Site-Directed Recombination Creates an Artificial Family of Cytochromes P450

Christopher R. Otey, Marco Landwehr, Jeffrey B. Endelman, Kaori Hiraga, Jesse D. Bloom and Frances H. Arnold

Experimental Methods

Library construction. The chimeric library was constructed following the sequence-independent site-directed chimeragenesis (SISDC) method [1], using the type IIb restriction endonuclease BsaXI. This required removal of a BsaXI site in parent A1 using a synonymous mutation at Gly368 (GGA to GGT). N- (blocks 1-4) and C-terminal (blocks 5-8) half-length parental genes were constructed separately with a tag sequence containing a BsaXI recognition site at each of the three crossover locations (DNA primers used for construction are listed in Table S4). The half-length genes were cloned into the pCR®-Blunt II-TOPO plasmid (Invitrogen) and their DNA sequences were confirmed by sequencing (Laragen, Los Angeles, CA). Digestion of the half-length parental genes with BsaXI to remove the tag sequences yielded DNA fragments with unique 3 bp overhangs at each crossover site. After column purification to remove the small (30 bp) tags, the fragments from three parents were mixed in equimolar amounts and ligated to make two half-length chimeric libraries. Digestion with NheI (restriction site designed into tag sequence) and BsaXI after ligation removed genes with residual tags from the final library. Each half-length library was PCR amplified, cloned back into the TOPO vector and transformed into Escherichia coli TOP10 cells (Invitrogen).

The N- and C-terminal half-libraries were digested with BglII/SalI, and SalI/MfeI, respectively, and cloned into the BamHI/EcoRI sites of the pCWori vector. The SalI recognition site corresponds to crossover site 216-217 and was introduced into all three P450 genes synonymously. The N- and C- terminal libraries were ligated at this site to yield the full-length library, which was transformed into the catalase-deficient E. coli strain SN0037 [2].

Probe hybridization analysis. Probe hybridization was performed as described [3,4]. Twenty-four unique oligonucleotide probes were designed to specifically recognize each parent fragment with a Tm ~63°C (Table S5). Probes were labeled using the terminal transferase reaction with either fluorescein (Amersham) or digoxigenin (Roche) following the manufacturer’s protocol. Anti-fluorescein (Amersham) or anti-digoxigenin (Roche) antibodies conjugated to alkaline phosphatase were used for chemiluminescent detection according to the manufacturer’s protocol and recorded with a Gel-Doc system from BIORAD or BIOMAX films from Kodak.

High-throughput carbon monoxide binding assay. CO binding assays were carried out as described [5] with minor modification. Lysate (160 µl) was added to wells of a 96-well plate followed by addition of 40 µl of freshly-prepared 0.1 M sodium hydrosulfite (in 1.3 M phosphate buffer, pH 8.0). Using a Spectra Max Plus 384 plate reader (Molecular
Devices), blank spectra were taken every 10 nm from 400 to 500 nm, as were readings for 450 and 490 nm. Plates were put in a vacuum oven and the atmosphere was removed (to ~350 mbar) and replaced with CO. Plates were allowed to incubate for 5 to 10 minutes, at which point the spectra and absorbencies at 450 and 490 nm were read. Assays were performed in duplicate.

Chimeras were assigned a folding status (folded P450 = 1, not folded = 0) based on the following. To be folded, the spectrum must have a distinct Soret peak within an absorbance range of -0.01 to 0.01 at 450 nm. In addition, the minimum CO difference value (A450 – A490) must be ≥ 0.0015. The background, as assayed using cells with a null vector (pCWori with no insert), was 4.9 x 10⁻⁴ ± 4.2 x 10⁻⁴, which set 1.5 x 10⁻³ as the minimum CO difference value giving greater than 95% confidence (greater than two standard deviations). If the sequence was not assigned folded status – 1, it was deemed ‘not folded’ = 0.


