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Isolation and some properties of lysine N^6-hydroxylase from *Escherichia coli* strain EN222

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Summary. Lysine N^6-hydroxylase was isolated as a soluble enzyme from the supernatant after ultrasonication of *Escherichia coli* strain EN222 which contained the structural gene on a multicopy plasmid (as described by Engelbrecht and Braun in 1986). The apoenzyme prepared by dialysis was purified by ammonium sulfate precipitation and fast protein liquid chromatography using Superose 12 and Mono Q columns. The molecular mass as determined by gel filtration was 200 kDa and 50 kDa by SDS/polyacrylamide gel electrophoresis. The enzyme binds 0.79 molecule FAD/50 kDa. The activity of the enzyme is strictly dependent on NADPH. Its properties are similar to other flavoprotein monooxygenases of the EC group 1.14.13.

Key words: Lysine N^6-hydroxylase — External flavoprotein (FAD) monooxygenase — Aerobactin — *Escherichia coli*

Introduction

In microorganisms, siderophores of the hydroxamate type are widely distributed (Neilands 1981). The first step in biosynthesis is the hydroxylation of a primary amino group, in the case of aerobactin biosynthesis the hydroxylation of L-lysine (Gross et al. 1985; Lorenzo et al. 1986). The cloned gene for the lysine N^6-hydroxylase was termed *aerA* by Gross et al. (1984) and *iucD* by Lorenzo et al. (1986); the gene product, showing a molecular mass of approximately 50 kDa in SDS/PAGE, was expressed in minicells. The nucleotide sequence of *iucD* was determined and the primary structure of the polypeptide deduced (Herrero et al. 1988). Little is known about the properties of the enzyme.

Earlier studies with cell-free systems of *Aerobacter aerogenes* 62-1 (Murray et al. 1977; Parniak et al. 1979) were hampered by low enzyme activities and the presence of all four enzymes of the biosynthetic sequence. The construction of strains in which single genes were cloned on plasmids opened the opportunity for studying the *N*-hydroxylase without interference with the second enzyme, *N*-transacetylase. In a previous study (Heydel et al. 1987), the activities of both enzymes were determined in a series of strains and a method for the cultivation in larger vessels was developed. In contrast to other strains where the enzyme was tightly bound to the cytoplasmic membrane and had to be solubilized, with strain EN222 (Engelbrecht and Braun 1986) most of the enzyme activity was soluble, and therefore this strain was selected for the characterization of the *N*-hydroxylase.

Materials and methods

Cultures and maintenance. Escherichia coli strain GR111 (plasmid pRG111, on pBR322, ampicillin-resistant, genotype $aerA^+B^-C^-D^+$; host *E. coli* H1443 $aroB^-$, source: K. Hantke) and strain EN222 (same plasmid as in strain GR111; host *E. coli* K12 lys^-) were kindly provided by V. Braun (Gross et al. 1984; Engelbrecht and Braun 1986). Strain maintenance, cultivation and cell disruption were described in an earlier publication (Heydel et al. 1987).

Enzyme assay (hydroxylysine production). To 200 µl 0.5 M potassium phosphate buffer pH 7.0 was added 50 µl 10 mM NADPH, 10 µl 0.5 mM FAD, 10 µl enzyme solution (containing about 0.1 mg protein in the case of the cell-free extract) and 580 µl deionized water. The reaction was started by adding 150 µl 10 mM L-lysine · HCl. Incubation was for 30 min at 37° C in microtiter plates (Nunclon) on a transversal shaker

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at 120 strokes/min. To stop the reaction, 0.5 ml 0.2 M perchloric acid was added, the suspension cooled in an ice bath for 15 min and centrifuged in 1.5-ml plastic vials in an Eppendorf centrifuge for 5 min. For control, 0.5 ml 0.2 M perchloric acid was added before the enzyme solution. 1 ml of the supernatant was used for the iodine oxidation test according to Csaky (1948) in the modification described earlier (Tomlinson et al. 1971; Heydel et al. 1987). An absorption coefficient of $\varepsilon = 17 \pm 0.32\ mM^{-1}\ cm^{-1}$ was used to calculate the amount of *N*-hydroxylysine formed in the test.

Enzyme assay (NADPH oxidation). The test composition was the same as described above. The mixture was filled into cuvettes of $d = 0.5$ cm and incubated at 37° C. After 2 min the reaction was started and the decrease in absorbance at 366 nm was measured. For the calculation of enzyme activity the decrease in NADPH absorbance in a blank containing the test mixture without added lysine was subtracted.

Enzyme purification. The cell-free extract (10 mg protein/ml) was dialyzed in a Visking tube 20/32 (from Serva, Heidelberg) against 0.1 M potassium phosphate buffer pH 7.0 containing 10 mM EDTA and 1.6 mM dithiothreitol overnight.

Chromatography. All column separations were performed with an FPLC unit from Pharmacia, consisting of a controller LCC-250, two pumps P-500, fraction collector Frac-100, ultraviolet-monitor UV-1. The buffer used for chromatography was 50 mM Tris/HCl pH 7.5 containing 1.6 mM dithiothreitol and 10% glycerol (by vol.). For the separation on Mono Q, 100 mM KCl was added to the same buffer and the enzyme eluted by a KCl gradient of 100–500 mM. The Superose 12 column was calibrated using a protein molecular mass standards kit, MSII (cytochrome *c* 12.3 kDa, *β*-lactoglobulin 35.5 kDa, ovalbumin 45 kDa, bovine serum albumin 67 kDa, rabbit aldolase 160 kDa, bovine catalase 232 kDa, horse ferritin 480 kDa) from Serva, Heidelberg. The molecular mass was determined by graphically plotting elution volumes against the logarithms of molecular mass.

Electrophoresis. SDS slab gel electrophoresis was performed according to Laemmli and Favre (1973) and Ames (1974) in a chamber according to Studier (1973) at room temperature. All chemicals used were from Bio-Rad Laboratories, München. Calibration proteins (LMW calibration kit, *α*-lactalbumin 14.4 kDa, trypsin inhibitor 20.1 kDa, carbonic anhydrase 30 kDa, ovalbumin 43 kDa, bovine serum albumin 67 kDa, phosphorylase *b* 94 kDa) were from Pharmacia, Freiburg. Protein was determined according to Lowry et al. (1951).

Chemicals. NADPH was from Boehringer Mannheim, ECTEOLA-cellulose from Serva Heidelberg and Bio-Gel P-2 from Bio-Rad München. L-Lysine and L-ornithine were from Merck Darmstadt, FAD, DL-2,6-diaminopimelic acid, DL-2,3-diaminopropionic acid, N^6-acetyl-L-lysine, dithiothreitol and *p*-chloromercuribenzoic acid from Sigma, St. Louis.

Results

In a preliminary search (Pfefferle 1987) for cofactors of the monooxygenase reaction, first evidence for the dependence of the reaction on NADPH as an electron donor came from the experiment shown in Table 1.

Table 1. Specific enzyme activities after addition of cofactors (all 1 mM except Fe^{2+} 0.5 mM) to the *N*-hydroxylase test (Heydel et al. 1987) in cell-free extract from *E. coli* GR111

Cofactors	Specific activity ($nmol \cdot mg^{-1} \cdot min^{-1}$)
Fe^{2+}, ascorbate, 2-oxoglutarate	1.07
Fe^{2+}, ascorbate	1.04
Fe^{2+}, NADPH	1.89
Fe^{2+}, NADH	0.94
Control (no cofactor addition)	1.45

In the experiments with the crude extract a slight increase in enzyme activity by 0.5 mM Fe^{2+} was observed. With the purified enzyme from *Escherichia coli* EN222 no activation or inhibition by Fe^{2+} was found at concentrations over 1–100 μM.

In extracts from *E. coli* EN222, more than 70% of the total activity after sonication was in the supernatant after a 1-h centrifugation at $100000 \times g$. When the cell-free extract was dialyzed overnight, only 20% of the activity remained. The activity could be restored by addition of a protein-free supernatant which had been prepared by boiling the cell-free extract for 5 min at 100° C and removal of the denaturated protein by centrifugation (Table 2).

From the protein-free supernatant, a yellow substance was isolated by chromatography on ECTEOLA-cellulose and gel filtration on Bio-Gel P-2, its absorption spectrum was identical with that of FAD. In the test with the dialysed extract, the protein-free supernatant could be replaced by FAD but not by FMN.

Strain EN222 was cultivated as described earlier (Heydel et al. 1987). The yield was 25 g frozen cells from 10 l culture fluid. The lysine N^6-hydroxylase was purified to homogeneity from 5-g portions of frozen cells using ammonium sulphate fractional precipitation, gel filtration on Superose 12 and ion-exchange chromatography on Mono Q

Table 2. Reconstitution of the activity in the dialyzed extract by the protein-free supernatant

Enzyme preparation	Specific activity ($nmol \cdot mg^{-1} \cdot min^{-1}$)
Cell-free extract	2.4
Dialyzed extract	0.67
Protein-free supernatant (PFS) 50 μl	0.056
Dialysed extract + 50 μl PFS	2.69

Table 3. Purification of lysine N^6-hydroxylase from *E. coli* EN222 starting from the dialyzed crude enzyme

Fraction	Volume (ml)	Protein (mg)	Activity (nmol · min^{-1})		Yield (%)
			(ml^{-1})	(mg^{-1})	
Dialysed enzyme	13	116	103	11.5	100
Ammonium sulphate precipitation (0.2–0.4 satd)	1.84	59	542	17	74
Superose fractions 7–9	4.5	15.8	162	46	55
Mono Q fractions 7+8	1.5	1.6	200	189	23

(Table 3). As an approximately 16-fold enrichement led to the purified enzyme, it amounts to almost 7% of the crude extract protein. The progress of purification to the homogeneous enzyme is demonstrated by SDS/polyacrylamide electrophoresis (Fig. 1).

The molecular mass of the purified FAD-free enzyme was determined by gel filtration on Superose 12 and found to be 200 kDa. The absorption spectrum revealed a symmetric band at 280 nm and gave no indication for the presence of a tightly bound cofactor. After incubation of the apoenzyme with a surplus of FAD, the holoenzyme was eluted from Superose 12 as a homogeneous band. The FAD content was estimated from the absorbance at 450 nm and was calculated as 0.79 molecule FAD/50 kDa.

With the purified enzyme the omission test showed a strict dependence of the lysine hydroxylase for L-lysine, NADPH and FAD (Table 4).

The value of 1.19 nmol/min in the NADPH oxidation test results from the non-enzymic decay of NADPH under test conditions. As the value of

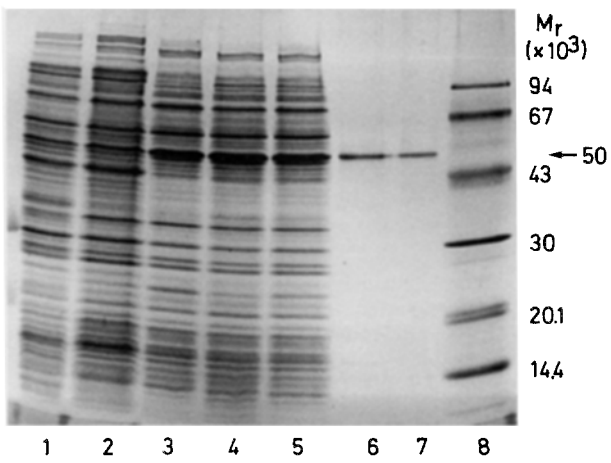

Fig. 1. SDS/PAGE of pools and fractions during purification of lysine N^6-hydroxylase. Lanes: 1, dialysed crude enzyme; 2, ammonium sulphate precipitation; 3 and 4, Superose 12 fractions 7 and 8; 5, pool of fractions 7 and 8; 6 and 7, Mono Q fractions 7 and 8; 8, standard proteins

Table 4. Omission test

Test composition	Hydroxylysine production (nmol/min)	NADPH oxidation (nmol/min)
Control	1.3	5.6
– L-lysine	0	1.73
– NADPH	0	0
– FAD	0.085	1.1
– enzyme	0	1.19

The tests are described in Materials and methods. In measuring NADPH oxidation, the decay of NADPH was not subtracted

NADPH oxidation in the absence of L-lysine (1.73 nmol/min) is somewhat higher, some unspecific NADPH oxidation by the enzyme must be assumed. No hydroxylated product could be detected with D-lysine or L-ornithine as a substrate. On the other hand, L-ornithine as well as DL-2,3-diaminopropionic acid, DL-2,6-diaminopimelic acid and N^6-acetyl-L-lysine stimulated the oxidation of NADPH but remained unchanged during the reaction. *p*-Chloromercuribenzoate inhibited the enzyme markedly (62% inhibition at 10 μM). The inhibition could be reversed by dithiothreitol.

Discussion

Secondary hydroxamates are synthesized by bacteria and fungi. From the early work of Emery (1971) with *Ustilago sphaerogena*, it was known that the precursors are first hydroxylated and then acetylated. Akers and Neilands (1978) demonstrated that the oxygen atom in the hydroxamic acid group of rhodotorulic acid comes from molecular oxygen. The characterization of the enzyme after purification almost to homogeneity led to the conclusion that the bacterial lysine N^6-hydroxylase belongs to the 'external' flavoprotein monooxygenases (Massey and Hemmerich 1975)

and has many common features with other monooxygenases of the EC group 1.14.13.

The best known enzyme of this group is *p*-hydroxybenzoate monooxygenase (EC 1.14.13.2) whose amino acid sequence and crystal structure is known (Wierenga et al. 1982). By comparing the amino acid sequence of this enzyme with those of glutathione reductase (Wierenga et al. 1983) and the *Acinetobacter* cyclohexanone monooxygenase (Chen et al. 1988), it was shown that the two bound nucleotides, FAD and NADPH, are similarly positioned.

The amino acid sequence of lysine N^6-hydroxylase as published by Herrero et al. (1988) shows a similar sequence of 13 amino acids starting from residue 4 at the NH_2 terminus with a consensus sequence Ile-Gly-Val-Gly compared to Ile-Gly-Ala-Gly in *p*-hydroxybenzoate monooxygenase and Ile-Gly-Gly-Gly in cyclohexanone monooxygenase as well as human and *E. coli* glutathione reductase. A sequence Val-Gly-Gly-Gly present at positions 187–190 in lysine N^6-hydroxylase could be part of the NADPH-binding site.

The apparent molecular mass of the native lysine N^6-hydroxylase was found to be 200 kDa as determined by gel filtration, whereas the FAD determination after saturation of the apoenzyme gave an FAD content of 3.16 molecules/200 kDa. SDS gel electrophoresis also indicates the presence of four subunits of molecular mass approximately 50 kDa.

It is remarkable that two other oxygenases exist which attack L-lysine, namely the lysine 2-monooxygenase (EC 1.13.12.2) which is an 'internal' monooxygenase and lysine, 2-oxoglutarate dioxygenase (EC 1.14.11.4) which hydroxylates the C-5 atom of L-lysine peptides. On the other hand, the lysine N^6-hydroxylase is clearly distinct from the *N*-oxide-forming monooxygenase (EC 1.14.13.8) and the drug-hydroxylating liver microsomal enzyme system which contains cytochrome P_{450} and is inhibited by carbon monoxide.

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