# Purification and characterization of morphinone reductase from *Pseudomonas putida* M10

Christopher E. FRENCH and Neil C. BRUCE*
Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, U.K.

The NADH-dependent morphinone reductase from *Pseudomonas putida* M10 catalyses the reduction of morphinone and codeinone to hydromorphone and hydrocodone respectively. Morphinone reductase was purified from crude cell extracts to apparent homogeneity in a single affinity-chromatography step using Mimetic Yellow 2. The purified enzyme was a dimeric flavoprotein with two identical subunits of $M_r$ 41100, binding non-covalently one molecule of FMN per subunit. The N-terminal sequence was PDTSFSNPGLFTPLQ. Morphinone reductase was active against morphinone, codeinone, neopinone and 2-cyclohexen-1-one, but not against morphine, codeine or isocodeine. The apparent $K_m$ values for codeinone and 2-cyclohexen-1-one were 0.26 mM and 5.5 mM respectively. The steroids progesterone and cortisone were potent competitive inhibitors; the apparent $K_i$ for cortisone was 35 $\mu$M. The pH optimum for codeinone reduction was 8.0 in phosphate buffer. No reverse reaction could be detected, and NADPH could not be used as a reducing substrate in place of NADH. Morphinone reductase activity was strongly inhibited by 0.01 mM $CuSO_4$ and *p*-hydroxymercuribenzoate, suggesting the presence of a vital thiol group. Steady-state kinetic studies suggested a Ping Pong (substituted enzyme) kinetic mechanism; however, product-inhibition patterns were inconsistent with a classical Ping Pong mechanism. Morphinone reductase may, like several other flavoprotein dehydrogenases, operate by a hybrid two-site Ping Pong mechanism.

## INTRODUCTION

Recent research on the microbial degradation of the morphine alkaloids by a strain of *Pseudomonas putida* resulted in the elucidation of a transformation sequence, whereby morphine and codeine were converted into hydromorphone and hydrocodone respectively as shown in Scheme 1 (Bruce et al., 1990; Hailes and Bruce, 1993). The first transformation of morphine and codeine carried out by *Pseudomonas putida* M10 is the oxidation of the C-6 hydroxy group by a highly specific $NADP^+$-dependent morphine dehydrogenase, yielding morphinone and codeinone respectively (Bruce et al., 1990). Morphine dehydrogenase has been purified from *P. putida* M10 and characterized in some detail (Bruce et al., 1991). Its structural gene, designated *morA*, has been cloned, sequenced and overexpressed in *Escherichia coli* (Willey et al., 1993). *morA* was located on a 165 kb plasmid in *P. putida* M10 and curing experiments suggest that some, if not all, of the enzymes mediating the degradation of morphine and codeine are encoded on this plasmid (Willey et al., 1993). The second reaction is the reduction of the 7,8-unsaturated bond of morphinone and codeinone by an NADH-dependent morphinone reductase, producing hydromorphone and hydrocodone respectively (Hailes and Bruce, 1993). These reactions are of potential industrial interest in that hydromorphone is a powerful analgesic, some seven times more potent than morphine, while hydrocodone is widely used as a cough suppressant.

The important potential industrial applications of morphinone reductase as a biocatalyst for the biosynthesis of powerful analgesics necessitated the characterization of this enzyme in more detail. The present paper describes a rapid one-step purification of morphinone reductase and the characteristic properties of the enzyme.

## MATERIALS AND METHODS

### Reagents

Morphine, codeine, codeinone, neopinone, and isocodeine were kindly given by Macfarlan Smith Ltd. (Edinburgh, U.K.). Morphinone was generated biologically as described by Hailes and Bruce (1993). Mimetic Yellow 2 was obtained from Affinity Chromatography Ltd. (Freeport, Ballasalla, Isle of Man, U.K.). Other reagents were of analytical or higher grade.

### Organisms and growth conditions

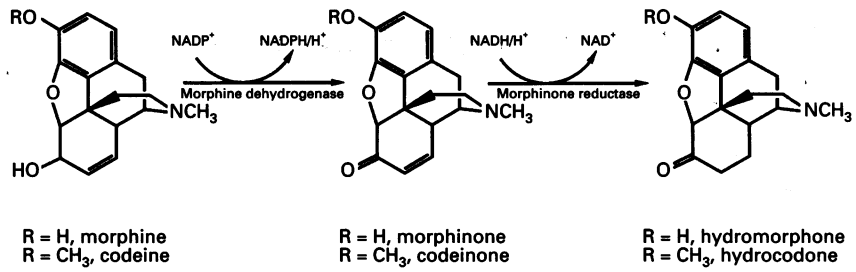
The organism used in this investigation, *Pseudomonas putida* M10, was originally isolated from industrial waste liquors (Bruce et al., 1990). Cultures of *P. putida* M10 were grown as described previously (Bruce et al., 1991).

### Purification of morphinone reductase

Cell extracts were prepared by sonication as described previously (Bruce et al, 1990). Morphinone reductase was purified by affinity chromatography using Mimetic Yellow 2. All steps were carried out at 4 °C, and 1 mM dithiothreitol was included in all solutions.

The cell-free extract (20 ml), containing up to 200 mg of protein, was loaded onto a 5 cm-diameter column containing 50 ml of Mimetic Yellow 2 (packed height 2.5 cm) which had been previously equilibrated with buffer A (50 mM phosphate, pH 7.0). The column was then washed with 100 ml of buffer A followed by 500 ml of 1 M NaCl in buffer A, to remove the majority of the bound protein. This was followed by a further wash with 100 ml of buffer A to remove the salt. Morphinone reductase was then eluted by reversing the direction of flow and

* To whom correspondence should be addressed.




**Scheme 1 Initial steps in the degradation of morphine and codeine by *Pseudomonas putida* M10**

washing the column with 100 ml of 5 mM NADH and 0.6 M NaCl in buffer A, followed by a further 100 ml of 0.6 M NaCl in buffer A. A linear flow rate of 15 ml/h per $cm^2$ was maintained. The eluate from these two washes, containing the morphinone reductase, was concentrated by ultrafiltration to a final protein concentration of 0.3–1.0 mg/ml and then diafiltered with buffer A to reduce the concentrations of NaCl and NADH 1000-fold, using an Amicon 8050 ultrafiltration cell with an Amicon Diaflo membrane with a nominal $M_r$ cut-off of 10000. The purified enzyme was stored at −20 °C.

### Morphinone reductase assay

Morphinone reductase activity was assayed by monitoring the oxidation of NADH spectrophotometrically at 340 nm, in a reaction mixture containing 0.3 mM codeinone and 0.3 mM NADH in buffer A, at 30 °C, unless otherwise specified. Codeinone was added as the water-soluble hydrochloride. The background rate of NADH oxidation in the absence of codeinone was measured and subtracted. Activities reported are the means of two to four replicate measurements. A unit of activity was defined as that amount of enzyme oxidizing 1 μmol of NADH/min under these conditions. Assays for morphinone reductase activities performed on separate batches of extracts generally agreed to within 10%. Kinetic equations were fitted to data using the Grafit software package (version 2.0) from Erithacus Software (Leatherbarrow, 1990).

### Protein assays

Protein concentration was routinely assayed by the method of Bradford (1976), using the Bio-Rad Protein Assay reagent according to the manufacturer's protocol, with BSA as a standard. To determine the relative responses of morphinone reductase and BSA to this dye reagent, the concentration of a sample of purified morphinone reductase was determined accurately by amino acid analysis by the Protein and Nucleic Acid Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Cambridge, U.K. The true protein concentration was found to be 91% of that given by reference to the standard curve for BSA, and this correction factor was applied to all results.

### Electrophoresis

SDS/PAGE was performed by the method of Laemmli (1970) using the Bio-Rad Mini-Protean II system. Polyacrylamide gels (12.5%) were run at 200 V, stained with 0.1% (w/v) Coomassie Blue R-250 in methanol/water/acetic acid (4:5:1, by vol.), and destained with several changes of the same solvent. The subunit $M_r$ of the purified enzyme was determined by using Bio-Rad Low Molecular Weight Standards. Native (non-denaturing) PAGE was performed using the same procedure with the omission of SDS and sample denaturation. Non-denaturing gels were run at 4 °C.

### $M_r$

The native $M_r$ of morphinone reductase was determined by gel-filtration chromatography as described by Andrews (1964). A column (1.6 cm diameter; 75 cm long) was packed with Sephacryl S-200 (Pharmacia) and run at a flow rate of 8 ml/h. The following standards were used: bovine liver catalase, $M_r$ 240000; yeast C300 hexokinase, 100000; BSA, 67000; cytochrome *c*, 14000. Catalase and hexokinase activities were measured as described by Bergmeyer (1986). BSA was detected by its absorbance at 280 nm and cytochrome *c* by its absorbance at 505 nm.

The subunit $M_r$ was determined by electrospray m.s. using a VG Bio-Q mass spectrometer by the Cambridge Centre for Molecular Recognition, Department of Chemistry, University of Cambridge, Cambridge, U.K. The sample was prepared by diafiltration to remove salt and buffer followed by addition of methanol to 50% (v/v) and acetic acid to 1% (v/v).

### Flavin identification

The flavin prosthetic group was dissociated from morphinone reductase by boiling 48 μg of morphinone reductase in 200 μl of buffer A for 3 min. Denatured protein was removed by ultrafiltration using a Filtron Microsep Centrifugal Concentrator with a nominal $M_r$ cut-off of 10000. Absorbance spectra were measured using a Hewlett–Packard 8452A diode-array spectrophotometer. T.l.c. of flavins was performed on silica plates using solvent systems B (n-butanol/water/acetic acid/methanol, 14:14:1:6, by vol.) and A [2% (w/v) $Na_2HPO_4$ in water] as described by Fazekas and Kokai (1971). Flavins were revealed on t.l.c. plates by their yellow fluorescence when illuminated by u.v. light at 366 nm.

Deflavo-morphinone reductase was prepared by treatment with acid and $(NH_4)_2SO_4$ as described by Husain and Massey (1978). To 800 μl of purified enzyme solution (0.7 mg/ml protein) in buffer A, on crushed ice, 280 μl of ice-cold saturated $(NH_4)_2SO_4$ was added, and then, slowly and with continuous swirling, 320 μl of ice-cold 0.1M HCl. The solution was then centrifuged in an Eppendorf centrifuge at 11600 *g* for 10 min at 4 °C. The supernatant was removed and the pellet dissolved in 800 μl of buffer A with 1 mM dithiothreitol. After 3 h on ice, the undissolved material was removed by centrifugation. Re-association was tested by incubation of the apoenzyme with various concentrations of flavin for 2.5 h on ice.

### N-terminal sequencing

Morphinone reductase was electroblotted on to poly(vinyl difluoride) membrane (ProBlott), and the N-terminal sequence was determined by automated Edman degradation by the Protein and Nucleic Acid Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Cambridge, U.K.

## RESULTS

### Purification of morphinone reductase

Morphinone reductase was purified rapidly from crude cell extract by affinity chromatography on Mimetic Yellow 2 with a typical yield of 40–60 %. Specific activities from different preparations ranged from 14 to 21 units/mg, and purification factors ranged from 70 to 90. The purified morphinone reductase was homogeneous as judged by SDS/PAGE (Figure 1). Purified morphinone reductase could be stored for several months at a protein concentration of 0.3–1.0 mg/ml in buffer A at −20 °C with no detectable loss of activity.

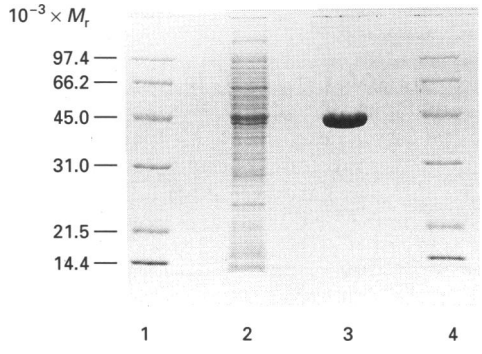



**Figure 1 SDS/PAGE gel showing purification of morphinone reductase**

Lanes 1 and 4, $M_r$ markers; lane 2, crude cell extract, 16 $\mu$g of protein; lane 3, purified morphinone reductase (1.5 $\mu$g).

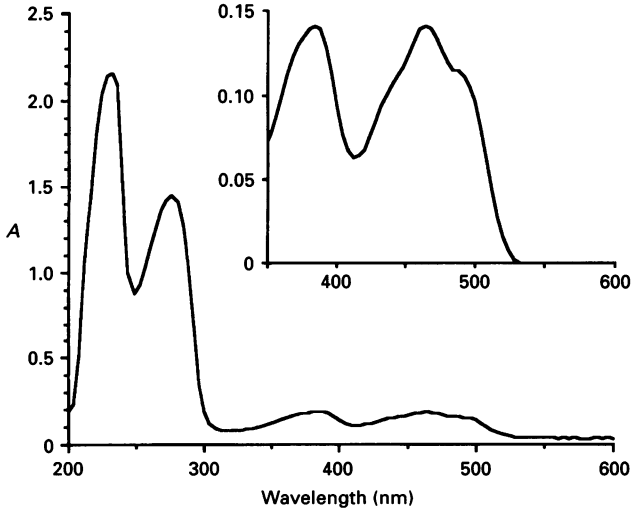



**Figure 2 U.v.–visible absorption spectrum of purified morphinone reductase**

The u.v.–visible absorption spectrum of 0.64 mg/ml purified morphinone reductase was measured in 50 mM phosphate buffer, pH 7.0, containing 1 mM dithiothreitol, against a blank consisting of the same buffer.

### $M_r$

The native $M_r$ of morphinone reductase was determined to be 80000 ± 5000 by gel-filtration chromatography, and the subunit $M_r$ measured by electrospray m.s. was 41120 ± 3, consistent with the migration position in SDS/PAGE gels (Figure 1). These results suggest that native morphinone reductase exists as a dimer.

### Identification of the prosthetic group

Purified morphinone reductase showed the visible absorbance spectrum characteristic of a flavoprotein (Figure 2). The flavin could be liberated by boiling and removal of denatured protein, indicating that the flavin is not covalently bound.

The liberated flavin was analysed by t.l.c. on silica plates, together with authentic flavin standards. In solvent A the $R_F$ values for riboflavin, FMN, FAD and morphinone reductase flavin were 0.34, 0.45, 0.60 and 0.45 respectively, while in solvent B the values were 0.62, 0.54, 0.48 and 0.54 respectively. Standards subjected to boiling in buffer A, as used to liberate the flavin from morphinone reductase, showed no sign of degradation; in particular, FAD was not hydrolysed to FMN. It was therefore concluded that the prosthetic group is non-covalently bound FMN.

### Preparation of deflavo-morphinone reductase and reconstitution of the active enzyme

Deflavo-morphinone reductase was prepared by $(NH_4)_2SO_4$ treatment at acid pH, as described in the Materials and methods section. Absorbance spectra were measured before and after removal of the flavin. Controls using authentic FMN confirmed that the presence of $(NH_4)_2SO_4$ and the altered pH had no effect on the visible absorbance spectrum of FMN. Deflavo-morphinone reductase was prepared from 12.5 nmol (subunit) of morphinone reductase. Determination of the protein concentration indicated that 70 % of the morphinone reductase was recovered as soluble apoprotein. The visible absorbance spectrum of the apoprotein indicated that the ratio of flavin to protein was approx. 20 % of that in the original morphinone reductase, indicating that approx. 1.8 nmol of FMN was still bound to soluble protein. The visible absorbance spectrum of the flavin-containing supernatant indicated that 10.1 nmol of flavin had been released as free FMN. If it is assumed that the protein lost as insoluble material had lost all flavin, these values suggest an original flavin content of 11.9 nmol, or 0.95 mol of FMN per mol of morphinone reductase subunit.

The specific activity of the deflavo-morphinone reductase was 4.7 units/mg, as compared with 21 units/mg for native morphinone reductase. Deflavo-morphinone reductase was incubated for 150 min on ice with various concentrations of FMN, and then assayed for activity. The specific activity increased linearly with increasing FMN. The peak specific activity of 22 units/mg was achieved with the addition of approx. 1.1 mol of FMN per mol of morphinone reductase subunit. Further analysis of the samples by native PAGE (Figure 3) resulted in three well-

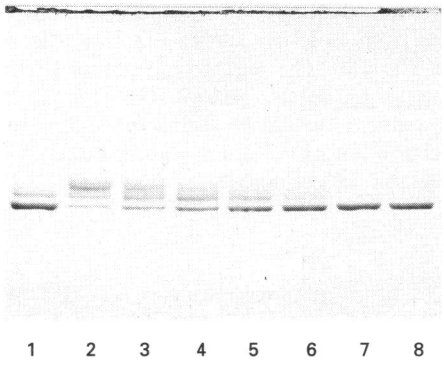



**Figure 3 Native PAGE gel showing titration of deflavo-morphinone reductase with FMN**

Reconstitution of deflavo-morphinone reductase was performed as described in the Materials and methods and Results sections. Each lane contained 4.5 $\mu$g of morphinone reductase. Lane 1, native morphinone reductase as produced by the purification protocol given in the text. Lanes 2–8, deflavo-morphinone reductase incubated with, respectively, 0, 0.22, 0.43, 0.65, 0.86, 1.1 and 1.3 mol of FMN per mol of enzyme subunit.

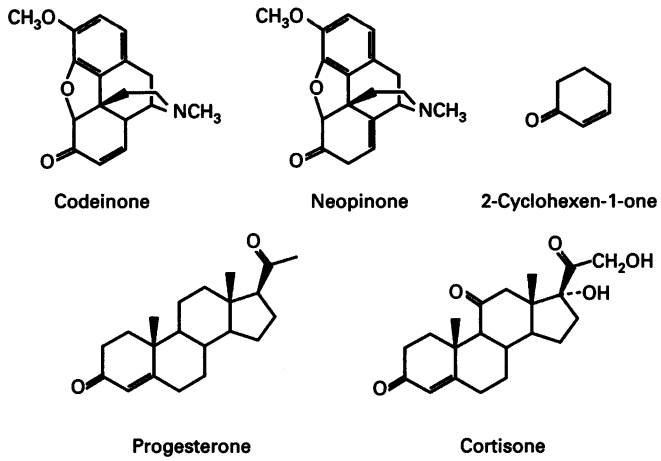



**Figure 4 Structures of compounds tested as substrates for morphinone reductase**

resolved bands with $R_F$ values of 0.57, 0.60 and 0.63. In native morphinone reductase, the highest-$R_F$ band was by far the most distinct, with the second band much weaker and the third absent. In deflavo-morphinone reductase, all three bands were present with the lowest $R_F$ being most distinct. With increasing addition of FMN, the highest-$R_F$ band increased at the expense of the others until finally it was the only band visible. The most obvious interpretation is that the three bands represent morphinone reductase dimers with no, one or two molecules of FMN bound. If this interpretation is correct, then the morphinone reductase prepared by the protocol described for the present study appears to contain a significant amount of dimer with only one molecule of FMN. This is consistent with the measured flavin content of 0.95 mol of FMN per subunit.

Finally, the deflavo-morphinone reductase was incubated with a slight molar excess of riboflavin, FMN or FAD. Increased specific activity and recovery of the non-denaturing PAGE migration pattern of native morphinone reductase were observed only with FMN (results not shown).

## pH optimum

The pH optimum for the morphinone reductase reaction was determined by measuring activity, at substrate concentrations the same as those for the standard assay, in the following range of buffers: 50 mM phosphate adjusted with NaOH to pH 6.0, 6.5, 7.0, 7.5 and 8.0; 50 mM Mops adjusted with NaOH to pH 6.5, 7.0, 7.5 and 8.0; 50 mM Tris adjusted with HCl to pH 7.5, 8.0, 8.5 and 9.0; and 50 mM glycine adjusted with NaOH to pH 9.0, 9.5, 10.0 and 10.5. The optimum pH was 8.0, with activity in phosphate buffer being higher than that in Mops. Activity at pH 8.0 in 50 mM phosphate buffer was approx. 20% higher than in the standard assay at pH 7.0.

## Substrate specificity

Various compounds were tested as substrates (replacing codeinone) and inhibitors in the standard assay (Figure 4). All compounds were added at 1 mM. In addition to being active towards morphinone, the enzyme was active with codeinone, neopinone and 2-cyclohexen-1-one. Morphine, codeine and isocodeine were neither substrates nor inhibitors at the levels tested. Bacterial flavoproteins have been described which saturate the olefin bonds of *trans*-buten-2-oate and orotate with oxidation of NADH or NADPH (Friedmann and Vennesland, 1960; Tischer et al., 1979). These compounds were also tested and found to be neither substrates nor inhibitors under the conditions used. The steroids progesterone and cortisone, which possess an unsaturated ring similar to that of codeinone, were not substrates, but were strongly inhibitory.

Activity with biologically generated morphinone was demonstrated previously by h.p.l.c. and $^1$H-n.m.r. with partially purified morphinone reductase (Hailes and Bruce, 1993). This has been confirmed unequivocally with the purified enzyme (A. M. Hailes and N. C. Bruce, unpublished work); however, owing to the difficulty of obtaining sufficiently large amounts of pure morphinone (Bruce et al., 1990), kinetic data have not been obtained for morphinone.

No activity was displayed when NADPH replaced NADH in the standard assay. Furthermore, no reaction in the reverse direction was detected with hydromorphone or hydrocodone and $NAD^+$ under the same assay conditions. Apparent $K_m$ and $k_{cat.}$ were measured for codeinone and 2-cyclohexen-1-one at 0.3 mM NADH. Codeinone concentrations ranging from 0.02 to 0.64 mM and 2-cyclohexen-1-one concentrations ranging from 1 to 8 mM were used. The apparent kinetic constants were, for codeinone: $K_m$ $0.26 \pm 0.02$ mM; $V_{max.}$ $35 \pm 1$ unit/mg; $k_{cat.}$ $24 \pm 1$ $s^{-1}$; $k_{cat.}/K_m$ $93 \pm 9$ $s^{-1} \cdot mM^{-1}$; and, for 2-cyclohexen-1-one: $K_m$ $5.5 \pm 1$ mM; $V_{max.}$ $0.98 \pm 0.09$ unit/mg; $k_{cat.}$ $0.67 \pm 0.06$ $s^{-1}$; $k_{cat.}/K_m$ $0.12 \pm 0.03$ $s^{-1} \cdot mM^{-1}$.

The apparent $K_m$ for NADH was measured as $0.050 \pm 0.003$ mM at 0.3 mM codeinone, with NADH concentrations ranging from 0.04 to 0.32 mM. All errors quoted are 1 S.E.M., and all $k_{cat.}$ values were calculated on the basis of one catalytic site per subunit of morphinone reductase.

The steroids cortisone and progesterone were found to be potent inhibitors of the morphinone reductase reaction. In kinetic studies of inhibition by cortisone, cortisone at concentrations of 15–45 $\mu$M was found to give competitive inhibition with respect to codeinone and predominantly uncompetitive inhibition with respect to NADH (results not shown). The apparent $K_i$ for

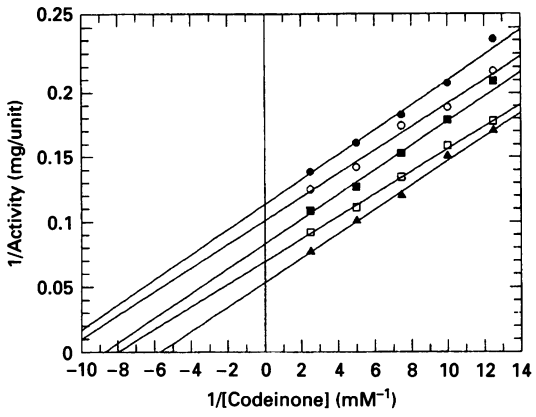



**Figure 5 Double-reciprocal plot showing initial rates of morphinone reductase activity in the presence of various substrate concentrations**

●, 0.04 mM NADH; ○, 0.05 mM NADH; ■, 0.067 mM NADH; □, 0.1 mM NADH; ▲, 0.2 mM NADH.Results are means for three independent assays. Lines shown were fitted to the data by non-linear regression using the Grafit software package of Erithacus Software Ltd. (version 2). Lines were fitted independently for each NADH concentration; 1 unit is that amount of activity oxidizing 1 $\mu$mol of NADH/min under the assay conditions.

competitive inhibition (with respect to codeinone) by cortisone, at 0.3 mM NADH, was 35 $\mu$M. Inhibition by progesterone was not characterized in detail, but the concentration required to halve activity in the standard assay was 1.3 $\mu$M.

### Kinetic mechanism

To investigate the kinetic mechanism of morphinone reductase, initial rates of NADH oxidation were measured at codeinone concentrations ranging from 0.04 to 0.4 mM and NADH concentrations ranging from 0.04 to 0.2 mM. Lineweaver–Burk double-reciprocal plots with either codeinone or NADH as the variable substrate gave a set of lines parallel within experimental error (Figure 5). This pattern is characteristic of a Ping Pong or substituted enzyme mechanism, although it can also be produced by sequential mechanisms under certain conditions (Cleland, 1970; Segel, 1975). Furthermore, the flavin prosthetic group could be reduced by excess NADH, as indicated by the loss of the characteristic visible absorbance peaks, in the absence of codeinone. This indicates that binding of the oxidizing (alkaloid) substrate is not required for the reductive half-reaction to occur.

To investigate further the kinetic mechanism, product inhibition by hydrocodone and $NAD^+$ was examined. With a constant NADH concentration of 0.1 mM, initial rates of NADH oxidation were measured at codeinone concentrations ranging from 0.08 to 2.56 mM, in the presence of either hydrocodone (1–4 mM) or $NAD^+$ (1–4 mM). Under these conditions, hydrocodone caused competitive inhibition and $NAD^+$ caused purely non-competitive inhibition (Figure 6). This is inconsistent with a classical one-site Ping Pong mechanism, in which hydrocodone should compete with NADH for the oxidized form of the enzyme, and $NAD^+$ should compete with codeinone for the reduced enzyme species (Cleland, 1970; Segel, 1975).

Inhibition by the same products with respect to NADH was also investigated. The same product concentrations were used with a constant codeinone concentration of 0.2 mM and NADH concentrations ranging from 0.03 to 0.24 mM. Under these conditions, hydrocodone caused uncompetitive inhibition and $NAD^+$ caused predominantly competitive mixed inhibition. However, a pronounced non-linearity was noted in linearized kinetic plots at NADH concentrations above 0.2 mM and, at concentrations above 0.3 mM, NADH appeared to cause inhibition of its own oxidation. It was not clear whether this inhibition was caused by the NADH itself or by some trace impurity in the NADH used. In view of this, these results must be treated with caution.

### Inhibition of activity

Morphinone reductase (3.3 $\mu$g/ml) was incubated in buffer A for 30 min at room temperature and 16 h at 4 °C with the following potential inhibitors: *p*-hydroxymercuribenzoate, $CuSO_4$, EDTA and 8-hydroxyquinoline sulphate. The reaction was then initiated by adding NADH (0.3 mM) and codeinone (0.3 mM). *p*-Hydroxymercuribenzoate (0.02 mM) caused complete inhibition within 30 min, and $CuSO_4$ (0.01 mM) caused complete loss of

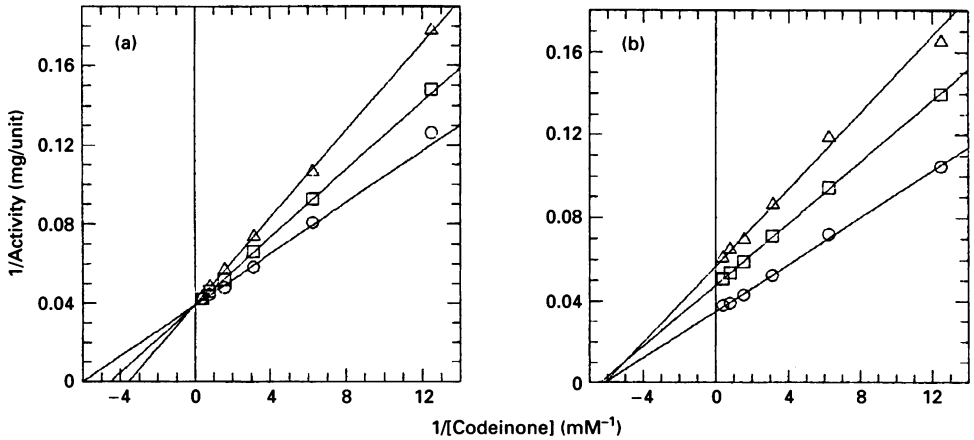



**Figure 6 Product-inhibition studies**

(**a**) Hydrocodone versus codeinone: ○, no hydrocodone; □, 2 mM hydrocodone; △, 4 mM hydrocodone. (**b**) $NAD^+$ versus codeinone: ○, no $NAD^+$; □, 2 mM $NAD^+$; △, 4 mM $NAD^+$. Results are means for two independent assays. Lines shown were fitted to the data by non-linear regression using the Grafit software package of Erithacus Software Ltd. (version 2.0). Lines were fitted independently for each inhibitor concentration; 1 unit is that amount of activity oxidizing 1 $\mu$mol of NADH/min under the assay conditions.

activity within a few seconds. This suggests that a reduced thiol group is important for activity. Incubation in the presence of one or other substrate did not appear to offer protection. The metal-complexing agents EDTA (0.5 mM) and 8-hydroxyquinoline (0.05 mM) caused no significant inhibition after 16 h.

### N-terminal sequence

The N-terminal sequence of morphinone reductase was found to be: Pro-Asp-Thr-Ser-Phe-Ser-Asn-Pro-Gly-Leu-Phe-Thr-Pro-Leu-Gln-. No significant sequence similarities were found in the Swiss-Prot database. This sequence will be used to design oligonucleotide probes in order to locate and clone the structural gene encoding this enzyme.

## DISCUSSION

Pharmaceutical compounds derived from morphine include widely used analgesics, antitussives and narcotic antagonists. Industrial syntheses of these alkaloids from morphine, codeine or thebaine are complex and require expensive catalysts. Recent studies on the microbial degradation of the morphine alkaloids are revealing important biological routes to a number of these pharmaceutical compounds (Bruce et al., 1993). Morphinone reductase catalyses the reduction of the 7,8-unsaturated bond of morphinone and codeinone during the metabolism of morphine and codeine in *P. putida* M10 (Hailes and Bruce, 1993). This reaction is required for the production of a number of semi-synthetic morphine derivatives, and this enzyme is therefore of potential industrial interest.

Morphinone reductase was purified from crude cell extract to apparent homogeneity, as judged by SDS/PAGE and N-terminal sequencing, in a single affinity-chromatography step using the affinity ligand Mimetic Yellow 2. Purified morphinone reductase had a subunit $M_r$ of 41100, and in gel filtration migrated at an apparent $M_r$ of approx. 80000, suggesting a dimeric structure. One molecule of FMN was bound non-covalently per subunit. The deflavoenzyme could be reconstituted with FMN but not with FAD, confirming the specificity of the enzyme for its prosthetic group. Morphinone reductase was also specific for NADH as the electron donor; no oxidation of NADPH was observed.

Purified morphinone reductase from *P. putida* M10 showed a high degree of substrate specificity, reducing the olefin bonds of codeinone, morphinone, neopinone and 2-cyclohexen-1-one. Although the enzyme has been designated 'morphinone reductase', since it was first investigated in connection with the catabolism of morphine, the majority of the characterization work has used codeinone as a substrate, since morphinone is not available commercially and is unstable and difficult to purify (Bruce et al., 1990).

Codeinone was a much better substrate than 2-cyclohexen-1-one, suggesting that the alkaloid structure is important for high activity. Interestingly, neopinone, which possesses an 8,14-unsaturated bond, was apparently a better substrate than 2-cyclohexen-1-one; however, reliable kinetic data could not be obtained since only a crude preparation of neopinone, containing significant levels of unidentified impurities, was available. No activity was detected with morphine, codeine or isocodeine, suggesting that the carbonyl group at C-6 is vital for activity.

The steroids progesterone and cortisone were found to be competitive inhibitors. Morphinone reductase appears to have a much higher affinity for these steroids than for the alkaloids tested, although no steroid tested so far has acted as a substrate. This may suggest that morphinone reductase originates from a steroid reductase or may itself be active against a steroid not tested. Several bacterial steroid reductases, catalysing reactions analogous to the morphinone reductase reaction, have been described; for example, *Clostridium innocuum* produces a moderately oxygen-stable steroid reductase which has an $M_r$ of 80000, uses NADH as an electron donor, and is stimulated by reduced FMN (Stokes and Hylemon, 1985). Morphinone reductase was susceptible to thiol-group inhibitors, suggesting that one or more reduced cysteine residues are important for activity; however, these may not be located at the active site, since protection from inhibition could not be afforded by the addition of codeinone or NADH. Morphinone reductase was not inhibited by metal-chelating agents.

Steady-state kinetic experiments suggested that morphinone reductase follows a Ping Pong kinetic mechanism. However, the product inhibition patterns observed were not consistent with a classical one-site Ping Pong mechanism; rather, they suggested that alkaloids and nicotinamide-nucleotide cofactors bound at separate sites. A number of flavoproteins have been reported to display kinetic patterns suggestive of a two-site Ping Pong mechanism, in which the two substrates bind independently at separate sites and reducing equivalents are passed between them by means of the flavin and other redox-active prosthetic groups. Examples include bovine mitochondrial dihydro-orotate dehydrogenase (Hines and Johnston, 1989), *Penicillium chrysogenum* nitrate reductase (Renosto et al., 1981), chicken liver xanthine dehydrogenase (Coughlan and Rajagopalan, 1980), and *Escherichia coli* glutamate synthase (Rendina and Orme-Johnson, 1978). The product-inhibition patterns observed for morphinone reductase suggest that morphinone reductase, like these other flavoenzymes, may follow a two-site Ping Pong mechanism.

In phosphate buffer at pH 7.0, morphinone reductase appeared to be incapable of catalysing the reverse reaction with $NAD^+$ and hydrocodone or hydromorphone, suggesting that the reaction equilibrium lies far to the right under the reaction conditions used. If the morphinone reductase reaction is essentially irreversible, this implies that, although the morphine dehydrogenase reaction is readily reversible (Bruce et al., 1991), a two-stage process using morphine dehydrogenase and morphinone reductase to produce hydromorphone and hydrocodone from morphine and codeine should result in high yields. Currently we are trying to clone the structural gene for morphinone reductase, designated *morB*, using oligonucleotide probes based on elements of the protein sequence. By expressing *morB* together with *morA* in a single host we hope to generate a recombinant organism capable of transforming morphine and codeine to hydromorphone and hydrocodone rapidly and in high yields.

C. E. F acknowledges the award of a Cambridge Commonwealth Trust Prince of Wales Scholarship. We warmly acknowledge gifts of chemicals from Macfarlan Smith Limited.

## REFERENCES

Andrews, P. (1964) Biochem. J. **91**, 222–223
Bergmeyer, H. U. (1986) Methods of Enzymatic Analysis, 3rd edn., V.C.H. Publishers, Weinheim
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Bruce, N. C., Wilmot, C. J., Jordan, K. N., Trebilcock, A. E., Gray Stephens, L. D. and Lowe, C. R. (1990) Arch. Microbiol. **154**, 465–470
Bruce, N. C., Wilmot, C. J., Jordan, K. N., Gray Stephens, L. D. and Lowe, C. R. (1991) Biochem. J. **274**, 875–880
Bruce, N. C., Caswell, D. A., French, C. E., Hailes, A. M., Long, M. T. and Willey, D. L. (1993) Ann. N.Y. Acad. Sci., in the press
Cleland, W. W. (1970) Enzymes 3rd Ed. **2**, 1–165

Coughlan, M. P. and Rajagopalan, K. V. (1980) Eur. J. Biochem. **105**, 81–84
Fazekas, A. G. and Kokai, K. (1971) Methods Enzymol. **18B**, 385–398
Friedmann, H. C., and Vennesland, B. (1960) J. Biol. Chem. **235**, 1526–1532
Hailes, A. M. and Bruce, N. C. (1993) Appl. Environ. Microbiol. **59**, 2166–2170
Hines, V. and Johnston, M. (1989) Biochemistry **28**, 1222–1226
Husain, M. and Massey, V. (1978) Methods Enzymol. **53**, 429–437
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Leatherbarrow, R. J. (1990) Grafit Version 2.0, Erithacus Software, Staines
Rendina, A. R. and Orme-Johnson, W. H. (1978) Biochemistry **17**, 5388–5393
Renosto, F., Ornitz, D. M. , Peterson, D. and Segel, I. H. (1981) J. Biol. Chem. **256**, 8616–8625
Segel, I. H. (1975) Enzyme Kinetics, John Wiley and Sons, New York
Stokes, N. A. and Hylemon, P. B. (1985) Biochim. Biophys. Acta **836**, 255–261
Tischer, W., Bader, J. and Simon, H. (1979) Eur. J. Biochem. **97**, 103–112
Willey, D. L., Caswell, D. A., Lowe, C. R. and Bruce, N. C. (1993) Biochem. J. **290**, 539–544

Received 15 December 1993/26 January 1994; accepted 28 February 1994