Escherichia coli dihydrodipicolinate synthase: characterization of the imine intermediate and the product of bromopyruvate treatment by electrospray mass spectrometry

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Simplified procedures for assaying and purifying dihydrodipicolinate synthase (EC 4.2.1.52), the first enzyme of the lysine biosynthetic pathway, have been developed and electrospray ionization m.s. has been used to observe the imine intermediate with pyruvate and to investigate the reaction of the enzyme with bromopyruvate and fluoropyruvate.

INTRODUCTION

The occurrence of the lysine biosynthetic pathway in microorganisms and plants but not in animals makes its enzymes attractive targets for antibacterials and herbicides. The first specific step of lysine biosynthesis in bacteria and plants (Cohen and Saint-Girons, 1987; Bonner and Lea, 1990), which involves the condensation of aspartic acid β-semialdehyde (ASA) and pyruvate to form dihydrodipicolinate, is catalysed by the enzyme dihydrodipicolinate synthase (DHDPS) (Shedlarski and Gilvarg, 1970). The gene encoding this enzyme in *Escherichia coli* (*dapA*) has been cloned and sequenced (Richaud et al., 1986) and the overexpressed enzyme has been purified and crystallized (Laber et al., 1992). The DHDPS gene has also been cloned and sequenced from *Corynebacterium glutamicum* (Bonnassie et al., 1990), and from two higher plant species: wheat (Kaneko et al., 1990) and maize (Frisch et al., 1991). To aid the design of inhibitors we have initiated a mechanistic study of the *E. coli* enzyme. Here we describe a simplified assay of the enzyme, and the use of electrospray ionization m.s. (e.s.m.s.) to observe the imine intermediate formed between the enzyme and pyruvate and to characterize the reaction of the enzyme with bromopyruvate and fluoropyruvate.

MATERIALS AND METHODS

Materials

Plasmid pDA2 carrying the *dapA* gene of *E. coli* was a gift from Dr. Patrick Stragier, Institut Pasteur, Paris, France (Richaud et al., 1986). It was transformed into *E. coli* MV1190, an M13-susceptible RecA strain obtained from Biorad, Watford, U.K. to produce MV1190/pDA2 which was used as the enzyme source. Cells for the enzyme preparation were grown on MM63 medium with ampicillin at 30 °C.

Aspartic acid β-semialdehyde was synthesized as the trifluoroacetate salt (Tudor et al., 1993).

Assay of DHDPS

The enzyme was assayed at 25 °C by monitoring the spontaneous formation of dipicolinic acid (formed without observable lag from the immediate reaction product dihydrodipicolinic acid, see the Results and Discussion section) at 270 nm (ϵ = 4000 $M^{-1} \cdot cm^{-1}$) using a Gilford model 2600 Spectrophotometer equipped with a slave recorder. The assay mixture (total volume 1 ml) contained (final concentrations) 100 mM imidazole buffer, pH 7.4, 5 mM sodium pyruvate, 2 mM aspartate semialdehyde and enzyme. The definition of 1 unit of enzyme activity is the amount of enzyme that catalyses the formation of 1 μmol of product/min.

Isolation of the assay product by h.p.l.c.

The reaction product was most conveniently isolated by incubating a 20 ml sample containing 5 mM sodium pyruvate, 2 mM ASA and 30 units of DHDPS in 100 mM Tris/HCl buffer, pH 7.4. (Preliminary experiments had shown that the assay worked not only in 100 mM imidazole buffer but also in 100 mM Tris/HCl buffer and in 100 mM potassium phosphate buffer although the observed rates in these two buffers were lower than those observed in imidazole buffer.) Samples (1 ml) were then run on a Biorad Aminex Ion Exclusion HPX-87H column (30 mm × 7.8 mm), eluting with 25 mM formic acid, using a Gilson h.p.l.c. system and monitoring with the detector set at 270 nm. Fractions containing the product peak (eluting at approx. 40 min) were pooled and freeze-dried.

Purification of DHDPS

All steps except the MonoQ chromatography were carried out at 4 °C. *E. coli* MV1190/pDA2 cells (24 g) were broken by three passages through an automatic French pressure cell at 95 MPa in 50 ml of 20 mM Tris/HCl, pH 7.4, extraction buffer containing 10 mM EDTA, 0.4 mM dithiothreitol, 100 mM KCl (Buffer A) and 10 mM sodium pyruvate. After centrifugation the supernatant was subjected to a heat step (70 °C for 2 min), centrifuged again and the supernatant dialysed overnight against buffer A. The dialysed protein was chromatographed on a DEAE-Sephacel (Pharmacia) column (30 cm × 3 cm) and eluted with a linear gradient (900 ml) of 0.1–0.5 M KCl in buffer A. The fractions

Abbreviations used: ASA, aspartic acid β-semialdehyde; DHDPS, dihydrodipicolinate synthase; e.s.m.s., electrospray m.s.

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with highest enzyme activity were pooled and dialysed overnight against buffer A. Ammonium sulphate was added to a final concentration of 0.5 M and the sample chromatographed on a phenyl-Sepharose (Pharmacia) column (15 cm × 4 cm) eluted with a decreasing linear gradient (500 ml) of 0.5–0.0 M KCl in buffer A. The fractions of highest activity were pooled and dialysed against buffer A. Finally the dialysed enzyme was applied to a Mono Q (Pharmacia) column (HR 10/10) and eluted with a two-phase gradient (180 ml) of 0.1–0.5 M KCl over 20 min followed by 0.5–1.0 M KCl over 40 min both in buffer A. The fractions with highest activity were pooled and dialysed overnight against buffer A. For long-term storage the enzyme was dialysed against buffer A containing 50 % (v/v) glycerol and kept at −20 °C. The enzyme retained essentially all its activity during 6 months storage in this manner.

E.s.m.s.

This was carried out with a VG BioQ quadrupole mass spectrometer as described previously (Shneier et al., 1991).

RESULTS AND DISCUSSION

Assay and purification of DHDPS

Three assays have been described for DHDPS (Yugari and Gilvarg, 1965): a spectrophotometric assay which monitors the change in absorbance at 270 nm when pyruvate and ASA are incubated with enzyme in the presence of imidazole buffer at pH 7.4; a spectrophotometric assay which monitors at 540 nm the formation of the adduct (of unknown structure) of the reaction product and *o*-aminobenzaldehyde; and a coupled assay with dihydrodipicolinate reductase. All three assays had disadvantages. The first two could not be used to determine precise kinetic constants since the products had not been characterized and the third one required the preparation of coupling enzyme. Previous versions of the 270 nm assay, which was the most convenient, involved the use of unpurified samples of aspartate semialdehyde produced by the ozonolysis of DL-allylglycine (Black and Wright, 1954a). ASA concentrations had to be determined enzymically using crude preparations of homoserine dehydrogenase (Black and Wright, 1954b). The availability of pure, stable preparations of ASA (Tudor et al., 1993) has now allowed this assay to be properly characterized. The product of the reaction was isolated by chromatography on an organic acids h.p.l.c. column eluted with 25 mM formic acid and its elution time from the column and its u.v. and n.m.r. spectra have shown it to be indistinguishable from those obtained with authentic samples of dipicolinic acid. The u.v. spectrum also underwent the characteristic shift on treatment with calcium chloride which has been previously reported for dipicolinic acid (Powell, 1953; Kimura, 1974). Progress curves have shown only a minimal lag (a few seconds) in the formation of the species absorbing at 270 nm. We conclude that under the conditions of the assay pyruvate and aspartate semialdehyde condense to form a cyclic product, presumably dihydrodipicolinate, which then immediately aromatizes in the presence of dissolved oxygen to dipicolinic acid. In earlier work where this assay was used enzyme activity was reported in absorbance units per minute; however, the absorption coefficient for dipicolinic acid is known (Powell, 1953) and it is therefore possible using this assay to measure dirctly the amount of enzyme in international units.

Using this assay procedure we have developed a simplified purification procedure for DHDPS which is essentially similar to that recently reported by Laber et al. (1992). The purification involved breakage of the cells in a French pressure cell, a heat step, chromatography on DEAE-Sephacel and then phenyl-Sepharose and finally chromatography on Mono Q. A typical purification table is shown as Table 1. It should be noted that the assay procedure consistently underestimated the enzyme activity in crude extracts. It is not known whether this was due to the presence of inhibitors which were later removed during purification or to a low activation of the enzyme during purification. The purification procedure gave 35 mg of protein of specific activity 52 units/mg from 24 g of cells. The enzyme was homogeneous as judged by PAGE in the presence of SDS. The subunit M_r, estimated by SDS/PAGE, was 33000, in good agreement with the M_r of 31372 predicted from the nucleotide sequence of the *dapA* gene (Richaud et al., 1986). The native M_r estimated by gel permeation chromatography on Superose 6 was 140000, confirming that the native enzyme is a tetramer as reported previously by Shedlarski and Gilvarg (1970) and Laber et al. (1992).

E.s.m.s. of DHDPS

The purified DHDPS was analysed by e.s.m.s. The observed M_r was 31272 (average of 11 determinations). Under the conditions of the e.s.m.s. only the dissociated enzyme subunits would be expected to be observed and not the native homotetramer.

The calculated M_r of DHDPS based on the DNA-derived amino acid sequence is 31372 (Richaud et al., 1986) (100 greater than observed). Unpublished amino acid composition data for the tryptic peptide containing residues 220 to 230 (inclusive) indicates that one of the three glutamic acid residues in this peptide must be a glycine (B. Laber, personal communication). This reduces the calculated M_r to 31300, still 28 larger than the observed mass. The difference of two methylene groups between the predicted and observed value is well within the resolution of the mass spectrometer, furthermore the deconvoluted spectrum shows no significant tailing. This indicates that the protein sample is pure and that the mass observed is real and not a consequence of poor resolution. The discrepancy between the experimentally observed M_r and the M_r calculated from the derived amino acid sequence indicates that the amino acid sequence must contain at least one more error.

E.s.m.s. of the incubation product of DHDPS with pyruvate

The enzyme mechanism is Ping Pong BiBi, with initial binding of pyruvate as an imine to an active site lysine (Shedlarski and Gilvarg, 1970; Kumpaisal et al., 1987; Laber et al., 1992) [Shedlarski and Gilvarg (1970) and Laber et al. (1992) both worked with the *E. coli* enzyme while Kumpaisal et al. (1987) worked with the wheat enzyme.] Water is lost and then ASA binds. The imine formation has been demonstrated by borohydride trapping experiments and lysine 161, the only conserved lysine residue between the bacterial and plant enzyme sequences, has been shown to form the imine (Laber et al., 1992). Imine formation on dehydroquinase has recently been observed directly by e.s.m.s. (Shneier et al., 1991). This has prompted us to undertake a study of the DHDPS reaction using e.s.m.s.

DHDPS was incubated with pyruvate and inactivated by the addition of sodium borohydride. The e.s.m.s. showed predominantly a single protein species indicating that all the active sites had been modified. The M_r of the modified protein was 31347 (four determinations) compared with a calculated molecular mass of 31345 for the reduced imine adduct with pyruvate. In a control experiment with borohydride but omitting pyruvate, no inactivation was observed and the e.s.m.s. was identical to native protein.

Table 1 Purification of DHDPS from *E. coli.* strain MV1190/pDA2

Typically, 24 g of cells were used; protein concentrations were measured by the method of Bradford (1976) and enzyme activities are given in i.u.

Step	Volume (ml)	Protein		Activity (i.u.)	Specific activity (units/mg)	Yield (%)	Purification (fold)
		Concentration (mg/ml)	Total (mg)				
Crude extract	38	31	1180	1240	1.1	100	1.0
Heat step	30	10	300	940	3.1	75	3.0
DEAE-Sephacel	170	0.43	72	2710	38	218	35
Phenyl-Sepharose	192	0.25	48	2110	44	170	42
Mono Q	35	1.0	35	1820	52	146	49

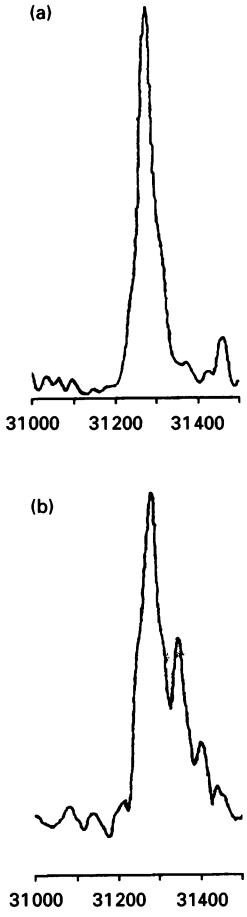

Figure 1 Observation of imine formation between DHDPS and pyruvate

These e.s.m.s traces show DHDPS alone (**a**) and DHDPS treated with 1 mM sodium pyruvate immediately before injection into the mass spectrometer (**b**).

The imine between pyruvate and DHDPS could be detected directly without borohydride treatment (Figure 1). When DHDPS was mixed with pyruvate immediately prior to injection into the mass spectrometer two peaks were seen, corresponding to native protein and the imine adduct (observed M_r 31346, calculated M_r 31343).

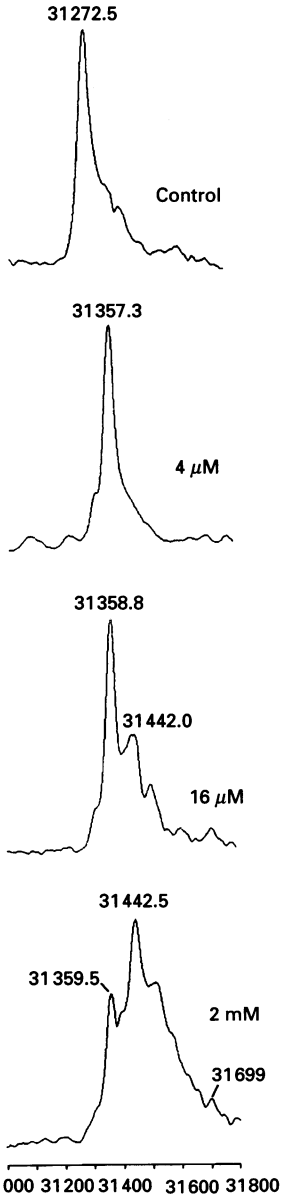

Figure 2 Reaction of DHDPS with bromopyruvate

These e.s.m.s. traces show the effect of treating DHDPS with bromopyruvate for 5 h at 20 °C at the concentrations shown.

E.s.m.s of incubations of DHDPS with ASA

The Ping Pong mechanism requires initial formation of the imine between the enzyme and pyruvate. Therefore it was not expected that any adduct between DHDPS and ASA would be seen when these were mixed in the absence of pyruvate. DHDPS was incubated with different concentrations of ASA prior to injection into the spectrometer, with and without borohydride treatment. In no experiment was there a significant signal in the e.s.m.s. corresponding to the adduct formed by covalent attachment of ASA to DHDPS.

E.s.m.s of DHDPS after inactivation with 3-bromopyruvate

Bromopyruvate has been reported to be an irreversible inhibitor of wheat DHDPS (Kumpaisal et al., 1989), and *E. coli* DHDPS (K_i = 1.6 mM) (Laber et al., 1992). In both cases, the kinetics of inactivation were not pseudo-first order. An initial phase of rapid inactivation was followed by a slower rate of inactivation. Inactivation was not complete even after treatment with high (30 mM) concentrations of bromopyruvate (Laber et al., 1992). It has been proposed that inactivation of DHDPS involves imine formation with bromopyruvate, followed by alkylation of the enzyme with concomitant loss of bromide at a slower rate (Kumpaisal et al., 1989).

The inactivation of *E. coli* DHDPS was investigated by using e.s.m.s. to follow the course of the inactivation and to characterize the covalent adducts formed between the enzyme and the inhibitor. *E. coli* DHDPS was incubated with various relatively low concentrations of 3-bromopyruvate (4 μM, 16 μM, and 2 mM, corresponding to 4, 16 and 2000 equiv. of inhibitor). The residual activity in these samples was 72, 57 and 27%, respectively, when the incubations were stopped after 5 h and the samples were analysed by e.s.m.s.

The e.s.m.s. of the 3-bromopyruvate incubations (Figure 2) give interesting results. The spectrum of the sample treated with 4 μM bromopyruvate shows a single modified protein species of M_r 31365. This corresponds to covalent modification of the protein by nucleophilic displacement of the bromide from bromopyruvate (calculated M_r 31358). This is not consistent with the previously proposed mechanism of inactivation which involved Schiff base formation between the enzyme and the inhibitor (Kumpaisal et al., 1989). This would give rise to adducts of calculated M_r values 31422 and 31344, before and after bromide displacement. The presence of a single modified protein species is highly significant. Despite the fact that the enzyme retains 74% of its activity, there is no unmodified protein. It suggests that there is one highly nucleophilic group on the enzyme which is easily alkylated, but which is probably not at the active site.

The number of modified species observed by e.s.m.s. increases with the 16 μM and 2 mM incubations. Two protein species of M_r 31359 and 31442 are observed in the 16 μM sample (corresponding to mono- and di-alkylation, calculated M_r 31358 and 31444). There is evidence of even more extensive modification of the enzyme (up to five alkylations) in the 2 mM incubation.

It is not possible to say if any of the alkylations are of active-site residues. The correlation between the loss of enzyme activity and alkylation detected by e.s.m.s. suggests rather that it is the cumulative effect of several alkyations which degrade the catalytic activity of the enzyme, perhaps by affecting its conformation or limiting access to the active site.

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Incubations of DHDPS with 3-fluoropyruvate

Fluoropyruvate has been shown to be an inhibitor of wheat DHDPS (Kumpaisal et al., 1989), but not the *E. coli* enzyme (Laber et al., 1992). Assuming that fluoropyruvate would alkylate DHDPS in an analogous way to the much more reactive bromopyruvate, the reaction was investigated using e.s.m.s.

Incubation of DHDPS with fluoropyruvate (4 μM and 5 mM) resulted in no significant loss of activity. The e.s.m.s. of the first incubation had two signals, the major one corresponding to unmodified DHDPS and a smaller signal due to the mono-alkylated enzyme (observed M_r 31352, calculated M_r 31358). The incubation with 5 mM fluoropyruvate showed the same signals but now the major species was the monoalkylated protein. These results were entirely consistent with the 3-bromopyruvate results, and supported the proposal that the initial alkylation was at a non-active site residue.

CONCLUSIONS

A procedure is described for the continuous spectrophotometric assay of DHDPS which gives the absolute enzyme activity rather than activity in arbitrary units. The proposed imine intermediate formed between the enzyme and pyruvate has been observed by e.s.m.s. which also demonstrates that there is no covalent reaction between the other substrate (ASA) and the enzyme. E.s.m.s. also indicated that the reaction of bromopyruvate with DHDPS involves direct alkylation of the enzyme; no evidence is obtained for imine formation prior to alkylation and it appears that alkylation occurs first at a highly reactive site, which is not essential for activity, and subsequently at several other sites. Substantial inactivation requires multiple alkylation of the enzyme.

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