5534 *Biochemistry* **1984**, *23*, 5534–5539

Mansuy, D., Thillet, J., Cendrier, B., Lallemand, J. Y., & Chottard, J. C. (1978) *Biochem. Biophys. Res. Commun. 83*, 217–225.
McIntosh, P. R., Kawato, S., Freedman, R. B., & Cherry, R. J. (1980) *FEBS Lett. 122*, 54–58.
Mieyal, J. J., Ackerman, R. S., Blumer, J. L., & Freeman, L. S. (1976) *J. Biol. Chem. 251*, 3436–3441.
Nakamura, T., Sugita, Y., & Bannai, S. (1973) *J. Biol. Chem. 248*, 4119–4122.
Ogawa, S., & Shulman, R. G. (1971) *Biochem. Biophys. Res. Commun. 42*, 9–15.
Olson, J. S., & Binger, C. (1976) *Biochim. Biophys. Acta 434*, 428–439.
Onady, G. M., Ferraiolo, B. L., & Mieyal, J. J. (1984) *Biochemistry* (following paper in this issue).
Perutz, M. F. (1970) *Nature (London) 228*, 726–734.
Riggs, A. (1981) *Methods Enzymol. 76*, 5–29.
Rossi-Fanelli, A., & Antonini, E. (1958) *Biochim. Biophys. Acta 30*, 608–615.
Salhany, J. M., Castillo, C. L., & Ogawa, S. (1976) *Biochemistry 15*, 5344–5349.
Starke, D. W., Blisard, K. S., & Mieyal, J. J. (1984) *Mol. Pharmacol. 25*, 467–475.
Tyuma, I., Benesch, R. E., & Benesch, R. (1966) *Biochemistry 5*, 2957–2962.
Van Kampen, E. J., & Zijlstra, W. G. (1961) *Clin. Chim. Acta 6*, 538–544.
Weber, G. (1971) *Int. Congr. Ser.—Excerpta Med., 1970 No. 219*, 58–65.
White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.
Winterhalter, K. H., & Deranleau, D. A. (1967) *Biochemistry 6*, 3136–3143.

Monooxygenase Activity of Human Hemoglobin: NMR Demonstration of Different Modes of Substrate Binding Corresponding to Different Activities of Hemoglobin Derivatives[†]

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ABSTRACT: In the accompanying paper [Ferraiolo, B. L., Onady, G. M., & Mieyal, J. J. (1984) *Biochemistry* (preceding paper in this issue)] we reported different aniline hydroxylase activities for ferrihemoglobin, its isolated subunits, and the converse pair of valency hybrids $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ and $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ in a reconstituted system containing reduced nicotinamide adenine dinucleotide phosphate (NADPH) and cytochrome P-450 reductase. To investigate the molecular basis for the different activities, ^{1}H NMR T_1 relaxation studies of aniline were performed in the absence and presence of each of the hemoglobin (Hb) species. The paramagnetic contribution of the ferric heme iron atoms of each Hb derivative to the enhanced relaxation of the proton nuclei of aniline was determined relative to control experiments in which the hemoproteins had been converted fully to the corresponding (carbonmonoxy)ferrous forms, which are diamagnetic. According to the known distance dependence of the paramagnetic effect and the relative changes in T_1 for the upfield and downfield signals in the spectrum of aniline, it was ascertained that aniline binds in the same manner to the β-ferric hybrid and to ferrihemoglobin. These two forms displayed equivalent hydroxylase activities that were the highest among the Hb derivatives for the same aniline concentration. The T_1 changes observed with the α-ferric hybrid suggest a different orientation for aniline in that complex. The T_1 data for the isolated subunits α^{3+} and $\beta^{3+}{}_4$ would indicate that overall binding of aniline includes a component of direct aniline–heme ligation in each case. For $\beta^{3+}{}_4$ this result is consistent with the marked substrate inhibition observed for the hydroxylase activity. These studies demonstrate that the specific mode of substrate association near the heme may determine the efficiency of monooxygenase catalysis.

Hemoglobin (Hb)[1] coupled to cytochrome P-450 reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) acts enzymically to hydroxylate aniline (Mieyal et al., 1976) and to catalyze other monooxygenase-like reactions such as the O-demethylation of *p*-nitroanisole and the N-demethylation of benzphetamine (Starke et al., 1984). Experiments with fetal Hb (HbF) (Blisard & Mieyal, 1980), which contains γ subunits instead of β subunits as in the adult form, demonstrated aniline hydroxylase activity 5-fold greater than that of adult Hb (HbA), suggesting a predominant contribution to the activity of the respective tetramers by the non α subunits. The prediction of differential activity of the α and β subunits within the $\alpha_2\beta_2$ tetramer of HbA was supported by two recent studies from our laboratory (Ferraiolo & Mieyal, 1982; Ferraiolo et al., 1984). In the first report, initial replacement of O_2 by the inhibitor CO on rabbit HbO_2 in hemolysates occurred exclusively on the β subunit with concomitant loss of aniline hydroxylase activity before CO

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[1] Abbreviations: Hb, hemoglobin; NMR, nuclear magnetic resonance; pAP, *p*-aminophenol; TSP, sodium 3-(trimethylsilyl)propionate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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ligation was detected on the α subunits. These results were corroborated with isolated human hemoglobin in the P-450-like reconstituted system by studying the aniline hydroxylase activity of a converse pair of hemoglobin hybrids in which either the α or the β subunits were prevented from expressing activity by CO ligation, i.e., $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ and $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ (Ferraiolo et al., 1984). At subsaturating aniline concentrations, the "β-active" hybrid was the much more efficient catalyst with V_{max}/K_M about 8 times greater than that of the "α-active" hybrid. Furthermore, the β-active hybrid was 40-fold more efficient than the separate β subunits ($\beta^{3+}{}_4$), suggesting that catalytic efficiency was conferred upon the β subunit by virtue of the local molecular environment associated with the quaternary structure of the heterologous tetramer $\alpha_2\beta_2$.

On a macromolecular scale, the structural differences between β_4 and $\alpha_2\beta_2$ that might account for the different expressed activities of the β chains must be subtle, because X-ray diffraction studies at low resolution show $(\beta^{2+}\text{-CO})_4$ and $(\alpha^{2+}\text{-CO})_2(\beta^{2+}\text{-CO})_2$ to be quite similar (Arnone & Briley, 1978). On the other hand, NMR studies reflecting the heme environments of deoxy-β_4 and deoxy-$\alpha_2\beta_2$ readily distinguish the two species (Perutz et al., 1974).

The kinetic data reported in the preceding paper (Ferraiolo et al., 1984) suggested that the nature of substrate binding was the decisive factor in determining the different aniline hydroxylase activities of the hemoglobin derivatives.

NMR spectroscopy is ideally suited to investigate such interactions of substrates with the hemoproteins that contain paramagnetic iron atoms, because marked changes may be observed in the NMR properties of those substrate moieties that most closely approach the paramagnetic site in the complex. In particular, NMR longitudinal relaxation time (T_1) measurements can be used to observe differential relaxation effects on different parts of a substrate molecule and thereby allow evaluation of the substrate orientation and distance from the paramagnetic center. Such data should allow evaluation of the type of substrate binding. For example, substrates may interact with the paramagnetic heme metal ion by direct coordination, by indirect or "outer sphere" coordination, or by association with a binding cavity in the protein that is located proximal to the heme moiety. We and others have used NMR techniques analogous to those described in this paper to study substrate and ligand interactions with myoglobin, hemoglobin, and cytochrome P-450 (Novak et al., 1977a,b; Hershberg & Chance, 1975; Novak & Vatsis, 1982).

In the present study, the T_1 technique was found to be particularly suitable for distinguishing the mode of interaction of substrate aniline with the individual subunits of the hemoglobin tetramer by examining the converse pair of valency hybrids $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ and $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$. Only one of the types of subunit in each has a paramagnetic ferric center, while the other exists as the (carbonmonoxy)ferrous diamagnetic center, which contributes negligibly to T_1 enhancement; i.e., only the subunit containing the ferric heme reports the substrate within its paramagnetic environment.

The T_1 data for normal Hb^{3+} and for the valency hybrids suggest that aniline effectively interacts exclusively with the β subunits of Hb^{3+} and that on the hybrids the aniline molecule assumes a different orientation near the β-hemes than near the α-hemes. These results are consistent with the greater catalytic productivity of the β sites (Ferraiolo et al., 1984).

Experimental Procedures

Materials. Reagent chemicals and preparation and characterization of the hemoglobin derivatives are described in detail in the preceding paper (Ferraiolo et al., 1984).

NMR T_1 Relaxation Studies. The longitudinal relaxation times (T_1) of the proton moieties of aniline, as well as that for tetramethylammonium chloride (TMA) added as a noninteracting control for nonspecific effects, were determined as follows: The Nicolet 1180 computer interfaced to the Bruker 270-MHz NMR instrument was used to carry out a series of 180°–τ–90° sequences (where τ refers to the interval between pulses) for each sample and to print out the spectra automatically as illustrated in Figure 1 (see Results). The 90° and 180° pulses were 7 and 14 μs, respectively. From these data, plots of ln $(M_\infty - M_\tau)$ vs. τ were prepared and T_1 values calculated from the slopes. M_∞ and M_τ refer to the maximal signal height and intermediate signal heights, respectively (observed after the 90° pulse), corresponding to particular moieties on the aniline molecule. These signal heights were measured manually in order to avoid artifacts due to base line or noise aberrations. These values of T_1 obtained in the absence of hemoprotein are termed $T_1{}^0$. The specific derivatives of hemoglobin at varying concentrations were then combined with aniline in the presence or absence of sufficient potassium cyanide to occupy the sixth ligand position of all the ferric heme sites in the solution. The apparent completeness of conversion and stability of the hemoglobin complexes were monitored separately by visible spectroscopy of aliquots of the same samples. The T_1 values for specific signals of aniline were then remeasured in the presence of the paramagnetic ferric heme species. The changes in relaxation rate ($T_1{}^{-1}$) were determined for each aniline moiety by subtracting the reciprocal of $T_1{}^0$ from the reciprocal of the T_1 observed in the presence of the paramagnetic species. The paramagnetic effect of the hemoprotein was determined from the difference between the changes, if any, in T_1 elicited by addition of diamagnetic heme species (Fe^{2+}-CO) and the changes elicited by paramagnetic heme species ($Fe^{3+}\text{-OH}_2$ or Fe^{3+}-CN). Each of the ferrihemoglobin derivatives (e.g., isolated subunits or valency hybrids) was converted to the corresponding diamagnetic form [completely (carbonmonoxy)ferrous] by treatment with sodium dithionite in the presence of CO.

Results

The ^1H NMR longitudinal relaxation times of the aniline signals were measured by using the standard 180°–τ–90° inversion–recovery sequence (Figure 1). Aniline assignments are made for the meta hydrogens at 7.25 ppm and the unresolved ortho–para hydrogens at 6.88 ppm relative to the methyl singlet of the sodium 3-(trimethylsilyl)propionic acid internal reference set at 0.0 ppm (Sadtler, 1967). The spectra in Figure 1 include the methyl signal (3.16 ppm) of the internal control $(CH_3)_4N^+$, which previously has been shown not to interact with ferrihemoglobin (Novak et al., 1977a). The upper and lower spectra in Figure 1 demonstrate the concentration-dependent effects of Hb^{3+} on the inversion intervals for the aniline signals. The approximate interpulse interval (τ) for inversion of the aniline signals was decreased from 5–10 s at 7 μM Hb^{3+} to 1–2.5 s at 70 μM Hb^{3+}, whereas the time of signal inversion for the noninteracting control $(CH_3)_4N^+$ remained essentially unchanged at nearly 5 s at both hemoglobin concentrations as expected.

Individual relaxation rates ($1/T_1$) were obtained from linear regression slopes of plots of ln $(M_\infty - M_\tau)$ vs. τ, where M_∞ corresponds to the maximum signal height measured at $\tau > 5T_1$, and M_τ refers to the signal heights measured at the individual τ values as illustrated in Figure 1. Diamagnetic contributions to the observed hemoprotein-induced changes in T_1 of the aniline signals were evaluated with the corresponding fully diamagnetic hemoprotein species as described

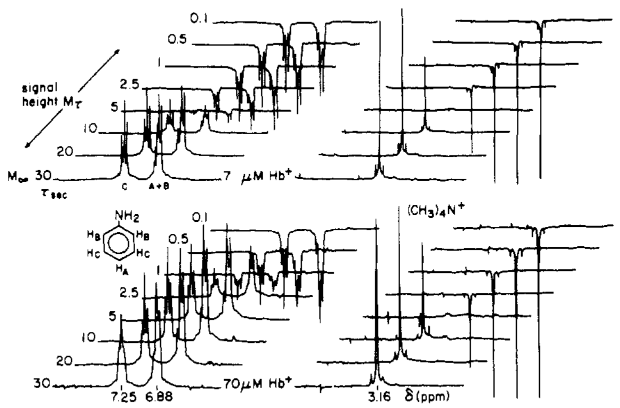

FIGURE 1: Inversion-recovery (180°–τ–90° pulse sequence) FT NMR spectra of aniline in the presence of ferrihemoglobin. Both sets of spectra shown were of samples that contained 50 mM aniline in 20 mM potassium phosphate buffer, pH 6.8. Tetramethylammonium chloride and sodium 3-(trimethylsilyl)propionate were 5–8 mM each. An appropriate aliquot of ferrihemoglobin (Hb^{3+}) was added to each sample in order to achieve the concentrations indicated. Spectra were accumulated at ambient temperature (~19 °C). The τ values were preset in a microprogram in the Nicolet computer, and the sequential spectra were automatically processed. T_1 data were obtained from these plots as described under Experimental Procedures.

under Experimental Procedures. The diamagnetic contribution (if any) was subtracted from the observed change in relaxation rate to give the paramagnetic change in relaxation rate, $1/T_{1p}$. Plots of $1/T_{1p}$ vs. hemoprotein concentration provide for the calculation of $\Delta(1/T_{1p})$ per unit of hemoprotein (tetramer). These values then serve as the basis for comparison of the relative effects of the various Hb derivatives on the aniline signals, reflecting the nature of the respective hemoprotein–substrate complexes (see Table I).

An illustration of the effects of increasing concentrations of paramagnetic centers on the relaxation rates of aniline is shown in Figure 2 for hemoglobin and the converse pair of valency hybrids. Analogous experiments were performed with the isolated subunits. Since aniline is also a potential ligand for the heme iron atom, it was necessary to distinguish whether such binding was a contributor to the observed T_1 changes. The iron site can be blocked, however, by the addition of a high-affinity ligand like cyanide. Therefore, a series of T_1 experiments were performed with the various hemoproteins in the presence of cyanide at a concentration that essentially saturated the heme sites. Association of cyanide converts the Fe^{3+} from a predominantly high-spin species to an essentially pure low-spin paramagnetic species ($S = {}^1/_2$), thereby diminishing the paramagnetism. This diminution should be predictable by the calculation of the ratio of $\Delta(1/T_{1p(-CN)})/\Delta(1/T_{1p(+CN)})$ (see Discussion). The effect of cyanide diminution of the $\Delta(1/T_{1p})$ values for aniline is illustrated for Hb^{3+} in Figure 3. The results of the complete set of experiments with aniline and the Hb derivatives are listed in Table I, expressed in terms of equivalent amounts of tetrameric hemoprotein, i.e., calculated from the slopes of $\Delta(1/T_{1p})$ vs. hemoprotein concentration (see Figure 2). The NMR results in Table I demonstrate that there is an aniline binding site(s) sufficiently close to the paramagnetic centers (ferric heme) in each of the hemoglobin derivatives so that changes in aniline relaxation rates are observed. When cyanide was added, there were diminished yet discernible changes in $\Delta(1/T_{1p})$. The magnitude of the $\Delta(1/T_{1p})$ values and the extent of the cyanide diminution were different for the different hemoglobin derivatives (Table I). The close correspondence of the data for ferrihemoglobin (line 3) and the β-ferric hybrid (line 4) is especially noteworthy in light of their nearly identical hy-

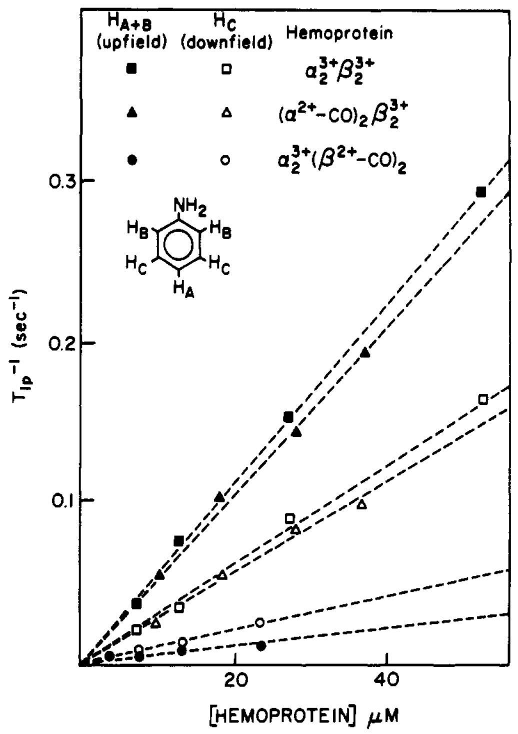

FIGURE 2: Dependence of the paramagnetic-induced acceleration of the relaxation rates (T_{1p}^{-1}) of the aniline proton signals on the concentration of various hemoglobin derivatives. All samples contained 50 mM aniline in 20 mM potassium phosphate, pH 6.8, and aliquots of the appropriate hemoprotein were added sequentially to achieve the concentrations indicated; T_1 experiments were performed after each addition. $T_{1p}^{-1} = T_{1,obsd}^{-1} - T_{1,dia}^{-1}$, where $T_{1,obsd}^{-1}$ represents the observed change in relaxation rate at each concentration of hemoprotein relative to that of aniline alone, and $T_{1,dia}^{-1}$ represents the difference (if any) between the relaxation rates of the aniline protons in the absence and presence of the various hemoproteins in their fully diamagnetic states (see Experimental Procedures). The closed and open circles are defined as shown.

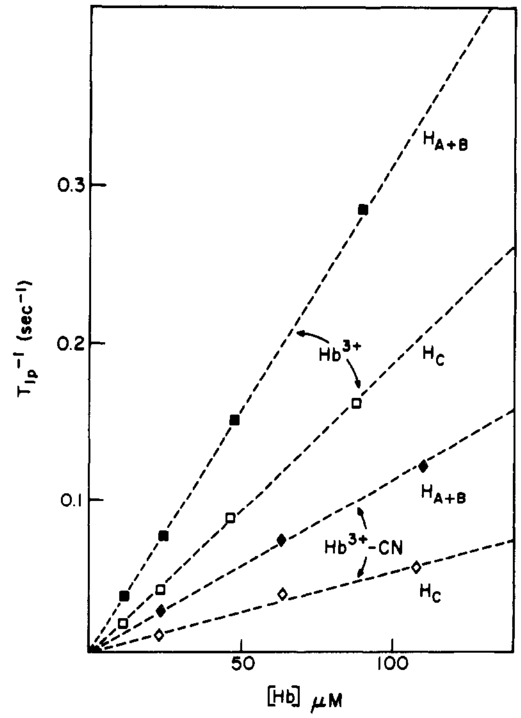

FIGURE 3: Dependence of the paramagnetic-induced acceleration of the relaxation rate (T_{1p}^{-1}) of the aniline proton signals on the concentration of Hb^{3+} in the absence and presence of CN^-. Conditions were as described under Figure 2, except that, for the experiment represented by the lower lines, sufficient NaCN was added to convert the Hb^{3+} to Hb^{3+}-CN as confirmed by visible spectroscopy.

Table I: Effect of Cyanide on $\Delta(1/T_{1p})$ Values for Aniline and Hb Derivatives[a]

hemoprotein	$\Delta(1/T_{1p})$ ($mM^{-1}\ s^{-1}$) upfield[b] −CN	upfield[b] +CN	downfield[c] −CN	downfield[c] +CN
α^{3+}	10.0[d]	<0.4[d]	8.1[d]	0.4[d]
$\beta^{3+}{}_4$	12.4	1.7	12.4	1.4
$\alpha^{3+}{}_2\beta^{3+}{}_2$	5.7	1.3	3.3	0.8
$(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$	5.3	1.2	2.9	0.7
$\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$	0.6		1.4	

[a] In all cases aniline was examined at 50 mM, and the hemoprotein concentration was varied up to at least 100 μM with respect to heme concentration, as illustrated in Figure 2. All solutions were in 20 mM potassium phosphate, pH 6.8, at ambient temperature (19–20 °C). ^{1}H NMR T_1 experiments were conducted, and the T_1 values were calculated from the slopes of the ln $(M_\infty - M_\tau)$ vs. τ plots by linear regression analysis as described under Experimental Procedures. The appropriate fully ferrous–CO complex served as the diamagnetic control in each case. [b] Ortho, para protons. [c] Meta protons. [d] In order to compare the data for monomeric α^{3+} to those for all the other tetrameric hemoproteins, the observed values of $\Delta(1/T_{1p})$ per millimolar hemoprotein in this case were multiplied by 4.

droxylase activities (Ferraiolo et al., 1984) (see Discussion).

Discussion

In order to utilize the relaxation rate data to compare the relative nature of the complexes of the substrate aniline with the various derivatives of hemoglobin, it is necessary to consider what factors contribute to the values of $1/T_{1p}$ that were obtained. The effect of electron spin–nuclear spin interaction on the longitudinal relaxation time (T_1) has been expressed according to the multiterm Solomon–Bloembergen equation (Solomon, 1953; Bloembergen, 1957), which can be simplified to the following relationship by appropriate assumptions and approximations (Novak et al., 1977a):

$$1/T_{1M} = [\text{const} \times S(S + 1)/r^6][f(\tau_c)] \qquad (1)$$

where $1/T_{1M}$ in the present case refers to the relaxation rate of individual proton nuclei of aniline, S is the paramagnetic electron spin state of the ferric heme iron atoms, r is the distance separating the paramagnetic center from the substrate nuclei, $f(\tau_c)$ represents a function of the correlation time that describes molecular motions that modulate the electron–nuclear dipolar coupling, and the constant contains values for Planck's constant and the nuclear and electron gyromagnetic ratios. For a given substrate moiety, the paramagnetic contribution $1/T_{1p}$ to the observed longitudinal relaxation rate $1/T_{1,\text{obsd}}$ is calculated by subtracting out the diamagnetic contribution (if any) $1/T_{1,\text{dia}}$:

$$1/T_{1p} = 1/T_{1,\text{obsd}} - 1/T_{1,\text{dia}} \qquad (2)$$

T_{1M} is directly related to T_{1p} according to the equation:

$$1/T_{1p} = \alpha/(T_{1M} + \tau_M) \qquad (3)$$

where α is the mole fraction of a given substrate complexed to the species containing the paramagnetic ion and τ_M is the mean residence time of the substrate interacting with the metal ion. When $\tau_M \ll T_{1M}$, "fast exchange" prevails and $T_{1M} = \alpha T_{1p}$ (Luz & Meiboom, 1964). This condition has been demonstrated previously for aniline interaction with hemoglobin at room temperature (Novak et al., 1977a). Equation 1 and the simplified eq 3 may be combined to define what factors contribute to $1/T_{1p}$:

$$1/T_{1p} = (\alpha/r^6)[S(S + 1)][f(\tau_c)] \qquad (4)$$

With one additional simplifying assumption, namely, that the correlation constants τ_c are essentially the same for the complexes of aniline with the various hemoglobin derivatives, it follows that the ratio of the $1/T_{1p}$ values for the interaction of aniline with any two of the hemoproteins would be

$$(1/T_{1p})_a/(1/T_{1p})_b = (\alpha r^{-6})_a/(\alpha r^{-6})_b \qquad (5)$$

Thus, greater $1/T_{1p}$ values may reflect a greater mole fraction of substrate in the complex, a shorter distance between substrate and heme, or both. This is the framework for a qualitative assessment of the aniline–hemoprotein complexes in light of their different hydroxylase activities (Ferraiolo et al., 1984).

First, let us consider the converse pair of valency hybrids and ferrihemoglobin (Table I, lines 3–5). The data for ferrihemoglobin and the β-ferric hybrid agree very closely, and this observation correlates with the nearly identical hydroxylase activities of these two species. It is noteworthy that the ratio $\Delta(1/T_{1p})_{\text{upfield}}/\Delta(1/T_{1p})_{\text{downfield}}$ is essentially the same, ca. 1.7, for the aniline–Hb^{3+} and aniline–β^{3+} hybrid interactions in the absence or presence of cyanide. Moreover, the diminutions of the $\Delta(1/T_{1p})$ values by CN$^-$ in these two cases (i.e., 4.1–4.4) are all close to the factor that would be predicted by the change in spin state alone. Mieyal & Freeman (1976) spectrophotometrically determined a spin-state change by following a shift in the Soret band, from 81% high spin for aquomethemoglobin to 16% high spin for anilinomethemoglobin at 0.3 M aniline. Under the conditions of the T_1 NMR experiments here at 50 mM aniline, spectral results indicate a 56% high-spin contribution. Such a spin-state equilibrium would predict a cyanide diminution of

$$\frac{1/T_1(-\text{CN})}{1/T_1(+\text{CN})} = \frac{S^{\text{hs}}(S + 1)^{\text{hs}} + S^{\text{ls}}(S + 1)^{\text{ls}}}{S^{\text{ls}}(S + 1)^{\text{ls}}} = \{[0.56(5/2)][0.56(5/2) + 1] + [0.44(1/2)][0.44(1/2) + 1]\}/[1/2(1/2 + 1)] = 4.8 \qquad (6)$$

where hs = high spin and ls = low spin. Moreover, if the added cyanide did not effect 100% conversion to low spin, this ratio would be smaller. For example, cyanide addition that resulted in 95% conversion to low spin would give a ratio of 4.3. Therefore, if aniline binds elsewhere than the sixth ligand position of the heme, a change in $\Delta(1/T_{1p})$ should be observed even in the presence of cyanide, and the magnitude of the diminution in $\Delta(1/T_{1p})$ by cyanide should approach the factor 4.8. A greater diminution (>4.8) would indicate competitive displacement of aniline from the sixth ligand position by cyanide. To confirm this analytical approach, an analogous set of T_1 experiments were performed with Hb^{3+} and imidazole in the absence and presence of cyanide, because imidazole has been demonstrated to directly ligate ferric heme in hemoglobin (Russel & Pauling, 1939; Bettlestone et al., 1968). As expected, cyanide displaced imidazole from the binding site and abolished the $\Delta(1/T_{1p})$ effect as observed previously (Novak et al., 1977a); i.e., $\Delta(1/T_{1p}) = 0$ for the ^{1}H NMR signals of imidazole in the presence of CN$^-$.

The overall T_1 results for Hb^{3+} and the β-ferric hybrid suggest that aniline is associated selectively with the β-globin protein moiety of both hemoproteins and its orientation relative to the ferric heme remains constant despite the replacement of H$_2$O by CN$^-$ and the change in spin state. Aniline is unlikely to be an outer-sphere ligand of the β-hemes because differences in the aniline interaction would be expected with the two different direct ligands H$_2$O and CN$^-$. Furthermore, the close correspondence between the $\Delta(1/T_{1p})$ data for $\alpha^{3+}{}_2\beta^{3+}{}_2$ and $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ indicates that neither direct ligation to the α-heme nor association of aniline with the α-globin near the heme occurs to a significant extent in ferri-

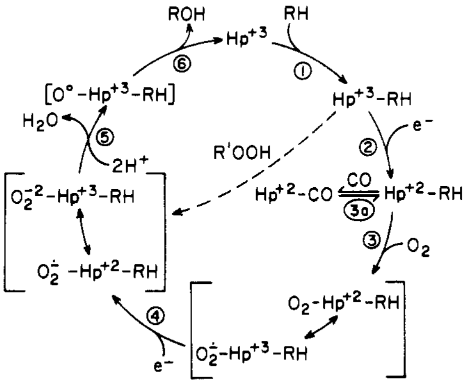

FIGURE 4: Schematic representation of the monooxygenase catalytic cycle. RH represents substrate and Hp stands for hemoprotein (P-450 or Hb). The formal charge indicated represents the formal charge on the heme iron atom; in effect, the actual valence state of the ferryl iron in the intermediate complex shown after step 5 may be more accurately represented as Fe^{5+}.

hemoglobin where the α^{3+} paramagnetic ferric heme would report an interaction if it existed. These results also indicate that any change in protein conformation upon changing the heme geometry does not significantly change the active site orientation of aniline at its protein binding site on the β-globin moieties. The consequence of this observation suggests that substrate arrangement remains the same for the high-spin complex, H_2O–Fe^{3+}–RH, at the initial stage of substrate binding (Figure 4, step 1) and for the low-spin complex, CN–Fe^{3+}–RH, which may serve as a model for the [O^0–Fe^{3+}–RH] complex that has been proposed as an intermediate preceding oxygen transfer to substrate in the scheme for the monooxygenase catalytic cycle (Figure 4, step 5).

As noted above, the signals for the ortho and para protons were not resolved, so we cannot assign a specific orientation of aniline relative to the heme. We propose, however, that the para position approaches most closely to the oxygen binding site on the basis of other information: (a) Para hydroxylation occurs regioselectively. (b) T_1 studies of the interaction of xylidine (*o*-dimethylaniline) with Hb showed the phenyl protons (meta and para) to be closer to the heme site than the methyl protons (Novak et al., 1977a). (c) Whereas *o*-methylaniline was para hydroxylated by hemoglobin about as readily as aniline, *m*-methylaniline showed extremely low activity, suggestive of steric hindrance (Starke et al., 1984). Further studies of other analogues, including [^{13}C]aniline, are necessary to define more accurately the substrate orientation.

The T_1 data for the α-ferric hybrid (Table I, line 5) are in contrast to the data for Hb^{3+} and the β-ferric hybrid in two ways. First, the magnitudes of the $\Delta(1/T_{1p})$ values are substantially smaller. This result is consistent with the higher K_M for aniline with this species (Ferraiolo et al., 1984) inasmuch as the K_M value appropriately reflects the affinity of aniline for this hemoprotein and thereby predicts a lower mole fraction of aniline in the complex. Interestingly, Olson & Gibson (1972) have reported more facile access of the bulky ligand butyl isocyanide to the β chains relative to the α chains. Second, the ratio $\Delta(1/T_{1p})_{\text{upfield}}/\Delta(1/T_{1p})_{\text{downfield}}$ (i.e., ortho–para proton effect/meta proton effect) is 0.4 in this case, suggesting a different juxtaposition of the aniline molecule relative to the α-ferric heme site compared to its arrangement near the β-heme site in Hb^{3+} and the β^{3+} hybrid. Hence, the lower hydroxylase activity displayed by the α-ferric hybrid may be explained by a combination of poor substrate affinity and a less favorable orientation of the molecule for the oxygen transfer reaction.

The NMR T_1 data for the isolated subunits of hemoglobin are clearly different from those for intact Hb and the hybrids. First, the $\Delta(1/T_{1p})$ values for $\beta^{3+}{}_4$ and α^{3+} in the absence of cyanide (Table I, lines 1 and 2) are much larger than those for Hb and the hybrids. Second, the magnitudes of the CN-induced diminutions in $\Delta(1/T_{1p})$ are larger than what would be predicted for the spin-state change alone; e.g., for $\beta^{3+}{}_4$ in the presence of CN^- the predicted $\Delta(1/T_{1p})$ values would be ca. 2.6, and for α^{3+} the $\Delta(1/T_{1p})$ values would be ca. 2.1 and ca. 1.7 for the upfield and downfield signals, respectively, according to the 4.8-fold diminution factor described above. These results suggest that for both $\beta^{3+}{}_4$ and α^{3+} there is a contribution of direct aniline–heme ligation to the observed T_1 changes. Such a mode of binding would be consistent with the substrate inhibition of the hydroxylase activity observed by Ferraiolo et al. (1984), where competition of aniline with O_2 for the heme binding site would be inhibitory. This inhibition was most pronounced for $\beta^{3+}{}_4$, where, under the conditions of the NMR experiment at 50 mM aniline, the hydroxylase activity was minimal. This result suggests that the heme sites on $\beta^{3+}{}_4$ approach full occupation by aniline and activity is lost irrespective of the degree of aniline binding at a productive substrate site. In the case of α^{3+} at an aniline concentration of 50 mM, there is still considerable hydroxylase activity (Ferraiolo et al., 1984). That result is surprising in light of the extent of the CN-induced diminution of the $\Delta(1/T_{1p})$ values, which would suggest that most of the aniline was ligated to the hemes or otherwise displaceable by CN^-. This is the only situation where the kinetic data for hydroxylase activity and the NMR T_1 data apparently are not compatible. It is conceivable that CN^- somehow causes displacement of aniline from substrate binding sites on the isolated α subunits as well as from the sixth ligand positions of the hemes. Additional experimental approaches are necessary, including the estimation of separate binding constants for aniline association with the hemes in the presence and absence of a variety of heme iron ligands. Fluoride anion may be effective for resolving the ambiguity with the CN^- effect since, in contrast to CN^-, F^- would enhance the paramagnetism by converting the α^{3+} hemes to pure high spin (Novak et al., 1977b).

The data reported in this paper along with those of the preceding paper (Ferraiolo et al., 1984) indicate that the differential activity of the hemoglobin derivatives appears to depend more on the mode of substrate binding than on intrinsic differences in O_2-activation capacity. With the heterologous tetramers, the T_1 NMR data suggest that the higher activity of the β subunits may reflect a more favorable interaction of aniline near the β-heme site of O_2 activation. In addition, the heterologous quaternary structure better protects both heme environments from direct aniline–heme ligation. With the isolated subunits, the limitation on activity may relate to lower substrate affinity and/or greater substrate inhibition. The different local heme environments apparently allow more or less facile direct ligation of aniline, blocking the O_2 binding site. Certain other substrates metabolized by hemoglobin (Starke et al., 1984) may display different patterns of relative activities with these various hemoglobins. For example, benzphetamine is a bulkier molecule than aniline and more hydrophobic, and anisole would be unlikely to coordinate directly to the hemes.

The current findings have significance as a model for the fine control of other oligomeric heme proteins also, especially the cytochrome P-450 system, which may exist in various states of aggregation within the endoplasmic reticulum.

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Registry No. Monooxygenase, 9038-14-6; aniline hydroxylase, 9012-80-0; aniline, 62-53-3.

References

Arnone, A., & Briley, P. D. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 93–107, Academic Press, New York.

Bettlestone, J. G., Epega, A. A., & Irvine, D. H. (1968) *J. Chem. Soc. A*, 1346–1351.

Blisard, K. S., & Mieyal, J. J. (1980) *Biochem. Biophys. Res. Commun. 96*, 1261–1266.

Bloembergen, N. (1957) *J. Chem. Phys. 27*, 572–573.

Ferraiolo, B. L., & Mieyal, J. J. (1982) *Mol. Pharmacol. 21*, 1–4.

Ferraiolo, B. L., Onady, G. M., & Mieyal, J. J. (1984) *Biochemistry* (preceding paper in this issue).

Hershberg, R. D., & Chance, B. (1975) *Biochemistry 14*, 3885–3891.

Luz, Z., & Meiboom, S. (1964) *J. Chem. Phys. 40*, 2686–2692.

Mieyal, J. J., & Freeman, L. S. (1976) *Biochem. Biophys. Res. Commun. 69*, 143–148.

Mieyal, J. J., Ackerman, R. S., Blumer, J. L., & Freeman, L. S. (1976) *J. Biol. Chem. 251*, 3436–3441.

Novak, R. F., & Vatsis, K. P. (1982) *Mol. Pharmacol. 21*, 701–709.

Novak, R. F., Kapetanovic, I. M., & Mieyal, J. J. (1977a) *Mol. Pharmacol. 13*, 15–30.

Novak, R. F., Kapetanovic, I. M., & Mieyal, J. J. (1977b) in *Microsomes and Drug Oxidations* (Ullrich, U., et al., Eds.) pp 232–239, Pergamon Press, Oxford and New York.

Olson, J. S., & Gibson, Q. H. (1972) *J. Biol. Chem. 247*, 1713–1726.

Perutz, M. F., Ladner, J. E., Simon, S. R., & Ho, C. (1974) *Biochemistry 13*, 2163–2173.

Russel, C. D., & Pauling, L. (1939) *Proc. Natl. Acad. Sci. U.S.A. 25*, 517–522.

Sadtler Research Laboratories (1967) Spectrum 191-M, Sadtler Research Laboratories, Philadelphia, PA.

Solomon, I. (1955) *J. Chem. Phys. 19*, 559–565.

Starke, D. W., Blisard, K. S., & Mieyal, J. J. (1984) *Mol. Pharmacol. 25*, 467–475.

Role of Water in Bacteriorhodopsin's Chromophore: Resonance Raman Study[†]

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ABSTRACT: The influence of water on the structure and photochemical behavior of the retinylidene Schiff base (SB) chromophore of *bacteriorhodopsin* (BR) in the *purple membrane* (PM) of *Halobacterium halobium* was studied by means of resonance Raman (RR) spectroscopy. The width of the C=N stretching band in the RR spectra of the parent chromophore, BR_{570}, considerably narrowed when H_2O as a solvent was replaced by D_2O. This is interpreted in terms of resonance energy transfer from the C=N stretching vibration (1641 cm^{-1}) to the bending vibration (1635 cm^{-1}) of H_2O molecules that are located close to the SB group. When these molecules are removed by a rigorous dehydration (dh) procedure, RR spectroscopic evidence indicates that the covalently bound proton at the SB group of the chromophore is removed and the configuration of its retinal moiety is changed. On illumination this new species, BR_{530}(dh), runs through a photoinduced cyclic process. Two intermediates of this cycle could be characterized by their RR spectra. On this basis it was concluded that, in contrast to BR_{570}, the primary photoinduced step in the dehydrated PM is proton transfer from a neighboring base to the SB group of BR_{530}(dh). Our experiments suggest that in the natural chromophore, BR_{570}, an ion pair structure—the positive charge at the SB group and a negative counterion—is stabilized by surrounding water molecules. It seems that this structure is a prerequisite for BR's function as a light-induced proton pump initiated by photoisomerization of the chromophore.

Bacteriorhodopsin (BR),[1] the major component of the purple membrane (PM) of *Halobacterium halobium*, acts as a light-driven proton pump (Oesterhelt & Stoeckenius, 1973). The active transport of protons across the membrane establishes a proton gradient that is used by the cell to drive ATP synthesis (Racker & Stoeckenius, 1974). The absorption of BR in the visible is due to its chromophoric center which contains a retinal molecule bound to the protein via a Schiff's base (SB) linkage. On illumination BR runs through a cyclic process with various intermediates (Figure 1) and there is no doubt that proton pumping is controlled by this "photochemical cycle" [for a review, see Stoeckenius et al. (1979); Ottolenghi, 1980].

In the dark BR exists in the two equilibrated forms, BR_{570} and BR_{548}, with maximum absorption at 570 and 548 nm, respectively. Under light-adapted conditions BR_{570} predominates. It could be shown by extraction and reconstitution experiments that in BR_{570} the retinal moiety is in the all-trans configuration while in BR_{548} the 13-cis form is prevalent (Oesterhelt et al., 1973; Sperling et al., 1977; Pettei et al., 1977) (Figure 2). The big red shift of BR chromophores with respect to the absorption of related model compounds in solution is due to the strong interaction the protein exerts on the

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[1] Abbreviations: RR, resonance Raman; BR, bacteriorhodopsin; PM, purple membrane; FWHH, full width at half-height; (dh), dehydrated.