MOLECULAR PHARMACOLOGY, 25:467–475

Substrate Specificity of the Monooxygenase Activity of Hemoglobin

DAVID W. STARKE, KAREN S. BLISARD,[1] AND JOHN J. MIEYAL

Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received November 1, 1983; Accepted January 20, 1984

SUMMARY

Hemoglobin has been characterized as a monooxygenase-like catalyst of aniline hydroxylation both in reconstituted systems [Mieyal *et al. J. Biol. Chem.* **251**:3436–3441 (1976)] and in intact erythrocytes [Blisard and Mieyal, *J. Biol. Chem.* **254**:5104–5110 (1979)]. In this report, the monooxygenase activity of isolated hemoglobin (Hb) in the reconstituted system, which includes NADPH and cytochrome P-450 reductase, was shown to include *N*- and *O*-demethylation reactions besides *p*-hydroxylation, and to extend to other typical cytochrome P-450 substrates such as benzphetamine and *p*-nitroanisole. Some substrates were tested also with intact erythrocytes. Those which were metabolized displayed relative activities qualitatively similar to the pattern with isolated Hb. With isolated hemoglobin, complete kinetic analysis was carried out for 10 different reactions. The K_m and V_{max} values varied broadly, so that the efficiencies of the reactions (V_{max}/K_m) encompassed a range greater than 40,000. The most efficient reaction was O-demethylation of *p*-nitroanisole; the highest V_{max} was observed for the *O*-demethylation of *p*-anisidine.The efficiencies (V_{max}/K_m) for a series of anisole derivatives (*p*-NH_2,*p*-OH,*p*-H,*p*-NO_2) was found to be quite sensitive to the electron-withdrawing effect of the *p*-substituent, i.e. a linear Hammett *sigma rho* relationship (log V_{max}/K_m versus σ) was observed ($\rho = 1.43$). Metabolism of *N*-methylaniline by hemoglobin displayed distinct regioselectivity, with *N*-demethylation being favored over *p*-hydroxylation. Separate K_m and V_{max} values were observed for these two reactions of the single substrate, suggesting that distinct ternary O_2-Hb-substrate complexes mediate the two reactions. In separate experiments, the various substrates were tested for their ability to accelerate autooxidation of HbO_2 in the absence of NADPH and reductase. Aniline and its derivatives induced autooxidation with a concentration dependence matching their K_m values for the corresponding hydroxylation reactions with the complete catalytic system. With the exception of *p*-hydroxyanisole, none of the other substrates accelerated autooxidation of HbO_2. Hence this phenomenon cannot be an indicator of potential monooxygenase reactivity with hemoglobin. The broad and regioselective activities observed for hemoglobin resemble the characteristics of the authentic monooxygenase enzyme cytochrome P-450.

INTRODUCTION

We and others have observed that Hb[2] from various species in isolated form as well as in erythrocytes and hemolysates catalyzes the *p*-hydroxylation of aniline in a manner typical of the monooxygenase reactions catalyzed by the liver microsomal cytochrome P-450 (EC 1.14.14.2) system (1–7). Previous studies by Mieyal *et al.* (2) showed that isolated human or bovine Hb could substitute one-for-one for solubilized rat liver cytochrome P-450 in a reconstituted aniline hydroxylase system also containing the P-450 reductase. The turnover numbers for the Hb-mediated reactions were comparable to those for the P-450 system. Hb in its natural environment within erythrocytes and hemolysates also displayed such activity, and NADPH was demonstrated to be the preferred cofactor (4,6). Aniline is only one prototype of the many diverse substrates of the cytochrome P-450 monooxygenase system, however, and it displays atypical behavior relative to other substrates with regard to complex formation with P-450 and its influence on P-450 oxidation reduction behavior (8, 9).

The wide specificity of the liver microsomal P-450 systems may be due in part to the many isozymes of P-

These studies were supported in part by National Institute of General Medical Sciences Grant 24076 and by a grant from the Rockefeller Foundation. Some of the results of these studies were presented in part at the 74th Meeting of the American Society of Biological Chemists, San Francisco, June 1983. They were also submitted in partial fulfillment of the requirements of the master's degree of David W. Starke, Case Western Reserve University, September 1983.

[1] Present address, Department of Pathology, University of New Mexico Affiliated Hospitals, Albuquerque, N. M. 87108.

[2] The abbreviations used are: Hb, hemoglobin; Hb^{3+}, ferrihemoglobin; $Hb^{2+}O_2$, oxyhemoglobin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography.

0026-895X/84/030467-09$02.00/0

450. Each appears to have its own subset of activities, including patterns of regioselective production of multiple metabolites from single substrates. The subsets of activities, however, overlap considerably. Hence we set out to determine whether the monooxygenase activity of hemoglobin also would extend to substrates other than aniline. Among the potential substrates chosen were analogues of aniline and other prototypic cytochrome P-450 substrates such as benzphetamine and *p*-nitroanisole. The reaction types represented were aromatic ring *p*-hydroxylation and both *N*- and *O*-demethylation. Additionally, intact erythrocytes were tested for activity with some of the same compounds to assess the ability of the physiological hemoglobin system to carry out these reactions.

We report here that, like the P-450 isozymes, Hb catalyzes a wide variety of monooxygenase reactions. Analysis of the kinetics for the different substrates gave K_m and V_{max} values which reflected a broad distribution of efficiencies (V_{max}/K_m) for the hemoglobin-catalyzed reactions, encompassing more than a 40,000-fold range. A distinct regioselectivity was observed for *N*-methylaniline metabolism, *N*-demethylation being much more favorable than *p*-hydroxylation both with respect to K_m and V_{max}.

METHODS

Chemicals. Aniline, phenol, potassium phosphate (mono- and dibasic), trichloroacetic acid, ammonium acetate, sodium hydroxide, sodium carbonate, potassium cyanide, potassium ferricyanide, diethyl ether, acetonitrile, *p*-dimethylaminobenzaldehyde, and acetic acid were purchased from Fisher Scientific Company (Pittsburgh, Pa.). NADPH was from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) or ICN Nutritional Biochemicals (Cleveland, Ohio). *p*-Anisidine, methoxyphenol, and *o*-toluidine were from Eastman Organic Chemical Company (Rochester, N. Y.). *m*-Toluidine and acetylacetone were from Aldrich Chemical Company (Milwaukee, Wisc.). Cytochrome *c* from horse heart and Tris-HCl were purchased from Sigma Chemical Company (St. Louis, Mo.). Most substrates were purified by recrystallization or fractional distillation in order to obtain products which displayed <2° m.p. or b.p. ranges.

Preparation of erythrocytes and Hb. Erythrocytes were prepared as previously described (4) from blood drawn from normal volunteers and anticoagulated with heparin. Human oxyhemoglobin was isolated according to the method of Eyer *et al.* (11). Ferrihemoglobin was prepared from oxyhemoglobin by oxidation using a 1.2-fold excess of potassium ferricyanide. Hb^{3+} was then purified by passage over two sequential Sephadex G-25 columns (1.5 cm × 15 cm) equilibrated with 20 mM potassium phosphate (pH 6.8) to remove ferricyanide and ferrocyanide. Hb concentrations were determined by the method of Van Kampen and Zijlstra (12) based on the cyanoferrihemoglobin absorbance at 541 nm, $\epsilon = 11\ mM^{-1}\ cm^{-1}$.

Preparation of cytochrome P-450 reductase. Partially purified cytochrome P-450 reductase was prepared by a modification of the method of Lu *et al.* (13) from sodium deoxycholate-solubilized, acetone-extracted rat liver microsomes as described previously (2). The cytochrome *c* specific activity of the reductase was 0.4 unit/mg (1 unit equals 1 μmole of cytochrome *c* reduced per minute), approximately 4-fold higher than that of microsomes. No cytochrome P-450 was apparent by CO difference spectra, and the preparation showed no monooxygenase activity in the reconstituted system in the absence of hemoglobin. The reductase was purged with N_2, and aliquots were stored frozen at −75°. It was confirmed that highly purified cytochrome P-450 reductase prepared by the method of Shephard *et al.* (14) (35 units/mg; single SDS-PAGE band) also was effective in coupling the Hb-catalyzed monooxygenase reaction.

Assays of product formation in the reconstituted system containing isolated Hb. All of the Hb-catalyzed reactions were conducted at 37° in a 1-ml total volume. As with cytochrome P-450 reconstituted systems, the hemoprotein was added in the ferric oxidation state, i.e. in this case ferrihemoglobin. The Hb^{3+} concentration was 1 μM. In all but the benzphetamine assay, the buffer was 20 mM potassium phosphate (pH 6.8). To improve the solubility range for bensphetamine, 0.1 M Tris-HCl (pH 6.8) was used. It was confirmed separately that equivalent results were obtained for demethylation of benzphetamine at the lower concentrations in potassium phosphate buffer. Reactions were carried out in the absence and presence of 0.02 unit of cytochrome P-450 reductase. The NADPH concentration was 0.2 mM except where indicated otherwise. The concentrations of substrates were varied.

The reaction was stopped by adding 0.3 ml of 20% (w/v) trichloroacetic acid and placing the resulting mixture on ice. These mixtures were then centrifuged for 10 min at top speed in a clinical centrifuge. One milliliter of supernatant was withdrawn from each sample and placed in a clean test tube. The appropriate colorimetric developing reagents were added, and the products were analyzed according to the following individual methods.

Quantitation of p-hydroxylated products. For aniline and aniline analogues, assays of *p*-hydroxylation were carried out according to a modification of the procedure of Brodie and Axelrod (15) in which the *p*-aminophenol congeners are converted to the corresponding indophenol chromophores for colorimetric assay as described in Mieyal *et al.* (2). Since *o*-aminophenols give indophenol derivatives that absorb near 400 nm (16), the assay is specific for *p*-aminophenols. Extinction coefficients of 14.8 $mM^{-1}cm^{-1}$ at 630 nm for *p*-aminophenol, 18.0 $mM^{-1}cm^{-1}$ at 630 nm for 4-hydroxy-N-methylaniline, and 8.8 $mM^{-1}cm^{-1}$ at 640 nm for 4-hydroxy-2-methylaniline were obtained from standard curves of the individual products. The indophenol derivative of the product of hydroxylation of *m*-toluidine absorbed maximally at 620 nm. Since an authentic sample of 4-hydroxy-3-methylaniline was not available, the extinction coefficient was estimated as the arithmetic mean of the values for *p*-aminophenol and 4-hydroxy-2-methylaniline, i.e., 12 $mM^{-1}\ cm^{-1}$.

Quantitation of formaldehyde. Assays of formaldehyde formed via the demethylation of benzphetamine, *N*-methylaniline, or the various anisole derivatives were performed by a modification of the assay of Nash (17). After obtaining the 1-ml supernatant of the terminated reaction mixture, 0.4 ml of a solution containing 0.4% acetylacetone in 4 M ammonium phosphate was added. Samples were mixed and kept at 37° for 45 min. After the samples were cooled, their absorbances at 412 nm were measured. From standard curves, the extinction coefficient for the formaldehyde-acetylacetone derivative was determined to be 3.8 $mM^{-1}cm^{-1}$.

Assays of autooxidation of oxyhemoglobin. The ability of each of the compounds to accelerate autooxidation of oxyhemoglobin was determined by visible difference spectroscopy and/or dual wavelength spectroscopy utilizing an Aminco DW-2 spectrophotometer. For difference spectroscopy, two cuvettes containing 1 ml of 1 μM HbO_2 were preincubated at 37° for two min, and a baseline was set to match the absorbances of the solutions in the sample and reference cuvettes over the region 350–500 nm. Microliter quantities of aniline or other test compounds in methanol were then added to the sample cuvette to give the appropriate final concentration. An equivalent amount of methanol alone was added to the reference cuvette. After mixing, the difference spectrum was recorded and the periodic changes in absorbance between 420 nm and 411 nm were measured from repetitive scans of the wavelength region.

Alternately, $\Delta A_{420-411}$ as a function of time was recorded directly by utilizing a single cuvette containing HbO_2 and substrate and operating the spectrophotometer in the dual wavelength and time base modes. Both assay types gave equivalent results.

Assays of product formation by Hb in intact erythrocytes. Experiments

with intact erythrocytes were performed as detailed previously for aniline hydroxylation whereby reaction mixtures contained a quantity of erythrocytes corresponding to 1 mM concentration with respect to HbO_2 (4). Each substrate was present at a concentration of 20 mM. This concentration was chosen both because of solubility limitations and in order to keep the amount of substrate-induced hemolysis at less than 5%. Incubations were carried out for 60 min at 37°, and the reactions were terminated by cooling in ice. Products were extracted with purified diethyl ether and back extracted into dilute HCl solutions as described previously (4). The products of p-hydroxylation of the aniline derivatives were assayed colorimetrically as described above, except that each extinction coefficient was multiplied by an appropriate recovery percentage. Average recovery for each compound was calculated from the ratio of the absorbances of the indophenol derivative of a known amount of the compound which had been carried through the extraction procedure and the same concentration of the compound which had been assayed directly. These values were determined over a wide range of concentrations, and are as follows: *p*-aminophenol, 50%; 4-hydroxy-2-methylaniline, 60%; recovery of 4-hydroxy-3-methylaniline (see above) was taken as the average for *p*-aminophenol and 4-hydroxy-2-methylaniline, i.e., 55%. Recoveries were found to be reproducible to within ±6%. Since *N*-methylaminophenol was observed to decay relatively rapidly in the presence of erythrocytes, its "recovery" was estimated to be approximately 60% by the following procedure. Sequential additions whereby a known amount of *N*-methylaminophenol was added to the incubation mixture in five equal aliquots at 0, 15, 30, 45, and 60 min were utilized to simulate product formation from *N*-methylaniline. After 60 min, the reaction mixture was cooled, extracted, and assayed as usual. Decay of *N*-methylaminophenol was not a significant problem in assays involving the reconstituted system because a shorter time course was monitored.

Demethylation of *N*-methylaniline by erythrocytes was measured by assaying for aniline. The samples and standards (prepared by adding a known amount of authentic aniline to an erythrocyte suspension) were processed in the same fashion as described previously (4), except that the reagent for development of color was a modified Ehrlich's reagent (18) consisting of 0.5% *p*-dimethylaminobenzaldehyde in a 5 M sodium formate buffer (pH 3.75). The adduct with aniline has a much higher extinction coefficient at 445 nm (ϵ = 10.0 $mM^{-1}cm^{-1}$) than *N*-methylaniline (ϵ = 0.02 $mM^{-1}cm^{-1}$) and could easily be detected in the concentration range of interest by subtracting out the absorbance due to the *N*-methylaniline (i.e., the absorbance of a control sample incubated at 0°). The resulting absorbance difference was then corrected for the average recovery of aniline (85%), which was determined separately. *N*-Methylaminophenol at a concentration of 20 μM did not give a measurable reaction with this reagent.

Verification of reaction products. HPLC was used to confirm that the products of hydroxylation of aniline, *o*-toluidine, *N*-methylaniline, and phenol co-eluted with authentic samples of the expected products. Either a Waters Associates high-pressure liquid chromatograph with a μBondapak C-18 column, or a Varian model 5000 instrument with a MCH-10 column was used. These "reverse-phase" systems were found to be interchangeable. The mobile phase used was 20% acetonitrile in water at a flow rate of 1 ml/min. The samples for analysis were extracted twice with ether, and the ether fractions were evaporated to dryness (except for *N*-methylaniline, where a second extraction into 1% acetic acid was used for injection). The residues were redissolved in the mobile phase solution, and a 25-μl aliquot was injected. Retention times were as follows: *o*-toluidine, 8.0 min; 4-hydroxy-2-methylaniline, 5.9 min; *N*-methylaniline, 30 min; *N*-methylaminophenol, 6.6 min; phenol, 5.7 min; hydroquinone, 2.8 min. Standard curves were constructed for these assays in the same way as for the colorimetric assays. Aniline, *o*-toluidine, and *N*-methylaniline co-chromatographed with authentic standards, and quantitative agreement with the colorimetric assays was obtained.

Gas chromatography was used to confirm that the metabolism of *N*-methylaniline by erythrocytes gave a product which co-eluted with authentic aniline. For this assay, supernatant from the reaction or standard sample was extracted with an equal volume of ether, and a small aliquot of the ether fraction was injected. A Packard Model 428 gas chromatograph fitted with a 6-foot 3% OV-17 (on 100/120 Gas-Chrom Q support) column and a flame ionization detector was used. Both the injection and detection ports were maintained at 250°; the column temperature was programmed from 50° to 150° at 10°/min, and the carrier gas flow rate was 30 ml/min. Aniline produced by erythrocyte metabolism of *N*-methylaniline co-chromatographed with authentic aniline, and the rate of metabolism agreed with that measured by the colorimetric assay.

RESULTS

The Hb-reconstituted monooxygenase system displays many cytochrome P-450-like reactions, including *p*-hydroxylation, *N*-demethylation, and *O*-demethylation (Table 1). Benzphetamine and various congeners of aniline and anisole were shown to be substrates. *N*-Methylaniline was metabolized to two different products in a distinctly regiospecific manner, *N*-demethylation being favored over *p*-hydroxylation.

Coupling of Hb with cytochrome P-450 reductase. As reported previously (2), the hydroxylation of aniline by Hb occurred to some extent with NADPH alone, in the absence of the reductase. This phenomenon was observed also with the other substrates. Figure 1 displays the dependence of two of the Hb-catalyzed reactions on NADPH concentration in the presence of the reductase. The biphasic nature of the curves is interpreted to reflect a high efficiency (low K_m for NADPH) phase ascribable to reductase-mediated electron transfer and a low-efficiency phase due to direct action of NADPH. Consistent with the K_m of the reductase for NADPH [5 μM (19)], the reductase-mediated component of the reactions appears to be essentially saturated near 200 μM NADPH. The non-reductase contribution at 200 μM NADPH was different for different substrates. For example, in the absence of reductase, no product was detectable from aniline whereas 920 pmoles/min/ml of *p*-aminophenol was produced from *p*-anisidine. For this reason, product formation in the absence of reductase was subtracted from total product formation for *p*-anisidine at 200 μM NADPH and for *N*-methylaniline and benzphetamine at 1 mM NADPH (values for the rates of the reaction in the absence of reductase, if any, are given in the legend to Table 1 for each of the substrates.) In all cases the K_m for substrates observed for the reactions in the absence of the reductase was found to be identical with the substrate K_m for the reductase-coupled reaction. This observation indicates that the substrate K_m values reflect the interactions between the substrates and Hb, and the reductase serves to catalyze electron transfer from NADPH to the Hb during the course of the reactions, analogous to its role in the P-450 system.

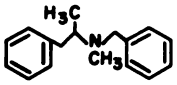
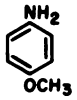
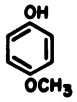
p-Hydroxylation reactions. We have reported previously (2) that *p*-aminophenol is the predominant product of aniline hydroxylation by Hb in this reconstituted system. *o*-Aminophenol was not detected by the indophenol colorimetric assay [i.e., no absorbance change near 400 nm; see Methods (16)] or by gas chromatographic-mass spectroscopic analysis. Likewise, in assays of the hydroxylation of the aniline analogues in the

TABLE 1

Monooxygenase activity of isolated Hb

Reaction mixtures (1 ml) contained 20 mM potassium phosphate (pH 6.8), 1 μM Hb^{3+}, 0.2 mM NADPH (unless otherwise indicated), and ± 0.02 unit of P-450 reductase; the rates (if any) in the absence of reductase were subtracted to express only the reductase-coupled reactions (see Fig. 1 and accompanying text). Non-reductase maximal rates at 1 mM NADPH: *N*-methylaniline *N*-demethylation, 92 pmoles/min-ml; benzphetamine *N*-demethylation, 147 pmoles/min-ml; and at 200 μM NADPH: anisidine, 920 pmoles/min-ml. All other substrates did not produce detectable product at 200 μM NADPH in the absence of reductase.

V_{max} and K_m values are from V versus $V/[S]$ plots for the reductase-coupled reactions (e.g., see Fig. 2). At least a 20-fold range in substrate concentration was tested in every case. The ideal of covering the range 0.1 K_m-10 K_m could not be accomplished for all substrates because of either limitations in the sensitivity of the assay system or solubility. In all cases, a sufficient number of experiments were performed (≥10), so that the regression coefficient for the slope of the V versus $V/[S]$ plot was ≥0.97. The standard error for each point on the graphs was ≤10% of the mean.

Substrate	Reaction type	V_{max} pmol/min-ml	K_m mM	V_{max}/K_m [c]
 Aniline	p-hydroxylation	64	5.7	11.2
 o-Toluidine	p-hydroxylation	68	65.4	1.0
 m-Toluidine	p-hydroxylation	4[a]	87[a]	0.05
 [b]N-Methyl-aniline	p-hydroxylation	25	3.6	6.9
	N-demethylation	164	0.52	315
 [b]Benzphetamine	N-demethylation	115	0.42	268
 p-Anisidine	O-demethylation	658	36.4	18.1
 Anisole	O-demethylation	439	0.89	493
 p-Methoxyphenol	O-demethylation	320	4.5	71.1
 p-Nitroanisole	O-demethylation	211	0.094	2244

[a] These values are estimates of K_m and V_{max}, because the limited reactivity and solubility of this substrate precluded more accurate determination of these values.

[b] NADPH (1 mM) was used in these cases. In other cases where both 1 mM and 0.2 mM NADPH were tested, the amount of product formation associated with the reductase-coupled reaction was found to be the same under both conditions; i.e., only the component rate in the absence of reductase was proportionally larger at 1 mM NADPH.

[c] The units of V_{max}/K_m as indicated are nM product formed/min/mM substrate at 1 μM Hb^{3+}.

present study, little if any absorbance change was detectable in the 400-nm region. Threfore, *p*-hydroxylation appears to be the predominant aromatic hydroxylation reaction catalyzed by Hb for these substrates.

The positioning of a methyl group on the aniline ring showed a varied effect on the *p*-hydroxylation reaction (Table 1). A methyl group *ortho* to the amine increased the K_m more than 10-fold without an effect on V_{max}. A *meta* methyl group decreased activity to the extent that a complete kinetic analysis could not be done. The estimates of K_m and V_{max} indicate a further increase in K_m by *meta* methyl substitution, and a large decrease in maximal activity. With *N*-methylaniline, two reactions occur, *N*-demethylation (see below) and *p*-hydroxylation, and these display separate K_m and V_{max} values (Fig. 2). *N*-Methyl substitution affected the apparent K_M for *p*-

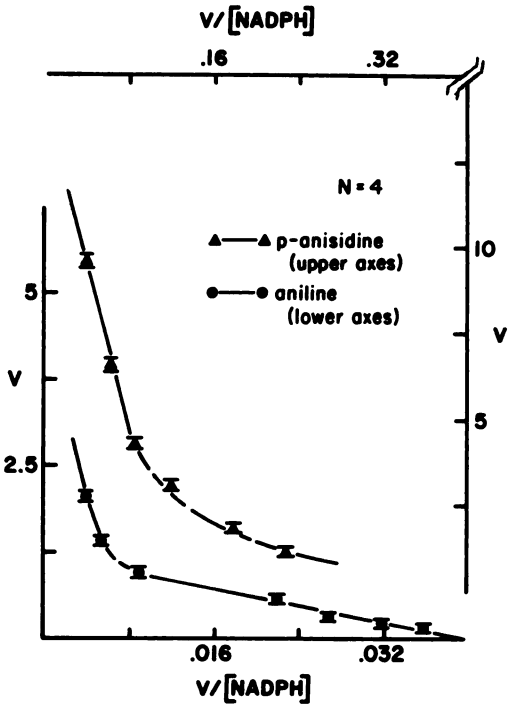


FIG. 1. *NADPH dependence of the aniline hydroxylase and p-anisidine O-demethylase activities*

Reaction mixtures contained 1 μM Hb, 20 mM potassium phosphate buffer (pH 6.8), 120 mM *p*-anisidine or 40 mM aniline, 0.02 unit of reductase, and 5–1000 μM NADPH. Data were plotted in the Eadie-Hofstee format and showed a biphasic relationship. The *y*-axis units are pmoles/min-ml. The *x*-axis units are pmoles of product/min-ml/μM NADPH.

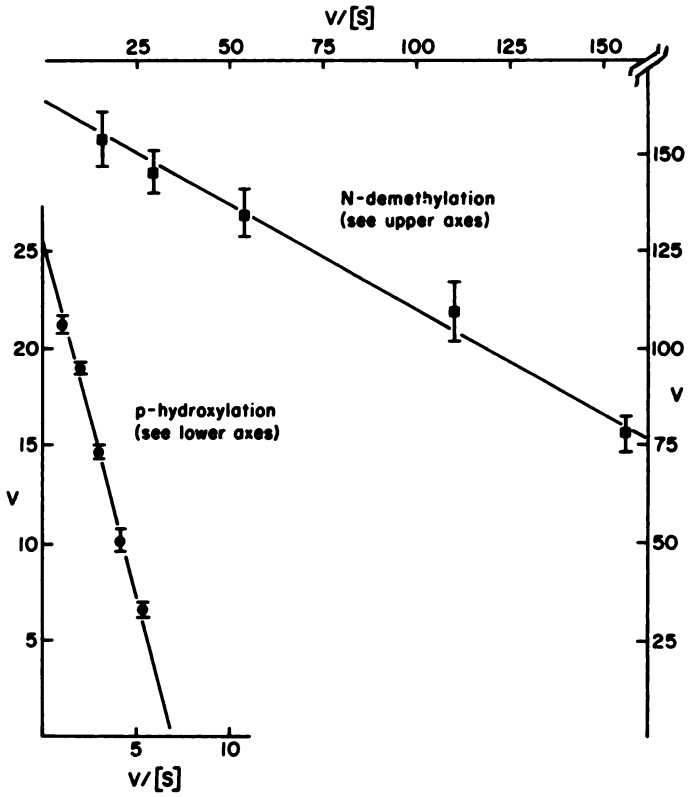


FIG. 2. *Dual metabolism of N-methylaniline by Hb*

Reaction mixtures contained 1 μM Hb, 20 mM potassium phosphate buffer (pH 6.8), 1 mM NADPH, 2–32 mM *N*-methylaniline for hydroxylation, and 0.5–10 mM *N*-methylaniline for *N*-demethylation. Plots of V versus $V/[S]$ yielded regression coefficients ≥0.97. The standard error of each point was within ±10%. The *y*-axis units for both plots are pmoles/min-ml. The *x*-axis units for both plots are pmoles of product formed/min-ml/mM *N*-methylaniline.

hydroxylation slightly, but decreased V_{max} approximately 2.5-fold relative to aniline. This may be due in part to the competing *N*-demethylation reaction, which proceeds much more efficiently.

Since phenol resembles aniline in size and electronic properties, it was also tested as a substrate, but complete and reliable kinetic analysis could not be accomplished, because phenol at higher concentrations caused denaturative precipitation of Hb. Catechol (*o*-hydroxyphenol) was separable but not detected in the HPLC assay for *p*-hydroxylation of phenol. The estimated maximal rate of *p*-hydroquinone formation appeared to be similar to *p*-aminophenol formation from aniline, but the K_m for phenol appeared to be >30 mM (data not shown).

N-Demethylation reactions. Benzphetamine and *N*-methylaniline have some molecular features in common, but they are quite different in other respects, such as molecular size and the nature of the nitrogen atom involved in the reaction. Benzphetamine is a bulky molecule with two aromatic rings situated at opposite ends of the molecule, whereas *N*-methylaniline is more compact. Benzphetamine is a tertiary aliphatic amine, while *N*-methylaniline is a secondary aryl amine. In spite of these differences, the K_m and V_{max} values are similar (Table 1), and both reactions are highly efficient relative to the other reactions.

O-Demethylation reactions. The *O*-demethylation of *p*-anisidine exhibited the highest maximal activity. Its high K_m value, however, limits the over-all efficiency of the reaction. The other *O*-demethylation substrates stand out as non-amines, and they form a homologous series of anisole derivatives, including anisidine. *p*-Nitroanisole, which is another prototypic P-450 substrate, gave the lowest K_m of all of the substrates tested in this Hb system, and this reaction displayed the greatest efficiency (V_{max}/K_m). Scrutiny of the kinetic data for the set of anisole homologues suggested a relationship to the electronic inductive effects of the substituents *para* to the methoxy group site of metabolism. It is evident that the V_{max} of the reaction is not sensitive to the inductive effect, whereas the K_m appears to be influenced markedly (Table 1). This relationship suggests that the rate determining step(s) of the *O*-demethylation reaction (V_{max}) is not sensitive to electronic effects of the *p*-substituent. Some important nonlimiting catalytic step, or the binding and/or orientation of the substrates must be favored by an electron-withdrawing substituent in the *para* position. Figure 3 displays a replot of the data in the format of the Hammett $\sigma\rho$ relationship, where V_{max}/K_m effectively represents the first-order rate constant for the reaction at low substrate concentrations (i.e., $S \ll K_m$). The slope of the plot ($\rho = 1.43$) indicates that the *O*-demethylation reaction of the anisoles is more sensitive to the inductive effects of the *para* substituents than is

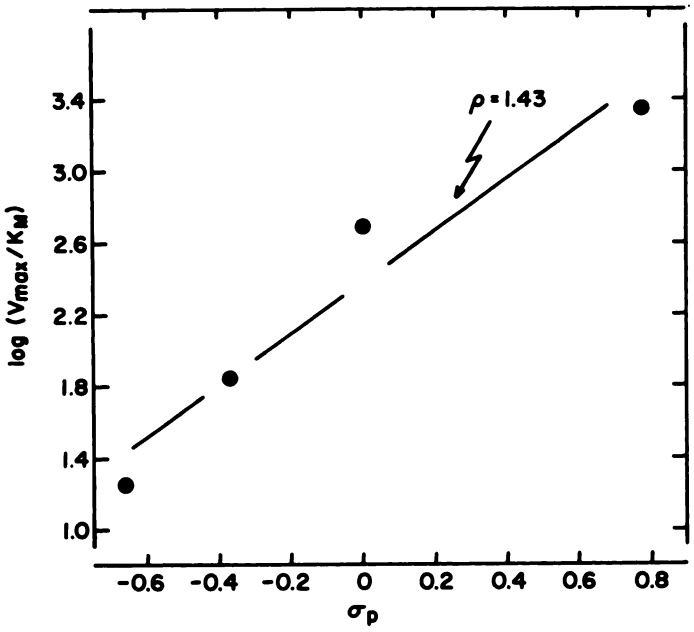


FIG. 3. *Hammett plot of kinetic data for anisole congeners*

Log (V_{max}/K_m), which represents the log of the pseudo-first order rate constant for the *O*-demethylation reaction at low substrate concentrations (i.e., $S \ll K_m$), was calculated from the data of Table 1. *Sigma-para* (σp) values were taken from Carey and Sundberg (20). The line indicated was calculated by regression analysis ($r = 0.97$).

TABLE 2

Monooxygenase activity of erythrocytes

Each sample contained 20 mM substrate and erythrocytes at 1 mM with respect to Hb (see Methods). Samples were incubated and products were extracted as described previously (5). Assays of individual products were conducted as described under Methods.

Substrate	Reaction type	Activity at 20 mM substrate
		pmoles/min/ml
Aniline	*p*-Hydroxylation	60.0 ± 3
o-Toluidine	*p*-Hydroxylation	50.0 ± 3
m-Toluidine	*p*-Hydroxylation	26.6 ± 3
Phenol	*p*-Hydroxylation	49.0 ± 4
N-Methylaniline	*p*-Hydroxylation	16.7 ± 2
	N-Demethylation	640 ± 0

the ionization of the corresponding benzoic acids (i.e., $\rho = 1.0$).

Substrate specificity of Hb within erythrocytes. A number of the substrates were tested also for metabolism in the erythrocyte system. The rates of metabolism at the same concentration (20 mM) of each of the substrates that did yield products are shown in Table 2. Two anisole derivatives, *p*-anisidine (20 mM) and *p*-nitroanisole (1 mM), gave little (if any) of the corresponding demethylated products (data not shown), even when the system was set up for maximal stimulation of monooxygenase activity by the addition of methylene blue and glucose (4). This result demonstrated a distinct qualitative difference between the reactivity of Hb in its native environment as compared with isolated Hb in the reconstituted system. The basis for the difference could range from a conformational difference in the Hb molecule to interference by other components of the erythrocyte, and further study is necessary. With those substrates that were metabolized by the erythrocytes (Table 2), the turnover numbers are obviously much lower than those for isolated Hb. Nevertheless, several similarities in the metabolism of these substrates are evident. The relative rates of *p*-hydroxylation reflect a comparable order, i.e., aniline ≈ *o*-toluidine > *N*-methylaniline. Moreover, *N*-methylaniline is dually metabolized, and a similar regiospecificity is displayed, *N*-demethylation > *p*-hydroxylation.

Acceleration of autooxidation. In a previous report, we demonstrated that aniline accelerated the autooxidation of oxyhemoglobin in the absence of NADPH, and no product *p*-aminophenol was formed under these conditions (21). The interaction constant (K_{act}) was identical with the Michaelis constant (K_m) for aniline hydroxylation in the complete system containing NADPH and reductase. We suggested that aniline, by binding to HbO_2, may distort the heme iron-oxygen bond, thereby activating the complex for metabolism and autooxidation. In order to test whether this hypothesis might be supported further, the other substrates were examined for their ability to accelerate HbO_2 autooxidation in a concentration-dependent manner (Table 3). The interaction constants (K_{act}) and maximal velocity (V_{max}^{autox}) are listed along with the K_m and V_{max}^{metab} values from Table 1 for product formation in the complete metabolic system. There was a close correlation between K_{act} and K_m for all of the aryl amine substrates (aniline, *o*-toluidine, *m*-toluidine, *p*-anisidine) and for *N*-methylaniline (*p*-hydroxylation only), and the phenol derivative, methoxyphenol. With the other three substrates, no acceleration of HbO_2 autooxidation was evident. For benzphetamine, no difference spectrum indicative of increased autooxidation of HbO_2 was obtained at 4 mM concentration (10 × K_m), which was the highest concentration attainable without exceeding 10% methanol in the solution. Likewise, anisole and *p*-nitroanisole did not accelerate Hb^{3+} formation from HbO_2 up to concentrations greater-than 10 K_m over at least a 1-hr period. These results indicate that substrate-induced autooxidation of HbO_2 is neither a prerequisite nor a reliable indicator of monooxygenase reactivity with hemoglobin. The equivalence of K_m and K_{act} for the amine and phenol substrates suggests a common enzyme-substrate complex for metabolism and autooxidation in those cases (see Discussion).

DISCUSSION

The expanded set of substrates for the Hb-reconstituted system indicates that Hb is a more general monooxygenase than was previously expected. It also displayed regioselective metabolism, another typical property of the microsomal P-450 enzymes. It will be interesting to discover whether substrates other than *N*-methylaniline are also metabolized to multiple products (e.g., *O*-dealkylation and *p*-hydroxylation of anisole). The catalytic activity of Hb exhibits all of the properties of an enzyme, i.e., typical Michaelis-Menten kinetics, dependence of activity on the native protein, etc. The simplest view of the interaction of Hb with substrate

TABLE 3

Relative substrate-dependent kinetics of Hb autooxidation and Hb-catalytic metabolism

Each sample was preincubated in a cuvette at 37° containing 1 μM oxyhemoglobin. Aliquots of the appropriate substrate were then added. After mixing, the change in absorbance between 420 nm and 411 nm was recorded as a function of time (see Methods).

Substrate	Metabolism			Autooxidation	
	Reaction type	K_m	V_{max}	K_{act}	V_{max}
		mM	*pmoles/min/ml*	*mM*	*pmoles/min/ml*
Aniline	*p*-Hydroxylation	5.7	63.7	7.8	17.3
o-Toluidine	*p*-Hydroxylation	65.4	68.0	77.7	14.5
m-Toluidine	*p*-Hydroxylation	87[a]	4[a]	>70[a]	3[a]
N-Methylaniline	*p*-Hydroxylation	3.6	25.3	2.9	4.6
	N-Demethylation	0.52	115		
Benzphetamine	*N*-Demethylation	0.43	164	ND[b]	ND[b]
p-Methoxyphenol	*O*-Demethylation	4.5	320	4.1	33.8
p-Anisidine	*O*-Demethylation	36.4	658	29.4	12.3
Anisole	*O*-Demethylation	0.89	439	ND[b]	ND[b]
p-Nitroanisole	*O*-Demethylation	0.094	211	ND[b]	ND[b]

[a] These values are estimates of K_m and V_{max}, because the limited reactivity and solubility of this substrate precluded more accurate determination of these values.

[b] No acceleration of Hb^{3+} formation was detected after a 1-hr incubation of HbO_2 with this substrate at concentrations ≥10 K_m.

involves the formation of one or more ternary substrate-hemoglobin-O_2 complexes. Such complexes may then proceed to react in two ways formally; namely, via transfer of electrons from the ferrous iron atom to oxygen (*autooxidation*) or via the acceptance of an exogenous electron (from NADPH and reductase) in order to complete the monooxygenase process of formation of hydroxylated substrate (*metabolism*). Interpretation of the kinetic data presented for the various substrates suggests that distinct and non-interconvertable ternary complexes are formed with different propensities for autooxidation and different modes of metabolism (*vide infra*).

Dual metabolism of N-methylaniline. The phenomenon of dual kinetics for a single substrate apparently represents an enzyme kinetic situation that has not heretofore been examined. The fact that separate K_m values were obtained for the *p*-hydroxylation and *N*-demethylation reactions of the single substrate *N*-methylaniline (see Fig. 2) is consistent with separate ternary O_2-Hb-substrate complexes mediating the two different reactions. If the contrary were true, i.e., both reactions shared a common intermediate, the individual kinetic constants of both reaction pathways would affect the steady-state levels of all intermediates; therefore, all of the kinetic constants would be included equally in both K_m values. Even though the intrinsic K_m of each pathway in the absence of the other reaction might be different because of differences in mechanism, the observed K_m for each pathway would be the same.

The nature of the two distinct ternary complexes and how they are formed cannot be gleaned from the current kinetic data; however, two interpretations are presented for further exploration. (a) There may be a slow conformational equilibrium between forms of the hemoprotein that favors different modes of substrate binding. Since Hb is a conformationally mobile molecule, it is conceivable that different forms of the hemoprotein (including the various oxidation and liganded states, e.g., oxyhemoglobin versus ferrihemoglobin) may present different binding modes for the same substrate. One mode would favor *N*-demethylation and the other would favor *p*-hydroxylation. For this to be the explanation, the rate of interconversion of the conformers must be slow on the time scale of the metabolic reactions. Additional studies are in progress to address this question further. These experiments include detailed two substrate kinetics (O_2 versus *N*-methylaniline) and experiments in which either Hb^{3+} or HbO_2 is used to initiate the metabolic reactions. (b) There may be two separate preexisting noninteracting substrate binding sites on the hemoprotein molecule. Since Hb is a tetrameric molecule, it is conceivable that such substrate sites could exist on separate subunits or, alternately, that both might be on the same subunit(s). Recent NMR and kinetic studies in our laboratory with valency hybrids of Hb have demonstrated a subunit selectivity in which the β-subunits of the $\alpha_2\beta_2$ tetramer are responsible for most of the aniline hydroxylase activity (22–24). Whether this subunit selectivity or another molecular mechanism pertains to the dual metabolism of substrates such as *N*-methylaniline remains to be elucidated. Analogous questions are pertinent to deriving the basis for the regioselective metabolism by cytochrome P-450 which also exists in oligomeric states in microsomes as well as in isolation (25, 26). For the most part, reports of the substrate specificities of the P-450 isozymes have compared different substrates at single concentrations, so that complete kinetic data are not available.

It will be interesting to discover whether regiospecific metabolism of various substrates by P-450 also displays separate K_m and V_{max} values for each of the modes of metabolism of a given substrate. In this regard, a recent report by Dieter and Johnson (27) included kinetic data for the 6β-hydroxylation and 16α-hydroxylation of progesterone by isolated isozymes of rabbit liver microsomal P-450. Because of unavoidable limitations in the data, however, it was not possible to ascertain whether the regioselective metabolism of progesterone displayed the

same or different K_m values for the two reactions if catalyzed by a single P-450 entity.

Autooxidation and its relationship to monooxygenase activity. As presented above, we once considered that the ability of aniline to accelerate autooxidation and serve as a hydroxylatable substrate might indicate a distortion of the geometry of the heme iron-dioxygen bond activating it for both reactions. Therefore, the event of substrate binding was postulated to activate Hb and divert it from its normal function as an oxygen carrier. It is apparent from the data of Table 3 that at least this is not generally true. The occurrence of mixed-function oxidase activity without the observation of substrate-induced autooxidation limits such a singular concept of Hb transformation.

In all cases where substrate-induced acceleration of autooxidation was observed, however, a close agreement was noted between K_{act} and K_m. This situation is consistent with a single ternary complex mediating both autooxidation and metabolism. It is noteworthy that the arylamines and the substituted phenol are the substrates that induce autooxidation. This observation is consistent with the hypothesis of Wallace and Caughey (28) that suggests that aniline and phenol derivatives may serve as electron donors (akin to $NaNO_2$) to accelerate autooxidation of HbO_2. With regard to the microsomal P-450 enzymes, an analogous situation exists in that there appears to be a mixture of productive and nonproductive oxidation of NADPH, the latter probably involving the autooxidation of $P\text{-}450^{2+}\text{-}O_2$. At the extreme, certain potential substrates have been reported to interact with the P-450 system to enhance nonproductive NADPH oxidation and O_2 consumption without being metabolized at all.

TABLE 4

Relative activities of Hb and Cytochrome P-450

Substrate	Hemoprotein	Turnover no.	Reference
		min^{-1}	
Aniline	P-450, LM2	1.0	10
	P-450, LM4	0.4	10
	P-450, LM1, 7	0.6	10
	Hemoglobin	0.063	This study
Benzphetamine	P-450, LM1	9.6	10
	P-450, LM2	56.8	10
	P-450, LM4	4.9	10
	P-450, LM1, 7	7.7	10
	P-450, LM7	13.6	10
	Hemoglobin	0.11	This study
	Mouse microsomes	16	29
	P-450, A_1	6	29
	P-450, A_2	5	29
	P-450, C_1	45	29
	P-450, C_2	8	29
Nitroanisole	P-450, LM1	2.5	10
	P-450, LM2	5.7	10
	P-450, LM4	3.4	10
	P-450, LM1, 7	3.3	10
	P-450, LM7	1.8	10
	Hemoglobin	0.21	This study
	P-450, LM2	1.24	7

Comparison of hemoglobin and cytochrome P-450. Hb demonstrates many properties in common with cytochrome P-450. These include most notably utilization of NADPH and cytochrome P-450 reductase as the electron transfer system, inhibition of activity by carbon monooxide, hydrophobic heme environment, and oligomeric quaternary structure. These similarities make Hb useful as a model for cytochrome P-450, especially since the former may be examined more conveniently in a stable oxyferrous form.

There are limitations to any model, however, and this is not an exception. The range of turnover numbers for Hb activity are in general lower than those for cytochrome P-450 for these same reactions, but remarkably in some cases the activities approach within the same order of magnitude (see Table 4).

Some fundamental physical differences exist between Hb and cytochrome P-450 which may explain the differences in activity. Monomeric cytochrome P-450 is a larger, more complex molecule than the subunits of Hb. More important, perhaps, is the fact that the reactive heme iron moieties of the two proteins are ligated differently. Cytochrome P-450 has as its fifth coordinating ligand a cysteinyl thiolate moiety, whereas the fifth ligand of Hb is a histidine nitrogen. The thiolate ligand may play an important role in the activation of oxygen (30), especially for the hydroxylation of relatively unreactive aliphatic substrates such as cyclohexane and testosterone. Although the substrates in the current study were diverse in size and electronic characteristics, all were aromatic. We have now set out to examine aliphatic hydroxylation by normal Hb, and to replace the proximal histidinyl residues of Hb with cysteines by site-specific mutagenesis. If thiolate ligation is the key to maximal monooxygenase reactivity, then the catalytic prowess of Hb should be enhanced after such mutation.

ACKNOWLEDGMENTS

We are grateful to Drs. L. M. Sayre, M. E. Maguire, and J. L. Blumer for critical evaluation of the manuscript prior to submission.

REFERENCES

1. Juchau, M. R., and K. G. Symms. Aniline hydroxylation in the human placenta: mechanistic aspects. *Biochem. Pharmacol.* **21**:2053–2064 (1972).
2. Mieyal, J. J., R. S. Ackerman, J. L. Blumer, and L. S. Freeman. Characterization of enzyme-like activity of human hemoglobin. *J. Biol. Chem.* **251**:3436–3441 (1976).
3. Jonen, H. G., R. Kahl, and G. F. Kahl. Enhancement of microsomal aniline and acetanilide hydroxylation by haemoglobin. *Xenobiotica* **6**:307–320 (1976).
4. Blisard, K. S., and J. J. Mieyal. Characterization of the aniline hydroxylation activity of erythrocytes. *J. Biol. Chem.* **254**:5104–5110 (1979).
5. Blisard, K. S., and J. J. Mieyal. Differential monooxygenase-like activity of fetal and adult erythrocytes. *Biochem. Biophys. Res. Commun.* **96**:1261–1266 (1980).
6. Blisard, K. S., and J. J. Mieyal. Role of NADPH and the NADPH-dependent methemoglobin reductase in the hydroxylase activity of human erythrocytes. *Arch. Biochem. Biophys.* **210**:762–769 (1981).
7. Ingelman-Sundberg, M., and G. Ekström. Aniline is hydroxylated by the cytochrome P-450 dependent hydroxyl radical-mediated oxygenation mechanism. *Biochem. Biophys. Res. Commun.* **106**:625–631 (1982).
8. Mannering, G. J. Role of substrate binding to P-450 hemoproteins in drug

metabolism, in *Drugs and Cell Regulation*. Academic Press, New York, 197–225 (1971).
9. Arrenius, S., B. J. Wilson, C. von Bahr, and J. B. Schenkman. On the significance of drug-induced spectral changes in liver microsomes. *Biochem. Soc. Symp.* **34**:55–77 (1972).
10. Coon, M. J., J. L. Vermillion, K. P. Vatsis, J. S. French, W. L. Dean, and D. A. Haugen. Biochemical studies on drug metabolism: isolation of multiple forms of liver microsomal cytochrome P-450, in *Drug Metabolism Concepts* (D. M. Jerina, ed.). ACS Symposium No. 44, American Chemical Society, Washington, D. C., 46–71 (1976).
11. Eyer, P., H. Hertle, M. Kiese, and M. Klein. Kinetics of ferrihemoglobin formation by some reducing agents, and the role of hydrogen peroxide. *Mol. Pharmacol.* **11**:326–334 (1975).
12. Van Kampen, E. J., and W. G. Zijlstra. Standardization of hemoglobinometry. II. The hemoglobincyanide method. *Clin. Chim. Acta* **6**:538–544 (1961).
13. Lu, A. Y. H., R. Kuntzman, S. West, M. Jacobson, and A. H. Conney. Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates. *J. Biol. Chem.* **247**:1727–1734 (1972).
14. Shephard, E. A., S. F. Pike, B. R. Rabin, and I. R. Phillips. A rapid one-step purification of NADPH-cytochrome c (P-450) reductase from rat liver microsomes. *Anal. Biochem.* **129**:430–433 (1983).
15. Brodie, B. B., and J. Axelrod. The estimation of acetanilide and its metabolic products, aniline, *N*-acetyl *p*-aminophenol and *p*-aminophenol (free and total conjugated) in biological fluids and tissues. *J. Pharm. Exp. Ther.* **94**:22–28 (1948).
16. Bray, H. G., and W. V. Thorepe. Analysis of phenolic compounds of interest in metabolism, in *Methods of Biochemical Analysis* (D. Glick, ed.), Vol. 1. Wiley Interscience Publishers, New York, 27–52 (1954).
17. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
18. Estus, G. S., and J. J. Mieyal. Structure-activity relationship for deacetylation of a homologous series of phenacetin analogs and their *N*-hydroxy derivatives. *Drug Metab. Dispos.* **11**:471–476 (1983).
19. Dignam, J. D., and H. W. Strobel. NADPH-cytochrome P-450 reductase from rat liver: purification by affinity chromatography and characterization. *Biochemistry* **16**:1116–1123 (1977).
20. Carey, F. A., and R. J. Sundberg. *Advanced Organic Chemistry*, Part A. Plenum Publishing Company, New York, 145 (1977).
21. Mieyal, J. J., and J. L. Blumer. Acceleration of the autooxidation of human oxyhemoglobin by aniline and its relation to hemoglobin-catalyzed aniline hydroxylation. *J. Biol. Chem.* **251**:3442–3446 (1976).
22. Ferraiolo, B. L., and J. J. Mieyal. Subunit selectivity in the monooxygenase-like activity of tetrameric hemoglobin. *Mol. Pharmacol.* **21**:1–4 (1983).
23. Ferraiolo, B. L., G. M. Onady, and J. J. Mieyal. Monooxygenase activity of human hemoglobin: role of quaternary structure in the preponderant activity of the beta subunits within the tetramer. *Biochemistry*, in press.
24. Onady, G. M., B. L. Ferraiolo, and J. J. Mieyal. Monooxygenase activity of human hemoglobin: NMR demonstration of different modes of substrate binding corresponding to different activities of hemoglobin derivatives. *Biochemistry*, in press.
25. Guengerich, F. P., and L. A. Holladay. Hydrodynamic characterization of highly purified and functionally active liver microsomal cytochrome P-450. *Biochemistry* **18**:5442–5449 (1980).
26. McIntosh, P. R., S. Kawato, R. B. Freedman, and R. J. Cherry. Evidence from cross-linking rotational diffusion studies that cytochrome P-450 can form molecular aggregates in rabbit liver microsomal membranes. *F. E. B. S. Lett.* **122**:54–58 (1980).
27. Dieter, H. H., and E. F. Johnson. Functional and structural polymorphism of rabbit microsomal cytochrome P-450 form 3b. *J. Biol. Chem.* **257**:9315–9323 (1982).
28. Wallace, W. J., and W. S. Caughey. Mechanism for the autooxidation of hemoglobin by phenols, nitrite and "oxidant" drugs: peroxide formation by one electron donation to bound dioxygen. *Biochem. Biophys. Res. Commun.* **62**:561–567 (1975).
29. Huang, M., S. B. West, and A. Y. H. Lu. Separation, purification, and properties of multiple forms of cytochrome P-450 from the liver microsomes of phenobarbital-treated mice. *J. Biol. Chem.* **254**:4659–4665 (1976).
30. White, R. E., and M. J. Coon. Oxygen activation by cytochrome P-450. *Annu. Rev. Biochem.* **49**:315–56 (1980).

Send reprint requests to: Dr. John J. Mieyal, Department of Pharmacology, Case Western Reserve University School of Medicine, 2119 Abington Road, Cleveland, Ohio 44106.