Biochem. J. (1986) 236, 447–451 (Printed in Great Britain)

Identification of lysine at the active site of human 5-aminolaevulinate dehydratase

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1. Reduction of human 5-aminolaevulinate dehydratase with $NaBH_4$ in the presence of ^{14}C-labelled substrate led to complete loss of catalytic activity and to incorporation of label into the enzyme protein. 2. By comparison with authentic lysyl-aminolaevulinic acid, prepared chemically, the modified active-site amino acid obtained by acid hydrolysis was shown to be lysine. 3. Sequencing of a CNBr-cleavage peptide isolated from the inactivated ^{14}C-labelled enzyme revealed that the lysine was present within the sequence M-V-K-P-G-M.

INTRODUCTION

5-Aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24) (Shemin, 1972) catalyses the dimerization of 5-aminolaevulinate to give porphobilinogen, the monopyrrolic precursor for haems, chlorophylls, corrins and other tetrapyrroles (Akhtar & Jordan, 1978).

The human enzyme has been purified to homogeneity (Anderson & Desnick, 1979; Gibbs *et al.*, 1985*b*), and shown to be a homo-octamer of apparent M_r 252000–285000.

Investigations with dehydratases from numerous sources have established the importance of thiol groups for activity, and it has been suggested that at least one of these residues may play an important role in the binding of the essential Zn^{2+} ion (Gibbs *et al.*, 1985*a*). In addition to thiol groups, histidine has been proposed as an important catalytic residue in the bovine enzyme (Tsukamoto *et al.*, 1975) and arginine has been implicated in the spinach enzyme (Liedgens *et al.*, 1983). The finding that pyridoxal 5′-phosphate may be present in the mammalian enzyme has also prompted the suggestion that the substrate may bind to this chromophore (Shemin, 1976).

Initial studies with the dehydratases from both bacterial (Nandi & Shemin, 1968) and mammalian sources (Cheh & Neilands, 1976; Jordan & Seehra, 1980) established that the enzymes can be inactivated with $NaBH_4$ in the presence of the substrate 5-aminolaevulinate, and that when ^{14}C-labelled substrate is employed the inactivation is accompanied by the incorporation of ^{14}C radioactivity into the dehydratase protein. More recently, mechanistic studies, in our laboratory, have established that, of the two substrate molecules required for the formation of porphobilinogen, it is the one giving rise to carbon atoms 2, 3, 8, 9 and 10 (see Scheme 1) that is irreversibly bound to the dehydratase protein by the borohydride treatment (Jordan & Seehra, 1980; Jordan & Gibbs, 1985).

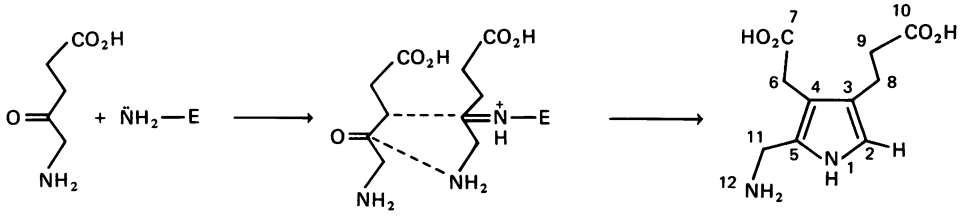
To date, the active-site amino acid that is involved in Schiff-base formation with 5-aminolaevulinate has not been fully characterized. In fact, it has been proposed that arginine fulfils the role in the spinach enzyme (Liedgens *et al.*, 1983) whereas in the bacterial enzyme lysine has been tentatively identified (Nandi, 1978). No equivalent studies have been carried out, however, on enzymes from mammalian sources.

This paper describes the inactivation of human 5-aminolaevulinate dehydratase with $NaBH_4$ in the presence of 5-amino[4-^{14}C]laevulinate, the isolation and sequencing of the active-site peptide and the identification of the modified active-site amino acid residue.

MATERIALS AND METHODS

Reagents

5-Aminolaevulinate hydrochloride, N^{α}-acetyl-L-lysine methyl ester hydrochloride and amino acid phenylthiohydantoin derivatives were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 5-Amino[4-^{14}C]-laevulinate hydrochloride was obtained from Amersham International (Amersham, Bucks., U.K.). *N*-Benzoyl-5-aminolaevulinate methyl ester was a gift from Dr. D. Evans (formerly of the University of Southampton). H.p.l.c. solvents and phenyl isothiocyanate were pur-



Scheme 1. Formation of porphobilinogen from 5-aminolaevulinate

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chased from Rathburn Chemicals (Walkerburn, Peebles-shire, Scotland, U.K.). Other chemicals were obtained from BDH Chemicals (Poole, Dorset, U.K.) except where the supplier is quoted in cited references.

Isolation of 5-aminolaevulinate dehydratase from human erythrocytes

The enzyme was purified to homogeneity from human erythrocytes and assayed according to the method of Gibbs *et al.* (1985*b*). The enzyme was always activated before use.

Modification of 5-aminolaevulinate dehydratase with $NaBH_4$ in the presence of 5-amino[4-^{14}C]laevulinate

Enzyme as $(NH_4)_2SO_4$ precipitate (approx. 940 nmol) was dialysed against 0.15 M-potassium phosphate buffer, pH 7.9, containing 10 mM-dithioerythritol. 5-Amino[4-^{14}C]laevulinate (940 nmol; 50 μCi) was mixed with the enzyme at 0 °C in a rapid-mixing apparatus (Jordan & Gibbs, 1985). After approx. 10 s 20 μl of 0.5 M-$NaBH_4$ was added and the pH was maintained at 7.9 by the careful addition of small volumes (20 μl) of 0.5 M-acetic acid. The reduction process was repeated three times, after which the inactive enzyme was precipitated by the addition of $(NH_4)_2SO_4$ (390 mg/ml of solution). The precipitate was collected by centrifugation, washed three times with saturated $(NH_4)_2SO_4$ and dialysed against 0.1 M-potassium phosphate buffer, pH 7.9, containing 20 mM-2-mercaptoethanol.

Complete modification of all available sites in the enzyme was ensured by repeating the reduction procedure described above in the presence of a large excess of unlabelled 5-aminolaevulinate (188 μmol).

Isolation of CNBr-cleavage fragments from modified human 5-aminolaevulinate dehydratase

The inactive ^{14}C-labelled protein (approx. 900 nmoles; 1.3×10^4 d.p.m./nmol) was carboxymethylated (Hirs, 1967), dialysed and freeze-dried. The residue was dissolved in 3.3 ml of aq. 70% (v/v) formic acid and stirred with CNBr (400-fold molar excess) for 18 h at room temperature in the dark. After being freeze-dried, the residue was suspended in 9% (v/v) formic acid and applied to a Sephadex G-50 (superfine grade) gel-filtration column (100 cm × 2.8 cm), which was developed at a flow rate of 0.25 ml/min. The radioactive fractions were identified by counting for radioactivity and were freeze-dried.

Purification and sequencing of the active-site peptide

The ^{14}C-labelled peptide was further purified by h.p.l.c. with a standard Waters Associates C_{18} μBondapak analytical column and a linear gradient from 100% solvent A to 90% solvent A/10% solvent B over a 20 min period at a flow rate of 1.2 ml/min [where solvent A is 0.12% (v/v) trifluoroacetic acid in water, and solvent B is 0.07% (v/v) trifluoroacetic acid in acetonitrile] (see Fig. 1*b*).

The purified ^{14}C-labelled peptide was sequenced by the method of Chang *et al.* (1978) except that the number of extractions used to remove excess coupling reagents was decreased to minimize losses of the peptide.

The amino acid dimethylaminoazobenzenethiohydantoin derivatives were identified by two-dimensional t.l.c. (Chang *et al.*, 1978), and the amino acid phenylthiohydantoin derivatives from the sequencing were identified by h.p.l.c. with a Waters Associates C_{18} μBondapak column developed essentially as described by Bloxham *et al.* (1982).

The ^{14}C-labelled peptide was hydrolysed for 18 h at 110 °C in 6 M-HCl (AnalaR), and after it had been freeze-dried the amino acid composition was determined by autoanalysis on a Rank Hilger (U.K.) amino acid analyser according to the manufacturer's instructions.

Synthesis of 'lysyl-5-aminolaevulinate'

Lysyl-5-aminolaevulinate (see Scheme 2) was prepared by reaction of 30 mg of *N*-benzoyl-5-aminolaevulinate methyl ester (5-benzoamido-4-oxopentanoate methyl ester) and 30 mg of N^{α}-acetyl-L-lysine methyl ester in 9 ml of methanol/10 mM-K_2HPO_4 (8:1, v/v) at a final pH of 9. After 8 h at room temperature the solution was treated with 15 mg of $NaBH_3CN$, and after 16 h the solution was freeze-dried. Deprotection was carried out by hydrolysis for 18 h at 110 °C in 6 MHCl (AnalaR). The hydrolysed product was dissolved in butanol/acetic acid/water (4:1:1, by vol.), applied to a silica column (2.5 cm × 1 cm) and eluted with the same solvent mixture to remove dihydro-5-aminolaevulinate and traces of 5-aminolaevulinate. The derivative was eluted with chloroform/methanol/aq. NH_3. (sp.gr. 0.880) (2:2:1, by vol.) and freeze-dried to yield 7 mg of lysyl-5-aminolaevulinate. Lysine remained bound to the silica.

The derivative was analysed, together with the ^{14}C-labelled modified amino acid obtained by the hydrolysis of labelled enzyme, by using high-voltage paper electrophoresis (pH 6.4) with pyridine/acetic acid/water (25:1:225, by vol.) as the buffer. Separation was performed for 1 h at 2 kV in a Gilson high-voltage electrophorator. Alternatively the derivative was analysed by two-dimensional t.l.c. on cellulose plates with chloroform/methanol/water (2:2:1, by vol.) in the first dimension followed by butanol/acetic acid/water (4:1:1, by vol.) in the second.

Radioactivity counting and protein determinations

These were carried out as described by Jordan & Gibbs (1985).

RESULTS AND DISCUSSION

Reaction of human 5-aminolaevulinate dehydratase with $NaBH_4$ in the presence of an equimolar quantity of 5-amino[4-^{14}C]laevulinate led to the incorporation of ^{14}C radioactivity into the protein and to a complete loss of catalytic activity within 2 min. The presence of the product, porphobilinogen, a competitive inhibitor, afforded almost total protection against inactivation (Fig. 1*a*). Borohydride treatment in the absence of substrate was also ineffective at inactivating the enzyme. The procedure resulted in the incorporation of 1.02 mol of 5-amino[4-^{14}C]laevulinate/mol of enzyme subunit, and a final specific radioactivity of 13400 d.p.m./nmol of protein was obtained. CNBr treatment of the inactive labelled protein, after prior carboxymethylation, yielded 12 peptides, one of which fractionated near the end of the Sephadex G-50 elution and contained most (approx. 75%) of the applied radioactivity.

After purification on a reverse-phase h.p.l.c. column (Fig. 1*b*) the ^{14}C-labelled peptide was subjected both to complete amino acid analysis and also to sequencing by the method of Chang *et al.* (1978). Amino acid analysis

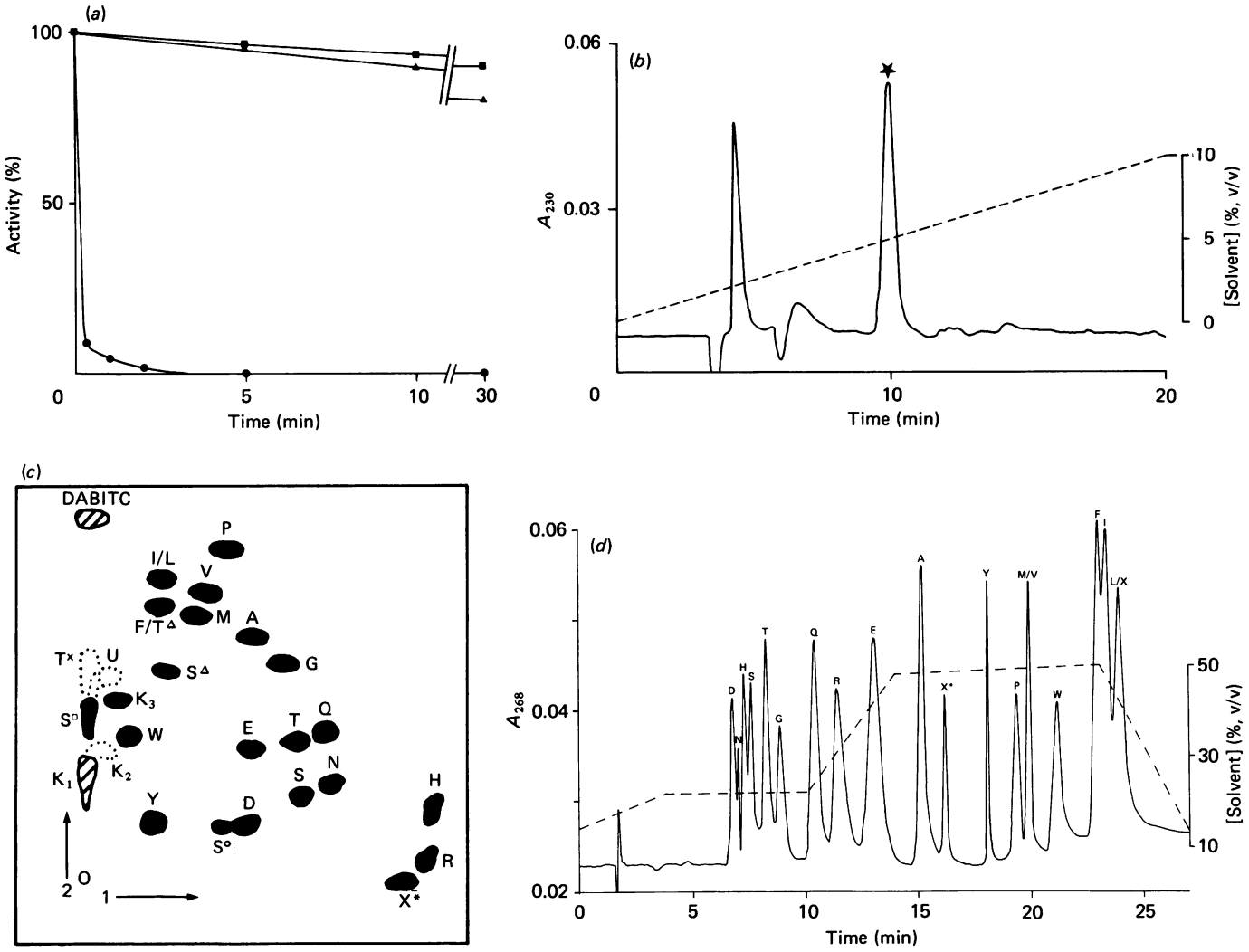
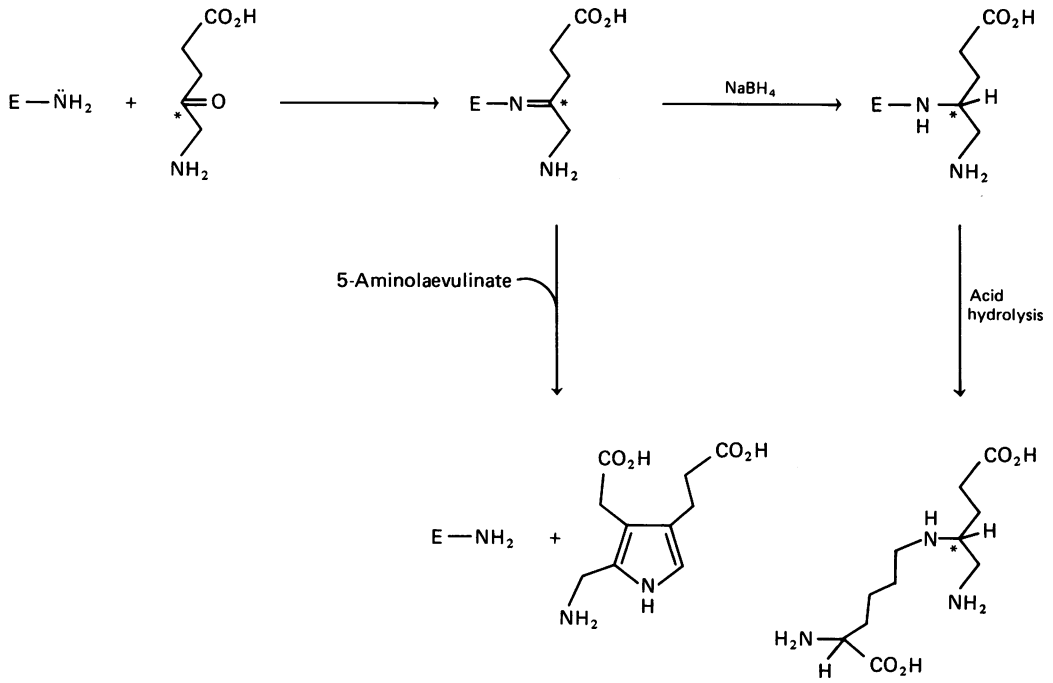


Fig. 1. Reduction of human 5-aminolaevulinate dehydratase with $NaBH_4$ in the presence of 5-amino[4-^{14}C]laevulinate, isolation of a labelled CNBr-cleavage peptide and identification of the modified active-site amino acid

(*a*) Protection by porphobilinogen of 5-aminolaevulinate dehydratase from inactivation by $NaBH_4$ in the presence of 5-amino[4-^{14}C]laevulinate. The human enzyme was mixed with a stoichiometric equivalent of 5-amino[4-^{14}C]laevulinate on ice at pH 7.9 in the presence (▲) and in the absence (●) of 10 mM-porphobilinogen. After the addition of 0.5 M-$NaBH_4$ (20 μl) followed by 0.5 M-acetic acid (20 μl), samples were removed at timed intervals and the enzyme activity was determined as described in the Materials and methods section. In the control experiment (■) only $NaBH_4$ (followed by acetic acid) was added. (*b*) Purification of the labelled human 5-aminolaevulinate dehydratase CNBr-cleavage peptide by h.p.l.c. The labelled peptide was purified by h.p.l.c. as described in the Materials and methods section. The symbol ★ indicates the location of the radioactive peptide. (*c*) Resolution of amino acid dimethylaminoazobenzenethiohydantoin derivatives by two-dimensional t.l.c. The amino acid derivatives were identified by exposure to HCl (red), dotted areas (blue) and hatched areas (purple). U is the blue-coloured thiourea formed by the coupling of phenyl isothiocyanate with hydrolysed dimethylaminoazobenzene isothiocyanate. X* is the modified ^{14}C-labelled amino acid. Multiple spots are produced by lysine (K_1, K_2 and K_3), serine (S, S□,S△ and S○) and threonine (T, T× and T△) [see Chang *et al.* (1978) for details]. (*d*) Resolution of amino acid phenylthiohydantoin derivatives by h.p.l.c. The phenylthiohydantoin derivatives were separated by h.p.l.c. in accordance with Bloxham *et al.* (1982) and as described in the Materials and methods section. A typical separation is shown in which the peak X* corresponds to the modified ^{14}C-labelled amino acid from turn 2 of the purified human 5-aminolaevulinate dehydratase CNBr-cleavage peptide and contained 95% of the applied radioactivity.

of the labelled peptide after acid hydrolysis revealed glycine, valine and proline as the major constituents in approximately equal proportions together with homoserine and homoserine lactone from the peptide *C*-terminus. In addition, a single peak, containing all the radioactivity, was identified that showed basic properties and chromatographed midway between histidine and lysine on the amino acid analyser (results not shown).

Sequencing of the peptide revealed that the active-site amino acid was the second residue in the peptide, since most (85%) of the ^{14}C radioactivity was lost during the second 'turn' when the thiazolinones were extracted into butyl acetate. After cyclization, the dimethylaminoazobenzenethiohydantoin and phenylthiohydantoin derivatives of the labelled active-site residue were characterized by t.l.c. and h.p.l.c. respectively, with the appropriate



Scheme 2. Formation of 'lysyl-5-aminolaevulinate' from modified 5-aminolaevulinate dehydratase

standard amino acid derivatives. The radioactive dimethylaminoazobenzenethiohydantoin derivative chromatographed near arginine on the two-dimensional system (Fig. 1*c*), whereas the ^{14}C-labelled amino phenylthiohydantoin derivative was eluted between alanine and tyrosine on h.p.l.c. (Fig. 1*d*). The amino acid sequence obtained by these two methods was found to be:

(M)-V-X*-P-G-(M)

where X* is the modified ^{14}C-labelled active-site amino acid. [A similar sequence was found for the bovine enzyme (Gibbs, 1984) except for the presence of arginine rather than methionine at the *C*-terminal position of the active-site peptide, a substitution satisfactorily explained on the basis of a single base difference (i.e. CTG for ATG) between the human and bovine genes (Gibbs, 1984).]

Both on intuitive grounds and from the studies carried out on the enzyme from *Rhodopseudomonas sphaeroides* (Nandi, 1978), we expected that lysine would be the amino acid at the active site of the human 5-aminolaevulinate dehydratase that is responsible for forming the covalent link with the substrate. Although histidine (Tsukamoto *et al.*, 1975) and arginine (Liedgens *et al.*, 1983) have also been implicated as being involved at the active site of 5-aminolaevulinate dehydratase, these conclusions were derived from the use of non-specific reagents such as pyrocarbonate and butanedione. In our studies we have modified the active-site amino acid by borohydride treatment in the presence of the substrate itself, ensuring that we are investigating a catalytically relevant species.

The preparation of an authentic reduced 5-aminolaevulinate–lysine adduct was central to the unambiguous identification of lysine at the human 5-aminolaevulinate dehydratase active site. Accordingly, this was prepared chemically by reaction of *N*-benzoyl-5-aminolaevulinate methyl ester and N^{α}-acetyl-L-lysine methyl ester under weakly basic conditions. The reduction of the Schiff base with $NaBH_3CN$, followed by deprotection, yielded a new amino acid, with the physical properties of a basic amino acid, which when analysed on the amino acid analyser chromatographed between histidine and lysine with exactly the same retention time as the ^{14}C-labelled peak that resulted from the hydrolysis of the ^{14}C-labelled active-site peptide. This provided strong evidence that the amino acid at the active site of the enzyme was indeed lysine.

Further corroboration was obtained by comparison of the dimethylaminoazobenzenethiohydantoin (Fig. 1*c*) and phenylthiohydantoin (Fig. 1*d*) derivatives of the radioactive active-site amino acid with the dimethylaminoazobenzenethiohydantoin and phenylthiohydantoin standards prepared from the chemically synthesized adduct. In both cases the authentic compounds had chromatographic properties identical with those of the ^{14}C-labelled amino acid derivatives released from the active-site peptide during sequencing. The modified ^{14}C-labelled amino acid also co-chromatographed with the synthetic adduct in the two-dimensional t.l.c. procedure detailed in the method.

Finally, the ^{14}C-labelled amino acid and the chemically synthesized adduct showed identical electrophoretic behaviour at pH 6.4, with a mobility of 0.81 when compared with lysine (1.0). We therefore conclude that lysine is the amino acid residue responsible for the initial formation of the Schiff base with the substrate molecule giving rise to carbon atoms 2, 3, 8, 9 and 10 in porphobilinogen.

Several features of the experimental conditions used in this work point to the reliability of this conclusion. Firstly, in using the substrate itself to label the active site, the possibility of indiscriminate reaction is minimized compared with non-specific reagents. Secondly, the product porphobilinogen, a competitive inhibitor, almost totally protects the enzyme from both inactivation and

^{14}C-labelling, suggesting we are dealing with an active-site phenomenon. Thirdly, and most important, the complex formed between enzyme and 5-aminolaevulinate that is stabilized by reduction with borohydride has been shown to be the same species that, on addition of further substrate, turns over to form the product porphobilinogen (see Scheme 2) (Jordan & Gibbs, 1985). There is little doubt, therefore, that we have by borohydride reduction trapped a mechanistically significant species rather than some non-physiological intermediate or an abortive complex.

Lysine has been shown to act as the active-site residue responsible for Schiff-base formation with the substrate in several other enzymes, most notably in aldolase, transaldolase and acetoacetate decarboxylase (Snell & di Mari, 1970). It is of some note that, although the 5-aminolaevulinate dehydratase would seem, from the above discussion, to belong to the Schiff-base aldolases (Class I), the mammalian enzyme also contains a metal ion similar to the Class II aldolases. There is evidence, in addition to that presented here, that is consistent with the suggestion that the metal ion is playing a structural rather than a catalytic role (Gibbs *et al.*, 1985*a*) and that the dehydratase is not a metalloaldolase, in the mechanistic sense at least.

The human 5-aminolaevulinate dehydratase cDNA has now been cloned and partially sequenced (Wetner *et al.*, 1986). Within the cDNA is an 18-base sequence coding for our active-site peptide sequence M-V-K-P-G-M. The DNA sequence data and peptide sequence data thus mutually reinforce one another, the DNA sequence providing additional evidence for the presence of lysine at the active site and our peptide sequence enabling the precise position of the active-site lysine to be placed within the primary structure of the enzyme.

We are grateful to the Science and Engineering Research Council for financial support, to Dr. M. G. Gore for assistance with amino acid analysis and to Professor Desnick for making results available before publication.

REFERENCES

Akhtar, M. & Jordan, P. M. (1978) Compr. Org. Chem. **5**, 1121–1163
Anderson, P. M. & Desnick, R. J. (1979) J. Biol. Chem. **254**, 6924–6930
Bloxham, D. P., Parmelee, D. C., Kumar, S., Walsh, K. A. & Titani, K. (1982) Biochemistry **21**, 2028–2036
Chang, J. Y., Brauer, D. & Wittman-Liebold, B. (1978) FEBS Lett. **93**, 205–214
Cheh, A. M. & Neilands, J. B. (1976) Struct. Bonding **29**, 123–170
Gibbs, P. N. B. (1984) Ph.D. Dissertation, University of Southampton
Gibbs, P. N. B., Gore, M. G. & Jordan, P. M. (1985*a*) Biochem. J. **225**, 573–580
Gibbs, P. N. B., Chaudhry, A. G. & Jordan, P. M. (1985*b*) Biochem. J. **230**, 25–34
Hirs, C. H. W. (1967) Methods Enzymol. **11**, 199–203
Jordan, P. M. & Gibbs, P. N. B. (1985) Biochem. J. **227**, 1015–1020
Jordan, P. M. & Seehra, J. S. (1980) FEBS Lett. **114**, 283–286
Liedgens, W., Lutz, C. & Schneider, H. A. W. (1983) Eur. J. Biochem. **135**, 75–79
Nandi, D. L. (1978) Z. Naturforsch. C. Biosci. **33**, 799–800
Nandi, D. L. & Shemin, D. (1968) J. Biol. Chem. **243**, 1231–1242
Shemin, D. (1972) Enzymes 3rd Ed. **7**, 323–337
Shemin, D. (1976) Philos. Trans. R. Soc. London Ser. B **273**, 109–115
Snell, E. E. & di Mari, S. J. (1970) Enzymes 3rd Ed. **2**, 335–370
Tsukamoto, I., Yoshinga, T. & Sano, S. (1975) Biohem. Biophys. Res. Commun. **67**, 294–300
Wetner, D., Bishop, D. F. & Desnick, R. J. (1986) Proc. Natl. Acad. Sci. U.S.A., in the press

Received 30 December 1985/17 February 1986; accepted 28 February 1986