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**Regulation of Polyamine
Synthesis in Plants**

Annual Progress Report

Russell L. Malmberg

**University of Georgia
Botany Department
Athens, GA 30602**

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Progress Report - DE-FG09-91ER20034

Regulation of Polyamine Synthesis in Plants

Russell L. Malmberg
Botany Department
University of Georgia
Athens, GA 30602

The goals of our research, in brief, are to understand the regulation of polyamine synthesis and the functions of polyamines in plants. In particular we have been interested in why plants have 2 pathways to putrescine synthesis, when mammals have only one pathway. We have been attempting to determine how arginine decarboxylase and ornithine decarboxylase are regulated, and what the functions of polyamines are, using a combination of tools, including antibodies, cDNA clones, and mutants.

Arginine Decarboxylase -- A novel proteolytic enzyme activation mechanism

ARGdc is clipped *in vivo* (Malmberg et al., 1992)

At the time we wrote the original proposal, our knowledge of arginine decarboxylase (ARGdc) structure was limited to finding a 24,000 Da polypeptide that bound the specific inhibitor DFMA (difluormethylarginine). In contrast, the cDNA clone we isolated for ARGdc contained an open reading frame encoding a 66,000 Da polypeptide, which we could not find in oat plant extracts. We hypothesized that the enzyme might be proteolytically processed from a 66,000 Da precursor polypeptide into the 24,000 Da fragment that we could find in plants. We did not know the fate of the missing 42,000 Da of the precursor polypeptide, nor did we know the structure of the active ARGdc enzyme. We proposed research to further investigate ARGdc by the generation of antibodies specific to different regions of the polypeptide precursor, as it was deduced to exist from the cDNA sequence.

By expressing separate regions of the cDNA individually in *E. coli*, we were able to produce polypeptides, and then generate antibodies specific to different regions of the precursor protein. These antibodies are indicated as anti-N and anti-C in the diagram. Western blot experiments, immunoprecipitation analysis, and reconstruction

experiments allowed us to establish the structure shown in the figure. Oat arginine decarboxylase is initially synthesized as a 66,000 Da precursor polypeptide, as encoded by the cDNA. This polypeptide is then cleaved at some point into 42,000 Da and 24,000 Da fragments that remain associated with each other, probably by a disulfide linkage, in the mature active enzyme. The DFMA inhibitor binding site is located on the 24,000 Da fragment, which is the portion that we originally identified.

ARGdc Activase is an enzyme that activates ARGdc by clipping (unpublished)

The next problem was to determine the mechanism by which the proteolytic processing occurred, and to determine if the processing was a required activation step. To study this we had to develop an in vitro clipping reaction which faithfully reproduced the 42,000 Da and 24,000 Da products seen in vivo. Since we could not find the full length precursor protein in oat plants, we tried several methods of generating it, eventually settling on the following method:

- in vitro transcription of the oat arginine decarboxylase cDNA clone
- in vitro translation of the mRNA via rabbit reticulocyte lysates
- test the translation product in various assays for in vitro processing / clipping.

Using this approach we have succeeded in defining an ARGdc Activase (ADCA) enzyme activity which has the following properties: It clips the 66,000 Da precursor polypeptide into 42,000 and 24,000 Da polypeptides, and simultaneously activates ARGdc. ADCA has a pH optimum of about 7.5, and a temperature optimum of about 28°C. The proteolytic activity is different from that of previously described plant proteases in that it is resistant to inhibitors of serine, cysteine, aspartate, and metallo proteases. We estimate its isoelectric point to be 5.6 and its molecular weight to be 36,000 Da. These last two physical properties establish ARGdc activase as a protein separate from arginine decarboxylase itself; in other words, the precursor protein does not cleave and activate itself.

The discovery of a separate enzyme (ADCA) to activate arginine decarboxylase by a proteolytic cleavage is quite a surprise. It is a novel result both in the characteristics of the protease and in the requirement for enzymatic cleavage for activation of another enzyme. One hypothesis we have to explain this is that ADCA may generate an appropriate prosthetic group for arginine decarboxylase by not only clipping it but also by modifying an amino acid. Most amino acid decarboxylases require pyridoxal phosphate as a co-factor, but the plant arginine decarboxylase does not (unpublished results). We would like to test this idea, and determine the mode of action by which ADCA activates arginine decarboxylase.

In the future we would also like to further characterize the activation reaction in terms of the overall regulatory pattern of arginine decarboxylase. We have discovered a novel means of regulating a protein, leading to the question as to how much, and under what circumstances does the plant use this mechanism? One possibility is that ADCA functions as an activase for a group of similar proteins, not just arginine decarboxylase.

Arginine Decarboxylase is a Phosphoprotein (Huber & Malmberg, unpublished)

In collaboration with Steve Huber at NC State University, we have discovered that arginine decarboxylase is phosphorylated. Specifically, the smaller 24 kDa fragment was found to contain a phospho-threonine residue. Further experiments are needed to establish if protein phosphorylation is a method by which arginine decarboxylase enzyme activity is

regulated, as it is for many other enzymes.

Taken together with the ADCActivase discovery, we have now documented two post-translation modifications of arginine decarboxylase. These discoveries suggest plants have complex, many-layered, means of regulating arginine decarboxylase and polyamine synthesis. In the future we will need to characterize regulation of the pathway at many levels to determine which modes of regulation are used in varying circumstances.

Characterization of Mutants in Polyamine Synthesis

Nicotiana tabacum (Trull et al. preprints)

One of the assets that we have is a collection of mutants, isolated from tobacco cell culture, that are resistant to inhibitors of polyamine synthesis. The philosophy behind these studies has been that perturbing the pathway, and then seeing the resulting morphological changes, would be a good way of determining the functions of polyamines in plant physiology and development. These 2 preprints describe characterization of polyamine levels and of the developmental phenotypes of two of these mutants. Another reprint (Canas and Malmberg) describes the development of monoclonal antibodies that are useful tools for studying the morphological changes in the polyamine mutants.

The mutant *Mgr27* has slightly lower levels of free polyamines than wild type, and dramatically lower levels of bound polyamines (polyamine-conjugates). The plant is dwarf, but analysis of its leaf growth patterns suggest that the basic phyllotaxy has not been disturbed. The flowers of the plant contain stigmatoid-growth on the anthers. The manner in which the stigmatoid growths appear on the anthers suggests the hypothesis that a diffusible substance is regulating stigma growth, both in the abnormal anthers and also in the normal stigma on the pistil. The mutant *Mgr21* has low levels of polyamines, about 1/4 those in wild type. The plant is an extreme dwarf, with irregular leaf growth patterns. The mutant flowers have a complex morphology that is intermediate between an inflorescence and a flower.

If we compare these two mutants, along with some previously characterized, we note the common themes of dwarf plants and altered flowers. The mutants suggest that polyamines are of particular importance in meristems, and that altered levels can either lead to, or be a consequence of disturbed cell division and elongation in the meristems.

Arabidopsis thaliana (unpublished new results)

So far we have not succeeded in isolating a similar collection of polyamine mutants in *Arabidopsis thaliana* by a direct selection strategy as was done in tobacco. We have instead started examining mutants isolated for other reasons, that we suspect might affect polyamine synthesis. One of the physiological links reported for polyamine synthesis, and arginine decarboxylase in particular, is an induction by potassium deficiency stress. This has been reported primarily in monocots, but we have found similar effects in *Arabidopsis* seedlings. We can induce a 5 fold increase in the level of arginine decarboxylase by potassium starvation or by using cesium, which inhibits the uptake of potassium.

Recently Mike Sussman (Wisconsin, unpublished results) has isolated *Arabidopsis* mutants that are defective in potassium uptake. We have obtained these mutants from Mike,

and have shown that they have constitutively higher levels of arginine decarboxylase than does wild type. These mutants thus support the idea of a link between potassium and polyamines, and also provide our first genetic method in *Arabidopsis* to regulate polyamine synthesis.

Ornithine Decarboxylase Purification

A variety of heterologous cloning strategies have failed. At various times we have used mammalian, yeast, and bacterial probes, or used PCR methods based on regions of conserved amino acid sequence. While we obtained several candidate clones and have sequence them, none of these approaches has lead to an authentic plant ornithine decarboxylase clone. Although all of these approaches were reasonable, we ended up wasting a fair amount of time sequencing clones that were not correct.

We therefore began plan B, which was to purify ornithine decarboxylase from plants, and then obtain an amino acid sequence. We have chosen tobacco cell cultures as a convenient source, and developed a standard column purification scheme that results in about a 100 fold purification of the enzyme. At this point there are about 50 protein spots on two dimensional protein gels. We succeeded in identifying which spot on the two dimensional gel was ornithine decarboxylase, using a labelling method that allowed us to specifically put a radioactive tag on our enzyme.

We then started with 60 liters of log phase tobacco cell cultures, and purified the protein 100 fold. We are now running a series of two dimensional protein gels to cut out the spot that is ornithine decarboxylase. It is possible that we have enough protein to sequence now, but we wish to do another 4 or 5 runs to be sure. With any luck at all, we should have the amino acid sequence quite soon..

Arginine Decarboxylase is highly conserved (some fun speculations).

Hierarchical Clustering of Sequences Using MultiAlin

When analyzing the arginine decarboxylase sequences we discovered that the enzyme is highly conserved. A sample cluster analysis is shown on the left. Remarkably, ARGdc from oat and *E. coli* are much more highly related than are actin protein sequences, and actin is considered one of the most slowly evolving of proteins.

One possible functional hypothesis to explain this observation is that arginine decarboxylase may interact directly with a variety of other proteins.

That is, the structure of arginine decarboxylase may need to be kept intact for more than just simple catalysis. In the results described earlier in this report, we presented evidence for 2 proteins that do interact with arginine decarboxylase, a proteolytic activating enzyme and a protein kinase.

Another possibility that we are considering for future research is the idea of metabolic channeling. Perhaps arginine decarboxylase is conserved because it occupies a central position in a multi-enzyme complex that channels metabolites through a series of reactions. I'm including this idea here (near the end) as a concept that may be worth exploring in future research.

Review Articles (Malmberg; Trull and Malmberg)

During the last year we have written two general review articles. One describes genetic approaches in *Arabidopsis thaliana*, the other reviews current progress in the genetic dissection of floral development.

Directions for the Next Year

We think clipping by arginine decarboxylase activase is an exciting, novel discovery, with implications for both enzyme structure and regulation. We wish to pursue it further. We would like to purify the enzyme, and determine more of its properties. Site specific mutagenesis of the target site in arginine decarboxylase should also provide valuable information. We also need to explore the second post-translation modification of arginine decarboxylase we discovered, namely phosphorylation, to determine if it activates or inactivates arginine decarboxylase. In the larger picture, we hope to determine how important each of these post-translational mechanisms are, by quantitatively determining how much and under what conditions, plants use each regulatory mechanism. The *Arabidopsis* potassium uptake mutants should provide a genetic handle on this problem. For ornithine decarboxylase we anticipate being able to finally determine some protein sequence, and then obtain a cDNA clone using this data.

The idea that arginine decarboxylase may interact with other proteins, as suggested by its sequence conservation, is worth some consideration. Genetic approaches have been developed in recent years to study proteins that interact with other proteins. We may give some of these approaches a try to see if there is some reality to our speculation.

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University of Georgia
Athens, GA 30602

Regulation of Polyamine Synthesis in Plants
R. L. Malmberg, Botany Department

Polyamines are small positively charged compounds that have been hypothesized to be involved in a wide variety of plant physiological and developmental functions. The regulation of the polyamine synthesis pathway is uniquely interesting because of the existence of two pathways to putrescine synthesis, and the consequent questions of how these two pathways are compartmentalized and how they interact with each other. The specific directions our research is taking are: (1) A characterization of arginine decarboxylase regulation; we have discovered two post-translational mechanisms for regulating arginine decarboxylase activity. One of these is a novel protease that clips the arginine decarboxylase pre-protein to activate it. We would like to understand this activating protease better, determine its mechanism of action, and determine its importance in the overall scheme of arginine decarboxylase regulation. (2) We have begun a similar characterization of ornithine decarboxylase by purifying it from plants. (3) We are characterizing the polyamine mutant collection we have developed. (4) Finally, we have begun to characterize the evolution of arginine decarboxylase, as an additional approach that could shed light on its functions in plants. Our intent is to understand arginine decarboxylase structure and regulation in detail, and then to further explore regulatory differences between ornithine and arginine decarboxylases.

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