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Microbial degradation of the morphine alkaloids

Purification and characterization of morphine dehydrogenase from *Pseudomonas putida* M10

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The $NADP^+$-dependent morphine dehydrogenase that catalyses the oxidation of morphine to morphinone was detected in glucose-grown cells of *Pseudomonas putida* M10. A rapid and reliable purification procedure involving two consecutive affinity chromatography steps on immobilized dyes was developed for purifying the enzyme 1216-fold to electrophoretic homogeneity from *P. putida* M10. Morphine dehydrogenase was found to be a monomer of M_r 32000 and highly specific with regard to substrates, oxidizing only the C-6 hydroxy group of morphine and codeine. The pH optimum of morphine dehydrogenase was 9.5, and at pH 6.5 in the presence of NADPH the enzyme catalyses the reduction of codeinone to codeine. The K_m values for morphine and codeine were 0.46 mM and 0.044 mM respectively. The enzyme was inhibited by thiol-blocking reagents and the metal-complexing reagents 1,10-phenanthroline and 2,2′-dipyridyl, suggesting that a metal centre may be necessary for activity of the enzyme.

INTRODUCTION

The morphine alkaloids have attracted considerable attention owing to their analgaesic properties and, consequently, much effort in the past has been directed at the production of new morphine alkaloids by micro-organisms (Iizuka *et al.*, 1960, 1962; Yamada *et al.*, 1962, 1963; Liras & Umbreit, 1975; Gibson *et al.*, 1984; Kunz *et al.*, 1985; Spassov *et al.*, 1986). It is therefore perhaps surprising that there have been few reports on the utilization by micro-organisms of these alkaloid compounds as sole carbon and energy sources.

Recently, the bacterium *Pseudomonas putida* M10 was isolated from industrial-waste liquors by its ability to utilize morphine as its sole carbon and energy source (Bruce *et al.*, 1990). So far as is known, the first step in the degradation of morphine by *P. putida* M10 is mediated by an $NADP^+$-dependent morphine dehydrogenase, which catalyses the oxidation of the C-6 hydroxy group of morphine, yielding NADPH and morphinone (Scheme 1). Morphinone was shown to be further degraded by cell-free extracts from morphine-grown cells of *P. putida* M10. Cultures of this organism could also utilize codeine as a growth substrate and, from preliminary results, codeine was shown to be dissimilated by a route similar to morphine, involving oxidation to codeinone, by the $NADP^+$-dependent morphine dehydrogenase (Bruce *et al.*, 1990). Unexpectedly this enzyme appears to be constitutive in *P. putida* M10; this is unusual, since constitutive synthesis of a catabolic enzyme is likely to prove disadvantageous when other compounds are available as growth substrates. However, it was considered conceivable that morphine dehydrogenase might be a non-specific alcohol dehydrogenase. Thus a more comprehensive knowledge of the properties and substrate specificity of the enzyme was deemed necessary in order to understand the role of this apparently novel $NADP^+$-dependent dehydrogenase in the degradation of morphine alkaloids.

Here we describe the purification to homogeneity of the $NADP^+$-dependent morphine dehydrogenase from *P. putida* M10. We present physical and chemical properties of the purified enzyme and provide evidence that the enzyme is a highly specific alkaloid dehydrogenase.

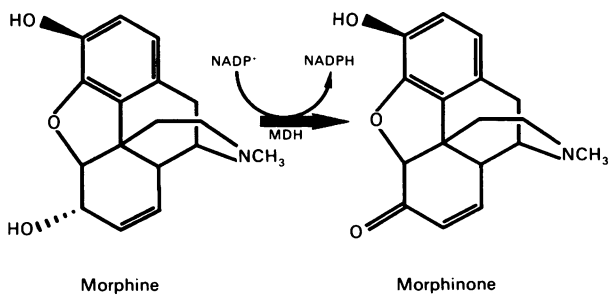
EXPERIMENTAL

Materials

Mimetic Orange 3 A6XL and Mimetic Red A6XL were obtained from Affinity Chromatography Ltd., Freeport, Ballasalla, Isle of Man, U.K. 6-Acetylmorphine was synthesized by the method of Wright (1941). Codeinone and dihydrocodeine were kindly given by Mr. J. R. Slater, Macfarlan Smith Ltd., Edinburgh, Scotland, U.K. Morphine, codeine and diacetylmorphine were purchased from Macfarlan Smith Ltd. $NADP^+$, NADPH and NAD^+ were obtained from Sigma (Poole, Dorset, U.K.). All reagents were of the highest grade that could be obtained commercially.

Organisms and growth of cells

The organism used in this investigation, *Pseudomonas putida* M10, was originally isolated from industrial-waste liquors (Bruce *et al.*, 1990). The defined minimal medium consisted of the following: $(NH_4)_2SO_4$ (0.5 g), K_2HPO_4 (2.0 g), KH_2PO_4 (0.2 g) and $MgSO_4$ (0.05 g), all per litre. Trace elements were as described by Barnet & Ingram (1955). Substrates were added in the following concentrations: 10 mM-glucose, 10 mM-diacetylmorphine and 7 mM-morphine, supplemented with 3 mM-glucose.



Morphine Morphinone

Scheme 1. Oxidation of morphine to morphinone catalysed by morphine dehydrogenase (MDH) from *P. putida* M10

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Diacetylmorphine was filter-sterilized and added to the medium aseptically before inoculation. Conditions for growth of the bacteria and preparations of cell-free extracts were described by Bruce *et al.* (1990). Bulk cultures of *P. putida* M10 (400 litres) were grown on the standard minimal media supplemented with 10 mM-glucose by Imperial Biotechnology Ltd., Imperial College Road, South Kensington, London, U.K. Cells were harvested and stored at −80 °C until required.

Enzyme assays

Morphine dehydrogenase activity was measured by monitoring the reduction of $NADP^+$ at 340 nm in 50 mM-glycine/NaOH buffer, pH 9.5, containing 3 mM-morphine, 3 mM-$NADP^+$ and enzyme in a final volume of 1 ml. In the reverse direction the reaction was measured in 50 mM-Mops buffer (pH 6.5)/0.5 mM-NADPH/1 mM-codeinone and enzyme in a total volume of 1 ml. The unit of enzyme activity is defined as the amount of enzyme necessary to reduce 1 μmol of $NADP^+$ or to oxidize 1 μmol of NADPH/min at 30 °C. Assays for morphine dehydrogenase activities performed on separate batches of extracts generally agreed to within 10%.

Protein was measured in solutions by the method of Bradford (1976), with BSA as the standard.

Purification of morphine dehydrogenase

All procedures were performed at 4 °C, and all centrifugations were at 30000 *g* for 20 min.

Preparation of cell-free extract. Crude extract was prepared from 40 g (wet weight) of frozen glucose-grown cells. The cells were resuspended in buffer A (50 mM-potassium phosphate/NaOH, pH 7.0, containing 1 mM-dithiothreitol) at a concentration of 0.5 g (wet wt.)/ml and were disrupted by 3 min ultrasonication in a Soniprep MSE Ultrasonic Disintegrator at an amplitude of 10 μm. The sonicated cell suspension was centrifuged to remove the cell debris.

Affinity chromatography on Mimetic Orange 3. The cell-free extract was applied to a Mimetic Orange 3 A6XL column (2.5 cm × 5.0 cm) that had previously been equilibrated with buffer B (20 mM-potassium phosphate, pH 7.0, containing 1 mM-dithiothreitol). After adsorption, the column was washed extensively with buffer B containing 0.25 M-KCl until no further absorbance at 280 nm was evident in the eluate (approx. 500 ml); then the morphine dehydrogenase was eluted batchwise with 400 ml of buffer B containing 0.8 M-KCl. Fractions (10.2 ml) were collected at a flow rate of 108 ml/h.

Affinity chromatography on Mimetic Red 2. Pooled fractions (54–61) from the Mimetic Orange 3 affinity-chromatography step containing the highest morphine dehydrogenase activity were dialysed overnight against 2 litres of buffer B. The dialysed sample was applied to an Mimetic Red 2 A6XL column (1.0 cm × 3.0 cm) that had previously been equilibrated with buffer B. After adsorption on to the affinity matrix, the column was washed with buffer B until no absorbance at 280 nm due to protein could be detected in the eluate, whence the enzyme was eluted batchwise with 0.1 M-KCl at a flow rate of 72 ml/h. The active fractions of the eluate (20–25) were pooled, then concentrated in an Amicon ultrafiltration cell fitted with a YM10 membrane. The purified enzyme was stored at −80 °C in buffer B.

SDS/PAGE

The purity of samples from various stages of the purification was monitored by the method of Laemmli (1970) on 0.75 mm-thick vertical slab gels (7.5 cm × 8.0 cm), containing 12.5% (w/v) polyacrylamide. Protein was detected by staining the gels with 0.1% (w/v) Coomassie Blue R-250 dissolved in a solvent system consisting of methanol/water/acetic acid (4:5:1, by vol.). Gels were destained by repeatedly washing in the above solvent mixture. M_r determinations of the purified enzyme were obtained by using Bio-Rad (Watford, Herts., U.K.) SDS/PAGE low-molecular-mass standards.

Non-denaturing gel electrophoresis was conducted as above, except that SDS was omitted and the samples were not boiled. Gels were either stained for protein, or a colorimetric stain was used for detecting morphine dehydrogenase activity. The gel was incubated in a mixture containing 50 mM-glycine/NaOH buffer, pH 9.0, 3 mM-$NADP^+$, 3 mM-morphine, 0.6 mM-Nitro Blue Tetrazolium and 16 μM-phenazine methosulphate.

M_r

The M_r of the enzyme was determined by the method of Andrews (1964) on columns (1.0 cm × 100 cm) of Sephacryl S-300 calibrated with marker proteins. After the column had been equilibrated with buffer A, a solution containing purified enzyme (3 units) was applied to the bed surface of the column and eluted with equilibration buffer at a flow rate of 8 ml/h, 1 ml fractions being collected. Catalase (M_r 240000), alcohol dehydrogenase (M_r 150000), hexokinase (M_r 110000), BSA (M_r 66000) and myoglobin (M_r 17000) were used as standards.

Determination of pI

Flat-bed isoelectric focusing was performed on an LKB Multiphor apparatus using pH 3.5–9.5 Ampholine PAG plates. A constant voltage (750 V) was applied for 5 h and the gel was maintained at 4 °C. Protein was detected by staining the gel for 10 min at 60 °C with Coomassie Blue R-250 dissolved in ethanol/water/acetic acid (25:67:8, by vol.). Gels were diffusion de-stained by repeated washing in the above solvent mixture.

N-Terminal amino acid determination

Automated *N*-terminal sequence analysis was performed on an Applied Biosystems 470A sequencer.

Protein sequence databases (PIR and DOOLITTLE) were searched using the program FASTP (Lipman & Pearson, 1985).

Absorption spectra

The absorption spectra of purified morphine dehydrogenase in 50 mM-potassium phosphate buffer, pH 7.0, were determined in 1 ml quartz cuvettes in a Perkin–Elmer Lambda 7 spectrophotometer.

Analytical techniques

The resolution and identification of morphine, codeine and codeinone was determined by h.p.l.c. analysis at 218 nm in a Waters 450 system linked to a Waters 740 Data Module. The 25-cm length (0.46 cm diameter) column contained 5 μm Spherisorb-ODS (C_{18}) reverse-phase packing. The solvent system was that described by Umans (1982).

RESULTS

Purification of morphine dehydrogenase

Significant levels of the $NADP^+$-dependent morphine dehydrogenase were produced constitutively in extracts of cells grown on different substrates (Table 1). This property enabled bulk cultures (400 litres) of *Pseudomonas putida* M10 to be grown on glucose for the purification of morphine dehydrogenase. Cells grown on diacetylmorphine, glucose and Luria–Bertani medium (Maniatis *et al.*, 1989) were found to have slightly elevated levels

Table 1. Induction of morphine dehydrogenase in extracts of cells of *P. putida* M10

Substrate for growth	Specific activity*
Glucose	0.059
Luria–Bertani medium	0.070
Diacetylmorphine	0.065
Morphine	0.020

* Specific activities are expressed as μmol NADPH·min^{-1}·mg protein^{-1}.

of morphine dehydrogenase activity compared with those cells grown on morphine. The enzyme activity was located in the soluble fraction of the cell. Table 2 summarizes the results of a typical purification protocol for morphine dehydrogenase from 40 g (wet wt.) of glucose-grown cells of *P. putida* M10. Morphine dehydrogenase was purified 1216-fold with an 84% overall recovery. The initial affinity-chromatography step (Fig. 1) on Mimetic Orange 3 resulted in separation of the enzyme from non-specific NADPH oxidases, thereby explaining the apparent increase in recovery of activity of over 100%. This step proved to be very effective at removing most of the contaminating proteins. The elution profile of morphine dehydrogenase activity from the second affinity column, Mimetic Red 2, coincided with a single peak in the 280 nm absorbance trace. Typically, this second affinity-chromatography step purified the morphine dehydrogenase to homogeneity, since SDS/PAGE analysis revealed a distinct single protein band after staining with Coomassie Blue R-250 (Fig. 2). Non-denaturing PAGE of the purified enzyme gave a single band stained for protein that coincided with morphine dehydrogenase activity when stained with the activity stain. Yields of activity were greatly improved by the addition of dithiothreitol to all the equilibration and elution buffers. Morphine dehydrogenase could not be eluted specifically from either of the affinity columns by the addition of 2 mM-NADH or -NADPH to the elution buffers.

The enzyme represents about 0.07% of the total soluble cell protein, since only 1.3 mg of pure morphine dehydrogenase was obtained from 1886 mg of starting protein.

Properties of morphine dehydrogenase

Stability. Morphine dehydrogenase was very unstable when stored at 4 °C, although there was only slow loss of activity on prolonged storage (approx. 10% loss of activity over 2 months) at −80 °C in buffer B.

Heat inactivation experiments with the purified enzyme indicated that morphine dehydrogenase had $t_{\frac{1}{2}} = 6.5$ min at 50 °C in 50 mM-potassium phosphate buffer, pH 7.0.

***M*$_r$.** The M_r of the native enzyme was determined by the method of Andrews (1964) on columns of Sephacryl S-300 to be 32000 ± 1000 as calculated from three independent determinations. Purified morphine dehydrogenase subjected to SDS/PAGE calibrated with standard proteins yielded a single

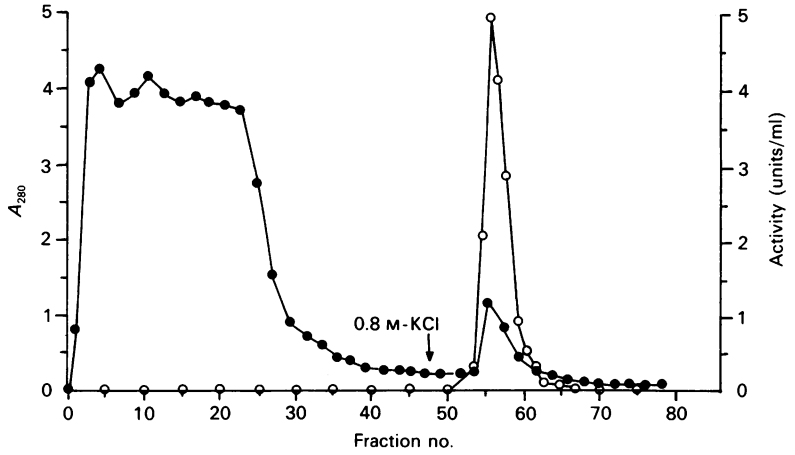

Fig. 1. Affinity chromatography of morphine dehydrogenase on Mimetic Orange 3

The enzyme was eluted batchwise with buffer B containing 0.8 M-KCl at a flow rate of 108 ml/h. Each fraction (10.2 ml) was assayed for dehydrogenase activity (○) and protein content (●).

Table 2. Purification of morphine dehydrogenase from crude extracts of *P. putida* M10

The starting material was 40 g of cell paste. Full experimental details are given in the Experimental section.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units·mg^{-1})	Recovery of activity (%)	Purification factor
Crude extract	100	116.5	1886	0.062	100	–
Mimetic Orange 3 affinity chromatography	60	161	24.1	6.7	138	108
Mimetic Red 2 affinity chromatography	2	98	1.3	75.4	84	1216

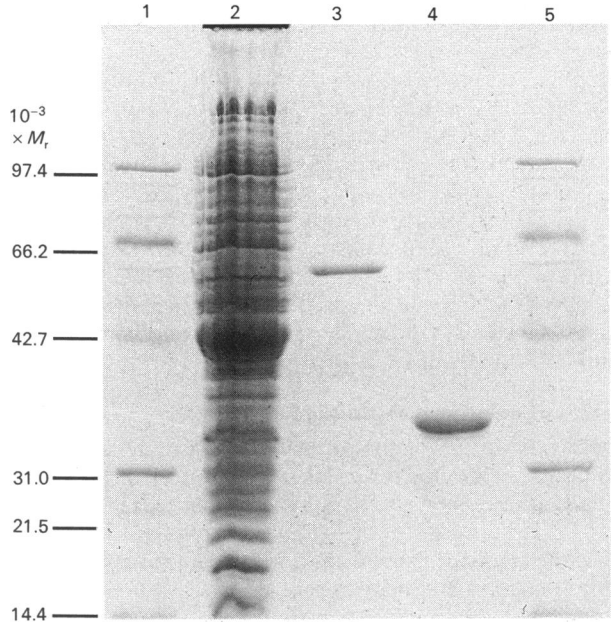

Fig. 2. Electrophoresis of morphine dehydrogenase from *P. putida* M10

The purification of morphine dehydrogenase was monitored on a 12.5% (w/v) polyacrylamide slab gel containing SDS. Lanes: 1 and 5, M_r markers; 2, cell-free extract (76 μg of protein); 3, Mimetic Orange 3 elution pool (3 μg of protein); 4, Mimetic Red 2 (3 μg of protein).

distinct band with an M_r of 31000 ± 1000 (Fig. 2). Comparison of the mean native M_r and the sub-unit M_r suggest that morphine dehydrogenase is monomeric.

pH optimum and pI. The effect of pH on morphine dehydrogenase activity over the range 7.0–10.5 showed an optimum of pH 9.5 in glycine/NaOH buffer. In the reverse direction, codeinone was reduced by NADPH with the enzyme over the pH range 6.0–8.5 with an optimum at pH 6.5 in Mops buffer. A single pI of 4.2 was obtained when morphine dehydrogenase was subjected to flat-bed isoelectric focusing.

Table 3. Substrate specificity of morphine dehydrogenase

Dehydrogenase activity was tested using 0.35 μg of purified enzyme with the alkaloid substrates at a final concentration of 3 mM, as described in the Experimental section. Alcohol substrates were at a final concentration of 50 mM, whereas DL-mandelic acid, testosterone and androsterone were at a final concentration of 10 mM. Activities are relative to that determined with 3 mM-morphine (0.0252 μmol of NADPH/min = 100%). No activity was shown by the enzyme towards 6-acetylmorphine (**IV**), cyclohexanol, benzyl alcohol, butan-2-ol, propan-2-ol, ethanol, propanol, testosterone, androsterone or DL-mandelic acid.

Substrate*	Relative activity (%)	K_m(mM)
Morphine (**I**)	100	0.46
Codeine (**II**)	120	0.044
Dihydrocodeine (**III**)	7.1	2.91

* Structures of the alkaloid analogues **I–IV** are shown in Fig. 3.

Substrate specificity. The ability of various compounds to serve as substrates was investigated by replacing morphine with each analogue in the reaction mixture. Dehydrogenase activity was expressed relative to morphine (**I**) (Table 3; Fig. 3). The enzyme showed a high degree of specificity, since only the analogues codeine (**II**) and dihydrocodeine (**III**) out of a wide range of compounds tested acted as substrates. The relative rate of activity of the purified enzyme from *P. putida* M10 with codeine as the substrate was higher (20%) than that determined with morphine itself. The enzyme showed low activity towards dihydrocodeine. Fig. 3 illustrates the structural features of morphine and codeine which differ from dihydrocodeine in

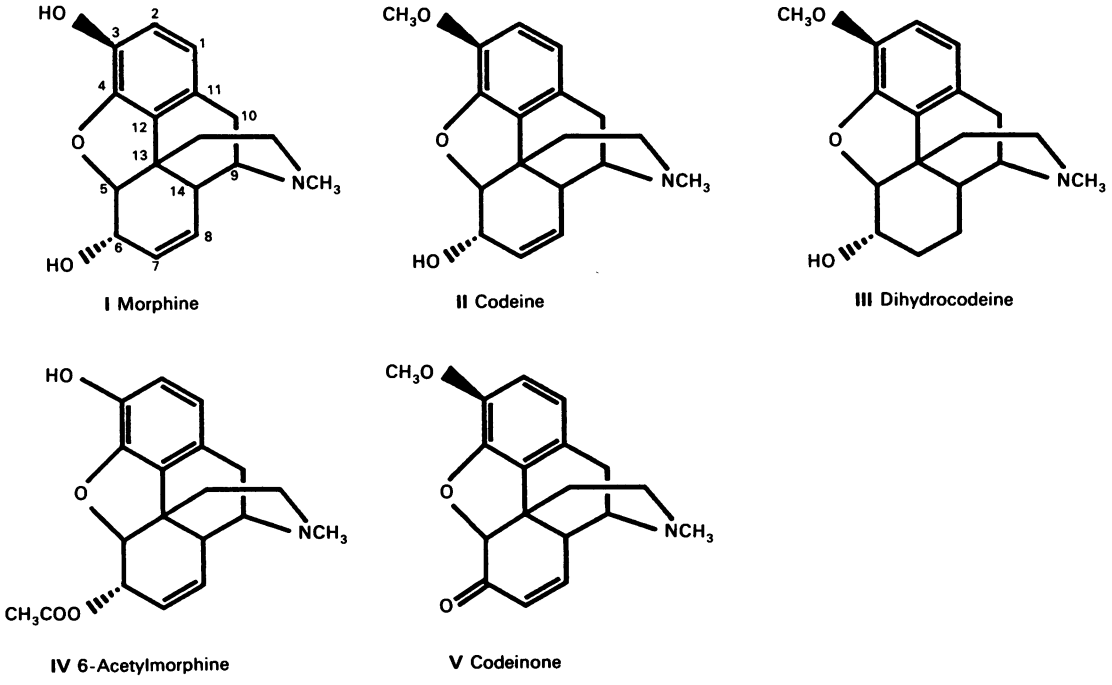

Fig. 3. Analogues of morphine

Table 4. Inhibition of morphine dehydrogenase by thiol blocking reagents, chelating agents and metals

The purified enzyme was incubated with the indicated reagents (*a*) for 10 min at room temperature and (*b*) for 16 h at 4 °C and then 10 min at 30 °C before enzyme activity was determined by the addition of 3 mM-$NADP^+$ to the reaction mixture. The specific activity, using 0.35 μg of enzyme, was 67 units/mg of protein (= 100%).

Addition to the assay mixture	Concn. (mM)	Relative activity (%) (*a*)	Relative activity (%) (*b*)
No addition	–	100	16
$CuSO_4$	0.1	34	5
p-Hydroxymercuribenzoate	0.01	0*	0*
N-Ethylmaleimide	0.05	100	–
	1.0	58	23†
Iodoacetate	0.1	61	–
	1.0	58	–
	10.0	54	30†
EDTA	0.5	104	18
8-Hydroxyquinoline	0.05	100	–
1,10-Phenanthroline	0.05	76	–
	0.5	61	14
2,2′-Dipyridyl	0.05	75	–
	0.5	67	9
Dithiothreitol	1.0	97	64
Mercaptoethanol	1.0	100	73

* After incubation with 3 mM-dithiothreitol for 10 min, 45% (*a*) and 23% (*b*) of the activity was recovered.

† After incubation with 3 mM-dithiothreitol for 10 min, no further activity was recovered.

possessing a cycloalkene rather than a cycloalkane ring. Morphine dehydrogenase could also reduce codeinone (**V**) to codeine (**II**) using NADPH as an electron donor. Production of codeine (**II**) was confirmed by h.p.l.c. with reference to authentic standards.

Effect of metals, chelating agents, thiol reagents and reducing reagents on morphine dehydrogeanse activity. Various potential inhibitors were tested against morphine dehydrogenase activity (Table 4). The chelating agents EDTA and 8-hydroxyquinoline had little or no effect on the enzyme activity, although the metal-complexing reagents 1,10-phenanthroline and 2,2′-dipyridyl caused inhibition. The metal copper significantly inhibited morphine dehydrogenase activity. Strong inhibition was achieved by the addition of the thiol-blocking reagent *p*-hydroxymercuribenzoate, which could be partially reversed by the addition of excess dithiothreitol. In contrast, *N*-ethylmalemide and iodoacetate could only inhibit activity when present at much larger concentrations, and protection from inhibition could be afforded by the presence of morphine in the incubation mixture (results not shown). The sensitivity of morphine dehydrogenase to *p*-hydroxymercuribenzoate implicates the importance of one or more reduced cysteine residues, possibly at or near the active site. Morphine dehydrogenase activity was considerably stabilized by the addition of the thiol reagents mercaptoethanol and dithiothreitol, with > 84% activity lost over 16 h in their absence.

Absorption spectra. No absorption maxima were observed when purified morphine dehydrogenase was scanned above 340 nm, suggesting that the enzyme does not contain a bound flavin, cytochrome or pyrroloquinoline quinone cofactor.

***N*-Terminal amino acid sequence.** The *N*-terminal amino acid sequence of morphine dehydrogenase was determined to be:

Ala-Gly-Lys-Ser-Pro-Leu-Ile-Asn-Leu-Asn-Asn-Gly-Val-Lys-Met-Pro-Ala-Leu-Gly-Leu-Gly-Val-Phe-Ala-Ala

Identification of the *N*-terminal sequence of morphine dehydrogenase enabled us to probe the protein sequence databases for dehydrogenases or other proteins that showed sequence identity. However, no significant sequence identity was encountered.

Kinetic properties of morphine dehydrogenase

Initial rates of oxidation of morphine and codeine were determined spectrophotometrically using reaction mixtures with all the components at the concentration of the standard assay (3.0 mM-$NADP^+$), except for the alkaloid substrates, which were varied within the range 0.15–2.0 mM for morphine, 0.015–0.5 mM for codeine and 0.5–10 mM for dihydrocodeine. Double-reciprocal and Eadie–Hofstee plots were linear throughout this range, and regression analysis of the data gave apparent K_m values of 0.46 mM, 0.044 mM and 2.91 mM for morphine, codeine and dihydrocodeine respectively.

The apparent K_m for $NADP^+$ was also measured using reaction mixtures with all the components of the standard assay. The Michaelis constant obtained for $NADP^+$ was 0.35 mM.

DISCUSSION

The majority of microbial alcohol and aldehyde dehydrogenases are inducible (MacKintosh & Fewson, 1987). It was therefore surprising that assays of the catabolic morphine dehydrogenase, in extracts of *Pseudomonas putida* M10 grown on various substrates, showed that the enzyme was constitutive (Bruce *et al.*, 1990; see also Table 1). This observation was supported by PAGE of cell-free extracts from cells grown solely on the substrates glucose and diacetylmorphine; activity staining revealed a single dehydrogenase band migrating to the same position in each lane on the gel (results not shown). It is not clear why this organism constitutively synthesizes morphine dehydrogenase. It was considered conceivable that the enzyme might be a non-specific alcohol dehydrogenase; however, recent studies with the purified enzyme have shown that this is not the case. Preliminary evidence seems to suggest that the second enzyme in the catabolic pathway is also constitutive (N. C. Bruce, unpublished work). Such a metabolic burden would place the organism with a distinct disadvantage in the natural environment. It might therefore be suspected that the prolonged selection process, which acts on the population to favour those variants with the faster growth rate, may well have resulted in a constitutive mutant of *P. putida* M10.

The enzyme was rapidly purified 1216-fold from *P. putida* M10 by applying two selective affinity-chromatography steps on immobilized dyes, namely Mimetic Orange 3 and Mimetic Red 2. The enzyme was homogeneous, as judged by both denaturing and non-denaturing PAGE. The M_r value for native morphine dehydrogenase determined by gel filtration was 32000. When the enzyme was analysed under denaturing conditions on PAGE, morphine dehydrogenase was found to be a monomer with an M_r of 31000. A very low M_r is, however, not unusual for catabolic dehydrogenases. Bacterial aromatic alcohol and aldehyde dehydrogenases have M_r values that vary substantially, with native M_r values ranging from as low as 27000 to as high as 200000 (MacKintosh & Fewson, 1987). Further, small monomeric

NAD^+-dependent dehydrogenases have been shown to mediate the catabolic hydro-aromatic pathways in micro-organisms. The NAD^+-dependent quinate dehydrogenase in *Rhodococcus rhodochrous* is known to have an M_r value of about 31 500 (Bruce & Cain, 1990), whereas the quinate dehydrogenase in *Neurospora crassa* has an M_r of 41 000 (Barea & Giles, 1978).

Purified morphine dehydrogenase was highly thermolabile ($t_{\frac{1}{2}} = 6.5$ min at 50 °C) and was found to lose activity rapidly during purification if thiol-protecting agents such as dithiothreitol were not included in all the elution buffers. Morphine dehydrogenase was inhibited by the metal-complexing reagents 2,2′-dipyridyl and 1,10-phenanthroline (Table 4), suggesting that a metal centre may either be involved at the active site for the oxidation of morphine or is necessary for the stability of the enzyme. However, much more detailed work is required before this can be established. Interestingly, the morphine dehydrogenase is quite distinct from most bacterial aromatic alcohol and aldehyde dehydrogenases, which are insensitive to inhibition by metal-chelating agents (MacKintosh & Fewson, 1987). The thiol-blocking reagent *p*-hydroxymercuribenzoate was highly inhibitory to *P. putida* M10 morphine dehydrogenase, possibly indicating the presence of functional thiol groups at the active site. However, both the alkylating agents iodoacetate and *N*-ethylmaleimide were less inhibitory.

Morphine dehydrogenase showed an absolute requirement for a nicotinamide nucleotide cofactor for the oxidation of morphine and codeine and was completely inactive when NAD^+ replaced $NADP^+$ in the reaction mixture. Furthermore, the purified enzyme from *P. putida* M10 showed a high degree of substrate specificity, oxidizing the C-6 hydroxy group of morphine and codeine to the corresponding carbonyl groups, giving morphinone and codeinone respectively. Interestingly, kinetic studies revealed that morphine dehydrogenase has a > 10-fold greater apparent affinity for codeine, which differs from morphine only by being methoxylated at the C-3 hydroxy group. It might be expected that differences should occur in the affinity of the purified enzyme for the substrates codeine and dihydrocodeine (Table 3; Fig. 3, compounds **II** and **III**). Dihydrocodeine differs significantly in configuration, since it possesses a cyclohexane ring, as opposed to the cyclohexene ring of codeine and morphine. No activity was observed with primary, secondary, cyclic or aromatic alcohols, showing that the constitutive morphine dehydrogenase from *P. putida* M10 is quite distinct from the general $NADP^+$-dependent alcohol dehydrogenase found in many organisms that falls into the category EC 1.1.1.2. Furthermore, morphine dehydrogenase showed no activity against the steroids testosterone and androsterone and is thus also distinct from the NAD^+-dependent hydroxysteroid dehydrogenase (EC 1.1.1.50 and 1.1.1.51) from *Pseudomonas testosteroni*, which Liras *et al.* (1975) showed to oxidize codeine readily to codeinone. Scrutiny of the protein sequence databases revealed no dehydrogenase or other enzyme exhibiting significant sequence identity with the *N*-terminal amino acid sequence of morphine dehydrogenase from *P. putida* M10.

In conclusion, the above results and the observation that morphinone is further degraded by crude cell extracts of *P. putida* M10 (Bruce *et al.*, 1990) suggest that this novel constitutive $NADP^+$-dependent alkaloid dehydrogenase mediates the first step in the catabolism of morphine in *P. putida* M10. However, only a morphine dehydrogenase-negative mutant of *P. putida* M10, incapable of utilizing morphine as its sole carbon and energy source, can demonstrate this unequivocally.

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