KINETICS OF HYDROLYSIS OF URIDYLIC OLIGONUCLEOTIDES WITH DIFFERENT DEGREE OF POLYMERISATION BY SNAKE VENOM PHOSPHODIESTERASE

L. B. DOLAPCHIEV

Biochemical Research Laboratory, Bulgarian Academy of Sciences, Sofia, Bulgaria

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1. Introduction

Snake venom phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) is an exonuclease, splitting successively 5′-mononucleotides from the polynucleotide end, bearing free 3′-hydroxyl group [1–3]. It has been reported that the reaction time course shows an atypical rate of liberation of the product from the polynucleotide substrate during the hydrolysis [3]. The molar concentration of the substrate remains constant for a long enough period of time, depending on the degree of polymerisation of the substrate molecule. Therefore, the increasing reaction rate with progress of the hydrolysis could be explained by the influence of the chain length of the substrate molecule.

In the present study we followed the change of some kinetic parameters of the enzyme reaction catalyzed by snake venom phosphodiesterase as a function of the length of the substrate molecule.

2. Materials and methods

Oligonucleotides of different chain lengths were prepared by column chromatography fractionation on DEAE-cellulose. A sample of poly U (Miles Chem. Comp., Elkhardt, Ind.) was hydrolyzed in 0.3 N KOH for 7 min after which it was treated with 0.1 N HCl for 1 h to hydrolyze 2′,3′-cyclic phosphates. The obtained oligonucleotides were dephosphorylated by a purified calf intestine alkaline phosphomonoesterase, kindly provided by Dr. A.A.Hadjiolov. The enzyme was eliminated by a three-fold treatment with water-saturated phenol [4] and the resulting mixture of oligonucleotides of the type $U(pU)_n$ was fractionated on a DEAE-cellulose column. The elution was made with a linear gradient of sodium acetate with pH = 7.6 [5]. In view of the homogeneity of the fractionated material a good separation of the oligonucleotides with $n = 1–6$, according to their chain length, was found in the absence of urea (fig. 1).

The fractions of each peak were pooled, desalted and evaporated to dryness [6]. Identification of every oligomer was made both by order of elution and by paper chromatography [7]. As markers for this chromatography and to control the results of the kinetic investigations an oligonucleotide set of the type $U(pU)_n$, where n is from 1 to 5 was used (Miles Chem. Comp., Elkhardt, Ind.). The purified phosphodiesterase preparation (from *Crotalus adamanteus* venom) was obtained from Worthington Biochem. Corp., Freehold, N.J. All other reagents used were analytical grade.

The enzyme reaction was carried out in glass redistilled water at constant pH, 25°, and in a final volume of 1 ml. The time course of the hydrolysis was recorded automatically by a microelectrode pH-stat titration assembly (Radiometer, Copenhagen, Denmark). The pH was kept constant by KOH with known concentration. The amount of KOH consumed corresponds to the quantity of phosphodiester bonds hydrolyzed. The interference of atmospheric CO_2 was eliminated by carrying out the reaction under a stream of purified nitrogen.

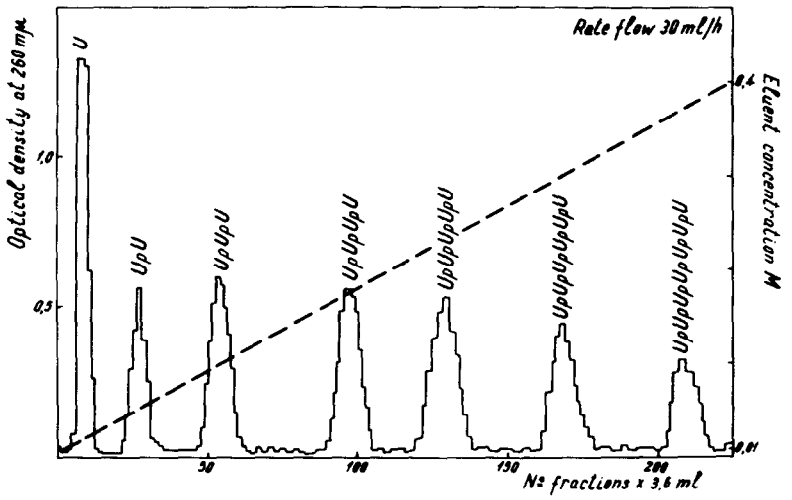

Fig. 1. Column fractionation of 90 O.U. of oligo U. Column (0.6 X 40 cm) packed with DEAE-cellulose (Bio-Rad Laboratories, Richmond, Calif.). The elution was made with 300 ml of CH_3COONa (linear gradient from 0.01 to 0.4 M) with pH = 7.6 at roon temperature.

3. Results and discussion

Initially the pH-optimum of every substrate was determined. For all substrates investigated the maximal rate of hydrolysis was obtained at pH = 9.7 ± 0.1. All other kinetic investigations were made at this pH.

Typical examples of reaction time curves for substrates with different chain length are shown in fig. 2. Under our conditions of substrate and enzyme concentrations the reaction rate remained constant for a long enough period of time. This allowed us to determine the initial rate accurately. This rate showed a linear dependence on the enzyme concentration for each oligomer. The Michaelis constant (K_m) and maximal velocity (V) were calculated from the plots of $1/v$ versus $1/s$ (v is the initial rate, obtained at substrate concentration s) using the least squares method according to the equation:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V}\frac{1}{s}.$$

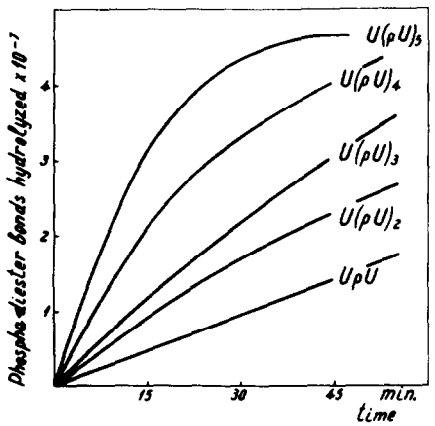

Fig. 2. Time course curves of enzyme hydrolysis of oligonucleotides with different chain length by snake venom phosphodiesterase. Incubation mixture 1.0 ml H_2O containing 0.4 μmoles substrate, 50 μg enzyme, pH = 9.7, 25° and chart speed 1 cm/4 min.

Since our enzyme attacks the substrate molecule only at the end bearing a free 3'-OH group of the pentose, the value of K_m expressed on a molar basis is of physical significance. It was found that with increasing the degree of polymerization of the substrate molecule K_m diminishes (fig. 3, solid line). On the contrary, increasing the substrate chain length brings about an increase of the maximal velocity (fig. 3, dotted line).

If we accept that determining the initial rate we get the rate of hydrolysis only of the first linkage then for the initial stages of the reaction the ordinary

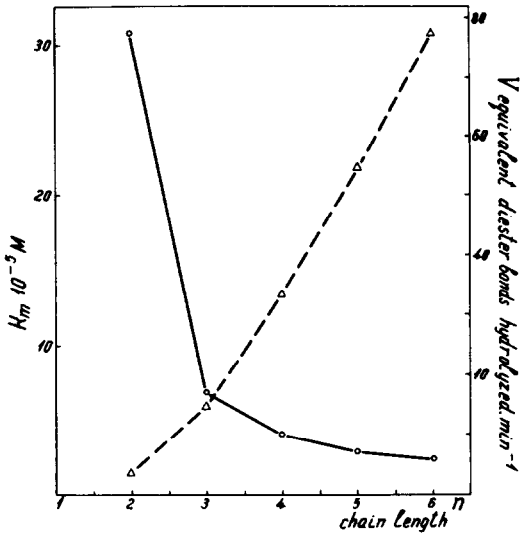

Fig. 3. Dependence of K_m (solid line) and V (dotted line) on the degree of polymerization (n) of the substrate.

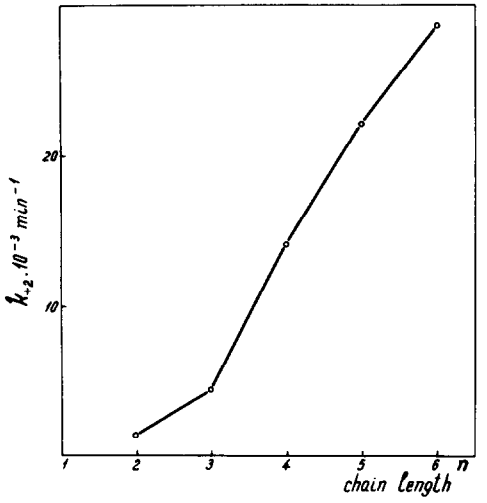

Fig. 4. Dependence of k_{+2} on the degree of polymerization (n) of the substrate molecule.

Michaelis-Menten scheme can be applied:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P.$$

According to this scheme on the basis of the following equation:

$$V = k_{+2}\, e\,,$$

we calculated the breakdown constant of the ES-complex k_{+2} dividing V by the enzyme concentration e. To calculate k_{+2} for each oligomer we used 20,000 as the molecular weight of the enzyme according to Singer and Fraenkel-Conrat [8]. In view of the linear dependence of k_{+2} on V, with increasing the degree of polymerization of the substrate molecule up to 6 the breakdown constant of the ES-complex shows the same relationship (fig. 4). Analogically, when the substrate chain is lengthened the $1/K_m$ increases (fig. 5).

Our preparation of phosphodiesterase is not crystalline, but in a previous study [9] we obtained evidence for the absence of enzyme contaminants, which can interfere with the investigated reaction. An oligonucleotide substrate of the type $(Ap)_nCP$ ($n \cong 15$)

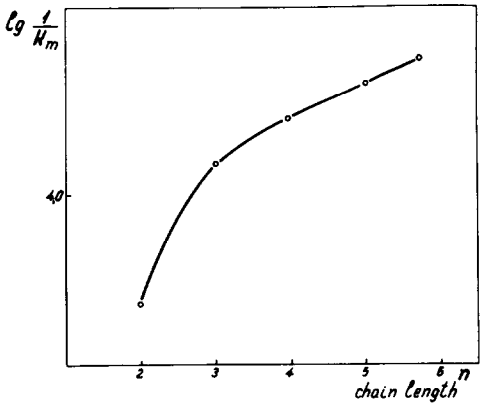

Fig. 5. Dependence of $\lg(1/K_m)$ on the chain length of the substrate.

was found to be resistant to the action of snake venom phosphodiesterase while $(Ap)_nC$ was rapidly degraded to give pA and pC [9]. This allowed us to calculate some thermodynamic parameters, characterizing the initial stages of the hydrolysis. Our view was to study their change as a function of the chain length of the substrate molecule. Assuming that $1/K_m$ represents approximately the dissociation constant of the ES-complex, i.e., $K_m = k_{-1}/k_{+1}$ (or $k_{+2} \ll k_{-1}$) the free energy change accompanying the ES-complex for-

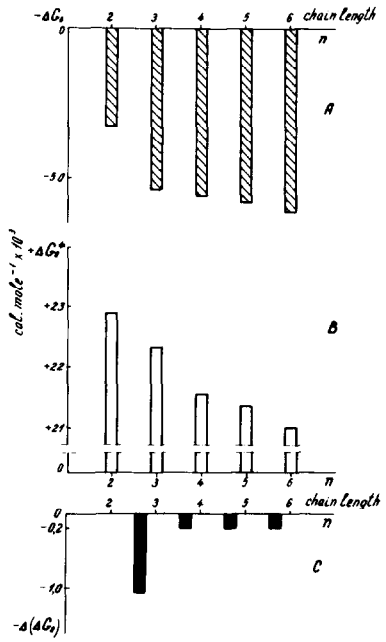

Fig. 6. Free energy change of the formation (A) and the degradation (activation energy) (B) of the ES-complex and increment of the free energy change per mole of residue (C).

mation is given by the equation [10]:

$$\Delta G_0 = -RT \ln(1/K_m),$$

where R and T are the gas constant and the absolute temperature, respectively. The free energy of activation for the breakdown of this complex can be calculated according to the transition state theory [11] as follows:

$$\Delta G_0^{\neq} = RT \ln(kT/hk_{+2}).$$

Here k and h are the Boltzmann constant and the Planck constant, respectively.

As can be seen in fig. 6 (A and B) both ΔG_0 and $\Delta G_0^{\neq}$ diminish with increasing the substrate chain length. In fig. 6C are presented the increments of the free energy change $\Delta(\Delta G_0)$ per mole of mononucleotide residue. The value for $\Delta(\Delta G_0)$ are obtained by subtracting ΔG_0 for the n-mer from ΔG_0 for the $(n+1)$-mer. These data suggest that all residues of the substrate oligomer (with $n = 2$–6) contribute to the binding of the enzyme molecule. The observed increment of ΔG_0 of about 200 cal per mole nucleotide residue, after the third one, indicate that these binding forces are most likely of Van der Waals type [12]. On the other hand the increment of ΔG_0 of 1100 cal per mole residue between the dimer and the trimer may be due eventually to some kind of chemical binding of the substrate and the enzyme's active site.

In conclusion we can state that there exists a strict correlation between the degree of polymerization of the oligonucleotide substrate and the kinetic and thermodynamic parameters of its hydrolytic degradation by the exonuclease snake venom phosphodiesterase. The exact quantitative evaluation of these data requires further studies with substrates with a higher degree of polymerization and a more precise estimation of the molecular weight of the enzyme.

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