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THE CONTROL OF LYSINE BIOSYNTHESIS IN MAIZE

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Abstract—Aspartate kinase has been partially purified and characterised from germinating maize seedlings. The K_m for aspartate was 9 mM. Out of several amino acids which are potential feedback regulators of the enzymes, only lysine is markedly inhibitory, having a K_i of 13 μM and causing 100% inhibition at 0·5 mM. Lysine also protects the enzyme against heat inactivation. Dihydrodipicolinic acid synthase isolated from the same tissue is also inhibited by lysine, 1 mM causing 95% inhibition.

INTRODUCTION

In bacteria, threonine, methionine, isoleucine and lysine all derive at least part of their carbon skeletons from aspartate and are considered to be members of the aspartate family of amino acids. In most fungi, excluding some of the lower orders, lysine is synthesised via the aminoadipic acid pathway [1]. Available evidence suggests that higher plants synthesize lysine from aspartate via the bacterial diaminopimelic acid pathway [2, 3]. Although the enzymes involved in the synthesis of the amino acids of the aspartate pathway-aspartate kinase (EC 2.7.2.4) [4, 5] and aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) [6] have been demonstrated in higher plants, little is known about them. Of the enzymes concerned solely with lysine synthesis, only diaminopimelate decarboxylase (EC 4.1.1.20) has been demonstrated [7]. To our knowledge no detailed report of dihydrodipicolinate synthase (EC 4.2.1.52) activity has been made in higher plants.

Both aspartate kinase and dihydrodipicolinate synthase are regulatory enzymes in bacteria [8]. In higher plants Bryan *et al.* [4] have reported that maize aspartate kinase is inhibited by lysine but that other amino acids have little effect. In most systems it is unusual for the first enzyme of a branched pathway to be regulated by only one of the end products. In contrast, Wong and Dennis [5] have suggested that concerted feedback inhibition by lysine and threonine occurs with the wheat enzyme. Unfortunately their results were obtained with preparations of low activity, so that the significance of small changes in rate is difficult to assess in the absence of statistical data. Even though there was some evidence for a concerted effect, the enzyme was still much more sensitive to lysine than to any other amino acid, and relatively high concentrations of threonine were required for an effect. Since both groups of workers used the hydroxamate assay, which is of low sensitivity and requires high concentrations of hydroxylamine and KCl in the assay medium, we have now reinvestigated the maize aspartate kinase using a more sensitive coupled-assay technique. We have also demonstrated and partially purified dihydrodipicolinate synthase and studied the effects of potential feedback regulators on its activity. The results of these studies are presented here. A preliminary note summarising part of the work has been published [9].

RESULTS

Aspartate kinase

The coupled assay depends on the reduction of β-aspartyl phosphate, produced by aspartate

kinase, to aspartic-β-semialdehyde in the presence of yeast aspartic-semialdehyde dehydrogenase. The maize preparation was free of the latter activity and also of homoserine dehydrogenase. Under saturating concentrations of aspartate and an equimolar mixture of Mg and ATP (Mg:ATP), and in the presence of 2 mg of aspartic-semialdehyde dehydrogenase, the rate of oxidation of NADPH was directly proportional to the quantity of extract present in the reaction mixture over the range of 0–3 mg of protein per ml. Moreover, the rate of reaction was independent of the amount of aspartic-semialdehyde dehydrogenase added over the range of 0·5–4 mg of protein for each of the preparations used. The sp. act. of aspartate kinase preparations from maize used in this study varied from 2·8–4·0 nmol/min/mg at 30° and pH 8·5. The K_m for aspartate was found to be about 9 mM, although the Lineweaver–Burk curve deviates from a straight line at low aspartate concns.

Preliminary experiments indicated that the addition of $MgSO_4$ before the ATP led to non-enzymatic and erratic changes in extinction values when adding ATP. This was eliminated by adding both together. The optimum activity for a given ATP concentration was achieved with an equal concn of Mg. The $MgSO_4$ and ATP were therefore added together in equimolar amounts. The apparent K_m for ATP under these conditions was 1·8 mM.

The effects of those amino acids that are synthesised from aspartate on the activity of aspartate kinase were studied to determine any pattern of feedback inhibition which might exist. Valine and leucine were also studied because of the interactions between these 2 amino acids and the biosynthesis of isoleucine. All the amino acids were tested for their effect on yeast aspartate-semialdehyde dehydrogenase at the same concentrations used in the coupled assay. They had little effect (a maximum of 15%) on this enzyme and, since it was present in at least two-fold excess in the coupled assay, any effects of the amino acids on the coupled assay system were due to their effect on aspartate kinase.

Lysine strongly inhibited aspartate kinase at concentrations of the order of 0·05 mM, but the effect was moderated by elevation of the aspartate concentration. The results of an experiment in which lysine inhibition was studied at two different aspartate concentrations indicated that inhibition by lysine is competitive with respect to aspartate, and a value of 13 μM was obtained for the apparent K_i for lysine from the point of intersection of the lines in a Dixon plot [10]. The effect of lysine at concentrations higher than 0·1 mM was tested to detect the possible presence of any aspartate kinase activity insensitive to lysine, as reported for *Escherichia coli* [8]. No evidence for this was found, since even at higher aspartate concentrations enzyme activity could be completely inhibited by lysine.

Table 1. The effects of various amino acids singly and in combination with lysine on aspartate kinase activity

Test amino acid	Lys alone (0·02) mM	Test amino acid (10 mM) alone	Lys + test amino acid
	(% of control rate in the absence of any amino acid)*		
Threonine	58	79	58
Methionine	72	88	64
Valine	75	111	92
Homoserine	73	92	75
Alanine	58	118	79
Isoleucine	73	126	103
Leucine	78	106	83

* The table is compiled from a number of experiments using different extracts. The activity of the control tube varied slightly around a mean of 3 nmol/min/mg.

The inhibition of aspartate kinase by threonine is a well established phenomenon in bacteria [8], and its occurrence has been implicated in *Marchantia* [11] and *Mimulus* [12]. At concentrations below 5 mM, threonine caused little inhibition, but at 10 mM some slightly inconsistent inhibition was observed of the order of 10–20%. Of the other amino acids tested individually, homoserine, methionine, isoleucine, valine, alanine and leucine did not inhibit the reaction at 5 mM. Some effects were observed at 10 mM and these are shown in Table 1. The effects of each of the amino acids in the presence of lysine (20 μM) are also shown. Apart from threonine and homoserine, where the effects are less than additive, the effect of each amino acid is essentially independent of the effect of lysine.

Allosteric modifiers are often capable of protecting an enzyme against thermal inactivation. The ability of lysine and threonine, individually and in combination, to prevent heat inactivation of aspartate kinase was examined by exposing the enzyme to a temperature of 45° for 15 min. After heating, the enzyme was rapidly cooled and dialysed over

night against 2 changes of the phosphate buffer used in the preparation of the maize extract. The unheated control and the heated sample to which no lysine or threonine had been added had specific activities of 4·0 and 2·5 nmol/min/mg respectively. Samples to which lysine (1 mM) was added prior to heating showed a specific activity of 3·5 nmol/min/mg. Threonine was without effect in the absence or presence of lysine.

Dihydrodipicolinate synthase

The *o*-aminobenzaldehyde assay of Yugari and Gilvarg [13] was used for the assay of this enzyme. The reaction between dihydrodipicolinic acid and *o*-aminobenzaldehyde is time dependent, and thus the enzyme reaction rate has a lag before becoming linear with time. This has been shown with the bacterial enzyme and is true also with the plant enzyme. The overall reaction depends on the presence of the substrates, aspartic-β-semialdehyde and pyruvate, and the enzyme. It is linear with respect to added enzyme over the range of 0–2·5 mg protein per assay. The preparations have been partially purified as indicated in the experimental section, although it is not possible to give meaningful figures for purification as there appear to be various non-specific colour reactions occurring in the crude preparations.

The K_m's for the substrates have been determined from conventional studies of the effect of concentration on the rates of the reaction. There is no evidence for any cooperativity with respect to pyruvate concentration, although it must be stressed that the nature and lack of sensitivity of the assay preclude the measurement of true initial reaction rates. The apparent K_m for pyruvate is *ca* 2 mM. The enzyme differs from that from *E. coli*

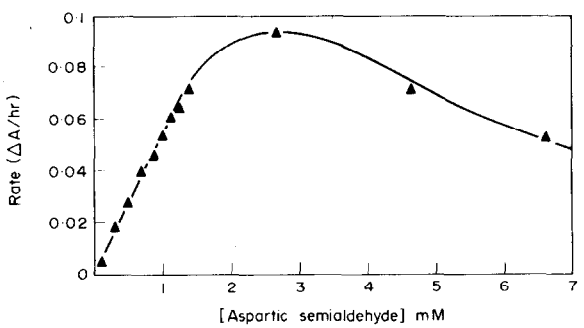

Fig. 1. The effect of increasing concentrations of aspartic-β-semialdehyde on the rate of dihydrodipicolinate synthase.

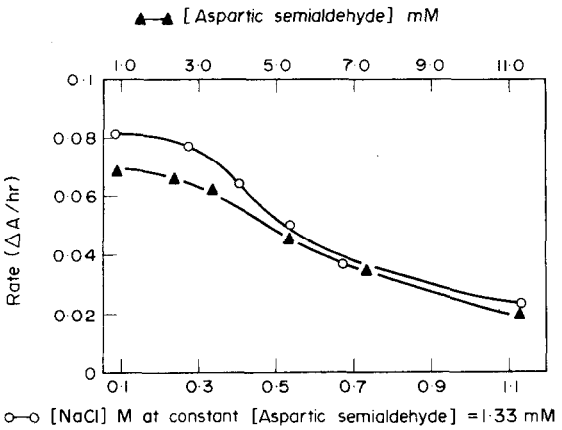

Fig. 2. The effect of increasing concentrations of aspartic-β-semialdehyde and NaCl, and of NaCl in the presence of a constant amount of aspartic-β-semialdehyde, on the rate of dihydrodipicolinate synthase.

in having a pH optimum of 6·5 compared with 8·4 in the bacterium. The results (Fig. 1) show that aspartic-β-semialdehyde, at high substrate concentrations, appears to inhibit the reaction. This is probably not due to the substrate itself, but to the presence of large amounts of NaCl formed during the neutralisation of the HCl in which the substrate must be kept. Fig. 2 shows the effect on the reaction of varying the NaCl concentration alone and demonstrates that this effect is sufficient to account for the inhibition produced by the aspartate-β-semialdehyde. The apparent K_m for aspartic-β-semialdehyde was *ca* 5·0 mM.

The enzyme is inhibited 40, 70 and 90% by 0·2 mM, 0·4 mM and 1 mM lysine respectively. Threonine, methionine, valine, homoserine, isoleucine and leucine at concentrations of 10 mM caused less than 10% inhibition of enzyme activity. These amino acids also had no effect on lysine inhibition of the enzyme.

DISCUSSION

The results presented here essentially confirm those of Bryan *et al.* on the control of aspartate kinase in maize [4]. They show that this enzyme is extremely sensitive to lysine with a K_i of approximately 13 μM. The effects of other amino acids are extremely small and require high concentrations of the amino acid to achieve them. The physiological significance of such high concentrations is uncertain. There is no evidence of any concerted effect of lysine or threonine in either

the inhibition of aspartate kinase or in its protection from heat inactivation. The protection of the enzyme from heat inactivation by lysine confirms the enzyme inhibition studies and suggests that the enzyme has a specific lysine binding site.

The results are unexpected in that control of the aspartate kinase step in bacterial systems is exerted by more than one of the aspartate family of amino acids. The explanation may lie in the fact that there exists a threonine and/or methionine isoenzyme in maize that both Bryan's group and ourselves have failed to isolate, despite various efforts to do this. If the control of the pathway is in reality solely by lysine, then it would suggest that the whole pathway could be shut off and the plant starved of threonine, methionine and isoleucine by the presence of excess lysine. It may be, however, that this is prevented *in vivo* by the control of lysine synthesis at the dihydrodipicolinate synthase step. Although this enzyme appears to be less sensitive to lysine, the situation *in vivo* will also depend on the relative substrate concentration.

The demonstration of dihydrodipicolinate synthase in maize and other higher plants (R. M. Wallsgrove and B. J. Miflin unpublished) confirms labelling and other studies indicating that plants synthesise lysine via the diaminopimelic acid pathway.

EXPERIMENTAL

Aspartate kinase. Maize (*Zea mays* cv Kelvedon 59A) shoots from seeds, germinated and grown at 28° for 3–4 days, were harvested and ground between a pestle and motar with an equal wt of 0·2 M Tris–HCl, 0·1 M KCl, 1 mM EDTA, 30% glycerol, 1·4 mM mercaptoethanol adjusted to pH 8·5 at 5°. After filtering through gauze the extract was centrifuged for 60 min at 20000 *g*. $(NH_4)_2SO_4$ was added to the extract to give 40% satn and the ppt collected by centrifugation. This was dissolved in 0·05 M KH_2PO_4, 1 mM EDTA, 20% glycerol and 1·4 mM mercaptoethanol, pH 7·5 and dialysed 18 hr against the same buffer. The extract was then centrifuged at 150000 *g* for 1 hr and used. This preparation, when stored at −12°, remained active for at least 4 months. The enzyme was assayed in a medium containing, in a final vol of 3 ml, 0·2 M Tris–HCl pH 8·5, 10 mM mercaptoethanol, 0·1 mM NADPH, aspartate and Mg:ATP as stated for each expt, 2 mg of yeast aspartate semialdehyde dehydrogenase and about 2 mg of maize enzyme. Oxidation of NADPH was followed at 340 nm and corrections made for background rates observed in absence of either aspartate or Mg:ATP. Aspartic-semialdehyde dehydrogenase was extracted from brewers yeast by a modification of the methods of Surdin [15] and Black and Wright [14]. The DL aspartic-β-semialdehyde was prepared from DL allyl-glycine by the method of Black and Wright [14]. All other amino acids were in the L form.

Dihydrodipicolinate synthase. Maize shoots were ground as above in a medium consisting of 0·2 M Pi buffer pH 8, 10 mM mercaptoethanol, 1 mM EDTA and 20% glycerol. The extract was filtered through cheesecloth and then centrifuged at 20000 *g* for 30 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 30% saturation. After 30 min, the ppt was centrifuged off and discarded. Further $(NH_4)_2SO_4$ to 60% saturation was added and the ppt collected and dissolved in the same medium as above except that glycerol was omitted. The extract was clarified by centrifugation and then passed through a Sephadex G-200 column (2·6 × 40 cm). The column was pre-equilibrated and eluted with the glycerol-free buffer. The active fractions were collected, made to 65% saturation with $(NH_4)_2SO_4$ and the resultant ppt collected. This was dissolved in buffer containing glycerol and dialysed 18 hr against the same buffer. The assay medium consisted of 0·2 M Pi buffer pH 6·5, 10 mM pyruvate, 10 mM mercaptoethanol, *ca* 2 mM aspartic-β-semialdehyde (0·2 ml of soln adjusted to pH 2 with Na_2CO_3), 1·5 mg of *o*-aminobenzaldehyde added in 0·1 ml of EtOH (freshly prepared) and about 2 mg of enzyme protein. The reaction mixture was made to 3 ml and incubated at 30° for 2 hr. The reaction was stopped by the addition of TCA, the soln clarified by centrifugation, and its A at 550 nm determined. Separate blanks lacking aspartic-β-semialdehyde and enzyme were used for correction of extinction changes.

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