Purification and characterisation of progesterone hydroxylase, cytochrome P-450 from Streptomyces roseochromogenes ATCC 13400

JAMES R. BERRIE, RALPH A.D. WILLIAMS and KELVIN E. SMITH

Department of Biochemistry, Queen Mary & Westfield College, University of London, Mile End Road, London, E1 4NS, U.K.

Cells of S. roseochromogenes were aseptically inoculated into 2.0 L flasks containing 2.0 l of liquid growth medium. The medium comprised yeast extract 4g/l, malt extract 10g/l and glucose 4g/l at pH 7.2. Each flask contained a coiled wire around the inner circumference to aid mixing and aeration. Cultures were incubated for 35 h at 25 C in an orbital incubator at 70 rpm. After 35 h cells were harvested by centrifugation at 0-4 C, 9000 rpm for 30 min. Pelleted cells were homogenised [1] in an MSE homogeniser for 6 x 30 s, then centrifuged at 15000 rpm for 30 min x 2. The supernatant was retained (S15). The S15 was assayed for hydroxylase activity in 1.0 ml incubations by the P-450 peroxide shunt pathway, using 4 mM progesterone and 2.5 mM NaOCl [4]. Incubation was for 3 h in 0.1 M phosphate buffer [2]. Steroidal products were extracted with an equal volume of chloroform by vortexing for 30 s. After 5 min, the chloroform layer was evaporated off and the steroid products re-suspended in 20 l methanol.

Biocatalysis was determined by TLC analysis on Kieselgel 60 F254 fluorescent high performance TLC plates run in ethyl acetate, toluene, petrol ether 4:3:3 v/v. The plate was observed under UV light.

Progestosterone transformation metabolites were identified by 1-D H NMR [3]. Progesterone was hydroxylated to 16α-hydroxyprogesterone (major metabolite) and 2β,16α-dihydroxyprogesterone (minor metabolite).

Abbreviations used: P-450 cytochrome(s) P-450 monooxygenase
DIT diithiothreitol TLC thin layer chromatography
EDTA ethylenediaminetetraacetic acid DEAE diethylaminoethyl
NaIO₃ sodium periodate NADH nicotinamide adenine dinucleotide

2β-monohydroxyprogesterone was not found in any incubation. Hydroxylase activity was found to be amphoteric by coumarin, which increased by 50% above that found constitutively.

The synthesis of both metabolites was inhibited by 3.5 μg/ml ketoconazole, consistent with the activity being P-450 catalysed.

The progesterone hydroxylase was purified by DEAE52 ion exchange chromatography, bed volume 100 ml. Progesterone hydroxylase was eluted from the column by 200 mM NaCl. After dialysis the hydroxylase was purified on a steroid affinity column (70 ml). This column comprised epoxy activated Sepharose 6B linked to 11α-hydroxyprogesterone. This linkage leaves the two replaceable protons (2β & 16α) maximally exposed within the mobile phase. Activity was eluted by 150 mM NaCl and the dialysed post steroid affinity fraction was then passed through a MIMETIC A6XL ligand affinity column. The M of the P-450 was determined as 65000 by 9% PAGE and by molecular sieve gel filtration.

After each purification dithionite reduced CO spectra were obtained. From these the P-450 concentration was determined. Total protein concentrations were determined by a modified method of the Bradford dye-binding procedure. Purity was determined by silver staining of non-denaturing gels and by two dimensional gel electrophoresis. When homogenous protein was assayed for activity as described above the two major metabolites were synthesised as the S15 fraction.

When the progesterone molecule is reversed and capsized, the two replaceable protons become virtually spatially coincident with 16α-hydroxyprogesterone being substrate for the synthesis of the dihydroxylated product.

This precursor / product relationship was confirmed by incubation of the P-450 with 1.0 mM [4-14C] 16αhydroxyprogesterone and unlabelled progesterone.

Table 1. Purification table. For cells grown with 1.5 mM coumarin.

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Total Protein (mg)</th>
<th>added P-450 (mg)</th>
<th>added P-450 ng protein</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge S15</td>
<td>10.0</td>
<td>2.01</td>
<td>0.019</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE ion exchange 100 ml fractions</td>
<td>42.59</td>
<td>20.02</td>
<td>0.47</td>
<td>95.29</td>
<td>24.7</td>
</tr>
<tr>
<td>Sepharose 6B 11α-hydroxyprogesterone affinity column 150 ml fractions</td>
<td>2.8</td>
<td>3.3</td>
<td>1.18</td>
<td>15.71</td>
<td>62.1</td>
</tr>
<tr>
<td>MIMETIC A6XL 11α-hydroxyprogesterone affinity column 120 ml fractions</td>
<td>0.08</td>
<td>0.27</td>
<td>3.46</td>
<td>1.29</td>
<td>182.1</td>
</tr>
</tbody>
</table>

Fig. 3. Dihydroxyprogesterone showing the 2β & 16α protons replaced by 14C. These are marked with circles upon rotation. The 11α position was found to the affinity column matrix.

Reconstitution of catalytic competence was obtained when P-450 was combined with the flow through fraction of the DEAE 52 column. Neither was independently capable of progesterone hydroxylation alone by the natural NADH-dependent pathway, but when combined, the NADH dependent hydroxylation of progesterone was then obtained.

References: