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SHORT COMMUNICATIONS

Hemoglobin catalysis of a monooxygenase-like aliphatic hydroxylation reaction*†

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Hemoglobin (Hb)‡ exhibits monooxygenase-like activity *in vitro* with a variety of substrates in a system containing O_2, NADPH, and cytochrome P-450 reductase [1–3]. The Hb system displays many properties in common with the liver microsomal cytochrome P-450 system, including relatively hydrophobic heme environments, oligomeric or aggregated structures of the respective hemoproteins, and utilization of the NADPH-reductase electron transfer system. In addition, Hb and cytochrome P-450_{LM} exhibit broad substrate selectivities, a property which contrasts to cytochrome P-450_{cam} and adrenal mitochondrial cytochrome P-450. The catalytic activity of Hb includes aromatic para-hydroxylations of aniline and congeners [1, 2]. O-demethylations of aromatic ethers [2], N-demethylations of *N*-methylaniline and benzphetamine [2], and N-hydroxylation of *p*-chloroaniline [3]. The versatility of the monooxygenase activity of Hb is qualitatively similar to that of the liver microsomal cytochromes P-450 including the prototype substrates aniline, *p*-nitroanisole and benzphetamine. Within the broader perspective of comparisons of the activities of Hb to those of cytochrome P-450 and the peroxidases, a number of distinctions have been made. For example, different patterns of kinetic isotope effects have suggested that the initial electron abstraction in N-dealkylation reactions may occur differently for Hb and cytochrome P-450 catalyzed reactions [4]. In another study, with styrene and H_2O_2 as co-substrates for Hb [5], evidence was reported for an unusual cooxidation mechanism involving an intermediate radical on the Hb protein reacting with O_2, in addition to the usual ferryl oxygen transfer mechanism associated with cytochrome P-450 reactions. All of the substrates for the various Hb-catalyzed reactions so far demonstrated [1–5] have in common the presence of unsaturated bonds or heteroatoms which may facilitate the reactions either by serving as sites of oxidation or by stabilizing the intermediates that lead to the oxidized product. Cyclohexane is a prototype aliphatic cytochrome P-450 substrate which has no double bonds or adjacent heteroatoms, and therefore its hydroxylation may be expected to require a more active oxidant. White and Coon [6] have suggested that catalysis of aliphatic hydroxylation may be limited among hemoproteins to cytochrome P-450 due to mechanistic differences in the activation of oxygen.

We report here that Hb catalyzes the hydroxylation of cyclohexane in an *in vitro* system analogous to reconstituted cytochrome P-450 systems. Thus, the qualitative similarities between Hb and cytochrome P-450 activities extend to aliphatic hydroxylation.

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‡ Abbreviations: Hb, hemoglobin; GC, gas chromatograph(ic); MS, mass spectrometer; P-450_{LM}, liver microsomal cytochrome P-450; P-450_{cam}, camphor-inducible cytochrome P-450 from *Pseudomonas putida*; and SDS, sodium dodecyl sulfate.

Materials and methods

Cyclohexane, cyclohexanol, and cycloheptanol were purchased from the Aldrich Chemical Co., Milwaukee, WI, and they were used directly without further purification. Gas chromatographic analysis indicated no detectable impurities or cross contaminants. NADPH was obtained from ICN Biochemicals, Cleveland, OH, and used directly. Diethyl ether from Fisher Scientific, Pittsburgh, PA, was treated with an equal volume of 5% ferrous sulfate solution to deplete peroxides, and then it was washed three times with water. Mono- and dibasic potassium phosphates were also obtained from Fisher Scientific.

Preparation of Hb. Human oxy-Hb was isolated according to the method of Eyer *et al.* [7]. Ferri-Hb was prepared from oxy-Hb by oxidation using a 1.2-fold excess of potassium ferricyanide. Ferri-Hb was then purified by passage over two sequential Sephadex G-25 columns 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 [8], which uses an absorptivity of 44 mM^{-1} cm^{-1} for cyanoferri-Hb.

Preparation of cytochrome P-450 reductase. Highly purified cytochrome P-450 reductase was prepared by the method of Shephard *et al.* [9], except that microsomes from mouse liver were used instead of microsomes from rat liver. The specific activities of the reductase preparations for reduction of cytochrome *c* at room temperature were 29–35 units/mg. With one of the preparations, it was confirmed that the enzyme migrated as a single band on SDS polyacrylamide gel electrophoresis. We demonstrated that none of the reductase preparations alone had contaminating cytochrome P-450 or monooxygenase activity (see Table 1).

Reaction mixtures. Each sample contained 20 mM potassium phosphate buffer, pH 6.8, 1 mM NADPH, 0.2 to 8 mM cyclohexane, and 0.02 units cytochrome P-450 reductase in a 1 ml total volume. This reductase concentration was determined separately to be non-limiting. Mixtures were preincubated for 5 min at 37°, and then the reactions were initiated by addition of ferri-Hb (final concentration, 1 μM). Controls omitting NADPH were incubated along with the samples. After 25 min of reaction time, a 20-μl aliquot of 250 μM cycloheptanol (internal standard) was added to the solution; 5 min later each reaction was terminated by shaking with 1 ml of ice-cold ether and immersion of the test tube in ice. NADPH was then added to the controls. Each sample was vortexed twice for 30 sec and then centrifuged at top speed in a clinical centrifuge for *ca.* 1 min. The ether layer was removed and placed in a conical vial. A second 1 ml of ether was added to the aqueous layer and the extraction procedure repeated. The two ether extracts were combined and placed on ice, and then evaporated to *ca.* 50 μl by a gentle stream of nitrogen. The concentrated ether solutions were then analyzed by the gas chromatographic procedure described in the legend of Fig. 1A. The extraction efficiencies for cyclohexanol and cycloheptanol were determined to be *ca.* 95%. Although controls lacking NADPH were used routinely, it was confirmed separately that no cyclohexanol product was detectable also in controls where Hb or

cyclohexane were omitted instead. Details of other procedures are described directly in the legends to the figures and table.

Results and discussion

Cyclohexane is oxidized to cyclohexanol by a catalytic amount of Hb (1 μM) in a reconstituted system also containing NADPH and cytochrome P-450 reductase. Figure 1A shows the gas chromatographic occurrence of cyclohexanol in the sample trace and its absence in the control trace. The product (cyclohexanol) was shown to co-chromatograph with authentic cyclohexanol. Furthermore, GC/MS analysis gave a molecular ion and fragmentation pattern for the product that were identical to those obtained for authentic cyclohexanol (see Fig. 1A, legend) and in agreement with literature values [10]. The amounts of cyclohexanol formed in the catalytic system were quantitated as described under Fig. 1B, which displays the standard curve for the GC analysis. The reaction was shown to require Hb, NADPH, cytochrome P-450 reductase, cyclohexane and O_2; increases in the concentration of O_2 enhanced the reaction, and it was inhibited by carbon monoxide (Table 1). In separate experiments performed in duplicate (data not shown), it was ascertained that the reaction was *not*

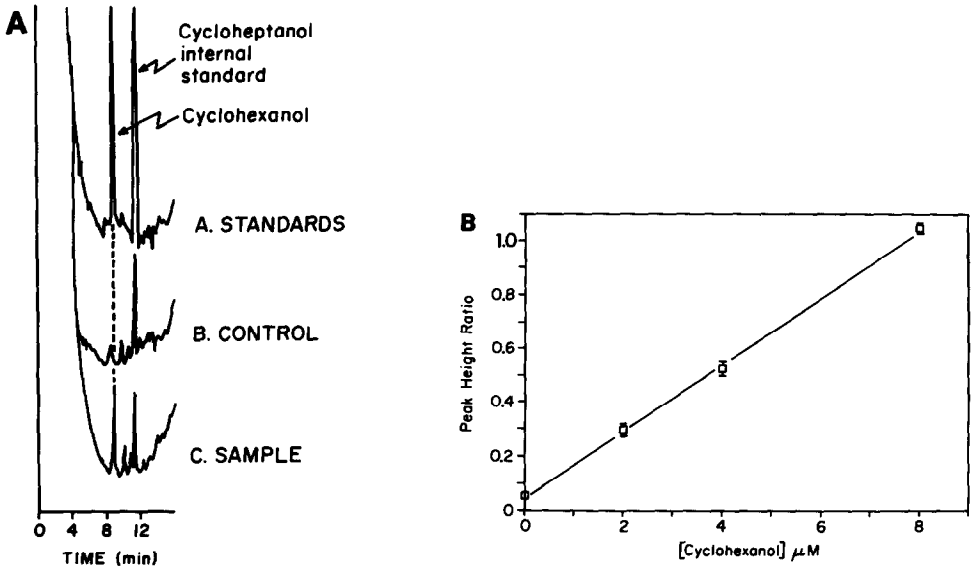

Fig. 1. (A) Typical gas chromatographic traces for separation of cyclohexanol and internal standard. Separation was achieved with a Hewlett Packard GC using a 10% Carbowax 20M/Diatoport S column (2 ft × ¼ in.) with the injector and detector ports maintained at 250° and column temperature programmed from 40° to 200° at 10°/min. Flow rates of helium, H_2 and air were 30, 30, and 200 ml/min respectively. Aliquots (5 μl) of the evaporated ether solutions (described under Materials and Methods, "Reaction mixtures") were injected, and the separated components were detected by flame ionization. The substrate cyclohexane eluted with the solvent front, and the retention times for cyclohexanol and cycloheptanol were 8.4 and 11.5 min respectively. The concentration of the standard cycloheptanol was 5 μM. Below is tabulated a comparison of mass spectral data for the putative cyclohexanol product and authentic cyclohexanol. The GC/MS spectra were obtained on an AEI MS-30 Dual Beam Mass Spectrometer coupled with a Pye-Unicam Gas Chromatograph and a Kratos Data System. The reaction product or authentic cyclohexanol (each eluting at 8.4 min) were carried through the same procedure (see Materials and Methods) and were detected by electron impact:

Reaction product		Authentic cyclohexanol	
m/e Ratio	% Base peak	*m/e* Ratio	% Base peak
100.087	5.4	100.087	3.9
82.076	49.6	82.077	55.8
71.051	17.5	71.054	13.2
57.027	100.0	57.042	100.0

(B) Standard curve for quantitation of cyclohexanol. Reaction mixtures, as described under Materials and Methods but excluding NADPH and containing various amounts of cyclohexanol, were incubated for 30 min. Before extraction, cycloheptanol was added (5 μM) and the samples were processed for GC analysis as described under Materials and Methods. The plot of peak height ratio (cyclohexanol/cycloheptanol) vs concentration was linear throughout the range of cyclohexanol concentrations obtained in the actual assays (Figs. 2 and 3). A contaminating peak (retention time 8.2 min) appeared in the chromatograms of all reaction samples and controls, accounting for the non-zero intercept of the standard curve. The intensity of the contaminant peak was the same in all analyses, and it was low (10–40%) in comparison to the intensity of product peaks. Product cyclohexanol was quantitated by GC analysis of paired control and sample incubations containing the same amount of substrate. Correction for the contaminating peak was made by subtracting the height of the control GC trace at the 8.4 min elution time from the height of the product peak in the GC trace of the corresponding sample.

Table 1. Dependence of cyclohexanol formation on the components of the reconstituted hemoglobin system

System	% Activity*
Complete	100 (1.19 nmol/ml/30 min)
Minus Hb	ND†
Minus reductase	ND
Minus NADPH	ND
0% O_2/100% N_2	ND
20% O_2/80% N_2	97 ± 9
50% O_2/50% N_2	132 ± 15
100% O_2/0% N_2	141 ± 16
20% O_2/80% CO	34 ± 5

* Percent activity is expressed relative to reactions run in air at 4 mM cyclohexane. Each value is the mean ± SE of at least four experiments.

† ND indicates that no product was detected. The practical detection limit of the assay was 0.1 μM.

inhibited by either 100 mM dimethyl sulfoxide (96% of control), a known hydroxyl radical scavenging agent [11], or 1 mM desferrioxamine (94% of control), a potent chelator of iron [12]. Thus, the possibility of non-enzymic formation of cyclohexanol in this system by hydroxyl radicals generated in solution is obviated. The cyclohexanol-forming reaction was linear for at least 30 min (Fig. 2), and the dependence on cyclohexane concentration displayed typical Michaelis–Menten kinetics (Fig. 3). It was confirmed separately that the reaction is linear with respect to Hb concentration up to 2 μM. The V_{max} for cyclohexanol formation was 52 pmol/min/nmol Hb (0.052 min^{-1} turnover), and the K_m was 1.0 mM, as estimated by non-linear least squares analysis of the primary data.

To the best of our knowledge, this is the first report that a non-P-450 hemoprotein is capable of catalyzing an O_2-dependent monooxygenase-like aliphatic hydroxylation reaction. The K_m and V_{max} for the cyclohexane reaction fall within the range of values observed previously for the other known monooxygenase substrates of Hb [2]. Reported values for the turnover number for cyclohexanol formation by a number of preparations of the well-characterized cytochrome P-450_{LM2} enzyme range from 8.8 min^{-1} [14] to 50 min^{-1} [15]. A value of 30 min^{-1} is considered typical for the turnover number, and 0.3 mM is considered the typical K_m for cyclohexane.* Thus, the K_m for cyclohexane oxidation by Hb (1.0 mM) is similar to that for the reaction

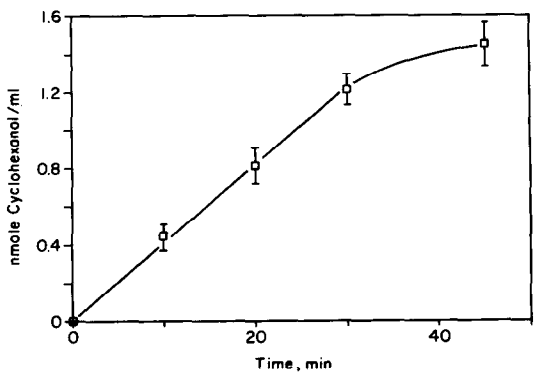

Fig. 2. Time course of cyclohexanol formation. Reaction mixtures with 4 mM cyclohexane were preincubated as described under Materials and Methods and then incubated for 10, 20, 30, and 45 min at 37°.

* D. R. Koop, personal communication, cited with permission.

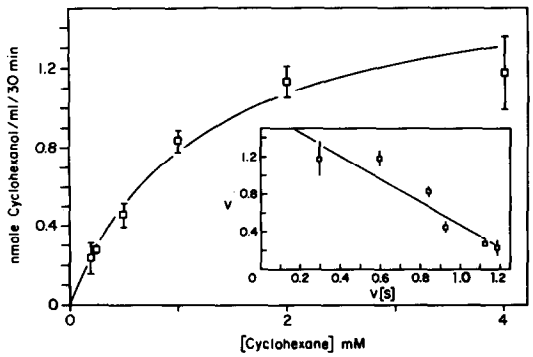

Fig. 3. Substrate dependence of cyclohexane hydroxylation. (Inset: Eadie–Hofstee replot of kinetic data (V vs $V/[S]$).) Reaction conditions were as outlined under Materials and Methods. Each point is the mean of at least six experiments ± SE. The K_m and V_{max} values determined via non-linear least squares analysis by an adaptation of the simplex curve-fitting method [13] were 1.0 mM and 52 pmol of cyclohexanol/min respectively.

catalyzed by cytochrome P-450_{LM2}. In contrast, the V_{max} for the Hb reaction (0.052 min^{-1}) is less than 1/500 of that for the cytochrome P-450 reaction (30 min^{-1}). A pattern of lower V_{max} values generally for the Hb reactions compared to those of cytochrome P-450 was observed previously for heteroatom-containing substrates as well [2]. In the case of cyclohexane oxidation, however, the difference in V_{max} values is much more pronounced. This intrinsic activity difference is likely due to one or both of the following possibilities: (1) The relative orientation of the bound substrate to the bound active oxygen is more reactive in cytochrome P-450 than in Hb, i.e. the "active site" of Hb may not be optimal for monooxygenase function. The concept of differentially optimized active sites may be illustrated by comparing cytochrome P-450 isozymes. For example, cytochrome P-450_{LM4} in general has a lower turnover for many prototypic substrates than does cytochrome P-450_{LM2} [16], even though both cytochrome P-450s probably have the same oxygen-activating system (i.e. a cysteine-ligated heme) and operate via the same mechanism. It follows that the hydroxylation sites on the substrates may be bound to LM4 in a less favorable geometry relative to the activated oxygen. By analogy, Hb may lie on one end of a continuum of hemoproteins with intrinsic monooxygenase reactivity, limited by its ability to orient substrates for optimal formation of product. (2) The second consideration regarding the different V_{max} values for Hb and cytochrome P-450_{LM2} reactions is that these two hemoproteins differ in their intrinsic abilities to activate oxygen. White and Coon [6] have postulated that the thiolate proximal heme ligand of cytochrome P-450 plays a primary role in the activation of oxygen. The lower activities of Hb would therefore be predicted due to the lack of a cysteine thiolate ligand. The results presented here and previously [2] indicate that Hb is able to activate oxygen sufficiently to mimic cytochrome P-450 activity in many cases. The quantitative difference between Hb and LM2 is consistent with the concept of the fifth ligand serving as a donor of electron density, i.e. both thiolate and histidinyl ligands have non-bonding electron pairs but the sulfur moiety is more polarizable and may be expected to stabilize a higher iron oxidation state more effectively.

Overall, the similarities and differences between Hb and cytochrome P-450 [17, 18] provide an opportunity to examine differential monooxygenase or peroxidase/peroxygenase activities in terms of structure, heme environment and interaction of the hemoproteins with other components of the oxygenase systems. Hb may be especially

useful in delineating the early events in the reaction scheme, because it can be examined in a stable oxyferrous form.

In summary, cyclohexane oxidation is a prototypic monooxygenase activity previously thought to be exclusive to cytochrome P-450 systems. In this report, hemoglobin (Hb) was shown to catalyze the hydroxylation of cyclohexane in a system containing O_2, NADPH, and cytochrome P-450 reductase. The reaction requires each component of the complete catalytic system; omission of Hb or cytochrome P-450 reductase or NADPH resulted in no measurable activity. The reaction is dependent on the O_2 content of O_2/N_2 gas mixtures, and substitution of O_2/CO (20/80) for O_2/N_2 (20/80) caused marked inhibition. The apparent K_m for Hb-catalyzed cyclohexane oxidation is similar to that for the corresponding cytochrome P-450_{LM2}-mediated reaction, but the V_{max} of the Hb reaction is approximately 500 times lower than that for P-450_{LM2}. The basis for this quantitative difference remains to be elucidated.

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Effects of maternally administered cimetidine during lactation on the development of drug metabolizing enzymes in mouse pups

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The H_2 antihistamine, cimetidine, is a drug of choice in duodenal ulcer and related disorders [1]. Despite the great attention and severe scrutiny to which it has been subjected, its use in the perinatal period is poorly documented except perhaps as it relates to prophylaxis against aspiration of acid stomach contents during obstetric anaesthesia [2]. However, cimetidine is known to cross the placenta and to be excreted in breast milk [3, 4]. It is not known what effects if any, such indirect exposure holds for the breastfed young of treated mothers.

Several cimetidine–drug interactions have been described in animals and man; the vast majority of which occur as a result of inhibition of microsomal oxidation mechanisms [5, 6]. Prolongation of barbiturate sleeping time has been attributed to this ability of cimetidine to inhibit drug metabolism [7]. Cimetidine has also been shown to inhibit aminopyrine *N*-demethylase in rodents [8, 9].

The objective of this study was to investigate the effects if any, of maternally administered cimetidine during lac-