Biochem. J. (1981) **196**, 781–786
Printed in Great Britain

Studies on the substrate specificity and inducibility of cytochrome *P*-450$_{meg}$

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(*Received 19 December 1980/Accepted 20 January 1981*)

The cytochrome *P*-450-dependent steroid 15β-hydroxylase system in *Bacillus megaterium* A.T.C.C. 13368 was investigated with regard to its appearance in the cell with respect to the growth curve of the organism, with regard to its inducibility by a number of agents (among them some of the classical inducers of the mammalian liver microsomal cytochrome *P*-450 system) and with regard to its capacity to convert non-steroidal substances into oxygenated compounds. The enzyme was found to reach a maximum concentration in the cell during the stationary phase of the growth curve. Of all the agents tested as inducers, none showed any capacity to induce cytochrome *P*-450$_{meg}$. Finally, of the substances tested as substrates only aniline (*p*-hydroxylation) was metabolized by the microbial enzyme system. This conversion might be related to the general oxygenase activity of haemoproteins. It is concluded that the substrate specificity of the *B. megaterium* hydroxylase system is narrow.

In previous publications, we have described the identification, resolution, characterization and purification of a cytochrome *P*-450-dependent steroid hydroxylase system in *Bacillus megaterium* A.T.C.C. 13368 (Berg *et al.*, 1975, 1976, 1977, 1979). Besides the well established phenomenon of induction of cytochrome *P*-450 in mammalian microsomal systems by a number of agents, several reports have indicated that it is also possible to induce cytochrome *P*-450 in micro-organisms (Ambike *et al.*, 1970; Broadbent & Cartwright, 1971; Peterson, 1971; Wiseman & Lim, 1975). Thus, as part of our ongoing studies with cytochrome *P*-450$_{meg}$ we thought it of interest to investigate whether this enzyme also possessed the capacity to be induced.

The cytochrome *P*-450-dependent hydroxylase system of *B. megaterium* only metabolizes steroids with a 3-oxo-Δ^4-structure and hydroxylation mainly takes place at position 15β in both C_{19} and C_{21} steroids. To obtain further information about the function and catalytic mechanism of the enzyme, we investigated its substrate specificity. The most extensively studied cytochrome *P*-450 species with regard to this characteristic are cytochromes *P*-450$_{LM2}$ and *P*-450$_{LM4}$ from rabbit liver. These enzymes have been found to have overlapping substrate specificities and to accept a variety of different compounds as substrates (Coon *et al.*, 1976). We therefore investigated whether the soluble cytochrome *P*-450$_{meg}$ had the capacity to metabolize compounds acting as substrates for mammalian liver microsomal cytochrome *P*-450.

Materials and methods

Chemicals

Bacto Yeast Extract and Bacto Soybean Extract were obtained from Difco Laboratories (Detroit, MI, U.S.A.). Polyglycol P 2000 ('PPG') was obtained from Dow Chemical Co. (Midland, MI, U.S.A.). All steroids used in the induction experiments were purchased either from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or from The Upjohn Company (Kalamazoo, MI, U.S.A.). NADPH, stearic acid, palmitic acid, lauric acid, cholesterol, β-naphthoflavone, 2-hydroxybiphenyl, 4-hydroxybiphenyl and benzo[*a*]pyrene were obtained from Sigma Chemical Co., and [9,10-^{3}H]palmitic acid (sp. radioactivity 500 Ci/mol), [1-^{14}C]stearic acid (sp. radioactivity 50 Ci/mol) and [G-^{3}H]benzo[*a*]pyrene (sp. radioactivity 20 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Corn oil was Mazola cooking oil. Cholestenone and β-sitosterol were gifts from Dr. P. Eneroth, Hormone Laboratory, Karolinska Hospital, Stockholm, Sweden. ^{14}C-labelled cholesterol (sp. radioactivity 55–61 Ci/mol) and ^{3}H-labelled β-sitosterol (sp. radioactivity 61 Ci/mol) were gifts from the same donor. Oxygenated benzo[*a*]pyrene derivatives (for use as standards) were obtained from the IIT Research Institute,

0306-3283/81/060781-06$01.50/1

Chicago, IL, U.S.A. Different hydroxylated fatty acids were gifts from Dr. Ingemar Björkhem, Huddinge Hospital, Huddinge, Sweden. Cholic acid, deoxycholic acid, lithocholic acid and chenodeoxycholiç acid were gifts from Dr. Björn Almé and prostaglandin $F_2\alpha$ a gift from Dr. Göran Hansson, both of the Department of Chemistry, Karolinska Institute. ^{3}H-labelled prostaglandin $F_2\alpha$ (sp. radioactivity 50 Ci/mol) was obtained from Dr. G. Hansson.

Aniline and biphenyl were purchased from Merck A. G. (Schuckardt, BRD), and 3-hydroxybiphenyl was obtained from ICN Pharmaceuticals (Plainview, New York, NY, U.S.A.). 7-Ethoxyresorufin was purchased from I.E. Pierce (Rotterdam, Holland).

Lidocaine was obtained as the hydrochloride from Astra Co. (Södertälje, Sweden) and [*carbonyl*-^{14}C]-lidocaine (sp. radioactivity 40 Ci/mol) from New England Nuclear Corp. (Boston, MA, U.S.A.). Different derivatives of lidocaine were gifts from Dr. Paul Skett, Department of Pharmacology, University of Glasgow, Scotland, U.K. Phenobarbital, ethylmorphine and aminopyrene were purchased from Karolinska Apoteket (Stockholm, Sweden). Lipidex 1000 was obtained from Packard Instrument Co. Inc. (Downers Grove, IL, U.S.A.).

The freeze-drying medium (100 ml) consisted of serum (75 ml), Difco-buljong [containing Bacto-Beef extract (0.3 g) + Bacto-Peptone (0.5 g/70 ml of water) (17.5 ml) and glucose solution (containing 7.5 g/30 ml of water (7.5 ml)]. The stock mineral solution contained 20 g of EDTA, 0.5 g of $CaCl_2$, 16.5 g of $FeCl_3,6H_2O$, 0.18 g of $ZnSO_4,7H_2O$, 0.16 g of $CuSO_4,5H_2O$, 0.15 g of $MnCl_2,4H_2O$ per litre of water. The vitamin mixture contained thiamin (20 mg/ml) and nicotinic acid (40 mg/ml).

Cultivation of micro-organism

B. megaterium A.T.C.C. 13368 was obtained from the American Type Culture Collection, Rockville, MD, U.S.A. It was stored in the freeze-dried state in freeze-drying medium in sealed glass tubes under N_2 at −70°C. Before a cultivation, the contents of one tube were added to 1 litre of medium (containing 40 g of Bacto Yeast Extract and 16 g of Bacto Soybean Extract per litre, adjusted to pH 7.2) in a 5-litre conical flask. This was incubated at 28°C on a shaking table for 15 h. The cells were then centrifuged at 8000 ***g*** for 10 min and the pellet was resuspended in 20 ml of the supernatant. This suspension was used to inoculate 8 litres of medium (as above, but containing 8 ml of a stock mineral solution) in a 10-litre Biotec fermentor containing baffles (LKB Biotec AB, Sundbyberg, Sweden). Temperature was maintained at 28°C; pH was kept at 7.2 ± 0.3 by automatic addition of 5 M-HCl; air supply was maintained at a constant 5 litres of air/litre of medium per min; medium was stirred at 1000 rev./min; foaming was controlled by automatic addition of Polyglycol P 2000; O_2 tension in the fermentor was monitored by using an oxygen electrode. To obtain a dry-weight curve, 10 ml portions were taken from the fermentor hourly, centrifuged in a bench centrifuge, dried overnight at 100°C and weighed. To monitor production of cytochrome *P*-450 in the fermentor, 100 ml portions were taken hourly and cell-free extracts were prepared as described previously (Berg *et al.*, 1976). Cytochrome *P*-450 in the cell-free extract was measured according to Omura & Sato (1964).

Screening of inducing agents

For these studies, cells were harvested (centrifuged at 8000 ***g*** for 10 min) under sterile conditions from the fermentor after 11 h or when the O_2-tension curve finally began to climb towards 100% (see Fig. 1). One-litre indented conical flasks (induction flasks) containing 100 ml of basic medium (0.2 M-potassium phosphate buffer, pH 7.4, containing 1 ml of stock mineral solution/litre and 100 mM-$MgCl_2$) were sterilized. After sterilization the compounds to be tested as inducing agents were added directly into the basic medium. All compounds were tested at two concentrations, i.e. 0.1 mM and 1.0 mM. Control flask contained only basic medium. Bacterial cells were then added to flasks to give a final concentration similar to that in the fermentor at harvesting, i.e. approx. 10 g wet wt./100 ml of medium. The flasks were then incubated on a shaking table at 28°C for 5 h. Cell-free extracts, which were prepared as described previously, were kept on ice until hydroxylase activity was assayed. Chromatography of cell-free extracts, which were obtained from flasks to which steroid had been added, was performed on the hydrophobic column-packing material (Lipidex 1000) before incubations. This step was included to remove unbound as well as protein-bound steroids from the aqueous solution (Dahlberg *et al.*, 1980) in order that they might not interfere with the subsequent incubation and assay procedure.

Incubations to assay extent of induction of cytochrome P-450$_{meg}$

Cell-free extract (3 ml, containing approx. 36 mg of protein) was added to the incubation flask. The substrate progesterone (50 mg, 400000 d.p.m.) was added in 50 μl of acetone and the flasks were pre-incubated for 3 min at 22°C. Incubations were started by the addition of NADPH (1 mg in 100 μl of 0.1 M-potassium phosphate buffer) and carried out for 30 min at 22°C. Control incubations were performed with boiled enzyme preparations. Incubations were terminated by the addition of 10 ml of chloroform/methanol (2:1, v/v). Incubation mixtures were extracted and 15β-hydroxylase ac-

tivity was assayed by t.l.c. as described previously (Berg *et al.*, 1976).

Preparation of cytochrome P-450$_{meg}$ for substrate-specificity studies

For these studies, cells were harvested from the fermentor after 13h or 2h after the O_2-tension curve began to rise finally towards 100% (Fig. 1). The cell-free extract was prepared as described above and cytochrome P-450$_{meg}$ was purified as described previously (Berg *et al.*, 1976, 1979) to a specific content of 3.5nmol/mg of protein. The preparation was free from haemoproteins other than cytochrome P-450 and contained no ferredoxin or ferredoxin reductase activity. *B. megaterium* ferredoxin reductase and *B. megaterium* ferredoxin were purified as described before (Berg *et al.*, 1976) to specific contents corresponding to 900nmol of dichlorophenol-indophenol reduced/min per mg of protein and 30nmol of acid-labile sulphur/mg of protein respectively. These preparations contained no measurable ferredoxin or ferredoxin reductase activity respectively, and no detectable cytochrome P-450.

Incubations to assay substrate specificity of cytochrome P-450$_{meg}$

The incubation mixture usually consisted of 0.3nmol of cytochrome P-450$_{meg}$, *B. megaterium* ferredoxin corresponding to 2nmol of acid-labile sulphur and *B. megaterium* ferredoxin reductase equivalent to 1000nmol of dichlorophenol-indophenol reduced per min (1000units) (Savage, 1957). The volume of the incubation mixture was adjusted to 1.9ml with 50mM-potassium phosphate buffer, pH7.4. The substrate (usually 150–200μM, except for benzo[*a*]pyrene, where a concentration of 12μM was used and 7-ethoxyresorufin, where a concentration of 10μM was used) was added in 50μl of acetone (for biphenyl, aniline, palmitic acid, stearic acid, prostaglandin $F_2\alpha$, β-sitosterol or cholesterol), methanol (for benzo[*a*]pyrene, cholic acid, deoxycholic acid, lithocholic acid, chenodeoxycholic acid), phosphate buffer (for aminopyrine, ethylmorphine, lidocaine) or Tris/HCl buffer (for 7-ethoxyresorufin) and the tubes were pre-incubated for 3min at 22°C. The incubations were started by the addition of 0.5μmol of NADPH in 50μl of water and carried out for 5–30min at 22°C. Control incubations were performed with boiled enzyme preparations.

Product analysis

Biphenyl incubations were terminated by the addition of 1ml of 2M-HCl and extracted with 2 × 5ml of n-hexane. The organic phases were combined and evaporated to dryness under N_2. The residue was dissolved in 0.1ml of TRI-SIL (Pierce) and after 30min at room temperature the sample was taken to dryness and dissolved in 0.1ml of n-hexane. Biphenyl metabolites were analysed by g.l.c. using a 1% OV-1 column.

Benzo[*a*]pyrene incubations were terminated by addition of 1ml of acetone. The incubation mixtures were extracted with 2 × 2ml of ethyl acetate containing 0.08% (w/v) butylated hydroxytoluene. The combined organic phases were dried with 0.5g of anhydrous Na_2SO_4 and the solvent was removed under a stream of N_2. The residue was stored dry in the dark at −20°C. The samples were dissolved in 50μl of dioxan and 20μl was injected into an LDC High Pressure Liquid Chromatograph equipped with two Constametric II pumps and a Nucleosil ODS column (1.52cm × 25cm). Elution was performed by using a linear gradient of 60% methanol in water to 100% methanol during 30min. The flow of eluent was 1.3ml/min and fractions were collected each 40s directly into counting vials for an Intertechnique SL-30 liquid-scintillation spectrometer.

Incubations with aniline as substrate were stopped by addition of 2ml of 10% (w/v) trichloroacetic acid. After centrifugation at 3000*g* for 10min, the supernatant was assayed colorimetrically for *p*-aminophenol as described by Kato & Gillette (1965).

Incubations containing lidocaine were stopped by adding 0.5M-NaOH to a pH of 9–11 and 10ml of chloroform/methanol (2:1, v/v), and the incubation mixture was extracted with 40ml of chloroform/methanol (2:1, v/v). The extract was evaporated to dryness in the dark. The residue was subjected to t.l.c. in the solvent system chloroform/methanol (19:1, v/v). The developed plates were scanned for radioactivity with a Berthold II thin-layer scanner.

Incubations containing fatty acids (palmitic acid or stearic acid) were stopped with 1M-HCl and extracted with chloroform/methanol (2:1, v/v). Fatty acid metabolites were analysed as methyl esters essentially by the method of Gustafsson & Bergman (1976).

Incubations with aminopyrine or ethylmorphine were ended by the addition of 2ml of 15% (w/v) $ZnSO_4$ in water followed by the addition of 2ml of saturated $Ba(OH)_2$ in water (Mazel, 1971). The precipitated $BaSO_4$ was sedimented by centrifugation and the supernatant was taken for spectrophotometric assay of formaldehyde using the Nash reagent (Nash, 1953).

Incubations with bile acids were terminated by the addition of 10ml of chloroform/methanol (2:1, v/v). The organic phase was evaporated to dryness. The residue was purified on a diethylaminohydroxypropyl-Sephadex LH-20 column and analysed by gas chromatography–mass spectrometry as methyl ester trimethylsilyl ether derivatives as described by

Almé *et al.* (1977). The gas-chromatography stationary phase was 1.5% SE-30.

Incubations with sterols were ended by the addition of 10ml of chloroform/methanol (2:1, v/v) and the organic soluble extract was run on t.l.c. in the solvent system diethyl ether/cyclohexane (9:1, v/v) before being scanned for radioactivity on the thin-layer scanner.

Prostaglandin $F_2\alpha$ incubations were ended by addition of 5ml of ethyl acetate. The contents of the incubation flask were placed in a separatory funnel containing 50ml of acidified ethyl acetate. After separation the organic phase was evaporated and subjected to t.l.c. after methylation with diazomethane. The plates were developed using the solvent system hexane/diethyl ether/acetic acid (140/60/3, by vol.).

O-De-ethylation of 7-ethoxyresorufin was measured as an increase in fluorescence at 585nm, with an excitation wavelength of 530nm. The instrument used was a JASCO FP-550 spectrofluorimeter.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

Fig. 1 shows the growth, O_2 tension and cytochrome *P*-450 concentration curves obtained from a typical cultivation. The initial fall in the O_2-tension curve, correlating with the rapid growth of the organism, probably reflects the utilization of some readily available energy source in the medium (possibly glucose). The subsequent rise and plateau of the O_2-tension curve, while growth continues, also probably reflects the adaptation of the organism to other energy sources. Interestingly enough, it is only when the stationary phase is reached that the cytochrome *P*-450 is produced within the cell. Several possible explanations exist for the synthesis of cytochrome *P*-450 at this relatively late stage of the growth curve. During the initial growth (i.e. the exponential growth phase) the organism uses readily available carbohydrates as carbon and energy sources, but when these have been exhausted and the culture changes to the stationary growth phase, *B. megaterium* uses some cytochrome *P*-450-mediated reaction for energy production. This might constitute an example of catabolite repression. Secondly, a suppressor of cytochrome *P*-450 synthesis might be present in the medium, and removed by the organism during growth. It might even be the case that accumulation of a variety of waste products or metabolites that appear during growth can give rise to induction of cytochrome $P\text{-}450_{meg}$. To test some of these possibilities the following experiment was performed. Cells were harvested from the fermentor after 11h, or just when the O_2-tension curve began its final rise (i.e. the organism had reached the stationary phase), and the supernatant was saved. In a parallel cultivation, the cells were harvested after 13h (i.e. when the cells contained maximal amounts of cytochrome *P*-450) and the cells were saved. These cells were then cultivated in the 11-h medium and the cellular cytochrome *P*-450 concentration was measured hourly for 5h. There was, however, no further increase in cytochrome *P*-450 concentration over that obtained after growth for 13h in the original culture. This could indicate that either the measured amount of cytochrome *P*-450 is already maximally induced or that the genetic structure of the organism gives this value as a maximum during the stationary phase (i.e. a non-inducible cytochrome *P*-450).

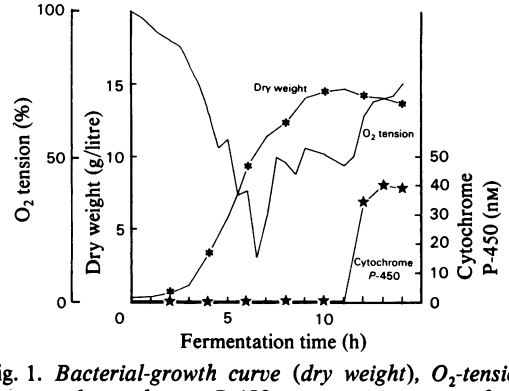

Fig. 1. *Bacterial-growth curve (dry weight), O_2-tension curve and cytochrome P-450-concentration curve for a typical 10-litre cultivation of B. megaterium*
For details concerning cultivation and assay procedures see the Materials and methods section.

Initial experiments to induce cytochrome $P\text{-}450_{meg}$ were performed in shaking-table cultures using the same medium as was used in the large fermentor. The inducers tried were the same as those listed in Table 1 and were present in the medium on inoculation. Since no induction was observed and in order to have more control over the inducing medium the experiments outlined below were performed. Thus, exploiting the knowledge obtained from the above experiments, a method was developed for the screening of possible inducing agents for cytochrome $P\text{-}450_{meg}$, the principle being to harvest the cells just as the enzyme synthesis began and allow synthesis to continue in a very simple medium (basic medium; see the Materials and methods section), on a shaking table, in the presence of various potential inducers. As can be seen from Table 1, when the harvested cells were simply resuspended in the basic medium and incubated on a shaking table at 28°C for 5h the 15β-hydroxylase turnover of the

Table 1. *Inducibility of cytochrome P-450$_{meg}$*
Incubations were performed as described in the Materials and methods section. Values are means for four experiments (the standard deviation was in no case greater than 0.02). The concentration of all agents tested for induction was 1 mM.

Source of cytochrome P-450	Turnover (nmol/mg of protein per 30 min)
(*a*) Fermentor after 11 h	0.08
(*b*) Induction flask after 5 h in basic medium	0.33
(*c*) As (*b*) but a boiled preparation	0.00
(*d*) As (*b*) + progesterone	0.22
(*e*) As (*b*) + 17α-hydroxyprogesterone	0.20
(*f*) As (*b*) + testosterone	0.31
(*g*) As (*b*) + corticosterone	0.20
(*h*) As (*b*) + deoxycorticosterone	0.20
(*i*) As (*b*) + cortisol	0.18
(*j*) As (*b*) + 11-deoxycortisol	0.18
(*k*) As (*b*) + cholesterol	0.25
(*l*) As (*b*) + cholestenone	0.30
(*m*) As (*b*) + lauric acid	0.15
(*n*) As (*b*) + palmitic acid	0.15
(*o*) As (*b*) + phenobarbital	0.17
(*p*) As (*b*) + β-naphthoflavone	0.33
(*q*) As (*b*) + 16α-cyanopregnenolone	0.33
(*r*) As (*b*) + corn oil (1 ml)	0.33
(*s*) As (*b*) + vitamin mixture (1 ml)	0.35

resulting cell-free extract was 0.33 nmol/mg of protein per 30 min. Boiled cell-free extract gave a turnover of 0.00 nmol of 15β-hydroxyprogesterone/mg of protein per 30 min.

Table 1 lists the compounds tested for induction. A number of 3-oxo-Δ^4-steroids were tried (*d–j*, Table 1) as cytochrome P-450$_{meg}$ has shown a preference for this structure in its steroid substrates (Berg *et al.*, 1976). Cholesterol and its 3-oxo-Δ^4-derivative, cholestenone, were also tested. The fatty acids palmitic acid and lauric acid were tested as it has been reported that a preparation of cytochrome P-450 from *B. megaterium* A.T.C.C. 14581 is active in the ($\omega-2$) hydroxylation of fatty acids (Miura & Fulco, 1974, 1975; Hare & Fulco, 1975; Ho & Fulco, 1976; Matson *et al.*, 1977). Some of the classical mammalian inducing agents were also tested, i.e. phenobarbital and β-naphthoflavone. In addition 16α-cyanopregnenolone was also tested. Corn oil and a vitamin mixture, often used to supplement bacterial cultivations, were also tested. As mentioned above, all compounds were tested at two concentrations, i.e. 0.1 and 1.0 mM. Only the results from the 1.0 mM induction trials are given in Table 1 as, in the case of each compound, the value obtained with the 0.1 mM concentration was either equal to or less than the value obtained with the 1.0 mM concentration. However, as is evident from Table 1, none of the compounds tested gave a significant induction of cytochrome P-450$_{meg}$. Indeed, the presence of a number of the compounds in the induction flasks appears to have inhibited the production of the cytochrome P-450. In some additional experiments a number of the above inducers were tested as major carbon sources in basic medium enriched with glucose. However, no growth occurred. Although we do not claim to have tested all possible inducers of the enzyme we do feel we have tried the more obvious compounds.

When the compounds listed under 'Incubations to assay substrate specificity of cytochrome P-450$_{meg}$' were investigated as possible substrates for the cytochrome P-450-dependent hydroxylase system in *B. megaterium*, only aniline was found to be metabolized and hydroxylated in the *para*-position. The reaction was inhibited in the presence of metyrapone (30% inhibition at 2 mM), a so-called type II inhibitor of cytochrome P-450 (Hildebrandt *et al.*, 1969). The rate of formation of *p*-aminophenol was linear up to 30 min and proceeded at an apparent rate of 0.43 nmol of product formed/min per nmol of cytochrome P-450. The enzyme activity was also linear with respect to concentration of cytochrome P-450 up to 1 nmol of the enzyme in the incubation mixture. The effect of increasing substrate concentration on the rate of *p*-hydroxylation of aniline by cytochrome P-450$_{meg}$ was studied and an apparent K_m of 68 μM was calculated. The capacity of cytochrome P-450$_{meg}$ to catalyse *p*-hydroxylation of aniline to *p*-aminophenol may be a function of the enzyme that is related to its general characteristics as a haemoprotein. This interpretation is supported by the findings of Mieyal and co-workers (Mieyal *et al.*, 1976; Mieyal & Blumer, 1976) that a system containing haemoglobin, NADPH, rat liver cytochrome P-450 reductase and atmospheric O_2-catalysed *p*-aminophenol formation from aniline with a turnover number (0.2 nmol/nmol of P-450 per min) similar to that found in the present study for cytochrome P-450$_{meg}$-catalysed aniline hydroxylation. Consequently, conclusions regarding the substrate specificity of cytochrome P-450$_{meg}$ from the data on aniline hydroxylation should be avoided.

The high selectivity of cytochrome P-450$_{meg}$ with regard to substrates renders this enzyme a suitable model system for studies on enzyme structure–function interrelationships in connection with monooxygenase-catalysed hydroxylations. A problem of great interest is what structural features distinguish the physically very similar species of cytochrome P-450 occurring in nature and form the basis of their different catalytic activities. Investigations on soluble bacterial cytochrome P-450 species with differing and narrow substrate specificities should be of great value in model studies aimed at understanding this issue.

We thank Dr. Jan-Åke Gustafsson, Dr. Magnus Ingelman-Sundberg and Dr. Rolf Eriksson for valuable discussions during the course of this investigation and Ms. Kaija Hyvönen for skilful technical assistance. This work was supported by a grant from the Swedish Medical Research Council (no. 13X-2819). We are also grateful to Stiftelsen Bengt Lundqvists minne for fellowships.

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