Isolation and characterization of genes coding for cytochrome $b_5$ and cytochrome-$b_5$ reductase

JACQUELINE S. RIGBY,* PETER C. BULL,* ALAN ASHWORTH,** ELIZABETH A. SHEPHERD,* INES SANTISTEBAN† and IAN R. PHILLIPS‡

*Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K. and †Department of Biochemistry, St Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London EC1M 6BQ, U.K.

Cytochrome $b_5$ and NADH-dependent cytochrome $b_5$-reductase (EC 1.6.2.2) are components of electron transport systems. In the endoplasmic reticulum of liver and many other tissues the proteins are involved in the desaturation of fatty acids (Oshino & Sato, 1971) and in the cytochrome $P_450$-mediated metabolism of endogenous compounds such as steroids, and of foreign compounds including many drugs, carcinogens and environmental pollutants (Ortiz de Montelana, 1986). In the cytosol of mature erythrocytes, cytochrome $b_5$ and cytochrome-$b_5$ reductase are present as smaller water-soluble species that are responsible for the reduction of methaemoglobin (Hultquist & Passon, 1971). The inherited disorder methaemoglobinaemia can be due to a homozygous deficiency of erythrocyte cytochrome-$b_5$ reductase (Scott & Griffith, 1959; Hultquist & Passon, 1971) or, in at least one case, to a deficiency of cytochrome $b_5$ (Heges et al., 1986).

In this paper we report the isolation and characterization of rat and human cDNA and rat genomic DNA clones coding for cytochrome $b_5$, and a human cDNA clone coding for cytochrome $b_5$-reductase.

**Cytochrome $b_5$**

cDNA clones coding for cytochrome $b_5$ were isolated by screening a rat liver $\lambda g t 11$ cDNA expression library with antibodies raised against cytochrome $b_5$ isolated from rat liver microsomal membranes. Sequence analysis of the cloned DNAs showed that cytochrome $b_5$ is encoded by two mRNA species of 720 and 820 nucleotides, respectively. The mRNAs code for identical proteins and differ only in their site of polyadenylation. Analysis of rat genomic DNA by Southern blot hybridization suggested that cytochrome $b_5$ is encoded by a large gene (Fig. 1A, track a). To investigate this further we used a cDNA to isolate genomic DNA clones. Restriction endonuclease mapping, hybridization analysis and DNA sequencing indicate a cytochrome $b_5$ gene containing at least five exons distributed over more than 25 000 base pairs.

A rat cytochrome $b_5$ cDNA was used as a molecular hybridization probe to screen a human liver cDNA library for cloned sequences coding for the corresponding protein of man. Comparison of the sequences of the rat and human cDNAs revealed a high degree of conservation in both their protein-coding and non-coding regions.

**Cytochrome-$b_5$ reductase**

A synthetic oligonucleotide was used to isolate a cloned cDNA coding for human cytochrome-$b_5$ reductase. Northern blot hybridization of human liver RNA showed that cytochrome-$b_5$ reductase is encoded by an mRNA of approximately 1900 nucleotides. The cDNA hybridized to only a single DNA fragment on Southern blots of human genomic DNA digested with either BamHI (Fig. 1B, track a), HindIII (Fig. 1B, track b) or EcoRI (Fig. 1B, track c). No additional hybridizing fragments were detected under lower stringency conditions [final wash at 50°C in 18 mm-NaCl, 1 mm-sodium phosphate, pH 7.7, 0.1 mm-EDTA, 0.1% (w/v) SDS at 65°C].

Genomic DNA was digested with HindIII (A and B, track b), BamHI (B, track a) or EcoRI (B, track c), electrophoresed through a 0.8% (w/v) agarose gel and transferred to a membrane filter (Hybond-N, Amersham). The membranes were hybridized with $^{32}P$-labelled cDNAs coding for rat cytochrome $b_5$ (A) or human cytochrome-$b_5$ reductase (B). Membranes were washed to a final stringency of 18 mm-NaCl, 1 mm-sodium phosphate, pH 7.7, 0.1 mm-EDTA, 0.1% (w/v) SDS at 65°C.

**Fig. 1. Southern blot hybridization analysis of rat (A) and human (B) genomic DNA**

We thank the M.R.C. for a grant in support of this work. J.S.R. and P.C.B. are recipients of M.R.C. post-graduate scholarships.


1989
Site-directed mutagenesis of the aromatic amino acid aminotransferase of *Escherichia coli*

MARTIN J. GARTLAND,* MICHAEL G. HUNTER,† IAN G. FOTHERINGHAM,‡ GEOFFREY C. ROWLAND* and ROBERT E. GLASS*

*Biochemistry Department, Nottingham University Medical School, Clifton Boulevard, Nottingham NG7 2UH, U.K. and †The Nutrasweet Company, 601 East Kensington Road, Mount Prospect, IL 60056, U.S.A.

In *Escherichia coli*, the aspartate and the aromatic amino acid aminotransferases are related enzymes (Fotheringham *et al.*, 1986) with overlapping, but different, substrate specificities. Both enzymes can synthesize tyrosine aspartate and phenylalanine, while the aromatic form can also synthesize leucine (Gelfand & Steinberg, 1977). In addition, there is evidence that the aromatic form is more thermostable (I. G. Fotheringham, personal communication). With the availability of a detailed crystal structure for aspartate aminotransferase from pig (Ford *et al.*, 1980), and the knowledge of the close homology of both prokaryotic and eukaryotic enzymes investigated, the aromatic amino acid aminotransferase provides an ideal opportunity to investigate the above properties.

Recent site-directed mutagenesis studies on aspartate aminotransferase have concentrated on residues known to be involved in the catalytic process. In particular, the critical active-site lysine (Malcolm & Kirsch, 1985) and the arginine residue involved in substrate specificity (Cronin *et al.*, 1987) have been altered and the properties of the resultant mutant enzymes investigated.

In this work, a different approach was adopted. Nonsense mutations were introduced into the structural gene for the aromatic amino acid aminotransferase (tyrB) to give an amber codon approximately every 50 residues. By using nonsense suppression to insert known amino acids at the mutant sites, a number of protein variants have been generated (see below).

During the process of selecting the sites for mutation, no consideration was given to their position in the 3-dimensional structure in order to prevent bias towards residues of known importance. This semi-random approach may expose important regions of the enzyme which are otherwise overlooked by the more conventional methods.

Site-directed mutagenesis (in M13mp18 with subsequent subcloning into a PAT153-derived vector) has been used to insert the sequence CTAG at six different sites (TAG being the stop codon). This allowed monitoring of the mutation at all stages as CTAG is the restriction target of *MaeI* as well as the core sequence of a number of other enzymes such as *NheI*, *SpeI* and *XbaI*. We have available ten different suppressors, permitting the creation of potentially 60 different enzyme variants. The preliminary results for one such position, Leu-115, making use of six amino acid substitutions are presented here.

The continuing ability of the different Leu-115 mutants to synthesize tyrosine, phenylalanine, aspartate and leucine in vivo has been investigated using a simple plate study. The plasmid-borne *tyrB* (Leu-115) nonsense mutation was transformed into a transaminase-deficient strain carrying a particular suppressor and the amino acid auxotropic requirements were tested at different temperatures.

Substitution of glutamine at position 115 (Leu115Gln) produced a variant incapable of growth at 37 and 42°C in the absence of aspartate. Substitution of tyrosine (Leu115Tyr) gave a similar result, albeit only at 42°C. It is possible, therefore, that Leu-115 may be in a region of the enzyme which affects the specificity for the precursor of aspartate, oxaloacetate.

The thermostability of the variant has also been studied in cell extracts. For this purpose, a simple linked assay was adopted: aspartate was used as the amino donor to α-ketoglutarate, the oxaloacetate produced being converted to malate via malate dehydrogenase. The drop in NADH levels can then easily be monitored spectrophotometrically. By using this system, it is also possible to get an indication of the aspartate/oxaloacetate specificity of the mutant enzymes. The results of these experiments are shown in Table 1.

In the Leu115Gln mutant, no activity was observed in any of the extracts prepared. This supports the results of the phenotypic tests that suggested a change in the specificity for oxaloacetate/aspartate.

In short, protein engineering using informational suppression of *in vitro* generated nonsense mutations in *tyrB* provides a powerful means for elucidation of residues involved in enzyme function.

Table 1. Thermolability of variants of the E. coli aromatic amino acid aminotransferase created by suppression of a nonsense mutation at position 115

<table>
<thead>
<tr>
<th>Mutant position</th>
<th>Amino acid substituted</th>
<th>Half-life of activity (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-115</td>
<td>Serine</td>
<td>37°C 42°C</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>47 6</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>65 39</td>
</tr>
<tr>
<td></td>
<td>Glutamine/tryptophan</td>
<td>—</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>70 35</td>
</tr>
</tbody>
</table>

Received 20 June 1988


Received 16 June 1988