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Lupanine hydroxylase, a quinocytochrome *c* from an alkaloid-degrading *Pseudomonas* sp.

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Lupanine 17-hydroxylase, the first enzyme in the pathway for bacterial degradation of the alkaloid, lupanine, was purified from a *Pseudomonas* sp. The enzyme acts by initial dehydrogenation of the substrate, and cytochrome *c* was used as electron acceptor in assays. It had an M_r of 66000 by ultracentrifuge studies and 74000 by gel filtration. The visible absorption spectrum was that of a cytochrome *c*, and a stoicheiometry of one haem group per molecule of enzyme was calculated. SDS/PAGE gave a single band of M_r 72000 containing the haem group. The enzyme also contained pyrroloquinoline quinone (PQQ), which could be removed by isoelectric focusing. The apoenzyme was reconstituted to full activity with addition of PQQ, and a stoicheiometry of one molecule of PQQ per molecule of enzyme was calculated. Steady-state kinetics gave values of 3.6 μM for the K_m for lupanine, 21.3 μM for the K_m for cytochrome *c* and 217 s^{-1} for the $K_{cat.}$.

INTRODUCTION

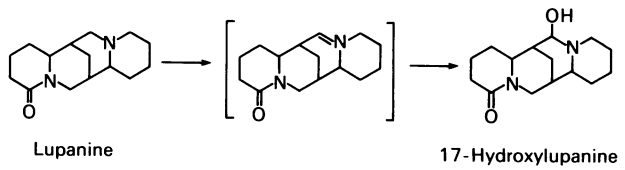
Lupanine is a quinolizidine alkaloid and is particularly abundant in species of the higher-plant genus *Lupinus* (lupins). It can be degraded by bacteria, and the first step in its catabolism by a *Pseudomonas* sp. is hydroxylation to 17-hydroxylupanine (Scheme 1; Toczko *et al.*, 1963). The hydroxylase for this reaction does not appear to be a mono-oxygenase, as it requires an electron acceptor rather than donor, and is thought to act by dehydrogenation of the substrate followed by hydration. A preparation of the enzyme purified almost to homogeneity had the spectrum of a *c*-type cytochrome (Rogozinski, 1975), and in this respect appeared similar to the bacterial anaerobic hydroxylases that hydroxylate the methyl group of *p*-cresol (McIntire *et al.*, 1985; Hopper, 1988) or the methylene group of 4-ethylphenol (Reeve *et al.*, 1989). However, the *p*-cresol methylhydroxylases and 4-ethylphenol methylenehydroxylase are flavocytochromes, with FAD covalently attached to the protein (McIntire *et al.*, 1981), whereas the small amount of peptide-bound flavin found in lupanine hydroxylase was ascribed to contamination of the preparation by some other flavin-containing protein (Rogozinski, 1975). If the enzyme does not contain flavin, then it must have another electron acceptor, because only one haem was found per molecule of lupanine hydroxylase and the reaction involved removal of two electrons from the substrate.

Here we describe the purification of the enzyme to homogeneity, and we show that it contains pyrroloquinoline quinone (PQQ) as the other electron acceptor.

MATERIALS AND METHODS

Organism

The organism was isolated from soil and is capable of growth on lupanine as sole carbon and nitrogen source (Mozejko-Toczko, 1960). It was identified as a *Pseudomonas* sp. and has been referred to as '*Pseudomonas lupanini*'. It produces abundant green–yellow fluorescent pigment on King's B agar, and in a range of cytological and biochemical tests it showed close similarity to *Pseudomonas putida*.



Scheme 1. Hydroxylation of lupanine

Growth of organism

Cells for enzyme purification work were grown in liquid medium containing (per litre) 4.8 g of Na_2HPO_4, 2.4 g of KH_2PO_4, 2.0 g of NH_4Cl, 4.0 ml of salts solution (Rosenberger & Elsden, 1960) and carbon source. Starter cultures of 100 ml in 250 ml Erlenmeyer flasks contained 0.05 g of lupanine as carbon source and were incubated at 30 °C on an orbital shaker. When grown, one of these was used to inoculate a larger volume in a 20-litre LHE 1/1000 fermenter (LH Fermentation, Maidenhead, Berks., U.K.) at 30 °C, with an aeration rate of 10 litres/min and a stirring rate of 300 rev./min. To make best use of the limited amounts of lupanine, succinate (2 g/l) together with lupanine (0.5 g/l) were added as carbon sources. When growth slowed, a further portion (0.5 g/l) of lupanine was added and, after growth on this, the cells were harvested with an Alfa Laval continuous-flow centrifuge. For the purification described the medium also contained 1 μM-PQQ. This was added as a filter-sterilized solution after autoclaving of the medium. The cell paste was stored at −20 °C.

Enzyme assay

The enzyme was assayed at 25 °C using horse heart cytochrome *c* as electron acceptor and monitoring the increase in A_{550} as it is reduced [$\Delta\epsilon_{mM}$(reduced − oxidized) = 21.1]. The reaction mixture in a 1 cm-light-path cuvette contained, in 1 ml of 10 mM-Tris/HCl buffer, pH 8.5 (I 0.01), 50 μM-cytochrome *c*, 200 μM-lupanine and enzyme. The rate decreases markedly with increasing ionic strength of the buffer. For assays of crude cell extracts 1 mM-KCN was included in the mixture to reduce reoxidation of reduced cytochrome *c* by membrane fragments. One unit of enzyme activity is defined as that amount of enzyme required to reduce 2 μmol of cytochrome *c*/min.

For kinetic studies the reaction mixtures contained cytochrome *c* in the range 6–60 μM and lupanine 1.5–5 μM and 0.93 pmol of enzyme. The data were analysed to obtain $V_{max.}$ and K_m values and their standard errors by using a non-linear-regression-

Abbreviation used: PQQ, pyrroloquinoline quinone.

analysis computer program ENZFITTER (Leatherbarrow, 1987).

Enzyme purification

All buffers used during purification contained 1 mM-2-mercaptoethanol.

Crude cell extract was prepared by thawing and resuspending frozen cells in 20 mM-Tris/HCl buffer, pH 8.0 (3 ml/g wet wt.), followed by disruption using ultrasonic disintegration at 4 °C with an MSE Soniprobe 150 in 30 s bursts for a total of 4 min. The extract was centrifuged at 22000 *g* for 15 min at 4 °C, and then the supernatant solution was centrifuged at 160000 *g* for 1 h at 4 °C, giving a supernatant solution that is termed 'crude extract'.

Crude extract from 60–70 g of cells was loaded on to a DE-52 DEAE-cellulose column (18 cm long × 5 cm diam.) pre-equilibrated with 20 mM-Tris/HCl buffer, pH 8.0. The column was washed with 1 column volume of buffer and then eluted with a linear gradient of 0–0.3 M-KCl in 2 litres of buffer; 10 ml fractions were collected. Fractions 89–121 were pooled.

The pooled fractions were concentrated to approx. 50 ml with a Minitran ultrafiltration system using a 30000 NMWL Polysulfone filter, and then to approx. 5 ml in an Amincon Ultrafiltration cell with a PM10 membrane. This was loaded on to a gel-filtration column of Sephacryl S-200 (91 cm long × 2.5 cm diam.) and eluted with 0.1 M-Tris/HCl buffer, pH 8.0; 5 ml fractions were collected. Fractions 40–47 were pooled.

The pooled fractions were dialysed against 2 litres of 1 mM-sodium/potassium phosphate buffer, pH 7.0, for 8 h with changes of buffer every 2 h, and then loaded on to a hydroxyapatite column (4.5 cm long × 2.5 cm diam.) equilibrated with 1 mM-sodium/potassium phosphate buffer, pH 7.0. The column was eluted with a linear gradient of 1–200 mM-sodium/potassium phosphate buffer, pH 7.0, in 250 ml; 3 ml fractions were collected. Fractions 38–47 were pooled.

The enzyme solution was concentrated to 8 ml in an Amicon Ultrafiltration cell with a PM10 membrane and then dialysed against 2 litres of 20 mM-Tris/HCl buffer, pH 8.0, for 4 h with one change of buffer after 2 h. The final step was by f.p.l.c. on a Hiload 16/10 Q Sepharose high-performance column (Pharmacia, Milton Keynes, Bucks., U.K.). It was eluted with a linear gradient of 0–0.4 M-KCl in 200 ml of 20 mM-Tris/HCl buffer, pH 8.0, at a flow rate of 2 ml/min; 2 ml fractions were collected. Fractions 60–63 were pooled.

Protein assays

Proteins were determined by the tannic acid turbidometric method of Mejbaum-Katzenellenbogen & Dobryszycka (1959), with BSA as standard. This method gives a linear relationship between A_{700} and protein concentration up to 100 μg of protein/ml. Elution of proteins from columns was monitored by A_{280}.

PAGE

Protein purity was assessed by electrophoresis on non-denaturing 7.5% (w/v) and 10% (w/v) polyacrylamide gels. Proteins were detected by staining gels with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol/water/acetic acid (5:4:1, by vol.) for 2 h and then destaining in ethanol/water/acetic acid (3:6:1, by vol.). Enzyme activity was detected by incubating gels in 15 mM-Tris/HCl, pH 7.6, containing 0.2 mM-lupanine, 0.05 mM-cytochrome *c*, 0.04 mM-PQQ and 2 mg/ml of Thiazoyl Blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylmonotetrazolium bromide; 'MTT']. This gave a blue colour with active enzyme.

Studies of subunit structure were performed by SDS/PAGE (Laemmli, 1970). Gels were stained for protein as described above and for haem by the method of Goodhew *et al.* (1986).

Isoelectric focusing

Preparative isoelectric focusing was performed with Sephadex G 200 SF as the support matrix. The Sephadex was swollen in a solution of 0.8 ml of pH 2.5–5.0, 0.8 ml of pH 5.8 and 0.5 ml of pH 3–10 Pharmalytes (Pharmacia ampholytes) in 33 ml of water. This was poured into a 10 cm × 8 cm gel casting mould and dried to give a firm gel. The gel was prefocused for 2 h at 250 V and, after addition of enzyme, was focused for a further 6 h at 250 V. Protein was recovered by removing the appropriate band from the gel and eluting the Sephadex with 20 mM-Tris/HCl buffer, pH 8.5, containing 1 mM-2-mercaptoethanol. The pH gradient was determined by measuring the pH of samples of the gel taken at 5 mm intervals after suspension in 2 ml of distilled water.

Determination of M_r

The M_r of the native protein was determined by gel filtration on a Sephacryl S-200 column (77 cm × 2.5 cm diam.) equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0. The column was calibrated with alcohol dehydrogenase (from yeast; M_r 150000), lactate dehydrogenase (from rabbit muscle; M_r 140000), BSA (M_r 66000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 29000), myoglobin (M_r 17000) and cytochrome *c* (M_r 12500).

The M_r was also determined by sedimentation-equilibrium experiments using a Beckman Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics. Before being centrifuged, samples were dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-2-mercaptoethanol, for 24 h at 4 °C. Meniscus-depletion experiments were performed according to the method of Yphantis (1964) at 26270 rev./min at 20 °C with an initial protein concentration of 0.52 mg/ml. A value of 0.73 was assumed for the partial specific volume of the protein.

For subunit M_r measurements, polyacrylamide gels were calibrated by electrophoresis of mixtures of protein M_r markers (30000–200000 and 14000–70000; Sigma Chemical Co. Ltd., Poole, Dorset, U.K.).

Spectra

Visible and u.v.-absorption spectra were recorded on a Uvikon 930 spectrophotometer (Kontron Instruments Ltd., Watford, Herts., U.K.).

Extraction and assay of PQQ

PQQ was detected and assayed by measuring the extent of reconstitution of glucose dehydrogenase (EC 1.1.99.17) apoenzyme originating from *Acinetobacter calcoaceticus* LMD 79.41 (van der Meer *et al.*, 1990). The apoenzyme came from an *Escherichia coli* strain which contains a plasmid with the gene for this enzyme and was a gift from Dr. J. A. Duine (Laboratory of Biotechnology, Delft University of Technology, 2628 BC Delft, The Netherlands).

The protein was extracted by addition of an equal volume of 1 M-sodium phosphate buffer, pH 1, followed by 5 vol. of methanol. After 1 h at room temperature the sample was centrifuged at 16000 *g* for 5 min to remove precipitated material. The methanol was removed by evaporation and the sample diluted to bring the PQQ concentration within the range of the calibration curve. Alternatively holoenzyme (50 μl) was denatured by incubating at 90 °C for 1 h with 150 μl of 0.1 M-sodium phosphate buffer, pH 6.6, containing 1% (w/v) SDS. This was centrifuged at 16000 *g* for 5 min. The supernatant solution was diluted to 20 ml with distilled water, and 20 μl of the diluted solution were used in the assay.

Chemicals

Lupanine was extracted from the seeds of *Lupinus angustifolius* by treating the minced seeds with 0.1 M-HCl. The HCl was strained through muslin, made alkaline and continuously extracted with diethyl ether. Evaporation of the ether gave a syrup containing mainly lupanine, but contaminated with other alkaloids such as 13-hydroxylupanine and angustifolin. This crude lupanine was used for large-scale growth of the organism. Alternatively, ground seeds were extracted with methanol and, after removal of the solvent and treatment with anhydrous Na_2SO_4, the lupanine was extracted with light petroleum (b.p. 30–60 °C). The petroleum was removed and the lupanine was precipitated from a methanol solution with $HClO_4$. The lupanine perchlorate crystals were recrystallized twice from water. This pure lupanine was used for small-scale growth of the organism and for enzyme assays.

PQQ was obtained from Fluka Chemicals Ltd. (Glossop, Derbyshire, U.K.) or as gifts from Dr. W. S. McIntire and Dr. J. A. Duine. Thiazoyl Blue was from Mann Research Laboratories (New York, NY, U.S.A.).

RESULTS

Purification of enzyme

Details of the purification of the enzyme are summarized in Table 1. This was from cells grown with PQQ added to the medium and yielded enzyme that required no added PQQ for full activity at any stage during the purification. Bacteria grown in the absence of PQQ gave variable results, sometimes giving enzyme requiring the addition of PQQ for full activity and leading to apparent multiple forms of the enzyme. Such preparations were assayed after a short preincubation with 0.4 mM-PQQ. Those preparations that could be stimulated by PQQ gave no increase in activity under similar conditions with FMN or FAD. The small apparent increase in number of units after the first step in the purification probably reflects incomplete inhibition of reoxidation of cytochrome *c* by remaining membrane fragments in crude extracts.

Electrophoresis of the pure enzyme on two gels containing different percentages of acrylamide gave a single band when stained with Coomassie Brillient Blue. This corresponded to the single band seen when gels were stained for enzyme activity.

Absorption spectrum

The purified enzyme was pink in colour and its u.v.–visible absorption spectrum is shown in Fig. 1. The visible spectrum with peaks 551, 552 and 416 nm is typical of that for a reduced cytochrome *c*, as too are the absorption bands in the u.v. between 300 and 350 nm. Oxidation with potassium ferricyanide resulted in a shift of the 416 nm peak to 412 nm, with a lowering of its intensity and the replacement of the other two peaks by a single peak at 528 nm. These changes are also characteristic of a cytochrome. The presence of flavin would be expected to give a shoulder at 450 nm in the oxidized spectrum, but no shoulder was observed. From the absorbance at 416 nm or 551 nm and using the absorbance coefficients for cytochrome *c* (Van Gelder & Slater, 1962) a ratio of one haem group per molecule of enzyme was calculated.

M_r of the enzyme

Gel filtration of the purified enzyme on a calibrated Sephacryl S-200 column gave an M_r of 74000. Meniscus-depletion sedimentation-equilibrium studies in the ultracentrifuge gave a value of 66000.

SDS/PAGE of the enzyme after pretreatment with 2-mercaptoethanol and SDS at 100 °C for 1 h gave a single protein band equivalent to an M_r of 72000. A single band corresponding to this M_r was also seen when a gel was stained for haem.

Preparative isoelectric focusing of the enzyme

Pure enzyme (2 mg) was subjected to preparative isoelectric focusing. After 6 h, two coloured bands could be seen, a red one at pI 5.3 and the other a pink one at pI 5.0. The bands were eluted and their spectra recorded. The red band gave a spectrum of oxidized enzyme and constituted about 65% of the total enzyme, whereas the pink band, about 35% of the total enzyme, gave the spectrum of reduced enzyme.

When assayed for lupanine hydroxylase activity the enzyme from the pink band had a specific activity of 68 units/mg of protein, comparable with that of pure enzyme. Preincubation of this enzyme with PQQ (4 μM) gave no increase in activity. By contrast, the enzyme from the red band was almost inactive, with a specific activity of less than 0.1 unit/mg. However, incubation with PQQ (4 μM) resulted in a large increase in activity, restoring the specific activity to that of the purified enzyme. Thus it appeared that isoelectric focusing resulted in loss of PQQ from the enzyme, and this was confirmed by procedures to release and assay the PQQ from the protein of the two bands. PQQ was

Table 1. Summary of the purification of lupanine hydroxylase from a *Pseudomonas* sp.

The hydroxylase was purified from 64 g of cells grown as described in the Materials and methods section. A unit of activity is that amount which will oxidize 1 μmol of substrate/min.

Purification step	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	217	3038	0.33	100	1
DEAE-cellulose chromatography	320	608	1.85	112	5.6
Sephacryl S-200 gel filtration	39	159	4.96	78.5	15
Hydroxyapatite chromatography	28	30.5	25.87	78.6	78.4
HL Q Sepharose HP f.p.l.c.	8	5.48	69.7	38	211

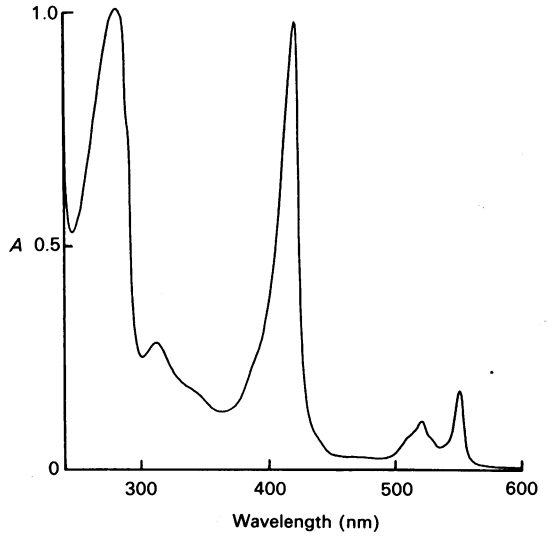


Fig. 1. U.v.–visible absorption spectra of purified lupanine hydroxylase

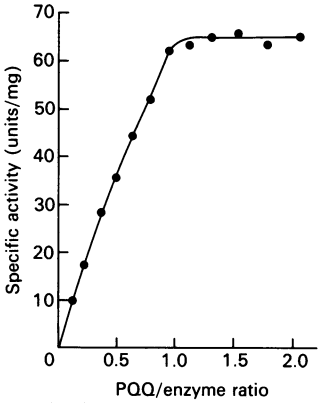


Fig. 2. Stoicheiometry of reconstitution of apoenzyme with PQQ

To a 34.9 μM solution of inactive apoenzyme in ice was added small volumes of 0.76 mM-PQQ. After each addition the mixture was incubated for 5 min and then assayed for enzyme activity.

assayed by its ability to reconstitute the activity of glucose dehydrogenase apoenzyme (van der Meer *et al.*, 1990). Samples of each protein were extracted with acid and methanol and portions of the extracts were incubated with the glucose dehydrogenase apoenzyme, which was then assayed for activity. The extract from the inactive, red-band, protein resulted in no detectable activation, whereas that from the active, pink-band, protein gave high activity. On a quantitative basis there was less than 0.01 μmol of PQQ extracted/μmol of the inactive enzyme, but 0.66 μmol of PQQ/μmol of active enzyme. Even better extraction of PQQ was obtained by heating the holoenzyme with SDS, resulting in the release of 0.87 μmol of PQQ/μmol of enzyme.

These results suggest the presence of one molecule of PQQ per molecule of enzyme, and this was confirmed by titration of the inactive preparation of the enzyme with PQQ and monitoring the restoration of activity. Full activity was regained at a ratio of added PQQ to enzyme of 1:1 (Fig. 2).

When monitored spectrophotometrically the haem group of the inactive oxidized enzyme from isoelectric focusing was not reduced on addition of lupanine. However, a further addition of PQQ resulted in the rapid reduction of the haem.

Steady-state kinetics

The enzyme was assayed over a range of pH from 7 to 10 in sodium/potassium phosphate, Tris/HCl and glycine/NaOH buffers. The highest activity was seen in Tris/HCl buffer at a pH optimum of 8.5.

A full two-substrate steady-state kinetic analysis of the enzyme was performed. The double-reciprocal primary plots of the results gave almost parallel lines, and the K_m values were 3.6 ± 0.7 μM and 21.3 ± 0.7 μM for lupanine and cytochrome *c* respectively. The $k_{cat.}$ value under the conditions of this assay was 217 ± 3 s^{-1}.

DISCUSSION

Lupanine hydroxylase was purified to homogeneity by the procedure described here. The enzyme was purer, as judged by electrophoresis and the relative heights of peaks at 280 nm and 416 nm in its spectrum, than that previously described (Rogozinski, 1975). It also had a much higher specific activity of 69.7 units/mg compared with 3.7 units/mg. This may, in part, be due to the realization, in the current work, of the importance of PQQ to enzyme activity and also to the different assay procedure used. Here the enzyme has been assayed with cytochrome *c* as acceptor and at its optimum pH of 8.5. Under the conditions used the enzyme showed low K_m values for both lupanine and cytochrome *c*, and the pattern of primary plots were suggestive of a Ping Pong mechanism.

Some of the properties of the enzyme reported previously have been confirmed here. A M_r of about 70000 was also found by Rogozinski (1975), who also calculated the presence of one haem group per molecule of enzyme. The spectrum is typical for a *c*-type cytochrome and, as expected for such a protein, the haem is not lost even during SDS/PAGE. The SDS/PAGE results suggest that the enzyme is monomeric, as this gave a single band corresponding to the same M_r as the native protein, despite prolonged preincubation at high temperature with SDS and 2-mercaptoethanol. The same band contained the haem group, discounting the presence of a separate low-M_r cytochrome *c*, as is found in some flavocytochromes *c* such as the *p*-cresol methylhydroxylases from various *Pseudomonas* species (Shamala *et al.*, 1986; Koerber *et al.*, 1985) and sulphide:cytochrome *c* oxidoreductase from *Chromatium* (Meyer *et al.*, 1985).

There was no evidence from spectra that the enzyme contained flavin, nor was apoenzyme stimulated by FMN or FAD. Again this contrasts with the anaerobic hydroxylase, *p*-cresol methylhydroxylase, which contains covalently bound FAD (McIntire *et al.*, 1980). However, evidence was obtained for the presence of the redox cofactor, PQQ, in the lupanine hydroxylase. This was not apparent from the spectrum of the enzyme because of the presence of the haem group, but was suggested by the finding that some preparations could be stimulated by added PQQ. This was confirmed by showing that inactive apoenzyme, produced by isoelectric focusing, contained no PQQ, whereas active enzyme contained the cofactor in amounts approaching 1 molecule per molecule of enzyme. The apoenzyme activity was fully reconstituted by addition of PQQ and the results confirmed a stoicheiometry of 1 molecule per molecule of enzyme (Fig. 2). This presumably acts as the primary electron acceptor, as there was no reduction of the haem in the presence of substrate unless PQQ was present.

Other quinocytochromes *c* have been described, including an amine dehydrogenase from a *Pseudomonas* sp. (Shinagawa *et al.*, 1988). This enzyme, however, consists of two non-identical subunits, a dehydrogenase of M_r 60000 and a cytochrome *c* of M_r 39000, and is reported to have covalently bound PQQ. Lupanine hydroxylase more closely resembles the alcohol dehydrogenase from *Pseudomonas testosteroni* (Groen *et al.*, 1986), which is monomeric with a M_r of 67000, contains a single haem group and requires PQQ for activity. Unlike the alcohol dehydrogenase the lupanine hydroxylase has no requirement for Ca^{2+} ions.

PQQ has now been identified as a component of a number of bacterial enzymes and particularly of dehydrogenases (Duine *et al.*, 1986). Lupanine hydroxylase, too, probably acts by dehydrogenation of the substrate, and hydroxylation is achieved by hydration of the product.

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