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Aromatic Hydroxylation Catalyzed by Toluene 4-Monooxygenase in Organic Solvent/Aqueous Buffer Mixtures

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Abstract

Toluene 4-monooxygenase is a four-protein component diiron enzyme complex. The enzyme catalyzes the hydroxylation of toluene to give *p*-cresol with ~96% regioselectivity. The performance of the enzyme in two-phase reaction systems consisting of toluene, hexane, or perfluorohexane and an aqueous buffer was tested. In each of the cosolvent systems, containing up to 93% (v/v) of solvent, the enzyme was active and exhibited regioselectivity indistinguishable from the aqueous reaction. Using the perfluorohexane/buffer system, a number of polycyclic aromatic hydrocarbons were oxidized that were not readily oxidized in aqueous buffer. An instability of the hydroxylase component and a substantial uncoupling of NADH utilization and product formation were observed in reactions that were continued for longer than ~3 min. More stable enzyme complexes will be needed for broad applicability of this hydroxylating system in nonaqueous media.

Index Entries: Diiron enzyme; monooxygenase; organic cosolvents; regioselective hydroxylation.

Introduction

Regioselective aromatic hydroxylations are challenging transformations for the chemical and pharmaceutical industries *(1)*. Current synthetic methods invariably involve harsh conditions, do not give selective hydroxylation in high yields, and can result in significant waste. By contrast,

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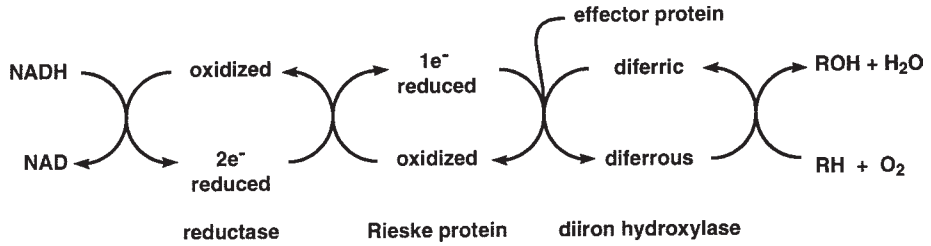


Scheme 1. Protein components of T4MO required for NADH- and O_2-dependent conversion of toluene to *p*-cresol.

a large number of microbial (typically fungal and bacterial) and mammalian (typically cytochromes P-450) monooxygenase enzymes have been identified that catalyze regioselective or enantioselective hydroxylation of hydrocarbons *(2–6)*. These multicomponent enzymes utilize an external reductant (typically NADH) and O_2 to catalyze the insertion of one O-atom into either aromatic or aliphatic substrates, while the other O-atom is reduced to water.

Toluene 4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR1 is one such multicomponent hydroxylating enzyme *(6)*. T4MO is a member of a diverse family of diiron-center-containing enzymes including ribonucleotide reductase, acyl-ACP desaturases, and bacterial hydroxylases such as methane monooxygenase, other isoforms of toluene monooxygenase, phenol hydroxylases, and alkene epoxidases. T4MO is composed of four protein components (Scheme 1) required for efficient multiple turnover catalysis *(7)*: an FAD- and a [2Fe-2S]-containing NADH reductase (T4MOF, 36 kDa); a Rieske [2Fe-2S] ferredoxin (T4MOC, 12.5 kDa); a catalytic effector protein containing no redox-active cofactors (T4MOD, 11.6 kDa); and a terminal hydroxylase component (T4MOH, with $[\alpha\beta\gamma]_2$ quaternary structure, 212 kDa). This complex has been cloned *(8,9)*, overexpressed in *Escherichia coli*, and purified *(7)*, and amino acid residues contributing to the hydroxylation regiospecificity have been identified *(10,11)*. T4MO catalyzes the hydroxylation of toluene to give *p*-cresol with ~96% regioselectivity *(10)*; notably, benzylic hydroxylation is not observed with this enzyme in contrast to the 70–100% benzylic hydroxylation typically observed with other monooxygenases including ammonia monooxygenase *(12)*, chloroperoxidase *(13)*, cytochromes P-450 *(14,15)*, methane monooxygenase *(16)*, and xylene monooxygenase *(2)*. Furthermore, although T4MO has a broad substrate specificity, a given substrate typically yields high regioselectivity for one product *(10,11)*. These characteristics make the T4MO complex an intriguing catalyst for large-scale, regiospecific hydroxylation of aromatic compounds.

In the present study, the catalytic function and stability of the purified, reconstituted T4MO complex were examined in the presence of various organic solvents. The results show that T4MO retained activity and regioselectivity for aromatic ring hydroxylation in two-phase reaction sys-

tems containing toluene, hexane, or perfluorohexane and an aqueous buffer. Furthermore, a number of polycyclic aromatic hydrocarbons (PAHs) not readily oxidized in aqueous buffer were oxidized using a perfluorohexane/buffer system. During these studies, hydroxylase instability and a substantial uncoupling of NADH utilization and product formation were observed. These complications likely represent the most severe constraints on developing and performing enzymic monooxygenase reactions in organic solvent systems.

Materials and Methods

Enzyme Preparations and Reagents

The T4MO complex was overexpressed *(7,17)*, purified, and characterized for iron content *(7)*, catalytic activity, coupling efficiency, and product regiospecificity *(7,10,11)* as previously described. Toluene; *o*-, *m*-, and *p*-cresols; benzyl alcohol, hexane, perfluorohexane; and all other chemicals were from Aldrich (Milwaukee, WI). All other reagents were of the highest purity available commercially.

Enzyme Reaction Conditions

A typical aqueous enzyme reaction contained 1.0 nmol of hydroxylase, 2.0 nmol of Rieske ferredoxin, 2.0 nmol of effector protein, 0.2 nmol of reductase, 9.4 mmol (1 µL) of toluene, and 250 µL of 50 m*M* potassium phosphate buffer (pH 7.5) in a Teflon-sealed 3-mL conical reaction vial. The reaction was initiated by the addition of NADH (250 nmol in 10 µL of buffer), and the reaction vial was shaken at 30°C and 100 rpm in a reciprocating water bath. At the appropriate time, a 50-µL aliquot of the reaction buffer was removed and extracted with 50 µL of chloroform and 50 µL of saturated NaCl at pH 1.0. The chloroform fraction was used for gas chromatographic analysis.

For reactions in the solvent/buffer mixtures, the same amounts of enzyme components just described were delivered in an appropriate volume of phosphate buffer to a reaction vial containing the organic solvent so that the nominal total reaction volume was 250 µL. For example, 125 µL of phosphate-buffered enzyme solution was added to 125 mL of the organic solvent to produce a reaction mixture containing 50% (v/v) of solvent. For each solvent investigated, the reaction mixture consisted of two phases. Toluene (or other substrates) was added to either the aqueous or the organic phase according to the experiment being performed. Oxidation reactions were initiated by the addition of NADH to the aqueous phase using a gastight Hamilton syringe.

One unit of hydroxylase activity is defined as the formation of 1 µmol of *p*-cresol/min in air-saturated 50 m*M* phosphate buffer, pH 7.5, at 25°C in the presence of optimal concentrations of the other T4MO components: toluene (5.8 m*M* at 25°C [18]) and NADH (0.5 m*M*). Specific activity was

determined relative to the initial amount of hydroxylase component in the assay mixture.

Product Distributions

Product distributions from toluene hydroxylation were determined by gas chromatography using a Hewlett-Packard 6890 gas chromatograph equipped with a 7683 Auto Injector and an EC-WAX column (30 m × 0.25 mm, 0.25-µm film thickness; Alltech, Deerfield, IL) connected to a flame ionization detector. The injector and detector were maintained at 250°C. The He carrier flow was 4.0 mL/min. For the separation of toluene oxidation products, the column temperature was initially started at 100°C, increased to 130°C at 5°C/min, then finally increased to 150°C at 2°C/min. Under these conditions, the benzyl alcohol and *o*-, *p*-, and *m*-cresols eluted at 7.3, 10.1, 12.1, and 12.3 min, respectively. All retention times were verified using neat compounds. PAH reactions were analyzed by liquid chromatography-mass spectrometry (LC-MS) (API 100; PE-Sciex, Norwalk, CT). The reaction mixtures were evaporated and the PAHs and their oxidation products were extracted into methylene chloride and then subjected to LC-MS analysis. Samples were separated using a Phenomenex Columbus 5 µ C_8 100 Å column (Torrance, CA) with dimensions of 50 × 2 mm using an isocratic elution consisting of 60% water (containing 0.1% trifluoroacetic acid)/40% CH_3CN (containing 0.1% trifluoroacetic acid). Confirmation of hydroxylated product was made by identification of the products' parent ion.

Results and Discussion

Catalysis by the T4MO complex was examined in the presence of organic solvents including *tert*-amyl alcohol, chloroform, toluene, hexane, and perfluorohexane. The reaction mixtures were assembled to contain 50 or 93% (v/v) of the organic solvent with the remainder of the volume provided by the aqueous buffer containing the enzyme. All reaction mixtures formed two-phase systems, with the reactions in 93% (v/v) organic solvent initially consisting of a single droplet of the buffer suspended within the solvent phase. The value of 93% (v/v) represented the highest organic solvent concentration that was attainable and was based on the need to add the monooxygenase components as an aqueous preparation. In reactions that were initiated by the addition of aqueous droplets of NADH-containing buffer to the organic phase, the NADH-containing droplets were retained within the organic phase, and, hence, the reductant was unavailable for the reaction. This method resulted in relatively low initial rates and product yields. When the NADH droplet was injected directly into the aqueous phase, higher initial rates and product yields were obtained. Except as noted, this latter method for the addition of NADH was used. The formation of solvent/buffer emulsions by vigorous agitation caused enzyme inactivation regardless of the solvent and was used to terminate the reaction in conjunction with acid precipitation.

Table 1
Comparison of Initial Rates and Product Distributions for T4MO-Catalyzed Hydroxylation of Toluene in Various Percentages of Solvent in Aqueous Buffer

		Regioselectivity		
Solvent/buffer (v/v [%])[a]	Rate (s^{-1})[b]	*p*-Cresol (%)	*m*-Cresol (%)	*o*-Cresol (%)
No organic solvent[c]	2.0	97	2	1
Toluene/buffer				
50/50	1.8	96	2	2
93/7	1.1	97	2	1
Hexane/buffer				
50/50[d]	1.8	99	1	<1
93/7[e]	1.6	97	3	<1
Perfluorohexane/buffer				
50/50[d]	1.9	100	<1	<1
93/7[e]	0.1	97	2	1

[a]Solvent/buffer composition reported as (v/v) percentage composition in the reaction vial.
[b]Rate with respect to an αβγ protomer of the hydroxylase.
[c]Aqueous reaction as described in Materials and Methods (9.4 µmol of toluene and 250 nmol of NADH).
[d]Reaction system with toluene added directly to the aqueous phase.
[e]Reaction system with toluene added directly to the organic phase.

No reaction was observed in the presence of *tert*-amyl alcohol or chloroform in either the 50 or 93% (v/v) reaction mixtures. Since these solvents have appreciable solubility in water (1.2 and 66 m*M*, respectively *[18]*), the high concentration of organic solvent in the aqueous phase likely led to enzyme inactivation *(19)*.

Reactions in Aqueous Buffer, Toluene/Buffer, and Hexane/Buffer Systems

Table 1 compares the initial rates for the T4MO-catalyzed hydroxylation of toluene in aqueous buffer and in various solvent/buffer mixtures. In a toluene-saturated aqueous buffer solution, the enzyme has a $k_{cat} = 2\ s^{-1}$ relative to an αβγ protomer of the hydroxylase component *(10,11)*. Time-course experiments (data not shown) revealed that *p*-cresol formation was maximal and linear for ~3 min in all solvent systems investigated, including the aqueous system alone.

In a reaction mixture containing 50% (v/v) toluene, k_{cat} was 90% of that observed in aqueous buffer. This result was consistent with previous catalytic studies *(10,11)* that were performed in a toluene-saturated aqueous buffer assembled without the presence of bulk, excess toluene. Since the apparent K_M value for toluene of 3 µ*M* at 25°C is roughly 1000-fold lower than the concentration of toluene in a saturated aqueous solution at the

same temperature (5.8 m*M [18]*), the active site should be saturated with toluene in a saturated aqueous solution. Furthermore, because the enzyme complex always remained in the aqueous phase, the maximum toluene concentration to which the enzyme could be exposed would be that of a saturated aqueous solution, regardless of the composition of the organic solvent phase. By increasing the volume percentage of toluene in the reaction vial from 50 to 93%, a decrease in k_{cat} of ~twofold was observed. However, it is unlikely that this represented a significant decrease in activity because substantial errors were introduced in the measurement of turnover number for the 93% toluene reaction mixture. This error was owing to a variable increase in the volume of the organic phase during extraction as the percentage of chloroform-extractable toluene (or other organic solvent) was increased in the reaction mixtures.

The 50 and 93% hexane mixtures (v/v) were also two-phase systems. The addition of toluene directly to the aqueous phase gave a k_{cat} value for the 50% mixture indistinguishable from that of the aqueous system; for the 93% mixture, the measured k_{cat} value was within a factor of 2 of that observed in aqueous buffer alone (data not shown). The addition of toluene (1 µL) directly to the hexane phase in the 93% mixture (232 µL of hexane) gave a k_{cat} value ~80% of the maximal value (Table 1). This shows that the partitioning of toluene between the organic and aqueous phases was sufficient to permit rapid catalysis and also implied that only a slightly higher concentration of toluene in the organic phase might be required to saturate the enzyme's active site. Furthermore, although hexane is sparingly soluble in water (139 µ*M* at 25°C *[18]*), hexane does not act as a substantial competitive inhibitor of T4MO-catalyzed toluene hydroxylation at high toluene concentrations. This conclusion is consistent with the low rate and yield of 1- and 2-hexanols obtained from T4MO-catalyzed oxidations of hexane (unpublished results), suggesting that a linear C6 hydrocarbon may not efficiently enter the enzyme's active site.

Reactions in Perfluorohexane/Buffer Systems

Perfluorohexane lacks a C-H bond required for monooxygenase reactivity, which effectively prevents this solvent from acting as an alternative substrate for T4MO. Furthermore, this solvent has extremely low solubility in water (46 µ*M [18]*), suggesting that it will not act as an inhibitor of enzyme reactions in the aqueous phase. These properties prompted the investigation of T4MO reactivity using perfluorohexane as the organic phase solvent. In the 50% perfluorohexane mixture (Table 1), T4MO had nearly identical activity to the buffer control reaction when toluene was added directly to the aqueous phase. However, when toluene was added directly to the perfluorohexane phase in both the 50 and 93% perfluorohexane reaction mixtures, the *p*-cresol formation rate was ~5% of that observed in the aqueous buffer reactions. Since toluene was not miscible in perfluorohexane, but remained as isolated droplets suspended in perfluorohexane, the low catalytic activity likely arose from limitations in mass transfer

Table 2
Conversion of Toluene and NADH in Perfluorohexane/Buffer Mixtures

Solvent/buffer (v/v [%])[a]	Toluene conversion (%)[b]	NADH conversion (%)
Aqueous buffer[c]	7.8	87.7
50/50	7.2	81.0
93/7	3.6	40.7

[a]Solvent/buffer composition reported as (v/v) percentage composition in the reaction vial.
[b]Toluene conversion based on available NADH (250 nmol).
[c]Aqueous reaction as described in Materials and Methods.

caused by the minimal surface area for contact between the aqueous phase and the toluene droplets.

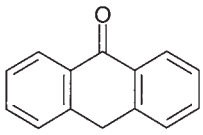
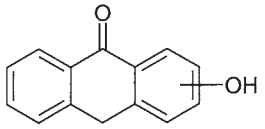
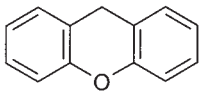
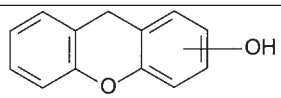
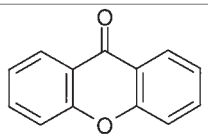
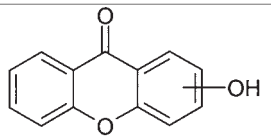
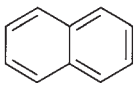
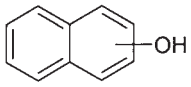
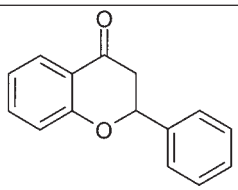
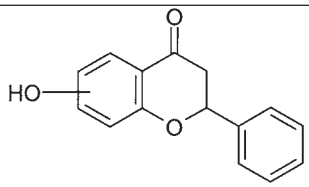
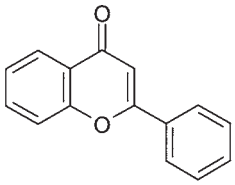
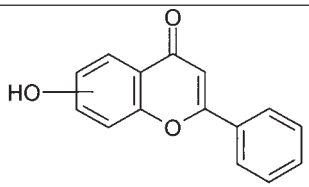
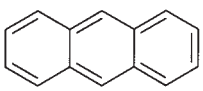
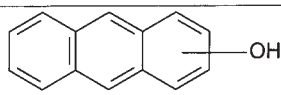
Coupling of NADH Utilization and Product Formation

Previous studies have shown that the T4MO complex is tightly coupled during maximal rate turnover *(11)*, which can typically be maintained for ~3 min. Table 2 shows the relationship between toluene oxidation and NADH utilization over a 30-min period in various perfluorohexane reaction mixtures and using identical amounts of toluene and NADH (250 nmol). Over this longer time period, ~8% conversion of toluene to *p*-cresol (based on available NADH) and ~80% consumption of NADH were observed in buffer. Moreover, relatively similar low conversion of toluene and high consumption of NADH was observed in the perfluorohexane reaction mixtures. Thus, it is likely that the hydroxylase was the least stable component of the enzyme complex over the 30-min time period, and that this inactivation resulted in a decrease in *p*-cresol yield. By contrast, the reductase apparently retained the ability to oxidize NADH and produce superoxide and peroxide during the longer time period. This would result in the continued, adventitious consumption of NADH, a result that would be exacerbated if the Rieske ferredoxin also retained the ability to accept electrons from the reductase.

Regioselectivity of Toluene Hydroxylation

Table 1 shows the results of a gas chromatographic determination of the regioselectivity of toluene hydroxylation in aqueous buffer and in the various solvent/buffer systems investigated. For each solvent system, the regiospecificity was indistinguishable from the 96% formation of *p*-cresol previously determined for the purified enzyme complex *(10)*. Thus, the presence of high concentrations of organic solvent does not disrupt the exquisite selectivity of T4MO. This suggests that the enzyme retains a catalytically competent active site in the presence of hydrophobic organic solvents.

Table 3
Polycyclic Aromatic Substrates and Proposed Products Obtained from T4MO-Catalyzed Oxidation in a Perfluorohexane/Buffer Mixture[a]

substrate	product
anthrone 	
anthene 	
anthone 	
aphthalene 	
flavonone 	
flavone 	
anthracene 	

[a]Reaction mixture of 93% perfluorohexane and 7% aqueous buffer.

Oxidations of PAHs in Perfluorohexane/Buffer Systems

T4MO is also capable of hydroxylating PAHs, with the conversion of indole to indoxyl the best-characterized reaction *(10,11)*. Since PAHs have a relatively high solubility in organic solvents, the approach of using an organic solvent to help enhance the T4MO reactivity with PAHs was tested. Table 3 shows that a wide variety of other PAHs can be hydroxylated using the mixed perfluorohexane/buffer system. Notably, as the compounds in

Table 3 have extremely low solubility in water, hydroxylated products were not observed from buffer reactions with the exception of the conversion of naphthalene to naphthol. For each of the products shown in Table 3, the presence of a hydroxyl group (corresponding to an *m/z* increase of 16 amu relative to the substrate) was confirmed by LC-MS analyses. Owing to extensive uncoupling observed during these oxidations (see above), the product yields for these oxidations were not determined. Furthermore, the positions of hydroxylation were not explicitly determined.

Not all PAHs tested could be hydroxylated by T4MO in the two-phase system. Specifically, paclitaxel, anthraquinone, and quinizarin were unreactive. The limited solubility of paclitaxel in both perfluorohexane and water may have been a major factor in preventing T4MO-catalyzed hydroxylation; however, the large size (854 Daltons) may also be a contributing factor in preventing access to the active site. The origin of the unreactivity of anthraquinone and quinizarin was unclear, although the polarity of the 1,4-benzoquinone groups may have prevented binding in the presumably hydrophobic active site. Alternatively, the electron-withdrawing nature of the quinone functional group may have deactivated these compounds toward hydroxylation.

Conclusion

The poor solubility of many aromatic compounds in water is an impediment to the large-scale utilization of monooxygenases in chemical synthesis. However, while there has been considerable interest using organic/aqueous systems for enzyme catalysis *(20–24)*, only a few examples of monooxygenase catalysis in nonaqueous media have been reported *(25–27)*. During monooxygenase catalysis, an organic cosolvent can potentially act as a hydroxylation substrate, which would cause competitive inhibition of the desired reaction. The presence of an alternative substrate (in this case, possibly a less easily oxidized organic solvent) could also cause an uncoupling of NADH utilization and substrate hydroxylation *(28,29)*. Furthermore, relative to enzymes consisting of a single polypeptide chain, multicomponent enzymes introduce problems of enzyme stability that are compounded by the requirement to maintain protein-protein interactions for subunit assembly; cofactor, coenzyme, and metal binding; electron transfer; and catalysis. Finally, turnover-dependent uncoupling leads to the production of hydrogen peroxide, which then adventitiously oxidizes one or more of the protein components (e.g., the [2Fe-2S] clusters in the reductase and ferredoxin). Uncoupling can be a manifestation of improper protein-protein interactions, which might be produced by organic solvent perturbations of the required protein interfaces. Other aspects, such as solvent denaturation, pH instability, and thermal stability, will also contribute to the observed inactivation, but first it will be necessary to ensure that the enzyme is functioning properly to avoid turnover-dependent inactivation.

The activity of T4MO in the presence of two-phase solvent/aqueous buffer systems suggests possibilities for future applications of this enzyme in biotransformations. The isolated enzyme system is capable of functioning in the presence of up to 93% (v/v) organic solvent. Moreover, to our knowledge, the reactivity of T4MO in the perfluorinated solvent described here represents the first use of this solvent to facilitate monooxygenation biotechnology. Perfluorohexane offers the advantages of being inert to monooxygenation reactions and of having an extremely low solubility in water. Through the use of perfluorohexane, the delivery of PAHs for monooxygenation was observed. However, as presently formulated, T4MO does not exhibit sufficient stability in either aqueous or solvent/aqueous two-phase systems for the long times likely required for effective, large-scale organic synthesis *(30)*. Therefore, further investigations are needed to improve the enzyme stability in both solvent systems.

Acknowledgments

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