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SHORT COMMUNICATION

Subunit Selectivity in the Monooxygenase-Like Activity of Tetrameric Hemoglobin

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SUMMARY

The aniline hydroxylase activity of adult rabbit hemolysates (1 mM with respect to hemoglobin concentration) was found to be 80 pmoles of *p*-aminophenol formed per minute per milliliter. This value is comparable to that observed for adult human hemolysates. The characteristics of this O_2-requiring aromatic *p*-hydroxylation reaction are typical of the monooxygenase reactions catalyzed by the liver microsomal cytochrome P-450 system. Both systems are inhibited by carbon monoxide, which coordinates directly with the heme iron atoms of the respective hemoproteins. With the use of ^{13}C-NMR spectroscopy, separate, well-resolved signals were observed for ^{13}C-enriched carbon monoxide bound to the α- and β-subunits of the tetrameric ($\alpha_2\beta_2$) rabbit hemoglobin. By appropriately adjusting conditions, the hemoglobin was converted into hybrids of ligation varying from full oxygenation to intermediate forms in which the oxygen was progressively replaced by ^{13}CO, first on the β-subunits, then on the α-subunits until full CO ligation was accomplished. The state of ligation of the hemoglobin in each case was determined from the integrated areas of the signals in the corresponding ^{13}C-NMR spectra. The corresponding aniline hydroxylase activity of the rabbit hemolysates containing such hybrids revealed that the monooxygenase activity of intact tetrameric hemoglobin is determined predominantly (if not exclusively) by the ligation of the β-subunits. To the best of our knowledge, this is the first report of differential subunit behavior for a monooxygenase-like enzymatic activity.

We and others (1–6) have observed that hemoglobin 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. Previous studies by Mieyal *et al.* (2) showed that isolated human or bovine hemoglobin 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 hemoglobin-mediated reactions were comparable to those for the P-450 system. Since oxygenated hemoglobin appeared to play a catalytically important role in the reconstituted system (2), further studies were directed toward the reactivity of hemoglobin in its natural environment within erythrocytes and hemolysates (4–6), where oxyhemoglobin was shown to be an absolute requirement for the hydroxylase activity, and NADPH was demonstrated to be the preferred cofactor. Blisard and Mieyal (6) discovered also that the hydroxylase activity of fetal hemolysates was almost 5 times greater than that of adult hemolysates. This difference in activities was ascribed to an intrinsic property of fetal hemoglobin itself, because a difference of similar magnitude was observed with the isolated and purified hemoglobins.

In light of this result, the structural difference between fetal hemoglobin ($\alpha_2\gamma_2$) and adult hemoglobin ($\alpha_2\beta_2$) suggested that the non-α-subunits might play a more important role in determining the over-all hydroxylase activity of tetrameric hemoglobin. In support of this hypothesis are numerous studies demonstrating differences between the α- and β-subunits of human hemoglobin, including primary and tertiary structure (7), physical properties as detected by separate ^{13}C-NMR resonances for binding of ^{13}CO (8), and [1-^{13}C]ethylisocyanide (9) and chemical properties as reflected by differential ligand binding (10–13) and oxidation-reduction behavior (14). The hypothesis regarding differential hydroxylase activities of the hemoglobin subunits in the tetramer was tested in the current study by using the hydroxylase inhibitor carbon

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monoxide, which displays sufficiently different affinities for binding to the heme moieties of the α- and β-subunits ($\beta > \alpha$) of adult hemoglobin (15) that complete saturation of the β-subunits was possible while maintaining the α-subunits in the fully oxygenated state. ^{13}C-Enriched carbon monoxide bound to the α- and β-subunits gives rise to separate resonances in the ^{13}C-NMR spectrum of hemoglobins from several species (8, 15). Adult rabbit hemolysates were used for this study because the α and β ^{13}CO resonances are very well resolved and thus suitable for quantitative analysis of the sequential replacement of the oxygen on the subunits with carbon monoxide. The aniline hydroxylase activity of these same hemolysates containing the hemoglobin forms of intermediate ligation (i.e., forms resulting from the stepwise replacement of the O_2 by ^{13}CO, first on the β-subunits and then on the α-subunits) revealed that the activity of tetrameric adult hemoglobin appears to be governed by the state of ligation of the β-subunits only.

Aniline and ethyl ether were obtained from Fisher Scientific Company (Pittsburgh, PA.). Aniline was distilled and stored frozen under nitrogen until used. Ether was prewashed with an $FeSO_4$ solution, then with water to remove any peroxide degradation products. Ninety per cent ^{13}C-enriched carbon monoxide was obtained through a supply grant from the Los Alamos Stable Isotope Resource. All other chemicals were reagent-grade.

Blood was obtained from adult male New Zealand White rabbits, and hemolysates were prepared as described by Blisard and Mieyal (4) and adjusted to 1 mM with respect to hemoglobin concentration.

The hydroxylation of aniline to *p*AP[1] by hemolysates was assayed by the colorimetric technique described by Blisard and Mieyal (4).

^{13}C-NMR spectra were obtained at 45.3 MHz (42-KG magnet) using the pulse Fourier transform technique, with a 5-sec delay between acquisitions, on a Bruker 180/270 superconducting spectrometer equipped with a Nicolet 1180 computer. Samples were contained in a specially designed 20-mm limited-volume (10 ml) anaerobic sample tube fabricated by Wilmad Glass Company (Buena, N. J.). Internal D_2O served as the field frequency lock. All spectra were obtained at room temperature using a 90° pulse width of 34.5 μsec and a spectral width of 15,000 Hz (330 ppm). [2-^{13}C]Acetone at a constant concentration was used as an internal reference for line position and for quantitation of percentage saturation of the α- and β-subunits with ^{13}CO. Internal 3-(trimethylsilyl)propionic acid was the chemical shift reference for 0 ppm.

The percentage saturation with CO was determined according to the integrals of the individual signals corresponding to ^{13}CO bound to the α- and β-subunits, normalized to the integral of the acetone internal reference.

To achieve CO ligation, the hemolysate sample in a round-bottom flask fitted with a stopcock was evacuated, exposed to ^{13}CO, and then transferred in a glove bag filled with nitrogen to an anaerobic NMR tube. An aliquot of the identical solution was analyzed for aniline hydroxylase activity.

The percentage inhibition of aniline hydroxylase activity was computed by subtracting the value for the activity of the CO-treated sample from that for HbO_2, dividing this result by the value for HbO_2 and multiplying by 100.

The aniline hydroxylase activity of adult rabbit hemolysates (100% HbO_2) was found to be 80 pmoles of *p*AP formed per minute per milliliter. This value is comparable to that observed earlier for adult human hemolysates [88 pmoles/min/ml (4)].

Figure 1 illustrates the sequential saturation with ^{13}CO of the α- and β-subunits of rabbit hemoglobin in hemolysates. In Fig. 1A, 40% of the β-subunits are saturated with ^{13}CO; the α-subunits remain fully saturated with O_2 and therefore do not give rise to a signal in the ^{13}C-NMR spectrum. After completion of the saturation of the β-subunits with ^{13}CO (Fig. 1B), the α-subunits proceed to bind ^{13}CO (Fig. 1C); finally, as represented by Fig. 1D, the α- and β-subunits are 100% saturated with ^{13}CO.

Figure 2 shows that, as the β-subunit is progressively saturated with CO in a series of hemolysate samples, there is a progressive increase in the inhibition of the aniline hydroxylase activity of these samples, even though the α-subunits remain saturated with O_2. In all cases where the β-subunits were completely saturated with CO (100% β-CO, 0–100% α-CO), *p*AP formation was essentially completely abolished; where the ligation of the β-subunits with CO was incomplete (0–100% β-CO, 0% α-CO), the inhibition of *p*AP formation appeared to be directly dependent on the percentage saturation of the β-subunits with CO. It appears that the state of ligation of the α-subunits does not affect the aniline hydroxylase activity. This interpretation is limited by the fact that oxygen on the α-subunits could not be replaced by CO until the β-subunits were fully saturated with CO (and the hydroxylase already abolished) owing to the greater CO affinity of the β-subunits (7).

The cytochrome P-450-like aniline hydroxylase activity of tetrameric adult rabbit hemoglobin appears to depend only on the state of ligation of the β-subunits, with the α-subunits apparently not participating (Fig. 2). To the best of our knowledge, this is the first report of differential subunit behavior for a monooxygenase-like enzymatic activity. This observation may be especially pertinent to further investigation of the function of microsomal cytochrome P-450, which also exists in polymeric forms in the membrane as well as after solubilization and isolation (16, 17).

The behavior we have observed for hemoglobin could represent a difference in the intrinsic monooxygenase-like activity of the α- and β-subunits, i.e., the α-subunits not being capable of performing the hydroxylation. It is more likely that the association into the tetrameric form may affect the intrinsic hydroxylase activities of the two types of subunits differentially. Pertinent to this issue are the following observations. (a) Nakamura *et al.* (13) found that association into the tetramer changed the ligand-binding properties of the α- and β-subunits differentially. In addition, Mansuy *et al.* (9) in a ^{13}C-NMR study observed significant differences between the mag-

[1] The abbreviations used are: *p*AP, *p*-aminophenol; HbO_2, fully oxygenated tetrameric hemoglobin; HbCO, tetrameric hemoglobin fully ligated with carbon monoxide.

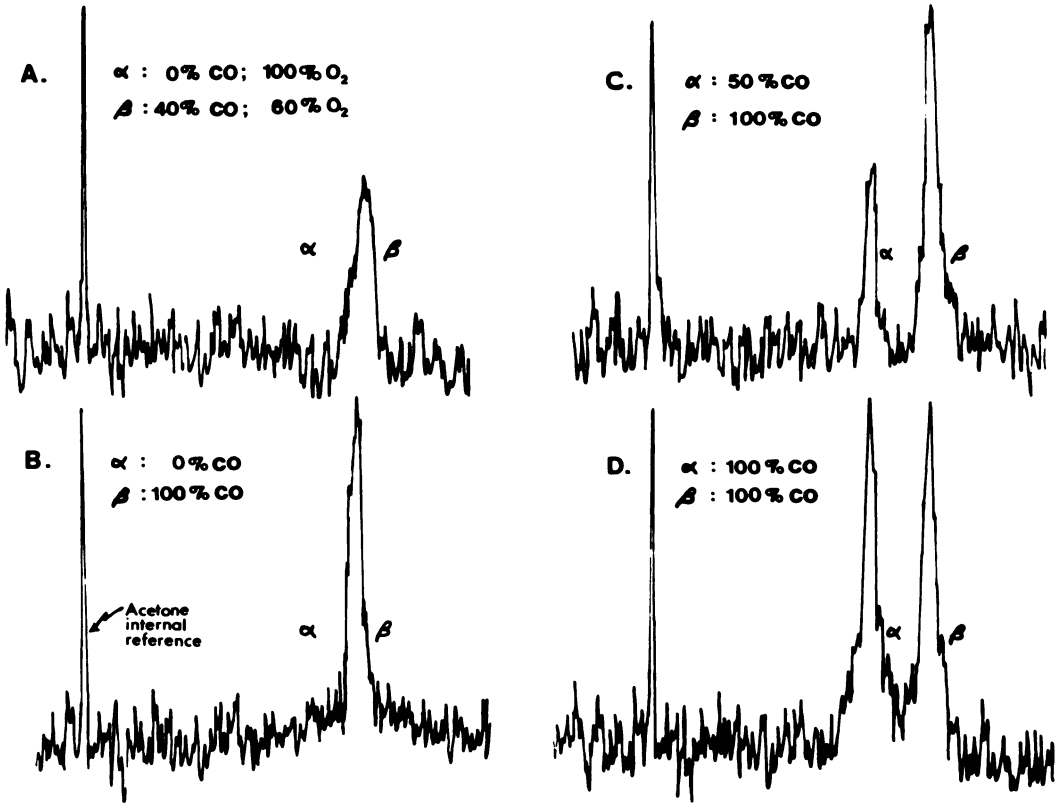

FIG. 1. *^{13}C-NMR spectra showing progressive conversion of HbO_2 to HbCO*

^{13}C-NMR spectra of adult rabbit hemoglobin in hemolysates illustrate progressive replacement of O_2 with ^{13}CO. In each case (A–D) the hemoglobin concentration was 1 mM, and the [2-^{13}C]acetone concentration was 1.24 mM, in 0.9% NaCl and 20% D_2O. Spectra were recorded at ambient temperature. The acetone signal was observed at 218 ppm and the α-^{13}CO and β-^{13}CO signals at 210 and 208 ppm, respectively, relative to the signal of internal 3-(trimethylsilyl)propionic acid, which was set at 0 ppm.

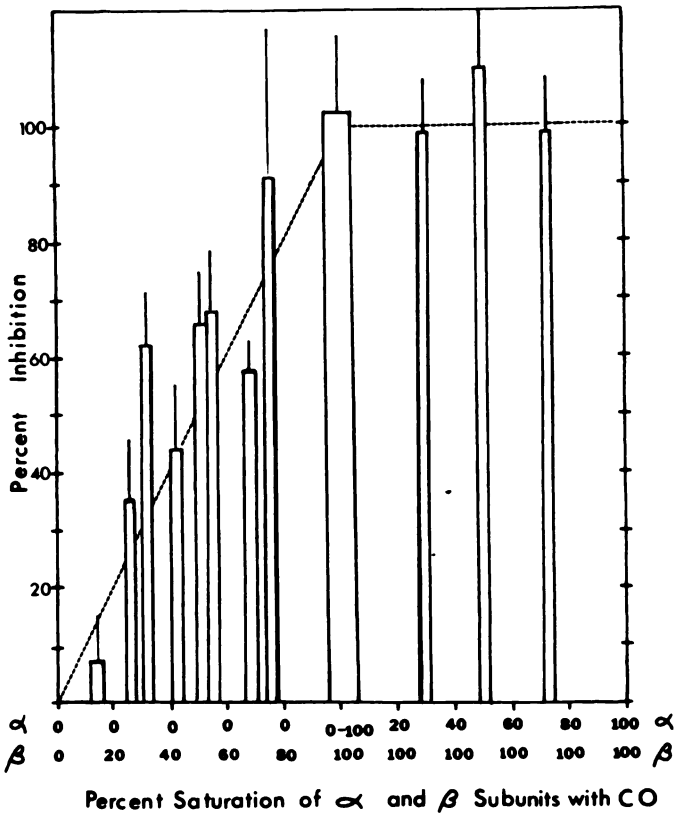

FIG. 2. *Inhibition of aniline hydroxylase activity of hemoglobin by progressive saturation with carbon monoxide*

The percentage inhibition of aniline hydroxylase activity (*p*AP formation) is plotted on the ordinate and the percentage saturation with CO of the individual subunits is plotted on the abscissa. The *dotted line* represents the theoretical relationship expected if *p*AP formation depended solely on the state of ligation of the β-subunits. The *thick bar* at 100% β-CO, 0–100% α-CO is a combination of the data for all cases in which the β-subunit was fully saturated with CO regardless of the percentage saturation of the α-subunits with CO. Hemolysates, adjusted to 1 mM Hb, 0.9% NaCl, and 20% D_2O and containing 45 mM aniline, were incubated for 1 hr at 37°.

netic environment experienced by ligand bound to isolated β-chains and ligand bound to β-chains within the hemoglobin tetramer. (b) Data reported by Smith and Beck (18) showed that both the isolated α- and β-subunits at concentrations equivalent to that of tetrameric hemoglobin A displayed peroxidase-like activity, but in all cases the activities of the subunits were lower than that of the intact tetramer. The approach used in that study could not distinguish the relative contributions of the subunit activities to the over-all activity displayed by the tetramer. (c) Sperm whale myoglobin is a monomeric hemoprotein whose amino acid sequence is quite different from that of the α- and β-subunits, but its quaternary structure and heme environment are remarkably similar to those of the α- and β-monomers. We observed that the intrinsic aniline hydroxylase activity (turnover number) of the myoglobin was approximately 40% of that of tetrameric human hemoglobin (2). Further study will be directed toward elucidating the basis for the inactivity of the oxygenated α-subunits as they exist in the hemoglobin tetramer when the β-subunits are complexed with CO.

The characterization of the unusual hydroxylase activity of hemoglobin contributes to the quest for an understanding of the factors which regulate the intrinsic reactivities among the various hemoproteins whose defined physiological functions range from simple electron or oxygen transport to the complex catalysis of peroxidase and monooxygenase reactions.

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