SECTION IV

INDUCTION KINETICS OF BENZO(a)PYRENE HYDROXYLASE IN ASPERGILLUS OCHRACEUS TS

Evidence Of Multiple Forms Of Cytochrome P-450
In Section II the presence of an inducible cytochrome P-450 (Cyt P-450) monoxygenase in \textit{A. ochraceus} TS capable of hydroxylating benzo(a)pyrene has been described. Attempts have been made to investigate the influence of different inducers of mammalian Cyt P-450 on kinetic parameters ($K_m$ and $V_{max}$) of BP-hydroxylation and also on different components of this hydroxylase system. Further to make a probe about the existence of multiple forms of Cyt P-450 in the test organism, transformation was tried with microsomal preparations obtained from cells induced by different agents and the role of flavone on the transformation(s) was also tested as depicted below.
MATERIALS AND METHODS

Cultivation and induction

Experiments on growth and induction were done in liquid medium as described previously in Section II. *A. ochraceus* TS was grown under liquid culture condition for 48 h. after which inducing agents were added followed by further incubation for 16-18 h. for induction.

Preparation of microsomes

Microsomes were prepared by differential centrifugation of cell homogenate suspended in buffer A as described in Section II. The microsomal fraction (105,000 g pellet) was washed and resuspended in buffer A containing 0.01 M KCl by the use of a hand held potter type homogenizer.

Enzyme assay

BP-hydroxylase activity was measured fluorometrically according to the method (vide Section II) of Nebert and Gelboin. Unless otherwise stated a typical incubation mixture contained 0.1 μ moles BP, 0.5 μ moles NADPH and microsomal protein (600-800 μg) in a total volume of 1 ml.
Effect of flavone on hydroxylation in vitro

The BP induced microsomes were preincubated with various concentrations of flavone (0-800 μM) dissolved in acetone before the addition of substrate and cofactors. Both NADPH and NaI\textsubscript{4} dependent hydroxylation were examined exactly in the same way described in Section II.

Effect of flavone on BP-hydroxylation by microsomes induced by different agents

Benzo(a)pyrene (BP), 3-methyl cholanthrene (3-MC), phenobarbital (PB), \β -napthoflanone (BNP) and polychlorinated biphenyl (PCB, Aroclor, 1254) induced microsomes were preincubated separately with different concentrations of flavone (0-1 mM) before the addition of substrate and NADPH. The reaction mixtures were then incubated at 30°C on a rotaty shaker for 15 min. and fluorescent phenolic metabolites were measured fluorometrically as described previously (Section II) in a Perkin-Elmer Spectrophotofluorometer (MPF 44B).

Assay of NADPH cytochrome c reductase activity

NADPH cytochrome c reductase activity was determined by the method of Williams and Kamin\textsuperscript{160} as described in Section II.
Measurement of metyrapone difference spectra

Phenobarbital (PB) and 3-methyl cholanthrene (3-MC) induced microsomes suspended separately in phosphate buffer (0.05 M, pH 7.5) containing KCl (15 mM) and glycerol (20% v/v) (final concentration of protein 2 mg/ml) were distributed in two cuvettes.

Dithionite (BDH, India) (few grains) was then added to both the cuvettes. This was followed by the addition of metyrapone dissolved in water (200 μM final concentration) to the experimental cuvette. The difference spectra were recorded in a Cary model 17D spectrophotometer.

Estimation of protein

Protein was estimated according to the method of Lowry et al., using bovine serum albumin as standard.

RESULTS

The results of effect of different classical inducer of mammalian cyt P-450 on BP-hydroxylation in vitro are presented in Table 4.1. They showed striking resemblance with those obtained with hepatic microsomes and hamster
fetus cells. 3-MC, BP, β-naphthoflavone and other aryl hydrocarbons used as inducers at concentration varying from 80-100 μM enhanced the hydroxylase activity which may be considered due to selective induction of a form or forms of cyt P-450 with a high activity towards BP-hydroxylation. On the other hand, a very moderate enhancement in BP-hydroxylation was observed with microsomes obtained from cells induced by phenobarbital (PB), polychlorinated biphenyl (PCB) and progesterone although a significant induction of NADPH-cyt c reductase activity (3 to 5 fold, Table 4.1) was possible with the above agents. Again, inducer (BP) when added at zero time did not cause any significant induction but caused a decrease in cell population.

Addition of BP at different concentrations (40-160 μM) during the induction period had a dramatic effect on the kinetics of BP-hydroxylase activity when measured by double reciprocal Lineweaver-Burk plot. The effect of different inducing agents on the kinetic parameters of BP-hydroxylase as well as on NADPH cyt c reductase activity are shown in Table 4.2. It was observed that with increase in concentrations of BP as inducer in the medium there was a lowering of Michaelis constant (K_m) i.e. increasing affinity for BP and elevation of maximal velocity (V_max).
Again, pretreatment with 3-MC, BNF and other aryl hydrocarbons, also exhibited a differential improvement in \( K_m \) and \( V_{max} \) for BP hydroxylation. On the other hand, PB, PCB and progesterone induced BP-hydroxylase activity to some extent and there was a slight modification in \( K_m \) and \( V_{max} \); although a significant induction of NADPH cyt c reductase (2.5 to 5 fold) was observed unlike the case where the heme component was induced selectively by the agents mentioned before.

The effect of flavone on NADPH and NaIO\(_4\) dependent BP-hydroxylation by microsomes from BP induced \textit{A. ochraceus} TS is presented in Figure 4.1. It was observed that flavone could inhibit NADPH dependent BP-hydroxylation while NaIO\(_4\) dependent activity was inhibited and stimulated by the same agent on the doses used (Table 4.3 and 4.4).

Again, the effect of flavone on NADPH dependent BP-hydroxylation by microsomes of \textit{A. ochraceus} TS induced by different agents were tested with a view to probe the multiplicity of cytochrome P-450 in this organism and the results are shown in Figure 4.2.

It was observed that the BP metabolism carried out by both 3-MC and BP induced microsomes were inhibited by
flavone (Figure 4.2c, Table 4.5 and 4.6). On the other hand, the addition of flavone (0.2-1 mM) to the reaction catalysed by BNF and PB induced microsomes stimulated the hydroxylation by 2 to 3 fold (Figure 4.2a, 4.2b; Table 4.7 and 4.8). The results of inhibition and/or stimulation by flavone on BP-hydroxylation by PCB induced microsomes was found to be inconclusive. The BP metabolism was observed to be inhibited at low and stimulated at high concentrations of flavone respectively (Figure 4.2d, Table 4.9).

Further, both PB and 3-MC induced microsomes exhibited dithionite reduced metyrapone difference spectra (Figure 4.3).
Table 4.1  Effect of inducing agents on different components of BP-hydroxylase system in *A. ochraceus* TS in vitro

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Concentration (μM)</th>
<th>BP hydroxylase activity (nmoles/min/mg)</th>
<th>NADPH cyt c reductase (nmoles/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo(a)pyrene</td>
<td>40</td>
<td>0.37</td>
<td>45.35</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.60</td>
<td>45.75</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.67</td>
<td>46.00</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.64</td>
<td>46.00</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>0.69</td>
<td>46.00</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>80</td>
<td>0.75</td>
<td>42.00</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.92</td>
<td>45.00</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>100</td>
<td>0.35</td>
<td>150.00</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>100</td>
<td>0.62</td>
<td>59.00</td>
</tr>
<tr>
<td>Benzanthracene</td>
<td>100</td>
<td>0.67</td>
<td>49.00</td>
</tr>
<tr>
<td>Polychlorinated biphenyl (Aroclor, 1254)</td>
<td>100</td>
<td>0.27</td>
<td>115.00</td>
</tr>
<tr>
<td>Pyrene</td>
<td>100</td>
<td>0.67</td>
<td>59.00</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>100</td>
<td>0.85</td>
<td>49.00</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>100</td>
<td>0.80</td>
<td>48.00</td>
</tr>
<tr>
<td>Anthracene</td>
<td>100</td>
<td>0.72</td>
<td>50.00</td>
</tr>
<tr>
<td>Progesterone</td>
<td>100</td>
<td>0.37</td>
<td>78.00</td>
</tr>
</tbody>
</table>
Table 4.2 Effect of inducing agents on NADPH-cyt c reductase and kinetics of BP-hydroxylase system in A. ochraceus TS in vitro

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Concentration (µM)</th>
<th>BP-hydroxylase</th>
<th>NADPH-cyt c reductase (nmoles/min, mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( K_m ) (µM)</td>
<td>( V_{max} ) (nmole hydroxy BP/min/mg protein)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>250</td>
<td>0.11</td>
<td>30.25</td>
</tr>
<tr>
<td>Benzo(a)pyrene BP</td>
<td>40</td>
<td>70</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>50</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>45</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>45</td>
<td>0.69</td>
</tr>
<tr>
<td>3-Methylcholanthrene (3-MC)</td>
<td>80</td>
<td>60</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>45</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>45</td>
<td>1.10</td>
</tr>
<tr>
<td>( \beta )-Naphthoflavone (BNP)</td>
<td>100</td>
<td>44</td>
<td>0.80</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>100</td>
<td>60</td>
<td>1.00</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>100</td>
<td>66</td>
<td>0.90</td>
</tr>
<tr>
<td>Phenobarbital (PB)</td>
<td>100</td>
<td>133</td>
<td>0.39</td>
</tr>
<tr>
<td>Polychlorinated biphenyl (PCB, Aroclor 1254)</td>
<td>100</td>
<td>125</td>
<td>0.32</td>
</tr>
<tr>
<td>Progesterone</td>
<td>100</td>
<td>150</td>
<td>0.39</td>
</tr>
</tbody>
</table>


Table 4.3  Effect of flavone on NADPH dependent BP-hydroxylation in vitro from BP induced A. ochraceus TS

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Relative enzyme activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>200</td>
<td>62.5</td>
</tr>
<tr>
<td>400</td>
<td>50</td>
</tr>
<tr>
<td>600</td>
<td>26</td>
</tr>
<tr>
<td>800</td>
<td>24</td>
</tr>
</tbody>
</table>

* Values are expressed relative to control

Table 4.4  Effect of flavone on NaIO₃ dependent BP-hydroxylation in vitro from BP induced A. ochraceus TS

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Relative enzyme activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>100</td>
<td>111.5</td>
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<tr>
<td>200</td>
<td>134</td>
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<tr>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>600</td>
<td>51</td>
</tr>
<tr>
<td>800</td>
<td>55</td>
</tr>
</tbody>
</table>

* Values are expressed relative to control
Fig. 4.1 Effect of flavone on NADPH and NaIO\textsubscript{4} dependent BP hydroxylation by microsome from BP-induced \textit{A. ochraceus} TS.
Table 4.5  Effect of flavone on NADPH dependent BP-hydroxylation in vitro from 3-MC induced A. ochraceus TS

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>BP-hydroxylase activity (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>200</td>
<td>0.80</td>
</tr>
<tr>
<td>400</td>
<td>0.62</td>
</tr>
<tr>
<td>800</td>
<td>0.30</td>
</tr>
<tr>
<td>1000</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Table 4.6  Effect of flavone on NADPH dependent BP-hydroxylation in vitro from BP induced A. ochraceus TS

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>BP-hydroxylase activity (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.60</td>
</tr>
<tr>
<td>200</td>
<td>0.44</td>
</tr>
<tr>
<td>400</td>
<td>0.31</td>
</tr>
<tr>
<td>600</td>
<td>0.17</td>
</tr>
<tr>
<td>800</td>
<td>0.15</td>
</tr>
<tr>
<td>1000</td>
<td>0.16</td>
</tr>
</tbody>
</table>
### Table 4.7 Effect of flavone on NADPH dependent BP-hydroxylation in vitro from BNF induced A. ochraceus TS

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>BP-hydroxylase activity (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.62</td>
</tr>
<tr>
<td>200</td>
<td>0.85</td>
</tr>
<tr>
<td>400</td>
<td>1.22</td>
</tr>
<tr>
<td>600</td>
<td>1.35</td>
</tr>
<tr>
<td>800</td>
<td>1.45</td>
</tr>
<tr>
<td>1000</td>
<td>1.40</td>
</tr>
</tbody>
</table>

### Table 4.8 Effect of flavone on NADPH dependent BP-hydroxylation in vitro from BP induced A. ochraceus TS

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>BP-hydroxylase activity (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.40</td>
</tr>
<tr>
<td>200</td>
<td>0.47</td>
</tr>
<tr>
<td>400</td>
<td>0.52</td>
</tr>
<tr>
<td>600</td>
<td>0.65</td>
</tr>
<tr>
<td>800</td>
<td>1.20</td>
</tr>
<tr>
<td>1000</td>
<td>1.25</td>
</tr>
</tbody>
</table>
Table 4.9  Effect of flavone on NADPH dependent BP-hydroxylation *in vitro* from PCB induced *A. ochraceus* TS

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>BP-hydroxylase activity (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.31</td>
</tr>
<tr>
<td>200</td>
<td>0.26</td>
</tr>
<tr>
<td>400</td>
<td>0.16</td>
</tr>
<tr>
<td>600</td>
<td>0.25</td>
</tr>
<tr>
<td>800</td>
<td>0.35</td>
</tr>
<tr>
<td>1000</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Fig. 4.2 Effect of flavone on NADPH supported BP hydroxylation by microsomes prepared after pretreatment of A. ochraceus TS with (a) BNF, (b) PB, (c) 3-MC and BP, (d) PCB.
Fig. 4.3 Difference Spectra of reduced microsomes from PB (-----) and 3-MC (-----) treated *A. ochraceus* TS with metyrapone.