

A MOLECULAR MODELING ANALYSIS OF POLYCYCLIC AROMATIC
HYDROCARBON BIODEGRADATION BY NAPHTHALENE DIOXYGENASE

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Abstract—A theoretical analysis was performed to examine the role of naphthalene dioxygenase (NDO) enzymes in determining differences in biodegradability and biodegradation rates of two- to four-ring polycyclic aromatic hydrocarbons (PAHs) via oxygenation and desaturation reactions. Investigation of the thermodynamics of PAH biodegradation reactions catalyzed by NDO revealed that enthalpies of reaction can explain reaction patterns or regioselectivity of the enzyme in limited cases. Molecular modeling analysis of the size and shape constraints of PAH–enzyme interactions suggests that PAHs bigger than approximately four rings and compounds with α substituents or other structural features contributing to increased width at the end of the substrate near the active site are expected to have binding difficulties. This explains some regioselectivity observations, in that thermodynamically favorable sites on some PAH molecules cannot be positioned correctly to be oxidized at the active site. The enzyme fit analysis also suggests that slower biodegradation rates are expected for compounds with larger widths because of the unique positioning that is required for reaction to occur. An inverse relationship between a molecular descriptor of compound width and previously obtained biodegradation rates suggests that this descriptor may be valuable for predicting relative biodegradation rates of PAHs with dioxygenases other than NDO.

Keywords—Polycyclic aromatic hydrocarbons Naphthalene dioxygenase Biodegradation

INTRODUCTION

Aerobic biodegradation is an important environmental fate for the class of pollutants known as polycyclic aromatic hydrocarbons (PAHs) [1], so reliable estimates of PAH biodegradability and biodegradation rates are necessary for predicting environmental recalcitrance and designing remediation efforts. Previous work has shown that in the absence of bioavailability constraints, small but statistically significant differences exist for biodegradation rates of two- to four-ring PAHs by a mixed culture [2,3]. These rates do not correlate with properties, such as hydrophobicity, that would be expected to represent the ability of the molecules to enter or move through the bacterial cell [2]. This suggests that steps involving the enzymes that are responsible for transformations of PAHs may be important for determining differences in biodegradability and biodegradation rates. For example, differences in thermodynamic favorability of the initial enzyme attack may make one reaction more likely than another, or ease of fit into the enzyme active site may affect transformation rates. The purpose of the present work was to study the interactions of PAHs with relevant enzymes to determine if observed differences in biodegradability can be explained and predictions made for other PAHs.

The known biodegradation pathways of PAHs by bacteria are initiated by a group of enzymes called aromatic hydrocarbon dioxygenases. These enzymes are part of a larger class of enzymes known as Rieske nonheme iron dioxygenases or aromatic ring-hydroxylating dioxygenases [4]. These enzymes are soluble, multicomponent systems that have one or two electron-transport proteins and a catalytic oxygenase component. The naphthalene 1,2-dioxygenase (NDO) from *Pseu-*

domonas sp. NCIB 9816-4 is the only member of this class of enzymes for which the complete structure has been resolved [5]. Numerous studies have shown high degrees of sequence homology between areas of the catalytic domains of NDO and other dioxygenases. Although these enzymes differ in subcomponent organization and overall structure, the reaction mechanism for dioxygenases is believed to be very similar [4,6]. For these reasons, studying NDO is valuable for understanding interactions of PAHs with this class of enzymes.

Naphthalene dioxygenase catalyzes addition of diatomic oxygen to one of the aromatic rings of naphthalene (naphthalene + $2e^- + 2H^+ + O_2 \rightarrow (+)-cis-(1R,2S)$ -dihydroxy-1,2-dihydronaphthalene). This reaction is highly regioselective and stereospecific, with an observed relative yield of 100% for the 1,2-oxidation product and an enantiomeric excess of greater than 98% [7,8]. Naphthalene dioxygenase catalyzes dioxygenation of several other aromatic compounds to produce *cis*-dihydrodiols, including a variety of heterocyclic compounds. Naphthalene dioxygenase also can catalyze other reactions for many substrates, including monooxygenation, desaturation, O- and N-dealkylation, and sulfoxidation. Many of these reactions that have been reported in the literature are summarized elsewhere [9].

In the present study, we used molecular modeling techniques to explore the role that enzyme/substrate interactions are expected to play in determining variations in biodegradability and biodegradation rates of PAHs by NDO. We focused specifically on the initial transformation reaction of the biodegradation process rather than on the collective reactions that result in complete mineralization of the PAH. We first present an analysis of the thermodynamics of the reactions that NDO has been observed to catalyze. A comprehensive list of NDO-catalyzed reactions (dioxygenation, monooxygenation, and desaturation) reported in the literature was compiled for 22 two-

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to four-ring PAHs; some hypothetical reactions that have not been observed also were included. Enthalpies of reaction were compared to determine whether they provide insight regarding which reactions are catalyzed, including why certain bonds of the molecules are attacked by the enzyme. We also present an analysis of the fit of potential substrates into the enzyme's active site and implications for relative biodegradation rates. Finally, we discuss the potential for applying what has been learned about NDO to other enzymes.

MATERIALS AND METHODS

A total of 22 two- to four-ring PAHs were included in the present study. These 22 compounds were selected because their biodegradation rates had been studied previously and laboratory data were available for comparison. Enthalpies of reaction were calculated for known NDO-catalyzed reactions gathered from the literature. Calculations also were performed for several reactions that were anticipated but have not been reported in the literature. The enthalpy of reaction, ΔH_R , is defined as

$$\Delta H_R = \sum n_p \Delta H_f(\text{products}) - \sum n_r \Delta H_f(\text{reactants}) \quad (1)$$

where n_p and n_r are the number of moles of each product and reactant, respectively, and ΔH_f is the enthalpy of formation for each of the compounds involved in the reaction. Enthalpies of formation for the products and reactants were estimated using Spartan 2002 (Ver 1.0.2; Wavefunction, Irvine, CA, USA). Calculations were performed at a semiempirical level of theory using PM3 parameterization. Enthalpies calculated for several of the reactants were compared to tabulated values determined by other methods to verify their accuracy. Hites and Simonsick [10] reported enthalpies of formation also calculated at a semiempirical level using MNDO parameterization rather than PM3, and their values agreed well with experimental values (mean error of 6.3 kcal/mol for 16 PAHs). Daubert and Danner [11] tabulated experimental values of enthalpies of formation for nine of the PAHs studied here; errors were reported as less than 3% for each of the tabulated values. Values calculated in the present study were slightly higher than the reported values in the databases, but agreement was quite good, with deviations of only a few kcal/mol for each compound. In addition, because estimates that are consistently slightly high are useful for relative comparison, calculated values were used with confidence for all relevant products and reactants.

Polycyclic aromatic hydrocarbon substrates were examined in the context of interactions with the enzyme active site. Naphthalene dioxygenase has been crystallized and its structure deposited in the Protein Data Bank (PDB) [12] in several forms, including structures with no substrate present (PDB code 107W) [5], with indole bound (PDB code 1EG9) [13], and with naphthalene bound (PDB code 107G) [14]. The crystallized structures of NDO were downloaded from the PDB and visualized using Rasmol 2.6 [15] and Spartan 2002.

To determine potential fit of substrates into the enzyme-binding cavity, it was first necessary to estimate the amount of space that various PAH substrates would occupy. Size estimates (length, width, and thickness) were generated using a system developed to calculate length to breadth ratios, which are useful for predicting chromatographic retention times [16,17]. For planar PAHs, a box is drawn enclosing the van der Waals surface of the PAH molecule such that the length to breadth ratio is a maximum and the length, width (breadth), and thickness of the box are then measured. For nonplanar

PAHs, the molecule is oriented such that the minimum dimension is aligned with the z -axis and the maximum dimension with the x -axis.

Overall geometries were determined from optimized structures. Structures of the PAH substrates were generated using Spartan 2002, and the geometries were optimized at a Hartree-Fock level using a 3-21G(*) basis set. Van der Waals radii used for the atoms were 1.92 Å for carbon, 1.52 Å for oxygen, and 1.20 Å for hydrogen.

RESULTS AND DISCUSSION

Summary of NDO-catalyzed reactions

For unsubstituted PAHs containing only six-carbon rings (alternant structure), dioxygenation (as described previously for naphthalene) is the only NDO-catalyzed reaction observed. Regioselectivity of the attack site varies by substrate, but stereospecificity is always very high [18–21]. For example, for phenanthrene, the major product is *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (>90%), with *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene as a minor product (<10%).

When naphthalene has alkyl substituents in the 2-position only (i.e., 2-methylnaphthalene and 2-ethylnaphthalene), dioxygenation is again the only reaction observed. The presence of alkyl substituents at other positions adds monooxygenation of the substituent as a potential NDO-catalyzed reaction. However, dioxygenation on the ring not containing the alkyl substituent is the dominant reaction for 1-methylnaphthalene and 1-ethylnaphthalene. It has been observed that yields for these compounds (and compounds with a variety of other non-alkyl 1-position substituents) are always lower than those obtained with the comparable 2-substituted naphthalenes. This has led researchers to suggest that the presence of a 1-substituent somehow interferes with dioxygenation [18]. Minor products are 1-hydroxymethylnaphthalene for 1-methylnaphthalene and 1-hydroxyethylnaphthalene for 1-ethylnaphthalene, which result from monooxygenation of the alkyl group. The minor reaction is not sufficient, however, to explain the yield difference. The major reaction for all disubstituted naphthalenes is monooxygenation of one of the methyl groups [18,22].

When a PAH contains a five-carbon ring (nonalternant structure), the potential reactions catalyzed by NDO become markedly more complex. The initial reaction can be of more than one type, including dioxygenation, monooxygenation, and desaturation reactions. In addition, NDO may catalyze subsequent reactions of the products of the initial reaction, which has not been the case for the reactions described thus far [9,22–24]. For example, for fluorene, the highest yield (\approx 85%) is observed for the dioxygenation product *cis*-3,4-dihydroxy-3,4-dihydrofluorene. A second, very minor dioxygenation product, *cis*-1,2-dihydroxy-1,2-dihydrofluorene, also is observed; relative yield was reported as less than 10% of the 3,4-diol in one study [9]. In addition to these two dioxygenation products, a monooxygenation product, 9-fluorenol, is observed at a yield of less than 15%. Subsequently, this product can undergo dioxygenation by NDO to yield *cis*-1,2-dihydroxy-1,2-dihydro-9-fluorenol and *cis*-3,4-dihydroxy-3,4-dihydro-9-fluorenol; both *syn*- and *anti*-dioxygenation with respect to the 9-hydroxyl group are observed [22]. Because NDO is capable of catalyzing secondary reactions, such as these for nonalternant PAHs, it is possible that the products may inhibit transformation of the parent PAH compound because of competitive effects.

Table 1. Enthalpies of reaction (ΔH_R) for known naphthalene dioxygenase-catalyzed and hypothetical transformations for the polycyclic aromatic hydrocarbons (PAHs) in the present study

PAH compound	Observed/hypothetical product	ΔH_R (kcal/mol)
Observed reactions		
Naphthalene	<i>cis</i> -1,2-Dihydroxy-dihydronaphthalene	-110.5
Anthracene	<i>cis</i> -1,2-Dihydroxy-dihydroanthracene	-115.4
Phenanthrene	<i>cis</i> -3,4-Dihydroxy-dihydrophenanthrene	-103.5
	<i>cis</i> -1,2-Dihydroxy-dihydrophenanthrene	-106.8
2-Methylnaphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-7-methylnaphthalene	-110.5
2-Ethyl-naphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-7-ethyl-naphthalene	-110.1
1-Methylnaphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-8-methylnaphthalene	-107.2
	1-Hydroxymethylnaphthalene	-111.5
1-Ethyl-naphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-8-ethyl-naphthalene	-108.6
	1-Hydroxyethyl-naphthalene	-111.4
2,6-Dimethylnaphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-3,7-dimethylnaphthalene	-111.0
	2-Hydroxymethyl-6-methylnaphthalene	-111.0
1,5-Dimethylnaphthalene	1-Hydroxymethyl-5-methylnaphthalene	-111.5
2,7-Dimethylnaphthalene	2-Hydroxymethyl-7-methylnaphthalene	-111.0
1,8-Dimethylnaphthalene	1-Hydroxymethyl-8-methylnaphthalene	-109.9
Fluorene	<i>cis</i> -3,4-Dihydroxy-dihydrofluorene	-100.5
	<i>cis</i> -1,2-Dihydroxy-dihydrofluorene	-101.3
	9-Fluoreneol	-111.9
Indene	<i>cis</i> -1,2-Indandiol	-129.0
	1-Indenol	-111.6
Indan	Indene	-95.8
	1S-Indanol	-110.5
Acenaphthene	1-Acenaphthenol	-110.6
	Acenaphthylene	-90.6
Hypothetical reactions		
Phenanthrene	<i>cis</i> -9,10-Dihydroxy-dihydrophenanthrene	-115.8
2-Methylnaphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-3-methylnaphthalene	-111.0
	<i>cis</i> -1,2-Dihydroxy-dihydro-6-methylnaphthalene	-110.5
	2-Hydroxymethylnaphthalene	-111.0
2-Ethyl-naphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-3-ethyl-naphthalene	-110.5
	<i>cis</i> -1,2-Dihydroxy-dihydro-6-ethyl-naphthalene	-110.5
	2-Hydroxyethyl-naphthalene	-112.5
1-Methylnaphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-4-methylnaphthalene	-110.7
	<i>cis</i> -1,2-Dihydroxy-dihydro-5-methylnaphthalene	-110.5
1-Ethyl-naphthalene	<i>cis</i> -1,2-Dihydroxy-dihydro-4-ethyl-naphthalene	-112.1
	<i>cis</i> -1,2-Dihydroxy-dihydro-5-ethyl-naphthalene	-112.2
Indan	<i>cis</i> -4,5-Dihydroxy-dihydroindan	-102.1
Indene	<i>cis</i> -4,5-Dihydroxy-dihydroindene	-102.6
	<i>cis</i> -6,7-Dihydroxy-dihydroindene	-100.9
Acenaphthene	<i>cis</i> -4,5-Dihydroxy-dihydroacenaphthene	-112.2

Thermodynamic analysis of reactions

All the theoretical and observed reactions of the types summarized thus far were compared on the basis of their thermodynamics for the 22 PAHs in the present study. The goal was to determine whether the change in enthalpy of the reactions could give insight regarding why some reactions are catalyzed whereas others are not, including why some bonds of the molecules are attacked by the enzyme whereas others are not. Examples of stoichiometric equations for each of the three reaction types include dioxygenation of naphthalene as described previously ($C_{10}H_8 + 2e^- + 2H^+ + O_2 \rightarrow C_{10}O_2H_{10}$), monooxygenation of fluorene ($C_{13}H_{10} + 2e^- + 2H^+ + O_2 \rightarrow C_{13}OH_{10} + H_2O$), and desaturation of indan ($C_9H_{10} + 2e^- + 2H^+ + O_2 \rightarrow C_9H_8 + 2H_2O$).

Table 1 lists the calculated enthalpies of reaction. Only transformations of the parent PAH compounds are included in the table; secondary reactions are not included. Compounds are organized by structure—that is, compounds with only six-carbon rings, followed by compounds with alkyl substituents and, finally, compounds with nonalternant ring structures. Included in Table 1 are reactions that reportedly have been observed as well as potential reactions that have not been ob-

served for the same compounds but that one might expect to occur. For example, dioxygenation of phenanthrene has been observed to occur only on two of the three rings, but we calculated the ΔH_R value for the hypothetical reaction product of *cis*-9,10-dihydroxy-dihydrophenanthrene. Another example is that side-chain monooxygenation has not been observed for 2-methylnaphthalene or 2-ethyl-naphthalene, but we calculated the enthalpies of reaction for formation of 2-hydroxymethylnaphthalene and 2-hydroxyethyl-naphthalene.

Analysis of the change in enthalpy associated with these reactions shows that all observed reactions were very favorable thermodynamically from an enthalpy standpoint, with high negative enthalpy changes. It also can be seen from comparing the values in Table 1 that differences in enthalpy changes for the various reactions are quite small and, in fact, may be the same within error when considering that the structures used for the enthalpy calculations likely contain some uncertainty. Enthalpy changes range from 100 to 130 kcal/mol for dioxygenation reactions and from 109 to 113 kcal/mol for monooxygenation reactions. The two desaturation reactions (acenaphthene to acenaphthylene and indan to indene) are only slightly less favorable (91 and 96 kcal/mol, respectively).

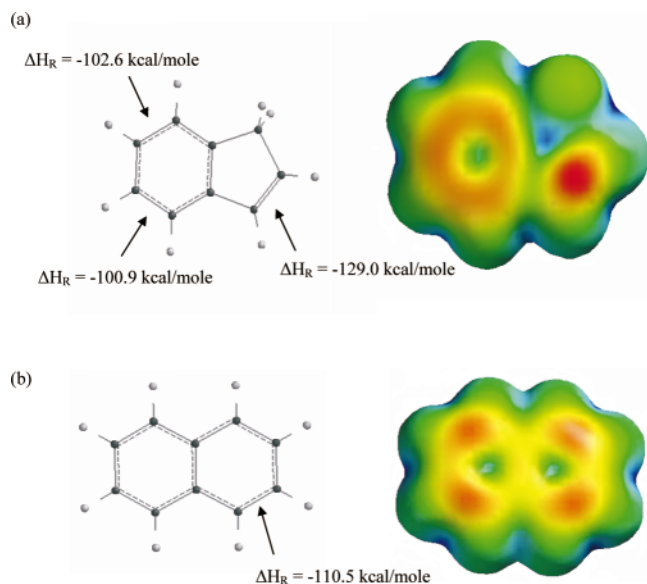


Fig. 1. The left half of the figure illustrates enthalpies of reaction (ΔH_R) for dioxygenation at different positions on the molecule for indene (a) and naphthalene (b). The right half of the figure shows ionization potential maps on constant electron-density surfaces. Values of ionization potential range from 12.2 eV (red) to 19.3 eV (blue).

Comparison of the enthalpies of reaction helps to explain a few observed NDO reactivity patterns, as illustrated by Figure 1. The dioxygenation reaction that occurs on the five-carbon ring of indene, which is the only dioxygenation reaction observed for that compound, has an enthalpy change of -129.0 kcal/mol. This makes it 26 and 28 kcal/mol more favorable than the two alternate dioxygenation reactions that are not observed for indene, as shown on the left side of Figure 1a. The right side of the figure shows the local ionization potentials of indene (Fig. 1a) and naphthalene (Fig. 1b) mapped on constant electron-density surfaces of the molecules. Ionization potential indicates the energy required to remove an electron from a molecule in its ground state at a given position on the molecule. Blue corresponds to high ionization potential; red indicates a low ionization potential. The dark red spot on the surface of indene corresponds to the double bond in the five-carbon ring; the less red ring corresponds to the aromatic ring. The double bond in indene clearly has the lowest ionization potential of any region in either molecule. In other words, the ionization-potential map illustrates what the difference in enthalpy change suggests: In indene, electrons are removed more easily from the double bond in the five-carbon ring than from the aromatic ring. Note also that comparison of indene and naphthalene reveals that the electrons are delocalized more completely on the aromatic ring of indene than are those in the aromatic rings of naphthalene. This also is true for indan (not shown graphically). Contributing resonance structures are equivalently stable for those two molecules, making none of the bonds on the ring more susceptible to attack, unlike the four regions clearly indicated on the rings of naphthalene. This provides a thermodynamic explanation of the lack of observed dioxygenation reactions on the aromatic rings of indan and indene.

In general, however, differences in the thermodynamics of the reactions do not explain the regioselectivity of the enzyme or the occurrence or absence of a reaction type for a given molecule. For example, as shown in Figure 2, differences

among three dioxygenation reactions for phenanthrene do not explain why 3,4-dioxygenation dominates (-103.5 kcal/mol), 1,2-dioxygenation is a minor reaction (-106.8 kcal/mol), and 9,10-dioxygenation does not happen at all (-115.8 kcal/mol). Shown as another example, the dioxygenation of acenaphthene (-112.2 kcal/mol), which does not occur, is more favorable than the dioxygenation of fluorene (-100.5 and -101.3 kcal/mol) that is the dominant reaction type for that molecule.

Enzyme fit analysis

The PAH substrates were examined in the context of interactions with the NDO enzyme active site. The first goal of this analysis was to determine approximate substrate-size limits based on the size and shape of the NDO active site. The second goal was to determine if space constraints could explain some of the reaction patterns not explained by the thermodynamic analysis and give insight regarding potential rate differences.

Table 2 lists the calculated dimensions for the 22 PAHs in the present study. A consistency of orientation was adopted such that for some of the compounds (1,4-dimethylnaphthalene, 1,5-dimethylnaphthalene, and 1-ethylnaphthalene) the length is smaller than the width. This was done for ease of comparison with other PAHs: The length refers to the dimension that is lengthwise for most substituted naphthalenes, although it is smaller than the width for these three compounds.

Based on the known crystal structure of NDO, the binding cavity of this enzyme is lined almost exclusively with hydrophobic residues, with the exception of a polar region near a nonheme iron at the active site at the bottom of the cavity. A phenylalanine (Phe224) and a leucine (Leu253) are at the entrance to the cavity and, apparently, are slightly mobile; Phe224 moves approximately 1 Å when indole is bound [13]. The approximate dimensions of the cavity are a height of 6 Å, a width of 8 Å, and a length of 10 Å, with the narrowest part of the cavity near the iron [5].

By examining the sizes of the compounds within the context of the active site of the enzyme, it can be seen that the dimension least likely to be problematic from a fit standpoint is the thickness. Most of the compounds are planar or nearly planar, so their thickness does not approach the roughly 6 Å height of the cavity. Two molecules have thicknesses that approach the thickness of the cavity: indan (5.4 Å), and 2-ethylnaphthalene (5.5 Å). However, flexibilities of the five-carbon ring of single bonds on indan and the ethyl group on 2-ethylnaphthalene mean that the thicknesses presumably could be altered if steric constraints were imposed by the amino acid residues lining the cavity.

The lengths of the compounds create potential fit problems. Even if a planar PAH fits completely diagonally into the cavity, the limit on length is roughly 12 Å if it is assumed that the PAH must be contained completely in the cavity for a reaction to take place. This available length corresponds to approximately three rings in a row; anthracene is 11.5 Å in length. Therefore, compounds with more than four rings almost certainly would not fit into the active site; fluoranthene, a four-ring compound, has dimensions of $11.4 \times 9.5 \times 3.8$ Å. This means that because of length constraints, the cutoff for acceptable NDO substrate size likely is approximately the same as the upper limit of the compounds included in the present study. Naphthalene dioxygenase probably is not capable of transforming PAHs with more than four rings; to our knowl-

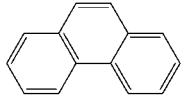
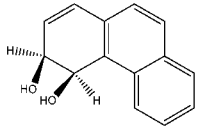
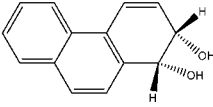
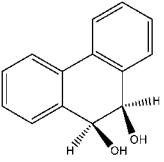
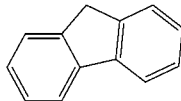
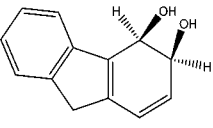
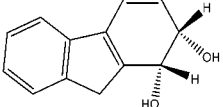
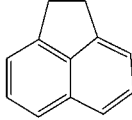
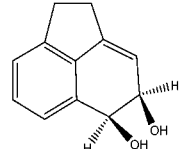
PAH Substrate	Product	ΔH_R	Yield
	 <i>cis</i> -3,4-dihydroxy-dihydrophenanthrene	-103.5 kcal/mole	>90%
	 <i>cis</i> -1,2-dihydroxy-dihydrophenanthrene	-106.5 kcal/mole	<10%
	 <i>cis</i> -9,10-dihydroxy-dihydrophenanthrene	-115.8 kcal/mole	not observed
	 <i>cis</i> -3,4-dihydroxy-dihydrofluorene	-100.5 kcal/mole	~85%
	 <i>cis</i> -1,2-dihydroxy-dihydrofluorene	-101.3 kcal/mole	minor
		 <i>cis</i> -4,5-dihydroxy-dihydroacenaphthene	-112.8 kcal/mole

Fig. 2. Enthalpies of reaction (ΔH_R) for potential naphthalene dioxygenase-catalyzed reactions of the polycyclic aromatic hydrocarbons (PAHs) phenanthrene (top), fluorene (middle), and acenaphthene (bottom).

edge, no such transformations have been reported in the literature.

The width of the compounds is a harder dimension to discuss succinctly, because the width at the position where the reaction is catalyzed is the most crucial. As mentioned in *Materials and Methods*, NDO has been crystallized with two different substrates bound, indole and naphthalene [13,14]. In both cases, the carbon atoms of the substrate that are involved in the reaction can be found at a distance of approximately 4.5 Å from the iron. A superposition of the two structures has been published comparing the C₁ and C₂ atoms of naphthalene with the C₂ and C₃ atoms of indole, which are the relevant atoms for the dioxygenation reactions [14]. The root mean square difference of 0.13 Å between the carbon atoms of the two substrates is smaller than the experimental error of the crystallizations. In other words, the atoms involved in the reaction are in essentially the same position for the two substrates. Based on this evidence, an assumption is made here

that the carbon atoms involved in the reaction would be required to bind at the same position for all substrates.

Examination of the substrate cavity in the vicinity of the iron atom reveals that the amount of space available for molecules that are much wider than indole and naphthalene is limited. Figure 3 illustrates an example using a ball-and-spoke model. When indole is bound at the active site, a distance of 3.4 Å exists between the nitrogen of indole and the carbonyl oxygen of an aspartate (Asp205). This leaves only approximately 0.5 Å of extra space available in this particular position. With constraints such as this, it appears that compound width could be problematic for larger bound substrates if the atoms involved in the reaction must be fixed at this position. Examination of the overall compound widths listed in Table 2 reveals values of up to 2.3 Å greater than that of naphthalene. These values, however, may or may not be important, because it is the width near the site of the reaction that is relevant. If the substrate is wide near the entrance to the cavity and narrow

Table 2. Calculated van der Waals dimensions (in Å) and substituent types present for the 22 polycyclic aromatic hydrocarbons in the present study

Compound	Length (Å)	Width (Å)	Thickness (Å)	Ring 1 width (Å)	Ring 2 width (Å)	Substituent types present
Indan	9.1	7.3	5.4	7.3	6.6	—
Indene	9.1	7.4	4.2	7.4	6.5	—
Naphthalene	9.1	7.3	3.8	7.3	7.3	—
1-Methylnaphthalene	9.1	8.3	4.2	8.3	7.3	α
2-Methylnaphthalene	10.1	7.3	4.2	7.3	7.3	β
1,2-Dimethylnaphthalene	10.4	8.3	4.2	8.3	7.3	α, β
1,3-Dimethylnaphthalene	10.1	8.3	4.2	8.3	7.3	α, β
1,4-Dimethylnaphthalene	9.1	9.3	4.2	9.3	7.3	α
1,5-Dimethylnaphthalene	9.1	9.4	4.2	8.3	8.3	α
1,6-Dimethylnaphthalene	10.1	8.3	4.2	8.3	7.3	α, β
1,8-Dimethylnaphthalene	9.1	8.3	4.2	8.3	8.3	α
2,6-Dimethylnaphthalene	11.4	7.3	4.2	7.3	7.3	β
2,7-Dimethylnaphthalene	11.3	7.3	4.2	7.3	7.3	β
2,3,5-Trimethylnaphthalene	10.1	8.3	4.2	8.3	7.3	α, β
1-Ethyl-naphthalene	9.1	9.7	4.2	9.7	7.3	α
2-Ethyl-naphthalene	11.2	7.3	5.5	7.3	7.3	β
Acenaphthene	9.1	8.3	4.2	8.3	8.3	—
Phenanthrene	11.6	7.9	3.8	7.3	7.3	—
Anthracene	11.5	7.3	3.8	7.3	7.3	—
Fluorene	11.4	7.3	4.2	7.3	7.3	—
1-Methylfluorene	11.4	8.3	4.2	8.3	7.3	α
Fluoranthene	11.4	9.5	3.8	9.5	7.4	—

near the catalytic site, fit problems would not be as likely. Therefore, it was decided to estimate the compound width at the end that could be positioned deepest in the cavity, nearest the iron.

Table 2 lists the widths of the terminal ring or rings for each compound, measured along an orthogonal to the dimension of the length. If one end of the compound is wider than the other, then the larger width is listed as the ring 1 width. For most compounds, width was simply measured as the van der Waals diameter of the terminal ring. For fluoranthene, the width at one end of the compound is of one ring; at the other end of the compound, it is the diameter of two adjacent rings. For acenaphthene, width includes contributions from an aromatic ring and a five-carbon ring at both ends of the compound. Also listed on the table are the substituent types that are present

in each molecule. We use α to denote substituents that are α to the bridgehead carbons (common to more than one ring) and β to denote substituents that are β to the bridgehead carbons.

Examination of the values in Table 2 reveals that none of the unsubstituted alternant compounds or the compounds with only β substituents are of widths that are anticipated to have fit problems. None of their terminal rings are more than 0.1 Å wider than those of naphthalene. Conversely, the presence of an α substituent increases the width of a terminal ring by approximately 1 Å, as is seen by comparison of 1-methylnaphthalene and naphthalene. Fluoranthene and acenaphthene, measured as described in the previous paragraph, also are substantially wider than naphthalene. Acenaphthene is 1 Å wider no matter which end enters the cavity first; fluoranthene is 2 Å wider if the two-ring end enters first. Therefore, for these compounds, it is probable that binding in the required position for reactivity could be difficult or impossible for at least one end of the molecule.

The analysis of substrate sizes in the context of the enzyme active site gives insight regarding some of the NDO reaction patterns that were not explained by the thermodynamic analysis. For example, no thermodynamic reasons were seen for the absence of reactions that would form *cis*-9,10-dihydroxy-dihydrophenanthrene or *cis*-4,5-dihydroxy-dihydroacenaphthene, as depicted in Figure 2. The fit analysis reveals that for the central ring of phenanthrene to be dioxygenated the entire length of the compound would have to fit into the cavity sideways, which is improbable given that the length of phenanthrene is more than 11 Å. Similarly, acenaphthene probably is too wide for the C₄ and C₅ carbons to bind in the proper location.

Width constraints also help to explain why smaller yields have been observed in reactions involving 1-substituted naphthalenes. The reactions may be hindered, because the substrate has to adjust to a nonideal binding position or even exit and re-enter the cavity with the other end entering first. Ultimately,

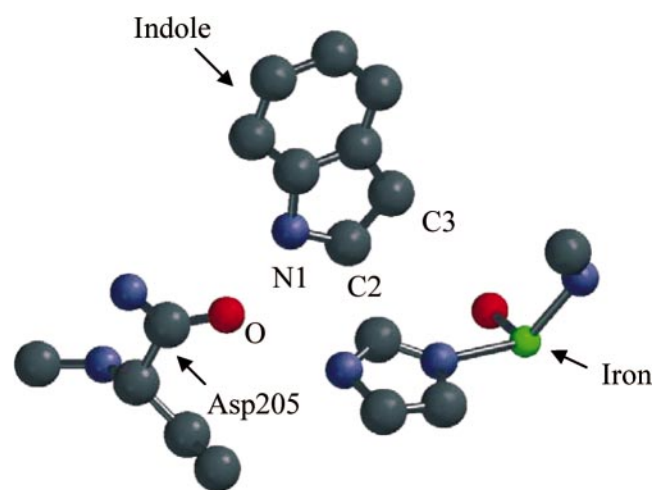


Fig. 3. A portion of the crystal structure of indole bound in the active site of naphthalene dioxygenase. Shown are indole, the active site iron, an aspartate (Asp205), and parts of the iron ligands His208 and His213. Structure from Berman et al. [12]; visualization generated using Spartan 2002 (Ver 1.0.2; Wavefunction, Irvine, CA, USA).

this may lead to slower biodegradation rates for compounds containing α substituents.

Some of the regioselectivity of the alkylated PAH reactions still cannot be explained. For example, it is not clear why the methyl group of 2-methylnaphthalene is not monooxygenated or why dioxygenation to form *cis*-1,2-dihydroxy-dihydro-6-methylnaphthalene is not observed. Unfortunately, less experimental data are available for alkyl-substituted PAHs compared with data for unsubstituted compounds. Perhaps these other reactions do occur but have not yet been reported. Or, perhaps some other factor not examined here is the reason, such as large activation energies for those particular reactions. Regardless, although width constraints help to explain some NDO reaction patterns, for now some of the regioselectivities that NDO exhibits toward alkylated compounds remain unexplained.

It should be noted that the binding position for the substrates likely is different than the position discussed for dioxygenation reactions when monooxygenation reactions are involved. With the same position, the products 1-acenaphthenol and 9-fluorene would not be expected because of width constraints. Crystallization of a bound monooxygenation substrate would be useful for exploring binding differences.

A final note is that this analysis of enzyme fit considers only molecular geometry and ignores the potential role of attractive and repulsive forces that may affect fit considerations. Although this is a limitation of the present study, we believe, because of the hydrophobic nature of the substrates and most of the binding cavity, as described previously, that the inferences drawn from this analysis are valid.

Extension to other enzymes

A secondary goal of the present work was to use NDO to understand PAH interactions with other dioxygenase enzymes. The question of how much of the previous analysis might be applicable to reactions catalyzed by other enzymes must be addressed. Evidence exists in the literature that the reactions catalyzed for PAHs are similar for a variety of dioxygenases. For example, Selifonov et al. [22] observed that reactions catalyzed for indan and indene by a NDO from *Pseudomonas aeruginosa* PAO1(pRE695) were very similar to those catalyzed by the NDO from *Pseudomonas* sp. strain 9816-4 [23] and by the toluene dioxygenase of *Pseudomonas putida* F1 [25]. In fact, observed products largely were the same except for variations in stereochemistry. Products of acenaphthene and acenaphthylene from reactions catalyzed by *Beijerinckia* sp. strain B8/36 (now reclassified as *Sphingomonas yanoikuyae* B8/36) also were very similar to the NDO-catalyzed products [26]. Anthracene and phenanthrene products from B8/36 were identical, including similar relative yields for the two phenanthrene products [21]. However, a few variations in the catalyzed reactions have been observed. Transformations of several alkylated PAHs by *Sphingomonas paucimobilis* 2322 revealed the capability of that organism to perform ring dioxygenation or alkyl group monooxygenation as reported here, but monooxygenation of the methyl group of 2-methylnaphthalene was observed in addition to dioxygenation of the unsubstituted ring [27]. Similarly, an enzyme in *Pseudomonas putida* CSV86 has been observed to perform ring dioxygenation and methyl group monooxygenation on both 2-methylnaphthalene and 1-methylnaphthalene [28]. In the case of fluorene, the three initial products that have been observed for NDO were the same as those observed for an unnamed enzyme

from *Arthrobacter* sp. strain F101 [29], but the dibenzofuran 4,4- α -dioxygenase from *Sphingomonas* sp. strain RW1 apparently is not capable of the monooxygenation reaction [30].

Although some variations in products are observed, the general reactions catalyzed for PAHs are consistent enough that extension of the findings of the NDO analysis to other enzymes seems to be promising. For example, the ability to catalyze multiple reactions for nonalternant PAHs appears to be distributed to dioxygenase enzymes other than NDO. This means that product inhibition may occur for dioxygenases other than NDO. As a note of interest, it appears that the presence of subsequent enzymes in the biodegradation pathway, which determines the ability of an organism to use PAH substrates as growth substrates, is more variable than the capability to catalyze these initial transformations. This variability, however, would not necessarily be expected to impact initial PAH transformation or transformation rates.

Because of the variability of amino acid residues that contribute to the substrate-binding cavity, especially in regions away from the active site, it might be expected that substrate range is more variable than the reaction types catalyzed. Yeates et al. [6] compared partial dioxygenase α -subunit sequences from 31 different reference bacteria and soil isolates. They found significant areas of sequence homology for portions of the residues that contribute to the active site. For example, Asp205, which is crucial for electron transfer in NDO [31], was conserved in all the enzymes, as were His208 and Asp362, two of the iron ligands. Several additional residues were wholly conserved, and many others were at least partially conserved, across the enzymes. There were also areas of high sequence divergence, however, especially in the region of NDO residues 221 to 297, which includes the residues at the opening of the cavity. This might mean that the size of the cavity and, therefore, the size of the substrates that it can accommodate varies for different dioxygenases.

Parales et al. [32] used mutagenesis to explore which NDO cavity residues were most critical for substrate specificity. They chose to check for changes in reactions of naphthalene, biphenyl, and phenanthrene after mutations of nine residues near the active site: Asn201, Phe202, Val260, Trp316, Thr351, Phe352, Trp358, Asp362, and Met366. With the exception of Asp362 mutations, which resulted in an inactive enzyme, the biggest effects were seen by mutating position 352. Reducing the size of the phenylalanine residue to two smaller hydrophobic residues (leucine and valine) increased the size of the cavity and resulted in decreased enantiomeric purity (92 and 96%, respectively) of the naphthalene dioxygenation product. It also changed relative yields of the phenanthrene products, and the F342V change even allowed formation of some of the *cis*-9,10-dihydroxy-dihydrophenanthrene product that is not observed in wild-type NDO, presumably because of width constraints, as discussed. The Phe352 was partially conserved in the enzymes of the Yeates et al. study; 23 of 31 enzymes had phenylalanine at that position. Enzymes that had variations at position 352 had a variety of hydrophobic and polar residues substituted: Serine, glutamine, isoleucine, threonine, and leucine were found.

These examples of variations in active-site residues and their observed effects illustrate that it is unclear how much of the enzyme fit analysis can be applied broadly to dioxygenases in general. Overall substrate-size constraint calculations likely will vary across different enzymes. The evidence for high conservation of several residues near the active site, however,

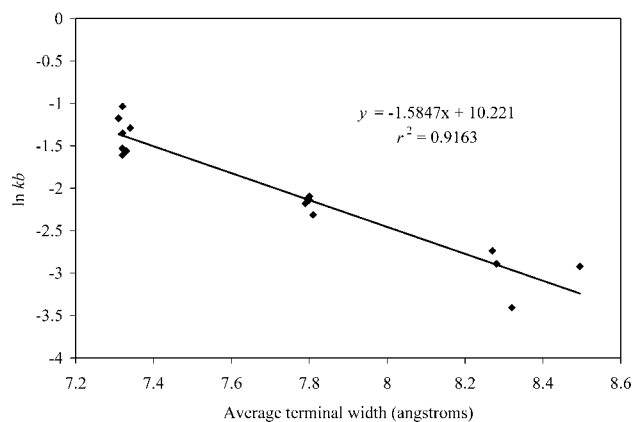


Fig. 4. Relationship between average terminal compound width (Å) and aqueous phase biodegradation rate by a mixed culture for alternant polycyclic aromatic hydrocarbons. Relative biodegradation rates are compared using a first-order, biomass-normalized rate coefficient k_b [1/(mg protein/L·h)].

supports an assumption that the width constraints discussed in the context of the NDO binding cavity may be applicable to a range of dioxygenase enzymes.

Correlation with biodegradation rate data

Premised on the assumption that width constraints near the active site are common to a broad range of dioxygenase enzymes, we examined the correlation of substrate width with biodegradation rate using available data for the 22 PAHs in the present study [2]. Specifically, the data used were measurements of the biodegradation rate in the aqueous phase by a mixed bacterial consortium at substrate concentrations designed to be sufficiently low such that substrate biodegradation kinetics were first order. Rates were reported as a first-order, biomass-normalized rate coefficient, k_b [1/(mg protein/L·h)]. Nonalternant compounds were excluded from this analysis, because their molecular structures have other features that inhibit the biodegradation rate. Rates of indene, indan, fluorene, and acenaphthene are slower than those of alternant compounds of comparable width, potentially because of competitive effects, as described previously.

Examination of the data shows that terminal compound width does correlate with biodegradation rate in this system. Figure 4 is a plot of the average terminal width (of the two ends of the compound) of the 16 alternant PAHs included in the present study versus the natural logarithm of k_b . A linear relationship is seen; the regression equation and its r^2 value are given in Figure 4. Interestingly, 1-methylfluorene and fluoranthene rates (not shown) do fall in line with the alternant compounds, suggesting that if product inhibition is, indeed, responsible for the rate deviations of the other nonalternant compounds, perhaps subsequent transformations are not catalyzed for those two compounds by the enzyme or enzymes of the mixed consortium.

The relatively strong relationship observed between terminal compound width and biodegradation rate data by a mixed consortium supports the idea that the width constraints of the NDO active site may be applicable to other enzymes. It appears that terminal compound width is a valuable molecular descriptor for predicting relative biodegradation rates of alternant PAHs, even for systems with enzymes other than naphthalene dioxygenase present, but that rates for nonalternant compounds may vary from predictions.

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REFERENCES

- Cerniglia CE. 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Curr Opin Biotechnol* 4:331–338.
- Wammer KH, Peters CA. 2005. Polycyclic aromatic hydrocarbons: A structure-based study. *Environ Sci Technol* 39:2571–2578.
- Knights CD, Peters CA. 2003. Aqueous phase biodegradation kinetics of ten PAHs. *Environ Eng Sci* 20:207–218.
- Gibson DT, Parales RE. 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* 11:236–243.
- Kauppi B, Lee K, Carredano E, Parales RE, Gibson DT, Eklund H, Ramaswamy S. 1998. Structure of an aromatic-ring-hydroxylating dioxygenase: Naphthalene 1,2-dioxygenase. *Structure* 6:571–586.
- Yeates C, Holmes AJ, Gillings MR. 2000. Novel forms of ring-hydroxylating dioxygenases are widespread in pristine and contaminated soils. *Environ Microbiol* 2:644–653.
- Jeffrey AM, Yeh HJC, Jerina DM, Patel TR, Davey JF, Gibson DT. 1975. Initial reactions in oxidation of naphthalene by *Pseudomonas putida*. *Biochemistry* 14:575–584.
- Jerina DM, Daly JW, Jeffrey AM, Gibson DT. 1971. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene—Bacterial metabolite from naphthalene. *Arch Biochem Biophys* 142:394–396.
- Resnick SM, Gibson DT. 1996. Regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4. *Appl Environ Microbiol* 62:4073–4080.
- Hites RA, Simonsick WJ. 1987. *Calculated Molecular Properties of Polycyclic Aromatic Hydrocarbons*. Elsevier Science, Amsterdam, The Netherlands.
- Daubert TE, Danner RP. 1989. *Physical and Thermodynamic Properties of Pure Chemicals: Data Compilation*. Hemisphere, New York, NY, USA.
- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. 2000. The Protein Data Bank. *Nucleic Acids Res* 28:235–242.
- Carredano E, Karlsson A, Kauppi B, Choudhury D, Parales RE, Parales JV, Lee K, Gibson DT, Eklund H, Ramaswamy S. 2000. Substrate binding site of naphthalene 1,2-dioxygenase: Functional implications of indole binding. *J Mol Biol* 296:701–712.
- Karlsson A, Parales JV, Parales RE, Gibson DT, Eklund H, Ramaswamy S. 2003. Crystal structure of naphthalene dioxygenase: Side-on binding of dioxygen to iron. *Science* 299:1039–1042.
- Sayle R, Milner-White EJ. 1995. RasMol: Biomolecular graphics for all. *Trends Biochem Sci* 20:374.
- Sander LC, Wise SA. 1997. *Polycyclic Aromatic Hydrocarbon Structure Index*. National Institute of Standards and Technology, Gaithersburg, MD, USA.
- Wise SA, Bonnett WJ, Guenther FR, May WE. 1981. A relationship between reversed phase C18 liquid chromatographic retention and the shape of polycyclic aromatic hydrocarbons. *J Chromatogr Sci* 19:457–465.
- Bestetti G, Bianchi D, Bosetti A, DiGennaro P, Galli E, Leoni B, Pelizzoni F, Sello G. 1995. Bioconversion of substituted naphthalenes to the corresponding 1,2-dihydro-1,2-dihydroxy derivatives. Determination of the regio- and stereochemistry of the oxidation reactions. *Appl Microbiol Biotechnol* 44:306–313.
- Deluca ME, Hudlicky T. 1990. Microbial oxidation of naphthalene derivatives—Absolute configuration of metabolites. *Tetrahedron Lett* 31:13–16.
- Koreeda M, Akhtar MN, Boyd DR, Neill JD, Gibson DT, Jerina DM. 1978. Absolute stereochemistry of *cis*-1,2-dihydrodiol, *trans*-1,2-dihydrodiol, and *cis*-3,4-dihydrodiol metabolites of phenanthrene. *J Org Chem* 43:1023–1027.
- Jerina DM, Selander H, Yagi H, Wells MC, Davey JF, Mahadevan V, Gibson DT. 1976. Dihydrodiols from anthracene and phenanthrene. *J Am Chem Soc* 98:5988–5996.
- Selifonov SA, Grifoll M, Eaton RW, Chapman PJ. 1996. Oxidation of naphthoaromatic and methyl-substituted aromatic

- compounds by naphthalene 1,2-dioxygenase. *Appl Environ Microbiol* 62:507–514.
23. Gibson DT, Resnick SM, Lee K, Brand JM, Torok DS, Wackett LP, Schocken MJ, Haigler BE. 1995. Desaturation, dioxygenation, and monoxygenation reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4. *J Bacteriol* 177:2615–2621.
 24. Lee K, Resnick SM, Gibson DT. 1997. Stereospecific oxidation of (R)- and (S)-1-indanol by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4. *Appl Environ Microbiol* 63:2067–2070.
 25. Wackett LP, Kwart LD, Gibson DT. 1988. Benzylic monoxygenation catalyzed by toluene dioxygenase from *Pseudomonas putida*. *Biochemistry* 27:1360–1367.
 26. Schocken MJ, Gibson DT. 1984. Bacterial oxidation of the polycyclic aromatic hydrocarbons acenaphthene and acenaphthylene. *Appl Environ Microbiol* 48:10–16.
 27. Dutta TK, Selifonov SA, Gunsalus IC. 1998. Oxidation of methyl-substituted naphthalenes: Pathways in a versatile *Sphingomonas paucimobilis* strain. *Appl Environ Microbiol* 64:1884–1889.
 28. Mahajan MC, Phale PS, Vaidyanathan CS. 1994. Evidence for the involvement of multiple pathways in the biodegradation of 1-methylnaphthalene and 2-methylnaphthalene by *Pseudomonas putida* Cvs6. *Arch Microbiol* 161:425–433.
 29. Casellas M, Grifoll M, Bayona JM, Solanas AM. 1997. New metabolites in the degradation of fluorene by *Arthrobacter* sp. strain F101. *Appl Environ Microbiol* 63:819–826.
 30. Bunz PV, Cook AM. 1993. Dibenzofuran 4,4- α -dioxygenase from *Sphingomonas* sp. strain Rw1—Angular dioxygenation by a three-component enzyme system. *J Bacteriol* 175:6467–6475.
 31. Parales RE, Parales JV, Gibson DT. 1999. Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity. *J Bacteriol* 181:1831–1837.
 32. Parales RE, Lee K, Resnick SM, Jiang HY, Lessner DJ, Gibson DT. 2000. Substrate specificity of naphthalene dioxygenase: Effect of specific amino acids at the active site of the enzyme. *J Bacteriol* 182:1641–1649.