Monooxygenase Activity of Human Hemoglobin: Role of Quaternary Structure in the Preponderant Activity of the β Subunits within the Tetramer†

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ABSTRACT: Catalysis of para hydroxylation of aniline was measured for human ferrihemoglobin and various derivatives in a reconstituted system consisting of the appropriate hemoprotein (at 4 μM heme), reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome P-450 reductase, and aniline under atmospheric O_2. The isolated subunits of hemoglobin (α^{3+} and $\beta^{3+}{}_4$) were prepared by treatment with *p*-(hydroxymercuri)benzoate. Semihemoglobin ($\alpha^{heme}{}_2\beta^0{}_2$) was prepared from ferrihemoglobin and apohemoglobin. Converse valency hybrids $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ and $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ were prepared from appropriately ligated α and β subunits. After chromatography, the hemoglobin derivatives were characterized by visible and ^{1}H NMR spectroscopy and electrophoresis. At the same concentration of aniline, the α and β subunits were much less active than the normal tetramer. α-Semihemoglobin and the $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ hybrid also displayed lower hydroxylase activity. The $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ hybrid was about as active as normal $\alpha^{3+}{}_2\beta^{3+}{}_2$. This result suggests that the activity of tetrameric hemoglobin primarily involves the β subunits. Also transfer of the β subunits from the β_4 molecular environment to the $\alpha_2\beta_2$ state enhances their monooxygenase activity approximately 15-fold. The hemoglobin derivatives were differently susceptible to substrate inhibition, the β_4 species being most sensitive. Estimates of V_{max} from the linear portions of the corresponding Lineweaver–Burk plots showed agreement within a factor of 2.5 for all of the hemoglobin derivatives, suggesting that the intrinsic O_2-activating capacities of the derivatives are similar. Their different hydroxylase activities may reflect different modes of substrate association.

Hemoglobin (Hb),[1] from various species in purified form as well as in erythrocytes and hemolysates, catalyzes the para 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 (Juchau & Symms, 1972; Mieyal et al., 1976; Jonen et al., 1976; Blisard & Mieyal, 1979, 1980, 1981). 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 similar to those for the P-450 system under comparable conditions (Mieyal et al., 1976). Moreover, Hb in the reconstituted system catalyzes at least 10 different monooxygenase-like reactions including N- and O-dealkylation reactions with a broad range of efficiencies (Starke et al., 1984). Hb in its natural environment within erythrocytes and hemolysates also displayed such activity, and NADPH was demonstrated to be the preferred cofactor (Blisard & Mieyal, 1979, 1981; Starke et al., 1984). The hydroxylase activity of fetal hemolysates was about 5 times greater than that of adult hemolysates (Blisard & Mieyal, 1980). This difference in activities was ascribed to an intrinsic property of fetal Hb itself, because a difference of similar magnitude was observed with the purified hemoglobins. The difference in subunit composition between fetal hemoglobin (HbF, $\alpha_2\gamma_2$) and adult hemoglobin (HbA, $\alpha_2\beta_2$) suggested that the non α subunits in each case might play a more important role in determining the overall hydroxylase activity of tetrameric hemoglobin. Previous studies have revealed physical differences between the α and β subunits of human Hb, including primary and tertiary structure (Perutz, 1970; Mansuy et al., 1978), and chemical differences, including ligand binding (Olson & Binger, 1976; Nakamura et al., 1973; Weber, 1971) and redox behavior (MacQuarrie & Gibson, 1971).

Direct support for a greater role of the β subunits in mediating the monooxygenase activity of Hb came from our recent ^{13}C NMR studies. Asymmetric displacement of O_2 by [^{13}C]carbon monoxide led to concomitant inhibition of the Hb-catalyzed hydroxylation of aniline in rabbit hemolysates (Ferraiolo & Mieyal, 1982). There was a nearly linear relationship between the degree of inhibition and the degree of CO saturation of the β subunits, regardless of the ligation of the α subunits. Therefore, the β subunits appeared to contribute most, if not all, of the activity of the tetrameric rabbit hemoglobin. The current studies were undertaken to resolve whether such selective action of the Hb subunits would represent a difference in the intrinsic monooxygenase-like activity of the α and β subunits or would reflect a modulation of the activity of one or both of the subunits due to quaternary structural effects. The data presented herein support the latter interpretation. The isolated α^{3+} and $\beta^{3+}{}_4$ subunits of human hemoglobin and the α-ferric hybrid $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ in which the β-heme sites are blocked by CO all displayed only low hydroxylase efficiencies (V_{max}/K_M). In contrast, the β-ferric hybrid $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ with the α subunits blocked was a more

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[1] Abbreviations: Hb, hemoglobin; NMR, nuclear magnetic resonance; pAP, *p*-aminophenol; PMB, *p*-(hydroxymercuri)benzoate (sodium salt); TSP, (trimethylsilyl)propionate (sodium salt); 2,3-DPG, 2,3-diphosphoglycerate; Tris, tris(hydroxymethyl)aminomethane; Mb, myoglobin; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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efficient catalyst by 15–40 times, essentially the same as normal ferrihemoglobin $\alpha^{3+}{}_2\beta^{3+}{}_2$. Different degrees of substrate inhibition by aniline at higher concentrations were observed for the various hemoglobin derivatives.

Experimental Procedures

Materials. Diethyl ether was prewashed with a 10% $FeSO_4$ solution and then washed with water to remove any peroxide degradation products. Aniline was fractionally distilled, purged with N_2 gas, and stored frozen (−70 °C) until used. PMB was purified by the method of Boyer (1954). Blood was obtained by venipuncture from normal volunteers (20–35 years old). Usually 50 mL of blood was drawn and mixed with 0.5 mL of heparin solution (1000 units/mL) as anticoagulant. When larger volumes were necessary, outdated blood was obtained from the Red Cross. For all preparations of isolated hemoglobin and its derivatives, the initial composite blood sample was pooled from those of several individuals. The erythrocytes were isolated by centrifugation at 4000*g* for 10 min at 4 °C. The packed cells were then washed 3 times with 0.9% NaCl. The packed, washed cells were lysed with 3 volumes of distilled water for 30 min at room temperature. Cell debris was removed by centrifugation at 30000*g* for 30 min. The Hb concentration was measured as the cyanoferrihemoglobin complex (extinction coefficient = 11 mM^{-1} cm^{-1} per heme at 541 nm) according to the procedure of Van Kampen & Zijlstra (1961). All other chemicals were reagent grade.

Hemoglobin Isolation and Purification. Hemoglobin was purified by the method of Riggs (1981). Ferric hemoglobin was prepared from oxy- or (carbonmonoxy)hemoglobin in 0.1 M potassium phosphate, pH 7, by the addition of a 1.2 molar excess of potassium ferricyanide under a nitrogen atmosphere. The mixture was then passed over Bio-Rad AG1-X8 (chloride form) equilibrated with 0.1 M potassium phosphate, pH 7, to remove residual ferricyanide and ferrocyanide. The α and β subunits of hemoglobin were separated by the method of Geraci et al. (1969) after reaction with PMB. PMB was removed from the α subunits by reaction with 2-mercaptoethanol by the method of Geraci et al. (1969). PMB was removed from the β subunits by the method of Tyuma et al. (1966). Mercury determinations by atomic absorption were performed at Lubrizol Corp. (Cleveland, OH) on the α-O_2 and β-O_2 subunits. No mercury was observed at the limit of detection, indicating less than 1 mol of Hg/735 mol of Hb chains. The purity of the separated, mercury-free subunits was confirmed by electrophoresis on cellogel (Isolab) on a horizontal apparatus with Tris–glycine buffer, pH 8.7, for 1.5 h at 200 V, 4 °C. The oxyferrous forms of the α and β subunits were visualized with benzidine stain (Broyles et al., 1979), and they were separated from each other by approximately 2 cm under these conditions. No cross-contamination was evident (Ferraiolo, 1982). The ferric subunits of Hb were prepared from the oxy subunits by the method of Banerjee & Cassoly (1969a,b), followed by passage over Bio-Rad AG1-X8. Apohemoglobin (globin) was prepared by the method of Rossi-Fanelli & Antonini (1958). Protein concentrations were determined according to Lowry et al. (1951), and molecular weights of proteins were estimated by gel filtration on a Sephadex G-100 column (Andrews, 1964).

Preparation of the Valency Hybrids. The hybrids $\alpha^{3+}{}_2$-$(\beta^{2+}\text{-CO})_2$ and $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ were prepared by the method of Cassoly (1981). Their composition and purity were confirmed in three ways: (a) Visible spectra were obtained on an Aminco DW/2 spectrophotometer operated in the split beam mode in the wavelength region 350–650 nm. Each of the hybrids gave spectra resembling a mixture of the ferric and (carbonmonoxy)ferrous forms of hemoglobin, consistent with their dual nature; i.e., the ratio of absorbance (408/420) approximated 1.0 (Ferraiolo, 1982) (see Figure 1). (b) Isoelectric focusing was performed either on agarose slab gels (Figure 2A) or in acrylamide tube gels (Figure 2B), utilizing a cathode buffer containing 0.2% pH 7–9 ampholytes (Isolab)/0.02 M $Ca(OH)_2$ and an anode buffer containing 0.2% pH 5–8 ampholytes or 0.033 M citric acid. Slab gels of 10% agarose containing 2% pH 6–8 ampholytes were focused for 20 min at a constant voltage of 500 V. The middle to lower portions of these gels (pH range = 7–8) were cut out, remelted, and re-formed to give gels with a narrower pH range. These gels were then used for testing the purity of the valency hybrids. With the Isolab Resolve apparatus, resolution of the valency hybrids from ferrihemoglobin and HbCO could be achieved in approximately 20 min (Figure 2A). Tube gels (5 mm i.d. × 125 mm) were 7.5% acrylamide, 2% ampholytes (60% pH 6–8, 40% pH 3–10), and 0.07% aluminum persulfate; after a 6-h prefocusing, samples were loaded and isoelectric focusing was performed for approximately 6 h at 400 V in the

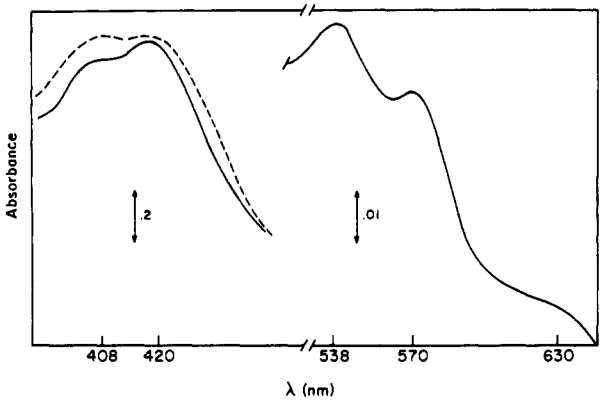

FIGURE 1: Tracings of visible spectra of the valency hybrids. Spectra (350–650 nm) were obtained at room temperature for solutions containing valency hybrids at approximately 4 μM concentration with respect to heme in 20 mM potassium phosphate, pH 6.8, in the presence of carbon monoxide. The dotted line in the Soret region represents the spectrum of the $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ hybrid, and the solid line represents the spectrum of the $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ hybrid, both in the Soret region and at longer wavelengths. During purification procedures, fractions were monitored by recording the visible spectra in the 350–450-nm region. Those fractions displaying absorbance ratios (408/420) near 1.0 were pooled, and the purity was confirmed by isoelectric focusing (see Figure 2).

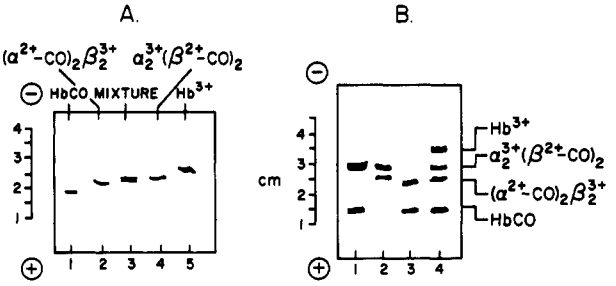

FIGURE 2: Tracings of the isoelectric focusing of the valency hybrids $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ and $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2 \pm Hb^{3+}$ and HbCO. (A) Isoelectric focusing was performed for approximately 20 min (to the time of visually observed maximum separation) on a 10% agarose gel containing an ampholyte gradient covering the range pH 7–8 as described in the text. The lanes depict the following components: (1) HbCO, (2) $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$, (3) a mixture of hybrids, (4) $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$, and (5) Hb^{3+}. (B) Isoelectric focusing was performed at 4 °C for 6 h with a 6-h prefocusing. Gels were pH 6–8; the cathode buffer was 0.2% pH 7–9 ampholytes/0.02 M $Ca(OH)_2$ and the anode buffer was 0.2% pH 5–8 ampholytes. Lane 1 depicts $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$ and HbCO, lane 2 shows the two hybrids, lane 3 shows $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$ and HbCO, and lane 4 shows all four species (HbO_2 comigrates with HbCO).

cold (4 °C) (Figure 2B). (c) ^1H NMR spectra were obtained on a Bruker 270-MHz (63-kG magnet) pulsed Fourier transform spectrometer. Usual conditions were a pulse width of 6 μs and a spectral width of 31 250 Hz; the delay between acquisitions was 2 s. Internal D_2O served as the frequency lock. All spectra were obtained at ambient temperature (~19 °C). Sodium (trimethylsilyl)propionate (TSP) served as the internal chemical shift reference for 0 ppm. The spectra were solvent suppressed by using a microprogram for homonuclear decoupling. All samples were prepared in 0.1 M potassium phosphate, observed pH 6.8, in D_2O. They were lyophilized to dryness and then reconstituted with D_2O in order to minimize H_2O content. It was especially critical to establish that the valency hybrids actually did possess heme in different valence and ligation states on the different subunits. This was accomplished conveniently by observing the 270-MHz ^1H NMR spectra of each of the hybrids after addition of KCN to ligate the respective ferric hemes, e.g., $(\alpha^{3+}\text{-CN})_2(\beta^{2+}\text{-CO})_2$ (Figure 3). In each case, only the paramagnetic ferric heme iron gives rise to contact-shifted resonances in the extreme regions of the spectra. The chemical shifts of these characteristic signals are given in the legend to Figure 3, and they agree with those reported previously by Ogawa et al. (1972) for analogous cyanoferric/(carbonmonoxy)ferrous hybrids and isolated cyanoferric subunits under identical conditions. After the spectra shown were recorded, both of the valency hybrids were then treated with a 1.2-fold excess of potassium ferricyanide and KCN to convert the remaining ferrous subunits to the cyanoferric state. A spectrum consistent with that of Hb^{3+}-CN (Figure 3, lower spectrum) was generated by this procedure from both hybrids, indicating that both subunits were present in each preparation, one in the ferric heme form and one in the ferrous heme form.

Ferric Semihemoglobin Preparation. Semi-Hb was prepared by mixing equivalent amounts of ferrihemoglobin and dimeric apohemoglobin, followed by purification according to the method of Winterhalter & Deranleau (1967). The semi-Hb was verified as $\alpha^{heme}{}_2\beta^0{}_2$ in three ways. (a) The heme absorbance to protein absorbance ratio was shown to be half that of normal ferrihemoglobin (Ferraiolo, 1982; Winterhalter & Deranleau, 1967). (b) Cellogel electrophoresis was performed as described above for the hemoglobin subunits. The semihemoglobin band was well resolved from that of normal hemoglobin (Ferraiolo, 1982). (c) The ^1H NMR spectrum of the cyanoferric derivative of the semihemoglobin displayed signals characteristic of the presence of the α-ferric heme and not the β-heme, analogous to Figure 3, middle spectrum.

Hydroxylation of Aniline by Hemoglobin and Its Derivatives. Aniline hydroxylase activity was measured in a reconstituted monooxygenase system as described by Mieyal et al. (1976). The reaction mixture consisted of a total volume of 1 mL containing aniline at the appropriate concentration, 20 mM potassium phosphate, pH 6.8, 4 μM hemoprotein (with respect to heme), 0.2 mM NADPH, and 0.02–0.03 unit (micromoles of cytochrome *c* reduced per minute) of partially purified rat liver cytochrome P-450 reductase (Mieyal et al., 1976). This amount of reductase was shown to provide maximum activity. As demonstrated previously, the reductase contains no P-450 and is inactive as a monooxygenase catalyst in the absence of Hb or P-450. We have confirmed separately that highly purified P-450 reductase also couples with Hb in this system. Reaction mixtures were preincubated for 3 min at 37 °C and reactions initiated with NADPH. The reactions were allowed to proceed for 15 min at 37 °C; then they were terminated by addition of 0.3 mL of ice-cold 20% trichloroacetic acid and submersion in an ice bath.

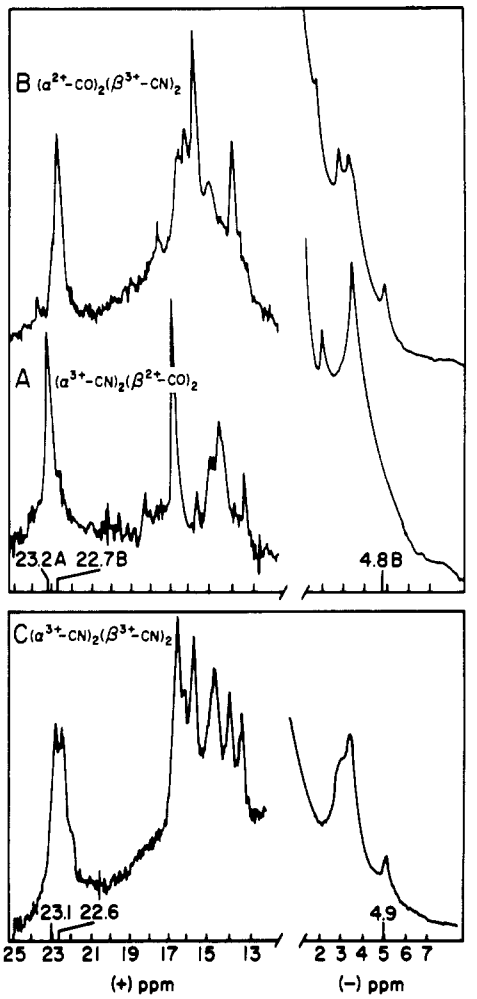

FIGURE 3: ^1H NMR spectra of human hemoglobin and its hybrids. The 270-MHz ^1H NMR spectra of the valency hybrids in the presence of 5 mM KCN were acquired at room temperature in 0.1 M potassium phosphate, pH_{obsd} 6.6 (in D_2O). TSP (0.4 mM) was the internal chemical shift reference for 0 ppm. The parts per million scale is interrupted on this display to delete the resonance at 4.86 ppm corresponding to residual water that was far off scale at the sensitivity setting required to visualize the heme-associated resonances. The region of 11–25 ppm is depicted at 4 times the sensitivity of the region of −1 to −8 ppm. Spectrum A represents 4 mM (with respect to heme) $\alpha^{3+}{}_2(\beta^{2+}\text{-CO})_2$. This spectrum was solvent suppressed and represents 11 500 acquisitions of a 31 250-Hz sweep width. The chemical shifts of the significant resonances were 23.2, 16.8, 14.5, 13.3, −1.9, and −3.3 ppm. Spectrum B represents 4.6 mM (with respect to heme) $(\alpha^{2+}\text{-CO})_2\beta^{3+}{}_2$. This spectrum was solvent suppressed and represents 1000 acquisitions of a 31 250-Hz sweep width. The chemical shifts of the significant resonances were 22.7, 15.7, 13.9, −2.8, −3.2, and −4.8 ppm. Spectrum C represents 6.4 mM (with respect to heme) Hb^{3+} in the presence of 12 mM KCN and 0.4 mM TSP. This spectrum was solvent suppressed and accumulated from 1000 acquisitions at a 31 250-Hz sweep width. The region of 17–26 ppm is depicted at 4 times the sensitivity of the region −1 to −8 ppm. The chemical shifts of the significant resonances were 23.1, 22.6, 16.6, 15.7, 14.5, 13.7, 13.0, −3.2, −3.5, and −4.9 ppm.

Results

Isolated Subunits of Hemoglobin. Human hemoglobin was chosen for this study, because the methodology for preparation of its derivatives has been established. The Hb subunits were isolated and characterized as pure monomeric α^{3+} and tetrameric $\beta^{3+}{}_4$ as described under Experimental Procedures. The isolated chains were tested for their ability to combine and re-form tetrameric Hb with the full catalytic activity and visible spectrum of untreated Hb^{3+}. These measures were used as the criteria to confirm that the subunits as isolated were

Table I: Aniline Hydroxylase Activity of Hemoglobin Derivatives[a]

hemoglobin derivative (4 μM with respect to heme)	activity[b] (pmol of pAP/min)
ferrihemoglobin, $\alpha^{3+}_2\beta^{3+}_2$	74.7 ± 2.9 (14)
α subunit, α^{3+}	31.3 ± 1.3 (5)
β subunit, β^{3+}_4	9.9 ± 1.1 (11)
α-semihemoglobin, $\alpha^{3+}_2\beta^{0}_2$	20.7 ± 3.3 (5)
α-ferric hybrid, $\alpha^{3+}_2(\beta^{2+}\text{-CO})_2$	38.1 ± 2.6 (15)
β-ferric hybrid, $(\alpha^{2+}\text{-CO})_2\beta^{3+}_2$	75.9 ± 2.0 (8)

[a] The experiments were performed with the reconstituted system containing in 1 mL the appropriate hemoprotein at a concentration of 4 μM with respect to heme, 0.2 mM NADPH, and 0.02–0.03 unit of cytochrome P-450 reductase in 0.02 M potassium phosphate, pH 6.8. Reactions were carried out at 37 °C for 15 min. The basis for these conditions was described previously (Mieyal et al., 1976). An aniline concentration of 40 mM represents approximately 5 times the K_M value determined with Hb^{3+}, i.e., an essentially saturating concentration. Therefore, this concentration was chosen for the initial comparison among the derivatives of Hb^{3+}. Product pAP was assayed as described previously. The number of determinations made (at least in triplicate) is indicated in parentheses. The mean values are presented ± SE. [b] The hemoprotein stock solution (40 μM) was bubbled with CO for 30 s at a flow rate of 20 (Matheson flow meter, no. 610) before dilution (1/10) into the reaction mixture. It was confirmed in separate experiments that this procedure ensured that the valency hybrids remained as constituted throughout the incubation period (see text). The activity of ferrihemoglobin was not altered by like treatment; i.e., the amount of unliganded CO in the stock solution diluted into the reaction mixture was insufficient to trap the new ferrous hemes formed via turnover of the originally ferric hemes during the course of the monooxygenase reaction cycle.

native and that the catalytic activity they displayed represented their intrinsic catalytic activity under the conditions employed. The aniline hydroxylase activity of Hb^{3+} made from the isolated subunits was indistinguishable from that of Hb^{3+} which was not split into subunits and recombined, and the visible spectra of the recombined and untreated hemoglobins were also identical. The activity data for the isolated subunits in comparison to normal Hb^{3+} are shown in the first three lines of Table I. Both subunits clearly possess intrinsic catalytic activity, but neither is comparable to the intact tetramer at equivalent heme concentrations. Moreover, the composite activity of the subunits (31.3/2 + 9.9/2 = 20.6) is less than one-third that of Hb^{3+} (74.7), suggesting that the activity of one or both of the subunits must be altered by incorporation into the $\alpha_2\beta_2$ tetramer.

Tetrameric Hybrids of Hemoglobin. As the first step in testing the interpretation that oligomeric structure might affect the activities of the subunits, tetrameric α-semihemoglobin ($\alpha^{3+}_2\beta^{0}_2$), which contains ferriheme only on the α subunits, was prepared; the β subunits are devoid of heme (see Experimental Procedures for characterization). Comparison of line 4 with line 2 (Table I) indicates that incorporation of the α-ferriheme subunits into the $\alpha_2\beta_2$ structure per se has little effect on their hydroxylase activity. The α-semihemoglobin appears to be about 70% as active as isolated α.

The situation was different for the converse pair of valency hybrids. Here, either the α-hemes or the β-hemes contain iron in the ferric (3+) state and are available for catalysis, while the other hemes in each case are blocked by the inhibitor carbon monoxide. As described under Experimental Procedures, the valency hybrids were characterized by electrophoresis and isoelectric focusing and by visible and ^1H NMR spectroscopy. Because of the possibility of CO/O_2 exchange with these derivatives, however, it was necessary also to establish a procedure to ensure that the ferrous subunits in each case would remain CO ligated during the reaction period. As described in the legend (Table I), stock solutions of the hybrids were bubbled with a metered amount of CO just prior to 10-fold dilution into the reaction mixture. If this step was omitted or if concentrated stock solutions of the hybrids were diluted by a much greater factor, then additional hydroxylase activity was observed, which could be ascribed to O_2 replacement of the CO on the ferrous subunits. Furthermore, it was confirmed in separate experiments that all of the hemoglobin derivatives have linear time courses over the 15-min period of observation, and half-concentrations of the hemoproteins gave half-activities as expected. In addition, admixtures of Hb^{3+} with each of the valency hybrids, and admixtures of the hybrids with each other, gave activities that agreed within 10% of the values predicted in each case from the average of the activities of the separate components. All of these tests documented that the various hemoglobin derivatives behaved as distinct entities during the course of the reactions and displayed activities unique to their separate molecular forms. The hydroxylase activities of the converse valency hybrids are given in lines 5 and 6 (Table I). When activity is calculated on a per ferriheme basis, the α-ferric hybrid (line 5, Table I) is about 2.4 times more active than the isolated monomeric α^{3+} subunits (line 2) (i.e., 38.1/2 vs. 31.3/4), indicating that the α subunits are activated when associated with CO-ligated ferroheme β subunits. The β-ferric hybrid (line 6) on the same basis remarkably is 15 times more active than the isolated tetrameric β^{3+} subunits (line 3) (i.e., 75.9/2 vs. 9.9/4). Hence the hydroxylase activity of the β subunits appears to be much more sensitive to changes in quaternary structure.

If the activities of the two hybrids simply represented the intrinsic contributions of the α- and β-ferric subunits to the net activity of Hb^{3+}, then their sum should equal the activity of Hb^{3+}. This cannot be the case, because the activity of the β-ferric hybrid alone is already essentially the same as that of Hb^{3+}. The similarity of the activities for the β-ferric hybrid and Hb^{3+} suggests either that the α subunits are inactive in Hb^{3+} or that the activities of both the α and β subunits have changed coincidentally to give a net result for Hb^{3+} that is the same as that for the β-ferric hybrid. The NMR data presented in the following paper (Onady et al., 1984) favor the former interpretation (see Discussion).

Dependence of Hydroxylase Activity of Hemoglobin Derivatives on Aniline Concentration. Comparison of the hydroxylase activities of the hemoglobin derivatives was originally conducted at a single concentration of the substrate aniline that had been shown previously to be essentially saturating, i.e., 5 times the K_M value for the ferrihemoglobin-catalyzed reaction (Mieyal et al., 1976). Marked differences in activities (Table I), however, suggested that the hemoglobin derivatives might differ either in their intrinsic activities (V_{max}) or in their interaction with substrate (K_M). Therefore, complete aniline concentration profiles were obtained for each Hb species (Figure 4). Substrate inhibition occurs with each of the Hb derivatives, albeit to different degrees. The β^{3+}_4 species appears to be most sensitive to this interference. If the linear portions of the Lineweaver–Burk double reciprocal replots of the data (Figure 4, inset) are extrapolated, apparent K_M and V_{max} values may be estimated. In contrast to the broad range of apparent activities observed at 40 mM aniline concentration (Table I), the V_{max} values (Table II) are all within a factor of 2.5. This result suggests that the various hemoglobin derivatives possess similar intrinsic aniline hydroxylase activities. Besides different degrees of substrate inhibition, the differences among the estimated K_M values covering a 40-fold range (Table II) for the different hemoglobin species suggest that the expression of hydroxylase activity for these hemoproteins

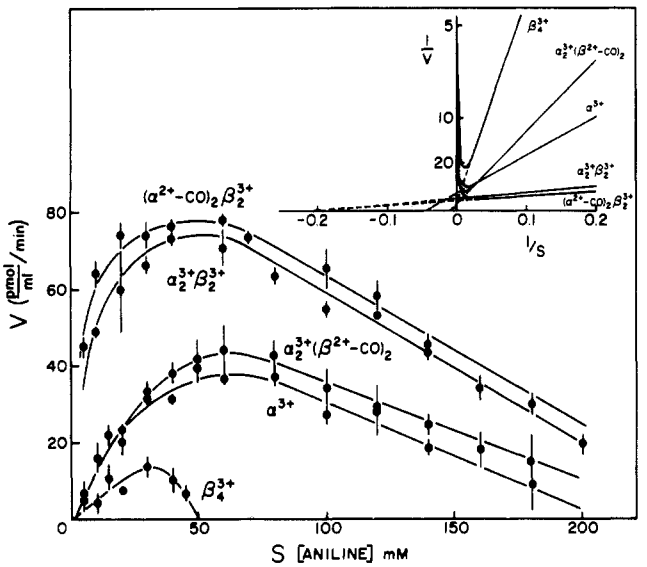

FIGURE 4: Dependence of hydroxylase activity of the hemoglobin derivatives on aniline concentration. Experiments were performed as described in Table I where all hemoproteins were tested at 4 μM concentration with respect to total heme, except that the aniline concentration was varied as indicated. The inset represents a double reciprocal ($1/V$ vs. $1/S$) replot of the V vs. S data. The linear portions of the double reciprocal curves were extrapolated by linear regression analysis (see r values, Table II) to the y axis and x axis to estimate $1/V_{max}$ and $-1/K_M$, respectively. The V_{max} and K_M values for each hemoglobin derivative are given in Table II.

Table II: Kinetic Values for Aniline Hydroxylation by Hemoglobin Derivatives[a]

hemoglobin derivative	V_{max} (pmol min^{-1} mL^{-1})	K_M (mM)	V_{max}/K_M	r
$\alpha^{3+}_2\beta^{3+}_2$	77	6	12.8	0.935
α^{3+}	52	22	2.4	0.960
β^{3+}_4	97	202	0.5	0.956
$\alpha^{3+}_2(\beta^{2+}\text{-CO})_2$	128	102	1.3	0.999
$(\alpha^{2+}\text{-CO})_2\beta^{3+}_2$	89	5	17.8	0.988

[a] Values were calculated from the data presented in Figure 4 as described in the legend to that figure (r refers to the regression coefficient).

is governed predominantly by the nature of the substrate association with the hemoprotein catalyst. [See Discussion and following paper (Onady et al., 1984).]

Discussion

Basis for Different Monooxygenase Activities of Hemoglobin Derivatives. Previous data on CO inhibition of tetrameric Hb in rabbit hemolysates suggested that the β subunits contributed most, if not all, of the monooxygenase-like activity (Ferraiolo & Mieyal, 1982). We set out in the present study to determine whether isolated hemoglobin displayed similar asymmetric catalysis, and whether this reflected a difference in the intrinsic hydroxylase activities of the individual subunits, or more likely whether the association into the specific $\alpha_2\beta_2$ tetrameric form affected the activities of the two types of subunits differently. The latter supposition apparently was borne out by comparison of various derivatives of hemoglobin at equivalent heme concentrations and a constant concentration of aniline (Table I). The isolated α subunits do display some aniline hydroxylase activity, and this is not much altered when they associate with β-globin to form α-semihemoglobin. In contrast, the β subunits are poorly active as the homologous tetramer β_4, but they apparently dominate the activity of the heterologous $\alpha_2\beta_2$ tetramer, as shown by the greater activity of the β-ferric hybrid relative to the α-ferric hybrid. These results support the hypothesis that the expressed activities of the individual subunits are affected differently by their pairing in the tetramer. The β activity is markedly increased upon association with the α subunits, whereas the α activity appears to be diminished slightly by association with β globin and enhanced by association with β^{2+}-CO, and it may be virutally abolished by association with β^{3+} (see below). The significance of these observations is highlighted by their contrast to the relatively small differential influence that the tetrameric structure seems to have on certain other properties of hemoglobin. Thus, molecules of the type $(\alpha^{2+}\text{-NO})_2(\beta^{2+}\text{-O}_2)_2$ or $(\alpha^{3+}\text{-CN})_2(\beta^{2+}\text{-O}_2)_2$ and their converse hybrids display similar properties (Brunori et al., 1970). Another example is the maintenance of the absolute values and the difference in the half-reduction potentials of the α and β subunits when they are associated into the tetrameric structure (Banerjee & Cassoly, 1969a). Also, although the β chains in Hb^{3+} and in hybrids of Hb react faster with ligands such as azide, these relative reactivities are similar to the relative behavior of the isolated subunits, so they do not represent a differential effect (Gibson et al., 1969). The properties of HbM Milwaukee [β67(E11) valine $\rightarrow$ glutamine], which is a naturally occurring valency hybrid of the form $\alpha^{2+}_2\beta^{3+}_2$ (i.e., low oxygen affinity relative to the isolated subunits and absence of cooperative interactions and Bohr effect), are similar to those of HbM Iwate [α87(F8) histidine $\rightarrow$ tyrosine] even though it is a valency hybrid of the converse type $\alpha^{3+}_2\beta^{2+}_2$ (Fung et al., 1976; Salhany et al., 1976). Therefore, although the α and β subunits of Hb differ in many respects and some of their properties are greatly altered in parallel by association into the tetrameric form, the differential effect that we have observed for the catalytic activity of the subunits stands out by virtue of its selectivity and magnitude, i.e., 15-fold higher in $\alpha^{3+}_2\beta^{3+}_2$ or $(\alpha^{2+}\text{-CO})_2\beta^{3+}_2$ relative to β^{3+}_4. Three main factors that might be responsible for the differential expression of the hydroxylase activities of the α and β subunits in the tetramers are (1) the relative affinities of the subunits for molecular O_2, which is the required second substrate for the monooxygenase reactions, (2) the relative ability of the subunits to activate O_2, and (3) the relative interactions of the subunits with the substrate aniline. These will be addressed in turn, the third factor being considered most likely.

(1) There are several reasons to dismiss the concept that differences in O_2 affinity could be the deciding factor for differences in monooxygenase activity. First, the isolated α and β subunits and myoglobin all have higher O_2 affinities than Hb; however, on a per heme basis the α and β subunits have lower monooxygenase activities than Hb (Table I) and Mb has a somewhat higher activity (1.6-fold; Mieyal et al., 1976). Second, the catalytic activity of HbF is the same in the presence of 2,3-DPG in hemolysates as it is in isolation, stripped of 2,3-DPG (Blisard & Mieyal, 1980). In contrast, the relative oxygen affinities of HbF and HbA are reversed under the two different conditions (i.e., in the presence of 2,3-DPG, the O_2 affinity is in the order HbF > HbA; without 2,3-DPG, HbA > HbF).

(2) The hydroxylase reaction scheme as conceived for Hb and cytochrome P-450 [Mieyal et al. (1976) and White & Coon (1980), respectively] involves the transfer of electrons from the iron atom (specifically in this case to bound O_2). Accordingly, differences in the oxidizability of the ferrous heme iron atoms on the separate subunits or, more particularly, differences in the autoxidizability of the respective oxyferrous hemes might be related to different O_2-activating capacities and monooxygenase activities. This hypothesis, however, does

not appear to be borne out by known facts. Although the isolated α subunits are oxidized at a faster rate than the β subunits, the difference in half-reduction potential (β = +0.113 V, α = +0.052 V) is maintained upon combination into the tetrameric form (Banerjee & Cassoly, 1969a,b); i.e., no selective change occurs. In addition, although the redox potential of myoglobin is similar to that of the α subunit of Hb, the catalytic activity of Mb is substantially higher (Mieyal et al., 1976). The redox potential and autoxidation rate of isolated HbF are essentially equivalent to those of HbA (Floke & Uehleke, 1966; Betke et al., 1956). However, the catalytic activity of HbF is 5 times greater than that of HbA (Blisard & Mieyal, 1980). Finally, the values of V_{max} estimated for all of the Hb derivatives agree within a factor of 2.5 and most agree more closely (legend, Figure 2), so it appears they may not differ substantially in intrinsic O_2-activating capacity.

(3) The third consideration here is the possibility of unique interactions of the substrate aniline with the various Hb derivatives. There appears to be no obvious relationship between catalytic activity and quaternary structure of the hemoproteins per se. The α subunits are monomeric and display low activity. In contrast, myoglobin has catalytic activity like that of HbA, yet it is monomeric. Moreover, there was little difference in the activities of the PMB α and PMB β subunits, which both exist as monomers.[2]

If there is a structural explanation for the differential effect on the catalytic activities, it must be on a more subtle molecular level than the mere formation of tetramers. The most attractive or productive binding site seems to be formed by conformational changes induced in the β subunits by interaction with the α subunits. Consistent with this interpretation is the marked substrate inhibition of the β_4 species that is relieved when the β subunits are associated with the α subunits (Figure 4). Also, the estimated K_M values are most favorable for Hb^{3+} and the $(\alpha^{2+}\text{-}CO)_2\beta^{3+}{}_2$ hybrid (Table II). NMR relaxation studies suggest that the observed substrate inhibition is due to direct ligation of aniline to the heme iron sites and that efficient catalysis is related to favorable orientation of the substrate on a protein site near the heme (Onady et al., 1984). The identity of the activities of $\alpha^{3+}{}_2\beta^{3+}{}_2$ and $(\alpha^{2+}\text{-}CO)_2\beta^{3+}{}_2$ (Table I) could be explained by these NMR studies, which showed that the substrate aniline approaches the heme moieties closely only on the β subunits in Hb^{3+}. Aniline does approach the α-hemes in the α-ferric hybrid where the α subunits are responsible for the hydroxylase activity (Table I).

Relationship of Hemoglobin Reactivity to Other Oligomeric Oxygen-Activating Hemoproteins. The observation of differential subunit participation in hemoglobin-mediated aniline hydroxylation may be especially pertinent to further investigation of the activity of microsomal cytochrome P-450, which also exists in oligomeric forms in the membrane as well as after solubilization and isolation (McIntosh et al., 1980; Guengerich & Holladay, 1979). Since cytochrome P-450 exists as multiple isozymes, these may associate as homologous oligomers analogous to β_4 or heterologous oligomers like the $\alpha_2\beta_2$ or $\alpha_2\gamma_2$ forms of Hb. The isolated P-450-LM_2 isozyme can be converted to the monomeric state by octyl glucoside (Dean & Gray, 1982). The monomers showed no monooxygenase activity, but the interpretation was ambiguous because of possible effects of the bound detergent on the native state of the monomers or their interaction with the P-450 reductase. In assessing subunit contributions to the overall expression of P-450 activity, it will be difficult to find conditions for preparation of native monomers or for estimation of their native properties by extrapolation to nondetergent conditions. The Hb system as a model for multiple heme oligomeric oxygenase activity avoids this difficulty, but it is lacking in other respects. Hb is a regular tetramer whereas P-450 is a less ordered aggregate. The subunit molecular weights differ (16 500 for Hb vs. 50 000 for P-450) as do the amino acid compositions. Most notably, the binding of the prosthetic heme to the respective hemoproteins differs fundamentally. Cytochrome P-450 has a cysteinyl thiolate as the fifth ligand to the heme iron atom, and this moiety has been implicated in the intimate molecular mechanism of oxygen activation by the P-450 system (White & Coon, 1980). In contrast, hemoglobin has a histidyl nitrogen as the fifth ligand. Nevertheless, Hb can catalyze at least 10 different P-450-like reactions (Starke et al., 1984).

[2] B. L. Ferraiolo, unpublished observations.

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

References

Andrews, P. (1964) *Biochem. J. 91*, 222–223.
Banerjee, R., & Cassoly, R. (1969a) *J. Mol. Biol. 42*, 337–350.
Banerjee, R., & Cassoly, R. (1969b) *J. Mol. Biol. 42*, 351–361.
Betke, K., Kleihauer, E., & Lipps, M. (1956) *Z. Kinderheilkd. 77*, 549–553.
Blisard, K. S., & Mieyal, J. J. (1979) *J. Bicl. Chem. 254*, 5104–5110.
Blisard, K. S., & Mieyal, J. J. (1980) *Biochem. Biophys. Res. Commun. 96*, 1261–1266.
Blisard, K. S., & Mieyal, J. J. (1981) *Arch. Biochem. Biophys. 210*, 762–769.
Boyer, P. D. (1954) *J. Am. Chem. Soc. 76*, 4331–4337.
Broyles, F. H., Pack, B. M., Berger, S., & Dorn, A. R. (1979) *Anal. Biochem. 94*, 211–219.
Brunori, M., Amiconi, G., Antonini, E., Wyman, J., & Winterhalter, K. H. (1970) *J. Mol. Biol. 49*, 461–471.
Cassoly, F. (1981) *Methods Enzymol. 76*, 106–112.
Dean, W. L., & Gray, R. D. (1982) *J. Biol. Chem. 257*, 14679–14685.
Ferraiolo, B. L. (1982) Ph.D. Thesis, Case Western Reserve University School of Medicine, Cleveland, OH.
Ferraiolo, B. L., & Mieyal, J. J. (1982) *Mol. Pharmacol. 21*, 1–4.
Floke, L., & Uehleke, H. (1966) *Life Sci. 5*, 1041–1045.
Fung, L. W. M., Minton, A. P., & Ho, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1581–1585.
Geraci, G., Parkhurst, L. J., & Gibson, Q. H. (1969) *J. Biol. Chem. 244*, 4664–4667.
Gibson, Q. H., Parkhurst, L. J., & Geraci, G. (1969) *J. Biol. Chem. 244*, 4668–4676.
Guengerich, F. P., & Holladay, L. A. (1979) *Biochemistry 18*, 5442–5449.
Jonen, H. G., Kahl, R., & Kahl, G. F. (1976) *Xenobiotica 6*, 307–320.
Juchau, M. R., & Symms, K. G. (1972) *Biochem. Pharmacol. 21*, 2053–2064.
Lowry, O. M., Rosebrough, J. T., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
MacQuarrie, R. A., & Gibson, Q. H. (1971) *J. Biol. Chem. 246*, 517–522.

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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