

CHAPTER IV

DISCUSSION

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P L A N T E N Z Y M E S

It has been known for the past decade and a half that in plants the biosynthesis of lysine, threonine, methionine and isoleucine involves the metabolism of aspartic acid and proceeds through the same initial sequence of reactions as in bacteria (Naylor et al., 1958; Dougall and Fulton, 1967). It has been noticed in several instances that, during the early growth of seedlings, homoserine which is a precursor of threonine and methionine is the dominant amino acid (Virtanen and Erikama, 1955; Larsen and Bowers, 1965). And, in the case of young pea seedlings, homoserine is produced more rapidly than it is consumed (Larsen and Bowers, 1965). In our laboratory, Renukrishna and Raghavendra Rao (personal communication) found that in seedlings of Bengal gram (Cicer arietinum) about 2 per cent of the label supplied in the form of aspartate- $U-^{14}C$ was converted to lysine. The label recovered in lysine corresponded to nearly 35 per cent of the total radioactivity in the amino acid pool.

Compared to the wealth of information available concerning the biosynthesis of the aspartate family of amino acids in microorganisms there have been very few detailed investigations on the

biosynthesis of these amine acids, particularly into the nature and extent of regulatory mechanisms such as feedback inhibition and repression in higher plants. Homoserine dehydrogenase has been identified in pea seedlings (Sasaka, 1961 cited by Bryan, 1969), and the roots of Zea mays (Bryan, 1969). Enzymes from both the sources exhibited the ability to utilize NAD^+ , the maize enzyme being able to act with $NADP^+$ as well. Studies on the regulatory properties of a partially purified preparation of the maize homoserine dehydrogenase (Bryan, 1969) indicated a strong inhibition of the enzyme activity by threonine. Such a sensitivity of the enzyme to threonine can provide an explanation for the observation made by Oaks (1965) that a supply of exogenous threonine prevents threonine biosynthesis in maize. Thus, according to Bryan (1969), the regulation of amine acid biosynthesis by feedback mechanisms such as those reported by him and by Oaks (1965a) does seem to be operational in higher plants, and particularly well adapted to the physiological needs of developing roots which are dependent upon the vascular system for a variable supply of metabolic precursors (Bollard, 1960).

But in our case studies on the enzymes of the aspartate pathway of biosynthesis in plants have always given very inconsistent and highly variable results so that it has not been possible to conduct

any detailed investigations on the regulatory properties of these enzymes. One of the reasons for such erratic behaviour, we presume, is due to varietal and cultural differences in the seeds (Rabin et al., 1947; Steward, 1965 cited by Larson and Beavers, 1965). In our initial studies with well-developed cotyledons of Phaseolus vulgaris, whenever an active enzyme preparation was obtained, the homoserine dehydrogenase was NAD(P)⁺-specific and was not sensitive to any of the end product amino acids. Aspartokinase activity, whenever it could be detected in the cotyledons of Phaseolus vulgaris, was found susceptible to individual inhibition by lysine (15 per cent) and threonine (45 per cent). With both the amino acids acting simultaneously, the value obtained was additive. So far, there does not appear to be any report in literature on the identification of aspartokinase in multicellular plants. Even in the maize enzyme preparation reported by Bryan (1969), no aspartokinase activity could be demonstrated in either the crude extracts or purified preparations.

On the other hand, in the other plant systems studied by us such as 2- 10 day-old seedlings of Phaseolus mungo, Phaseolus radiatus and Cicer arietinum, it was never possible to detect homoserine dehydrogenase activity. Extremely low amounts of aspartokinase activity not regulated by any end-product, could be detected only in the

extracts of seedlings of Phaseolus mungo and Phaseolus radiatus and not in Cicer arietinum. Different procedures adopted for preparing plant extracts such as inclusion of polyvinyl pyrrolidone, sucrose, higher concentration of KCl, etc. did not help. It is rather difficult to explain our observations in view of the findings of Ramakrishna and Raghavendra Rao (personal communication) of this laboratory in their studies with seedlings of Cicer arietinum. Lysine in the total amine acid pool was found to receive from aspartate-U-¹⁴C the major portion of the label in the amino acid fraction. They also observed the biosynthesis of lysine in these seedlings to proceed via the diamino pimelic acid pathway and not through the intermediate formation of α -aminoadipic acid.

In the experiments with plants, there does not seem to be any clear cut explanation for the variability and more often, the apparent absence/inability to detect the enzymes, although the use of tracers have provided valuable information concerning metabolic pathways.

EXPERIMENTS WITH BACTERIA

In all bacteria studied so far, the synthesis of the four "essential" amino acids of the aspartate family, viz, lysine, threonine, isoleucine and methionine involves the highly branched chain of reactions starting from aspartic acid (Datta, 1969). The biosynthetic pathway concerned is subject to primary regulation at two points. The first one is the initial step in the reaction sequence - the conversion of aspartate to β -aspartyl phosphate, mediated by aspartokinase which controls the primary flow of carbon to all the amino acids of this family. The other point of control is the third step in the reaction sequence involving the reduction of aspartic- β -semialdehyde to homoserine, catalysed by homoserine dehydrogenase. Many different systems of control of these enzymes have been discovered in different species of bacteria and these have been described in detail in Chapter I. In E. coli K12 and B, strains in which the regulation of these enzymes has been studied in great detail (Stadtman et al., 1961; Patte et al., 1966 and 1967; Falco-Kelly et al., 1969), isoenzymic control seems to be the principal mode of control. No detailed study of the regulation of this pathway seems to have been made in any other related bacteria

except Salmonella typhisaurium (Freundlich, 1933; 1934; Cafferata and Freundlich, 1939). Isoenzymic pattern of control seems to be operative in S. typhisaurium also as seen by the presence of two distinct and separable homoserine dehydrogenases, the activity of one isoenzyme controlled by threonine and the formation of the other by methionine (Cafferata and Freundlich, 1939). In our investigations, the patterns of regulation adopted by Micrococcus glutamicus, and Serratia marcescens were looked into.

In our preliminary studies with M. glutamicus ATCC 15032 the presence of a homoserine dehydrogenase susceptible to feedback inhibition by threonine was detected in conformity with the finding by Nara et al. (1961). But aspartokinase of the strain of M. glutamicus behaved rather differently from the one described by Nakayama et al. (1966). According to this report, the enzyme was susceptible only to concerted feedback inhibition by lysine plus threonine and not to inhibition by the individual amino acids. However, we obtained an aspartokinase whose activity was inhibited by lysine and threonine individually to an extent of 70 and 59 per cent respectively. When both the end products were present, the resultant inhibition was

total (100 per cent). Further experiments on the M. glutamicus enzyme(s) could not, however, be done due to the instability of the enzyme preparations which lost all activity on ammonium sulphate precipitation.

Incorporation of ^{14}C -aspartate - The existence of the aspartate pathway of biosynthesis of amino acids in S. nassosensis was ascertained by experiments with radioactive aspartic acid. When cells of S. nassosensis were grown in presence of ^{14}C -U-aspartate, about 90 per cent of the label present in the amino acid fraction of the cells was accounted for by the four amino acids of the aspartate family. The individual distribution was: lysine, 20 per cent; threonine, 28 per cent; isoleucine, 27 per cent and methionine, 15 per cent. In contrast, there was no incorporation of radioactivity into lysine, threonine and methionine (results reminiscent of those of Roberts et al. (1955) with E. coli), when the growth medium containing labelled aspartate was supplemented with those amino acids. This suggested an active involvement of the amino acids in their own syntheses. In early studies on the biosynthesis of amino acids in E. coli, Roberts et al. (1955) found that the addition of threonine, aspartate, etc. to the growth medium containing glucose-U- ^{14}C abolished completely the incorporation of ^{14}C into the respective amino acids in the cell protein.

It is known that certain strains of E. coli have the ability to synthesize isoleucine from β -methyl aspartate which can be derived from L-glutamate (Abramsky and Shemin, 1965; Meister, 1965; Phillips et al., 1972). Presumptive evidence for the existence of this alternate pathway for the biosynthesis of isoleucine in S. marcescens, in addition to the aspartate pathway was obtained with studies involving the metabolism of glutamate-U-¹⁴C by rapidly-growing cells of ^{the} bacterium in the presence and absence of threonine. The isoleucine and threonine portions of protein hydrolysates of cells supplied with ¹⁴C-glutamate plus threonine got diluted by 75 and 40 per cent, respectively. Possible reversibility of the reaction was indicated by results obtained with cells supplied with ¹⁴C-threonine, viz, dilution to an extent of 40 per cent of the threonine and isoleucine components of cell protein caused by ¹²C-glutamate plus ¹⁴C-threonine.

Preliminary studies have shown that ¹⁴C-labelled glutamate is partly converted to β -methyl aspartate by fresh cell-free extracts of the organism. Of the total radioactivity present in the amino acid pool in the reaction medium, 15.5 per cent was present in glutamic acid and 10 per cent in β -methylaspartic acid. These results also indicated the presence of the alternate pathway for isoleucine biosynthesis in S. marcescens.

Repression studies - Our results with the metabolism of ^{14}C -aspartate (U) by S. marcescens were substantiated by studies on the extent of repression of aspartokinase and homoserine dehydrogenase by the end-product amino acids. Aspartokinase concentration was reduced by about 10 per cent when the cells were grown in presence of lysine; growth in presence of methionine or of a mixture of lysine, threonine, methionine and isoleucine (all amino acids used at a concentration of 10 mM) resulted in a very strong repression of aspartokinase to an extent of about 90 and 100 per cent respectively. Also, the aspartokinase produced when cells were grown in presence of methionine was completely inhibited by lysine. In the case of E. coli, Stadtman et al. (1961) and Falcoz-Kelly et al. (1969) have reported complete repression of the lysine-sensitive enzyme by 10 mM L-lysine in the growth medium. The other aspartokinases behave somewhat differently. The threonine-sensitive aspartokinase of both E. coli and S. typhimurium are repressed only by a mixture of threonine and isoleucine (Freundlich, 1963).

Yeast aspartokinase is repressed about 40 - 50 per cent when yeast is grown in presence of 20 mM L-threonine (Stadtman et al., 1961). The activity of aspartokinase of yeast when grown in the presence of threonine plus methionine or high methionine (20 mM) alone,

is significantly inhibited by L-methionine and by L-lysine (Stadtman et al., 1961). The authors, however, could not say from their results whether or not the observed effect was due to the synthesis of a new aspartokinase or to a modification in the sensitivity of the enzyme produced on minimal media. A somewhat similar situation occurs in the case of the single aspartokinase present in Pseudomonas putida (Robert-Cero et al., 1970). The enzyme is repressed when the cells are grown in presence of the inhibitors lysine and threonine, and the enzyme synthesized under such conditions is sensitive neither to individual inhibition by lysine and threonine nor to their concerted feedback inhibition.

The effect of methionine on the concentration of aspartokinase of S. aureus could not be corroborated by experiments with radioactive aspartate: growth of the cells in the presence of aspartate- $U-^{14}C$ and ^{12}C -methionine did not affect the normal incorporation of label into lysine. This may be explained by the facts that there is still residual aspartokinase activity (5 - 10 per cent) and the cells grow normally and therefore synthesize their lysine requirement. A 50 per cent reduction in incorporation was seen only in the case of threonine. Studies on repression of the enzyme systems by methionine

revealed the existence of two isoenzymes of homoserine dehydrogenase: one dependent on NAD^+ for its activity and susceptible to strong repression by methionine (100 per cent), homoserine (100 per cent) and cysteine (95 per cent) and the other, dependent on NADP^+ for activity and subject to inhibition (55 per cent) by threonine. The synthesis of this latter enzyme was also partially repressible by methionine (35 - 40 per cent), homoserine (80 per cent) and a mixture of lysine, threonine, methionine and isoleucine (75 per cent). Homoserine dehydrogenases of many other organisms are also known to be repressed by methionine: E. coli (Patte et al., 1967); S. typhimurium (Cafferata and Freundlich, 1969); S. cerevisiae (Karassevitch and de-Robichen Szalmajster, 1965).

The physiological age of the cells at the time of harvesting is quite important in reference to the total enzyme content and the extent of repression by metabolites. The enzyme content of the cells harvested at the stationary phase goes down almost to 2 to 5 per cent of those obtained at the log phase. These are in keeping with what is to be expected. During stationary phase, it is known that there is degradation of enzymes in bacteria as in higher cells (Mandelstam, 1958).

The decrease in the extent of repressibility observed in older cells is best explained by the catabolism/utilization of the concerned metabolite and hence lowering its effective concentration.

The individual enzymes - It was found early in the studies on aspartokinase and homoserine dehydrogenase of Serratia marcescens that these enzymes differed markedly in two properties from the corresponding enzymes of E. coli. The aspartokinase activity of S. marcescens was susceptible only to inhibition by lysine and the aspartokinase and homoserine dehydrogenases were separable from one another unlike in E. coli (Pette et al., 1966; Falcoz-Kelly et al., 1969).

It has already been stated that the two aspartokinase activities of E. coli K₁₂ are associated with homoserine dehydrogenases whereas another aspartokinase is both repressible and inhibitable by lysine (Truffa-Bachi and Cohen, 1968). The two isoenzymes of homoserine dehydrogenase and the aspartokinase present in Serratia marcescens have been separated from one another by chromatography on DEAE-cellulose. The activity of the aspartokinase separated from homoserine dehydrogenase activity is strongly affected by L-lysine

(85 per cent) and thus resembles aspartokinase III of E. coli K12. Of the two homoserine dehydrogenases, one is susceptible to inhibition by threonine and requires specifically NADP⁺ for its activity; the homoserine dehydrogenase I of E. coli K12 behaves similarly except that it also has aspartokinase activity. The activity of the second homoserine dehydrogenase, on the other hand, is not sensitive to any of the end products of the pathway but its synthesis is susceptible to repression by methionine.

Cofactor requirement - The two homoserine dehydrogenases of S. marcescens are similar, in a number of their properties, to the corresponding enzymes, homoserine dehydrogenase I and II, respectively, of E. coli, e.g., in their cofactor requirements the threonine-sensitive enzymes from both the organisms are similar. With respect to their NADP⁺-specificity, the threonine-sensitive enzymes of a number of different genera of bacteria are similar. The enzymes of E. coli K12 (Patte et al., 1966), B. rubrum (Datta and Gest, 1964), B. sphaeroides (Gibson et al., 1962) and M. glutamicus (Nara et al., 1961), require NADP⁺ for their activity. The corresponding enzyme of yeast (Black and Wright, 1955a) and Pseudomonas putida (Robert-Gero et al., 1970a), however, can act with both the cofactors.

Threonine-sensitive enzyme: Potassium ions - As we have found in the case of the S. marcescens enzyme, the threonine-sensitive homoserine dehydrogenases of most other bacterial sources have been found to be activated in the presence of potassium ions. The activity of the S. marcescens enzyme could be enhanced by increasing the concentration of KCl up to 0.5 M. But above this concentration, KCl became inhibitory whereas in the case of E. coli, the threonine-sensitive aspartokinase I - homoserine dehydrogenase I complex can be activated even by 1 M KCl. (Patte et al., 1966). Also, the sensitivity to modifiers of the enzyme from R. rubrum is greatly influenced by K^+ concentration in that in low K^+ condition inhibition and stimulation of enzyme activity are significantly reduced (Datta and Gest, 1965). In the case of S. marcescens, K^+ to some extent, has a protective action against threonine inhibition.

Molecular weight - The molecular weight of the enzymes from E. coli and S. marcescens are similar: 360,000 (Truffa-Bachi et al., 1968) and 338,000 respectively. However, unlike the E. coli K_{12} enzyme, the threonine-sensitive homoserine dehydrogenase of S. marcescens is not associated with aspartokinase activity. Cohen et al. (1969) could demonstrate in two strains of S. marcescens,

2-68 and 5-68 individual inhibition of aspartokinase activity by lysine (63 and 61 per cent) and threonine (28 and 33 per cent). With both the effectors acting simultaneously, the inhibition was 90 per cent. Contrary to the above findings, in the strain of S. marcescens that we studied, S. marcescens 3a-3, we could detect only an activity sensitive to lysine and not threonine despite all precautions taken while preparing the cell-free extracts and during subsequent purification of the enzyme. On the other hand, the aspartokinase activity demonstrable in the cell-free extracts and at all stages of enzyme purification was enhanced by about 6 per cent (admittedly, an insignificant effect) in the presence of threonine in the reaction medium. This could mean that either we have not been able to detect the threonine-sensitive aspartokinase in our strain of S. marcescens for unknown reasons or it is not present in the strain at all. Variations have indeed been observed in the relative activities of the three types of aspartokinase from strain to strain, even within a single species such as E. coli (Cohen et al., 1969). On the other hand, this could also mean that S. marcescens 3a-3 has either lost the threonine-sensitive aspartokinase cistron during evolution or has

not yet developed it as has been speculated in the case of E. coli B in which the methionine-repressible homoserine dehydrogenase activity is absent (Patte et al., 1967).

Molecular weight and subunit structure - In E. coli B, the apparent molecular weight of aspartokinase II alone was only 70,000 as compared to 180,000 of the E. coli K12 aspartokinase II - homoserine dehydrogenase II complex (Patte et al., 1967). But in view of the fact that the threonine-sensitive homoserine dehydrogenase of S. marcescens Sa-3 has a molecular weight roughly equal to that of the threonine-sensitive enzyme complex of E. coli K12, our latter speculation about the loss or non-development of the particular cistron in S. marcescens Sa-3 during evolution can perhaps be discounted. From comparative study of the primary regulation of aromatic biosynthetic pathway in bacteria by Jensen et al. (1967) and on the regulation of biosynthesis of amino acids of the aspartate family in coliform and pseudomonads by Cohen et al. (1969), a common evolutionary origin for the members of large taxa can be postulated.

Urea effect - Urea (8 M) had no effect on the activity of the threonine-sensitive homoserine dehydrogenase of S. marcescens, even

when the enzyme was incubated with it up to 48 hr. While the considerably high molecular weight of the enzyme (398,000 as determined by gel filtration on Sephadex G-200) would suggest the presence of subunits, it is not clear whether the enzyme disaggregated into subunits in the presence of urea at all: no specific tests were made towards the this end. If this did happen, then perhaps the subunits are probably individually active. This could have been checked by determining the molecular weight in presence of urea and also the number of binding sites. The instability of the threonine-sensitive enzyme and the lack of a good quantity of enzyme (especially in the case of the methionine-repressible homoserine dehydrogenase where also a similar result with urea was obtained) precluded these experiments. The presence of KCl in the enzyme preparations might have also prevented the breakdown of the enzyme into its subunits. Other compounds such as guanidine hydrochloride and sodium dodecyl sulphate could not be used since they were found to form a precipitate in presence of KCl. The threonine-sensitive enzyme complex of *E. coli* K12 has been found to be a tetramer with a subunit molecular weight of 86000 ± 4000 (Falcoz-Kelly *et al.*, 1972; Clark and Ogilvie, 1972).

Stability - The S. marcescens enzyme could not be purified any further beyond DEAE-cellulose fractionation as it is rather unstable, especially at the later stages of purification and no satisfactory means of stabilisation could be determined. At this stage of purification, it was purified 40-fold but still contained 2-3 other proteins as noticed in disc gel electrophoresis.

Methionine-repressible enzyme - The second isoenzyme of homoserine dehydrogenase of S. marcescens was found to be susceptible only to repression by methionine. It could function with both NAD^+ and NADP^+ . The methionine-repressible homoserine dehydrogenase II of E. coli K₁₂ has also been shown to act with both the cofactors (Falcos-Kelly et al., 1969) and in both the bacteria, the rate of reaction with NAD^+ is higher than that with NADP^+ . The ratio of the dehydrogenase activity with NADP^+ to that with NAD^+ in S. marcescens Sa-5 varies from 0.5 to 0.65 while with a homogeneous enzyme from E. coli K₁₂ the ratio of activity with NADPH to NADH has been reported to be about 0.47 (Patte et al., 1967). The molecular weight of the S. marcescens enzyme was obtained as 155,000 by gel filtration on Sephadex G-200. SDS-polyacrylamide gel electrophoresis indicated the presence of two subunits of identical molecular weight (75,800) in the enzyme. The E. coli K₁₂ enzyme, on the other hand, has been reported

to have a molecular weight of 189,000 \pm 9000 with 4 identical subunits of 45,000 \pm 5000 molecular weight each (Falces-Kelly et al., 1969). The methionine-repressible enzyme accounts for nearly 50 per cent of the total homoserine dehydrogenase activity of the S. marcescens cells. This is considerably higher than that obtained with wild-type S. typhimurium in which it is approximately 12 per cent of the total activity (Cafferata and Freundlich, 1969) and in E. coli K12 the concentration of this enzyme is so low as to be practically undetectable in crude extracts of wild cells (Patte et al., 1967).

"Inhibitor" of the methionine-repressible homoserine dehydrogenase - In the fresh cell-free extracts of S. marcescens, the methionine-repressible activity (acting with NAD⁺) could not be detected. But on "ageing" the cell-free extracts or on precipitation of the proteins by ammonium sulphate the activity became detectable presumably due to the removal of an inhibitor of the enzyme activity in the cell-free extracts. Preliminary experiments conducted to identify the nature of the inhibitor revealed it to be a non-dialysable compound. As its activity was not affected by trypsin digestion and because of its susceptibility to periodate oxidation, the inhibitor was assumed to be a carbohydrate rather than a polypeptide. It is not known whether a similar situation obtains in the fresh cell-free

extracts of wild cells of E. coli K₁₂ in which the enzyme activity could not be demonstrated. It is also not known whether this "latency" of the enzyme in S. marcescens is another probable mechanism of regulation of enzyme activity.

Like the threonine-sensitive enzyme, the methionine-repressible homoserine dehydrogenase also did not show aspartokinase activity.

Aspartokinase - The strong repression of aspartokinase synthesis (95 per cent) observed when the bacterial cells were grown in presence of methionine can be explained if we assume that the synthesis of the apparently single aspartokinase of S. marcescens susceptible to inhibition as well as repression by lysine is repressible also by methionine. The very low aspartokinase activity remaining in the methionine-grown cells was completely inhibited by lysine. Through the various steps of purification employed, apparently a single aspartokinase gets purified in S. marcescens as indicated by its increasing susceptibility to inhibition by lysine. But in the absence of any satisfactory spot test to determine the number of activity bands in the crude extracts submitted to gel electrophoresis as to whether there is only one or more aspartokinases present in the S. marcescens strain used, nothing conclusive can be said yet.

The aspartokinase was purified about 45-fold; and at this stage of purification, it was associated with one other protein as seen by disc gel electrophoresis. The activity of the enzyme was sensitive only to lysine (85 per cent inhibition) and the inhibition due to lysine was of a competitive type. Thus, it differed from the corresponding enzymes of E. coli K12 (Stadtman et al., 1961) and Pseudomonas putida (Robert-Gere et al., 1970) in the nature of inhibition. In E. coli, a strictly non-competitive type of inhibition was observed and in P. putida, a mixed type of inhibition. The various bacterial aspartokinases studied so far have been found to have similar pH optima - around pH 8.0. Potassium ions have also been seen to enhance enzyme activity in most cases. At concentrations of KCl higher than 200 mM, however, the activity of the S. marcescens enzyme was reduced.

A 60 per cent reduction in activity resulted when aspartokinase was incubated with 8 M urea. The original activity could not be restored after removing urea by dialysis which indicates a non-reversible disaggregation of the enzyme into inactive subunits in the presence of urea.

On the basis of the results obtained in our studies on S. marcescens Sa-5 we could suggest that the following possible scheme of regulation of biosynthesis of the aspartic acid family of amino acids in that organism. The apparently single aspartokinase of Serratia marcescens Sa-5 which is subject only to partial inhibition by lysine (85 per cent)(or two aspartokinases - one repressible by methionine and the other repressible and also inhibitable by lysine) feeds a common pool of aspartyl phosphate from which the other end products of the pathway are derived. Thus, even in the presence of excess of lysine, the synthesis of threonine, methionine and isoleucine would never fall below a critical level necessary for optimal growth rate. Repression and feedback control of the enzymes involved in later steps of the biosynthetic pathway and the first enzymes of the terminal branches would then direct the ultimate fate of aspartyl phosphate. The two isoenzymes of homoserine dehydrogenase individually regulated by threonine (inhibition) and methionine (repression) will thus regulate the flow of carbon from aspartyl phosphate to those amino acids. The pattern of regulation of the initial enzymes of the different branches of the pathway needs to be examined before any definite conclusion can be drawn regarding the regulation of biosynthesis of the aspartate family of amino acids in S. marcescens.