Plant C-N Hydrolases and the Identification of a Plant
N-Carbamoylputrescine Amidohydrolase Involved in
Polyamine Biosynthesis*

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A nitrilase-like protein from Arabidopsis thaliana (NLP1) was expressed in Escherichia coli as a His,
-tagged protein and purified to apparent homogeneity by Ni2+-chelate affinity chromatography. The purified en-
zyme showed N-carbamoylputrescine amidohydrolase activity, an enzyme involved in the biosynthesis of poly-
amines in plants and bacteria. N-carbamoylputrescine amidohydrolase activity was confirmed by identification of two of the three occurring products, namely putrescine and ammonia. In contrast, no enzymatic activity could be detected when applying various compounds including nitriles, amines, and amides as well as other N-carbamoyl compounds, indicating the specificity of the enzyme for N-carbamoylputrescine. Like the homol-
ogous β-alanine synthases, NLP1 showed positive coop-
erativity toward its substrate. The native enzyme had a molecular mass of 279 kDa as shown by blue-native poly-
acrylamide gel electrophoresis, indicating a complex of eight monomers. Expression of the NLP1 gene was found in all organs investigated, but it was not induced upon osmotic stress, which is known to induce biosyn-
thesis of putrescine. This is the first report of cloning a plant N-carbamoylputrescine amidohydrolase and the first time that N-carbamoylputrescine amidohydrolase activity of a recombinant pro-
tein could be shown in vitro. NLP1 is one of the two missing links in the arginine decarboxylase pathway of putrescine biosynthesis in higher plants.

The term “C-N Hydrolases” was introduced by Bork and Koonin in 1994 (1) describing a protein superfamily whose members are all involved in the cleavage of C-N bonds but display only moderate homology (12–24% amino acid identity). Members of this family are found from bacteria to man and can be classified into 13 branches (2, 3). The family includes the following: (i) nitrilases (and cyanide hydratases) and aliphatic amidases, which hydrolyze nitriles (−CN) or amides (−CONH₂), respectively, to the corresponding carboxylic acids; (ii) β-alanine synthases (also called β-ureidopropionases), which produce β-alanine from N-carbamoyl-β-alanine (β-urei-
dopropionic acid); and (iii) other amidohydrolases whose natu-
ral substrates are largely unknown. The genome of Arabidopsis

thaliana encodes eight C-N hydrolases: (i) four nitrilases, one of which (NIT4) was identified as β-cyanoalanine hydratase/ nitrilase (4); (ii) one β-alanine synthase involved in pyrimidine catabolism (5); and (iii) three other nitrilase-like proteins (NLPs) whose function is yet unknown. One of these, provi-
sionally called NLP1, shows high homology to a recently char-
acterized protein of Pseudomonas aeruginosa, the function of which was defined as N-carbamoylputrescine amidohydrolase, involved in polyamine biosynthesis in this species (6).

Polyamines are low molecular mass polycations of vital func-
tion for all living organisms. A key intermediate in polyamine biosynthesis is putrescine (H₂N(CH₂)₄NH₂), which is already a polyamine and is further converted to higher polyamines like spermidine and spermine. In principle, two different biosyn-
thetic pathways lead to the formation of putrescine: (i) the ornithine decarboxylase (ODC) pathway through the decar-
boxylation of ornithine by ODC; and (ii) the arginine decarboxylase (ADC) pathway through the successive reactions of arginine decarboxylase (producing agmatine from arginine), agmatine iminohydrolase (producing N-carbamoylputrescine from agma-
tine), and finally N-carbamoylputrescine amidohydrolase (pro-
ducing putrescine from N-carbamoylputrescine). Both path-
ways are described as occurring in plants (reviewed in Ref. 7), but recently it was shown that in A. thaliana no ODC gene exists and that the ornithine decarboxylating activity found in extracts of this plant was non-enzymatic (8). This stresses the importance of the ADC-pathway for plants, at least with re-
spect to A. thaliana. Many genes of plant polyamine biosyn-
thesis have been cloned (for review see Ref. 9), but until now the genes of the ADC-pathway encoding agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase were not known. We will show here that the C-N hydrolase NLP1 of A. thaliana encodes a functional N-carbamoylputrescine amidohydrolase.

EXPERIMENTAL PROCEDURES

Synthesis of N-Carbamoylputrescine—Synthesis of N-carbamoyl-
putrescine-HCl was done according to Smith and Garraway (10). Puri-
fication of N-carbamoylputrescine was performed as follows. After re-
moval of the side product NN-dicarbamoylputrescine by filtration, the filtrate was evaporated to dryness. The residue was extracted with ethanol, and the extract again was evaporated to dryness. The residue was then dissolved in 50 ml of water, neutralized using 2 M HCl, and loaded on a column (25 cm x 1.5 cm) of Dowex 50 X 8 (Li-form). Elution was done with 3-aminopropyl-1,3-diaminopropane (0.2 M, neutralized with 2 M HCl). The eluting fractions were tested by thin layer chroma-
tography for ninhydrin-positive substances. Fractions showing only one ninhydrin-positive spot were combined, evaporated to dryness, and

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‡ The abbreviations used are: NLP, nitrilase-like protein; ADC, argi-
nine decarboxylase; CPA, N-carbamoylputrescine amidohydrolase, ESA-
tof, electrospray ionization-time-of-flight; EST, expressed sequence tag; ODC, ornithine decarboxylase.
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RESULTS AND DISCUSSION

Identification of Arabidopsis NLPs—In the course of our studies on plant nitrilases (4, 11) we detected genes from A. thaliana coding for proteins homologous to nitrilases, which we subsequently called provisionally nitrilase-like proteins. Of the three NLPs existing in A. thaliana (NLP1, gene At2g27450; NLP2, gene At4g08790; and NLP3, gene At5g12040), NLP1 is annotated as a putative nitrilase and shows high homology to an EST clone from tomato, which is annotated as β-alanine synthase (88% identity, GenBank accession number Y19104). However, NLP1 showed no β-alanine synthase activity (vide infra), and recently the real β-alanine-synthase from A. thaliana was cloned (5).

Phylogenetic analysis of a large sample of C-N hydrolases, wherein 31 nitrilases (including two cyanide-hydrolases), three aliphatic amidases, four β-alanine synthases (β-ureidopropionases), one N-carbamoyl-α-amino acid amidohydrolase, one putative N-carbamoylputrescine amidohydrolase, and 15 C-N hydrolases of yet unknown substrate-specificities were aligned using the DNAMAN software (version 5.2.2, Lynnon Biosoft, Vaudreuil, Quebec, Canada), and a phylogenetic tree was constructed using the same software with a maximum likelihood algorithm and 100 bootstrap trials.

Mass Spectrometric Analysis of the Reaction Product—A standard reaction as described above using N-carbamoylputrescine as the substrate was performed with the modification that the buffer concentration was reduced to 5 mM. After 2 h, a 100-μl aliquot was withdrawn, and 400 μl of ethanol were added. After boiling for 10 min, the denatured protein was removed by centrifugation (15 min at 13,000 rpm in a tabletop centrifuge), and the supernatant evaporated to dryness. The purified protein was desalted using a PD-10 column (Amersham Biosciences), which was equilibrated in 50 mM potassium phosphate, pH 8.0, and 1 mM dithiothreitol. Protein concentrations were between 8 and 12 μg/μl. The purified protein was shock frozen in liquid nitrogen and stored at −80 °C.

Activity Measurements—The standard reaction contained 1 μg of protein in 50 mM potassium phosphate, 1 mM dithiothreitol, pH 8.0, in a final volume of 1 ml. Substrates were added at a final concentration of 1–3 mM. Reactions were carried out for 1–2 h at 37 °C or, for determination of the kinetic parameters of NLP1, for 30 min at 30 °C. Ammonia production was measured using the Berthelot reaction as described elsewhere (4). Production of putrescine was shown qualitatively and semiquantitatively by thin layer chromatography. Aliquots (5 μl) of the sample were spotted on silica plates (Polygram SIL G/UV 254, Macherey-Nagel, Düren, Germany), which were subsequently developed in ethanol/NH₄OH (25% (v/v)) 1:1. N-Carbamoylputrescine and putrescine were visualized with ninhydrin.

Blue-native Polyacrylamide Gel Electrophoresis—Blue-native polyacrylamide gel electrophoresis was performed as described by Schagger et al. (12) for soluble proteins using a 4–15% (w/v) separating gel.

Conconstruction of Phylogenetic Tree—Fifty-five amino acid sequences of C-N hydrolases, therein 31 nitrilases (including two cyanide-hydrolases), three aliphatic amidases, four β-alanine synthases (β-ureidopropionases), one N-carbamoyl-α-amino acid amidohydrolase, one putative N-carbamoylputrescine amidohydrolase, and 15 C-N hydrolases of yet unknown substrate-specificities were aligned using the DNAMAN software (version 5.2.2, Lymonne Biosoft, Vaudreuil, Quebec, Canada), and a phylogenetic tree was constructed using the same software with a maximum likelihood algorithm and 100 bootstrap trials.

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Blue-native Polyacrylamide Gel Electrophoresis—Blue-native polyacrylamide gel electrophoresis was performed as described in Materials and Methods. After 2 h of reaction, the reaction mixture was subjected to Blue-native polyacrylamide gel electrophoresis (15% (w/v) separating gel, 5% (w/v) stacking gel) in a Bio-Rad discontinuous system. After electrophoresis, the gel was stained with Coomassie blue R-250.
number of C-N hydrolases revealed that NLP1, together with the above mentioned C-N hydrolase from tomato, lies on one branch with β-alanine syntheses and an N-carbamoyl-α-amino acid amidohydrolase from *Agrobacterium* sp. (AspDCase in Fig. 1). These enzymes have in common the fact that they hydrolyze N-carbamoyl compounds, and it could therefore be speculated that the natural substrate for NLP1 and its tomato homolog is also an N-carbamoyl compound. Recently, the gene of a N-carbamoylputrescine amidohydrolase from *P. aeruginosa* was identified (6) (*PaCPA* in Fig. 1), and the coded amino acid sequence shows high similarity to NLP1 (67% identity). This is not only in line with the above mentioned hypothesis but moreover points to N-carbamoylputrescine as a possible substrate of NLP1. *PaCPA* belongs to the same family (family 11) of C-N hydrolases (as classified by Pace and Brenner, Refs. 2 and 3) as NLP1. The function of the members of this family was formerly identified (6) (*polyamine biosynthesis* (Ref. 6, and vide infra)).

**NLP1 Is a Functional N-Carbamoylputrescine Amidohydrolase**—The cDNA of NLP1 was obtained from the Arabidopsis Biological Resource Center as an EST clone and was subsequently sequenced. It contains a proposed complete open reading frame of 900-bp coding for 299 amino acids and 31 bp of the 5′-UTR. The open reading frame, which is 100% identical to the published sequence derived from the genome data (GenBank™ accession number NM_128305) was amplified by PCR and cloned into the pET-21b (+) vector. NLP1 was expressed as His6-tagged protein in *E. coli* and purified using Ni2+-chelate affinity chromatography to apparent homogeneity as judged by Coomassie-stained SDS-polyacrylamide gels. The purified enzyme displayed N-carbamoylputrescine activity as shown by different methods as follows. (i) By thin layer chromatography it could be shown that N-carbamoylputrescine vanished during the time course of the reaction while a product, showing the same Rf value as authentic putrescine, was formed (Fig. 2A). (ii) This product could be identified as putrescine by mass spectrometric analysis, because it showed the same ESI-TOF spectrum as authentic putrescine (Fig. 3). (iii) In addition to putrescine, NH4+ was formed as shown by the indophenol blue method (Fig. 2B).

NLP1 showed no activity toward the N-carbamoyl compounds N-carbamoyl-β-alanine (β-ureidopropionic acid) and N-carbamoyl-D,L-aspartic acid as judged by thin layer chromatography (data not shown) and the measurement of liberated NH4+ (Table I). Of some other substances tested, including nitriles, amides, and intermediates of polyamine biosynthesis, none were substrates of NLP1 (Table I).

This is the first time that cloning of an in vitro active N-carbamoylputrescine amidohydrolase is reported. This enzyme is of vital importance for *A. thaliana*, because putrescine bio-

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**Fig. 3.** Mass spectrometric identification of the reaction product as putrescine. A, ESI-TOF spectrum of authentic putrescine. The quasi-molecular ion ([M + H]+, m/z 89.1) as well as a fragment of putrescine (m/z 72.0) is visible. B, ESI-TOF spectrum of an aliquot of the same reaction as shown in Fig. 2A. The signals at m/z 132.1 and m/z 115.1 are derived from residual N-carbamoylputrescine (quasi-molecular ion and fragment, respectively).

**Fig. 4.** Dependence of velocity on substrate concentration. NLP1 was incubated with N-carbamoylputrescine at the indicated concentrations. The curve was calculated by non-linear regression to the Hill equation: $V = \frac{V_{\text{max}}[S]^n}{[S] + K_m}$. The dotted line represents the non-linear regression to the Michaelis-Menten equation $V = \frac{V_{\text{max}}[S]}{[S] + K_m}$.

| Substrate | Relative activity | Typical substrate for |
|-----------|------------------|----------------------|
| N-Carbamoylputrescine | 100 | *Agrobacterium* sp. DCase |
| N-Carbamoyl-D,L-aspartic acid | N.D. | β-Alanine synthase |
| N-Carbamoyl-β-alanine | N.D. | *A. thaliana* NIT1 |
| 3-Phenylpropionitrile | N.D. | *A. thaliana* NIT4 |
| Allylcyanide | N.D. | NIT1 |
| β-Cyano-L-alanine | N.D. | NIT1 |
| Indolyl-3-acetonitrile | N.D. | NIT1 |
| Agmatine | N.D. | Agmatine iminohydrolase |
| L-Asparagine | N.D. | Asparaginase |
| L-Glutamine | N.D. | Glutaminase |
| L-Arginine | N.D. | Arginine deiminase |
| L-Citrulline | N.D. | Citrullinase |
synthesis via the ODC pathway (vide supra) does not occur in Arabidopsis because of the absence of ornithine decarboxylase (8). Using BLAST search (15) sequences for homologues of NLP1 can be found for many plant species, indicating that the ODC pathway of putrescine biosynthesis may be widespread among higher plants (data not shown). To completely uncover the putrescine biosynthesis pathway of Arabidopsis, cloning a functional agmatine iminohydrolase (agmatine deiminase) is necessary.

Biochemical Characterization of NLP1—NLP1 showed maximal activity at ~40 °C and between pH 8 and 9. Determination of kinetic parameters revealed that NLP1 showed no Michaelis-Menten kinetics toward N-carbamoylputrescine (Fig. 4, dotted line). The $V_{\max}$ graph was found to be sigmoid and could be described by the Hill equation $V = (V_{\max} S^h)/(K + S^h)$. Non-linear regression of the data to this equation (Fig. 4, solid line) gave a Hill coefficient ($h$) of 2.2, indicating positive cooperative binding of the substrate, and a $V_{\max}$ value of 86 nanokatals (mg of protein)$^{-1}$. Half maximal velocity was reached at 135 μM substrate. Cooperative binding of the substrate with comparable h values was also found for β-alanine synthases of man and rat (16, 17). Further studies regarding allosteric behavior of NLP1 are currently underway.

Expression of NLP1—We investigated the expression of the NLP1 gene in comparison to the two arginine decarboxylase genes of Arabidopsis (ADC1 and ADC2, Ref. 18) in different organs by Northern-blot analysis (Fig. 6A). Flowering plants were divided into roots, stems, rosette leaves, and complete flowers. In all organs investigated, the mRNA of ADC1, ADC2, and NLP1 was detectable. The highest expression for all three genes was found in the roots and the flowers, whereas expression in stems and leaves was lower. These results are in contrast to those shown by Soyka and Heyer (14), who found only weak expression of ADC1 and ADC2 in roots and a strong expression of ADC1 in rosette leaves. However, the stronger expression of ADC pathway genes in roots compared with rosette leaves and stems shown here is in agreement with the ~2-fold higher putrescine levels of roots compared with shoots reported by Watson et al. (18). It is also worth mentioning that most of the Arabidopsis EST clones coding for NLP1 were made from root mRNA. We next investigated the expression of NLP1 in leaves under osmotic stress, which leads to a strong increase of putrescine levels in Arabidopsis, concomitant with an increase of ADC activity caused by a strong induction of the ADC2 gene (14, 19). As expected, we could show a strong induction of the ADC2 gene upon osmotic stress treatment (Fig. 6B), whereas the expression of ADC1 was very low in buffer-treated leaves and reached comparable levels in stressed leaves and freshly harvested leaves. The expression of NLP1 kept constant under the conditions tested, indicating that N-carbamoylputrescine amidohydrolase is not the rate-limiting enzyme in osmotic stress-induced putrescine biosynthesis. The expression of NLP1 was found to be very low; we did not detect the protein in crude extracts of leaves by use of an α-NLP1 antibody, nor could we show N-carbamoylputrescine amidohydrolase activity using the NH$_3$ assay (data not shown).

Name for NLP1—“NLP1” was only a provisional name based on the homology of this enzyme to nitrilases. Because enzyme names defined by function are preferable, we propose to call this enzyme CPA (for N-carbamoylputrescine amidohydrolase). We could also show that the tomato NLP, which was annotated as β-alanine-synthase, is a functional CPA (data not shown).

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