J. Biosci., Vol. 11, Numbers 1–4, March 1987, pp. 571–579. © Printed in India.

Arginine decarboxylase is a component activity of the multifunctional enzyme putrescine synthase in cucumber seedlings

G. L. PRASAD and P. R. ADIGA*
Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Abstract. A homogenous preparation of putrescine synthase, the versatile multifunctional enzyme involved in agmatine→putrescine conversion in *Cucumis sativus* was found to catalyze enzymatic decarboxylation of arginine also. Similarly, the purified arginine decarboxylase mediated the component as well as the complete set of coupled reactions harboured by putrescine synthase. Both the enzyme preparations exhibited identical electrophoretic and chromatographic behaviour and were immunologically indistinguishable. All the enzymic activities are stabilized concurrently by feeding arginine to the intact seedlings. Therefore, it is concluded that the multifunctional putrescine synthase in *Cucumis sativus* seedlings also harbours arginine decarboxylase activity unlike its counterpart in *Lathyrus sativus.*

Keywords. Putrescine biosynthesis; arginine decarboxylase; multifunctional enzyme.

Introduction

Earlier studies in this laboratory (Adiga and Prasad, 1985) on enzymatic aspects of polyamine biosynthesis in higher plant systems have led to purification and characterisation of the first enzyme of the biosynthetic pathway *viz.,* arginine decarboxylase (ADC, EC 4·1·1·19), the multifunctional enzyme putrescine synthase (PS) involved in agmatine→putrescine conversion from the seedlings of *Lathyrus sativus* (Ramakrishna and Adiga, 1975; Srivenugopal and Adiga, 1981) as well as *Cucumis sativus* (Prasad and Adiga, 1985, 1986b). The molecular characteristics of these two enzymes from the above plant sources were found to differ significantly in terms of finer details of their structural and functional characteristics. Thus, while ADC from *L. sativus* did not catalyze ornithine decarboxylation, the cucumber enzyme exhibited both these activities (Prasad and Adiga, 1986a). Furthermore, despite the fact that the polycephalic enzyme, PS from two plant species differed structurally, the cucumber enzyme nevertheless harboured as expected all the 4 constituent activities *viz.,* agmatine iminohydrolase (AIHase, EC 3·5·3·12), ornithine transcarbamylase (OTCase, EC 2·1·3·3), putrescine transcarbamylase (PTCase, EC 2·1·3·6) and carbamate kinase (CKase, EC 2·7·2·2) and the complete reaction resulting in agmatine→putrescine transformation as the direct consequence of coupling either OTCase or CKase activities to PTCase component of PS. During these studies it was observed rather unexpectedly, that the purified ADC and PS of cucumber seedlings exhibited identical chromatographic and electrophoretic properties. Hence a detailed investigation was undertaken to probe in to the discrete possibility that each of the two enzyme preparations purified through two distinct

*To whom all correspondence should be addressed.
Abbreviations used: ADC, Arginine decarboxylase; PS, putrescine synthase; AIHase, agmatine iminohydrolase; OTCase, ornithine transcarbamylase; PTCase, putrescine transcarbamylase; CKase, carbamate kinase; PAGE, Polyacrylamide gel electrophoresis; NCP, N-carbamyl putrescine; SDS, sodium dodecyl sulphate; ODC, ornithine decarboxylase.

protocols, catalyze the reactions catalyzed by the other. This paper deals with these investigations which support the concept that in *C. sativus* seedlings, ADC activity is also integral constituent of the multifunctional putrescine synthase.

Materials and methods

The source of the seeds and growth conditions of *C. sativus* seedlings and the reagents used have been described in detail earlier (Prasad and Adiga, 1985). The enzyme ADC and PS have been purified from *C. sativus* seedlings as detailed elsewhere (Prasad and Adiga, 1985b, 1986b). The ADC was purified by a 3-step procedure involving ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-150. PS was isolated by affinity chromatography on putrescine carboxyhexyl Sepharose. ADC activity was quantified by measuring the CO_2 evolved when [U-^{14}C]-arginine was used as the substrate in Warburg flasks (Prasad and Adiga, 1985b). The constituent and the complete reactions catalyzed by PS were assayed according to Prasad and Adiga (1986b). Ouchterlony immuno-double diffusion (Ouchterlony, 1967), Polyacrylamide gel electrophoresis (PAGE) on non-denaturing slab gels (Davis, 1964) and on denaturing gels (Laemmli, 1970) were performed according to the standard procedures. Protein estimation using bovine serum albumin as the standard was carried out according to Lowry *et al.* (1951) and the amines were estimated in the deproteinized enzyme reaction mixtures by the standardized procedures employed earlier (Srivenugopal and Adiga, 1981; Prasad and Adiga, 1986b).

Unit activity

Unless otherwise stated, one unit of enzyme activity is defined as the amount of enzyme required to produce 1 μ mol of product (NCP citrulline or ATP) under standard assay conditions. Specific activity is expressed in units/mg protein.

Results

A careful scrutiny of the behaviour of ADC and PS of cucumber during purification revealed that both of them behave identically during ion-exchange chromatography, gel filtration and during electrophoresis on non-denaturing as well as denaturing polyacrylamide gels (Prasad and Adiga, 1985b, 1986b). Thus both, these enzyme activities are elutable with about 0·2 M KCl at pH 7·6 from DEAE-cellulose columns and both are excluded in the void volume of Sephacryl S-300 gel filtration columns. Furthermore, the two enzymes comigrate as single species on non-denaturing polyacrylamide gels at pH 8·3, yet resolve into 48 K, 44 K and 15 K polypeptides under reducing conditions on sodium dodecyl sulphate (SDS)-PAGE. Under non-reducing conditions these two enzyme preparations are found to band with an apparent molecular mass of 150 KD during SDS-PAGE (data not given). In line with these similarities, homogenous ADC preparation could cross-react with the immunoglobulin fraction prepared from the anti-serum raised against PS showing thereby that they share immunological cross-reactivity (figure 1). All these observations have prompted a further probe into the possibility that ADC activity in the cucumber seedlings, is a component activity of the purified multifunctional enzyme, PS.

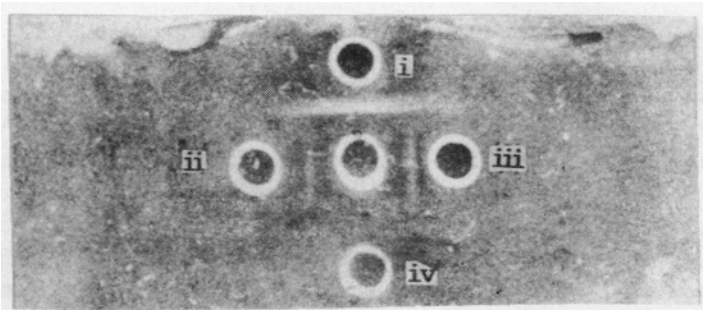

Figure 1. Cross-reactivity of ADC with specific immunoglobulin-G against putrescine synthase.

Wells (i) and (ii) ADC, (iii) PS, (iv) BSA and centre well contained immunoglobulin-G.

Product isolation studies

A more convincing proof for the above premise stems from the product isolation from enzyme reaction mixtures. When the amine products from ADC reaction mixture were isolated and separated on paper chromatograms, it could be shown that putrescine, rather than agmatine accounts for more than 90% of the product derived from arginine decarboxylation (table 1). This unexpected, yet intriguing finding could be further confirmed by using non-radioactive arginine as the substrate at saturating concentrations and identifying ninhydrin-positive putrescine on the chromatograms (data not shown). Addition of inorganic phosphate, which was earlier shown to enhance ADC activity (Prasad and Adiga, 1986a), gave rise to enhanced production of putrescine. Inclusion of agmatine (10 mM), the immediate product of arginine decarboxylation, in the ADC assay mixture could trap significant amount of radioactivity in agmatine fraction, subsequently purified on paper chromatograms; nevertheless significant amount of radioactive putrescine could still be recovered as one of the products under these conditions.

Table 1. Identification of product amines in ADC reaction mixture. Enzyme source: Pure ADC (Sephadex G-150 eluate).

Additions (mM)	Radioactivity (cpm)	
	Putrescine	Agmatine
Nil (Complete system)	2,050	250
P_i(5)	5,300	500
P_i(5) + agmatine(10)	3,300	1,300

The enzyme reaction was carried out according to Prasad and Adiga (1985b). P_i or P_i + agmatine were included in the standard assay mixture at indicated concentrations. ADC activity was measured by counting the $^{14}CO_2$ trapped in KOH. Amines were isolated and quantitated as described by Srivenugopal and Adiga (1981).

Energetics of arginine →putrescine conversion by ADC preparation

The enzymatic conversion of NCP→putrescine as catalyzed by PTCase has been earlier shown to be thermodynamically unfavourable (Srivenugopal and Adiga, 1981) (for enzymatic reactions, see figure 2). It may be recalled that in order to drive the reaction in favour of putrescine formation, the putrescine synthase utilizes two coupled enzymatic reactions, involving either OTCase or CKase. However, the results of the present study show that under the assay conditions employed, the arginine→putrescine conversion, which occurs *via* agmatine and NCP, can proceed even in the absence of either added ornithine or ADP + P_i. This observation is in apparent contradiction with that made with *L. sativus* PS (Srivenugopal and Adiga, 1981). Of relevance in this context, are the earlier reports that decarboxylation reaction could yield energy of ≃7 K cals (Jencks, 1970). Therefore, an attempt was made to investigate whether the decarboxylation of arginine is also an exergonic reaction, in which case the thermodynamic constraint referred to above could be overcome and the putrescine production from arginine by the multifunctional enzyme be easily explained.

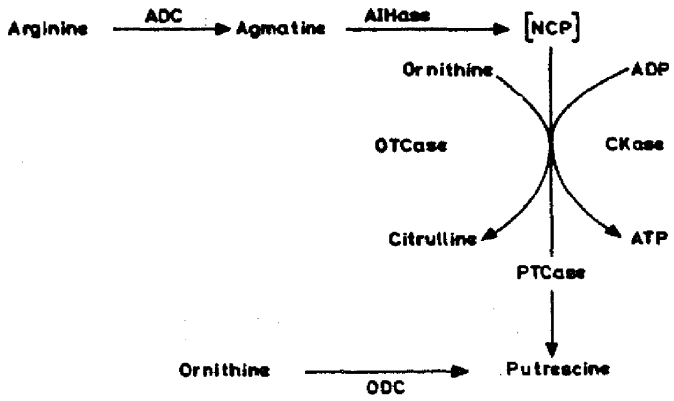

Figure 2. Reactions catalyzed by PS from cucumber seedlings.

Putrescine synthase from *C. sativus* in addition to mediating agmatine→putrescine conversion, unlike its counterpart from *L. sativus* (Srivenugopal and Adiga, 1981), also catalyzes decarboxylation of arginine and ornithine.

Data of table 2, clearly indicate that the decarboxylation can be coupled to the production of ATP and that at least a major part of the energy generated during arginine decarboxylation could be conserved. Production of ATP was maximum when ornithine and agmatine were included in the assay mixture. However, addition of ornithine alone decreased ATP generation, presumably by competing with arginine as observed earlier (Prasad and Adiga, 1985b). It may be noted that agmatine which is inhibitory to ADC activity, also decreased the yield of ATP. It could be shown that the K^+ and Mg^{2+} ions are required for optimal activity and are probably involved at the level of coupling of the component activities or kination, since ADC activity assayed in isolation was independent of added metal ions (Prasad and Adiga, 1985).

Table 2. Decarboxylation of arginine coupled to ATP synthesis (catalyzed by pure ADC preparation).

Additions (mM)	Activity units (nmol of ATP produced/mg protein)
Arginine(5)	157·5
Arginine(5) + Agmatine(5)	65·6
Arginine(5) + Ornithine(5)	0
Arginine(5) $-Mg^{2+}$	109·0
Arginine(5) −KCl	97·5
Agmatine(5) + Ornithine(5)	341·2
Agmatine(5) + Ornithine(5) $-Mg^{2+}$	223·0

The ADC assay was carried out as described in table 1. Indicated additions were made to the ADC reaction mixture prior to the commencement of assay. Standard assay mixture also consisted of 2mM ADP; 5 mM P_i, 5 mM Mg^{2+} and 1 mM K^+. After terminating the ADC reaction, the ATP generated was assayed by hexokinase-glucose-6-phosphate dehydrogenase coupled assay as described by Srivenugopal and Adiga (1981).

Agmatine →putrescine conversion by ADC preparation

The complete reaction which is a measure of the activities catalyzed by PS (agmatine + ornithine $\xrightarrow{P_i}$ putrescine+ citrulline), was also mediated by the ADC preparation. This reaction also displayed the same requirements as those by the reaction mediated by PS (table 3).

Decarboxylation of arginine catalyzed by PS

Since all the above reactions were observed with ADC preparation, it was considered necessary to demonstrate decarboxylation of arginine by purified PS also. For this purpose, the putrescine–Sepharose eluate was employed. As expected, it could be clearly shown that putrescine-Sepharose eluates decarboxylated arginine enzymatically and efficiently and this was inhibited by agmatine (table 4), a phenomenon demonstrated earlier with purified ADC.

Stabilization of component activities of PS by feeding arginine to cucumber seedlings

Cucumber seedlings were fed with 20 mM in solution with arginine on 3rd and 5th days of their germination and harvested on day 8. Unfed seedlings served as controls.

Table 3. Agmatine to putrescine conversion linked to OTCase activity catalyzed by pure ADC preparation (Sephadex G-150 eluate).

Agmatine + ornithine $\xrightarrow{P_i}$ citruline + Putrescine.

Assay condition	Activity units (nmol of citrulline produced)/mg protein
Complete system*	206
$-P_i$	6
$+P_i$(2 mM)	100
− Ornithine	5
− Agmatine	0

*Complete system contains: Tris-HCl pH 8·8; 50 μmol; agmatine 2·5 mM; ornithine 0·5 mM; Mg^{2+} 10 mM; P_i 5 mM; enzyme 80 μg.

Purified ADC was used as the enzyme source. Citrulline was quantitated (Prasad and Adiga, 1986b).

Table 4. Decarboxylation of arginine by PS (putrescine-CH-Sepharose eluate)

Enzyme protein added (μg)	Additions	Activity units*
12	Nil	2·4
48	Nil	8·3
48	Agmatine	2·2 (75)

The value in parenthesis is percent inhibition. *picomoles of $^{14}CO_2$ liberated in a 2 h assay. Agmatine (10 mM) was included to the standard assay mixture prior to ADC assay.

All the component activities tested were stabilized upon feeding with arginine. Day 3 seedlings which were exposed to arginine for a longer time exhibited maximum activities, followed by day 5 seedlings. Untreated seedlings processed on day 8 had relatively the least activity (table 5).

Product switch

Analysis of the total amine fraction of cucumber seedlings showed the presence of significant amounts of agmatine along with spermidine, spermine and putrescine (Suresh *et al.,* 1978). If the cucumber PS converts arginine to putrescine, through a series of coupled reactions then the accumulation of agmatine in significant amounts in the seedlings cannot be easily explained. Towards this end, yet another regulatory feature of the cucumber enzyme became evident, while assessing the stoichiometry of the ADC reaction. With crude enzyme extracts, the major product of the arginine decarboxylation turned out to be agmatine, unlike the case with the purified enzyme

Table 5. Effect of arginine feeding on the component activities of PS from cucumber seedlings.

Enzyme activity	Activity of control (%)	
	Arginine fed for 3 days	Arginine fed for 5 days
ADC* (arginine→agmatine + CO_2)	130	253
Complete reaction (agmatine + ornithine→citrulline + putrescine)	111	285
OTCase (ornithine + carbamyl-phosphate →citrulline)	117	182
PTCase (putrescine + carbamyl-phosphate →NCP)	150	200

Untreated, 8-day-old seedlings served as control. Enzyme activities were assayed in crude extracts.
*From the data presented it is evident that ADC is also a component activity of PS in *C. sativus*.

Table 6. Identification of product amines in ADC reaction mixture. Enzyme source: Crude extracts.

Enzyme source	Radioactivity (cpm)	
	Putrescine	Agmatine
Control	1980	8250
Homoarginine	5610	21450
KCl	990	1485

Cotyledons were treated either with homoarginine (10 mM) for 12 h, or K^+ (30 mM for 72 h) and crude ADC was prepared as described by Prasad and Adiga (1985a). ADC assay was carried out as described by Prasad and Adiga (1985a). Product amines were isolated from the reaction mixture as already detailed.

with which putrescine is the major end product. This was found to be the case when the cotyledons were cultured with homoarginine and KCl also (table 6). It is clear that the switch over from agmatine→putrescine as the major product of the reaction occurs as a result of purification of the enzyme with the elimination of other controlling factors which presumably modulate the *in vivo* elaboration of the various amine intermediates.

Discussion

The multifunctional enzyme, PS of cucumber seedlings exhibits higher degree of structural complexity than its *L. sativus* counterpart (Prasad and Adiga, 1986b).

Results presented in this paper clearly show that it also displays equally complex functional versatility, in that, unlike in *L. sativus,* the enzyme harbours ADC activity also. Thus it appears that in cucumber, the whole of biosynthetic machinery required for the biogenesis of putrescine is organized into a single yet multifunctional unit in the form of PS. This rather unexpected finding has been supported by several pieces of circumstantial evidence such as; (i) identical elution pattern of ADC and putrescine-Sepharose eluate activities during purification by ion-exchange and gel filtration chromatography, (ii) indistinguishable electrophoretic behaviour on non-denaturing and denaturing gels, (iii) complete immunological cross-reactivity, (iv) susceptibility to proteolysis and (v) co-ordinate stabilization of the activities in the seedlings by feeding arginine. Unequivocal proof favouring ADC as the constituent activity of putrescine as the sole product of enzymic reaction catalysed by the purified ADC which sharply contrasts with the situation observed with the *L. sativus* system.

A major question that stems from such a situation is concerned with the nature and source of the driving force required to 'push' the energitically unfavourable phosphorylitic cleavage of NCP→putrescine in the absence of added other substrates to support the coupling reactions. The demonstration that enzymatic decarboxylation of arginine *per se* could meet this energy demand is evident from the data of table 2. This accords with the postulate of Rabinovitch and Flowers (1964) with regard to the functioning of multifunctional/multienzyme systems in that the energy generated in a reaction could be utilized by the subsequent one. It is noteworthy that when either agmatine or NCP acts as the substrate, the PTCase should obligatorily be coupled to either OTCase or CKase ensure substantial putrescine production.

Another important issue that merits attention is the *in vivo* accumulation of agmatine in the plant, which is in apparent contradiction to the data discussed above. However, the enzyme in the crude extracts, gives rise to agmatine from arginine, while in purified form produces putrescine (tables 6 and 1). It would, therefore appear, that the AIHase activity of the multifunctional enzyme remains partly suppressed in the crude extracts, presumably by a regulator, that determines the extent of conversion of agmatine→putrescine, through NCP, while accounting for the release of agmatine into the reaction medium. Our unpublished results (Prasad and Adiga) indicate that the regulator is lost at the DEAE-cellulose chromatography step during purification. An analogous situation is the finding that in *Salmonella typhimurium,* prephenate produced by the action of chorismate mutase component of chorismate mutase-prephenate dehydratase complex has been shown to diffuse into the medium before entering prephenate dehydratase site for further metabolism (Schmit and Zalkin, 1969). If such a regulator for AIHase exists and is functional *in vivo,* then AIHase reaction assume the role of the 'committed step' in putrescine biosynthesis. In other words, although ADC happens to be the rate limiting step, agmatine produced may not be obligatorily converted *in toto* to putrescine, not withstanding the fact that the whole of the biosynthetic machinery for the diamine is organized into a single enzyme. Vance (1976) has argued that multifunctional enzymes have distinct advantages in catalyzing those metabolic sequences wherein the rate limiting reaction is not the committed step, so that the unnecessary accumulation or intermediates is prevented. This kind of situation is reminiscent of the 'Sluice gate' type regulation discussed for the arom conjugate of *Neurospora*

crassa (Welch, 1977; Welch and Gaertner, 1980). In terms of cellular economy, the benefits that accrue to the plant by recruiting the multifunctional PS for arginine→ putrescine conversion apparently arise from chanelling the intermediate metabolites through the coupled reactions discussed earlier (Srivenugopal and Adiga, 1981).

From the forgoing, it is abundantly clear that the putrescine synthase of *C. sativus* significantly differs from its *L. sativus* counterpart, despite the fact that both the enzymes mediate the diamine biosynthesis. It is pertinent to mention in this context, that the enzyme 'arom' conjugate investigated in detail in *N. crassa* also exhibits a broad spectrum of differential association of constituent activities in several other organisms hitherto examined (Welch and Gaertner, 1980). Similarly, the anthranilate synthase is bifunctional in some microbes, whereas in several others it is monofunctional (Zalkin, 1980). Another classical example worthy of attention in this context is the difference enzyme activities associated with the fatty acid synthase system (Schewizer, 1980; Wakil *et al.,* 1983). Whatever may be its ultimate organisational complexity, it is clear from the foregoing that the PS from the cucumber seedlings is a highly versatile multifunctional protein and a comprehensive appreciation of its catalytic, structural and regulatory aspects needs a clearer understanding of the competing demands for its products and substrates *in vivo* and the connecting metabolic sequences utilizing these amine intermediates in the plant.

Acknowledgement

The financial assistance received from University Grants Commission, New Delhi is gracefully acknowledged.

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