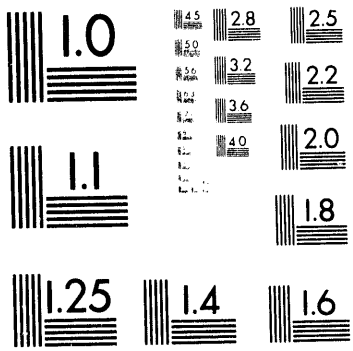
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ON USING RATIONAL ENZYME REDESIGN TO IMPROVE ENZYME-MEDIATED MICROBIAL DE-HALOGENATION OF RECALCITRANT SUBSTANCES IN DEEP-SUBSURFACE ENVIRONMENTS

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Abstract

Heavily halogenated hydrocarbons are one of the most prevalent classes of man-made recalcitrant environmental contaminants and often make their way into subsurface environments. Biodegradation of heavily chlorinated compounds in the deep subsurface often occurs at extremely slow rates because native enzymes of indigenous microbes are unable to efficiently metabolize such synthetic substances. Cost-effective engineering solutions do not exist for dealing with disperse and recalcitrant pollutants in the deep subsurface (i.e., ground water, soils, and sediments). Timely biodegradation of heavily chlorinated compounds in the deep subsurface may be best accomplished by rational redesign of appropriate enzymes that enhance the ability of indigenous microbes to metabolize these substances. The isozyme family cytochromes P450 are catalytically very robust and are found in all aerobic life forms and may be active in may anaerobes as well. We are attempting to demonstrate proof-of-principle rational enzyme redesign of cytochromes P450 to enhance biodehalogenation.

Background

The Problem and Overview

During the last half century, organic chemists have synthesized many new halogenated organic compounds with the highly "desirable" property of extra stability; thus environmental recalcitrance was engineered into these synthetic compounds. These substances are frequently used as solvents, degreasing agents, intermediates of synthesis, and a variety of speciality purposes. Halogenated compounds derived from industrial wastes, agricultural practices, waste products of energy production, and national defense activities have entered the environment through routine disposal practices and accidents. An EPA listing of 129 *Priority Pollutants* contained 62 halogenated organic compounds, while 27 out of 65 compound classes on a list of toxic pollutants were halogenated hydrocarbons (1). The extent of chemical contamination on U.S. Department of Energy (DOE) lands was recently summarized; heavily halogenated hydrocarbons also constitute one of the largest classes of contaminants on DOE lands (2). Only more recently have the toxicological and carcinogenic properties of halogenated organics become significantly understood (3,4). Thus, contamination of water and soil/sediments by heavily chlorinated and synthetic hydrocarbons is a serious environmental problem.

Underground aquifers are a major source of fresh water in many countries and supply 90 to 95% of the drinking water of rural populations in the United States (5). Such aquifers are becoming contaminated with the toxic by-products of human activities (6). Most aquifers harbor significant numbers of microorganisms, and these populations often have the ability to degrade otherwise harmful organics (reviewed in 7). The problems associated with environmental restoration on contaminated DOE lands (reviewed in 8) has spurred an interest in the microbiology of the deep terrestial subsurface. Subsurface sediments have been collected from deep boreholes on DOE lands in South Carolina (7,9) and Washington (10,11). Initial studies these samples contain diverse populations at virtually all levels examined down to 1,000 meters. Organisms collected from these environments may prove useful for remediation.

A recent survey of lakes in Sweden and soil samples from different parts of the world concludes

that halocarbons are more widespread in nature than can be explained by human activities (12). This finding is consistent with the fact that over 700 halometabolites have been identified as natural products of terrestial bacteria, fungi, and plants, as well as of marine algae and animals (13). Several reviews on microbial biodegradation of halocarbons have recently appeared (14-17). That microorganisms can rather readily degrade many man-made halogenated compounds with few halogens is therefore not so surprising. However, the more heavily chlorinated compounds generally appear to be man-made in origin and extremely resistant to biodegradation.

The nature of deep contamination, often highly disperse and inaccessible, substantially limits or precludes the successful application of many strategies currently being applied to remediate surface and near-surface contamination. Remediation of subsurface contamination has proven to be problematic. "Given the magnitude of subsurface contamination problems and the rather poor success rate for current remediation technologies, it is obvious that an improved understanding of the processes governing subsurface transport and fate of contaminants and limiting ground water and soil remediation is needed" (18). Moreover, "the case for biodegradation of recalcitrant, synthetic and sometimes toxic components associated with hazardous wastes and environmental contamination problems is not transferable immediately from our knowledge of conventional waste treatment process engineering" (19). Cost-effective remediation of heavily halogenated synthetic substances in deep subsurface environments may require novel bioremediation alternatives (20,21). This report describes the basis and progress of a new approach, "rational enzyme redesign," which has considerable promise as a cost-effective and timely process for solving many environmental problems associated with heavily halogenated hydrocarbons in the deep subsurface.

The first step of the rational enzyme redesign process is to engineer into an appropriate enzyme(s) improved catalytic ability for degrading one or more heavily halogenated target compounds. This involves a coupled experiment-theory research effort that fully exploits biotechnology and computed-based molecular simulations. The second step will employ indigenous microflora from a variety of niches from the subsurface. Appropriate organisms could be cultured in large numbers (on the surface); the gene for a redesigned enzyme(s) inserted into a plasmid or the genomic DNA; and the engineered cells then delivered back to the subsurface. Presumably, such cells returning to a familar niche will have a reasonable chance of survival and increasing biodegradation of the targeted compound(s).

Biochemistry of Microbial Dehalogenation

Four mechanisms of biodehalogenation of chlorinated organic compounds have been identified: substitutive, oxidative, reductive, and eliminative (22). Some aerobic microorganisms can dehalogenate a limited number of chlorinated organics, but often cannot metabolize the most heavily chlorinated substances. For instance, bacteria capable of aerobically and cometabolically dehalogenating trichloroethylene, dichloroethylenes, vinyl chloride, and monochlorinated benzenes are usually unable to dehalogenate the most heavily chlorinated aliphatic or aromatic analogues (23-27).

Reductive dehalogenation is the only known biodegradative mechanism for perchlorinated

compounds (with all hydrogens bonded to a carbon replaced by a halogen) such as hexachlorobenzene, tetrachloroethylene, pentachlorophenol, and carbon tetrachloride (22). Different methanogenic bacteria have been shown to slowly reductively (anaerobically) dechlorinate tetrachloroethylene; oxidative dehalogenation of trichloroethylene can be ~100 to 15,000 times faster (see Table 2 of reference 22).

The environmental and public health significance of microbial-catalyzed reductive dehalogenation reactions has generated much interest to understand the molecular basis of these transformations. Reductive dehalogenation of carbon tetrachloride was found to occur in an *E. coli* (28), *Pseudomonas* (29), and other bacteria (30). Although there is evidence that transition-metal coenzymes, without enzymes, can reductively dechlorinate various heavily chlorinated compounds (30-33), there is also clear evidence that enzymes may be involved (30; and references therein). Microorganisms native to the DOE's Hanford Site in Washington State have been shown to degrade carbon tetrachloride when supplied with an electron donor such as acetate (34); although, the active biochemical group (i.e., coenzymes and/or enzymes) has not been studied. *Pseudomonas putida* G786 and purified cytochrome P450cam have been demonstrated to catalyze anaerobic reductive dehalogenation of perchlorinated hydrocarbons such as carbon tetrachloride (35) and hexachloroethane (36). It should be appreciated that P450s contain an essential transition-metal coenzyme, heme. For each chlorine atom that is replaced by a hydrogen atom, there is a requirement for a two-electron reduction step (36). The reduction step is an energy-consuming step and usually is not accompanied by energy gain as a result of breaking the carbon-halogen bond. An ideal substrate enzyme pair can approach 100% coupling of electron transfer and product formation; pairs without lock-and-key regiospecificity often have very low coupling and, thus, are energetically very inefficient.

Bioremediation of heavily chlorinated organics by microorganisms currently relies heavily on cometabolic degradation; that is, fortuitous degradation of the target contaminant by organisms that are utilizing another compound as a growth substrate (37; and references therein). Under nutrient-limiting conditions, as is generally the case in the deep subsurface, the simultaneous availability of primary metabolite(s) and heavily chlorinated compounds will generally be rare. Thus, in order to have significant rates of biodehalogenation of heavily chlorinated compounds under nutrient-limiting conditions, it is beneficial to have highly coupled electron transfer and product formation. Since transition-metal coenzymes have a solvent accessible reactive center, it is expected that coupling of electron transfer and dehalogenation cannot attain values possible by an equivalent enzyme-coenzyme complex containing a buried active site with "lock-and-key" specificity for a particular heavily chlorinated compound(s).

Enzyme Evolution and Timely Biodegradation

It is generally assumed that the genetic traits necessary for biodegradation of synthetic compounds can be selected for, in relatively short time intervals, under laboratory or field conditions. But since enzyme "lock-and-key" or "induced-fit" mechanisms have generally evolved to be very specific for their particular substrate(s) and product(s), microbial degradation of mankind's synthetic substances will probably only occasionally occur at significant levels due to (coincidental) cometabolism, gene recruitment, or timely (fortuitous) selection/evolution. While it is less than straightforward to obtain a quantitative time

estimate, genealogical analysis of different evolutionarily related proteins suggests that enzymes evolve at more or less characteristic rates. The unit evolutionary period (UEP) is the time in millions of years needed to establish a 1% difference between the amino acid sequence between divergent lines; the UEP range for several enzymes is 2 to 20 (38). Thus, the evolution of an enzyme with enhanced specificity and efficiency for a non-native substrate may proceed very slowly if multiple and specific genetic changes are simultaneously required for adaptation (20,39). On the one hand, microorganisms with rapid generation times, in the presence of appropriate selection pressure, could perhaps evolve new enzyme functions in a much shorter period than suggested above. On the other hand, microorganisms existing under nutrient-limited conditions may require extra long periods to evolve. Therefore, "designing" multiple changes into an enzyme may be the only systematic and timely alternative to waiting for essentially geological timescales for such rare changes to occur *in vivo* under environmental conditions, or under selection conditions in the laboratory or field which may also require a very long period of time based on statistical probabilities.

Protein Engineering, Dynamics and Rational Redesign

The term protein engineering means changing a protein to make it serve a different purpose. In principle, one may change the functional characteristics of an enzyme by altering its substrate specificity, catalytic action, pH, or temperature optima (40-45). Redesigning enzyme catalytic specificities and physical features is a relatively new enterprise with far reaching consequences; some very significant results have already been obtained (41,46-49). It is especially noteworthy that rational site-directed mutagenesis has been used to alter the functional class of an enzyme from a cytochrome to a demethylase (45).

The field of protein engineering developed in the 1980s as advances in recombinant DNA techniques made it possible to specifically modify the coding sequence of a cloned gene. Obviously one cannot make all possible combinations of amino acid changes for even the smallest enzyme, so the essential question is which changes to make. To have a reasonable chance to successfully carry out rational redesign, to alter an enzyme's specificity, and or enhance its efficiency, it is necessary to have adequate structural data about the native enzyme and native enzyme-substrate interactions, an understanding of the underlying catalytic mechanism(s), a cloned gene for site-directed mutagenesis experiments, and robust simulation methods for studying protein dynamic fluctuations. Starting with a three-dimensional x-ray crystal and/or NMR structure as input, computer modeling and simulations can play a valuable if not essential and unique role in this process (20).

"Any attempt to understand the function of proteins requires an investigation of the dynamics of the structural fluctuations and their relation to activity and conformational change" (50). "Many protein 'engineers' fail to appreciate the subtle ways that protein dynamics are altered by mutagenesis and the effects of these alterations on the thermodynamics and kinetics of chemical steps" (51). Although experimentally determined enzyme-protein structures are invaluable in understanding structure-function relations, they generally suffer from the following limitations that can be significantly resolved from a self-consistent, coupled experiment-theory analysis:
1.) The structural image obtained is an average in time and space and, therefore, some atomic

positions may be due to mathematical averaging.
2.) Conformational details are usually nonresolvable for structures less than 5 to 10 percent in population. Understanding these lower populated conformations may be important since the bioactive conformer(s) may be a less populated or transient conformation.
3.) Molecular surface properties can be significantly deformed due to crystal packing in the solid state, or due to different solution conditions from those of *in vivo*. How surface distortions impact more interior residues is also not currently well understood.
4.) Proton locations are generally not experimentally determined. Two such related problems may seriously impact the quality of a simulation. Inappropriate choices of local pKa effects on potentially ionizable groups and/or the conformation of protons on side chain hydroxyl groups of buried residues (52) can lead to very serious consequences in simulations that do not first correct for these situations.

A self-consistent, coupled experiment-theory analysis, starting from a high-resolution experimental structure, can potentially help resolve the above limitation. This analysis will lead to a better understanding of the dynamic mobilities and fluctuations inherent in enzyme structure and function. Moreover, these experimentally unexpected, but testable, hypotheses will often be crucial to understanding the mechanisms essential for enzyme function alteration. It should be stressed that current redesign objectives are to increase regiospecificity and efficiency for an enzyme with experimentally demonstrated inherent chemical capacity for carbon-halogen bond cleavage. At the present time, we are not attempting to alter the inherent bond making-breaking mechanism of an enzyme, only the physico-chemical components of catalysis.

Cytochrome P450: An Ideal Enzyme for Rational Redesign To Improve Environmental Biodegradation

Background

What is now known to be the cytochrome P450 gene superfamily consists of numerous enzymes that are remarkable in the variety of chemical reactions catalyzed and in the number of substrates attacked (53,54; and references therein). Animals, plants, and microorganisms all contain cytochromes P450; in mammals, isozymes of P450 have been found in all tissues examined, and individual cells may produce dozens of different types of P450 (53). There are two major functions of P450 enzymes: (1) some isozymes have critical and specific roles in metabolism of endogeneous substances (i.e., conversion of cholesterol to corticoid and sex hormones) and (2) other isozymes process the burden of natural (and man-made) "foreign" chemicals in a relatively nonselective manner (54; and references therein). Metabolism of xenobiotics often leads to the formation of more polar compounds that are more readily excreted; this is usually a detoxication process, but sometimes foreign compounds are converted to products with much greater cytotoxicity, mutagenicity, or carcinogenicity. There is broad interest in the P450s because of the significance of these enzymes in a wide variety of disciplines ranging from the environmental sciences, agriculture, inorganic chemistry, and medical genetics.

The known substrates of P450s range in size from ethylene to that of cyclosporin A (molecular

weight of 1201) (54). Most of the substrates are lipophilic. "The number of man-made 'environmental chemicals' has been estimated as greater than 200,000, most of which are thought to be potential substrates for P450" (53).

Sequence data from several dozen P450 sequences indicate that the P450 gene superfamily diverged more than 2 billion years ago from a single ancestral gene. The emergence of many new genes during the last 800 million years most likely reflects "animal-plant warfare" (55). As animals began to ingest plants, plants developed new noxious metabolites; animals responded by developing novel enzymes to degrade the new plant metabolites. Can mankind's synthetic compounds induce a similar process? Can rational enzyme redesign be used to significantly reduce the time required for adaptation?

P450 Biochemistry and Structure

Cyochrome P450cam

P450 is a generic term applied to a group of hemoproteins defined by unique spectral properties (near-UV λ_{max} at 450 nm for the Fe^{II}-CO complex) imparted by the presence of an axial cysteinyl thiolate ligand to the heme iron (56). Most of the reactions begin with the transfer of electrons from NAD(P)H to either NADPH-cytochrome P450 reductase in microsomal systems or a ferredoxin reductase and a nonheme iron protein in the mitochondrial and bacterial systems, and then to cytochrome P450. Many P450 reactions proceed with the stoichiometry characteristic of monooxygenases (57):

$$2NAD(P)H + O_2 + RH \rightarrow 2NAD(P)^+ + H_2O + ROH$$

In a reaction of this type, hydroxylation usually occurs at a carbon atom, but is also seen at heteroatoms such as N, S, and I; dealkylation of amines and ethers, and epoxidation as well as reduction reactions are also catalyzed by P450s (reviewed in 57; 58).

Most P450 reactions are relatively slow and rates of ~1 nmol of product formed/nmol of P450/min are common for many substrates. The catalytically fastest P450 enzymes are those in bacteria, such as P450cam (see next section), which form products at rates greater than 10^3/min. The native P450cam reaction cycle is believed to be rate limited by electron-transfer, and the chemical intermediates have been well characterized to the point where the input of the second reducing equivalent initiates dioxygen bond scission (reviewed in 59). In the faster bacterial P450 systems, the enzyme is very selective with respect to substrate stereochemistry. In other P450 systems that are typically slower, however, the rate-limiting step varies. For instance, studies have indicated that hydrogen atom abstraction can be rate-limiting, as can product release, and oxygen availability (reviewed in 54). This suggests that the P450cam system is ideal for learning the principles by which nature has designed a selective and efficient P450 enzyme.

In general, P450 systems are bound to an intracellular membrane and thus are refractory to extensive solubilization; whereas, P450cam is soluble and easily purified. Among the various P450s that have been isolated, P450cam, a cytosolic enzyme expressed by the soil bacterium *Pseudomonas putida* that catalyzes the first step in the degradation of camphor (60; reviewed in 61), is the first P450 for which crystallographic structures have been solved. These

structures include substrate-free P450cam refined at 2.20 Å (62), camphor-bound P450cam refined to 1.63 Å (63), and the ferrous carbon-monoxide- and camphor-bound ternary P450cam complex refined to 1.9 Å (64). In addition, crystal structures have been determined for P450cam complexed with different inhibitors and alternative substrates (65-68) as well as an active site mutant (69). The three-dimensional x-ray structure of P450cam in both substrate-bound and free forms has served as an excellent road map for structure-function investigations of the active site. The position of the camphor molecule and its interactions with the protein heme group in the P450cam complex are understood in atomic detail as depicted in Table 3 and Figure 4 of the 1.63 Å resolution structure report (63). The heme provides the largest contact surface for interactions with the substrate. The remainder of the camphor atoms are in contact with a limited number of protein active site amino acid side chains: Phe-87, Tyr-96, Thr-185, Leu-244, Val-247, Thr-252, and Val-295. The side chain hydroxyl group of Tyr-96 forms a hydrogen bond with the keto group of the camphor molecule, but because this interaction is on the opposing face of camphor to that which is catalytically attacked, it is apparent that this hydrogen bond is mainly just for steering purposes (63). P450cam is unlike many other enzymes in that its active site does not contain acidic or basic amino acids sidechains involved in binding the substrate. P450cam has been cloned and expressed in *Escherichia coli* (70,71). This has made possible a number of site-directed mutagenesis studies of P450cam (72-74; reviewed in 59).

The mechanistic pathways and intermediates for P450cam catalysis are reasonably well understood. When camphor binds inside the active site pocket, the active site waters (62) are displaced resulting in a pentacoordinate high-spin heme iron atom. Water access (75) to the heme iron has been shown to regulate both the spin state equilibrium (76) and the redox potential (77) of P450cam. The substrate-free enzyme possesses a low-spin (S=1/2), hexacoordinated, aquo-liganded ferric heme, and a network of hydrogen-bonded solvent molecules occupies the buried substrate binding pocket (62). The major structural alteration associated with substrate binding results from the camphor-induced expulsion of six water molecules from the active site, yielding a high-spin (S=5/2) pentacoordinated heme (62,63). The change from low-spin to high-spin heme is accompanied by a change in reduction potential from -300 to -170 mV, resulting in a favorable driving force for electron transfer from the physiological redox partner putidaredoxin (78).

Aerobically, the one-electron reduction of P450cam is followed by binding of dioxygen, resulting in dioxyferrous P450cam; the subsequent second electron-transfer step initiates O-O bond scission, with formation of water and a high-valent, iron-oxo intermediate suggested to be the active hydroxylating agent (79; reviewed in 59). Substrate hydroxylation follows, most likely by a radical mechanism; under tightly coupled reaction conditions, 100 percent of the NADH-reducing equivalents are used for product formation (reviewed in 59). If substrate hydroxylation and electron flux are decoupled, nonproductive shunting may occur through either autoxidation pathways and hydrogen peroxide formation or an oxidase pathway yielding excess water (reviewed in 59).

New P450 Structures

The only bacterial P450 that is known to utilize a FAD- and FMN-containing flavoprotein

reductase is $P450_{BM-3}$. The active monomer has a molecular weight of 120 kD and is a soluble monooxygenase from *Bacillus megaterium* that catalyzes the hydroxylation and epoxidation of several fatty acid substances (80). The crystallization and preliminary x-ray diffraction analysis of the heme containing domain of $P450_{BM-3}$ (55 kD) was reported in 1992 (81) and the structure has been solved to 2.0 Å resolution (82). The crystal structure of $P450_{BM-3}$ is very interesting in that the unit cell contains two forms of the enzyme: one with the active site relatively closed and the other with the active site relatively open. The access channel in the open form is relatively large, but this is not surprising since many of the fatty acid substrates of $P450_{BM-3}$ have molecule weights about twice that of camphor. Fatty acid monooxygenases have also been reported to be present in plants, yeast, bacteria, and mammals (reviewed in 83). P450 monooxygenases are believed to play an important role in mammalian hydroxylation of endogenous fatty acids, prostaglandins, and leuotrienes. The gene encoding this catalytically self-sufficient polypeptide was engineered into two separate functional domains: the NH_2-terminal domain (55 kD) that contains the heme and functions as the monooxygenase and the COOH-terminal domain (66 kD) that contains both FAD and FMN and functions as a NADPH:cytochrome reductase (84).

Peterson, Deisenhofer and coworkers have also solved the x-ray diffraction structure of another cytochrome P450 from soil *Pseudomonad* which can grow on α-terpineol as its sole source of carbon and energy (81).

We believe that these new P450 structures present excellent opportunities for rational redesign to efficiently dehalogenate substances with sizes and shapes more analogous to the natural substrate of the individual P450. For instance, $P450_{BM-3}$ is expected to be more suitable for redesigning its specificity to dehalogenate hydrocarbons that are twice the size of camphor than would be redesigning P450cam.

P450s and Dehalogenation

It is well known that polyhalogenated hydrocarbons undergo one- and two-electron reductions mediated by cytochromes P450 (reviewed in 3). A purified reconstituted P450 system has been shown to reductively convert carbon tetrachloride to chloroform (85). It has been shown that cytochrome P450cam is capable of dehalogenating some hydrocarbons oxidatively and reductively under the same conditions, although the rate of conversion is relatively slow (86); the latter study indicates that some halogenated hydrocarbons permeate bacterial cells more rapidly than they can be metabolized, and the resulting buildup leads to toxicity. Recently, a series of halogenated methane and ethane substrates were studied, and many of them were found to be significantly dehalogenated by a reconstituted P450cam system (36,87). Others have also explored the dehalogenation potential of P450s (35,88-93). The potential of bacterial P450s for bioremediation has been realized (94). Interest in recruiting P450 into bioremediation processes has heightened since the discovery of a P450 that catalyzes the initial dehalogenation of pentachlorophenol in *Rhodococcus rhodochrous* (95); this class of P450 has been recently purified and characterized (96).

Agents that selectively inhibit P450 are known to hinder reduction of halogenated hydrocarbons

(reviewed in 97). Reductive dehalogenation under anaerobic conditions by P450 is believed to proceed by radical formation (98) and then a carbanion (99,100). The first step in the reaction is the enzyme-catalyzed transfer of a single electron to the antibonding or d-orbital of the carbon-halogen bond to yield a radical anion as a transient intermediate. Oxygen binding in the substrate binding site prevents substrate access to the second electron; hence, little carbon tetrachloride reduction is seen in the presence of dioxygen (3). Cytochromes P450 have enormous catalytic versatility but active site regioselectivity (complementarity between the enzyme's active site and the substrate) and dynamics are critical to the observed efficiency of dehalogenation (Paulsen and Ornstein, unpublished data).

Structure-Function-Dynamic Relationships

An important general finding from a variety of data is the crucial importance of substrate mobility at the active site; this mobility is linked both to the regiospecificity and the efficiency of the reaction (reviewed in 59). Recent molecular dynamics simulations focused on obtaining a fundamental molecular understanding of the complementarity and dynamic motions involved in P450cam interactions with its native substrate camphor (101-103) and the substrate analogues norcamphor (103-105) and thiocamphor (103,106). These simulations considered all published coordinates of P450cam including 405 amino acids (with all hydrogens), the heme, substrate and waters of crystallization. On the one hand, these reduced solvent model simulations are much less computationally intensive than heavily solvated simulations, but nevertheless require about 0.8 CPU hours of a CRAY XMP for each psec using highly vectorized code (Discover from Biosym Technology, Inc.). On the other hand, because we were primarily interested in addressing questions about the deeply buried active site, a reduced solvent model simulation seemed adequate, as was demonstrated in simulations with a large solvent shell (Paulsen and Ornstein, unpublished data). While cytochrome P450cam catalyzes the hydroxylation of camphor to 5-*exo*-hydroxycamphor with 100% stereospecificity and 100% efficiency, norcamphor is hydroxylated by this enzyme yielding 45% 5-*exo*-, 47% 6-*exo*-, and 8% 3-*exo*-hydroxynorcamphor with only 12% overall efficiency (73). Simulations with camphor-bound or norcamphor-bound P450cam were performed and compared in order to better understand the underlying factors controlling P450cam regiospecificity and catalytic efficiency (103-105). These data suggest that the lack of product specificity is due, at least partially, to the mobility of the substrate within the active site. Therefore, it is not necessary for substrate to enter this deeply buried active site prealigned for catalytic activity. Second, the high mobility of norcamphor in the active site leads to an average increase in separation between the heme iron and the substrate of about 1.0 Å; this increase in separation may be the cause of the uncoupling of electron transfer from product formation (reduced efficiency) when norcamphor is the substrate (1). Not surprisingly, camphor has a significantly reduced level of rotational mobility in the active site compared to norcamphor (102-104). On the basis of the x-ray crystal structure (63) and several 150 to 200 psec molecular dynamics simulations (101,103), camphor-bound P450cam appears very stable.

"One puzzling question regarding substrate binding is how the camphor molecule gains access to the active site. Unlike many enzymes, the active site of P450cam does not define an open cleft at the molecular surface but is well sequestered with no obvious route to the pocket" (63). Thus, significant dynamic fluctuations of the enzyme may be required for camphor, or other

substrates, to bind and for product to exit. We have analyzed the detailed dynamic motion of each amino acid residue that lines the buried active site in substrate-bound P450cam. Most of the backbone and sidechain dihedral angles associated with the active site residues have low fluctuations, as is the case for the chi-2 dihedral angle of Tyr-96, but chi-2 of Phe-87 (in van der Waals contact with Tyr-96) exhibits very significant flexibility whether the bound substrate is camphor (102), norcamphor (104), or thiocamphor (103).

The x-ray crystal structure of thiocamphor-bound P450cam (68) gave the most unexpected orientation in the active site of any substrate analogue considered to date. Unlike camphor, carbons 5 and 6 of thiocamphor are positioned away from the heme iron, while carbon 3 is positioned close to the heme iron. While camphor is hydroxylated with 100% regiospecificity at carbon 5 and 100% efficiency with respect to NADH consumption, thiocamphor is hydroxylated at carbons 5 (64%), 6 (34%) and 3 (2%) with 98% efficiency (72). Therefore, the crystallographically observed thiocamphor-bound P450cam structure is not consistent with the catalytic observation.

In an attempt to better understand this puzzling situation, we performed molecular dynamics simulations on thiocamphor-bound P450cam starting with the x-ray structure, with and without the presumptive heme iron-oxo reactive intermediate (103,106). The variation over the simulation for the distance between the heme iron and the 3- and 5-position carbons of thiocamphor for the case without an oxygen ligated to the heme iron is similar to that in the x-ray crystal structure. In 100% of the computed structures, carbon 3 is closer to the heme iron. In the thiocamphor simulation with the presumptive heme iron-oxo intermediate present, the distance between the oxygen (of the iron-oxo intermediate) and the 3- and 5-position carbons switches. During the warmup, the two carbons "switch" such that now the 3-position carbon face is away from the heme, while the 5-position carbon face is close to the heme. Not only do these simulations offer a consistent explanation for the structural and catalytic experiments, but they suggest a unique role for the simulations since the iron-oxo intermediate has to date proved too unstable to be observed for P450cam. Such simulation data are expected to be invaluable in predicting the regiospecificity and efficiency of P450cam mutants for alternative substrates. From these results involving thiocamphor as substrate, it appears that a simulation without the iron-oxo oxygen is predictive of the x-ray binary structure, while a simulation with an iron-oxo intermediate is predictive of the experimental catalytic product regiospecificity. (Interestingly, simulations with and without the oxygen on the heme iron yield similar results when the substrate is camphor or norcamphor (103; and references therein).

Simulations with norcamphor-bound P-450cam and thiocamphor-bound P450cam indicate that statistical sampling, starting geometry, and random velocity seed significantly influence computed regiospecificity and efficiency predictions. This is not surprising because these non-native complexes are inherently less complementary so that initial positions are not necessarily the best or only stable enzyme-substrate geometry. On the one hand, we are currently studying such effects to access ways to minimize the error in non-native complex simulations of limited duration [i.e., the effect of different starting geometries, the effect of different random velocities, and the effect of simulation duration (105; Paulsen and Ornstein, unpublished data)]. On the other hand, this problem can potentially be minimized by appropriately packing the active site for a non-native substrate. In particular, to minimize

this error, the density of the active site (substrate and protein) should approach that of the native complex. We are developing methods to choose mutants with ideal complementarity and density prior to starting the molecular dynamics simulations.

P450 Rational Redesign

Proof-of-Principle Redesign of P450cam

Before attempting to redesign the activity of P450cam for a heavily halogenated substrate, we have chosen to first modify the regiospecificity and/or efficiency of P450cam for camphor analogues. We are currently atternpting to design mutants of P450cam that will (1) have increased efficiency for hydroxylating norcamphor, and (2) alter the regiospecificity of norcamphor hydroxylation. Achieving these intermediate redesign goals will give us considerable confidence and guidance toward the goal of modifying the specificity of P450cam to initiate the degradation of targeted pollutants. We now describe our first prediction involving the former goal.

We noticed in our simulations on camphor-bound (102), norcamphor-bound (104), and thiocamphor-bound (103) P450cam that the sidechain of Phe-87 exhibited unexpectedly large conformational mobility for a deeply buried and bulky active site residue. On the basis of these results and preliminary modeling and simulations, we proposed replacing Phe-87 by the bulkier aromatic residue Trp to increase the coupling when norcamphor is the substrate. The simulation of this mutant showed reduced mobility of norcamphor in the active site as compared to the wild type simulation. The prediction (in advance of experimental evidence) suggested that norcamphor would be more tightly coupled to electron transfer as is indicated by the shorter separation between the substrate center-of-mass and the heme iron for the mutant (107). This shortening in the substrate heme separation is a direct result of the tighter packing of residue 87 in the mutant. When the theoretical prediction was tested, the mutant was found to have twice the coupling exhibited by the wild enzyme when the substrate was norcamphor (Bass, Filipovic, Sligar and Ornstein unpublished data).

It has been predicted from simulations that Thr-185 when mutated to a Phe would result in a large increase in coupling and reduction in 3-hydroxy product when the substrate is norcamphor; both results have been experimentally confirmed (108). These results suggest that our presently employed computational methods are useful for characterizing and predicting substrate-P450cam interactions and dynamics in the binding pocket.

Redesign of P450cam for Dehalogenation of Heavily Chlorinated Compounds

Although cytochromes P450 have enormous catalytic versatility, the lack of adequate substrate steric complementarity for the active site, not inherent chemical reactivity, may often greatly limit the efficiency of the enzyme. Thus, we are pursuing the redesign of P450cam to improve complementarity for heavily chlorinated target compounds in order to enhance dehalogenation efficiency. The five basic design steps are outlined below:

1. Graphically position a target halocarbon inside the active site in a mechanistically ideal

orientation for reductive (or oxidative) dehalogenation. Maintaining a hydrophobic active site, graphically make appropriate substitutions of amino acids that sterically 'lock' in the target substrate. The principle we plan to follow is to use hydrophobic amino acid side chains to build a tight-fitting ("repacked") active site that reduces dynamic motions and has a density similar to that observed in the wild type active site. If possible, amino acid residues with a weak hydrogen bond donor will be aligned for possible H-bonding to one or more chlorine atoms (weak acceptor) of the target substrate. Visually repacked active sites will be energy minimized. Only those repacked structures maintaining the essence of the original structure upon minimization will be considered further.

2. A series of short molecular dynamics trajectories will be run (from 5 to 20 psec) to see if the energy-minimized geometry is maintained. Only those repacked structures with suitable geometry and dynamic properties will be considered further. Longer molecular dynamics simulations (50 to 200 psec) to further test for steric stability in mechanistically relevant configuration(s) will then be run.

3. Propose additional amino acid replacements (repeat steps 1 and 2) or propose mutants for site-directed construction.

4. Experimentally construct mutant(s) and test for binding and catalytic activity under aerobic, limited-aerobic (less than or equal to 5% O_2) and/or anaerobic environments. If significant binding and/or activity is observed, attempt to crystallize and determine the binary x-ray crystal structure of halocarbon-bound P-450cam mutant(s).

5. If *in vitro* activity is adequate, then begin *in vivo* and environmentally relevent experiments.

Detoxification and mineralization (to inorganic salts, carbon dioxide, and water) and not just disappearance of the parent halogenated compound must be considered since the breakdown intermediates may be of equal or greater concern (109). For example the degradation pathway of a single compound such as perchloroethylene can lead to the production of several chlorinated hydrocarbons (110). In principle, a series of enzymes can be designed with different specificities and then inserted into one or more microbes in order to effectively dehalogenate the parent and the intermediate dehalogenation products. Once the first redesigned P450cam mutant enzyme that can efficiently dehalogenate a target substance has been accomplished further redesign for other halogenated hydrocarbons should proceed more readily.

For reasons noted above, rational enzyme redesign may be the only practical scientific-technology for remediation of deep and disperse subsurface halogenated hydrocarbons. In comparsion to more conventional engineering remediation solutions, the application of "designer" microbes is relatively cost effective. Moreover, this technology may result in a permanent solution rather then transfering the substance from one location to another, as often occurs with conventional "cleanup" methods. For instance, soil venting often just transfers the contaminants to the air. An interesting spin off of this technology is that the biotransformation of halogenated hydrocarbons using redesigned enzymes and/or microbes may offer new routes for the synthesis of useful intermediates and novel products.

Perspective and Summary

The ability of microorganisms to adequately degrade the growing array of synthetic compounds

generated by the activities of mankind is uncertain. Although microorganisms have tremendous capacity to evolve to meet new selection pressures, the time scale required to do so, depending on the complexity of the required underlying metabolic modifications, may be unacceptably long as measured by human time scales when hazardous substances are involved. The unique advantage of rational design is its ability to foster discovery of new enzymatic forms that would otherwise have an extremely low probability of evolving biologically under either laboratory-controlled or natural conditions.

Cytochromes P450 are an ideal enzyme for rational redesign studies because of the robust catalytic properties of this family, the availability of structural and mechanistic data, as well as the availability of clones. We have described our progress in studying fundamental structure-function-dynamic relationhips to design cytochrome P450cam mutants that are stereochemically and dynamically engineered to efficiently bind and degrade halocarbons. Improving (by design) the catalytic (bond-making and bond-breaking) mechanism of the enzyme may soon also be possible. Our expectation for redesign at this time, however, is only at the level of optimization of the substrate geometry and dynamics in the binding pocket (i.e., stereochemical complementarity). Mutants that look promising after theoretical analysis will then be constructed, expressed, and tested for *in vitro* activity. Mutants that exhibit adequate *in vitro* activities can then be tested under laboratory-simulated environmental conditions.

Moreover, not only is there broad interest in the cytochrome P450 superfamily of enzymes because of their significance in a wide variety of disciplines ranging from the environment, inorganic chemistry, and medical genetics, but the principles learned will lead future studies with other key enzymes. With the growing number of 3-dimensional structures of enzymes determined by x-ray crystallography and the prospects of high-field NMR enzyme structure determination, rational design can be expected to play an increasing role in enzyme engineering for enhanced remediation, biotechnology, and industrial uses.

Numerous concerns have been raised over the ecological impact of the release of genetically engineered microorganisms into the environment (111-113). In view of the natural diversity of cytochromes P450, it seems that the intentional redesign of additional cytochromes P450 for incorporation into microorganisms for environmental bioremediation purposes could be a potentially acceptable risk, especially in cases where no other acceptable means exist for degrading known hazardous contaminants. The use of engineered microorganisms to assist in the removal of anthropogenic environmental contaminants is becoming a viable alternative for economic, regulatory, and social reasons. The burgeoning costs of remediation technologies and site monitoring and the increasingly stringent regulatory requirements on contaminant concentrations for site closure demand relatively rapid remediation technologies that effectively remove trace levels of contaminants. With time, in the face of increasing concern over the effects and fate of environmental contaminants, both society as a whole and the Environmental Protection Agency appear to be displaying a more lenient attitude toward the selected release of microorganisms engineered to degrade contaminants. Redesigned biodegradative enzymes could also be used as part of an extracellular enzyme treatment strategy. In addition to engineered enzymes potentially having desirable catalytic properties, a single enzyme could theoretically be made more resistant to harsh environments where microorganisms may be unable to grow at adequate levels. Since viable organisms are not needed, the use of extracellular enzymes could not possibly result in significant negative ecological impact.

Chakrabarty (114) has discussed the biological imperative associated with genetic selection experiments (i.e., a microorganism grown in a chemostat with only one source of carbon and energy, the recalcitrant substance). The urgency of any type of effective metabolic advantage, whether by gene recruitment or evolution of new biodegradative genes, is likely to be more severe than in nature where alternative solutions to growth are often possible because of the availability of a mixture of carbon and energy sources. Therefore, the nature of the environment plays a large role in dictating the mode and speed with which evolution of new degradative genes may arise in microorganisms (114). On the other hand, it is also recognized that the number of genetic mutations possible for any enzyme is too immense to be optimized by nature; nature can never globally optimize a protein with more than about 30 residues (115). The best approach to evolve new enzyme function is by an iterative combination of knowledge-based design and directed evolution. If current and future environmental remediation problems, as well as many of industrial and health related goals, are to be effectively resolved, the science and art of rational enzyme redesign will play an increasing role.

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