of aliquots of 37 mM benzene in aqueous medium to a solution of 1 μM methemoglobin in 0.02 M phosphate buffer, pH 7.5 elicited a UV-Vis difference spectrum characteristic of a decrease in extinction of the Soret band at 406 nm. The change in optical density, ΔOD, of the difference spectrum appears to be saturable with increasing concentration of benzene. Observation of the UV-Vis absolute spectrum of 1 μM methemoglobin before and after addition of the benzene solution confirmed that a decrease in the intensity of the Soret band (406 nm) occurred; small changes in the absorbance of the bands at $\sim$ 500 nm, $\sim$ 540 nm and $\sim$ 630 nm were also observed.

rate reflects the continued interaction of benzene with cyanomethemoglobin, or represents a specific interaction with one or more protein moieties in a specific region of the hemoglobin molecule or a non-specific generalized interaction involving a large number of binding sites on the apoprotein remains to be elucidated. These results, when compared to previous studies, do suggest however, that a basic site on a molecule (such as nitrogen) may not be an absolute requirement for binding. The use of such NMR relaxation time measurements in studying ligand hemoprotein interactions as presented here may provide basic information on the role of substrate binding as well as molecular mechanisms active in hemoprotein catalyzed substrate oxidation such as those mediated by cytochrome P450.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Paul F. Hollenberg and Dr. Eugene M. Silinsky for critical review of this manuscript. The authors are indebted to Dr. Hollenberg for use of the Aminco-Chance DW-2 double beam spectrophotometer.

REFERENCES

1. Novak, R.F., Kapetanovic, I.M., and Mieyal, J.J. (1977) Mol. Pharm. 13, 15-30.
2. Novak, R.F., Kapetanovic, I.M. and Mieyal, J.J. (1977) "NMR Relaxation Studies of Substrate-P450 Interactions" in: Microsomes and Drug Oxidations, eds. Estabrook, R.W., Hildebrandt, A.G. and Ullrich, V., Pergamon Press, Oxford, 232-239.
3. Novak, R.F. and Mieyal, J.J. (1976) The Pharmacologist 18, 242.
4. Novak, R.F. and Clarkson, C.W. (1978) Federation Proceedings 37, 1123.
5. Van Kampen, E.J. and Zijlstra, W.G. (1961) Clin. Chim. Acta 6, 538-544.
6. Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, North Holland Publishing Co., Amsterdam.
7. Gupta, R.K. and Mildvan, A.S. (1975) J. Biol. Chem. 250, 246-253.
8. Gupta, R.K. (1976) J. Biol. Chem. 251, 6815-6822.
9. Luz, Z. and Meiboom, S. (1964) J. Chem. Phys. 40, 2686-2692.
10. Swift, T.J. (1974) "The Paramagnetic Linewidth" in: NMR of Paramagnetic Molecules, eds. LaMar, G.N., Holm, R. and Horrocks, W.D., Academic Press, New York, 53-83.
11. Solomon, I. (1955) Phys. Rev. 99, 559-565.
12. Bloembergen, N. (1957) J. Chem. Phys. 27, 572-573.