Bacterial morphinone reductase is related to Old Yellow Enzyme

Christopher E. FRENCH and Neil C. BRUCE*

Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, U.K.

Morphinone reductase, produced by *Pseudomonas putida* M10, catalyses the NADH-dependent saturation of the carbon–carbon double bond of morphinone and codeinone, and is believed to be involved in the metabolism of morphine and codeine. The structural gene encoding morphinone reductase, designated *morB*, was cloned from *Ps. putida* M10 genomic DNA by the use of degenerate oligonucleotide probes based on elements of the amino acid sequence of the purified enzyme. Sequence analysis and structural characteristics indicated that morphinone reductase is related to the flavoprotein α/β -barrel oxidoreductases,

and is particularly similar to Old Yellow Enzyme of *Saccharomyces* spp. and the related oestrogen-binding protein of *Candida albicans*. Expressed sequence tags from several plant species show high homology to these enzymes, suggesting the presence of a family of enzymes conserved in plants and fungi. Although related bacterial proteins are known, morphinone reductase appears to be more similar to the eukaryotic proteins. Morphinone reductase was overexpressed in *Escherichia coli*, and has potential applications for the industrial preparation of semisynthetic opiates.

INTRODUCTION

The requirement for enzymes specifically active against morphine for use in a biosensor for the detection of illicit heroin [1,2] led to the isolation of the Gram-negative bacterium *Pseudomonas putida* M10 from opiate factory waste liquor [3]. This organism was shown to transform morphine initially through the action of two constitutively expressed enzymes, morphine dehydrogenase [4] and morphinone reductase [5] (Scheme 1). Morphine dehydrogenase has been purified and characterized [4] and the structural gene, located on a large natural plasmid, has been cloned and sequenced [6]. This enzyme now forms the basis for a highly sensitive and specific biosensor for morphine and heroin [2].

Morphinone reductase catalyses the saturation of the carbon–carbon double bond of morphinone and codeinone with oxidation of NADH, yielding hydromorphone and hydrocodone (Scheme 1). The enzyme has been purified to homogeneity and characterized [7], and has potential application as a biocatalyst for the biological production of semisynthetic opiate drugs [8]. Here we present the nucleotide sequence of the structural gene *morB* and the deduced amino acid sequence of morphinone reductase, and show that morphinone reductase is a member of the α/β -barrel flavoprotein family, and in particular is highly similar to Old Yellow Enzyme in sequence as well as in physical properties and in the reaction catalysed. Furthermore, expressed sequence tags derived from both monocotyledonous and di-

cotyledonous plants suggest that a family of proteins related to Old Yellow Enzyme is widely distributed. These findings may have implications for the physiological role of Old Yellow Enzyme, which remains uncertain.

EXPERIMENTAL

Organisms, plasmids and growth conditions

Ps. putida M10 was isolated from industrial waste effluent on the basis of its ability to metabolize morphine [3]. The organism was grown as previously described [3,4]. *Escherichia coli* JM109 was obtained from Promega (Southampton, Hants., U.K.) and was grown according to standard procedures [9]. pBluescript SK+, a standard cloning vector carrying a *lac* promoter, was obtained from Stratagene (Cambridge, U.K.). pT7-7, an expression vector carrying a T7 RNA polymerase promoter [10], and *E. coli* BL21(DE3), which expresses T7 RNA polymerase from a *lac* promoter, were obtained from United States Biochemical Corporation (obtained through Amersham International plc, Little Chalfont, Bucks., U.K.).

Reagents

Codeinone was a gift from Macfarlan Smith Ltd. (Edinburgh, Scotland, U.K.). Other reagents were of analytical or higher grade.

Enzyme and protein assays and SDS/PAGE

Morphinone reductase activity was assayed as described previously [7]. Protein concentrations were determined by the method of Bradford [11] using the commercially available reagent of Pierce (Rockford, IL, U.S.A.) according to the manufacturer's protocol. SDS/PAGE was performed by the method of Laemmli [12] using the Bio-Rad (Hemel Hempstead, Herts., U.K.) Mini-Protein II apparatus according to the manufacturer's instructions. Samples were denatured by heating to 100 °C for 2–5 min after dilution with 1–4 vol. of sample buffer consisting of 60 mM Tris/HCl, pH 6.8, 1 % (w/v) SDS and 30 mM dithiothreitol with a small amount of Bromophenol Blue as tracking dye. The

Scheme 1 Reactions catalysed by morphine dehydrogenase and morphinone reductase

* To whom correspondence should be addressed.

The nucleotide sequence data reported have been submitted to GenBank and assigned the accession number U37350.

acrylamide mixture used consisted of 29.2:0.8 (w/w) acrylamide/*N*, *N'*-methylenebisacrylamide. Gels of 12% (w/v) acrylamide, unless otherwise specified, were run at 200 V, stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in a solvent consisting of 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) water, and destained in several changes of the same solvent.

Determination of protein sequence

To obtain the N-terminal sequence of morphinone reductase, purified enzyme (29 µg) was transferred from an SDS/polyacrylamide gel to a poly(vinylidene difluoride) membrane (ProBlott; Applied Biosystems, Foster City, CA, U.S.A.) using an LKB 2005 Transphor Electroblotting Unit with a transfer buffer consisting of 10 mM 3-cyclohexylamino-1-propanesulphonic acid (CAPS) adjusted with NaOH to pH 11, 10% (v/v) methanol and 0.5 mM dithiothreitol. Transfer was with a current of 1.2 A for 60–90 min. The N-terminal sequence was determined by automated Edman degradation in the Protein and Nucleic Acid Chemistry Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge.

Internal sequence elements were obtained by N-terminal sequencing of peptide fragments resulting from digestion with CNBr and trypsin [13]. For CNBr digestion, 130 µl of reaction mixture containing 0.3 mg/ml morphinone reductase and 1.3 µg/ml CNBr in 70% (v/v) formic acid/30% (v/v) water was incubated under argon at 30 °C for 48 h and then lyophilized to remove formic acid and CNBr. The lyophilized product was dissolved in 60 µl of SDS/PAGE sample buffer and 25 µl samples were subjected to SDS/PAGE using a 16.5% (w/v) acrylamide gel. The gel was electroblotted to Problott membrane for N-terminal sequencing as described above. Digestion appeared to be incomplete. The N-terminal sequences of three well-resolved fragments were determined; two of these gave the same N-terminal sequence and the third gave the N-terminal sequence of the intact protein. For partial tryptic digestion, 60 µl of 0.3 mg/ml undenatured morphinone reductase was digested with 0.3 µg of trypsin in 20 mM Tris/acetate buffer, pH 8.0, at 37 °C for 2 h. The digestion mixture was diluted with 1 vol. of SDS/PAGE sample buffer and denatured as described above. Samples of 40 µl were subjected to SDS/PAGE using a 15% (w/v) acrylamide gel, followed by electroblotting to Problott membrane as described above. The N-terminal sequences of three well-resolved fragments were determined; one of these gave the same N-terminal sequence as intact morphinone reductase.

Cloning and sequencing of *morB*

Genomic DNA from *Ps. putida* M10 was prepared as described by Ausubel et al. [14]. Megaplasmid DNA from M10 was prepared as described previously [6]. Restriction endonucleases were obtained from Boehringer-Mannheim (Lewes, E. Sussex, U.K.), Gibco/BRL (Paisley, Scotland, U.K.), Promega and New England Biolabs (Beverly, MA, U.S.A.). DNA fragments were separated by agarose-gel electrophoresis using 0.8% (w/v) agarose gels and TAE buffer [9]. Oligonucleotides were synthesized in the Protein and Nucleic Acid Chemistry Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge. For Southern blots, DNA was transferred from agarose gels to nylon membrane (Hybond N+; Amersham) by alkaline capillary blotting according to the manufacturer's protocol. For colony blots, the same membrane was used according to the manufacturer's protocol. Oligonucleotide probes were end-labelled using T4

polynucleotide kinase (Promega) and [γ - 32 P]ATP (Dupont/NEN). Filters were prehybridized at the hybridization temperature in 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) with 5 × Denhardt's solution (where 1 × Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA) and 0.02 mg/ml sonicated denatured salmon sperm DNA [9]; hybridization was overnight in the same solution with the addition of 1 pmol/ml labelled oligonucleotide probe. After hybridization, filters were washed four times for 10 min in 0.2 × SSC. Probes N1 and T1 (see the Results section) were hybridized at 42 °C and washed at 50 °C; probe T2 was hybridized at 50 °C and washed at 60 °C. Fuji RX100 X-ray film was used for autoradiography.

For cloning, DNA fragments were eluted from agarose gels using the US-Bioclean system (United States Biochemical Corporation). Alkaline phosphatase treatment was not used as it was found to decrease transformation efficiencies greatly. Ligation reactions were carried out for 16–24 h at 16 °C using T4 DNA ligase (Promega or Gibco/BRL). *E. coli* was transformed with plasmid DNA by CaCl₂ treatment as described by Sambrook et al. [9]. Where possible, recombinant plasmids were detected by blue-white selection [9].

DNA was sequenced by dideoxynucleotide chain termination [9] using the reagents of United States Biochemical Corporation and T7 DNA polymerase from Boehringer-Mannheim. Reaction mixtures were labelled with [α - 35 S]thio[dATP (Dupont/NEN). Electrophoresis was performed in 50 cm 6% (w/v) acrylamide gels containing 7 M urea. A voltage of 2000–2400 V was used. Fuji RX100 X-ray film was used for autoradiography.

DNA sequence data were analysed using the GCG package [15].

PCR was performed using Vent DNA polymerase, with proof-reading activity, from New England Biolabs according to the manufacturer's protocol. Reaction mixtures contained each deoxynucleotide at 0.2 mM and 50 pmol of each primer in 50 µl. DNA polymerase was added to reaction mixtures after 5 min at 95 °C. Primers were then extended over 30 cycles, each consisting of 30 s at 95 °C, 30 s at 61 °C and 1 min at 72 °C.

Purification and characterization of morphinone reductase from recombinant *E. coli*

Morphinone reductase from *E. coli* JM109/pMORB3 was purified as previously described for purification from *Ps. putida* M10 [7].

RESULTS

Cloning and sequencing of *morB*

The N-terminal sequence of morphinone reductase from *Ps. putida* M10 was found to be:

P-D-T-S-F-S-N-P-G-L-F-T-P-L-Q-

After CNBr cleavage, the following N-terminal sequence was obtained from one fragment:

A-P-L-T-R-S-R-T-P-P-S-V-P-G-R-L-Q-Q-I-

Two partial tryptic cleavage fragments gave the following sequence elements:

R-T-D-Q-Y-G-G-S-I-E-N-R-A-R-F-P-L-E-V-?-D-A-A/V-A-E
and

G-L/D-T-D-D-E-N/P-P/E-A-M-A/F-F-Y-L-A-G-E-L-D-R-
R-G-L-A-Y-L-(H)-F-N-(E)-P-D-

Figure 1 Restriction map of *morB* and surrounding DNA

Nucleotides are numbered with 1 being the A of the ATG initiating *morB*. Restriction site positions between -283 and 1283 were determined by sequencing; those outside this region are approximate and were estimated by agarose-gel electrophoresis. Plain arrows indicate sequence elements derived by sequencing of subclones using vector-based primers; arrows preceded by open circles indicate sequence elements derived using primers based on previously determined sequence.

where residues shown in parentheses indicate uncertain assignments. The typical codon bias in *Ps. putida* was determined from 40 *Ps. putida* genes in the Gen/EMBL Database using the CODONBIAS program of the GCG package. The degenerate oligonucleotide probe

AAC-CC(G/C)-GG(C/T)-CT(G/C)-TTC-AC(G/C)-CC(G/C)-CT

(designated N1) was designed on the basis of the N-terminal sequence and two further probes

AC(G/T/C)-GA(T/C)-CAG-TA(T/C)-GG(G/T/C)-GG

(designated T1) and

AG-(G/C)GG-GAA-(G/C)CG-(G/C)GC-(G/C)CG-GTT-(T/C)TC-GAT

(designated T2) were designed on the basis of the first tryptic fragment sequence. Southern blots showed that all three probes bound specifically to the same region of *Ps. putida* M10 genomic DNA, whereas binding to M10 megaplasmid DNA appeared to be non-specific, suggesting that *morB* was located on the bacterial chromosome rather than on the natural plasmid which bears *morA* [6].

Two 5.0 kb genomic *PstI* fragments, one binding probe N1 and the other probes T1 and T2, were cloned in pBluescript SK+ to give pMORB-G2 and pMORB-G1 respectively; an overlapping 1.5 kb *ClaI*-*XhoI* fragment was cloned to give pMORB-G3 and was found to bind all three probes. An alignment of these three genomic fragments is shown in Figure 1

together with the sequencing strategy employed. Sequencing revealed the presence of a single open reading frame encoding a polypeptide of 376 amino acids, excluding the initiating methionine. All protein sequence elements known from morphinone reductase were present in the deduced protein sequence, and the deduced M_r of the putative protein, as calculated by the PEPTIDESORT program of the GCG package, was 41118.5, consistent with the subunit M_r of 41120 ± 2.8 (S.E.M.) determined for morphinone reductase by electrospray MS [7]. It was concluded that this open reading frame, designated *morB*, represents the structural gene encoding morphinone reductase. The sequence of *morB* and flanking DNA, together with the deduced amino acid sequence of morphinone reductase, is shown in Figure 2.

A putative *E. coli* σ^{70} -like promoter sequence and ribosome-binding site were located immediately upstream of *morB*, and a 10 bp inverted repeat indicating a putative transcription termination sequence was detected immediately downstream.

Comparison of the deduced amino acid sequence of morphinone reductase with related sequences

The GCG program TFASTA was used to screen the Gen/EMBL Database for proteins related to morphinone reductase. It was found that morphinone reductase is related to the flavoprotein α/β -barrel enzymes exemplified by trimethylamine dehydrogenase from the Gram-negative bacterium *Methylophilus methylotrophus* W₃A₁ [16] and Old Yellow Enzyme from *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* [17]. Other proteins known to belong to this family include bile acid-induced proteins C and H from *Eubacterium* sp. VPI12708 [18,19], an NADH oxidase of unknown physiological function from *Thermoanaerobium brockii* [20] and oestrogen-binding protein from the yeast *Candida albicans* [21]. In addition to these previously described sequences, the database search yielded expressed sequence tags from the plants *Arabidopsis thaliana* (accession numbers T04750, T22704), *Brassica campestris* (accession number L33532) and *Oryza sativa* (accession numbers D24670, D21963), which appear to represent partial sequences from closely related proteins. A multiple sequence alignment generated by the GCG program PILEUP is shown in Figure 3, and a sequence similarity dendrogram based on these data is shown in Figure 4. The deduced amino acid sequence of morphinone reductase shows 39–40% sequence identity with and 60–62% similarity to those of oestrogen-binding protein and isoenzymes of Old Yellow Enzyme as determined by the GAP program of the GCG package.

Assembly of an intact *morB* gene and expression of morphinone reductase in recombinant *E. coli*

To assemble an intact *morB* gene adjacent to the T7 RNA polymerase promoter of pT7-7, PCR with pMORB-G3 as a template was used to generate a 0.7 kb fragment bearing the 5'-end of *morB* and to introduce an *NdeI* site at the start codon. The forward primer

5'-TGCTACTCATATGCCGGATACATCCTTCTCC-3'

consisted of seven random bases to allow effective cutting by *NdeI*, followed by the introduced *NdeI* site and the first 21 bases of *morB*. The reverse primer

5'-AACACCTCGGCCACGGCGTC-3'

was complementary to bases 670–689 of *morB*, immediately downstream of a *SalI* site. The resulting PCR product was

-283 cgaggaagcctccacgaaggcgatcagg -255
SmaI

-254 tcgaggttggaatcccgaggagcgtgggaagccaccaggcggtcgagc -204
SmaI

-203 atctcggcggcggtcatggcgctccttgggtcacgtttcgggaaatccta -153

-152 tcacgcccggggcgagggcgggcggtggcgctcatagcccagactatc -102

-101 cggtcgatgccgtcactctttttgacagcagtcattacaggcctaagatg -51
 -35 -10

-50 tcgggagcagcgggatacccgctccattcactcttcatctgagggcggtattg 0
 rbs

M P D T S F S N P G L F T P L Q L
 1 atgccggatacatctcttccaacccgggctcttcaccccgctgcagctg 51
SmaI PstI

G S L S L P N R V I M A P L T R S
 52 ggcagcctcagcctgcccaacccgctgatcatggcgcgctgacccgctcg 102

R T P D S V P G R L Q Q I Y Y G Q
 103 cgcacgcggacagcgttcccgcgaggttcagcagatctactatggccag 153
BglI

R A S A G L I I S E A T N I S P T
 154 cgcgcagcgcggggtgatcatcagcagggccaccaatatctcgccacc 204

A R G Y V Y T P G I W T D A Q E A
 205 gcccgcggtacgtctacacgcggggatctggaccgacgcgcaggaagcc 255

G W K G V V E A V H A K G G R I A
 256 ggctggaagggcgctcgtagggcggtgcacaaagggcgggcgatcgcc 306
SphI

L Q L W H V G R V S H E L V Q P D
 307 ctgcagctgtggcagctcgccggtgtctccacagcgtggtgcagccgac 357
PstI EagI

G Q Q P V A P S A L K A E G A E C
 358 ggccagcagcccggtggcaccgagcgcctcaaggccgagggggcggaatgc 408

F V E F E D G T A G L H P T S T P
 409 ttcgtcagttcgaggacggcacggcggggtgcacccaccagcacgcg 459
SacI

R A L E T D E I P G I V E D Y R Q
 460 cggcgcttgagaccgacgagatccccggcatcgctcagaggactaccgccag 510

A A Q R A K R A G F D M V E V H A
 511 gctgcgacgcgcgcaagcgtgccgcttcgacatggtcgagggtccacgcc 561

A N A C L P N Q F L A T G T N R R
 562 gccaacgcctgctgcccacagttcttcgcccacggcaccacacggcg 612

T D Q Y G G S I E N R A R F P L E
 613 accgaccagtcaggcggtccatcgagaacggggcgcgcttcccgctggag 663

V V D A V A E V F G P E R V G I R
 664 gtggtcgacgcctggccgaggtgttcgggcccagcggtcgccatccgc 714
SacI

L T P F L E L F G L T D D E P E A
 715 ctgaccccttctcagcgtcttcggcctcaccgacgacgagcccgaggcg 765
XhoI, SacI

M A F Y L A G E L D R R G L A Y L
 766 atggccttctactggcggcgagctcgaccgcgcggcctggcctacctc 816
SacI

H F N E P D W I G G D I T Y P E G
 817 cacttcaacgagcccgactggatcggtggcgatatacctacccgaagcg 867
EcoRV

F R E Q M R Q R F K G G L I Y C G
 868 ttccgggagcagatgcgcagcgttcaagggtgggctgatctactcggcg 918

N Y D A G R A Q A R L D D N T A D
 919 aactacgatgcggggcgcccagggcgctggatgacaacacggcgac 969

A V A F G R P F I A N P D L P E R
 970 gccgtggccttcggcgcccttcatcgccaacccgatctgcccagcgc 1020
EagI

F R L G A A L N E P D P S T F Y G
 1021 ttccgcctggggggcgccctcaacgagcccgacccagcacttctacggc 1071

G A E V G Y T D Y P F L D N G H D
 1072 ggcgccgaggtcggtacacgactacccttctcgcacaacggccacgac 1122

R L G End
 1123 cggtcggtgagtcagcgtcgccctggaagcatcagcaaccccgccca 1173
 Terminator

1174 gcgtgcccagcttctggtgatggggaggggtggtcggggcatcgctggt 1224

1225 gattggcgacatcaacacccggcgctcagatccacagaacgcattccgag 1275

1276 ggaccgccc

Figure 2 Nucleotide sequence of *morB* and deduced amino acid sequence of morphinone reductase

The putative promoter —35 and —10 regions, ribosome-binding site (rbs) and transcription termination sequence are indicated. Regions of amino acid sequence that are known from protein sequencing experiments are shown in italics. Nucleotides are numbered from the first base of the ATG initiating *morB*. The region between —277 and 1212, including *morB*, was sequenced on both strands; the regions flanking this are known only from one strand.

digested with *NdeI* and *SalI* and ligated together with a 2.6 kb *SalI*–*Clal* fragment of pMORB-G1, bearing the 3'-end of *morB* (Figure 1), and pT7-7 cut with *NdeI* and *Clal*. The resulting construct was designated pMORB1.

pMORB1 was introduced into *E. coli* BL21(DE3), which expresses T7 RNA polymerase from a *lac* promoter. Crude extract prepared from mid-exponential-phase cells of *E. coli* BL21(DE3) showed morphinone reductase activity of 2.0 units/mg in the absence of isopropyl thioalactopyranoside; with induction by isopropyl thioalactopyranoside the activity increased to 3.5 units/mg, as compared with 0.2 unit/mg for *Ps. putida* M10 and < 0.02 unit/mg for untransformed *E. coli*.

To construct an intact *morB* gene together with the native flanking DNA, a 1.5 kb *EcoRI*–*XhoI* fragment from pMORB-G3, consisting of the 1.5 kb *Clal*–*XhoI* insert bearing the 5'-end of *morB* and the *EcoRI*–*Clal* region of the vector multicloning site, was ligated with a 2.5 kb *XhoI*–*Clal* fragment from pMORB-G2 bearing the 3'-end of *morB*, and pBluescript SK+ cut with *EcoRI* and *Clal*. The resulting construct contained, in effect, the 4.0 kb genomic *Clal* fragment bearing *morB*; this construct was designated pMORB2.

Crude cell extract from saturated overnight cultures of *E. coli* JM109/pMORB2 was found to contain morphinone reductase activity at 3.5 units/mg. SDS/PAGE showed the presence of large amounts of a protein co-migrating with authentic morphinone reductase as purified from *Ps. putida* M10 (Figure 5). In pMORB2, *morB* is in the correct orientation to be expressed from the *lac* promoter of pBluescript SK+, although 0.8 kb of DNA separates this promoter from the start of *morB*. When the 4.0 kb *Clal* insert of pMORB2 was inverted to create pMORB3, so that expression of *morB* could not be driven by the *lac* promoter, equivalent expression was observed, suggesting that expression was driven by a promoter on the insert, presumably the *E. coli* σ^{70} -like promoter detected by sequencing.

Recombinant morphinone reductase was purified from *E. coli* JM109/pMORB3, using the affinity-chromatography procedure devised for morphinone reductase from *Ps. putida* M10. The procedure resulted in apparently homogeneous protein (Figure 5) with a specific activity of 14.8 units/mg, similar to that found

Figure 3 Alignment of the predicted amino acid sequence of morphinone reductase with those of related proteins from the Gen/EMBL Database

This alignment was generated using the GCG PILEUP program with the following parameters: gap weight = 3, gap length weight = 0.1. Positions where only one or two different residues occur are shown in bold type. Stop codons are indicated by an asterisk. This alignment was generated using the entire sequences of trimethylamine dehydrogenase, bile acid-induced proteins C and H and NADH oxidase, but only the region believed to be involved in the formation of the N-terminal α/β -barrel domain is shown here; an alignment of the remaining portions of these sequences may be found in [19]. at750, at7043, expressed sequence tags from *Arabidopsis thaliana* (accession numbers T04750, T22704); osc101111, osr23491a, expressed sequence tags from *Oryza sativa* (D24670, D21963); bceftf165, expressed sequence tag from *Brassica campestris* (L33532); morb, morphinone reductase; scnadphor, scoyle, sccox1a, isoenzymes of Old Yellow Enzyme (L06124, X53597, L29279); caebp1a, oestrogen-binding protein (L25759); baic, esbai, bile acid-induced proteins C and H (M36292, L11069); tbnadhox, 'NADH oxidase' (X67220); mmtmda, trimethylamine dehydrogenase (X68079). Note that the original alignment of morb with osc101111 suggested a reading frame error in osc101111; the sequence shown here was derived by rectifying this putative error by the insertion of G after nucleotide 220 of osc101111. The positions of the α -helices and β -sheets forming the α/β -barrel in trimethylamine dehydrogenase are shown [16,22].

with *Ps. putida* M10 [7]. The activity yield was 67% and the purification factor was 6.4. The N-terminal sequence (10 residues) of this protein was determined and found to be identical with that of authentic morphinone reductase. The K_m of the recombinant protein for codeinone was measured as 0.19 ± 0.03 mM (S.E.M.), consistent with the value of 0.24 ± 0.03 mM measured for authentic morphinone reductase [7].

DISCUSSION

A fragment of DNA from *Ps. putida* M10 was cloned on the basis of hybridization to three oligonucleotide probes based on elements of the amino acid sequence of morphinone reductase. An open reading frame on this fragment encodes a putative protein of the same M_r as morphinone reductase, with a predicted amino acid sequence containing all known elements of the amino acid sequence of morphinone reductase. Introduction of this DNA fragment into *E. coli* resulted in the expression of a protein with M_r and N-terminal sequence identical with those of

Figure 4 Dendrogram showing similarity among proteins related to morphinone reductase

This dendrogram was derived using the PILEUP program of the GCG package on the deduced amino acid sequences shown in Figure 3.

Figure 5 Purification of morphinone reductase from recombinant *E. coli* (SDS/PAGE)

Lanes 1 and 6, M_r markers; lane 2, cell extract from *E. coli* JM109/pBluescript SK+ (23 μ g of protein); lane 3, cell extract from *E. coli* JM109/pMORB3 (23 μ g of protein); lane 4, morphinone reductase purified from *E. coli* JM109/pMORB3 (3.8 μ g of protein); lane 5, morphinone reductase purified from *Ps. putida* M10 (3.8 μ g of protein).

morphinone reductase and the presence of high levels of morphinone reductase activity. These data led us to conclude that this open reading frame is *morB*, the structural gene encoding morphinone reductase.

In *E. coli*, morphinone reductase appeared to be expressed from a promoter present on the DNA insert. A sequence typical of *E. coli* σ^{70} promoters was located immediately upstream of *morB*. This putative promoter sequence is similar at the -35 and -10 positions to the *E. coli* *trp* promoter and would therefore be expected to result in high expression in *E. coli*. Similar promoters have been reported for other *Pseudomonas* genes [23,24] and are associated with constitutively expressed or negatively regulated genes.

Oligonucleotide hybridization showed that *morB* was located on the chromosome of *Ps. putida* M10, but showed no sign of a copy of this gene on the large plasmid which has been shown to encode *morA*, the structural gene encoding morphine dehydrogenase [6], which is believed to initiate the metabolism of morphine alkaloids in *Ps. putida* M10 [4]. Although both morphine dehydrogenase and morphinone reductase clearly participate in the transformation of morphine and codeine *in vivo* in *Ps. putida* M10, and both enzymes appear to be specific for alkaloid substrates, we have been unable to demonstrate the introduction of carbon from morphine alkaloids into central metabolism via this route, and it is therefore impossible to conclude with certainty that reduction of morphinone and codeinone is the physiological function of morphinone reductase.

Sequence comparisons showed that morphinone reductase is a member of the family of α/β -barrel flavoprotein oxidoreductases [25]. Other known bacterial members of this family are: trimethylamine dehydrogenase from *Methylophilus methylotrophus* W₃A₁, which initiates the catabolism of trimethylamine [16]; bile acid-induced protein C from *Eubacterium* sp. VPI 12708, which is believed to be involved in the 7-dehydroxylation of exogenous steroids [18]; bile acid-induced protein H from the same organism, which reduces unbound flavins with NADH oxidation [19]; and an NADH oxidase of unknown physiological function from *Thermoanaerobium brockii* [20]. The crystal structure [26] and complete primary sequence [16] of trimethylamine dehydrogenase have been determined. In this enzyme the N-terminal flavin-binding domain forms an eight-stranded α/β -barrel. Two other domains, resembling elements of the flavoprotein disulphide oxidoreductases, form the C-terminal portion of the protein. The other proteins mentioned also possess sequence elements homologous to those encoding these C-terminal domains, as well as a cluster of cysteine residues which in trimethylamine dehydrogenase form an iron-sulphur centre.

By contrast, the known eukaryotic members of this group, including Old Yellow Enzyme from *Saccharomyces* spp. [17] and oestrogen-binding protein from *Candida albicans* [21], appear to consist only of the N-terminal α/β -barrel domain, lacking the iron-sulphur centre and C-terminal domains of their more complex relatives. The crystal structure of Old Yellow Enzyme from *Saccharomyces carlsbergensis* has recently been determined [27] and shows that an eight-stranded α/β -barrel similar to the N-terminal domain of trimethylamine dehydrogenase is in fact formed.

Morphinone reductase, like these eukaryotic proteins, appears to consist solely of the N-terminal α/β -barrel domain. In other respects, too, morphinone reductase appears to be highly similar to Old Yellow Enzyme and oestrogen-binding protein. The known physical characteristics of the members of this enzyme family are shown in Table 1. Like Old Yellow Enzyme and oestrogen-binding protein, morphinone reductase exists as a homodimer and reduces the double bond of 2-cyclohexenone with oxidation of a reduced nicotinamide cofactor [7]. Furthermore, all three enzymes are known to have an affinity for steroids. In the case of oestrogen-binding protein, the binding of oestrogens prevents the reduction of 2-cyclohexenone. Madani et al. [21] speculated that this was due to an allosteric effect; in the light of our observation that morphinone reductase activity is competitively inhibited by progesterone and cortisone [7], it seems more likely that the inhibition observed is competitive in nature.

We previously reported, on the basis of product inhibition studies, that morphinone reductase showed kinetics inconsistent with a simple one-site Ping Pong mechanism but consistent with a hybrid two-site Ping Pong mechanism [7]. However, the crystal

Table 1 Known physical characteristics of the α/β -barrel flavoenzymes

CHEO, 2-Cyclohexen-1-one; ETF, electron-transfer flavoprotein; FeS*, iron-sulphur centre deduced from sequence similarity.

Enzyme	Subunits, subunit M_r	Prosthetic groups	Known substrates	Reference
Morphinone reductase	α_2 , 41 100	FMN	Alkaloids and CHEO; NADH	This study and [7]
Old Yellow Enzyme	α_2 , 44 900	FMN	CHEO; NADPH	[17]
Oestrogen-binding protein	?, 46 000	?	CHEO; NADPH	[21]
Trimethylamine dehydrogenase	α_2 , 81 600	FMN, ADP, FeS	N(CH ₃) ₃ ; ETF	[16]
Bile acid-induced protein C	?, 59 500	?, FeS*	Steroids?	[18]
Bile acid-induced protein H	α_3 , 72 000	Flavin, FeS*	FAD and FMN; NADH	[19]
NADH oxidase	α_6 , 71 400	2 $\times$ FAD/subunit, FeS	?, NADH	[20]

structure elucidated by Fox and Karplus [27] shows that Old Yellow Enzyme possesses a single binding site which accommodates both nicotinamide nucleotides and substrate analogues. Our kinetic results can be explained by a mechanism in which hydrocodone binds unproductively to the reduced form of the enzyme with very much higher affinity than to the oxidized enzyme form. Unproductive binding of NADH to the reduced enzyme form would also explain the apparent substrate inhibition by NADH; such substrate inhibition is common in one-site Ping Pong enzymes [28]. Such a mechanism is also consistent with the observation that two-site Ping Pong mechanisms are typically associated with flavoproteins which, unlike morphinone reductase, possess additional redox centres (see, for example, refs. [29,30]). However, we have not yet sought to confirm this speculation.

Our sequence search also revealed a number of expressed sequence tags from both monocotyledonous and dicotyledonous plants which appear to represent partial sequences from proteins related to Old Yellow Enzyme and morphinone reductase. Sequence considerations suggest that morphinone reductase and the putative plant proteins are more closely related to the Old Yellow Enzyme branch of this family than to the trimethylamine dehydrogenase branch. For example, Thr-37 of Old Yellow Enzyme, which is important in FMN binding [27], is conserved in the rice expressed sequence tag, in oestrogen-binding protein and in morphinone reductase but not in the other bacterial enzymes.

These data invite speculation that Old Yellow Enzyme, the physiological function of which has long been mysterious, is a member of a group of related proteins conserved in fungi and plants. Scrutton [25] has speculated that the related multidomain prokaryotic proteins are derived from an Old Yellow Enzyme-like ancestor which underwent domain fusion with a member of the flavoprotein disulphide oxidoreductase family. Morphinone reductase appears to be more similar to related eukaryotic proteins than to the other bacterial enzymes. The structural gene *morB* may have been acquired from a eukaryotic source, as has been suggested for glutamine synthase II of *Bradyrhizobium* spp. and related bacteria [31], Cu-dependent superoxide dismutase of *Photobacterium leiognathi* [32], β -glucosidase B of *Clostridium thermocellum* [33] and α -amylase of *Streptomyces limosus* [34]; fluorescent pseudomonads are ubiquitous in soil and are commonly found associated with plants. Alternatively a similar protein may also be widespread in bacteria. Desrut et al. [35] have reported that the saturation of the double bond of a cyclohexenone-type ring is found in a variety of bacteria. However, Hailes [36] investigated several other fluorescent pseudomonad strains and found no proteins cross-reacting with antibodies raised against morphinone reductase. This question is

currently under investigation in our laboratory. We are also working towards the use of morphine dehydrogenase and morphinone reductase as biocatalysts for the preparation of semisynthetic opiate pharmaceuticals such as hydromorphone and hydrocodone [5].

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