INTRAMOLECULAR DETERMINATION OF PRIMARY KINETIC ISOTOPE EFFECTS IN HYDROXYLATIONS CATALYZED BY CYTOCHROME P-450

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SUMMARY: Isotope effects for hydroxylation reactions catalyzed by cytochrome P-450 have usually been measured by comparing the overall reaction velocities of deuterated and nondeuterated substrates. Since the rate-limiting step is probably not the single reaction involving covalent bond cleavage, such an approach does not yield information about the primary isotope effect. We measured the primary kinetic isotope effect for benzylic hydroxylation by a method utilizing intramolecular competition, using the symmetrical substrate 1,3-diphenylpropane-1,1-d_2. These experiments yield a value of k_H/k_D = 11, a larger effect than has previously been reported for benzylic hydroxylations.

INTRODUCTION

Several in vitro measurements of the primary kinetic isotope effect for aliphatic hydroxylation reactions catalyzed by cytochrome P-450 have been made. In general, low values ($k_H/k_D \leq 2.0$) were found (1-9), including several cases where no isotope effect could be detected (10, 11). The methodology of virtually all of these studies involved comparisons between the measured velocities of reactions with deuterated and nondeuterated substrates. The velocities of such reactions, however, are probably complex functions of many of the individual rates which compose the total reaction cycle. One electron reduction of the P-450 substrate complex has been proposed as the rate-limiting step in the reaction cycle (12-14), but if this were strictly true, then no isotope effect on the overall reaction should be observable. In a recent paper on the steady-state analysis of kinetic isotope effects, Northrop (15) suggests that: "It now appears likely that the maximal velocity of most enzymes is dependent upon several rate-contributing or partially rate-limiting steps."

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If this is the case with hydroxylations catalyzed by P-450, then a distinction must be made between isotope effects which are measured as the ratio of the overall enzyme reaction velocities (apparent isotope effects) and a measure of the effect of deuterium substitution on the rate of the individual step involving covalent modification of the substrate (true isotope effects).

Northrop suggested a steady-state method for determining true isotope effects from comparisons of the apparent deuterium and tritium effects on V_{max}/K_m. Another possibility, however, is the use of intramolecular competition at sites on the same molecule differing only by isotopic substitution (16). In this fashion, isotope effects can be measured by examining the relative rates of reaction at the labeled and unlabeled sites.

1,3-Diphenylpropane-1,1-d_2 is ideally suited for an intramolecular study of primary kinetic isotope effects associated with hydroxylations catalyzed by cytochrome P-450, due to symmetric 1 and 3 benzylic positions which also lack chirality. This communication presents our initial results concerning intramolecular isotope effects observed in the hydroxylation of this compound by rat liver microsomes.

MATERIALS AND METHODS

Microsomes. Adult male Sprague Dawley rats were fasted 12 hours prior to decapitation and exsanguination, after which the liver microsomal fraction was isolated according to Mazel (17). Pellets were washed once in 50 mM phosphate buffer pH 7.4 and stored at -20° C. Protein was determined by a modified Lowry method (18).

Standard incubation and assay conditions. Assay media contained in a total volume of 4.7 ml: 50 mM phosphate buffer pH 7.4, 0.30 mM NADP (SIGMA), 2 units of Sigma type V glucose-6-phosphate dehydrogenase, 8 mM glucose-6-phosphate (SIGMA), 5 mM $MgCl_2$, 4.7 mg protein, 0.002% by weight Tween 80, and 1 mM substrate unless otherwise noted. Reactions were carried out in 25 ml Erlenmeyer flasks and the assay media were preincubated for 5 minutes at 37° C prior to the addition of microsomes to start the reaction. After incubation with air as the gas phase for 15 min, the reaction was stopped by the addition of 1 ml of 0.25 M zinc sulfate and the resulting suspension was cooled on ice. The contents of each flask were then transferred to 15 ml glass stoppered centrifuge tubes and 2 ml of benzene containing 50 µg of o-phenethylbenzyl alcohol (Aldrich) was added as an internal standard for subsequent assay by gas chromatography. Extractions were carried out with a rocking mechanical extractor for 20 minutes, after which the contents of each tube were centrifuged, and the benzene layer drawn off and placed in 4 ml plastic vials. The benzene extract was then taken to dryness with a stream of dry nitrogen in a heated sand bath and redissolved in 0.1 ml benzene for chromatography.

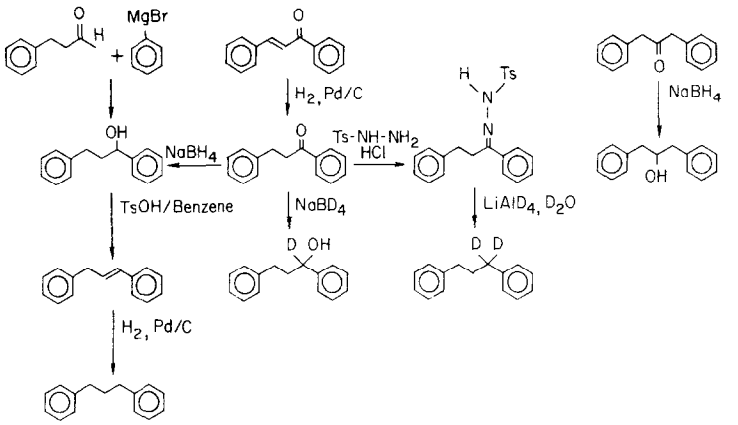

Figure 1: Synthetic routes used in the preparation of substrates and analytical standards.

Gas chromatography. Routine chromatograms were run at 150° C on a Varian 200 gas chromatograph equipped with flame ionization detectors. A 2m x 2mm glass column of 3% OV-225 on Gas Chrom Q, 100-120 mesh (Applied Science) was used with a flow rate of 30 ml/min. Peak areas were quantitated with an Omni Scribe recorder equipped with an electronic integrator (Houston Instruments).

Gas chromatography-mass spectroscopy. GC-MS was performed on a combined Varian 2100 gas chromatograph and Varian CH-7 mass spectrometer. A 2m x 2mm glass column of 3% OV-101 on Gas Chrom Q 100-120 mesh was used at 180° C with a flow rate of 20 ml/min. Spectra were recorded at 25 electron volts and 100 μamp filament current.

Synthesis of substrates and analytical standards. All possible aliphatic hydroxylation products of 1,3-diphenylpropane, including the two possible sets of alcohols and corresponding ketones, were readily prepared through the synthetic routes indicated in Figure 1. Starting materials were commercially available and were used without further purification. A possible phenolic metabolite, 1-p-hydroxyphenyl-3-phenylpropane, was also synthesized but was found not to be extracted from the incubation medium under the standard conditions we employed. After final purification, all compounds showed one spot by thin layer chromatography with benzene on silica gel GF, and no impurities were detected by gas chromatography on OV-225. Deuterated compounds showed isotope incorporations in excess of 98% by mass spectroscopy.

RESULTS

Figure 2 is a chromatogram of the metabolic products formed after a 20-minute incubation of 1,3-diphenylpropane with rat liver microsomes under standard conditions. This figure clearly demonstrates the separation of the substrate, the four metabolic products, and the internal standard. 1,3-Diphenylpropane is a typical type I substrate and after mixing with microsomes a characteristic difference spectrum with a peak at 390 nm and a trough at

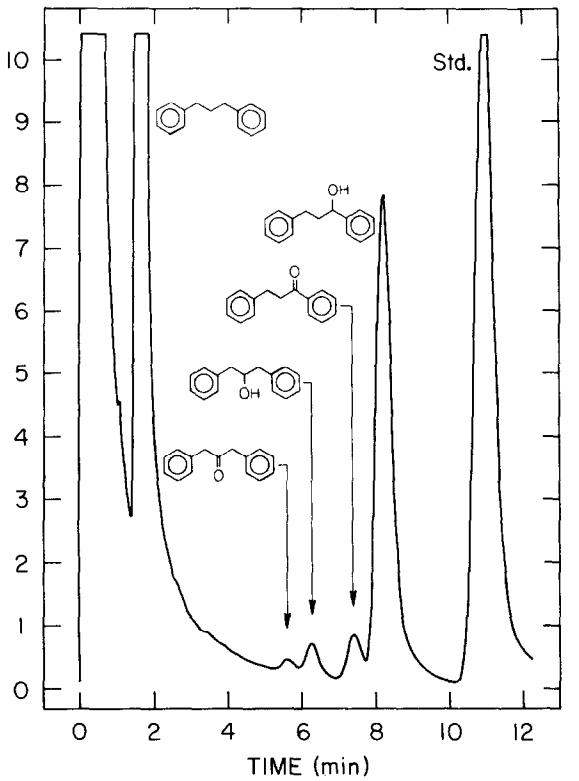

Figure 2: Gas chromatogram of the products of microsomal oxidation of 1,3-diphenylpropane. Incubation, extraction, and chromatography were performed as indicated in Materials and Methods. The internal standard (retention time = 11 min) was o-phenethylbenzyl alcohol.

420 nm is obtained. The time course of hydroxylation is linear for at least 15 minutes, with a specific activity of 2 nmoles product/mg microsomal protein/minute. This is a typical value for microsomal hydroxylations (17).

Although Figure 2 shows 1,3-diphenyl-1-propanol to be the major enzymatic product, the corresponding ketone, 1,3-diphenyl-1-propanone, was also found in small amounts (< 5%). The possibility therefore existed that the alcohol and ketone were involved in some kind of equilibrium. Were this the case, any alcohol with a geminal deuterium might lose this label by oxidation to the ketone and subsequent reduction to the alcohol. We examined this possibility by performing 15-minute incubations with either 1,3-diphenyl-1-propanol or 1,3-diphenyl-1-propanone as the substrate at a concentration of 50 μM. This

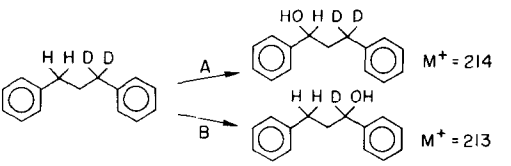

Figure 3: Microsomal hydroxylation reactions of 1,3-diphenylpropane-1,1-d_2 leading to alcohols having parent ions of m/e 214 or m/e 213.

value was chosen since it represents the approximate final concentration of products formed under our normal assay conditions. In each case, less than 20% of the substrate was either oxidized or reduced. This indicates that the alcohol and ketone formed at the benzylic position are not involved in a process of equilibration in the time period of our assay and, in addition, that the trace amount of ketone present in normal incubations is probably derived from the alcohol. The ketone could therefore be safely ignored in our subsequent calculations.

The isotope effect was measured as the relative abundance of ions at m/e 214 and 213 in the chromatographic peak corresponding to 1,3-diphenyl-1-propanol, using 1,3-diphenylpropane-1,1-d_2 as the substrate. Figure 3 shows pathways leading to the formation of alcohols having parent ions at either m/e 214 or 213. In the event of hydroxylation involving a carbon-hydrogen bond, both deuteriums are retained and the parent ion is m/e 214. If the oxidation involves a carbon-deuterium bond, however, the resulting secondary alcohol will have lost one deuterium atom either by displacement or by exchange of a hydroxyl deuterium with the aqueous medium giving an alcohol with a parent ion at m/e 213.

In order for the ratio of abundances at m/e 214 and 213 to accurately represent the isotope effect, a correction must be made for the amount of the m/e 213 ion present as a normal M-1 peak related to parent ions at m/e 214. We measured the abundance of M-1 relative to M^+ for both 1,3-diphenyl-1-propanol and 1,3-diphenyl-1-propanol-1-d and found .070 $\pm$ .001 to be a reproducible value for both compounds.

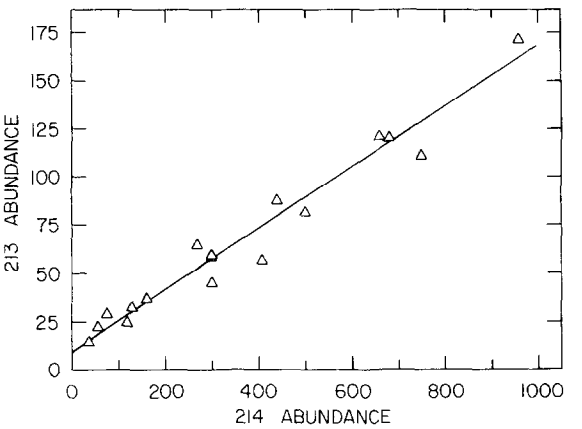

Figure 4: Relative abundance of m/e 213 vs m/e 214 in the benzylic alcohol formed during hydroxylation of 1,3-diphenylpropane-1,1-d_2 by rat liver microsomes. Units are volts/cm and are a measure of peak heights in the mass spectrum. The spectra were obtained from several parts of the chromatographic peak appearing at 8.2 min (see Fig. 2) following incubation, extraction, and GC-MS as described in Materials and Methods.

Figure 4 gives the results of seventeen separate determinations of the relative abundance of m/e 214 and 213 after incubation of 1,3-diphenylpropane-1,1-d_2 as described in Methods. These values were obtained from three different experiments and the spectra taken from all areas of the chromatographic peak of the product alcohols (retention time = 8.2 min, Fig. 2). Regression analysis indicates that the abundance of M-1 = 0.16 M^+ + 9.8 v/cm with a correlation coefficient of 0.95. The finding that the y intercept is 9.8 rather than zero is consistent with our measurement of background from substrate blanks. We find a sizable peak at m/e 213 even in the absence of substrate and product. The slope of the regression line is 0.16 with a standard error of 0.01. Subtracting a value of 0.07 M^+ as a normal abundance of the M-1 ion (see above), these data then give 0.09 $\pm$ 0.01 as the abundance of m/e 213 relative to m/e 214 due to the isotope effect. Expressed as a ratio, k_H/k_D = 11 $\pm$ 1.

This result must be qualified, however, due to possible inaccuracies in our method of calculating the isotope effect from simple m/e 214/213 ratios.

We have assumed that the relative abundance of the M-1 and M^+ species for the alcohol containing two deuterium atoms and having M^+ = 214 (see Fig. 3) is 0.07, as was measured for the alcohols having only one or no deuterium labels, respectively. If this assumption is not correct, we can still estimate a lower limit for the isotope effect by assuming no contribution to m/e 213 from the m/e 214 parent ion (i. e., M-1 = 0 for the alcohol containing two deuteriums). This gives a value of $\sim$ 6 for the kinetic isotope effect. In the other extreme case, where all of the m/e 213 is related to the m/e 214 ion as an M-1 species, the value of the isotope effect approaches infinity. We conclude on this basis that the true value of the isotope effect is not less than six and that our measured value of eleven is a reasonable estimate of the true isotope effect.

DISCUSSION

Although most previous studies have reported low isotope effects in the range of k_H/k_D = 1.0 - 2.0 (1-11), a large value is not without precedence. Foster et al. (9) found an isotope effect of 10 for the mono-O-demethylation of p-trideuteromethoxyanisole. McMahon also reported 87% retention of deuterium in the hydroxylation of S(+)-ethylbenzene-α-d_1 (5). Although McMahon proposed that this result reflects a preference of the enzyme for the formation of S(+) alcohols, more recent investigations suggest an alternate interpretation. Maylin et al. (19) have shown that the stereospecific formation of S(+) alcohols in crude homogenates is the result of the oxidation of racemic alcohol formed by P-450 to ketone, with subsequent stereospecific reduction back to the S(+) alcohol. If McMahon's data are now viewed as a measure of the isotope effect by intramolecular competion, the results would yield a value of k_H/k_D = 6.7.

The results of McMahon and Foster can therefore be interpreted as support for our determination of a large isotope effect in the benzylic hydroxylation of 1,3-diphenylpropane-1,1-d_2, which in turn provides clear evidence for a difference between inter- and intramolecular isotope effects in oxidations

catalyzed by cytochrome P-450. A large value of the isotope effect also has implications for understanding the mechanism of the hydroxylation reaction. Although "insertion" is commonly accepted as the mechanism for aliphatic hydroxylation by P-450 (20), model systems exist which are known to have radical hydroxylation mechanisms and yet display retention of stereochemistry, the major feature of P-450 catalyzed aliphatic hydroxylations. Chromyl chloride is such a model (21), and one of the major differences between this model and P-450 has been the observation of large isotope effects ($k_H/k_D > 6$) associated with benzylic oxidations by chromyl chloride (22). Our measurement of a large isotope effect for the P-450 catalyzed reaction therefore suggests that radical abstraction-recombination be considered as a possible mechanism for hydroxylation in light of the similarity of these two systems (23).

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