Chemical modifications of Pokeweed antiviral protein: Effects upon ribosome inactivation, antiviral activity and cytotoxicity

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Pokeweed antiviral protein (PAP) is a protein known to inactivate eukaryotic ribosomes by an unknown enzymatic action and inhibit the production of mammalian viruses in tissue culture. This protein was subjected to a variety of chemical modifications to determine their effects upon ribosomal inactivation, antiviral action, and cytotoxicity. It was found that modifications of a number of different amino acid residues had similar effects upon all 3 activities. Also the inactivation of PAP with diethylpyrocarbonate was not due to its reaction with a histidine residue but to a modification of an unidentified amino acid residue.

Pokeweed antiviral protein *Herpes simplex virus* *Protein synthesis* *Ribosome* *Cytotoxicity* *Chemical modifications*

1. INTRODUCTION

Pokeweed antiviral protein (PAP) is a single chain protein which inactivates eukaryotic ribosomes by an unknown enzymatic action on the larger ribosomal subunit [1]. This effect of PAP has been shown to specifically affect the interaction of elongation factors with ribosomes [2,3], the major effect being the inhibition of the translocation step of peptide chain elongation [4]. Also, the effects of PAP upon protein synthesis have been found to be identical to those caused by the A chain of the toxin ricin [4].

The antiviral action of PAP has been shown to be very general in that it inhibits the multiplication of such diverse viruses as poliovirus [5], influenza virus [6], and herpes simplex virus [7] in cultured mammalian cells. One form of the antiviral protein obtained from pokeweed seeds has been shown to be cytotoxic to HeLa cells at high concentrations and lethal to mice at a LD_{50} of >1 mg/kg [8].

Chemical modification studies upon PAP were undertaken to determine:

(i) The nature of essential amino acid residues which may provide some insight concerning the enzymatic action of PAP;
(ii) If ribosome inactivation is the direct cause of PAP's antiviral and cytotoxic activities;
(iii) Amino acid residues which can be chemically modified without affecting PAP's activity in order to prepare conjugates of PAP.

2. MATERIALS AND METHODS

PAP was prepared as in [9]. The chemical modification reagents 2,4,6-trinitrobenzene sulfonyl chloride, tetranitromethane, phenylglyoxal, and iodoacetamide were obtained from Sigma, diethylpyrocarbonate was from Aldrich and [^{14}C]phenylglyoxal was from Research Products International.

Chemical modifications were performed at 25°C with 5×10^{-5} M PAP under conditions shown in table 1. Modified PAP was separated from excess reagent by gel filtration through Sephadex G-25, concentrated by ultrafiltration, and protein concentration determined as in [10] using unmodified PAP as a standard.

The extent of chemical modifications caused by 2,4,6-trinitrobenzene sulfonyl chloride, tetranitromethane, and diethylpyrocarbonate were determined spectrophotometrically using the respective extinction coefficients: $\varepsilon_{345} = 1.4 \times 10^4\ M^{-1} \cdot cm^{-1}$ [11], $\varepsilon_{381} = 2.2 \times 10^3\ M^{-1} \cdot cm^{-1}$ [12] and $\varepsilon_{242} = 3.2 \times 10^3\ M^{-1} \cdot cm^{-1}$ [13]. The reaction of

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Table 1

Conditions used to modify PAP

Reagent	mM	pH	Time (h)	Modified residues
2,4,6-Trinitrobenzene sulfonyl chloride	20	6	1	4.3 Lys
Tetranitromethane	50	8	16	4.2 Tyr
Diethylpyrocarbonate	5	5	0.5	0.9 His
Iodoacetamide	5	6	2	0.9 His
Phenylglyoxal	10	9	1	2.3 Arg

Chemical modifications were performed at 25°C with 5×10^{-5} M PAP under the indicated conditions. The extent of each modification was determined as in section 2.

PAP with phenylglyoxal was monitored by both amino acid analysis and incorporation of [^{14}C]-phenylglyoxal. The extent of alkylation with iodoacetamide was determined by amino acid analysis and the failure of diethylpyrocarbonate to react with the alkylated PAP.

The inhibition of [^{14}C]polyphenylalanine synthesis by modified PAP was determined on 100 μg wheat germ ribosomes in 0.25 ml reaction volumes as in [14].

Antiviral activity was determined by the ability of modified PAP to inhibit herpes simplex virus yields in Vero cells as in [7].

The cytotoxic action of PAP was determined by measuring the inhibition of cellular protein synthesis in Vero cells ($\sim 10^5$ cells) incubated with modified PAP for 48 h. Protein synthesis was measured with 1 h pulses of [^{14}C]leucine (312 Ci/mol) contained in Eagle's medium followed by precipitation of the 0.1 N KOH cell digest with 10% trichloroacetic acid. The precipitate was collected on nitrocellulose filters and radioactivity determined by liquid scintillation spectrometry.

PAP activity in all three assays was determined in duplicate for three concentrations. The average of these values was used to determine the dose which produced 50% inhibition (ID_{50}) of either viral yields or protein synthesis using linear regression analysis.

3. RESULTS AND DISCUSSION

A number of chemical modifications were performed on PAP using the reagents and conditions specified in table 1. In all cases, conditions were chosen to modify either a minimum number of specific residues or a number of residues in excess of which had no effect upon PAP's ability to inhibit polyphenylalanine synthesis. Thus, we have found that the modification of 3 lysine, 3 tyrosine or 1 arginine residues had no effect upon the ribosome inactivating ability of PAP. These lower levels of modification were obtained by performing reactions with either lowered reagent concentrations or shorter reaction times.

The modifications of lysine, tyrosine and arginine with the reagents tested required modification of at least 2 residues to reduce PAP's activity (table 2). The modification of 4 lysine residues slightly reduced the ability of PAP to inactivate ribosomes and inhibit cellular protein synthesis; antiviral activity was stimulated but the observed activity was still within the variability of the dose–response observed in the series of experiments. Nitration of four tyrosine residues nearly abolished all 3 activities of PAP. Nitration probably caused denaturation of the protein's structure rather than the modification of an active site residue unless a tyrosine residue of low reactivity is present.

The chemical modification of the arginine residues of PAP with phenylglyoxal consistently caused a large reduction in PAP activity but the quantitiation of the number of modified residues was variable. Arginine modification was determined both by amino acid analysis and [^{14}C]phenylglyoxal incorporation with the latter technique producing the most consistent results. The results in table 2 show good correlation for the loss of all 3 activities upon the modification of 2–3 arginine residues. The results also suggest that one or two arginine residues may be essential for PAP's activity.

A most noteworthy finding of these studies is the results obtained upon modification of PAP with the two histidine specific reagents diethylpyrocarbonate and iodoacetamide. The results in table 1 show that both reagents react with a single histidine residue. When both of these PAP derivatives were tested for activity it was found that iodoacetamide modified PAP retained all 3 activities but the diethylpyrocarbonate modified PAP was almost completely inactivated (table 2). Such results could be due to the modification of different his-

Table 2

The effect of chemically modified PAP on ribosome inactivation, herpes simplex virus yields and cellular protein synthesis

Reagent	Ribosome inactivation		Herpes simplex virus yields		Cellular protein synthesis	
	ID_{50} (pmol)	% act.	ID_{50} (μM)	% act.	ID_{50} (μM)	% act.
2,4,6-Trinitrobenzene sulfonyl chloride	7.1	58	1.5	153	4.3	67
Tetranitromethane	14.6	21	–	0	60.7	5
Iodoacetamide	5.4	72	0.8	145	4.4	84
Phenylglyoxal						
trial 1	–	0	7.0	37	8.1	31
trial 2	7.9	25	37.3	3	5.6	48
Diethylpyrocarbonate						
trial 1	11.5	28	11.2	13	14.7	16
trial 2	–	0	120	1	126.5	3

Chemically modified PAP was assayed for its ability to inhibit the rate of polyphenylalanine synthesis on wheat germ ribosomes, herpes simplex virus yields in infected Vero cells after 24 h, and protein synthesis in uninfected Vero cells after 48 h as in section 2. The doses of unmodified PAP which produced 50% inhibition were 1.1–3.9 pmol/100 μg ribosomes for polyphenylalanine synthesis, 1.2–3.0 μM for virus yields, and 2.3–3.7 μM for cellular protein synthesis

tidine residues by the 2 reagents. To test this possibility, PAP was modified with iodoacetamide and then the reaction of diethylpyrocarbonate with the alkylated PAP was tested. The data in table 3 show that the alkylated histidine residue of the modified PAP is no longer susceptible to acylation with diethylpyrocarbonate. These results show that the 2 reagents are reacting with the same histidine and indicate that diethylpyrocarbonate is reacting with some other amino acid residue in PAP which does not cause an increase in absorbance at 242 nm characteristic of ethoxyformylhistidine [13]. Also, the reaction of diethylpyrocarbonate with denatured PAP results in the acylation of ~3 histidine residues, the number previously determined to be present in PAP [3].

These results indicate that the ribosome inactivating ability of PAP is the direct cause of this protein's antiviral and cytotoxic activities. At μM levels of PAP, maximal antiviral activity is observed in 24 h but a 48 h exposure is required to obtain similar levels of inhibition of cellular protein synthesis in uninfected cells. It can be concluded, therefore, that PAP must enter cells to express its antiviral and cytotoxic effects and that the process of viral infection enhances PAP entry into cells although PAP may enter uninfected cells only upon prolonged incubation.

These studies failed to specifically identify any amino acid residue required for the enzymatic action of PAP but implicate one or more arginine residues as being essential for PAP's activity. Also the results obtained with diethylpyrocarbonate suggest that PAP may contain an unusual amino acid residue or at least one or more residues which react with diethylpyrocarbonate in an unexpected manner.

The relative resistance of PAP to inactivation following modification of its lysine residues make them excellent sites for the introduction of cross-

Table 3

Reaction of diethylpyrocarbonate with native, alkylated and denatured PAP

	ΔA_{242}	His modified
Native	0.14	0.9
Alkylated	0	0
Denatured	0.43	2.7

PAP was treated with diethylpyrocarbonate as in table 1. Where indicated PAP was alkylated with iodoacetamide under the conditions in table 1 or denatured in 6 M guanidinium hydrochloride

linking reagents for the preparation of 'target-directed' toxins. In fact, we have used the reagent *N*-succinimidyl 3-(2-pyridyldithio)-propionate to introduce a free sulfhydryl group into PAP and subsequently coupled this derivative to a monoclonal antibody against a mouse lymphoma which has been used to inhibit in vivo lymphoma growth [15]. Other authors have used similar methodology to link PAP to a F_{ab}' fragment of an antileukemic IgG [16] which was found to be cytotoxic to L1210 cells.

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