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$P450_{BM-3}$ AND OTHER INDUCIBLE BACTERIAL P450 CYTOCHROMES: Biochemistry and Regulation

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INTRODUCTION[1]

Cytochrome P450 (1), initially identified as a pigment from liver with a characteristic absorption peak at 450 nm when bound in the reduced state to carbon monoxide (2, 3), is actually a multigene family of heme proteins that are found in essentially all eukaryotes and in some bacteria (4–8). The ancestral P450 gene is probably more than two billion years old (9, 10) and it has been suggested that P450s may have played an early role in oxygen-detoxification during chemical evolution (11). In general, the P450 moieties, in the presence of O_2 and NAD(P)H and one or more electron-transfer proteins, can function as monooxygenases and are implicated in the oxygenation of a vast array of hydrophobic carbon compounds of both endogenous and environmental origin.

Distribution and Function of P450 Cytochromes in Nature

In humans, P450-dependent monooxygenases are essential for several vital metabolic pathways including bile acid formation, steroid hormone synthesis,

[1]The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase.

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vitamin D metabolism and certain polyunsaturated fatty acid oxygenations (12, 13), and for the oxidative detoxification and elimination of many potentially dangerous hydrophobic pollutants in our food, water and air (14, 15). This family of enzymes also inactivates and causes the development of tolerance to many therapeutic agents and drugs (16, 17). Similar functions are served in other higher animals and in many invertebrates (18–23). Insects, for example, may use P450-dependent systems to develop tolerance to various insecticides (24). In some fungi and bacteria, P450 enzymes are used to initiate the oxidation of hydrocarbons or other recalcitrant compounds for use as carbon sources for growth (6, 7). These P450-dependent monooxygenase systems can be broadly segregated into three types based on the minimum number of protein components necessary to reconstitute monooxygenase activity. These three categories are illustrated in Figure 1.

Figure 1A illustrates the three-protein component type that occurs in the mitochondria of mammalian cells (normally those involved in steroid metabolism) and typically in those bacteria shown to contain P450-dependent monooxygenase systems. The mitochondrial P450s are intrinsic membrane proteins while the associated electron-transfer components are isolated in

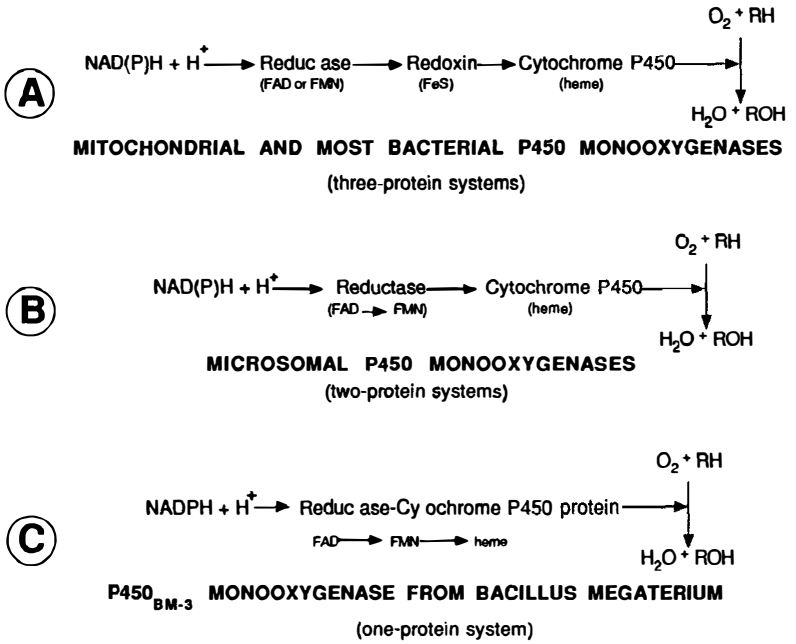

Figure 1 Comparisons of mitochondrial, microsomal, and bacterial P450 monooxygenase systems.

soluble form; the bacterial proteins, including the P450 components, are generally (but not always) soluble (4, 5). For example, the steroid hydroxylase P450s isolated from adrenal cortex mitochondria require the mediation of both a reductase, which contains FAD and interacts directly with NADPH, and a small ferredoxinlike protein (adrenodoxin), which shuttles electrons between the reductase and the P450 cytochrome (25). Analogous systems found in bacteria are considered in detail later in this review.

Figure 1B depicts the two-protein P450-dependent monooxygenase system found in the endoplasmic reticulum of higher animal cells as well as in most other eukaryotic cells (5). With respect to the number of distinct species, this is by far the largest class of P450s found in mammals (14, 18, 26) but has not yet been clearly established in prokaryotes that, as a group, lack true membranous organelles. These so-called "microsomal" P450 systems have only one electron-transfer protein that interacts with NADPH and transfers electrons directly to the various P450 cytochrome species. This protein, NADPH-P450 oxidoreductase, contains both FAD and FMN (but no iron-sulfur center) and is an integral membrane protein that exists in only one major form in contrast to the multitude of distinct P450 species. In this protein, FAD serves as the initial electron acceptor from NADPH whereas FMN can be considered analogous to the redoxin component of the three-component system in that it interacts with and reduces the P450 moiety (27, 28). Figure 1C illustrates the only known example to date of a one-protein P450-dependent monooxygenase system, that isolated from *Bacillus megaterium* (29). This single soluble polypeptide contains FAD, FMN, and a P450 heme moiety (but no Fe-S center): after initial reduction by NADPH, all electron transfers are intramolecular.

Mechanisms of Induction of P450 Cytochromes

Most cytochrome P450 systems are inducible and many excellent reviews have covered this subject in depth (8, 12, 30–34). This section briefly describes the method used to classify the types of induction in this discussion of bacterial P450s. Unfortunately, no completely satisfactory system exists for classifying types of P450 induction. Ideally, classification should be based on the mechanism of the induction process, optimally at the molecular level. Despite considerable progress in dissecting the nature and sequence of events that lead to the receptor-mediated induction of certain liver microsomal P450s (specifically those involving the Ah receptor; for current reviews see refs. 31, 32), many other eukaryotic and prokaryotic P450 systems are induced by essentially unknown mechanisms (18, 30). A less elegant but more universally applicable classification scheme involves categorizing induction by the chemical (or biochemical) nature of the inducer (i.e. polycyclic aromatics, phenobarbital, ethanol, PCN-glucocorticoids, etc.), a system used to advan-

tage by Okey in his recent review emphasizing mammalian liver P450s (30). This method is too specific, however, to be useful in categorizing bacterial systems.

For this survey, a classification scheme has been formulated that recognizes three types of induction among P450-producing bacteria. These include: (*a*) substrate induction, (*b*) nonsubstrate induction by exogenous or endogenous substances, and (*c*) induction by environmental factors. The first category includes not only true substrates for the induced enzyme but also substrate analogs, i.e. substances that, although not metabolized by the enzyme, mimic a substrate by interacting with the enzyme at the substrate-binding site and, like a true substrate, cause induction, presumably by the same mechanism. In bacteria, these substrate-type inducers are usually exogenous (i.e. "xenobiotics") but may be endogenous and, by definition, do not include oxygen. The second category involves induction mediated by a substance (again, excluding oxygen) that is neither a substrate nor a substrate analog and may be a xenobiotic or a cell product. The final catch-all category includes such factors as temperature, pressure, light, oxygen tension, pH, and the depletion of carbon sources leading to a release of catabolite repression. Naturally, combinations of these three groupings are theoretically possible.

Scope of this Review

Although more than a dozen inducible bacterial P450 cytochromes have now been identified (7, 35), and are considered here, this study emphasizes the six that have been characterized, cloned and sequenced. These incude: $P450_{cam}$ from *Pseudomonas putida,* the most comprehensively studied of any P450 cytochrome; $P450_{soy}$ from *Streptomyces griseus;* $P450_{SU1}$ and $P450_{SU2}$ from *Streptomyces griseolus;* and $P450_{BM-3}$ and $P450_{BM-1}$ from the same strain of *Bacillus megaterium*. Kanemoto et al (35a) reported that the nucleotide sequence of the plant-inducible locus *pinF* from *Agrobacterium tumefaciens* contained two open reading frames *(pinF1* and *pinF2)* that coded for polypeptides with relative molecular weights of 47,519 and 46,740, respectively. A comparison of the deduced amino acid sequences of these open reading frames indicated that they are similar to known polypeptide sequences for cytochrome P450 enzymes, especially $P450_{cam}$. However, since the proteins themselves have not yet been isolated or characterized nor have their substrates been determined, they will not be further considered in this review. $P450_{cam}$ has been extensively reviewed recently (35–38) and is thus treated here only briefly. Compared to $P450_{cam}$, much less is known about $P450_{soy}$. Although it has been cloned and sequenced (F. S. Sariaslani, personal communication), the information has not been released as of this writing and hence an analysis of structure-function relationships and sequence homologies of $P450_{soy}$ with other P450s is precluded from this survey. The cloning and

sequencing of $P450_{SU1}$ and $P450_{SU2}$ were reported as this review was being written and a summary of this most recent work has been included with a discussion of previous work on these herbicide-metabolizing monooxygenases.

This review examines in-depth cytochrome $P450_{BM-3}$, and, to a lesser extent, $P450_{BM-1}$, not only because of the author's research interests but also because these proteins have not yet been comprehensively reviewed.[2]

MULTI-COMPONENT INDUCIBLE P450 SYSTEMS

In general, the bacterial P450 systems whose components have been purified and well characterized require three proteins for monooxygenase activity. However, there is no a priori reason why this should always be so; the existence of a one-component bacterial system has been clearly established (29) and several inducible bacterial P-450 systems that have not yet been completely characterized may in fact be two-protein component monooxygenases.

Cytochrome $P450_{cam}$ from Pseudomonas putida

COMPONENTS OF THE CATALYTICALLY ACTIVE COMPLEX AND THEIR INTERACTION The $P450_{cam}$ system isolated from strain C1 from *Pseudomonas putida* (now designated ATCC 17453) contains three soluble protein components (39) that function according to the scheme shown in Figure 1A to carry out the stereospecific 5-*exo*-hydroxylation of camphor. These include the hydroxylase component, $P450_{cam}$ (M_r = 47,000), putidaredoxin (M_r = 12,500), a 2 Fe-2S protein that directly interacts with and reduces the P450 component and the NADH-specific putidaredoxin reductase (M_r = 43,000), a FAD-containing component that couples with and reduces putidaredoxin. The initiating step, substrate binding by $P450_{cam}$, is followed by the first one-electron reduction by putidaredoxin; interaction with molecular oxygen then yields a P450-substrate-oxygen complex that may now accept a second electron from reduced putidaredoxin; and then rearrange to yield the oxidized form of $P450_{cam}$, water, and hydroxycamphor. (For a comprehensive discussion of the structure-function relationships of the $P450_{cam}$ system with emphasis on electron transfer and catalysis, see ref. 35.) The $P450_{cam}$ component has been cloned, sequenced, and the complete three-dimensional X-ray structure determined (40–44). $P450_{cam}$, containing 414 amino acids, is smaller

[2]In general, the P450s reviewed here are designated by the name commonly used in the scientific literature and/or assigned by those who discovered or characterized the protein. On occasion, when there is no consensus, the terms used are those employed in reviews by Sariaslani (7) or by Sligar & Murray (35). When neither option is available, a name is provided for a P450 cytochrome based either on its major substrate or on the bacterium from which it was isolated.

than the typical membrane-associated P450s of mammalian liver, in part attributable to its lack of a large N-terminal hydrophobic amino acid sequence for membrane-insertion. Some $P450_{cam}$ sequences indicate homologies with the mammalian P450s, particularly around the heme-binding region (for detailed discussion, see refs. 9, 37, 38, 45). Sequence comparisons of $P450_{cam}$ with other bacterial P450s are considered later.

SUBSTRATE SPECIFICITY AND INDUCTION OF THE $P450_{cam}$ SYSTEM The functional role of the $P450_{cam}$ monooxygenase system is to initiate the oxygenation of camphor and certain related compounds so that they may eventually serve as carbon sources for growth (35). Although D-camphor (2-bornanone) is both the prototypical substrate and inducer for $P450_{cam}$ (thus classifying this as a substrate-inducible P450), the specificity of the system is not absolute. Although D-camphor is probably the best monooxygenation substrate, several other bornane derivatives are almost as active and at least a dozen (including several nonsubstrates) are as good as or better than camphor as inducers (46, 47). The mechanism of induction of the $P450_{cam}$ system is complex and not yet completely understood. Early genetic studies showed that the $P450_{cam}$ system as well as many other enzymes involved in camphor degradation were encoded on a large (240 kb) transmissible plasmid. Substrate induction by camphor led to the simultaneous induction of the three components of camphor 5-*exo*-hydroxylase (i.e. the $P450_{cam}$ system) as well as 5-*exo*-alcohol dehydrogenase and several other of the early catabolic enzymes (46, 48, 49). More recently, Koga et al reported (50) the cloning of three genes (*cam*R, *camD,* and *cam*C) from the CAM plasmid. The *cam*C gene encodes the $P450_{cam}$ protein whereas *cam*D specifies the 5-*exo*-alcohol dehydrogenase. The *cam*R gene is a camphor-responsive negative regulator residing upstream of the strutural gene. When camphor is added the P450 and dehydrogenase proteins are expressed coordinately; when a portion of the *cam*R gene at the 5'-end containing an open reading frame is deleted in the construct, these two proteins are expressed constitutively. Reinsertion of the deleted fragment restores substrate control. This finding implies that the *cam*R gene encodes a repressor protein that negatively controls expression of *cam*C, *cam*D and other genes in the same operon. Presumably, inducers like camphor cause the dissociation of this repressor or in some other way prevent it from strongly inhibiting transcription.

Cytochrome $P450_{soy}$ from Streptomyces griseus

COMPONENTS OF THE CATALYTICALLY ACTIVE COMPLEX AND THEIR INTERACTION Cytochrome $P450_{soy}$ (so named because it is induced when *S. griseus* (ATCC 13273) is grown on complex media enriched with soy flour) is probably the most promiscuous of any known eukaryotic or bacterial

P450 with respect to substrate specificity (7, 51, 52). Like most other bacterial P450-dependent monooxygenases, the native $P450_{soy}$ system requires three-protein components for catalytic activity that appear to function according to the scheme in Figure 1A (52). The P450 component has been purified to homogeneity and characterized as a soluble polypeptide (M_r = 47,500) in association with one ferriprotoporphyrin IX group (53). A second component of this system, a 7 Fe ferredoxin that transfers electrons to $P450_{soy}$, has also been purified and characterized (54) and its amino acid sequence determined (55). This protein contains 105 amino acids, has a calculated molecular weight of 12,291, and is highly homologous (>50%) with the six other 7 Fe ferredoxins sequenced from different bacteria. The third component, which has not yet been purified to homogeneity or completely characterized, is a soluble, flavin-containing ferredoxin reductase specific for NADH (56). In addition to ferredoxin from *S. griseus* this reductase can couple electron flow to other ferredoxins such as adrenodoxin or the ferredoxins from spinach and *Clostridium pasteurianum* (56). In reconstitution experiments, the protein from *S. griseus* can also be replaced by NADPH-specific spinach ferredoxin reductase in the $P450_{soy}$ monooxygenase system (54).

SUBSTRATE SPECIFICITY AND INDUCTION OF THE $P450_{soy}$ SYSTEM As noted above, the range of substrates transformed by the $P450_{soy}$ system is enormous. These include, for example, such diverse compounds as benzene, chlorobenzene, toluene, naphthalene, biphenyl, 17β-estradiol, precocene II, cyclohexane, 7-ethoxycoumarin, and pyridine (51), as well as a variety of promutagenic chemicals (57). Although the rates of these oxygenations are very low relative to the activities of other soluble bacterial P450 systems, they are in the same range as similar reactions carried out by the liver microsomal systems (52). Surprisingly, none of the known substrates for $P450_{soy}$ appear to act as inducers. Genistein, an isoflavinoid compound found in soy flour, is the sole inducer so far characterized and it does not appear to be a substrate. One would thus classify $P450_{soy}$ as inducible by an exogenous nonsubstrate (i.e. type 2 induction). Soy flour not only induces the P450 component but also the 7 Fe ferredoxin and the ferredoxin reductase, even though the ferredoxin component, at least, is not on the same operon as $P450_{soy}$ (F. S. Sariaslani, personal communication).

Other Multi-component Inducible P450-dependent Systems

CYTOCHROME $P450_{MEG}$ FROM *BACILLUS MEGATERIUM* Various strains of *Bacillus megaterium* are the source of at least four distinctive P450 cytochromes. The first to be characterized, $P450_{meg}$, was detected as a 15 β-steroid hydroxylase activity in a cell-free extract from *Bacillus megaterium* ATCC 13368 (58). The partially purified P450 component catalyzed

hydroxylation of progesterone in the presence of $NaIO_4$ or $NaClO_2$ but not in the presence of reduced pyridine nucleotides. Further fractionation and purification of components of the cell-free extracts led to the characterization of three soluble proteins that together could reconstitute steroid hydroxylase activity (59–62). These included $P450_{meg}$ (M_r = 52,000), a ferredoxin component, megaredoxin, (M_r = 13,500) that reduced the P450 moiety and a NADPH-specific, FMN-containing protein, megaredoxin reductase (M_r = 55,000–60,000). The specificity of $P450_{meg}$ for electron-donor proteins is relatively low, however, since either adrenal ferredoxin reductase or microsomal cytochrome P450 reductase can transfer electrons to the P450 via adrenal ferredoxin (60). Conversely, the substrate specificity of $P450_{meg}$ is much narrower that of $P450_{soy}$, with only a limited number of steroids (including progesterone, testosterone, 4-androstene-3,17-dione, 17α-hydroxyprogesterone, deoxycorticosterone, and corticosterone) being hydroxylated (59). Despite this high substrate specificity, affinity for these substrates is relatively low as measured by their K_m values (approximately 100–1000 μmolar). The rates of hydroxylation are also much lower than for many other bacterial P450-dependent monooxygenases. None of these substrates served as inducers nor did a variety of nonsubstrates such as phenobarbital, β-naphthoflavone, 16α-cyanopregnenolone and other known inducers in microsomal P450 systems (63). Nevertheless, cytochrome $P450_{meg}$ was not synthesized in culture during the log phase of growth but showed a dramatic rise after the cells entered stationary phase. The factors responsible for this stationary-phase induction are unknown but the release of catabolite repression must be considered as a possibility.

CYTOCHROMES $P450_{BM-1}$ AND $P450_{BM-2}$ FROM *BACILLUS MEGATERIUM* Growth of *B. megaterium* ATCC 14581 (or most other strains of this bacterium) in the presence of phenobarbital or other barbiturates induces at least three distinct P450 cytochromes, designated $P450_{BM-1}$, $P450_{BM-2}$, and $P450_{BM-3}$ (64–66). Cytochrome $P450_{BM-3}$ (a single-protein monoxygenase) and the induction mechanisms governing all three P450s are discussed in detail in a later section. $P450_{BM-2}$ is a relatively small polypeptide (M_r = 48 kd) that has not yet been cloned intact. It remains poorly characterized except for some partial amino acid sequences and the detection of fatty acid hydroxylation activity in the presence of either the bypass reagent, iodosylbenzenediacetate, or NADPH and the trypsin-generated reductase domain of $P450_{BM-3}$ (L. O. Narhi, R. T. Ruettinger, H.-M. Liu, A. J. Fulco, unpublished data; see also ref. 65, 67). The still smaller $P450_{BM-1}$ was initially described as a 38-kd protein with no detectable fatty acid oxygenase activity except in the presence of iodosylbenzenediacetate (65). However, it later became apparent that the 38-kd P450 is a product of proteolysis, with about the same molecular weight as $P450_{BM-2}$ (H.-M. Liu, R. Ruettinger, A. J.

Fulco, unpublished data). The uncertainty was definitively resolved when the gene encoding $P450_{BM-1}$ was cloned, sequenced, and the amino acid sequence deduced (68). $P450_{BM-1}$ is a polypeptide of 414 amino acids with a calculated M_r of 47,439. Although clearly belonging to a new P450 gene family, distinct especially from $P450_{BM-3}$, its sequence is most closely related to $P450_{cam}$ (27.3% identity). Electron-transfer proteins have not yet been isolated from the strain of *B. megaterium* harboring $P450_{BM-1}$ but its structure and lack of activity in the presence of the reductase fragment from $P450_{BM-3}$ (L. Narhi, A. Fulco, unpublished data) suggest that it is the catalytic unit of a three-protein monooxygenase system (see Figure 1A.). Finally, note that 11 of the 12 *B. megaterium* strains examined contained all three BM P450s; only ATCC 13368, a variety known to harbor the $P450_{meg}$ steroid hydroxylase system (58), contained none.

INDUCIBLE CYTOCHROME P450 SYSTEMS INVOLVED IN HYDROCARBON OXIDATIONS Several bacterial systems are relatively specific for initiating the oxidation of various hydrocarbons and in each case they are substrate-induced. These include $P450_{oct}$ from a species of *Corynebacterium,* now classified as *Rhodococcus rhodochrous* ATTC 19067 (7, 69, 70), $P450_{non}$ from strain EB104 from *Acinetobacter calcoaceticus* (71–76), $P450_{cym}$ from several strains from *Pseudomonas putida* (77–81) and $P450_{cyc}$ from a species from *Xanthobacter* (82). $P450_{oct}$ was obtained as a particulate preparation by ammonium sulfate fractionation of a cell-free extract from *R. rhodochrous*. In combination with NADH and a soluble fraction containing a flavoprotein, it catalyzed the conversion of n-octane to 1-octanol (69). $P450_{oct}$, as measured by its reduced CO difference spectrum, was induced about eightfold when grown in a medium containing octane as a sole carbon source (70). The $P450_{oct}$ system merits further investigation, especially since the crude system appears to resemble the microsomal P450 monooxygenases more than the soluble, three-protein systems typical of many bacteria. $P450_{non}$ from *A. calcoaceticus,* which hydroxylates and is induced by alkanes from C_6 to C_{16} (71, 73), is more characteristic of most bacterial or mitochondrial P450s. Catalytic activity requires three proteins including the P450 component (M_r = 52,000), which was soluble in the presence of Triton X-100 but denatured when the detergent was removed (76), an iron-sulfur protein (a ferredoxin), and an NADH-specific ferredoxin reductase (72). The latter two proteins are soluble but have not yet been purified to homogeneity or characterized in detail. $P450_{cym}$ was originally identified as the catalytic component that was induced by and initiated the catabolism of p-cymene and other terpenes in a PI strain of a soil pseudomonad (78). The genetic organization of an identical or very similar system has more recently been studied in various mutants of *Pseudomonas putida* JT810 and suggests that three protein components, not yet characterized, are involved in p-cymene hydroxylation (79, 80). As with

many bacterial systems that oxidize hydrocarbons, the enzymes for the p-cymene pathway are apparently encoded on transmissible plasmids (81). $P450_{cyc}$ was isolated as a NADPH-dependent cyclohexane hydroxylase activity from a species of *Xanthobacter* that had been grown on cyclohexane as a sole carbon source; only alicyclic hydrocarbons closely related to cyclohexane supported growth (82). Sariaslani (7) reported a personal communication from one of the discoverers that the $P450_{cyc}$ system contains three protein components necessary to convert cyclohexane to cyclohexanol.

INDUCIBLE CYTOCHROME P450 SYSTEMS INVOLVED IN ALCOHOL AND ETHER OXIDATIONS Several bacterial P450 systems have been described that further oxygenate various alcohols and ethers. These include $P450_{lin}$ from *Pseudomonas putida* (incognita) strain PpG777 (83–86), $P450_{ver}$ from *Streptomyces setonii* (87), $P450_{gua}$ from a species of *Moraxella* (88, 89), $P450_{npd}$ from a species of *Norcardia* (90–93), and $P450_{SU1}$ and $P450_{SU2}$, from the same strain of *Streptomyces griseolus* (94–95a). Each P450 is induced by its substrate. The $P450_{lin}$ monoxygenase, consisting of three soluble proteins, is the best characterized in this category (86). The P450 component (M_r = 44,800) hydroxylates the 8-methyl group of linalool, a monoterpene alcohol, in the presence of a 2Fe-2S* redoxin (M_r = 12,800) and a NADH-specific, FAD-containing redoxin reductase (M_r = 43,700). The $P450_{lin}$ proteins closely resemble the components of the $P450_{cam}$ system in physical and chemical properties and both systems can further oxidize the initial alcohol products to the oxo-derivatives (i.e. 8-oxolinalool and 5-ketocamphor, respectively). Despite these many similarities, however, they exhibited no immunological cross reactivity and very little catalytic activity occurred in heterologous reconstitutions where the P450 component of one system was tested with the electron-transfer proteins of the other (86). $P450_{ver}$ has not been characterized but was discovered as a soluble protein in extracts from cells of veratrole-grown *S. setonii*. The experimental data suggests that this catalytic entity sequentially demethylates veratrole to guaiacol and hence to catechol (87). $P450_{gua}$ (M_r = 52,000) was partially purified from a strain of *Moraxella* that grew on guaiacol as a sole carbon source and, when recombined with a protein fraction from the soluble extract from the bacterium, demethylated guaiacol to catechol in the presence of NADH. Other 2-alkoxyphenols up to 5 carbons in the side chain, like guaiacol, caused a low-spin to high-spin shift in the Soret band of $P450_{gua}$ and presumably could also be converted to catechol in the complete system (88, 89). $P450_{npd}$ (M_r = 45,000) is the soluble catalytic component of a system from the NH 1 strain of *Norcardia* that O-dealkylates p-alkylphenyl ethers such as 4-methoxybenzoate and 4-methoxy-3-hydroxybenzoate (91, 92). A NADH-specific protein (M_r = 60,000–80,000), isolated from an extract of cells ruptured in a press,

served as a reductase component in this system but was relatively unstable and could not be separated into two constituents (93). Although Cartwright & Broadbent (93) hypothesized that the reductase protein was actually a complex between two smaller proteins (i.e. a redoxin and a redoxin reductase), $P450_{npd}$ monooxygenase could equally be a two-component system analogous to the liver microsomal P450s of liver (Figure 1B).

$P450_{SU1}$ (M_r = 44,300) is the major species of P450 induced in *S. griseolus* by several sulfonylurea herbicides; $P450_{SU2}$ (M_r = 44,400) is a minor component found only in cells grown in the presence of chlorimuron ethyl. A third P450 has been found in very low levels in all cultures, independent of the presence of sulfonylurea inducers, and has been designated $P450_{CON}$. These three proteins, all soluble, are immunologically distinct, and can also be distinguished by their spectral and chromatographic properties, substrate specificities, and specificity of induction (94, 95) as well as by their amino acid sequences (95a). Although the electron transfer components of the system have not been identified, both $P450_{SU1}$ and $P450_{SU2}$, in the presence of NADH and a soluble supernatant fraction, carry out O-demethylations, methyl hydroxylations, and deesterifications on many sulfonylurea herbicides (7, 95). In a recent report O'Keefe and coworkers (95a) described the cloning and sequencing of both monooxygenases. $P450_{SU1}$ and $P450_{SU2}$ are similar, with the DNA of the genes showing an overall 65.4% homology. The two proteins show a 44.4% identity with each other, but only 29.7% and 29.4% identity, respectively, to $P450_{BM-1}$ (68) and 25.9% and 27.8% identity to $P450_{cam}$ (42). These results establish $P450_{SU1}$ and $P450_{SU2}$ as members of a new gene family that is, nevertheless, much closer to the $P450_{BM-1}$ and $P450_{cam}$ families than to any eukaryotic P450 family or to $P450_{BM-3}$ (see next section).

A number of bacterial P450s have not been included in this review because they are apparently noninducible. These include two P450s from *Saccharopolyspora erythraea* that are involved in the synthesis of the antibiotic, erythromycin (96, 97) and a group of six distinct bacterial P450s, all isolated from *Rhizobium japonicum* (98–101). The catalytic activities and functions of this group are unknown. The properties and possible metabolic roles of these mysterious proteins from *Rhizobium* have recently been summarized by Sligar & Murray (35); Sariaslani (7) has briefly reviewed the P450s of *Saccharopolyspora*.

$P450_{BM-3}$ FROM *BACILLUS MEGATERIUM* ATCC 14581

General Properties

$P450_{BM-3}$, which functions as a catalytically self-sufficient fatty acid monooxygenase, is a single peptide containing 1,048 amino acid residues with a

peptide molecular weight of 117,641 daltons. It is by far the most catalytically active P450 reported to date; with long-chain fatty acids (C_{14} to C_{16}), it exhibits a specific activity of > 4500 moles substrate oxygenated/mole of heme/minute. $P450_{BM-3}$ was first detected as a fatty acid hydroxylase activity in cell-free preparations from *Bacillus megaterium* (102) and only later identified as a P450 cytochrome (103, 104). In the presence of O_2 and NADPH, this enzyme hydroxylated (in the ω minus 1, 2, and 3 positions) long-chain saturated fatty acids and other long-chain carbon compounds such as fatty amides and alcohols (105, 106) and epoxidated and/or hydroxylated unsaturated fatty acids (107, 108). Various mid-chain hydroxy-substituted fatty acids were also hydroxylated (109, 110). The methyl group of these substrates is never oxygenated and it appears to be sequestered in a hydrophobic pocket on the enzyme (105). Initial efforts at purifying and characterizing the protein responsible for this activity were hampered by its being present normally at very low concentrations in the cell and not inducible by its substrates. However, in 1982 it was found that phenobarbital, a nonsubstrate in our system but a well-recognized specific P450 inducer in the livers of higher animals, could also induce the bacterial monooxygenase (111). Subsequently, many other barbiturates and barbiturate analogs were also shown to be inducers; indeed, some barbiturates were much more active than phenobarbital (112–115).

Structure

Using pentobarbital, we were able to induce $P450_{BM-3}$ about 400-fold in shake cultures of *Bacillus megaterium* and this made it possible to isolate, purify and characterize this monooxygenase (29, 67, 116, 117) and, using an immunochemical screening technique, to eventually clone it and sequence the gene (118, 119). The structural and functional properties of $P450_{BM-3}$ were unique. In striking contrast to the P450 systems already discussed, $P450_{BM-3}$ consists of a single peptide-chain, soluble, catalytically self-sufficient protein that combines both the electron transport functions and substrate oxygenation function in one large molecule. As a consequence, perhaps, $P450_{BM-3}$ is the most catalytically active P450 known—several orders of magnitude more active than most liver microsomal systems. Nevertheless, except for its solubility, $P450_{BM-3}$ resembles the liver microsomal systems (Figure 1B) more than the mitochondrial or soluble bacterial $P450_{cam}$ monooxygenases (Figure 1A). In the presence of substrate, $P450_{BM-3}$ was cleanly cut into two fragments by trypsin and these, on analysis, proved to be functionally analogous to the two components of the liver system (67).

As schematically indicated in Figure 2, one trypsin peptide formed a domain containing the heme moiety and the substrate binding site. It showed a typical P450 spectrum and, upon the addition of fatty acid substrate, it

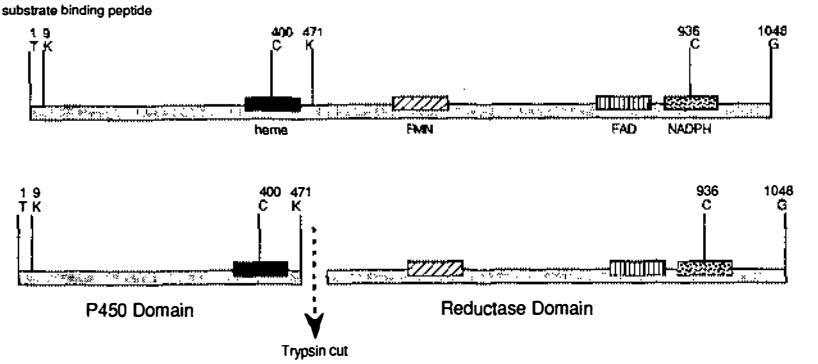

Figure 2 A schematic representation of the structure of cytochrome $P450_{BM-3}$ showing regions involved in substrate, heme, flavin, and NADPH binding. The formation of separate but functional P450 and reductase domains by trypsin treatment of $P450_{BM-3}$ in the presence of fatty acid substrate is also illustrated.

underwent a low-spin to high-spin shift. It is thus analogous in size, structure, and function to typical liver microsomal P450s. The second domain, containing both FAD and FMN as prosthetic groups and an NADPH-binding site, is analogous in structure and function to the liver reductase but somewhat smaller in size. Like the liver enzyme, the $P450_{BM-3}$ reductase domain can function as a cytochrome c reductase as well as a protein transferring electrons to P450 (67). The extent of these analogies became clear, however, only after the $P450_{BM-3}$ gene was sequenced. The complete sequence of a 5-kb fragment of DNA from *B. megaterium* that was cloned into *E. coli* and that contained the $P450_{BM-3}$ structural gene, and the complete regulatory region involved in expression and barbiturate induction of the enzyme in *B. megaterium* was determined (119). By analogy with previously characterized P450s and reductases (8, 120, 121), the heme, FAD-, FMN-, and NADPH-binding sites were more precisely located and, in each case, the analogy with the liver system holds. The reductase domain of $P450_{BM-3}$ lacks the large, extremely hydrophobic string of residues found at the N-terminal region of the microsomal reductases (121), but this is not surprising. This hydrophobic region presumably allows the mammalian reductase to insert into the endoplasmic reticulum membrane whereas the enzyme in *B. megaterium* is soluble. In addition to the analogous location and function of the prosthetic groups, the amino acid sequences of the $P450_{BM-3}$ heme and reductase domains also most obviously resemble the proteins of the liver system (excluding the N-terminal hydrophobic residues of the mammalian proteins). For example, using standard sequence-similarity comparison methods (122, 123), the heme domain of $P450_{BM-3}$ (residues 1–460) showed a 41% similarity with rat $P450_{pcn}$, a

34% similarity with either rabbit $P450_{p\text{-}2}$ (prostaglandin ω-hydroxylase) or rabbit $P450_{LAW}$ (lauric acid hydroxylase) but only a 20% similarity with yeast $P450_{dm}$ (lanosterol demethylase), a 22% similarity with bacterial $P450_{cam}$ and a 24% similarity with mitochondrial $P450_{SCC}$ (119). The $P450_{BM\text{-}3}$ reductase domain overall shows strong sequence similarity (i.e. 33–35%) with mammalian microsomal reductases but the resemblance is especially striking in regions putatively involved in NADPH, FAD and FMN binding. For example, in comparing the last 150 residues of the bacterial reductase domain with the same segment of rat microsomal reductase (regions that putatively contribute to reduced pyridine nucleotide and FAD binding), 51% of amino acid residues are identical (119, 121).

Substrate Binding and Specificity

As noted above, when $P450_{BM\text{-}3}$ is treated with trypsin in the presence of fatty acid, two peptides are formed, corresponding to a substrate-binding heme domain and a reductase domain. In the absence of fatty acid, however, an additional trypsin-catalyzed cleavage takes place between lysine 9 and threonine 10 of the heme domain that abolishes the low-spin to high-spin shift upon substrate addition, despite the other spectral properties of this domain remaining unaltered (67). This result not only demonstrated that fatty acid substrate binding protects lysine 9 from trypsin proteolysis but also strongly suggested that one or more of the first 9 residues at the N-terminal region of $P450_{BM\text{-}3}$ are involved in substrate binding. This hypothesis was recently tested by the use of site-directed mutagenesis to replace lysine 9 with various other amino acids and in several instances, dramatic effects on enzymatic activity were noted (124).

Based on K_m values, the most active group of substrates tested to date are fatty acids of chain-lengths from 12 to 18 carbons, either saturated or unsaturated (29, 105, 108). The order of activity for the saturated series is $C_{15} = C_{16} > C_{14} > C_{17} > C_{13} > C_{18} > C_{12}$, with the K_m values ranging from about 2 μM for C_{15} to 110 μM for C_{12} (29). Although stearic acid itself is a relatively poor substrate for hydroxylation ($K_m = 80 \pm 20$), 6-hydroxystearic acid is a better substrate than palmitate ($K_m = 2 \pm 1$) and several other hydroxystearates (which are converted into dihydroxy derivatives) are also relatively high-affinity substrates (109, 110), as are stearic acids substituted near mid-chain with keto, acetoxy or halogen groups (A. Fulco, unpublished data). Unsaturated fatty acids can be both monohydroxylated at methylene groups near the ω-end and epoxidized at the double bond, but the evidence strongly indicates that both epoxidation and hydroxylation involve identical binding and active sites on the enzyme (29, 128). Although K_m values for long-chain alcohols and amides have not been measured because solubility problems prevent enzyme saturation, they are probably lower than the K_m

values for the corresponding fatty acids (105). Neither long-chain hydrocarbons nor fatty acid methyl esters are substrates for the enzyme; amines and other end-chain polar-group substituted alkyl derivatives have not yet been adequately tested.

Electron Transfer Properties

As already noted, cytochrome $P450_{BM-3}$ is the most active of the known P450-dependent monooxygenases, perhaps because a putative rate-limiting step, the transfer of electrons to the active site (35, 125) is intramolecular. However, the rate-limiting step in substrate oxygenation in the $P450_{BM-3}$ system has not yet been identified. The kinetics of fatty acid hydroxylation clearly demonstrate that substrate structure, binding conformation or product dissociation are not rate-limiting factors since, under identical assay conditions, the large number of fatty acids tested (29, 108) exhibited a wide range of K_m values but gave the same V_{max} values. On the other hand, pretreatment of the enzyme with NADPH, which is both an obligate cofactor and a negative effector of monooxygenase activity, had the opposite effect. The enzyme does not substantially oxidize NADPH unless a substrate such as fatty acid or cytochrome c is present, indicating that substrate binding must occur before a significant flow of electrons from NADPH to the protein can begin. In the absence of substrate, a brief incubation of NADPH with the enzyme causes a conformational change that, while not altering substrate K_m, results in a 30-fold decrease in the V_{max} of fatty acid oxygenation. Under the same conditions, however, the rate of cytochrome c reduction is not changed, an indication that the rate-limiting steps in the two reactions must be different (29). In support of this conclusion, we have also observed that the addition of oxidized cytochrome c to the reaction mixture does not affect the rate of fatty acid hydroxylation (R. S. Matson, L. O. Narhi, A. J. Fulco, unpublished observations).

Inducers of the P450 Cytochromes of Bacillus megaterium

Cytochrome $P450_{BM-3}$ as well as the two smaller P450 cytochromes of *B. megaterium* ATCC 14581 are induced by barbiturates (disubstituted with alkyl or aryl groups at the 5-position), disubstituted acetyl ureas, and certain disubstituted acetamides. These inducers are not substrates for $P450_{BM-3}$ nor do they show any interaction with this enzyme in vitro (65, 66, 111–114, 116, 126, 127). A strong correlation (within class) was noted between increasing lipophilicity and inducer potency, a trend that is apparent from the data on barbiturate inducers as shown in Table 1.

Among nonbarbiturate inducers such as the acyl ureas and several other barbiturate analogs (114), a similar trend was demonstrated. The potencies (in brackets following name) of these barbiturate analogs relative to phenobarbi-

Table 1 Relationship between barbiturate structure and induction of monooxygenase activity (113)

Barbiturate[1] Tested	R_1	R_2	X	Y	Relative Specific Activity[2]
Barbituric acid	H	H	O	H	0.0
2-Thiobarbituric acid	H	H	S	H	0.0
Barbital	ethyl	ethyl	O	H	0.0
Butabarbital	ethyl	1-methylpropyl	O	H	2.3
Amobarbital	ethyl	isopentyl	O	H	4.3
Pentobarbital	ethyl	1-methylbutyl	O	H	10.6
Thiopental	ethyl	1-methytlbutyl	S	H	16.1
Phenobarbital	*ethyl*	*phenyl*	*O*	*H*	*1.0*
Mephobarbital	ethyl	phenyl	O	CH_3	0.0
5-Ethyl-5-p-tolylbarbiturate	ethyl	tolyl	O	H	0.8
4-Methylprimidone	ethyl	tolyl	2H	H	0.0
p-Hydroxyphenobarbital	ethyl	p-hydroxyphenyl	O	H	0.0
Aprobarbital	allyl	isopropyl	O	H	1.1
Allobarbital	allyl	allyl	O	H	3.2
Talbutal	allyl	1-methylpropyl	O	H	14.2
Secobarbital	allyl	1-methylbutyl	O	H	33.3
Thiamylal	allyl	1-mcthylbutyl	S	H	33.5
Methohexital	allyl	1-methyl-2-pentynyl	S	CH_3	71.0
Hexobarbital	methyl	1-cyclohexenyl	O	CH_3	1.4

[1]R_1 and R_2 are the substitutents at the 5-position of the barbiturate ring, Y is the substituent at the 2-position (ring nitrogen) and X is the group at the 3 position.

[2]Relative Specific Activity: Expressed as specific myristate hydroxylase activity induced by the compound tested at 2 mM relative to the specific activity induced by 2 mM phenobarbital.

tal were as follows: 2-phenyl-3-methylpentenoylurea [29]; 2-phenylbutyryl-thiourea [15]; 2-isopropyl-4-pentenoylurea [8.5]; 2-ethylhexanoylurea [7.5]; 2-phenyl-4-pentenoylurea [7]; 2-phenylbutyrylurea [5]; 2-isopropyl-4-pentenoylurea [5]; 2-ethylhexanoylurea [5]; (+)-2-phenylbutyrylurea [3.6]; (−)-2-phenylbutyrylurea [1.9]; methylpropionylurea [1.4]; 2-phenylbutyramide [0.9]; 2-isopropyl-4-pentenamide [0.4]; 2-ethylhexanoic acid [<0.05]; 2-phenylbutyric acid [<0.04]. Although some of the acylureas tested can be formed from the analogous barbiturates by autoclaving the parent compound in aqueous solution at pH 8.0, these transformations do not occur under normal culture conditions; the evidence is now compelling that the induction of P450s in *B. megaterium* ATCC 14581 by barbiturates is not dependent upon their conversion to the analogous acylureas but that the barbiturates themselves are active inducing substances.

Regulation of Barbiturate-Inducible P450 Expression

GENERAL CHARACTERISTICS OF INDUCTION The molar ratio of synthesis of the three different P450s in response to inducers can vary with inducer

structure. The effect can be dramatic (114, 115, 127), an indication that the regulatory systems controlling the expression of the three genes encoding $P450_{BM-1}$, $P450_{BM-2}$ and $P450_{BM-3}$ must also differ at the molecular level. The observed stimulation of P450 synthesis in *B. megaterium* after barbiturate inducers were added to log-phase cultures was rapid (<5 min); the maximum rate of synthesis was achieved in less than 30 min in such cultures. Induction was blocked by inhibitors of protein or RNA synthesis but not by DNA synthesis inhibitors (118, 128, 129); specific mRNAs were induced by barbiturates but RNA stability was not affected (128; M. Ashby, J.-S. He, A. J. Fulco, unpublished data).

INITIAL STUDIES ON $P450_{BM-3}$ In a study of barbiturate-mediated induction of $P450_{BM-3}$ in *B. megaterium*, a DNA insert was obtained that contained, in addition to the structural gene, the complete regulatory region (118). When *E. coli* was transformed by a vector containing this 5-kb segment of DNA from *B. megaterium*, the synthesis of $P450_{BM-3}$ was directed by its own promoter included in the insert (118, 119, 130). $P450_{BM-3}$ synthesis proceeded constitutively at a very high rate in transformed *E. coli* but was not stimulated by barbiturates. When this 5-kb fragment was inserted into a high copy number shuttle vector and introduced into *B. megaterium*, the cloned gene was expressed at a very low basal level but appeared to be highly induced by barbiturates (118). However, quantitative work with this system was difficult because the chromosomal gene product could not be distinguished from that produced by the insert contained in the shuttle vector (i.e. both were $P450_{BM-3}$). Thus, a shuttle vector was used containing a promoterless CAT gene because it was easy to measure and distinguish the activity of the CAT gene product, chloramphenicol acetyl transferase, from that of $P450_{BM-3}$-catalyzed oxygenase activity. Moreover, since no promoter was included in this plasmid, it could only be expressed when an active promoter was inserted at a polylinker site 5' to the CAT gene. When a 1.3-kb piece of DNA that forms the 5'-flanking region of the $P450I_{BM-3}$ structural gene was inserted in the correct orientation into the CAT vector and introduced into *B. megaterium*, CAT activity was expressed at a relatively low basal level but was highly induced by barbiturates. Furthermore, the dose-response curves in response to pentobarbital and the relative inducer potencies of other barbiturates (including 2-thiobarbiturate, phenobarbital, secobarbital, and methohexital) were the same for the induction of CAT in this construct, when introduced into *B. megaterium*, and for the expression of $P450_{BM-3}$ in untransformed *B. megaterium* (130). However, when $P450_{BM-3}$ monooxygenase activity was measured in *B. megaterium* transformed by this vector, $P450_{BM-3}$ gene expression was sharply inhibited, both in the presence and absence of pentobarbital. Furthermore, unlike CAT expression, the competition effect of

the DNA insert on $P450_{BM-3}$ expression was independent of the orientation of the insert. After preparing various deletion derivatives of the regulatory region of $P450_{BM-3}$, it was concluded that the synthesis of $P450_{BM-3}$ in *B. megaterium* is under positive control and requires gene interaction with at least one transacting factor, presumably a protein, to activate transcription from the $P450_{BM-3}$ promoter. The binding of this putative protein is mediated by at least two regulatory regions (R_1 and R_2) that span about 1 kilobase of the 5'-flanking region of the $P450_{BM-3}$ gene (130).

CURRENT STATUS Recent work has clarified and expanded knowledge of the regulation of expression of cytochrome $P450_{BM-3}$ (G.-W. Shaw, A. J. Fulco, unpublished data). The current interpretation of the regulation of $P450_{BM-3}$ is illustrated in Figure 3, which is a schematic representation of the complete regulatory region (approximately 1000 bp 5' to the ATG start codon of the $P450_{BM-3}$ structural gene).

The $P450_{BM-3}$ gene has been proposed to exist within a bicistronic operon. The segment of the 5'-flanking region spanning base pairs -29 to -605 (Figure 3) contains an open reading frame that encodes a peptide of 192 residues and also, near the 3' end, contains sequences for R_1, a putative binding site for a factor acting as a positive regulator in $P450_{BM-3}$ expression, and for a promoter, P_1, that is responsible for basal $P450_{BM-3}$ expression (i.e. expression in the absence of barbiturates) in *B. megaterium*. One hypothesis suggests that, in the absence of barbiturates, the 192 residue peptide is a repressor that negatively controls both its own expression and the inducible expression of $P450_{BM-3}$ by impeding the function of P_2. Barbiturate-mediated induction, then, would involve the binding of an as yet unidentified protein to R_1, R_2 and perhaps other segments of the regulatory region to displace the repressor or in some other way activate P_2 and thus permit the "induced" expression of $P450_{BM-3}$.

A RELATIONSHIP BETWEEN BARBITURATE-MEDIATED INDUCTION IN RAT AND BACTERIUM? Although research efforts on barbiturate-mediated in-

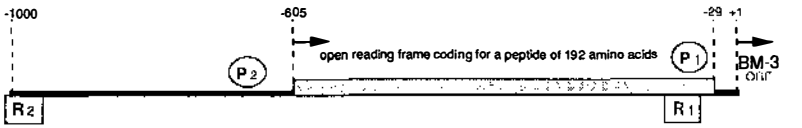

Figure 3 The complete regulatory region of the $P450_{BM-3}$ gene. P_1 and P_2 are promoter regions, R-1 and R-2 are segments of DNA implicated in the binding of a positive regulatory factor and BM-3 ORF marks the beginning of the open reading frame that encodes the 1048 amino acids residues of $P450_{BM-3}$.

duction of P450 cythochromes have focused on $P450_{BM-3}$, recent work with $P450_{BM-1}$ (J. S. He, A. J. Fulco, manuscript submitted) strongly suggests that this process in bacteria and in mammals is mechanistically related. Once the $P450_{BM-1}$ gene was sequenced, including the 5' regulatory region (68), this region was compared with the regulatory sequences of $P450_{BM-3}$ (119) and the published 5'-flanking regions of the rat P450b and P450e genes (131, 132). This analysis revealed a string of 17 bases among the 4 genes that shared a high degree of sequence identity (Table 2). Also, a small segment of $P450_{BM-1}$ 5'-flanking DNA that contained this homologous 17 bp sequence was bound in gel-retardation assays to a soluble protein component obtained from *B. megaterium*. Labeled oligonucleotide probes of the four sequences shown in Table 2 were prepared and tested in gel retardation assays with soluble protein obtained from *B. megaterium* grown either in the presence or absence of phenobarbital. The 17-mers from $P450_{BM-1}$ and P450b bound strongly to the same protein component from cells grown in the absence of barbiturate. The homologous sequences from $P450_{BM-3}$ and P450e also complexed with this protein, but less tenaciously. When protein from cultures grown in the presence of phenobarbital was used, the binding of all four homologous sequences was dramatically reduced. It thus seemed possible that these barbiturate-responsive elements were interacting with a *B. megaterium* repressor protein. The opposite phenomenon was observed when nuclear extracts were prepared from rats that were left untreated or injected with phenobarbital 16–20 hr before they were killed and gel-retardation assays were performed with the same four oligonucleotide probes. All of the probes complexed weakly but distinctly to onc protein band in nuclear extracts from untreated rats; in extracts from rats treated with phenobarbital, the amount of probe bound to the same protein band dramatically increased in each case. Surprisingly, a similar effect could be obtained by prolonged incubation with phenobarbital of either soluble protein from the bacteria grown in the absence of barbiturates or nuclear extract protein from untreated rats. That is, such treatment resulted in decreased binding by the protein from *B. megaterium* and increased binding by the rat protein, all to the same four oligonucleotides. This result suggests that de novo protein synthesis may not be involved in the observed effect and with the rat protein, a phenomenon different from that demonstrated by Rangarajan & Padmanaban (133) may be occurring. They showed that the binding of a putative transcription factor from rat nuclear extracts to a 5'-flanking region of the P450b/e gene was significantly enhanced by pretreatment of the animals with phenobarbital but that the effect was blocked by cycloheximide treatment. The evidence indicated that this factor may be a 85-kd protein. Parenthetically, these researchers obtained a DNase footprint with the nuclear extract from phenobarbital-treated rats that revealed a 32-bp protected region containing the 17-bp homologous sequence

Table 2 Homologous 5' flanking sequences in *B. megaterium* and rat

Gene	Organism	Sequence[1]	5' Location[2]	Reference
$P450_{BM-1}$	*(B. megaterium)*	CCATAAAAAGCTGGTGC	−318 to −302	68
$P450_{BM-3}$	*(B. megaterium)*	ATATCAAAAGCTGGTGG	−243 to −227	119
P450b	(rat)	ATAGCTAAAGCAGGAGG	−119 to −103	131
P450e	(rat)	ATAGCCAAAGCAGGAGG	−116 to −100	132

[1]Bases with double underlining are identical in all four sequences; bases with single underlining are identical in the $P450_{BM-3}$ sequence and in both rat sequences.

[2]In each case, location is determined by counting backwards from the translation start site (+ 1) of the gene.

from P450e (Table 2). Although molecular weights have not yet been estimated, the rat and bacterial oligonucleotide-binding proteins in the author's system appear to be identical size to each other but significantly smaller than 85 kd.

SUMMARY

When I began this review my goal was to present a coherent overview of the biochemistry and regulation of the inducible P450 cytochromes of bacteria. Now, at the end, I wonder if a unified perspective is possible at this time. On the basis of admittedly limited data, bacterial P450 systems seem as different from each other as they are, as a group, from the mammalian P450 cytochromes. The most obvious physical difference between the bacterial monooxygenases and their mammalian counterparts is solubility; with several possible exceptions (69, 70, 76), bacterial P450s are soluble whereas the microsomal and mitochondrial P450s are membrane-associated proteins. In structure and organization, however, the few well-characterized prokaryotic P450-dependent systems vary widely. The three-component arrangement is probably most common but even here variation is apparent. The $P450_{cam}$ putidaredoxin reductase contains only FAD and is quite specific for NADH (35, 39); the $P450_{meg}$ megaredoxin reductase contains only FMN and is specific for NADPH (59, 60). Putitive two-component P450 systems in bacteria have not yet been adequately characterized but the $P450_{oct}$ and $P450_{npd}$ monooxygenases (69, 70, 93) could well be organized in this way. The catalytically self-sufficient $P450_{BM-3}$ is currently the only single-component P450-dependent monooxygenase known but additional examples of this arrangement may well be found in other bacteria. Paradoxically, $P450_{BM-3}$ is structurally much more analogous to liver microsomal P450 systems than to any other bacterial P450 monooxygenase characterized to date.

Another generally recognized difference between prokaryotic and eukaryotic P450s pertains to function; most known bacterial P450-dependent systems initiate the oxidation of recalcitrant carbon compounds so that the hosts can utilize them as sole carbon sources for growth. Some lower eukaryotes [certain yeasts, for example (134)] also employ P450-dependent systems in this way but, among most fungi as well as in higher eukaryotes, P450 cytochromes are involved in specific pathways of sterol or other lipid syntheses or, as in the mammalian liver microsomal systems, in detoxification reactions. This apparent general difference in function is partly artifactual, however, since many of the bacterial systems were discovered by enrichment techniques that depended on the ability of an organism to use a component of the growth medium as a carbon source (7). There are now several examples of bacteria that use P450 cytochromes for other purposes (as documented in this review) and the number in this category will undoubtedly expand significantly as more attention is directed towards elucidating the mechanisms of antibiotic synthesis and resistance in bacteria (97, 135). Finally, bacterial P450s may be sorted and categorized based on the type of induction. Using the three broad categories of induction set forth in this review, most bacteria as well as the preponderance of eukaryotic P450s can apparently be classified as substrate-inducible (30). Nevertheless, the enormous differences in the levels of organization between eukaryotic and prokaryotic cells assure that this grouping is superficial at best. For example, the extremely complex receptor-mediated Ah system that regulates the substrate-induction of microsomal P450s involved in the oxygenation of aromatic hydrocarbons (30, 31) cannot exist in prokaryotic cells that lack endoplasmic reticulum and a nucleus; substrate induction of P450s in bacteria must be a fundamentally different process. A second type of P450 induction, that mediated by environmental factors, has not been commonly observed in bacteria but this situation may simply reflect a lack of interest in this specific area by researchers. Temperature, oxygen tension, light, and other environmental factors have all been implicated in the induction and regulation of enzymes involved in the biosynthesis of unsaturated fatty acids and other lipids in bacteria (136) and this will undoubtedly also hold true for P450s involved in lipid pathways. The third category, nonsubstrate induction of P450s, is also relatively uncommon in bacteria. Two examples are known: the induction of $P450_{soy}$ in *S. griseus* by the isoflavinoid, genistein (137), and the induction of three P450s in *B. megaterium* by barbiturates (66). In neither case is the induction mechanism known but, as reported in this review, it now seems likely that barbiturate-mediated induction in rat liver and in *B. megaterium* may be in some way related. Indeed, in both eukaryotic and bacterial systems, the detailed elucidation at the molecular level of various induction mechanisms represents the new frontier in P450 research and I hope for and predict extraordinary advances in this area within the next few years.

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