Cytochrome P-450 in drug-resistant *Mycobacterium tuberculosis*

Geetha Ramachandran[†], Prema Gurumurthy[†],

P. R. Narayanan[†,#] and Usha Mahadevan*

[†]Tuberculosis Research Centre (ICMR), Mayor V. R. Ramanathan Road, Chetput, Chennai 600 031, India *Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Methods for the isolation and spectrophotometric determination of cytochrome P-450 in mycobacteria were standardized. Cytochrome P-450 levels were estimated in *Mycobacterium tuberculosis* organisms sensitive to both isoniazid and rifampicin and resistant to any of the two drugs. Cytochrome P-450 was isolated and its presence was shown in *M. smegmatis*, *M. fortuitum*, *M. chelonae* and *M. tuberculosis* H$_{37}$Rv. The cytochrome P-450 content was significantly elevated in *M. tuberculosis*, resistant to both isoniazid and rifampicin when compared with the corresponding sensitive strains. It therefore appears that cytochrome P-450 might play a role in causing drug resistance in tuberculosis.

TUBERCULOSIS is one of the most alarming infectious diseases facing the world today, particularly with the advent of multi-drug resistant organisms. Bacteria develop resistance to drugs via a limited number of mechanisms[1], some of which apply to mycobacterial drug resistance[2] and this appears to be a multi-locus phenomenon and several factors contribute to the development of resistance. The role of drug-deactivating enzymes in the development of drug resistance is well established in bacteria[3] and insects[4]. This phenomenon

[#]For correspondence. (email: icmrtrc@ren.nic.in)

usually involves increased activity of a particular enzyme responsible for degrading the active drug.

Cytochrome P-450, earlier referred to as the CO-binding pigment, was first described by Klingenberg[5]. It belongs to a group of hemoproteins and forms a vital component of the mixed-function oxidase system of the endoplasmic reticulum. Cytochrome P-450 constitutes the most powerful oxidizing enzymes and is involved in the metabolism and biotransformation of a wide variety of drugs and endogenous compounds. It is found in abundance not only in the hepatic microsomes, but also in the microsomes and mitochondria from the adrenal cortex. The hemoprotein is found in liver microsomes of a variety of vertebrates, including mammals, birds, snakes, frogs and fishes, plants, bacteria[6], bacteroids[7] and yeast[8].

An association between drug resistance and cytochrome P-450 drug-metabolizing enzyme activity has been reported in certain species of Plasmodium[9,10], *Drosophila melanogaster*[11], *Musca domestica*[12], *Trypanosoma cruzi*[13], *Leishmania mexicana*[14], *Heliothis virescens*[15], *Lucilia cuprina*[16], certain Candida species[17–19], *Cryptococcus neoformans*[20], etc. In all these studies, it has been clearly shown that the resistant species had elevated levels of cytochrome P-450 than the corresponding sensitive ones. Indirect evidence relating to mechanism of resistance in *M. avium* with respect to quinolones has been shown to be associated with bacterial P-450 activity[21]. For example, the resistant strains might have enhanced P-450 activity in the periplasm, resulting in an increased rate of metabolism of quinolones, which, therefore, cannot reach the critical

concentration needed to be effective. However, when cytochrome P-450 is inactivated, the resistance mechanism gets defeated.

Recently the complete genomic sequence of *M. tuberculosis* has been reported by Cole *et al.*[22]. A large
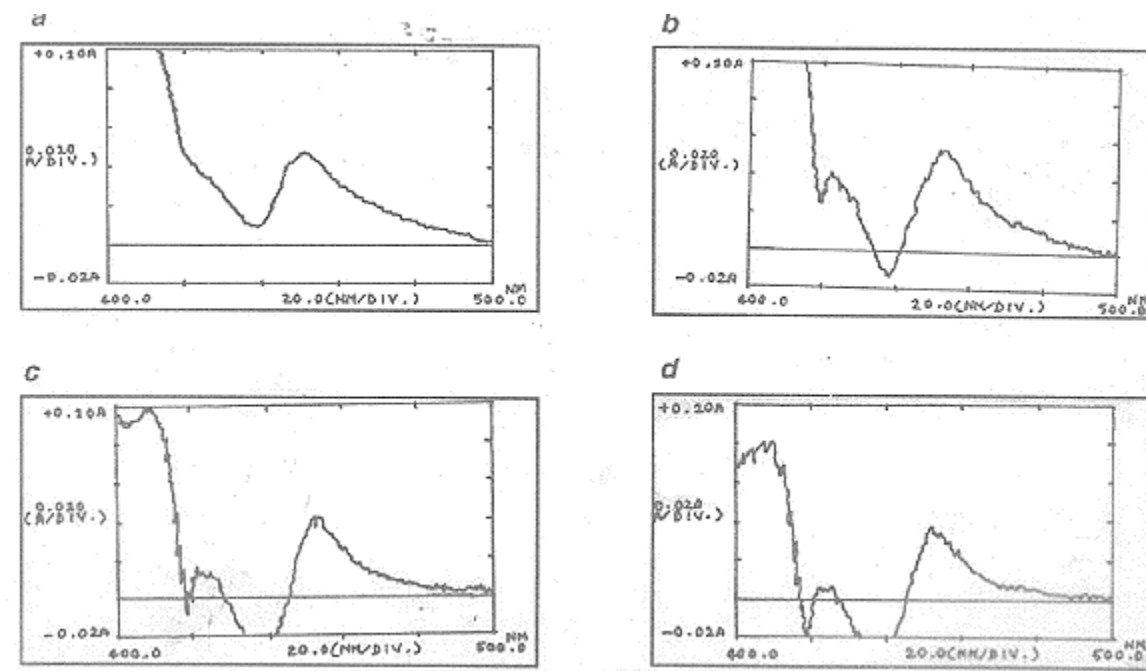

Figure 1. Spectral activity of cytochrome P-450 in different mycobacteria, *a*, *M. smegmatis*; *b*, *M. tuberculosis* H$_{37}$Rv; *c*, *M. fortuitum*; *d*, *M. chelonae*. The experiments were repeated on three different occasions with different batches of bacteria.

number of oxygenases containing cytochrome P-450 have been predicted to be present in *M. tuberculosis* and about 22 genes coding for cytochrome P-450 have been identified. However, an association between cytochrome P-450 and drug resistance in mycobacteria has not been studied. It was therefore decided to isolate this protein in certain mycobacterial species and estimate its content in *M. tuberculosis* strains, sensitive and resistant to certain anti-tuberculosis drugs, to see its possible involvement in causing drug resistance in tuberculosis.

The mycobacterial strains used for isolation of cytochrome P-450 were *M. smegmatis* (ATCC607), *M. tuberculosis* H$_{37}$Rv (standard strain), *M. fortuitum* (TMC 1529) and *M. chelonae* (clinical isolate). Clinical isolates of *M. tuberculosis* strains, resistant to isoniazid alone (n = 12) and resistant to rifampicin and isoniazid (n = 9) were also used. The drug sensitivity pattern was determined by the indirect drug sensitivity test.

DNaseI and PMSF from Sigma Chemical Co. USA, sodium dithionite from Riedel-DeHaan Ag. Sealze, Hannover, b -mercaptoethanol from Fluka Buchs, Switzerland and lysozyme from Hi-media, India, were used for the above experiments. All other chemicals used were of analytical grade.

The organisms were maintained on LJ Slopes by regular subculturing. For experimental purposes, they were grown in modified Sauton's medium at 37°C. The cells were harvested during the log phase. The wet weight of the cells was determined.

Since cytochrome P-450 is a membrane-bound protein, the membranous fraction of the bacteria (sensitive and resistant strains) was prepared as follows: A 100% suspension of the cells was prepared in 10 mM potassium phosphate buffer pH 7.4. The cells were treated with lysozyme in the presence of a protease inhibitor namely, phenyl methyl sulfonyl fluoride (PMSF) and lysed by extensive sonication for about 20 min. The sonicate was treated with DNaseI (10 mg/ml) and further sonicated for 10 min. The cell suspension was centrifuged at 6000 rpm for 15 min to get rid of the cell debris. The resultant supernatant was again centrifuged at 40,000 rpm for 60 min. The pellet was washed with 10 mM potassium phosphate buffer pH 7.4 and suspended in the same buffer containing 1 mM EDTA, 20% glycerol, 100 mM PMSF and b -mercaptoethanol.

Cytochrome P-450 was determined according to the method of Omura and Sato[23]. In brief, the samples were suitably diluted in 10 mM potassium phosphate buffer pH 7.4. Equal volumes of the diluted sample were taken into two test tubes marked 'blank' and 'test'. A few grains of sodium dithionite were added to both the tubes. Carbon monoxide gas was bubbled into the tube marked 'test'. The contents of both the test tubes were then transferred into two matched cuvettes and the spectrum was recorded between 500 and 400 nm in a double-beam spectrophotometer (ATI Unicam). The protein content in the pellet was determined by the method of Lowry et al.[24].

A distinct peak at 450 nm was obtained in the membranous pellet in all the mycobacterial species tested (Figure 1 a–d). The presence of cytochrome P-450 in *M. smegmatis*, *M. tuberculosis* H$_{37}$Rv, *M. fortuitum* and *M. chelonae* was thus established. The quantity of cytochrome P-450 in the pellets of the different organisms was calculated from the difference in the optical density between 450 and 490 nm and using the molar extinction coefficient of 91 mM^{-1} cm^{-1}. Table 1 gives the quantity of cytochrome P-450 present in the various avirulent and virulent mycobacterial species.

M. tuberculosis H$_{37}$Rv, sensitive to isoniazid and rifampicin (n = 12) were processed simultaneously along with resistant bacteria. Clinical isolates of *M. tuberculosis* strains, resistant to isoniazid alone (n = 12) and resistant to isoniazid and rifampicin (n = 9) were processed for the cytochrome P-450 content. Table 2 gives the hemoprotein levels in these strains. The isoniazid-resistant organisms had a more than 2-fold increase in the P-450 content when compared to the sensitive bacteria. A similar trend was seen in the isoniazid and rifampicin-resistant bacteria too. The differences in the cytochrome P-450 content between the sensitive and both the resistant bacteria were highly significant (P < 0.001) (Figure 2).

mycobacterial species

ganism	Mean (range)* Cytochrome P-450 (nmol/mg protein)
smegmatis	0.39 (0.19–0.78)
tuberculosis H$_{37}$Rv	0.60 (0.44–0.97)
fortuitum	0.30 (0.22–0.49)
chelonae	0.47 (0.29–0.60)

can of 3 experiments.

ble 2. Cytochrome P-450 content in drug resistant and sensitiv bacteria

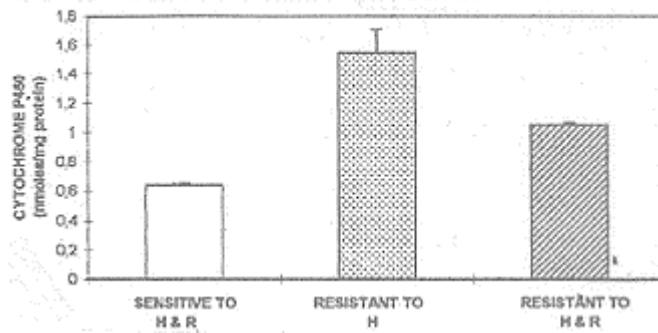| ganism tuberculosis)	n	Cytochrome P-450 (nmol/mg protein) mean ± SD
and R-sensitive	12	0.64 ± 0.24
esistant	12	1.55 ± 0.31
and R-resistant	9	1.05 ± 0.32

Figure 2. Cytochrome P-450 levels in drug sensitive and resistant *M. tuberculosis*. *M. tuberculosis* H_{37}Rv sensitive to H and R (*n* = 12), *M. tuberculosis*, resistant to H (*n* = 12) and resistant to H and R (*n* = 9) were processed. H, isoniazid; R, rifampicin.

Although mutations in the *katG* (ref. 25), *inhA* (ref. 26) and *ahpC* genes[27] have been associated with resistance to isoniazid, the exact mode of action of isoniazid is not known. Likewise mutation in the *rpoB* gene that codes for RNA polymerase has been reported to be the cause of resistance to rifampicin[28]. However, some bacterial strains with low to intermediate resistance to rifampicin have been noticed with the *rpoB* gene intact, suggesting that there may be other cellular components which are able to mutate and confer a degree of resistance[29]. Studies conducted on rifampicin resistance in *Neisseria meningitidis* suggest that different levels of resistance to rifampicin can be imparted by mechanisms other than mutations in the *rpoB* gene[30].

The results reported here, i.e. elevated levels of cytochrome P-450 in isoniazid and rifampicin-resistant
M. tuberculosis than the corresponding sensitive bacteria are similar to those reported in various other living species. Enhanced elimination of drugs by detoxification and metabolic pathways mediated by cytochrome P-450 is more likely to be responsible for increased resistance to the drug. Since the metabolism of isoniazid and rifampicin is cytochrome P-450-mediated, it is logical to assume that development of resistance to these drugs could possibly be due to increased cytochrome P-450 content in the bacteria. Moreover, an isoniazid-inactivating substance has been isolated from isoniazid-resistant avian mycobacteria which decomposed isoniazid to hydrazine and isonicotinic acid[31]. In a study conducted to see the effect of chronic retrovirus infection in the P-450-mediated activation of acetaminophen in mouse liver microsomes, it was observed that enhanced resistance to acetaminophen-induced hepatotoxicity could be due to increased excretion of the drug by P-450 mediated metabolic pathways[32]. In another study, patients with acute promyelocytic leukemia treated with retinoic acid, developed resistance to retinoic acid despite an initial good response[33]. One potential mechanism for clinical retinoic acid resistance is the pharmacologic alteration in the metabolism of retinoic acid. Induction of cytochrome P-450 resulted in lower plasma and cellular levels of active retinoids. Thus, acquired resistance to retinoic acid may be explained at least in part by drug metabolism in leukemic cells.

In an *in vitro* study carried out to assess the anti-*M. avium* activities of quinolones, it was found that a cyclopropyl group substituted at the N1 position of a quinolone is crucial against all of the resistant *M. avium* strains but not the susceptible ones[21]. For example, resistant strains might have enhanced P-450 activity in
the periplasm, resulting in an increased rate of metabolism of quinolones, which therefore cannot

reach the critical concentration needed to be effective. The substituted group induces suicidal inactivation of the metabolizing enzyme namely, cytochrome P-450, which in turn reduces its activity and defeats the resistance mechanism.

It therefore appears that cytochrome P-450 also plays a role in causing drug resistance in *M. tuberculosis*. It would be of interest to study the isoform pattern of cytochrome P-450 in isoniazid and rifampicin resistant *M. tuberculosis* and compare the findings with the sensitive organism. This might throw light on whether different isoforms of cytochrome P-450 exist in drug-sensitive and resistant bacteria. Attempts on these lines are in active progress.

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