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Purification of cytochrome P-450 in mycobacteria

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Purification of cytochrome P-450 from *Mycobacterium smegmatis, M. chelonae, M. fortuitum and M. tuberculosis* $H_{37}R_V$ was undertaken. The electrophoretic pattern revealed a single band corresponding to a molecular weight of 66 kDa in all the four species. Cytochrome P-450 purified from drug-resistant *M. tuberculosis* showed a different pattern from that of the sensitive bacteria, and the former was similar to the purified product obtained from phenobarbital-induced cytochrome P-450 in *M. tuberculosis* $H_{37}R_V$. It therefore appears that different forms of cytochrome P-450 are present in drug-sensitive and resistant *M. tuberculosis*, and that there is similarity in the pattern between drug-resistant and phenobarbital-induced *M. tuberculosis*.

A wide variety of drugs, chemical carcinogens and xenobiotics are metabolized by enzymes which belong to a family of hemoproteins with the collective name, cytochrome P-450. *M. tuberculosis*, the causative agent of tuberculosis (TB), has re-emerged as a global threat to human health. An unusual feature of the proteome of this bacteria is the large number of cytochrome P-450 enzymes, about 22 in number, that is more than in any other bacterial genome to date[1].

The role of cytochrome P-450 in the development of drug resistance has been well established in bacteria[2], insects[3] and other living species[4–6]. This phenomenon usually involves increased activity of cytochrome P-450, which brings about biotransformation of the active drug. In an attempt to elucidate the association between cytochrome P-450 and drug resistance in *M. tuberculosis*, we had previously isolated this protein in certain mycobacterial species, including *M. tuberculosis* $H_{37}R_V$ and demonstrated enhanced cytochrome P-450 activity in isoniazid-resistant and isoniazid and rifampicin-resistant *M. tuberculosis*, implicating a role for this protein in causing drug resistance in *M. tuberculosis*[7].

Cytochrome P-450 is known to exist as multiple isozymes which differ functionally. Sequencing of the *Aspergillus fumigatus CYP51* gene encoding cytochrome P-450 sterol 14*a*-demethylase in azole-susceptible and resistant forms showed point mutations in the latter[8], thereby demonstrating that different forms of cytchrome P-450 might exist in drug-susceptible and resistant bacteria. Since drug-resistant *M. tuberculosis* had increased cytochrome P-450 activity, it is likely that different isoforms of this protein might exist in sensitive and resistant *M. tuberculosis*.

In an attempt to investigate this aspect, we purified to homogeneity cytochrome P-450 in the standard strain of *M. tuberculosis* $H_{37}R_V$ that is sensitive to all anti-TB drugs and compared its protein profile with that obtained from isoniazid-resistant and isoniazid and rifampicin-resistant *M. tuberculosis*. In addition, cytochrome P-450 was purified in *M. smegmatis, M. chelonae* and *M. fortuitum.*

Cytochrome P-450 levels in hepatic microsomes are known to increase markedly in the presence of substances such as phenobarbital, 3-methyl cholanthrene, *b*-naphthoflavone, dexamethasone, ethanol, etc.[9]. We also conducted induction studies with phenobarbital on cytochrome P-450 in *M. smegmatis*, *M. tuberculosis* $H_{37}R_V$, *M. chelonae* and *M. fortuitum*, and purified the induced protein in *M. tuberculosis* $H_{37}R_V$.

The mycobacterial strains used in this study were *M. smegmatis* (ATCC 607), *M. tuberculosis* $H_{37}R_V$ (standard strain), *M. fortuitum* (TMC 1529), *M. chelonae* (clinical isolate) and clinical isolates of *M. tuberculosis* resistant to isoniazid alone and to isoniazid and rifampicin.

The organisms were maintained on Lowenstein–Jensen slopes by regular sub-culturing. For experimental purposes, they were grown in Sauton's liquid medium at 37°C. *M. smegmatis*, *M. fortuitum* and *M. chelonae* were harvested at the end of 3–4 days and *M. tuberculosis* at the end of 6–8 weeks.

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Phenyl methyl sulphonyl fluoride (PMSF), cholic acid (sodium salt), Triton X-100, (3–3[(cholamido propyl) dimethyl ammonio]) 1-propane sulfonate (CHAPS), *w*-amino octyl agarose and phenobarbital (sodium salt) from Sigma Chemical Co, USA, sodium dithionite from Riedel–DeHaan Ag, Hannover and Emulgen 913 from Kao Atlas, Japan were used in this study. All other chemicals used were of analytical grade.

Cytochrome P-450 is a membrane-bound protein. Therefore, the membranous fraction of the bacteria was prepared and cytochrome P-450 activity was estimated according to the method standardized in our laboratory[7]. In brief, a 100% bacterial suspension in 10 mM potassium phosphate buffer, pH 7.4, containing lysozyme, *b*-mercapto ethanol and PMSF was sonicated for 30 cycles of one minute each with an interval of one minute, followed by centrifugation at 6000 rpm for 15 min at 4ºC to get rid of the cell debris. The supernatant which had the cytosol proteins along with cell membrane was further centrifuged at 40,000 rpm for 1 h at 4°C. The resultant pellet was washed with 10 mM potassium phosphate buffer and suspended in the same buffer containing 1 mM EDTA, 20% glycerol, 100 mM PMSF and *b*-mercapto ethanol.

The activity of cytochrome P-450 was determined by suitably diluting the samples in 10 mM potassium phosphate buffer, pH 7.4, followed by addition of sodium dithionite. The reduced protein was made to bind with carbon monoxide and the spectrum was recorded between 500 and 400 nm in a dual-beam spectrophotometer. The cytochrome P-450 concentration was calculated by taking the difference in the optical density between 450 and 490 nm, and using a molar extinction coefficient of 91 mM^{-1} cm^{-1}.

Phenobarbital solutions were prepared fresh under sterile conditions. A stock solution of phenobarbital was prepared by dissolving the substance in the required volume of sterile distilled water. About 5 µl of 10 N sodium hydroxide solution was added to facilitate complete dissolution of the substance. The pH of the solution was made neutral by adding a few drops of glacialacetic acid. The required volume of phenobarbital solution was added to the flasks containing the organisms grown in Sauton's medium during the growth phase of the bacteria.

Initial experiments were performed in *M. smegmatis*. Three concentrations of phenobarbital, viz. 1.0, 2.0 and 4.0 mM were tested. Organisms grown in the absence of phenobarbital served as 'controls' and were processed simultaneously. After the addition of phenobarbital solutions, incubation of bacteria was continued at 37°C. At the end of 24 h, the cells were harvested and the activity of cytochrome P-450 was determined in the membranous pellet of the organism.

On the basis of the results obtained with *M. smegmatis*, the optimal concentration of 4.0 mM phenobarbital was used to carry out induction experiments in *M. fortuitum*, *M. chelonae* and *M. tuberculosis* $H_{37}R_V$.

Cytochrome P-450 that is tightly bound to membrane proteins, was solubilized using 20% sodium cholate solution. The sodium cholate to protein ratio was maintained at 1.5 : 1.0. The requisite volume of sodium cholate was added dropwise to the pellet suspended in 10 mM potassium phosphate buffer, pH 7.4, kept in ice and sonicated at regular intervals. This suspension was kept on a magnetic stirrer at 4°C overnight and centrifuged at 40,000 rpm for 1 h at 4°C. Spectral activity was determined in the pellet and supernatant after reduction with sodium dithionite and passing CO. An absorption peak at 450 nm in the supernatant indicated good solubilization. The detergent was removed from the solubilized supernatant solution by dialysis against 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 1 mM dithiothreitol and 20% glycerol.

Further purification of cytochrome P-450 was carried out at 4°C. This was done by passing the dialysate through a column packed with *w*-amino octyl agarose. After washing the column with 10 mM potassium phosphate buffer, pH 7.4, the dialysate was loaded onto the column. This was followed by washing the column with 10 mM potassium phosphate buffer, pH 7.4, containing 1% sodium cholate. Cytochrome P-450 was then eluted from the column with 100 mM potassium phosphate buffer, pH 7.4, containing 1% sodium cholate and 0.1% Emulgen 913. Fractions of about 300–400 µl were collected. The spectral activity of cytochrome P-450 was determined spectrophotometrically in all the fractions collected. The fractions which exhibited an absorption peak at 450 nm after reduction and treatment with CO were taken to be rich in cytochrome P-450 activity, and such fractions were pooled and run on SDS–PAGE. Samples were electrophoresed on a vertical slab gel apparatus and stained with 0.2% Coomassie brilliant blue (CBB). Purification of cytochrome P-450 was carried out in *M. smegmatis*, *M. tuberculosis* $H_{37}R_V$, *M. fortuitum* and *M. chelonae*. The hemoprotein was purified in *M. tuberculosis* strains resistant to isoniazid alone and isoniazid and rifampicin. Cytochrome P-450 induced with 4.0 mM phenobarbital in *M. tuberculosis* $H_{37}R_V$ was also purified.

The cytochrome P-450 values following induction with different concentrations of phenobarbital in *M. smegmatis*, *M. fortuitum*, *M. chelonae* and *M. tuberculosis* $H_{37}R_V$ are given in Table 1.

In the case of *M. smegmatis*, 1.0 and 2.0 mM concentrations of phenobarbital were not able to cause an increase in the cytochrome P-450 content, although at 2.0 mM concentration an enhanced activity could be seen. However, this difference in the hemoprotein content between the control and phenobarbital-treated bacteria was not statistically significant ($P = 0.061$). But, at 4.0 mM concentration, phenobarbital was able to cause a four-fold enhancement of cytochrome P-450 activity. The difference in the hemoprotein content between the control and

Table 1. Cytochrome P-450 levels (mean ± SD) in different mycobacterial species treated with phenobarbital

Concentration of phenobarbital	Cytochrome P-450* (nmol/mg protein)			
	M. smegmatis	*M. fortuitum*	*M. chelonae*	*M. tuberculosis* $H_{37}R_V$
Nil	0.47 ± 0.24	0.30 ± 0.06	0.47 ± 0.08	0.55 ± 0.11
1.0 mM	0.34 ± 0.11	–	–	–
2.0 mM	0.66 ± 0.19	–	–	–
4.0 mM	1.80 ± 0.91**	0.75 ± 0.12**	2.24 ± 0.37**	1.31 ± 0.39**

*Mean of 6 experiments; **Highly significant ($P < 0.05$).

the bacteria exposed to 4.0 mM phenobarbital was statistically significant ($P = 0.030$).

With respect to the other bacteria, significant increase in the cytochrome P-450 content was observed following exposure to 4.0 mM phenobarbital. Maximal induction was observed in *M. chelonae*, followed by *M. smegmatis* and *M. tuberculosis* $H_{37}R_V$, and it was least in *M. fortuitum*.

Fractions which did not exhibit an absorption peak at 450 nm were run along with cytochrome P-450-rich fractions and served as controls. The eluted fractions rich in cytochrome P-450 gave a single prominent band corresponding to molecular weight of about 66 kDa, while the controls did not give such a pattern. Cytochrome P-450 purified from *M. fortuitum*, *M. smegmatis*, *M. chelonae* and *M. tuberculosis* $H_{37}R_V$ gave uniform electrophoretic pattern, i.e. a single band corresponding to a molecular weight of 66 kDa (Figures 1 and 2). The recoveries (%) were 16, 12, 12 and 13% for *M. smegmatis*, *M. fortuitum*, *M. chelonae* and *M. tuberculosis* $H_{37}R_V$, respectively.

When the electrophoretic patterns of the purified cytochrome P-450 from isoniazid-resistant and isoniazid and rifampicin-resistant *M. tuberculosis* were compared with standard *M. tuberculosis* $H_{37}R_V$ that was sensitive to all anti-TB drugs, a striking difference was noticed. While the cytochrome P-450 purified from *M. tuberculosis* $H_{37}R_V$ displayed a single band corresponding to a molecular weight of 66 kDa, both the resistant bacteria gave an additional band (Figure 3). The additional band obtained in the case of the isoniazid-resistant bacteria had a molecular weight of approximately 50 kDa and that of the isoniazid and rifampicin-resistant bacteria had a molecular weight of approximately 55 kDa.

Cytochrome P-450 was also purified from *M. tuberculosis* $H_{37}R_V$ treated with 4.0 mM phenobarbital. The purified product on SDS–PAGE showed a pattern similar to that obtained with the resistant bacteria, i.e. two discrete bands corresponding to molecular weights of 66 and 50 kDa (Figure 4).

Purification of cytochrome P-450 in different forms of mycobacterial species was undertaken and is reported here. The ability of phenobarbital to induce levels of drug metabolism in mammals has been known for over 40 years. We observed significant induction of cytochrome

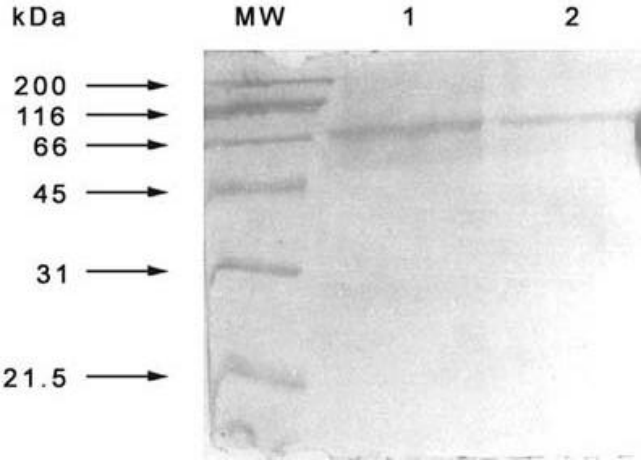

Figure 1. Cytochrome P-450 purified from *M. smegmatis* and *M. tuberculosis* $H_{37}R_V$. The proteins were separated on 12.5% SDS–PAGE, run alongside molecular weight markers and stained with CBB. Lane 1, *M. smegmatis*; and lane 2, *M. tuberculosis* $H_{37}R_V$.

P-450 in the presence of 4.0 mM phenobarbital at 24 h in *M. smegmatis*, *M. fortuitum*, *M. chelonae* and *M. tuberculosis* $H_{37}R_V$. A dose-dependent induction was noticed in *M. smegmatis* as reported by others in *Bacillus megaterium*[10,11].

The purification and characterization of membrane-bound forms of cytochrome P-450 from different sources have been in steady progress during recent years. Reports on purification of the recombinant protein in *B. megaterium*[12], *Streptomyces* sp.[13], *Corynebacterium* sp.[14], *E. coli*[15] and *B. subtilis*[16] have emerged in the recent past. Based on the mobility of the protein on SDS–PAGE, its molecular weight was reported to range from 44 to 50 kDa in the different species. Similar studies are, however, lacking in mycobacteria.

O'Keeffe *et al.*[17], and Narhi and Fulco[10] have purified cytochrome P-450 in *Pseudomonas putida* and *B. megaterium* respectively. However, both methods were quite cumbersome and lengthy, involving the use of three to four different columns. The method described here for purifying cytochrome P-450 in mycobacteria is relatively simple, involving a single column that yielded a recovery of about 12–16%.

We tried solubilization of cytochrome P-450 from the membrane with several detergents such as sodium cholate 20%, Emulgen 0.1%, Triton X-100 1% and CHAPS 0.1% at different concentrations, and found 20% sodium cholate solution to be most effective.

Amino octyl sepharose has been used in a few studies for purification of cytochrome P-450 from rat liver microsomes[18] and from the mitochondria of adrenal cortex of rats[19]. In using amino octyl derivatives of sepharose for purification of the microsomal enzymes, the selection of detergents included in the elution buffers is important to obtain satisfactory separation of the enzymes. Cytochrome P-450 gets adsorbed onto the amino octyl sepharose column when solubilized with cholate. Cytochrome P-450 could then be eluted with buffer containing Emulgen 913 in addition to cholate, as shown in this study and that of Imai[18] which was performed in rat liver microsomes. Cytochrome P-450 can be distinguished by a number of criteria, including spectral properties, molecular weight, amino acid composition, substrate specificity and immuno chemical properties[20]. We have identified cytochrome P-450 by virtue of its characteristic spectral property, i.e. an absorption peak at 450 nm.

Upon purification of cytochrome P-450 from isoniazid-resistant and isoniazid and rifampicin-resistant *M. tuberculosis*, a different electrophoretic pattern was observed when compared to that obtained with the standard, sensitive strain of *M. tuberculosis* $H_{37}R_V$. The additional band obtained in the case of isoniazid and isoniazid and rifampicin-resistant bacteria had molecular weights of about 50 and 55 kDa respectively, suggesting that multiple forms of cytochrome P-450 are present in *M. tuberculosis* resistant to different drugs. Another interesting finding in this study is the similarity in the protein profiles of resistant *M. tuberculosis* and phenobarbital-induced *M. tuberculosis*. Both forms of bacteria had increased cytochrome P-450 activity.

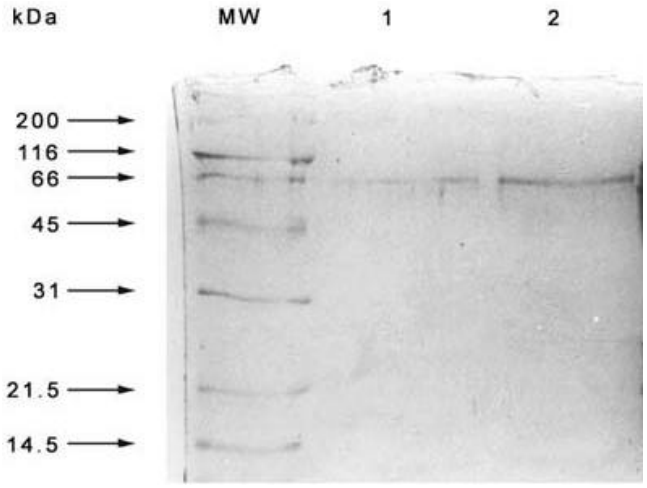

Figure 2. Cytochrome P-450 purified from *M. chelonae* and *M. fortuitum*. The proteins were separated on 12.5% SDS–PAGE, run alongside molecular weight markers and stained with CBB. Lane 1, *M. chelonae*; and lane 2, *M. fortuitum*.

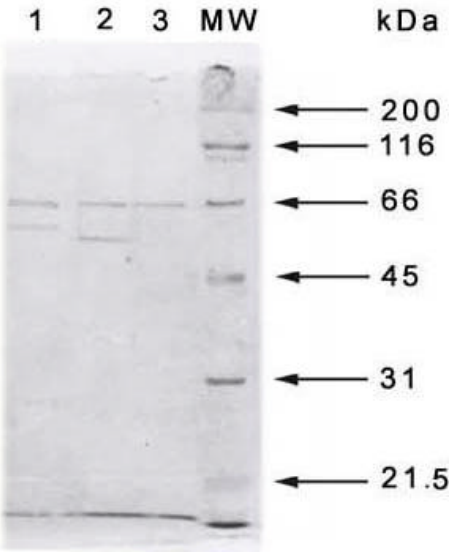

Figure 3. Cytochrome P-450 purified from drug-sensitive and resistant *M. tuberculosis*. The proteins were separated on 12.5% SDS–PAGE, run alongside molecular weight markers and stained with CBB: Lane 1, Isoniazid (INH)-resistant *M. tuberculosis*; lane 2, INH and Rifampicin (RMP)-resistant *M. tuberculosis*; and lane 3, *M. tuberculosis* $H_{37}R_V$ (sensitive to INH and RMP).

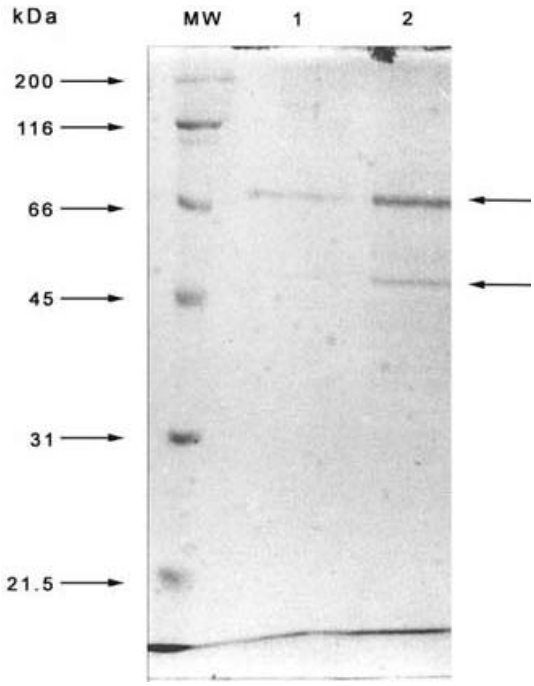

Figure 4. Cytochrome P-450 purified from phenobarbital-treated *M. tuberculosis* $H_{37}R_V$. 4.0 mM concentration of phenobarbital solution was added to the bacteria during their growth phase. At the end of 24 h, the organisms were harvested and cytochrome P-450 was isolated and purified. The proteins were separated on 12.5% SDS–PAGE, run alongside molecular weight markers and stained with CBB. Lane 1, Uninduced *M. tuberculosis* $H_{37}R_V$ (Control); and lane 2, Phenobarbital (4.0 mM)-induced *M. tuberculosis* $H_{37}R_V$.

As early as 1959, it was suggested by Conney *et al.*[21] that multiple forms of drug-metabolizing enzymes catalysing the same reaction exist in the liver of a given species. The criteria adopted for the presence of multiple forms have been mobility on SDS–PAGE, spectral and catalytic properties, immunological relatedness, peptide mapping and finally the protein sequence. In this study, based on the mobility of the protein on SDS–PAGE, it appears that different forms of cytochrome P-450 are present in drug-sensitive, isoniazid-resistant and isoniazid and rifampicin-resistant *M. tuberculosis* and also the same isoform patterns of the protein are present in the resistant and phenobarbital-induced *M. tuberculosis*. Further studies at the molecular level would confirm these findings and give a better understanding of the significance of this study.

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Estimation of monthly rain rate over Indian Ocean region using MSMR data

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India is surrounded by ocean in three directions. The land and ocean interaction controls the climatic conditions over the Indian subcontinent. The study of meteorological parameters over ocean has paramount importance in understanding the interaction between ocean and land. In the present study, efforts have been made to deduce the monthly variations of the rain rate over the oceanic region adjacent to India from July 1999 to June 2000 using brightness temperature data observed by the Multi-channel Scanning Microwave Radiometer (MSMR) sensor on-board IRS-P4 (OCEANSAT). Using brightness temperature measured at 10 and 18 GHz frequencies in horizontal and vertical polarizations, rainfall rate has been compared. The rain rate deduced from MSMR brightness temperature data shows a high value over the ocean during June and July. The rain rate shows moderate to low values during October–November and increases in January, followed by a decrease in March and April, again it increases from May. Variation of rain rate is mainly controlled by summer and winter monsoon. The southwest summer monsoon hits India in May/June; as a result high rain rate, which is responsible for higher rainfall, has been observed over the ocean. Due to the northeast winter monsoon, rain rate increases over the ocean during January–February. The rain rate retrieved from MSMR data is compared with NCEP monthly averaged rain rate.

THE passive microwave remote sensing technique has proved to be a powerful tool in monitoring spatial and temporal behaviour of the earth surface. Numerous studies have been carried out to explore the use of microwave remote sensing technique to retrieve information regarding the physical state of ocean and land surfaces. All-time and all-weather operational advantage of microwave remote sensing allows monitoring of the earth with better temporal resolution compared to the sensors in the visible portion of the electromagnetic spectrum. A number of space-borne sensors have shown sensitivity to rain rate or to other features associated with rain[1]. The retrieval and monitoring of the rain rate over ocean surrounding India has made significant progress after the launching of OCEANSAT-I (IRS-P4). Multi-channel Scanning Microwave Radiometer (MSMR), one of the sensors of IRS-P4, has the ability to penetrate through clouds and is also highly sensitive to rain[2]. IRS-P4 is a polar orbiting satel-

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