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HYDROLYSIS OF PHOSPHORIC ACID DIESTERS BY SNAKE VENOM PHOSPHODIESTERASE AND 5'-NUCLEOTIDE DIESTERS OF SINAPIS ALBA GERMS VIA A COVALENTLY ENZYME-BOUND INTERMEDIATE

Chr.-U. Rugevics and H. Witzel

Institut für Biochemie
Westfälische Wilhelms-Universität
Orléansring 23a, D-4400 Münster

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Summary - Snake venom phosphodiesterase from Crotalus durissus terrificus and 5'-nucleotide phosphodiesterase from Sinapis alba were incubated with the 1-naphthyl ester of 5'-[methyl-^{3}H]thymidylic acid. After short-time incubation the enzymes were denatured by extraction into phenol and chromatographed on Sephadex G-25. Protein fractions containing radioactivity were collected, dialysed and subjected to ultra-thin-layer isoelectric focussing and autoradiography. The results obtained indicate a hydrolytic course via a covalently-bound intermediate.

A 5'-nucleotide phosphodiesterase from bovine intestine was demonstrated by Landt and Butler (1) to hydrolyse its substrates via a covalently bound 5'-nucleoside monophosphate. An active site serine or threonine is suggested to be involved in the catalytic step. Burgers et al. (2) and Bryant and Benkovic (3) proved that snake venom 5'-nucleotide phosphodiesterase (SVPDE) cleaves asymmetrically labeled thiophosphat substrates with retention of configuration at the phosphorous atom, indicating a double-replacement mechanism. These results were confirmed recently by Jarvest and Lowe (4) and Mehdi and Gerlt (5) using ^{31}P-NMR-spectroscopy and ^{16}O, ^{17}O, ^{18}O-substituted nucleotidylic products for determination of configuration. Retention indicates an enzyme bound intermediate for which however no direct evidence could be found.

Since there are profound similarities between these animal enzymes and the 5'-nucleotide phosphodiesterases of plants concerning physical properties and substrate specificities, we studied SVPDE and the corresponding enzyme of Sinapis alba (Sa-PDE) with respect to common features. In this paper we report the isolation and characterization of an intermediate

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that was trapped by incubating SVPDE and Sa-PDE respectively with the radioactive 1-naphthyl ester of 5'-[methyl-^{3}H]thymidylic acid (1-Np[^{3}H]T) which is a substrate for both enzymes.

MATERIALS AND METHODS

Materials: [methyl-^{3}H]T with a specific activity of 45 Ci/mmol was supplied by Amersham Corp., thymidine from Pharma Waldhof. Commercial grade 1-naphthyl phosphate, disodium salt, was purified by ion-exchange chromatography on Whatman DE 32 with a linear gradient of triethylammonium hydrogencarbonate at pH 7.5 and reacted as the bis-triethyl ammonium salt. Commercial grade phenol was further purified by distillation. All other chemicals used were reagent grade.

Enzyme sources: SVPDE of Crotalus durissus terrificus and alkaline phosphatase of E. coli were from Boehringer. Sa-PDE was isolated by a method that will be described elsewhere.

Synthesis of 1-Np[^{3}H]T: 1 mCi[methyl-^{3}H]T with a specific activity of 45 Ci/mmol in ethanol was diluted with 100 mg (0.4 mmol) thymidine and rendered anhydrous with pyridine. The reaction was carried out in 5 ml of anhydrous pyridine with 0.45 mmol 1-naphyl phosphate and 0.6 mmol of 2,4,6-triisopropyl-benzenesulfonyl chloride. After 20 hours at room temperature in the dark, the reaction was stopped by addition of 1 ml water and stirred for another 15 hours. The pyridine was evaporated under diminished pressure, the residue taken up in 10 ml water and titrated to pH 7.5 with sodium hydroxide. The aqueous solution was extracted two times with 5 ml of diethyl ether and applied to a column (24 cm x 3 cm) packed with DE32 anion exchanger equilibrated with hydrogencarbonate and washed to neutrality. Unreacted thymidine was eluted with water. When the elution of radioactive thymidine ceased, the products were fractionated by applying a linear gradient of triethylammonium hydrogencarbonate (total volume 2000 ml, from 0,02 M to 0.08 M).

Elution of 1-Np[^{3}H]T was centered at 0.036 M. The substance was identified by the ultraviolet spectrum and chromatographic behaviour. Its hydrolytic products after incubation with SVPDE and E. coli alkaline phosphatase were identified by thin-layer chromatography as 1-napthol, 5'-thymidylic acid and thymidine as compared with authentic reference substances. The isolated yield was 59 %, the radiochemical purity was 93 %. The remaining 7 % were thymidylylthymidine (5 %) and the 1-naphthyl ester of 3'-thymidylic acid (2 %).

Ultra-violet spectra and activity assay were performed on a Perkin Elmer type 320 double beam spectrophotometer. Radioactivity was determined with a Packard Tricarb Model 3320 liquid scintillation counter using Zinsser Unisolve scintillation cocktail at an efficiency of 36-38 %.

Isolation of covalently-bound intermediates - The method of Landt and Butler (1) was used with some modifications of the original procedure.

A solution of 0.5 mg SVPDE and Sa-PDE respectively in 0.7 ml reaction buffer (0.1 M Tris/HCl, pH 8.5, 0.25 M NaCl) was used in each labelling experiment. To this incubation mixture was added quickly 0.2 ml of a solution containing a total of 4.49×10^{-5} mole 1-Np[^{3}H]T. 10 seconds after injection of the

radioactive substrate the enzymatic hydrolysis was stopped by injection of 0.1 ml phenol. Under these conditions the enzymes lost their catalytic activity instantaneously. The emulsion obtained was brought to a homogenous phase by dropwise addition of elution buffer (0.1 M Tris/HCl, pH 7.0, 0.1 % SDS). The total volume comprised about 2.5 ml. This solution was applied on a pre-equilibrated Sephadex G-25 (Pharmacia Corp.) column (116 cm x 1.5 cm) and eluted at a rate of 12 ml/h with elution buffer. Blanks were obtained by first denaturating the enzymes through the addition of phenol and then adding the substrate solution. All other operations were run in the same way. Identification of eluted substances was done spectrophotometrically and by running a test mixture of a known composition over the same column before and after the incubation mixture.

Protein fractions containing radioactivity were pooled and thereafter dialysed against 10 litres of bidestilled water for 15 hours with one exchange of water. The radioactive protein containing solution was lyophilized. With SVPDE radioactivity incorporated amounted to 9570 dpm/mg. Incorporation of radioactivity into Sa-PDE was 6125 dpm/mg.

Ultra-thin-layer isoelectric focussing and autoradiography - Ultra-thin-layer isoelectric focussing was performed according to Radola (6) on a 0.1 mm Servalyt pH 3-6 (Serva) ampholyte layer, prefocussed at 600 V for 4 hours. The electrodes were immersed into solutions of 25 mM aspartic acid/25 mM glutamic acid (anode) and 25 mM arginine/25 mM lysine/2 M ethylendiamine (cathode) respectively. The sample (0.04 ml) was applied at pH 4.6 and focussed at 26 V/cm for 4 hours. The pH-gradient was determined with a calibrated surface electrode and the layer was thereafter dried at 90° C for 15-20 minutes. After spraying with EN^{3}HANCE-spray (NEN Corp.) and covering with an Agfa Gaevert Osray T 4 x-ray film the autoradiogram was recorded at -80° C for 15 hours.

The protein was stained according to Holbrook and Leaver (7) on the gradient layer after autoradiography had been performed.

Non-enzymatic hydrolysis of enzyme-bound intermediates - 0.2 mg denatured radioactive labeled protein was incubated in 0.1 ml 1 mM HCl (pH 3), 0.1 ml Tris buffer (pH 7) and 0.1 ml 0.1 M NaOH (pH 13) at 50° C for varying time intervals. The protein was extracted into 0.1 ml of phenol and the radioactivity determined which remained within the aquous phase after phase separation. Blanks were obtained by extraction of the protein into the organic phase prior to incubation and counting the phenol saturated aqueous phase.

RESULTS AND DISCUSSION

Short-time incubations of SVPDE and Sa-PDE respectively with the radioactive substrate 1-Np[^{3}H]T resulted in the formation of protein associated radioactivity after denaturation with phenol and SDS and separation on Sephadex G-25. Fig. 1a shows the elution profile obtained with SVPDE. Associated with the protein peak - as determined from its ultraviolet spectrum - was eluted a total of 9670 dpm/mg of protein. Recalling the

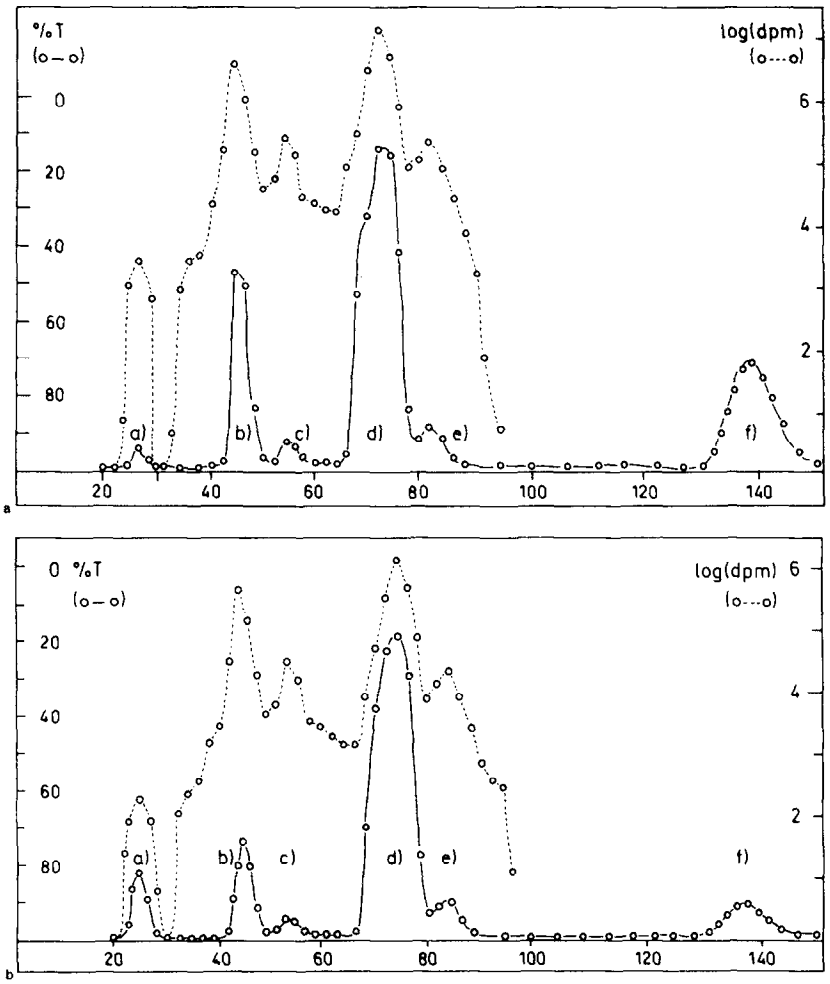

Fig. 1. Separation of denatured 5'-nucleotide phosphodiesterase and low molecular weight components by gel chromatography on Sephadex G-25. Column: 116 cm x 1.5 cm. Elution buffer: 0.1 M Tris/HCl, pH 7.0, 0.1 % SDS at 4°C. Elution rate: 12 ml/h, fractions containing 3.3 ml. Ordinate: per cent transmission at 280 nm and logarithm of total decompositions per minute (dpm) and fraction. Column was calibrated prior and after fractionating the incubation mixture with following substances: a) dextrane blue and protein, b) 5'-thymidylic acid, c) 3'-5'thymidylyl-thymidine, d) phenol + 1-Np[^{3}H]T, e) 1-naphthyl ester of 3'-thymidylic acid and f) 1-naphthol. Abscissa: fraction number.
Fig. 1.a. Incubation mixture containing SVPDE (0.5 mg).
Fig. 1.b. Incubation mixture containing Sa-PDE (0.5 mg).
Blank: Elution profile missed peaks b) and f) and contained no radioactivity associated with protein peak a).

specific activity of the substrate and the moles of enzyme in this experiment incorporation of 5'-thymidylic acid was calculated to be 26 %. In a similar experiment, with protein first denaturated and radioactive substrate then added, no radioactivity was eluted with the protein peak and no product formation was observed.

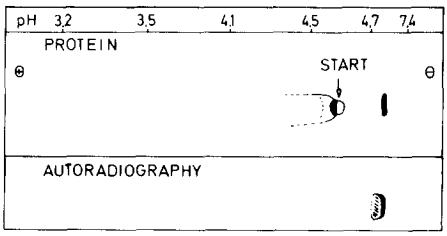

Fig. 2. Schematic representation of ultra-thin-layer isoelectric focussing (top) and autoradiography (bottom) of trapped and with 5'-thymidylic acid labelled Sa-PDE. Servalyt pH 3-6 ampholyte layer, 0.1 mm, prefocussed at 600 V for 4 hours. The sample was applied at pH 4.6 and focussed at 26 V/cm for 4 hours. After the pH was measured, the dried layer (15-20 minutes at 90° C) was sprayed with EN^{3}HANCE-spray. Autoradiography on Agfa Gaevert Osray T4 exposed for 15 hours at -80°C.

The same results were obtained when incubating Sa-PDE under the same conditions and separating the incubation products on Sephadex G-25 as shown in fig. 1 b. The protein peak contained 6125 dpm/mg of protein - that is 17 % incorporation of 5'-thymidylic acid.

We used the type G-25 Sephadex for separation of protein and low molecular weight constituents within the incubation mixture. We found that under the elution conditions of 0.1 M Tris/HCl of pH 7.0 containing 0.1 % SDS at 4°C the low molecular weight components were not only separated very efficiently from the protein but also from each other. Therefore it was possible to determine the overall conversion of substrate to products from the distribution of radioactivity on the different low molecular weight substances. From calibration runs of authentic compounds over the same chromatographic column it was possible to identify each of the separated peaks.

To determine whether or not the protein associated radioactivity was covalently bound the protein containing fractions were pooled and dialysed against water and lyophilized. Part of the freeze dried residue in an aqueous solution was checked by ultra-thin-layer isoelectric focussing (fig 2). A test run of non-radioactive samples showed a depression of the isoelectric points from 4.8 to 4.2 for SVPDE and from 5.4 to 5.1 for Sa-PDE respectively compared with denatured free enzyme samples.
As is schematically demonstrated for Sa-PDE in fig. 2, autoradiography revealed the superposition of the protein band and darkening on the x-ray film. As radioactivity and protein are not separabel on dialysis and ultra-thin-layer isoelectric focuss-

Table 1. Non-enzymatic hydrolysis of enzyme-bound intermediate.

	pH of hydrolysis	time of hydrolysis (hours)	decompositions per minute and mg protein in aqueous phase
SVPDE	13 a)	12	31
		60	132
	7 b)	12	8
	3 c)	12	74
		60	328
Sa-PDE	13 a)	48	922
	3 c)	48	682

Incubation was carried out in 0.5 ml Eppendorf-tubes at 50°C. The incubation mixture was extracted with an equal volume of phenol and radioactivity within the aqueous phase was determined against a blank at pH 7. Incubation buffer: a) 0.1 M NaOH, b) 0.1 M Tris/HCl, c) 1 mM HCl. Radioactivity within the aqueous phase could be bound completely to Dowex 2 x 8 anion exchange resin.

ing, we suggest that there really exists a covalent bond between protein and 5'-nucleotide.

A radioactive protein sample was hydrolysed at different pH values non-enzymatically to determine the nature of the protein-thymidylate-bond. As is demonstrated in tab. 1 hydrolytic products are merely observed at neutral conditions, but are found after acidic and alkaline hydrolysis which remained within the aqueous phase on extraction with phenol after neutralization. This behaviour seems to be compatible with a bond between a nitrogen atom of histidine and a 5'-nucleotide. A tyrosine as nucleotide aceptor can not be excluded strictly wherease the formation of a mixed anhydride between a carboxylate group and the nucleotide can be ruled out, since reduction with sodium borohydride did not liberate radioactivity from the protein.

Our results demonstrate that SVPDE and Sa-PDE hydrolyse their diester substrates like the bovine intestinal protein via a covalently bound intermediate. This is in agreement with retention of configuration at the phosphorous atom, already found by other workers (2-5).

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