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INHIBITION OF PLANT NUCLEASE I BY ATP AND OTHER NUCLEOTIDES

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Abstract—Nuclease I (nucleate endonuclease, EC 3.1.4.9) from tobacco cell cultures is inhibited by several nucleotides. The purine nucleoside 5′-triphosphates are highly inhibitory, whereas *c*AMP and *c*GMP have little effect on the activity of the enzyme. ATP, which affects both the endonuclease and 3′-nucleotidase activities of tobacco nuclease I, is a competitive inhibitor with a K_i of 0.5 μM at pH 5.8. This nucleotide also strongly inhibits nuclease I from mung bean and barley.

INTRODUCTION

The activity of many plant enzymes is regulated by the concentration of certain small molecules within the cell [1]. Although the controls on the enzymes of biosynthetic and energy-producing pathways have been extensively investigated, little work has been done on the regulation of nucleolytic enzymes that may affect the nucleic acid economy of the plant cell. One of the major nucleolytic enzymes in plants is nuclease I (nucleate endonuclease, EC 3.1.4.9), an enzyme that catalyzes the hydrolysis of phosphodiester bonds in polynucleotides and 3′-phosphomonoester bonds in mono- and polynucleotides [2]. Nuclease I has been isolated and partially characterized from several species of higher plants [3–8]. Thus, it is of interest to ascertain the response of this enzyme to compounds that may be modulators of its activity. In this report I describe the effect of various nucleotides and related compounds on the activity of nuclease I from tobacco cultures. One of these compounds, ATP, is present at high concentrations in plant cells, and is a particularly potent inhibitor of nuclease I from tobacco cultures, mung bean sprouts, and barley malt.

RESULTS

Plant nuclease I preferentially cleaves bonds on the 3′-side of adenosine residues in mono- and polynucleotides [2]. Many adenine nucleotides inhibit the ability of tobacco nuclease I to degrade single-stranded (ss) DNA particularly at high concentrations (Table 1). Several of the nucleotides bear a 3′-phosphomonoester group, and are substrates of the 3′-nucleotidase activity of this enzyme; a marked depression of the rate of DNA hydrolysis by these compounds is not unexpected. Inhibition of nuclease I by 5′-AMP has also been reported by Suno *et al.* [6].

At a concentration of only 0.05 mM, ADP and ATP inhibited the tobacco nuclease almost completely (Table 1). The effect of nucleotide concentration on the degree of inhibition of the ss-DNase activity by 5′-AMP, ADP and ATP was studied. The inhibitory actions of ADP and ATP in this system were virtually identical; a concentration of 1.7 μM was required for 50% inhibition. The concentration of 5′-AMP required to produce this level of inhibition was *ca* 30-fold greater.

A kinetic study of the rate of hydrolysis of ss-DNA by tobacco nuclease as a function of substrate concentration was performed in the presence and absence of ATP. Double reciprocal plots [10] of the data indicated that ATP is a competitive inhibitor of the nuclease. Values of 17 and 0.5 μM were obtained for the apparent K_m of denatured T5 DNA and K_i of ATP, respectively.

The inhibitory effects of P_i, PP_i, PPP_i and the corresponding nucleoside 5′-mono- and polyphosphates on the ss-DNase activity are presented in Table 2. Hanson

Table 1. Effect of adenine nucleotides on the hydrolytic activity of tobacco nuclease I with denatured T5 DNA

Compound added	Residual DNase activity (% of control) 0.05 mM	0.5 mM
2′-AMP	82	34
3′-AMP	49	3.0
5′-AMP	43	5.6
5′-ADP	2.0	0.5
5′-ATP	2.0	0.4
2′,5′-ADP	16.7	2.1
3′,5′-ADP	7.6	1.3
coenzyme A	4.1	1.0
2′:3′-cyclic AMP	89	41
3′:5′-cyclic AMP	96	76
NAD^+	101	95
$NADP^+$	67	22
FAD	52	6.4
ADP-glucose	68	17.1

and Fairley [4] have also observed an inhibition of nuclease I by P_i. At the mono- and di-phosphate level only the adenine nucleotides were highly inhibitory, whereas at the triphosphate level all purine nucleotides were strong inhibitors. The action of deoxyribonucleotides was similar to that of ribonucleotides. Several of the pyrimidine nucleotides were less inhibitory than the corresponding inorganic phosphate compounds. The level of inhibition produced by PP_i and PPP_i was *ca* 25% at 5 μM and >90% at 100 μM. By contrast UDP-glucose at 0.5 mM produced only 13% inhibition, and adenosine, *D*-ribose 5-phosphate and 3':5'-cyclic GMP caused less than 10% inhibition at this concentration.

The tobacco nuclease degrades RNA, ss-DNA and double-stranded (ds) DNA at a high rate from pH 5 to 6, and also hydrolyzes 3'-nucleotides efficiently over a broader pH range [8]. The data presented in Table 3 show that the potent inhibitory effect of ATP is apparent with each of these substrates in the pH range of 5.2–5.8. However, the effect of ATP on the rate of hydrolysis of 3'-AMP at pH 7.6 is much less than that observed at pH 5.8. ATP also inhibited nuclease I from mung bean sprouts and barley malt; at 10 μM it decreased the ss-DNase activity of each enzyme by 92–94%.

DISCUSSION

The results of this study indicate that nuclease I is sensitive to inhibition by a variety of nucleotides. This inhibitory effect should be considered when one is measuring nuclease I activity in crude extracts of plant materials; dialysis or gel filtration may be necessary to prevent partial inhibition by endogenous nucleotides in the extract. The degree of inhibition is dependent upon the nature of the nucleoside and the position of phosphoryl groups. In general, the order of inhibitory effectiveness of nucleoside phosphates is A > G > C ≅ U. Among the isomers of AMP, the effects of the 3' and 5' phosphates are similar, whereas 2'-AMP is less inhibitory. The observations that 3'-AMP inhibits the DNase activity of the enzyme and that ATP inhibits both the 3'-nucleotidase and the nuclease activities support the proposals of other investigators [6,9] that hydrolysis of phosphodiester bonds and 3'-phosphomonoester bonds occurs at the same site(s) on nuclease I.

Table 2. Effect of inorganic phosphate and nucleoside 5'-phosphate compounds on the hydrolytic activity of tobacco nuclease I with denatured T5 DNA

Parent compound	Residual DNase activity (% of control) monophosphate*	diphosphate†	triphosphate†
(Inorganic)	80	74	71
Adenosine	22	22	20
Deoxyadenosine	20	n.t.‡	26
Guanosine	68	73	26
Cytidine	91	98	70
Uridine	100	101	91
Deoxythymidine	98	n.t.‡	76

* P_i and 5'-NMPs were tested at 150 μM. † All polyphosphate compounds were tested at 5 μM. ‡ Not tested.

Table 3. Effect of 0.1 mM ATP on rate of hydrolysis of various substrates of tobacco nuclease I

Substrate	pH of assay	% inhibition
ds-DNA	5.2	99
ss-DNA	5.8	99
rRNA	5.8	96
3'-AMP	5.8	90
3'-AMP	7.6*	39

* The buffer in the standard assay for 3'-nucleotidase was replaced with Tris–acetate buffer (pH 7.6) at the same concn.

From a kinetic study of the effects of nucleosides and 5'-NMPs on the 3'-nucleotidase activity of nuclease I from potato tubers, Suno *et al.* [6] hypothesized that the enzyme possesses one binding site for a nucleoside and two binding sites for phosphate groups, one for 3'-phosphate and another for 5'-phosphate. This is consistent with the present observation that 3',5'-ADP is much more inhibitory than either monophosphate isomer. The inhibitory effect of 5'-ATP, which is a competitive inhibitor of the enzyme, is greater than that of 2',5'-ADP or 3',5'-ADP. This may reflect the presence on the enzyme of another phosphate binding site that recognizes an additional phosphate group on the 5'-side of the nucleotide.

Inhibition of another plant nuclease by nucleoside 5'-polyphosphates has been reported. Baumgartner *et al.* [11] have isolated an RNase from senescing morning glory corollas that is strongly inhibited by 5'-ADP and GDP. The lower inhibitory activity of ATP suggests that this RNase is distinct from nuclease I. Reports of inhibition of microbial nucleases by ATP have also appeared [12–14], although the physiological significance of these inhibitory effects has not been established.

ATP is the main nucleotide in plant cells; it is estimated that the average ATP content in plant tissue is 0.1 μmol/g fr. wt [15]. Plant nuclease I is almost completely inactive at this concentration of ATP (*ca* 0.1 mM) in solutions buffered at pH 5–6. At pH 7.6, which is perhaps somewhat nearer to the pH occuring in the cytoplasm and some organelles of plant cells, the enzyme is much less affected by 0.1 mM ATP. Nuclease I from corn roots [5] and oat leaves [7] is associated with a particulate fraction in homogenates prepared at low ionic strength; this may reflect a physical compartmentation of the enzyme in the plant cell. Since the inhibition is of a competitive type, the affinity of nuclease I for substrates and ATP at the pH of the compartment of the cell in which the enzyme is located and the relative concentration of each in this region of the cell will control the rate of substrate degradation. The values of many of these parameters have not been determined, and the physiological significance of the inhibition of nuclease I by ATP is uncertain.

EXPERIMENTAL

Enzymes. Extracellular nuclease I from suspension cultures of *Nicotiana tabacum* L. cv. Xanthi was isolated by the method of ref. [8] and subjected to affinity chromatography with NADP–agarose [16]. The ATPase activity of the resulting preparation, measured at pH 6 by the method of ref. [17], was below the limit of detection (<0.2% of the nuclease activity observed with ss-DNA). Mung bean (*Phaseolus aureus*) nuclease I, prepared by the method of ref. [3], was supplied

by Dr. Laskowski. Barley (*Hordeum vulgare*) nuclease I was purified 200-fold from malt by $(NH_4)_2SO_4$ fractionation and chromatography with SP-Sephadex and Sephadex G-75 (unpubished, M. Sasakuma and A. Oleson).

Radioactive substrates. Radioactive DNA (sp. act. 1.15 × 10^6 dpm/μmol) from T5 bacteriophage and radioactive ribosomal RNA (sp. act. 3 × 10^5 dpm/μmol) from *Escherichia coli* were prepared by the methods of ref. [17]. In each preparation the radioactive precursor was uracil-[2-^{14}C]. Quantities of radioactive DNA and RNA are expressed as moles of mononucleotide residues. Denatured T5 DNA was prepared by heating a 1 mM soln of native DNA in 0.01 M Tris–HCl buffer (pH 7.5), 0.01 M NaCl for 10 min at 100° and then cooling rapidly to 0°. *Nucleotides* were tested for purity by UV spectral measurements and by TLC.

Enzyme assays. Nuclease activity on *r*RNA or denatured T5 DNA was measured in a standard reaction mixture (0.3 ml) that contained 20 nmol ^{14}C-labeled polynucleotide, 15 μmol Tris–acetate buffer (pH 5.8) and sufficient enzyme to convert 5 to 50% of the radioactivity to an acid soluble form during the reaction. For assay of DNase activity on native T5 DNA the same reaction mixture was used, except that the Tris–acetate was buffered at pH 5.2. The reaction mixture was incubated for 30 min at 37° and then chilled in ice. The acid-soluble fraction was isolated as described in ref. [17], and 0.7 ml was mixed with 0.3 ml N NH_3 and 4 ml scintillation soln [0.4% Omnifluor in toluene–Triton X-100 (2:1)]. Radioactivity was measured in a liquid scintillation spectrometer. The standard reaction mixture for assay of 3′-nucleotidase activity contained, in a vol of 1 ml, 0.4 μmol 3′-AMP, 50 μmol Tris–acetate buffer (pH 5.8) and enzyme. The soln was incubated for 30 min at 37°, chilled and treated with 0.5 ml cold 10% TCA. A portion of the soln was removed for analysis of P_i by the method of ref. [18].

One unit of enzyme activity (*U*) is defined as the amount that catalyzes the formation of 1 μmol of product per min under the standard conditions of assay.

Inhibition of nuclease I by nucleotides and other compounds was tested in the standard reaction mixture supplemented with the test compound at the concn indicated in the Tables or text. The amount of enzyme employed was 0.2 mU when DNA or RNA was the substrate, and 1.1 mU when 3′-AMP was the substrate. The values reported represent the mean of duplicate or triplicate expts.

Kinetic studies. The rate of the reaction catalyzed by tobacco nuclease I (0.08 mU) was measured at pH 5.8 in reaction mixtures that contained denatured T5 DNA at initial concns ranging from 6–30 μM, and ATP at concns of 0, 0.4 or 1.0 μM. Double reciprocal plots [10] of reaction rate as a function of substrate concn were used to evaluate the kinetic constants. Each plot was linear over the range of substrate concns that were tested.

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