Purification and properties of porphobilinogen deaminase from *Arabidopsis thaliana*

Russell M. JONES and Peter M. JORDAN

School of Biological Sciences, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K.

Porphobilinogen deaminase (EC 4.3.1.8) has been purified to homogeneity (16000-fold) from the plant *Arabidopsis thaliana* in yields of 8%. The deaminase is a monomer of M_r 35000, as shown by SDS/PAGE, and 31000, using gel-filtration chromatography. The pure enzyme has a $V_{max.}$ of 4.5 μmol/h per mg and a K_m of 17 ± 4 μM. Determination of the pI and pH optimum revealed values of 5.2 and 8.0 respectively. The sequence of the N-terminus was found to be NH_2-XVAVEQKTRTAI. The deaminase is heat-stable up to 70 °C and is inhibited by NH_3 and hydroxylamine. The enzyme is inactivated by arginine-, histidine- and lysine-specific reagents. Incubation with the substrate analogue and suicide inhibitor, 2-bromoporphobilinogen, results in chain termination and in inactivation.

INTRODUCTION

Porphobilinogen deaminase (EC 4.3.1.8) catalyses the deamination and polymerization of four molecules of porphobilinogen (Scheme 1) resulting in the formation of a highly unstable 1-hydroxymethylbilane, preuroporphyrinogen (Battersby et al., 1979; Burton et al., 1979). Preuroporphyrinogen acts as the substrate for uroporphyrinogen III synthase (cosynthase) resulting in uroporphyrinogen III (Jordan et al., 1979) the common precursor of the haem, chlorophyll and cobalamin biosynthetic pathways [see Warren and Scott (1990), Battersby and Leeper (1990), Jordan (1990, 1991) for reviews].

The exact sequence of steps involved in the assembly of the 1-hydroxymethylbilane at the porphobilinogen deaminase active site has been investigated extensively (Jordan and Warren, 1987; Warren and Jordan, 1988). Deaminases are unique in that they all utilize a dipyrromethane cofactor as a primer to which the four substrate molecules are covalently attached (Jordan and Warren, 1987; Hart et al., 1987; Warren and Jordan, 1988). The dipyrromethane cofactor is covalently attached to Cys-242 in the *Escherichia coli* deaminase sequence (Jordan et al., 1988a; Miller et al., 1988; Scott et al., 1988).

Porphobilinogen deaminase has been purified from a variety of prokaryotic and eukaryotic organisms including *Rhodobacter sphaeroides* (Jordan and Shemin, 1973), *E. coli* (Hart et al., 1986; Thomas and Jordan, 1986; Jordan et al., 1988b), *Euglena gracilis* (Williams et al., 1981), *Chlorella regularis* (Shioi et al., 1980), human erythrocytes (Anderson and Desnick, 1980), rat liver (Mazzetti and Tomio, 1988) and yeast (Correa-Garcia et al., 1991). The enzyme has also been isolated from plant sources including spinach (Higuchi and Bogorad, 1975) and pea (*Pisum sativum* L.) (Spano and Timko, 1991). In all cases the deaminase was found to be a monomer of M_r ranging from 34000 to 44000.

The genes/cDNAs encoding deaminases have been isolated and sequenced from a variety of organisms including *E. coli* (Thomas and Jordan, 1986), human erythrocytes (Raich et al., 1986), rat spleen (Stubnicer et al., 1988), mouse (Beaumont et al., 1989), *E. gracilis* (Sharif et al., 1989), *Bacillus subtilis* (Petricek et al., 1990), pea (*P. sativum* L.) (Witty et al., 1993) and Thame cress (*Arabidopsis thaliana*) (A. G. Smith, personal communication). The primary structures of the deaminases, derived from these nucleotide sequences, indicate that the enzyme is structurally conserved throughout the biosphere.

The crystallization of *E. coli* porphobilinogen deaminase (Jordan et al., 1992) and the determination of its three-dimensional structure at a resolution of 0.17 nm (1.7 Å) (Louie et al., 1992) has increased considerably the understanding of how the enzyme functions in the polymerization of porphobilinogen. Site-directed mutagensis of the *E. coli* deaminase (Jordan and Woodcock, 1991; Lander et al., 1991) has highlighted the role of a number of invariant arginine residues, both for the assembly of the dipyrromethane cofactor and the tetrapolymerization reaction. In addition, site-directed mutagenesis has also revealed the importance of Asp-84 in the catalytic mechanism (Woodcock and Jordan, 1994).

The present paper describes the purification to homogeneity and subsequent characterization of the porphobilinogen deaminase from *A. thaliana*, previously unpurified from this source.

MATERIALS AND METHODS

Materials

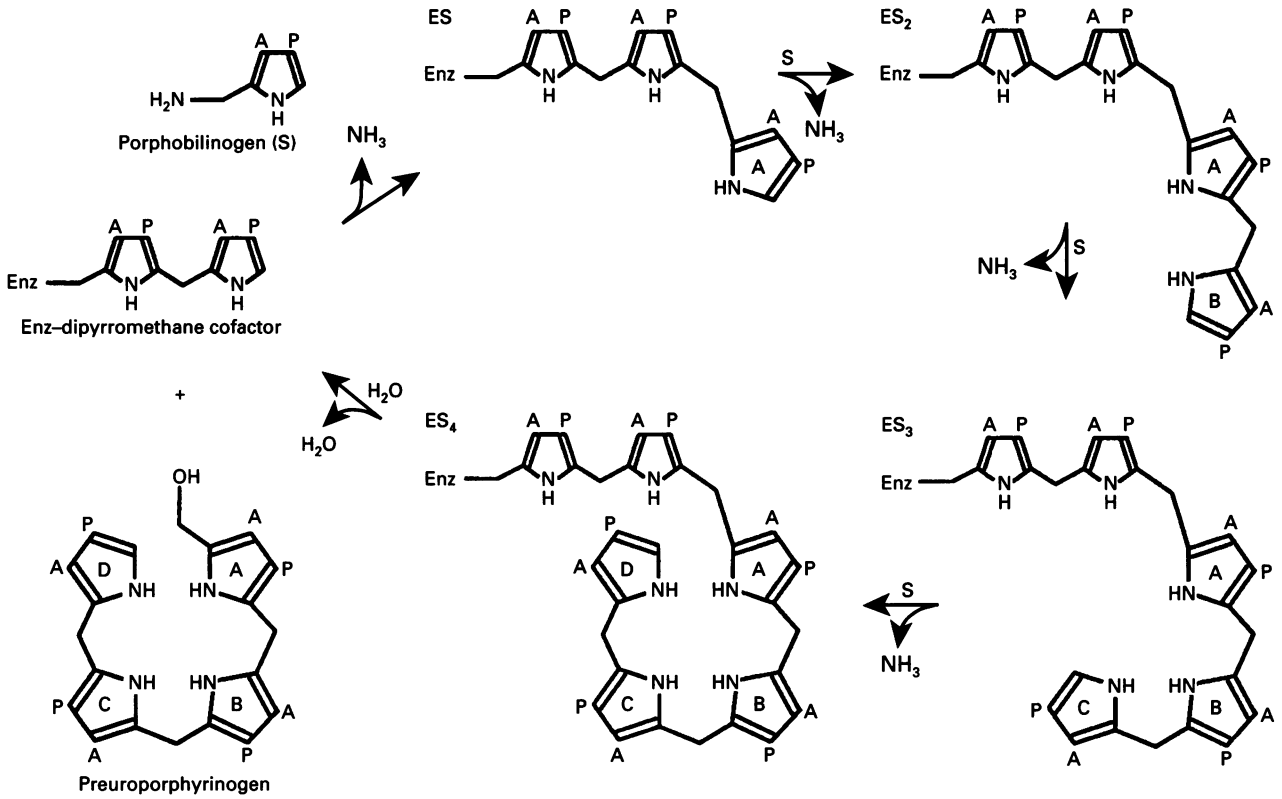
Porphobilinogen was synthesized enzymically using purified 5-aminolaevulinic acid dehydratase (Jordan and Seehra, 1986). The f.p.l.c. system was purchased from LKB/Pharmacia. Dalton VII M_r standards, Sephadex G-100, DEAE-Sephacel and general laboratory chemicals were purchased from Sigma. The affinity-chromatography material MIMETIC RED3 was from Affinity Chromatography. 2-Bromoporphobilinogen was a gift from Professor A. I. Scott (Texas A&M University, College Station, TX, U.S.A.) Uroporphyrin, coproporphyrin and protoporphyrin were purchased from Porphyrin Products and reduced to their porphyrinogens as previously described (Jones and Jordan, 1993). Miracloth was purchased from Calbiochem Novachem.

Purification of porphobilinogen deaminase from *A. thaliana*

Growth of *A. thaliana* Columbia

Plants were grown below 15 °C in greenhouses until just before

Abbreviation used: Ches, 2-(*N*-Cyclohexylamino)ethanesulphonic acid.



Scheme 1 Conversion of porphobilinogen into preuroporphyrinogen catalysed by porphobilinogen deaminase

A, acetic acid; P, propionic acid.

flowering (4–6 weeks). The optimal period for growth was from October until March.

Preparation of crude extracts of *A. thaliana*

Leaves (1 kg) were separated from the plants immediately before the purification of the enzyme and blended in batches of approx. 30 g using 30 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM benzamidine and 1 mM phenylmethanesulphonyl fluoride by means of a Polytron homogenizer fitted with a 30 mm probe. Both the probe and the vessel were precooled at 0 °C and all homogenization was carried out at 4 °C. The plant extract was strained through four layers of Miracloth (Calbiochem), and the filtrates were pooled and centrifuged at 10000 ***g*** for 15 min at 4 °C. The supernatant was collected and the pellet was discarded.

$(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was added to the supernatant (approx. 1 litre) to 40% saturation. The extract was stirred slowly for 10 min at 4 °C and then centrifuged for 15 min at 7000 ***g***. The supernatant was carefully decanted and a further quantity of $(NH_4)_2SO_4$ was added to 70% saturation. The extract was again stirred slowly for 10 min at 4 °C and then centrifuged, as before. The resulting pellet, containing porphobilinogen deaminase activity, was resuspended in a minimum volume (approx. 50 ml) of 50 mM Tris/HCl buffer, pH 8.0, containing 2 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM benzamidine. The resulting suspension was then dialysed overnight, at 4 °C, against two changes of 4 litres of the same buffer.

Anion-exchange chromatography

The dialysed extract was applied to a column (50 cm × 3.5 cm) of DEAE-Sephacel equilibrated with 25 mM Tris/HCl buffer, pH 8.0, containing 2 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM benzamidine. The column was then washed with 150 ml of the same buffer and protein was eluted with a linear gradient of KCl (0–300 mM; 500 ml total volume in buffer) at a flow rate of 1 ml/min. Fractions (8 ml) were collected and assayed for deaminase activity as above. Active fractions were pooled and the resulting solution was concentrated to approx. 5 ml using an Amicon ultrafiltration cell fitted with a PM-10 membrane.

Gel-filtration chromatography

The enzyme was applied to a column (100 cm × 3.5 cm) of Sephadex G-100 which had previously been equilibrated with 25 mM Tris/HCl buffer, pH 8.0, containing 1 mM 2-mercaptoethanol and 1 mM benzamidine. Fractions (6 ml) were collected and assayed for activity. The fractions containing deaminase activity were pooled and concentrated to 5 ml by means of an Amicon ultrafiltration cell, as above.

F.p.l.c.

Fractions containing porphobilinogen deaminase, from the previous stage, were applied to a Mono Q (HR 5/5) column fitted to an LKB/Pharmacia f.p.l.c. system previously equilibrated with 25 mM Tris/HCl buffer, pH 8.0. The protein was eluted with a linear gradient of KCl (0–200 mM; 20 ml total volume in buffer) at a flow rate of 1 ml/min. Fractions containing deamin-

Table 1 Purification of porphobilinogen deaminase from 1 kg of *A. thaliana*

Stage	Volume (ml)	Protein (mg/ml)	Activity (units/mg)	Total (units)	Yield (%)	Purification (fold)
Crude extract	1660	38.72	0.27	17290	100	1
Centrifuge extract	1690	16.00	0.64	17278	98	2.4
$(NH_4)_2SO_4$ fraction	117	50.56	2.03	12026	70	7.6
DEAE-Sephacel chromatography	13	55.80	13.45	9756	56	50
Gel filtration	10.2	3.12	114.7	3651	21	427
F.p.l.c.	8.5	0.79	350.2	2088	12	1153
Affinity column chromatography	3.45	0.084	4393.5	1273	8	16333

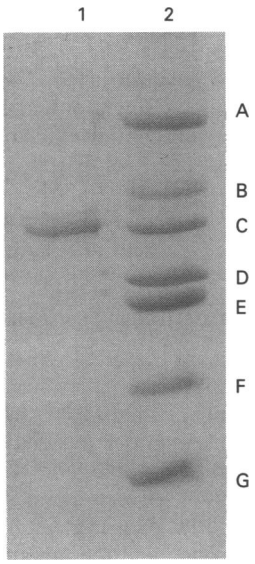


Figure 1 Purity of purified porphobilinogen deaminase from *A. thaliana*

Porphobilinogen deaminase was subjected to PAGE in the presence of SDS (Weber and Osborn, 1969). Lane 1, homogeneous porphobilinogen deaminase; lane 2, monomeric protein M_r standards: A, BSA (66000); B, ovalbumin (45000); C, glyceraldehyde-3-phosphate dehydrogenase (36000); D, carbonic anhydrase (29000); E, trypsinogen (24000); F, trypsin inhibitor (20100); G, α-lactalbumin (14200).

ase activity were pooled and desalted using a Pharmacia PD-10 column.

Affinity chromatography

The porphobilinogen deaminase was purified to homogeneity by a final chromatography step using a MEMETIC RED3 affinity column previously equilibrate with 25 mM Tris/HCl buffer, pH 8.0 containing 1 mM 2-mercaptoethanol. Unwanted protein was eluted with 4 ml of 25 mM Tris/HCl buffer, pH 8.0, containing 1 mM 2-mercaptoethanol, followed by 4 ml of the same buffer containing 100 mM KCl. Porphobilinogen deaminase was then eluted with 4 ml of the same buffer containing 200 mM KCl. Fractions (500 μl) were collected and those containing enzyme activity were pooled. The total purification is summarized in Table 1. The porphobilinogen deaminase was judged to be homogeneous by PAGE in the presence of SDS, followed by staining with Coomassie Brilliant Blue (Figure 1). The pure porphobilinogen deaminase was desalted into 25 mM Tris/HCl buffer, pH 8.0, containing 1 mM 2-mercaptoethanol and stored at 4 °C until required.

Assay of porphobilinogen deaminase activity

Porphobilinogen deaminase activity was determined in 25 mM Tris/HCl buffer, pH 8.0, by incubation at 37 °C in the presence of 2 mM porphobilinogen in a final volume of 50 μl. The reaction was terminated by the addition of 30 μl of 5 M HCl and 30 μl of benzoquinone (1 mg/ml) in methanol. Samples containing porphobilinogen deaminase produced preuroporphyrinogen that was cyclized by the acid to uroporphyrinogen I and oxidized by the benzoquinone to uroporphyrin I (ϵ_{405} 548 litre·mmol^{-1}·cm^{-1}; Rimington and Sveinsson, 1950). One unit of deaminase catalyses the formation of 1 nmol of uroporphyrin I/h under the standard assay conditions at 37 °C.

Assay of protein

An estimate of protein concentration was obtained at 280 nm where an absorbance of 1 corresponded to a protein concentration of 1 mg/ml. Protein was also routinely determined by the method of Bradford (1976), using BSA as standard.

M_r determination

Denaturing SDS/PAGE

The M_r of porphobilinogen deaminase was determined by SDS/PAGE under denaturing conditions as described by Weber and Osborn (1969).

Gel-filtration chromatography

The M_r of native porphobilinogen deaminase was determined by gel-filtration chromatography using a Superose 12 HR10/30 f.p.l.c. column (10 mm × 30 mm) fitted to an LKB/Pharmacia f.p.l.c. system and equilibrated with 25 mM Tris/HCl buffer, pH 7.5, containing 300 mM KCl and developed at a flow rate of 0.4 ml/min. The column was standardized by means of protein M_r standards. The standards used were: apoferritin (450000), alcohol dehydrogenase (150000), BSA (66000), ovalbumin (45000), carbonic anhydrase (29000) and cytochrome *c* (12000).

Sequencing of the N-terminus of porphobilinogen deaminase

Native porphobilinogen deaminase (40 μg, approx. 1 nmol) in 25 mM potassium phosphate, pH 7.0, was applied to Polybrene filter discs (Applied Biosystems) and sequencing was performed using an Applied Biosystems 477A protein sequencer fitted with a phenylthiohydantoin 120A analyser.

Determination of the isoelectric point of porphobilinogen deaminase

The isoelectric point of porphobilinogen deaminase was determined by means of an LKB/Pharmacia flat-bed isoelectric-focusing apparatus, using LKB Ampholine PAGE plates according to the manufacturer's instructions (pH range 3.5–9.5) and standards of known pI. The gel was focused at 30 mA constant current for 2 h and protein was visualized by staining with Coomassie Brilliant Blue. A standard curve of R_F against isoelectric point was plotted, and the isoelectric point of porphobilinogen deaminase was calculated. The protein standards used were: trypsinogen (pI 9.3); lactate dehydrogenase (pI 8.4); carbonic anhydrase (pI 6.6); β-lactoglobulin A (pI 5.1); glucose oxidase (pI 4.2).

Reaction of Ehrlich's reagent with porphobilinogen deaminase

A sample of porphobilinogen deaminase was desalted into 25 mM Tris/HCl buffer, pH 8.0, using a PD-10 column (Pharmacia), to remove 2-mercaptoethanol. The resulting sample was allowed to react with an equal volume of modified Ehrlich's reagent (Mauzerall and Granick, 1956) and scanned between 400 nm and 600 nm, using a Hitachi U2000 spectrophotometer. The reaction was followed to completion, by scanning every 3 min.

Modification of porphobilinogen deaminase by protein group-specific reagents

Porphobilinogen deaminase was preincubated at 37 °C for 20 min in the presence of the reagent at a final concentration of 1 mM, and the effect on activity was determined, after separation of the reagent using a Pharmacia PD-10 minicolumn, as indicated in the legend to Table 3.

RESULTS AND DISCUSSION

Stability and storage of porphobilinogen deaminase

The *A. thaliana* porphobilinogen deaminase was found to be stable to heat-treatment up to 70 °C for 10 min. The deaminase could be stored, with no loss of activity, for several weeks at 4 °C in 25 mM Tris/HCl filter-sterilized buffer, pH 8.0, containing 2 mM 2-mercaptoethanol. The purified deaminase was stable for several months at −20 °C.

M_r determination of porphobilinogen deaminase

SDS/PAGE

SDS/PAGE of the deaminase and staining with Coomassie Brilliant Blue revealed a single protein band (Figure 1) with a mobility similar to that of glyceraldehyde-3-phosphate dehydrogenase (M_r 36000). A plot of log M_r against mobility (results not shown) yielded a value of 35000 ± 2000.

Superose 12 gel-filtration chromatography

The M_r of the deaminase was determined under non-denaturing conditions by gel-filtration chromatography, using a Superose 12 column. The deaminase was eluted just before carbonic anhydrase (M_r 29000), and a plot of log M_r against elution volume (not shown) gave a value for M_r of 31000 ± 4000. This result, together with the value obtained by SDS/PAGE, indicates that *A. thaliana* porphobilinogen deaminase is a monomeric protein. This is consistent with observations on porphobilinogen deaminases purified from other sources, which are also reported to be monomers of M_r ranging from 34000 to 44000 (Jordan, 1991).

Kinetic properties of porphobilinogen deaminase

The apparent K_m for porphobilinogen was found to be 17 ± 4 μM measured as uroporphyrin I, using substrate concentrations from 1 to 50 μM. This value is of a similar order to those reported for the deaminases from *R. sphaeroides* [20 μM (Jordan and Shemin, 1973)], human erythrocytes [6 μM (Anderson and Desnick, 1980)], rat liver [17 μM (Mazzetti and Tomio, 1988)], *E. coli* [19 μM (Jordan et al., 1988b)] and yeast [19 μM (Correa-Garcia et al., 1991)].

Determination of pH optimum and isoelectric point

The *A. thaliana* porphobilinogen deaminase exhibited a broad pH optimum between pH 7.7 and 8.5 (Figure 2a). This value is in a similar range (pH 7.4–8.2) to that reported for the deaminases from a variety of organisms (Anderson and Desnick, 1980; Shioi et al., 1980; Mazzetti and Tomio, 1988; Correa-Garcia et al., 1991; Spano and Timko, 1991). The isoelectric point (pI) of the deaminase, determined by isoelectric focusing on polyacrylamide gels, was found to be 5.2. A single protein band was obtained confirming that the porphobilinogen deaminase was homogeneous. pI values determined for porphobilinogen deaminase from other sources are 4.2 for the *Chlorella* deaminase (Shioi et al., 1980), 4.5 for the *E. coli* enzyme (Jordan et al., 1988b) and 4.9 for the rat liver enzyme (Mazzetti and Tomio, 1988).

Thermal stability of porphobilinogen deaminase activity

Porphobilinogen deaminase exhibited remarkable thermal stability with little loss of activity even after heating to 70 °C (Figure 2b). At temperatures higher than 80 °C, however, there was a rapid loss in activity. Deaminases isolated from other sources have also shown remarkable stability to elevated temperatures (Jordan and Shemin, 1973; Anderson and Desnick, 1980; Shioi et al., 1980; Williams, 1984; Jordan et al., 1988b). The heat-stability of both the enzyme and the potentially labile dipyrromethane cofactor are explained by the large number of protein–cofactor interactions revealed in the X-ray structure of the *E. coli* porphobilinogen deaminase (Louie et al., 1992). In contrast, the apodeaminase, which lacks the dipyrromethane cofactor, is denatured above 40 °C (Scott et al., 1989; Jordan and Woodcock, 1991).

Presence of the dipyrromethane cofactor in *A. thaliana* porphobilinogen deaminase

Porphobilinogen deaminases contain a novel dipyrromethane cofactor (Jordan and Warren 1987; Jordan, 1991) attached covalently to an invariant cysteine (Cys-242 in the *E. coli* enzyme). An equivalent cysteine was found to be present in the primary sequence of *A. thaliana* at position 254 (A. G. Smith, personal communication). The reaction of purified porphobilinogen deaminase from *A. thaliana* with Ehrlich's reagent resulted in the characteristic spectral change (Figure 2c) on reaction with a dipyrromethane (Pluscec and Bogorad, 1970; Jordan and Warren, 1987; Warren and Jordan, 1988).

Determination of the N-terminal sequence of porphobilinogen deaminase

The N-terminus of *A. thaliana* porphobilinogen deaminase, sequenced by Edman degradation, was found to be as follows: XVAVEQKTRTAI. This sequence is in agreement with that derived from the *A. thaliana* cDNA sequence (A. G. Smith, personal communication) except for the N-terminal amino acid,

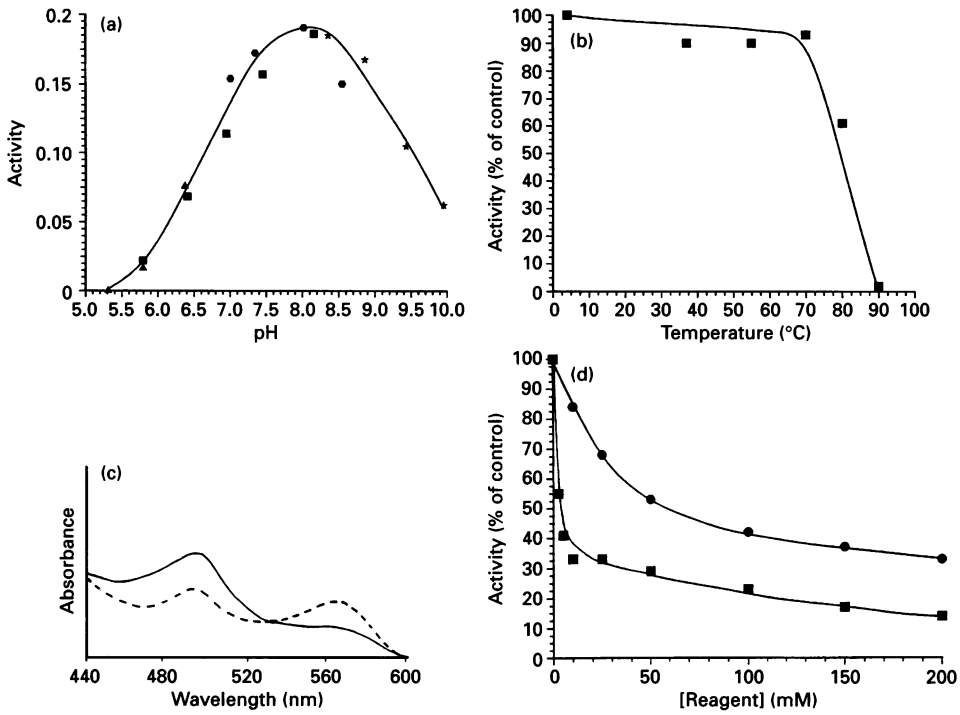


Figure 2 Properties of purified porphobilinogen deaminase from *A. thaliana*

(**a**) The pH optimum of porphobilinogen deaminase was determined by assay of enzyme activity, as described in the Materials and methods section, using porphobilinogen as substrate, at a range of different pHs from 5.5 to 10.0, in 100 mM buffer. The buffers used were as follows: Mes (pH 5.5–6.5), potassium phosphate (pH 6.0–8.0), Tris/HCl (7.5–8.5) and Ches (pH 8.5–10.0). (**b**) Effect of thermal denaturation on deaminase activity. Porphobilinogen deaminase was preincubated for 10 min at a variety of temperatures and the effect on activity determined, as described in the Materials and methods section. (**c**) Change in absorbance spectrum on reaction of modified Ehrlich's reagent with porphobilinogen deaminase. The change was determined between 400 nm and 600 nm over a period of 15 min after the addition of modified Ehrlich's reagent, as described in the Materials and methods section. The spectra shown are initial time (——) and after 15 min (– – –) (**d**) Effect of NH_4Cl (●) and hydroxylamine (■) on the activity of purified porphobilinogen deaminase. Purified porphobilinogen deaminase was assayed for activity, as described in the Materials and methods section, using porphobilinogen as substrate, in the presence of the range of neutralized NH_4Cl and hydroxylamine hydrochloride concentrations indicated.

cysteine, which could not be identified unambiguously because of being masked by traces of other amino acid contaminants, a common finding with the first Edman reaction. The presence of an N-terminal cysteine cannot therefore be confirmed.

The N-terminal sequence of the porphobilinogen deaminase from *A. thaliana* is similar to that deduced from the pea cDNA sequence (SLAVEQQTQQNKTAL), with five of the first eight amino acids being identical. A gap of three amino acids in the *Arabidopsis* sequence (QQN in pea) is the main difference. Thereafter the sequence similarity resumes with a basic amino acid (R/K) followed by TAL/I. The similarities in the N-termini of the *Arabidopsis* and pea enzymes probably relate to similarities in the recognition sequence for the protease responsible for processing the precursor protein into the mature enzyme after import in the chloroplast. Both mature *Arabidopsis* and pea enzymes have N-terminal extensions compared with other deaminases, Ala-14 and Ala-11 respectively representing the start of the first β-strand of the protein indicated from the X-ray structure of the *E. coli* enzyme (Louie et al., 1992), in which Asn-4 is the equivalent position.

Effect of NH_3 and hydroxylamine on porphobilinogen deaminase activity

NH_3, the enzymic product from the deamination of the substrate, porphobilinogen, is known to inhibit the deaminase, as is the NH_3 analogue, hydroxylamine (Jordan, 1991). The inhibitory base causes the release of the bound enzyme–substrate intermediates and is incorporated into the released pyrrole (Davies and Neuberger, 1973). The effects of NH_3 and hydroxylamine on the activity of porphobilinogen deaminase from *A. thaliana* were determined by assaying the enzyme in the presence of a range of concentrations of these bases (Figure 2d). As measured by the formation of uroporphyrin I, the deaminase activity was reduced to 50% at concentrations of 100 mM NH_4Cl and 2.5 mM hydroxylamine hydrochloride. The small amount of enzyme available did not permit a full detailed study of the effects of these inhibitory bases, but the *A. thaliana* deaminase appears to resemble those from other sources in this respect.

Effect of metal ions on activity

Porphobilinogen deaminase activity was determined in the presence of various metal ions as shown in Table 2. The presence of Cd^{2+} and Hg^{2+} at concentrations of 100 μM, and Zn^{2+}, Ag^+, and Co^{2+}, and to a lesser degree Cu^{2+}, at concentrations of 1 mM, inhibited the enzyme. Similar observations have been made on the porphobilinogen deaminases from rat liver (Farmer and Hollebone, 1984), yeast (Correa-Garcia et al., 1991) and pea (Spano and Timko, 1991) where activity was particularly susceptible to inhibition by heavy-metal ions. The presence of K^+ or Na^+ ions decreased uroporphyrin I production at high concentrations, with Na^+ having a greater effect than K^+ ions (results not shown). This observation was important in subsequent column purification procedures where K^+ was used in preference to Na^+ during salt gradient elution to avoid undue inhibition. This

Table 2 Effect of metal ions on the activity of porphobilinogen deaminase

Purified porphobilinogen deaminase was incubated with porphobilinogen in the presence of a variety of metal ions at the concentrations shown. The metals were all chloride salts except for Cu^{2+}, for which the sulphate was utilized. The effect on the activity was determined as described in the Materials and methods section, using porphobilinogen as substrate. All data are from a minimum of three determinations.

Metal ion	Concentration (mM)	Activity (% of control)
Control	0	100
Li^+	1	100
K^+	1	100
Mg^{2+}	1	92
Sr^{2+}	1	100
Mn^{2+}	1	85
Fe^{3+}	1	43
Co^{2+}	1	5
Ni^{2+}	1	58
Cu^{2+}	1	34
Ag^+	1	10
Zn^{2+}	1	22
Cd^{2+}	0.1	45
Hg^{2+}	0.1	24
EDTA	25	110

Table 3 Effect of protein-modification reagents on the activity of porphobilinogen deaminase

Purified porphobilinogen deaminase (0.2 nmol) was preincubated with various reagents (1 mM concentration) for 20 min at 37 °C in the presence or absence of porphobilinogen (PBG; 1 μM). The effect on activity was determined as described in the Materials and methods section, using porphobilinogen as substrate, in the absence of 2-mercaptoethanol. All data are from a minimum of three determinations.

Reagent	Activity (% of control)	
	−PBG	+PBG
Control	100	100
Phenylglyoxal	82	94
Diethyl pyrocarbonate	45	55
2,4,6-Trinitrobenzenesulphonic acid	61	75
Pyridoxal 5-phosphate	78	75
N-Ethylmaleimide	90	90
Iodoacetic acid	100	100
Iodoacetamide	100	100
2-Mercaptoethanol	170	170

finding contrasts with that reported for the human deaminase, for which there was no effect of Na^+ ions (Fumagalli et al., 1985).

Effect of protein-modification reagents on *A. thaliana* porphobilinogen deaminase

The effects of preincubation of porphobilinogen deaminase with a variety of reagents specific for functional groups in proteins are shown in Table 3. Lysine-modification reagents such as 2,4,6-trinitrobenzenesulphonic acid (1 mM) and pyridoxal 5-phosphate (5 mM) were shown to inhibit activity. Modification of lysine residues by pyridoxal 5-phosphate in the porphobilinogen deaminase of *E. gracilis* has also been shown to inactivate the enzyme (Hart et al., 1984). Diethyl pyrocarbonate reduced the deaminase activity by 50 % with the inactivation being reversible on the addition of hydroxylamine, implicating histidine as a possible amino acid target. Arginine modification by phenylglyoxal also resulted in some reduction in activity. This latter result was expected in view of the evidence that several essential and invariant arginine residues are located in the active-site cleft (Jordan and Woodcock, 1991; Lander et al., 1991). The above data demonstrate that both arginine and lysine residues are involved in the functioning of the deaminase catalytic site. The fact that modification of the deaminase in the presence of porphobilinogen resulted in a degree of protection from inhibition by both the lysine- and arginine-modifying reagents suggests that the residues involved may be located in the enzyme active site. These observations are consistent with information obtained from the three-dimensional structure of the *E. coli* deaminase (Louie et al., 1992) in which lysine and arginine residues play key roles in the enzyme mechanism.

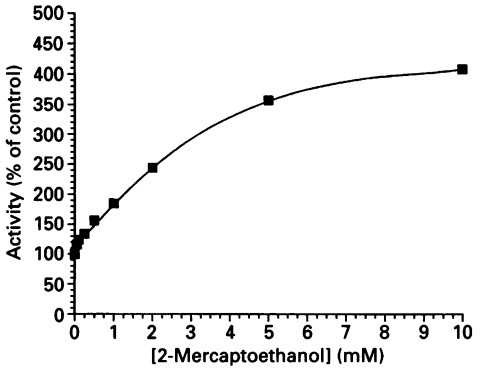


Figure 3 Effect of 2-mercaptoethanol concentration on the activity of purified *A. thaliana* porphobilinogen deaminase

Purified porphobilinogen deaminase was assayed for activity, as described in the Materials and methods section, using porphobilinogen as substrate, in the presence of the range of 2-mercaptoethanol concentrations indicated. All data are from a minimum of three determinations.

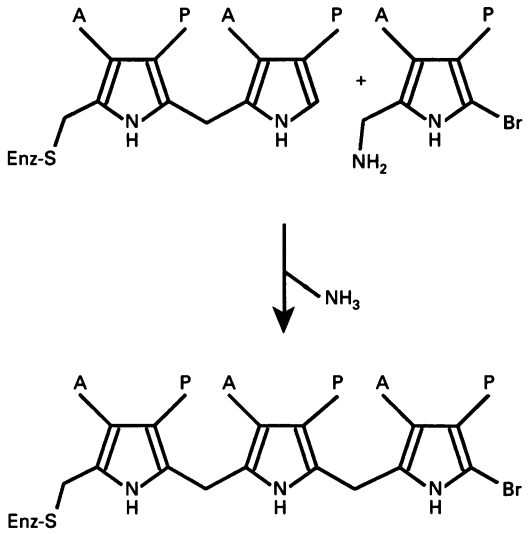
No appreciable inactivation was observed with the cysteine-specific modification reagent *N*-ethylmaleimide in either the presence or absence of porphobilinogen. This contrasts with the dramatic inactivation observed with the *E. coli* porphobilinogen deaminase caused by *N*-ethylmaleimide in the presence of porphobilinogen (Warren and Jordan, 1988). The reactive thiol of the *E. coli* enzyme, Cys-134 (Louie et al., 1992), is substituted by Ser-141 in the *A. thaliana* enzyme (A. G. Smith, personal communication) thus accounting for its lack of reaction with the reagent.

Little effect on enzyme activity was seen with other cysteine-modification reagents, consistent with previous observations made on the rat liver (Mazzetti and Tomio, 1988) and yeast enzymes (Correa-Garcia et al., 1991).

The reducing agent 2-mercaptoethanol stimulated the activity of the deaminase and was used subsequently during the purification. Further investigation revealed that the degree of stimulation was concentration-dependent, reaching a maximum at 10 mM (Figure 3). The presence of a thiol could enhance the activity of the deaminase by either maintaining the dipyrromethane cofactor in a reduced state or keeping the product, preuroporphyrinogen, in the reduced form so that optimal cyclization to uroporphyrinogen I may occur.

Effect of porphyrins and porphyrinogens on deaminase activity

Porphobilinogen deaminase was preincubated with a variety of porphyrins and porphyrinogens at concentrations of 4 μM, and



Scheme 2 Structure of 2-bromoporphobilinogen and its suicide reaction with the enzyme-bound dipyrromethane cofactor to yield a termination complex

A, acetic acid; P, propionic acid.

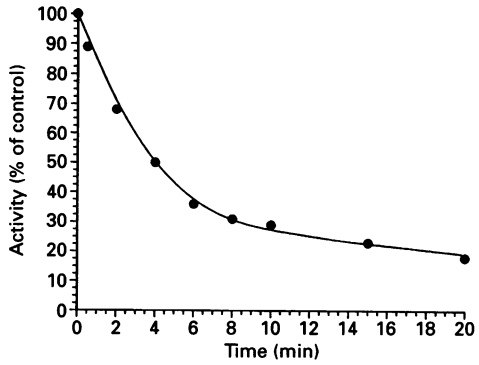


Figure 4 Effect of incubation time with 2-bromoporphobilinogen on the activity of purified *A. thaliana* porphobilinogen deaminase

Porphobilinogen deaminase was preincubated for a range of times with 2-bromoporphobilinogen (12 μM) at 37 °C. The effect on the activity was determined using porphobilinogen as substrate. All data are from a minimum of three determinations.

the effect on activity determined (results not shown). All the porphyrins and porphyrinogens tested inhibited activity to some extent (5–15%), although none caused marked inhibition.

Inhibition of porphobilinogen deaminase by 2-bromoporphobilinogen

2-Bromoporphobilinogen is structurally closely related to the substrate porphobilinogen (Scheme 2) and, as such, is deaminated and linked to the dipyrromethane cofactor of the holoenzyme in the same way as the substrate. However, once incorporated, the presence of the bromine atom at the 2-position blocks further addition of substrate, leading to an inactive enzyme. The inactivation of *E. coli* porphobilinogen deaminase with this suicide inhibitor has been studied extensively (Warren and Jordan, 1988). Purified *A. thaliana* porphobilinogen deaminase was preincubated with 12 μM 2-bromoporphobilinogen at 37 °C for various lengths of time, and the resulting effect on activity was determined by incubation with porphobilinogen (Figure 4). The results indicate that the plant enzyme is also sensitive to the inhibitor and is inactivated with a $t_{\frac{1}{2}}$ of 4 min.

Preincubation of the enzyme with a range of 2-bromoporphobilinogen concentrations for 10 min at 37 °C indicated that inactivation was concentration-dependent, with 50% inhibition occurring at concentrations of 4 μM. If a correction was made for the inactivation, 2-bromoporphobilinogen could also be shown to act as a competitive inhibitor with a K_i of 30 ± 5 μM.

Separation of porphobilinogen deaminase complexes by non-denaturing PAGE

Porphobilinogen deaminases from several sources have been shown to assemble the linear tetrapyrrole, preuroporphyrinogen, in a stepwise manner, through enzyme–intermediate complexes with one, two, three and four molecules of the substrate linked covalently to the dipyrromethane cofactor (Scheme 1). These complexes are referred to as ES, ES_2, ES_3 and ES_4 respectively (Jordan and Warren, 1987; Warren and Jordan, 1988). When *A. thaliana* porphobilinogen deaminase was incubated in the presence of porphobilinogen (2 μM) and the enzyme was subjected to PAGE under non-denaturing conditions, a second more negatively charged protein band of higher mobility appeared below the native deaminase (results not shown). The appearance of the second band was accompanied by a decrease in the intensity of the native deaminase band, indicating that it was derived from the native enzyme. The mobility of the complex was consistent with the formation of an enzyme–intermediate complex with one molecule of bound substrate (ES). The complex is more negatively charged because of the additional acetate and propionate groups of the bound substrate (see Scheme 1).

Final conclusions

The porphobilinogen deaminase purified from *A. thaliana* has been shown to possess properties very similar to other porphobilinogen deaminases. In common with the enzyme from *E. coli*, the *A. thaliana* deaminase contains the novel dipyrromethane cofactor which acts as a primer in the tetrapolymerization reaction. The amino acid sequence of the *A. thaliana* deaminase, derived from the cDNA sequence (A. G. Smith, unpublished work), indicates the probable covalent attachment site for the dipyrromethane cofactor as Cys-254, equivalent to Cys-242 in *E. coli* deaminase. The presence of a serine residue at position 141, instead of the Cys-134 found at the equivalent position in the *E. coli* deaminase, explains satisfactorily the lack of inhibition of the *A. thaliana* deaminase by cysteine-directed chemical reagents. The small amount of pure enzyme isolated prevented further detailed studies, but the construction of a recombinant strain of *E. coli* harbouring the *A. thaliana* deaminase cDNA will permit more extensive studies in the future. Nevertheless, the results reported in the present paper suggest that the *A. thaliana* enzyme is closely related to deaminases from other sources, showing that porphobilinogen deaminases are highly conserved in both structure and mechanism throughout the biosphere.

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