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Purification, crystallization and properties of porphobilinogen deaminase from a recombinant strain of *Escherichia coli* K12

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Porphobilinogen deaminase has been purified and crystallized from an overproducing recombinant strain of *Escherichia coli* harbouring a *hemC*-containing plasmid which has permitted the purification of milligram quantities of the enzyme. Determination of the M_r of the enzyme by SDS/polyacrylamide-gel electrophoresis (35000) and gel filtration (32000) agrees with the gene-derived M_r of 33857. The enzyme has a K_m of 19 ± 7 μM, an isoelectric point of 4.5 and an *N*-terminal sequence NH_2-MLDNVLRIAT. The substrate, porphobilinogen, binds to the active-site dipyrromethane cofactor to form three intermediate complexes: ES, ES_2 and ES_3. The gene-derived primary structure of the *E. coli* deaminase is compared with that derived from the cDNA of the human enzyme.

INTRODUCTION

Porphobilinogen deaminase (EC 4.3.1.8), together with uroporphyrinogen III synthase, catalyses the transformation of the pyrrole porphobilinogen into uroporphyrinogen III, the common tetrapyrrole precursor to all haems, chlorophylls and corrins (see Akhtar & Jordan, 1979; Leeper, 1985 for reviews). Porphobilinogen deaminase is responsible for the assembly of the hydroxymethylbilane intermediate, preuroporphyrinogen, which is then rearranged and cyclized by the synthase to yield uroporphyrinogen III (Burton *et al.*, 1979; Battersby *et al.*, 1979*a*; Jordan *et al*, 1979).

The mechanism by which porphobilinogen deaminase catalyses the tetrapolymerization of porphobilinogen has been investigated by several workers, although the precise details are still far from clear. Single turnover reaction studies (Battersby *et al.*, 1979*b*; Jordan & Seehra, 1980) have established the order in which the four substrate molecules are incorporated into the tetrapyrrole product preuroporphyrinogen. Investigations with the purified enzyme from *Rhodobacter sphaeroides* have revealed that the reaction proceeds in discrete stages through covalently bound enzyme–intermediate complexes (Berry *et al.*, 1981). Extension of these studies using the enzyme isolated from recombinant strains of *Escherichia coli* has provided strong evidence that the deaminase contains a dipyrromethane cofactor, linked to cysteine-242, and it is to this that the substrates are covalently attached (Jordan & Warren, 1987). This is contrary to the conclusions, from binding studies with pyridoxal phosphate, that lysine is responsible for the covalent binding of the substrate molecules (Battersby *et al.*, 1983).

Porphobilinogen deaminases have been isolated from a wide range of prokaryotic and eukaryotic sources. Among the prokaryotic deaminases, the *R. sphaeroides* enzyme is one of the best characterized (Jordan & Shemin, 1973; Jordan & Berry, 1981). Porphobilinogen deaminase has also been isolated from mammalian sources (Anderson & Desnick, 1980; Sancovich *et al.*, 1969) and from plants (Higuchi & Bogorad, 1975; Williams *et al.*, 1981). All the deaminases appear to be monomeric enzymes with M_r values ranging from 33000 to 44000.

Detailed structural studies on porphobilinogen deaminase have always been hampered by the very low levels of the enzyme occurring naturally. However, since the identification, cloning and sequencing of the porphobilinogen deaminase gene (*hemC*) from *E. coli* (Jordan *et al.*, 1986; Thomas & Jordan, 1986) it has been possible to obtain genetically engineered strains which produce almost 100 times the porphobilinogen deaminase found in the wild-type *E. coli* (Jordan *et al.*, 1986). This knowledge has already permitted the isolation of the *E. coli* enzyme (Hart *et al.*, 1986).

In this paper we report the isolation and crystallization of the enzyme from a recombinant strain of *E. coli* and a detailed study of its properties.

MATERIALS AND METHODS

Chemicals

[^{14}C]Porphobilinogen and unlabelled porphobilinogen were synthesized enzymically using purified 5-aminolaevulinate dehydratase (Jordan & Berry, 1981). Trizma base, 2-(*N*-cyclohexylamino)ethanesulphonic acid (Ches), Coomassie Brilliant Blue, protein standards, SDS-7, and antibiotics were obtained from Sigma Chemical Co. Sephacryl S-200 and Mono Q were purchased from Pharmacia Fine Chemicals. DEAE cellulose DE-52 was obtained from Whatman and hydroxyapatite HTP was obtained from Bio-Rad. Tryptone yeast extract and agar were obtained from Difco Laboratories. Other chemicals were supplied by B.D.H.

Bacteria

E. coli strain HB101 (Boyer & Roulland-Dussoix, 1969) was a gift from Dr. W. T. Drabble (this Department). Wild-type *E. coli* JA200 (F^+, *trpE5*, *recA*, *thr*,

Abbreviations used: SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid.

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leu, *lae Y*) and a strain carrying plasmid pLC41-4 were kindly provided by Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510, U.S.A. *E. coli* strains ST1046, ST1047 and ST1048 were generated as described previously (Jordan *et al.*, 1986). Bacteria were grown aerobically in 1 l batches in 2 l flasks of Luria broth (Lennox, 1955) supplemented with glucose (1 g/l) from 10% (v/v) inocculae.

Bacterial strains used for the isolation of the enzyme (ST1046, ST1047, ST1048) were grown in media containing ampicillin (30 μg/ml). The development of enzyme activity with growth of the cultures is discussed in the Results section.

Bacteria were harvested by centrifugation and the cells were washed in 20 mM-Tris/HCl buffer, pH 8.2, before use.

Determination of enzyme activity

The enzyme was assayed by incubation with 100 nmol of porphobilinogen in a final volume of 450 μl of 0.1 M-Tris/HCl, pH 8.2, at 37 °C. The reaction was stopped after 5 min by the addition of 125 μl of 5 M-HCl. The porphyrinogens formed were oxidized to porphyrins by the addition of 50 μl of 0.1% (w/v) benzoquinone in methanol. After 20 min at 0 °C, samples were centrifuged and 0.1 ml aliquots of the supernatants were made up to 1 ml with 1 M-HCl. Absorbance was measured at 405.5 nm ($\epsilon = 5.48 \times 10^5$ M^{-1} cm^{-1}) against a blank in which the reaction was quenched with acid at time zero. One unit of porphobilinogen deaminase is defined as that amount of enzyme which will catalyse the utilization of 1 μmol of porphobilinogen in 1 h.

Specific activities were calculated after determination of protein by the methods of Bradford (1976) and Lowry (Lowry *et al.* 1951) using bovine serum albumin as a standard.

Purification of porphobilinogen deaminase from *E. coli* K12 ST1048

All procedures were carried out at 4 °C unless stated otherwise. The bacteria were grown aerobically from 10% (v/v) inocula in fifty 2 l flasks each containing 1 l of medium. Cells were harvested after 55 h using a Sorvall RC-3B centrifuge. The cells were washed in 20 mM-Tris/HCl buffer, pH 8.2. Fifty litres of culture yielded approximately 100 g wet weight of cells.

Sonication of bacteria. Bacteria were suspended in 1 l of ice-cold 0.1 M-Tris/HCl buffer, pH 8.2, and were sonicated in 50 ml aliquots using an M.S.E. Soniprep ultrasonic disintegrator with 4 × 45 s bursts at an amplitude of 8–10 μM (peak to peak).

Heat treatment. The extract (1 l) was quickly heated to 60 °C in a boiling water bath with rapid swirling. The temperature was maintained at 60 °C for 10 min and the solution was then rapidly cooled to 0 °C in an ice/salt water slurry. The extract was centrifuged in an M.S.E. 21 centrifuge at 10000 rev./min for 20 min at 4 °C (6 × 500 ml rotor). The heat treatment inactivated uroporphyrinogen III synthase and removed a great deal of protein.

Ion-exchange chromatography. The supernatant was applied to a column of DEAE cellulose (10 cm × 10 cm) which had been equilibrated in 50 mM-Tris/HCl buffer, pH 8.2. The column was then washed with 1 l of the same buffer followed by 0.5 l of 25 mM-KCl. The enzyme was eluted by the application of a linear KCl gradient (25–400 mM-KCl; 1000 ml in total volume) in 50 mM-Tris/HCl buffer, pH 8.2. Active fractions were pooled and concentrated to 20 ml in an Amicon ultrafiltration cell fitted with a PM-10 membrane.

Sephacryl S-200 gel filtration. The concentrate prepared by ultrafiltration (20 ml) was applied to the bottom of a vertical column (75 cm × 5 cm) of Sephacryl S-200, equilibrated in 20 mM-Tris/HCl buffer, pH 8.2. The column was pumped upwards at a flow rate of 60 ml/h. Fractions (10 ml) were collected and those with a high level of enzymic activity were pooled.

Phenyl-Sepharose chromatography. The pooled active fractions from the gel-filtration step were made up to 30% saturation with respect to ammonium sulphate (w/v). The solution was then applied to a column of phenyl-Sepharose (1.5 cm × 10 cm) which had been equilibrated in 20 mM-Tris/HCl buffer, pH 8.2, with 30% (w/v) saturated ammonium sulphate. The column was then washed consecutively with 100 ml of the same buffer, 100 ml of buffer with 20% (w/v) saturated ammonium sulphate, and finally 100 ml of buffer with 15% (w/v) saturated ammonium sulphate. The enzyme was eluted with a linear gradient of ammonium sulphate from 15% to 0% to saturation (total volume 600 ml) in 20 mM-Tris/HCl buffer, pH 8.2. Fractions (10 ml) were collected and those with enzyme activity were again pooled, concentrated and dialysed against 20 mM-Tris/HCl buffer, pH 8.2. The enzyme was eluted at approximately 5% (w/v) saturated ammonium sulphate.

High-resolution anion-exchange chromatography. The enzyme solution was applied to a high-resolution anion-exchange Mono Q HR5/5 column attached to a Pharmacia f.p.l.c. system which had been equilibrated in 20 mM-Tris/HCl buffer, pH 7.5. The enzyme was eluted using a sodium chloride gradient by a pre-programmed method (0–350 mM-NaCl; 30 ml total volume) at a flow rate of 1 ml/min. The absorbance at 280 nm was monitored and individual peaks were collected and assayed for deaminase activity. The active fractions were pooled, dialysed against 10 mM-Tris/HCl buffer, pH 8.2, and stored frozen at 20 °C.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis under non-denaturing conditions (in the absence of SDS and 2-mercaptoethanol) was carried out by the method of Laemmli & Favre (1973). Enzyme activity was determined by incubation of the gel for 15 min at 37 °C with 0.5 mM-porphobilinogen in 0.1 M-Tris/HCl buffer, pH 8.2, followed by exposure of the gel to iodine solution [0.01% (w/v) in 0.1 M-HCl] for 5–10 min. Fluorescence due to the formation of uroporphyrin was detected using a long-wavelength u.v. lamp. Gels were then stained for protein using Coomassie Brilliant Blue [0.2% (w/v) in acetic acid/methanol/water (1:4:10, by vol.).

Polyacrylamide-gel electrophoresis under denaturing conditions was carried out according to Laemmli & Favre (1973).

M_r determinations. The M_r of the deaminase was determined using the gel system of Laemmli & Favre (1973) which had been calibrated using molecular mass markers (see Results and discussion). Alternatively, the M_r was determined by chromatography through Sephacryl S-200 SF (100 cm × 2.5 cm). The column was developed in 0.1 M-Tris/HCl, pH 8.2, at a flow rate of 15 ml/h. The M_r standards used were alcohol dehydrogenase (150000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000) and cytochrome *c* (12500).

Formation of enzyme substrate complexes with [^{14}C]porphobilinogen. Enzyme (0.1 nmol) was mixed with [3,5-$^{14}C_2$]porphobilinogen (90 nmol/μCi; 1.5–30 nmol) in 0.1 M-Tris/HCl buffer, pH 8.2, at 4 °C in a rapid-mixing device. The enzyme and its substrate complexes were subjected to electrophoresis under non-denaturing conditions and the bands were visualized both by assay of the gel for enzymic activity and staining with Coomassie Brilliant Blue. Radioactivity was determined by counting 2.5 mm gel slices of duplicate gels run under identical conditions. Alternatively, the enzyme and its substrate intermediates were separated and isolated by high-resolution ion-exchange chromatography using a Mono Q HR5/5 column attached to a Pharmacia f.p.l.c. system. In this case, the enzyme was eluted with a linear salt gradient (0–350 mM-NaCl; 30 ml total volume). All isolated peaks were collected and assayed for both enzyme activity and radioactivity.

Amino acid composition. A total amino acid analysis was carried out on 6 nmol samples of both native and performic acid-oxidized protein which allowed a measurement of the cysteine residues as cysteic acid to be made. The amino acid analysis was carried out on a Rank Hilger J120 amino acid analyser. The protein samples were freeze-dried in 6 M-HCl with 0.2% (w/v) phenol at 110 °C for 16–20 h *in vacuo*. The hydrolysed sample was dried in a vacuum dessicator and the amino acids resuspended in 10 mM-HCl for application to the analyser.

Isoelectric focusing. Flat-bed isoelectric focusing was performed on an LKB Multiphor apparatus using LKB polyacrylamide gels of pH 3–9 according to the manufacturer's instructions.

Protein *N*-terminal sequencing. Porphobilinogen deaminase (1 mg/30 nmol) was carboxymethylated (Hirs, 1967) and dialysed against 0.01 M-Tris/HCl buffer, pH 8.5. The modified protein was precipitated in 80% (v/v) ethanol, dried, and resuspended in 500 μl of 5% (v/v) ethylmorpholine buffer, pH 8.8, containing 45% (v/v) methanol and 25% (v/v) propanol, after which it was incubated at 56 °C for 2 h with 40 mg of phenylisothiocyanate-derivatized control pore glass. The mixture was then filtered and a further 700 μl of buffer, together with a drop of propylamine, was added to the glass beads, after which they were incubated at 55 °C for a further 20 min. The glass-coupled protein was then washed consecutively with ethylmorpholine buffer and dry methanol and the beads were dried *in vacuo*.

Sequencing was performed using an LKB Sequenator or an Applied Biosystems Model 477A sequencer. Phenylthiohydantoin derivatives were analysed by h.p.l.c. according to Bloxham *et al.* (1981).

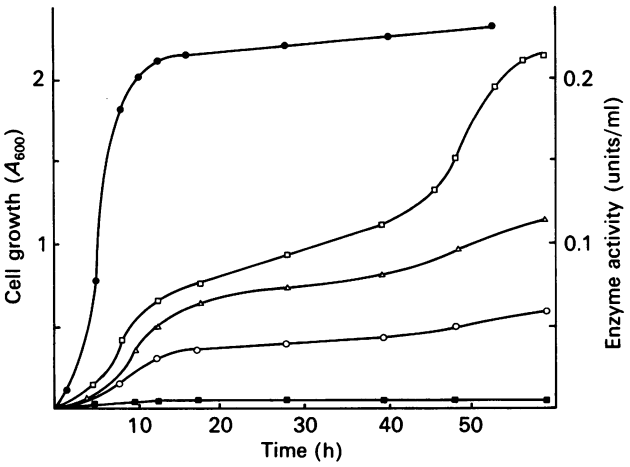


Fig. 1. Time course of bacterial cell growth and porphobilinogen deaminase activity in strains harbouring plasmids encoding *hemC*

Cell growth (●); porphobilinogen deaminase activity in ST1048 (□); in ST1047 (△); in ST1046 (○) and in HB101/pBR322 (■).

Chemical cleavage of the porphobilinogen deaminase, by formic acid. Carboxymethylated enzyme (50 μg; 1.5 nmol) was dissolved in 300 μl of 70% (v/v) aqueous formic acid and was incubated for 36 h at 37 °C. The reaction was terminated by the addition of distilled water (1 ml) and the solution was freeze-dried.

Crystallization of *E. coli* porphobilinogen deaminase. The hanging-drop vapour-diffusion method (McPherson, 1982) was used to prepare crystals of the *E. coli* deaminase.

RESULTS AND DISCUSSION

Porphobilinogen deaminase levels in genetically engineered strains of *E. coli*

Previous studies (Jordan *et al.*, 1986) have led to the identification of the *hemC* gene coding for *E. coli* porphobilinogen deaminase. Three recombinant strains of *E. coli* were produced (ST1046, ST1047 and ST1048), each of which contained the *hemC* gene in pBR322-derived plasmids and which produced levels of porphobilinogen deaminase 25–90 times that of the parental strain HB101/pBR322. To determine the optimum growth conditions for maximal deaminase production for these strains, the levels of the deaminase during the growth of the genetically engineered strains were compared with the wild-type strain.

During the exponential phase of growth, porphobilinogen deaminase activity in all four strains increased in parallel with the cell density (see Fig. 1). After about 16–20 h, the cultures showed little further increase in cell density indicating that the bacteria had entered the stationary phase. However, the strains harbouring plasmids containing *hemC* continued to show a steady increase in porphobilinogen deaminase activity over the next 25 h. After this period, a brief acceleration in the rate of increase of enzyme activity was seen in ST1048, until a maximum was reached at approximately 60 h after innoculation. For optimum

Table 1. Purification of porphobilinogen deaminase from 50 l of *E. coli* ST1048

Purification stage	Volume (ml)	Total protein (mg)	Total units (μmol/h)	Specific activity	Yield (%)
Sonication	1000	40000	6500	0.14	100
Heat treatment and centrifugation	1000	4200	6350	1.5	97
Ion-exchange (DE-52)	220	2000	6000	3.0	92
Gel filtration (S-200)	200	260	5200	20.0	80
Phenyl-Sepharose	100	150	4600	30.0	71
High-resolution ion-exchange Mono Q	40	70	3000	43.0	46

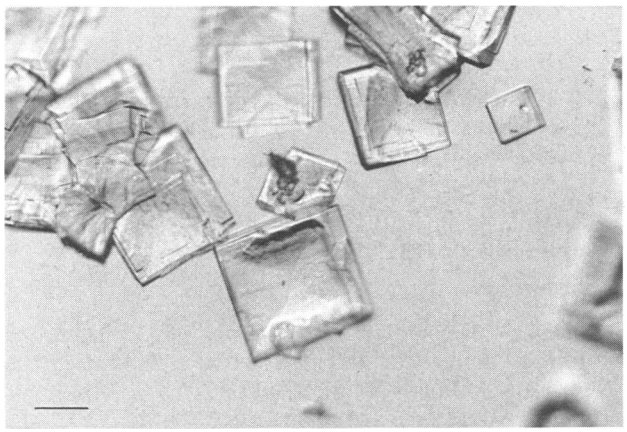


Fig. 2. Crystals of porphobilinogen deaminase

Scale: 1 cm = 100 μm.

deaminase levels, organisms were harvested after 55–60 h, as described in the Methods section.

The unexpected increase in the expression of the deaminase found in ST1048 may be attributed to the fact that the locus coding for the *rom* (previously *rop*) gene has been deleted during the construction of plasmid pST48 (Jordan *et al.*, 1986). The *rom* gene has been shown to direct the synthesis of a 63-amino-acid polypeptide, the repressor primer, which is known to regulate DNA replication by modulating the initiation of transcription of the RNA precursor molecule necessary to prime DNA replication (Cesareni *et al.*, 1982). Twigg & Sherratt (1980) have shown that plasmids deleted in DNA encoding the *rom* gene show an increase in copy number consistent with porphobilinogen deaminase activity assayed in the strain containing pST48. In agreement with this view is the observation that ST1048 yields approximately three times the plasmid DNA compared with ST1046, in which the plasmid pST46 contains the intact *rom* locus.

Purification and crystallization of *E. coli* porphobilinogen deaminase

The enzyme was isolated from 50 l of *E. coli* K12 ST 1048 as described in the Materials and methods section. The enzyme was judged to be homogeneous by electrophoresis on polyacrylamide gels containing SDS (SDS/PAGE). A summary of the purification is shown in Table 1. The purified enzyme had a specific activity of 43 μmol/h per mg of protein, when protein was determined by the methods described. When protein was determined by measuring the absorption at 280 nm and

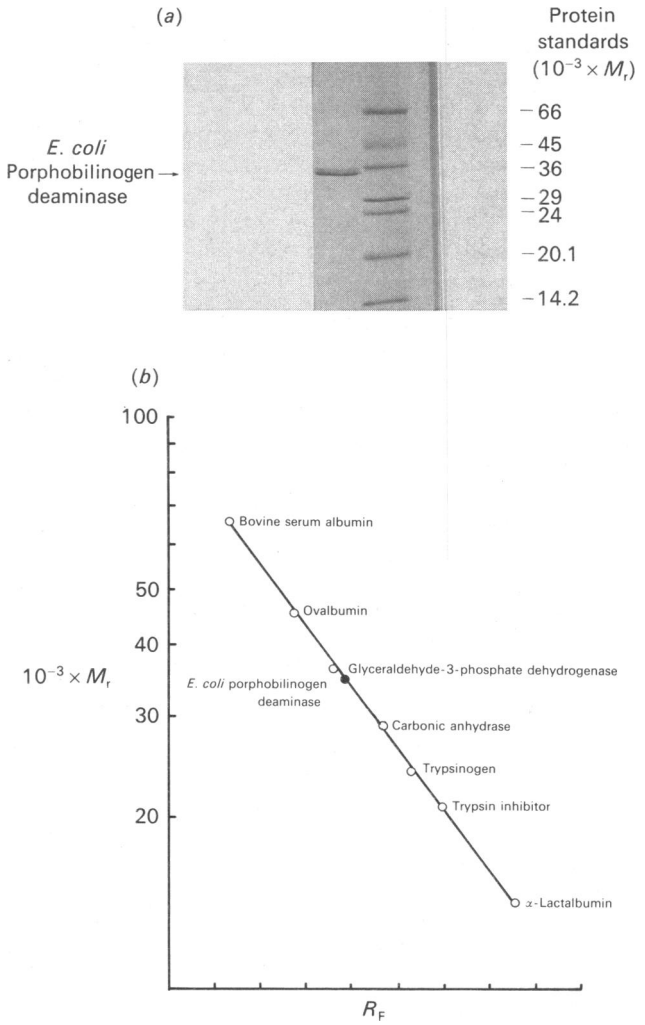


Fig. 3. Determination of M_r of porphobilinogen deaminase using SDS/PAGE

(*a*) Purified porphobilinogen deaminase (10 μg, left lane); M_r standards (right lane). (*b*) Mobility of *E. coli* porphobilinogen deaminase compared with the protein standards.

assuming an $A^{1\%}_{1\,cm}$ value of 10, as used by other workers (Hart *et al.*, 1986), the specific activity was 93 μmol/h per mg of protein. This is, however, an overestimate of the specific activity, since our determinations have shown that for the deaminase enzyme $A^{1\%}_{1\,cm}$ at 280 nm is substantially lower than 10.

Crystallization of the *E. coli* deaminase was ac-

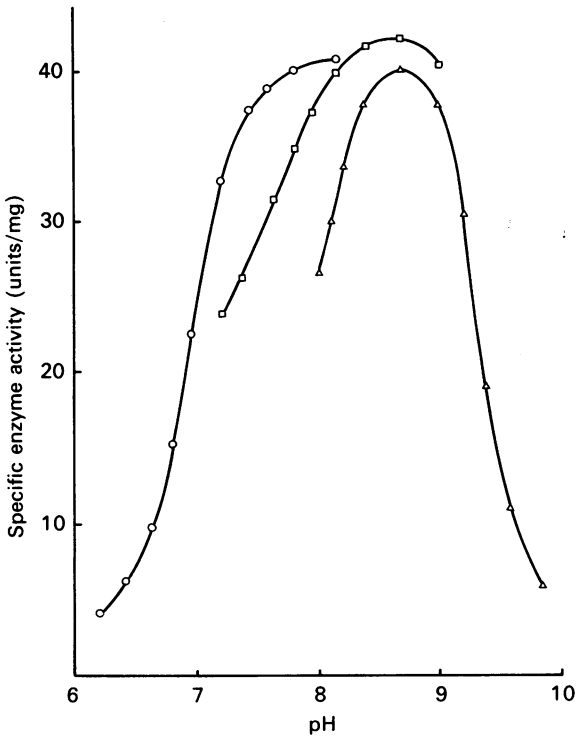


Fig. 4. Effect of pH on the activity of porphobilinogen deaminase

Porphobilinogen deaminase activity was determined as described in the Materials and methods section over the pH range 6–10 using 0.1 M-phosphate (○), Tris/HCl (□) and Ches (△) buffers.

complished by means of the hanging-drop vapour-diffusion method as described in the Materials and methods section. Crystals appeared in the droplets after several weeks. The crystals grew to about 300 μm in length, as shown in Fig. 2.

M_r determination

SDS/PAGE. The enzyme, when subjected to SDS/PAGE (Laemmli & Favre, 1973), migrated as a single protein band with a mobility slightly greater than glyceraldehyde-3-phosphate dehydrogenase (M_r 36000). A plot of $\log_{10} M_r$ of protein standards against mobility established the subunit M_r as 35000±3000 (Figs. 3*a* and *b*).

Sephacryl S-200 chromatography. The M_r of the deaminase was determined by chromatography using Sephacryl S-200. The enzyme migrated as a single peak with an M_r of 32000±2000. There was no evidence for higher M_r forms under any conditions. The *E. coli* enzyme therefore appears to exist as a monomer, as do all other deaminases isolated thus far.

pH optimum, K_m and isoelectric point

The pH optimum was found to be a broad peak from 8.4 to 9 (see Fig. 4), with some enzyme activity detectable even at pH 10. The enzyme was inactive below pH 6 and was found to be irreversibly inactivated below pH 4. The K_m for porphobilinogen was found to be 19±7 μM at its pH optimum. This is of a similar order to that for the *R. sphaeroides* enzyme [K_m for uroporphyrin formation 20 μM (Jordan & Shemin, 1973)] and 16 μM for that of the

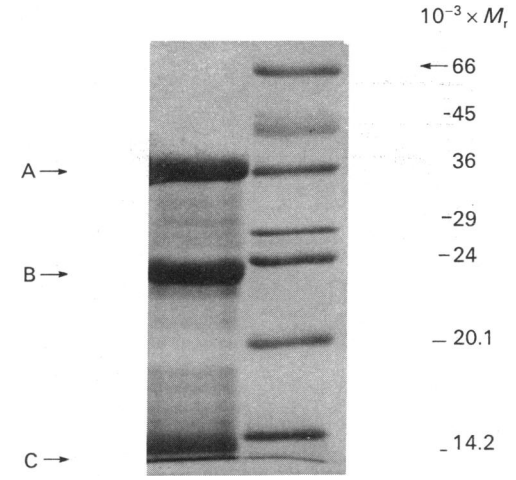


Fig. 5. Formic acid cleavage of porphobilinogen deaminase

SDS/PAGE analysis of 50 μg of porphobilinogen deaminase after cleavage with formic acid (left lane). A, uncleaved enzyme; B, 24000 M_r fragment; C, 11000 M_r fragment. M_r standards (right lane).

Table 2. Amino acid composition of purified *E. coli* porphobilinogen deaminase

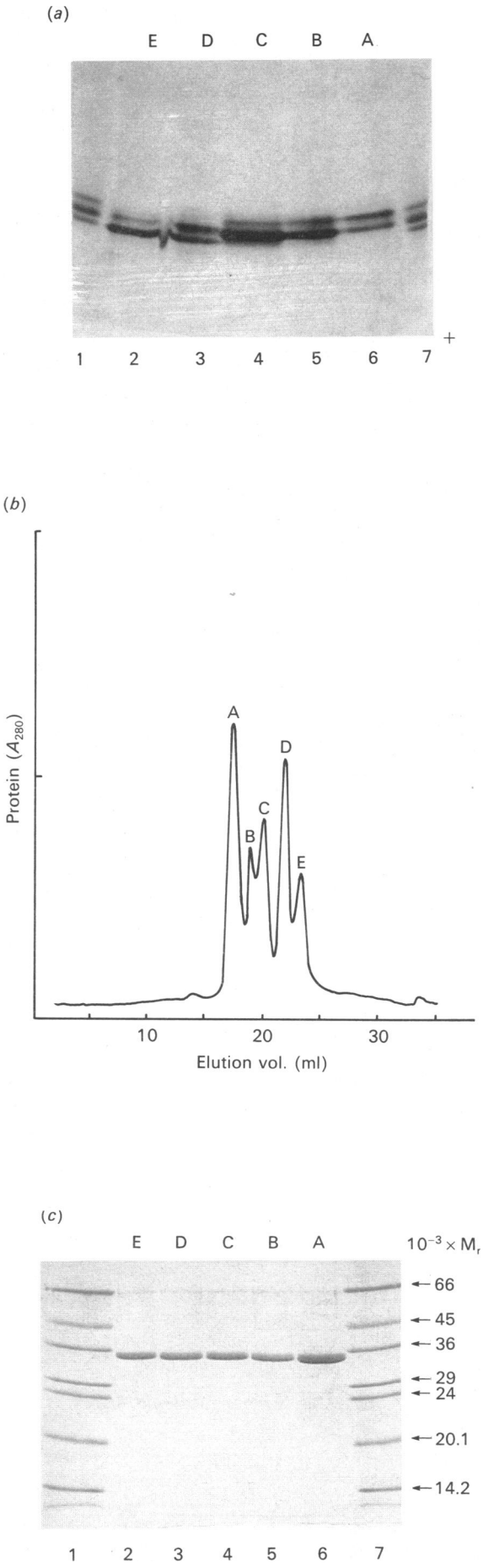
Comparison is made to the amino acid composition predicted from the gene sequence.

Amino acid	No. of residues	
	Observed	Predicted
Aspartate/asparagine	30	30
Threonine	10	10
Serine	13	16
Glutamate/glutamine	27	34
Glycine	28	28
Alanine	30	34
Cysteine	3	4
Valine	20	24
Methionine	5	6
Isoleucine	16	19
Leucine	40	42
Tyrosine	5	5
Histidine	7	5
Lysine	8	8
Arginine	28	27
Phenylalanine	4	3
Proline	ND	16
Trytophan	ND	2

E. coli enzyme (Hart *et al.*, 1986). Flat-bed isoelectric focusing of the purified deaminase gave three major bands very close together with an average isoelectric point value of 4.5.

Molecular properties of *E. coli* porphobilinogen deaminase

***N*-terminal analysis**. The porphobilinogen deaminase was carboxymethylated and linked to derivatized control pore glass as described in the Methods section. The *N*-terminal portion of the enzyme was sequenced by a semi-automated Edman procedure. The *N*-terminus was



shown to be as follows: NH_2–MLDNVLRIAT. This sequence confirms and extends that previously reported (Thomas & Jordan, 1986) and is consistent with that predicted from the complete gene sequence data reported previously (Thomas & Jordan, 1986). The sequence, however, differs from that reported by Hart *et al.* (1986), in which the *N*-terminus appears to be glutamate.

Formic acid cleavage. Analysis of the products of formic acid cleavage by SDS/PAGE showed two new polypeptides of M_r 24000 and 11000, corresponding to the cleavage at the single aspartic acid–proline link (Fig. 5). This observation lends further support to the protein primary structure predicted from the gene sequence. Formic acid treatment also resulted in the cleavage of dipyrromethane cofactor and the formation of uroporphyrin in a non-enzymic reaction (see also Jordan & Warren, 1987).

Amino acid composition. Amino acid analysis of the purified protein is shown in Table 2. The composition is in close agreement to that predicted from the full gene sequence.

Enzyme heterogeneity

Although the purified deaminase existed as a single protein band after electrophoresis in the presence of SDS, under non-denaturing conditions three major protein bands could be visualized (Fig. 6*a*, tracks 1 and 7). These bands all exhibited the same deaminase specific activity. When the same sample of purified deaminase was applied to a high-resolution anion-exchange Mono Q column, five peaks of protein were detected (Fig. 6*b*). The peaks from f.p.l.c. also exhibited the same deaminase specific activity. All the isolated peaks from f.p.l.c. ran as a single band of M_r 35000 on denaturing electrophoresis gels (Fig. 6*c*), eliminating the possibility that they represented proteolytic degradation products. Surprisingly, even peaks derived from f.p.l.c. appeared to exist in at least two forms when run under non-denaturing conditions (Fig. 6*a*, A–E).

It was important to determine whether these multiple species were due to enzyme heterogeneity or to the presence of enzyme–intermediate complexes. It is well established that the deaminases isolated from other sources show multiple bands on non-denaturing polyacrylamide gels (Anderson & Desnick, 1980; Berry & Jordan, 1981). These bands have been interpreted as

Fig. 6. Analysis of the different forms of the enzyme by high-resolution ion-exchange chromatography and electrophoresis

(*a*) Non-denaturing polyacrylamide-gel electrophoresis of unresolved enzyme and the five peaks isolated by high-resolution ion-exchange chromatography (*b*). Lanes 1 and 7 contain 30 μg of unresolved porphobilinogen deaminase. Lanes 2–6 contain 30 μg of protein from peaks A–E in (*b*). (*b*) High-resolution ion-exchange chromatography of purified porphobilinogen deaminase using Mono Q. The enzyme eluted in five separate peaks marked A–E, as shown. (*c*) SDS/PAGE of each of the five peaks isolated by high-resolution ion-exchange chromatography in (*b*). Lanes 1 and 7 contain M_r standards. Lanes 2–6 contain 10 μg of protein from each peak, A–E, respectively.

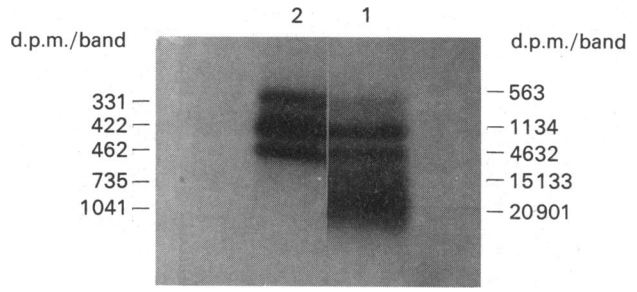


Fig. 7. Non-denaturing polyacrylamide-gel electrophoresis showing the effect of hydroxylamine treatment on [^{14}C]-enzyme–intermediate complexes derived from purified porphobilinogen deaminase and [^{14}C]porphobilinogen

Track 1: protein profile of the ^{14}C-labelled enzyme–intermediate complexes before treatment with 0.2 M-hydroxylamine. Track 2: similar sample to track 1, but after hydroxylamine treatment.

being due to enzyme together with enzyme–intermediate complexes representing different stages of the catalytic cycle *en route* to the tetrapyrrole, i.e. E, ES, ES_2, ES_3, and ES_4. Such complexes are susceptible to treatment with hydroxylamine (Davis & Neuberger, 1973) and under these conditions the bound intermediate is released regenerating the free enzyme. The purified *E. coli* deaminase was therefore treated with 0.2 M-hydroxylamine and re-analysed by both non-denaturing gel electrophoresis and f.p.l.c. The hydroxylamine-treated enzyme showed no change in mobility indicating that the multiple species were not due to enzyme–intermediate complexes. This was further confirmed by incubating the purified enzyme with [^{14}C]porphobilinogen to generate the enzyme–intermediate complexes (Berry *et al.*, 1981). The resulting enzyme complexes were subjected to non-denaturing gel electrophoresis and stained for protein (Fig. 7, track 1). A duplicate gel was analysed for radioactivity showing that the new bands representing enzyme–substrate complexes, were radioactive. Treatment of these complexes with hydroxylamine resulted in the liberation of the ^{14}C radioactivity and the regeneration of the original enzyme pattern of three bands, all of which were essentially devoid of radioactivity (Fig. 7, track 2). This firmly establishes that the multiple species observed in the purified enzyme were not enzyme–intermediate complexes.

Since all the forms of the enzyme encountered have the same specific activity and the fact that we have eliminated the possibility of both proteolytic degradation products and enzyme–substrate intermediates as candidates, we investigated the possibility that the heterogeneity may be due to variation in the nature of the covalently bound cofactor. Accordingly, we reacted each of the five species obtained from f.p.l.c. with Ehrlich's reagent, as described previously (Jordan & Warren, 1987). Each species exhibited the typical spectral profile of a dipyrromethane (data not shown). Furthermore, when each of the five peaks isolated from Mono Q chromatography was treated with formic acid (50 %, v/v) for 2 h, uroporphyrin was formed in each case from the enzyme-bound dipyrromethane cofactor. These data establish that each form of the native enzyme contains a functional dipyrromethane cofactor.

We are therefore of the opinion that the heterogeneity

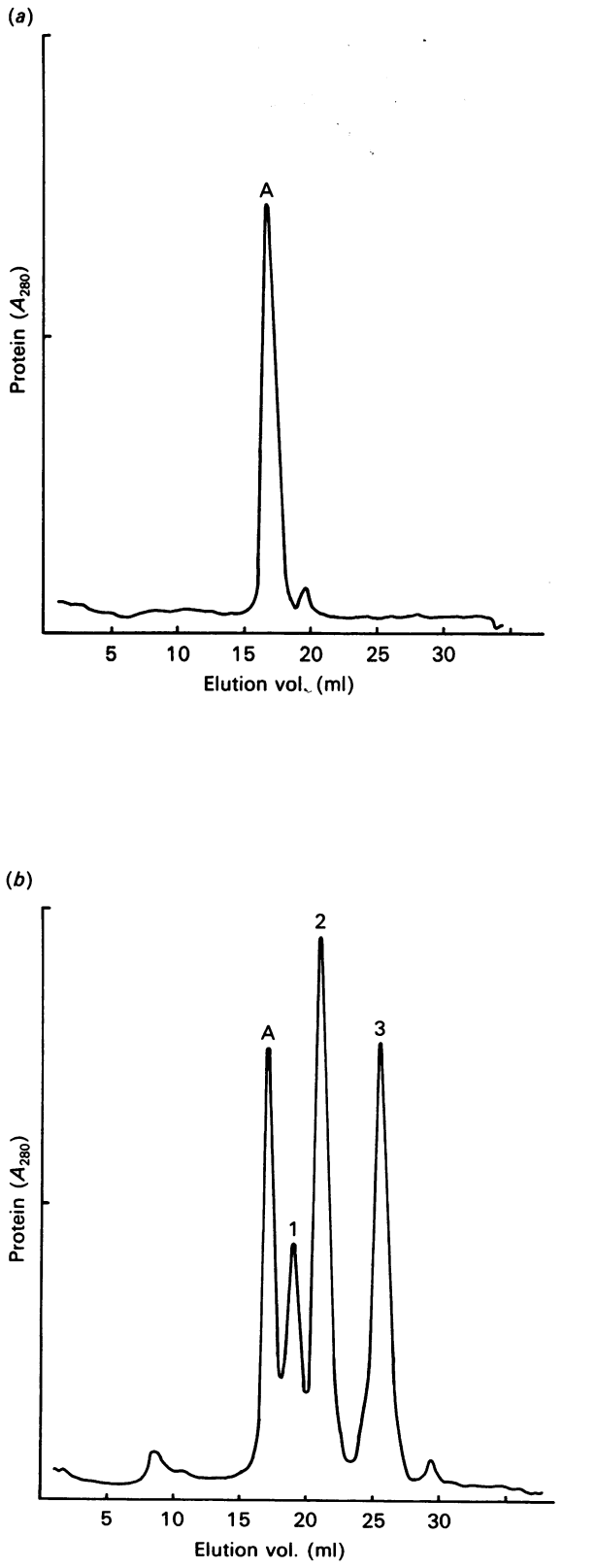


Fig. 8. Isolation and identification of enzyme–intermediate complexes

High-resolution ion-exchange chromatography of a single peak of porphobilinogen deaminase isolated from a Mono Q column (A in Fig. 6*a*). (*a*) Before treatment with porphobilinogen; (*b*) after mixing with a 10-fold molar excess of porphobilinogen. Unchanged enzyme (A); enzyme–intermediate complexes (peaks 1–3).

observed in the purified enzyme probably represents slightly different structural forms of the deaminase, possibly due to deamidation of asparagine residues. Whatever the reason for these enzyme forms, it does not seem to affect the ability of each of them to behave as an enzyme with normal activity.

Table 3. Ratio of substrate (porphobilinogen)/enzyme in isolated peaks obtained from high-resolution chromatography using Mono Q (see Fig. 8*b*) after mixing enzyme with [^{14}C]porphobilinogen

Peak	Ratio of substrate/enzyme	Assignment of peak
A	0.04	E
1	1.01	ES
2	2.10	ES_2
3	3.19	ES_3

Isolation and characterization of enzyme-bound substrate intermediates

Previous studies with deaminases isolated from *R. sphaeroides* and human sources have shown the formation of covalent enzyme–intermediate complexes on incubation of the native enzyme with the substrate porphobilinogen. From the studies of Jordan & Warren (1987), it has been established that these enzyme-bound intermediates are all covalently linked to the free α-position of a dipyrromethane cofactor covalently linked at the active site of the enzyme. The two pyrrole rings which make up the cofactor have been shown not to be incorporated into the product and serve only to anchor the growing tetrapyrrole chain to the 'holoenzyme'. The intermediate complexes may be separated by polyacrylamide-gel electrophoresis or ion-exchange chromatography, although there is some decomposition under these conditions. The availability of f.p.l.c. has allowed us to carry out a rapid separation of the enzyme-intermediate complexes and has permitted a detailed evaluation of their properties.

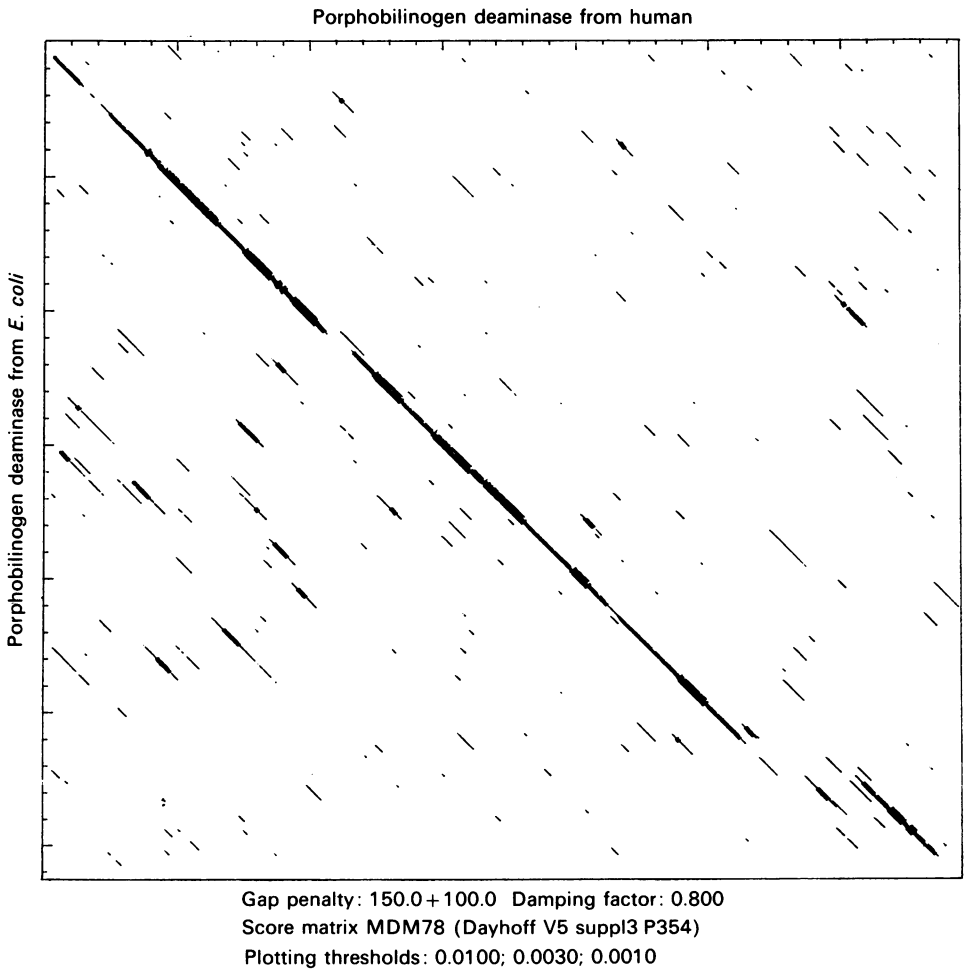


Fig. 9. Plot to show the sequence homology between *E. coli* and human porphobilinogen deaminase

Exponentially damped alignment comparison of the predicted amino acid sequence of *E. coli* porphobilinogen deaminase (sequence runs from top to bottom) and the predicted amino acid sequence from the human porphobilinogen deaminase cDNA as described by the method of Boswell & McLachlan (1984). The line thickness corresponds to sequence similarity, the thicker the line the greater the similarity.

When a single peak of purified *E. coli* porphobilinogen deaminase, isolated by f.p.l.c., was rapidly mixed with a 10-fold molar excees of [^{14}C]porphobilinogen in order to generate enzyme–intermediate complexes, further f.p.l.c. showed that the enzyme peak had diminished and three new species had been formed (Fig. 8*a* and *b*). Each species was assayed separately for enzyme activity, radioactivity and protein content.

As expected, all the peaks showed the same enzyme specific activity; however, the ^{14}C/protein ratios of peaks 1, 2 and 3 increased with values of 1.01, 2.10 and 3.19, respectively (Table 3). This suggests that peak 1 was enzyme with a single bound pyrrole unit, peak 2 contained enzyme with two bound pyrrole units and the final peak, 3, represented an enzyme species with three pyrrole units. No evidence was found for a species with four pyrrole units, as is noted with the human deaminase (Anderson & Desnick, 1980). When any of the enzyme–intermediate species was treated with hydroxylamine, a reagent known to displace the enzyme-bound pyrroles (Davis & Neuberger, 1973; Radmer & Bogorad, 1972), a single non-radioactive peak of free enzyme was regenerated (data not shown) and the ^{14}C label appeared in the liberated polypyrroles. Hydroxylamine treatment did not release the dipyrromethane cofactor from the enzyme (Jordan & Warren, 1987).

Comparison of the *E. coli* and human primary sequence

The deaminase primary structure predicted from the sequence of the *E. coli* gene and the human cDNA when compared show remarkable similarity. Almost 50% of the amino acids are identical after alignment of the sequences and nearly 80% of the amino acids are structurally related. Expression of the two sequences in the form of an alignment plot is shown in Fig. 9. Very few gaps are found, the most notable being a sequence of 28 amino acids in the *E. coli* sequence which accounts for the higher M_r of the human enzyme (Grandchamp *et al.*, 1984, 1986).

The cloning of the *hem*C gene of *E. coli* into an expression system which yields almost 100 times the wild-type levels of deaminase has permitted a detailed evaluation of the properties of the purified enzyme and much preliminary data regarding the structure of the protein. Currently, X-ray diffraction studies are underway, which, together with the existing protein chemistry, will assist in the elucidation of the detailed mechanism of this remarkable enzyme.

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