Supplementary material


Materials

*Escherichia coli* strains AN1459 [1], BL21(DE3)/pLysS [2] and BL21(DE3)*recA* [3] were as described. Plasmid pGP1-2 [4] was used as source of phage T7 gene 1. The phage λ-promoter vector pMA200U [5] and the T7-promoter vectors pETMCSI and pETMCSIII [6] have been described previously. Plasmid pCM910 is a derivative of pETMCSIII with an ~1 kb DNA fragment containing an *Eco*RI site between the *Nde*I and *Nco*I sites (C.S. Miles and N.E. Dixon, unpublished). Insertion of a gene with its start codon as part of the *Nde*I site of pETMCSI and pETMCSIII (or pCM910) leads to production of the protein without and with an N-terminal Met-His$_6$ tag, respectively.

Construction of the synthetic s-CYPA gene

The nucleotide sequence of h-CYPA, the gene that encodes human cyclophilin A (*hCypA*), was modified to maximize its identity with the *E. coli* homologue, *ppiB*, without changing the sequence of the encoded protein (Fig. S1). The modified synthetic gene was named s-CYPA. About 50% of its codons are different from the h-CYPA gene. This biased the codon usage of the human gene to maximize *hCypA* synthesis with the *E. coli* extract, in particular by eliminating three rare codons (two for Arg, one for Leu). 5’-Phosphorylated oligonucleotides (15 to 64 nucleotides in length) comprising the entire sequence of both strands of the gene were designed with 5–10 nucleotide overhangs (Fig. S1). The oligonucleotides were chemically synthesized and purified by reverse-phase liquid chromatography. Annealing of pairs of complementary oligonucleotides was carried out by heating stoichiometric amounts at 95 °C for 15 min in 10 mM Tris/HCl (pH 7.6), 1 mM
EDTA, followed by slow cooling to room temperature. A mixture of the annealed oligonucleotide pairs (~0.1 µM each) was treated overnight at 14 °C with 200 units.mL⁻¹ of T4 DNA ligase in 30 mM Tris/HCl (pH 7.6), 10 mM MgCl₂, 10 mM dithiothreitol and 1 mM ATP. The annealed and partially-ligated fragments were then recombined by a strand-extension polymerase chain reaction (PCR) using 40 units.mL⁻¹ of Vent DNA polymerase with 250 µM dNTPs and 16.25 nM of each oligonucleotide. The PCR thermocycling conditions were 94 °C for 2 min, followed by 20 cycles of overlap extension at 94 °C for 15 s, 35 °C for 60 s, and 65 °C for 30 s. The reaction was stopped at this point, and 1 µM of each flanking primer was added. Amplification of the full-length gene was achieved by a further PCR of 32 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 10 min. The amplified DNA fragments were digested with EcoRI and NdeI; the 497-bp s-CYPΑ fragment was isolated and inserted between the corresponding sites in pCM910. Plasmid DNA was isolated from selected transformants of strain AN1459, and the identity of the inserted fragment was verified by Big Dye Terminator DNA sequencing (Applied Biosystems) with vector primers, as described [6]. Although no single plasmid gave the required DNA sequence over the entire gene, three had overlapping sections that together made up all of the required gene sequence. The ApaI and FokI sites within the gene were used to recombine these three fragments to yield plasmid pBH950.

The s-CYPΑ gene in pBH950 (Fig. S1) was constructed without a stop codon. To enable expression of authentic hCypA, a TAA codon was incorporated at the end of the synthetic gene by insertion of a short double-stranded oligonucleotide linker between the XhoI site near the 3’ end of the gene and an NcoI site in the downstream multiple cloning site derived originally from pETMCSIII (Fig. S1). Plasmid DNA (pBH951; 5154 bp) was isolated from selected transformants of strain AN1459, and the identity of the inserted linker was verified by nucleotide sequencing. For expression of s-CYPΑ under the control of the T7 promoter, but without the N-terminal His₆ tag, the 501-bp NdeI–EcoRI fragment of pBH951 was isolated and inserted between the corresponding sites in pETMCSI to yield plasmid pBH964 (5144 bp).
Purification of cyclophilin A

hCypA, produced in vivo, was purified simply by passing it successively through columns of anion- and cation-exchange resins, and was used for comparison with hCypA produced using the cell-free system. E. coli strain BL21(DE3)/pLysS containing pBH964 was grown at 37 °C in four 1-L cultures, in Luria Bertani (LB) medium supplemented with 50 mg.L⁻¹ ampicillin and 25 mg.L⁻¹ thymine. When $A_{595}$ reached 0.5, synthesis of hCypA was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. After a further 3 h of incubation, the cells (6.2 g) were harvested by centrifugation (8000 × g; 15 min) and suspended in 90 mL of a lysis buffer comprised of 20 mM Tris/HCl (pH 8.0), 1 mM dithiothreitol, 10 mM spermidine. Subsequent steps were carried out at 0–4 °C. The cells were lysed by passage (twice) through a French press at 12 000 psi and the cell debris was removed by centrifugation (38 000 × g; 1 h). Proteins in the supernatant were precipitated with 0.45 g.mL⁻¹ of ammonium sulphate and collected by centrifugation (38000 × g; 30 min). The protein pellet was dissolved in 22 mL of Buffer A (50 mM imidazole/HCl, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol), and dialyzed against two changes of 2 L of the same buffer. The solution was clarified by centrifugation and diluted 4-fold with Buffer A before being applied at 1 mL.min⁻¹ to a column (2.5 × 16 cm) of Toyopearl DEAE-650M anion-exchange resin (Tosoh, Japan) that had been equilibrated in Buffer A. The hCypA passed directly through the column on washing with Buffer A. Fractions were collected, pooled (total volume, 105 mL), and dialyzed against two changes of Buffer B (20 mM potassium phosphate, pH 6.8, 1 mM EDTA and 1 mM dithiothreitol). After clarification of the suspension by centrifugation, the hCypA fraction was loaded onto a column (2.5 × 18 cm) of CM-Fractogel (Merek) that had been equilibrated in Buffer B. Fractions containing hCypA, which again passed directly through the column, were combined and stored at −70 °C. Concentrations of samples of pure hCypA were determined spectrophotometrically at 280 nm, using a value of $\varepsilon_{280} = 8250$ M⁻¹cm⁻¹ [7]. The yield of the pure protein was 34 mg per litre of culture medium, which was about twice that obtained with an earlier method using the wild-type h-CYPA gene expressed under control of the tac promoter [8].
Expression and purification of T7 RNA polymerase

Plasmid pGP1-2 [4] was digested with BamHI and PstI. The isolated 4.3 kb fragment containing cI857 and T7 gene 1 under control of the phage λ pL promoter was inserted between these sites in the high-copy number plasmid vector pMA200U. The resulting plasmid, pKO1166 (Fig. S2) was transformed into E. coli BL21(DE3)recA. T7 RNA polymerase (T7 RNAP) was purified using a published procedure [4], with some modifications.

Two litres of cells E. coli BL21(DE3)recA/pKO1166 were grown aerobically at 30 °C in LB medium (pH 7.4) supplemented with thymine (50 mg.L⁻¹) and ampicillin (100 mg.L⁻¹). At A₅₉₅ of 1.0, the temperature was raised to 42 °C for 30 min and subsequently lowered to 40 °C for 120 min. The cells (5.0 g) were harvested by centrifugation, frozen in liquid nitrogen and stored at –70 °C. Thawed cells were resuspended in 40 mL of grinding buffer comprising 50 mM Tris/HCl (pH 7.9), 5% (v/v) glycerol, 2 mM EDTA, 0.5 M NaCl, 1 mM dithiothreitol, 23 µg.mL⁻¹ α-toluenesulphonyl fluoride, 130 µg.mL⁻¹ lysozyme and 10 mM spermidine and blended at low speed in a Waring Blendor at 4 °C for 2–3 min, until the cells were completely resuspended. The cell mixture was cooled in ice for 20 min and 4% (w/v) sodium deoxycholate was added with stirring to a final concentration of 0.05%. The mixture was blended for 30 s at low speed and after 20 min at 8–12 °C, it was again blended for a further 30 s at high speed to shear the DNA. The clear supernatant obtained after centrifugation (11 000 × g; 45 min) at 2 °C was collected, and protein was precipitated from it by addition of 0.35 g.mL⁻¹ of ammonium sulphate. After centrifugation (11 000 × g; 45 min), the pellet containing T7 RNAP was dissolved in 100 ml of TGED buffer (10 mM Tris/HCl, pH 7.9, 5% (v/v) glycerol, 0.1 mM EDTA and 1 mM dithiothreitol), and loaded onto a column (2.5 × 13 cm) of Toyopearl DEAE-650M resin that had been equilibrated with TGED buffer with added NaCl (0.15 M). After the column had been washed with the same buffer (70 mL), the bound protein was eluted with a linear gradient (200 mL) of 0.15–0.58 M NaCl in TGED buffer. Aliquots of each fraction were analyzed by 10% SDS-PAGE. The first large peak that eluted between 0.17 and 0.20 M NaCl contained an ~100 kDa protein corresponding to the expected size of T7 RNAP. Pooled fractions containing T7 RNAP (30 mL) were diluted with an equal volume of Buffer P (10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA and 1 mM dithiothreitol) and applied to a column (2.5 ×
2 cm) of phosphocellulose (Whatman P-11) that had been equilibrated with Buffer P. The column was washed with 40 mL of Buffer P containing 200 mM KCl, and the bound proteins were eluted with a linear gradient (80 mL) of 0.2–1.0 M KCl in Buffer P. Fractions containing T7 RNAP eluted at 0.5–0.8 M KCl; they were pooled and dialyzed against 20 mM potassium phosphate (pH 7.5) buffer containing 50% (v/v) glycerol, 0.1 mM EDTA and 1 mM dithiothreitol and stored at –20 °C. This procedure yielded 25 mg of highly-purified T7 RNAP.

References:

Fig. S1. Construction of the synthetic CYP4 gene (s-CYP4). The gene was constructed following manipulation of the nucleotide sequence to increase its identity with the E. coli ppiB gene, using a combination of ligation and recursive overlap extension of complementary synthetic oligonucleotides as described above. Oligonucleotides used for the construction of the s-CYP4 are identified by arrows above the complete gene sequence. The NdeI, ApaI, FokI, XhoI, EcoRI and NcoI restriction endonuclease sites are boxed. The start codon (ATG) is within the NdeI site and the stop codon (TAA) is identified by a black box in the linker.
Fig. S2. Plasmid pKO1166. This plasmid, which directs overproduction of T7 RNA polymerase, was constructed by insertion of a DNA fragment bearing T7 gene 1 under control of the bacteriophage λ pL promoter into vector pMA200U [5].