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STERIC AND CHARGE FACTORS IN THE RESISTANCE OF URIDYLYL(3′ — 5′)N^6-(*N*-THREONYLCARBONYL)ADENOSINE TO VENOM PHOSPHODIESTERASE HYDROLYSIS

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Summary

The contribution of steric and negative charge factors to the resistance of uridylyl(3′ — 5′)N^6-(*N*-threonylcarbonyl)adenosine to venom phosphodiesterase was investigated. The hydrolysis rates of uridylyl(3′—5′)N^6-(*N*-threonylcarbonyl)-adenosine, its model derivatives, methyl ester and *O*-benzyl ester, together with unmodified uridylyl(3′—5′)adenosine, were studied. It was found that the contribution of both factors is of the same order. The steric inhibition of digestion is distinctly higher than that confirmed by N^6-(Δ^2-isopentenyl)adenosine [1], which is ascribed to the rigid conformation of the threonylcarbonyladenosine side chain.

Introduction

It has been noted in a number of cases that oligonucleotides containing some modified units are digested by phosphodiesterases much slower than their nonmodified analogues, e.g. pseudouridine [2], L-enantiomers of β-ribonucleosides [3], α-anomers of ribonucleosides [4, 5] and nucleosides in *syn* conformation [6], when present in oligoribonucleotides, distinctly inhibit their susceptibility to phosphodiesterase action.

The resistance imposed on phosphodiester bond by hypermodified nucleoside N^6-(*N*-threonylcarbonyl)adenosine (Compound I) resulted in the isolation of uridylyl(3′ — 5′)N^6-(*N*-threonylcarbonyl)adenosine (Compound

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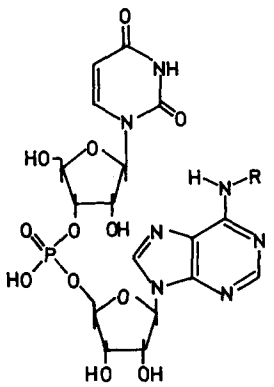
Abbreviations: Compound I, N^6-(*N*-threonylcarbonyl)adenosine; Compound II, uridylyl(3′ — 5′)N^6-(*N*-threonylcarbonyl)adenosine; Compound III, methyl ester of Compound II; Compound IV, *O*-benzyl ester of Compound II; UpA, uridylyl(3′ — 5′)adenosine (see Fig. 1).

II) [7] and its respective 5′-phosphate [8]. These compounds seem to be of significant importance as they occur in the same location of the anticodon section of a broad variety of tRNAs in both procaryotic and eucaryotic organisms [9,10].



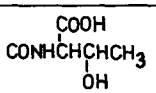
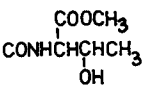
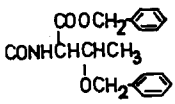
COMPOUND No.	SIDE CHAIN SUBSTITUENT R	ABBREVIATION
II		Upt^{6}A
III		Up(Met)^{6}A
IV		Up(Bzl$_2$t)^{6}A
V	H	UpA

Fig. 1. Uridylyl(3′ — 5′)N^6-(*N*-threonylcarbonyl)adenosine and its side chain modified analogs. Compounds II, III and IV were synthesized chemically, V enzymatically.

Opinions on the basic reasons for the inhibitory action of Compound I have been diverse so far.

Cunningham and Gray [8] suggested that the phosphodiester bond linking 5′-hydroxyl of Compound I to the 3′-hydroxyl of the adjacent nucleoside is exceptionally resistant to phosphodiesterase hydrolysis due to the presence of the bulky side chain in Compound I. Strikaitis and Schweizer [7] believed rather that the negatively charged carboxyl group had an important contribution to the resistance. This assumption has been based on the data of Richards and Laskowski [11] who observed in the case of unmodified oligonucleotides a distinct inhibition of phosphodiesterase action by a charged primary phosphate at the 3′ position.

It has been also found by Holy [12] in the case of uridylyl(3′ — 5′)5-carboxyuridine that the presence of a charged carboxyl group in this substrate results in an inactivity of snake venom phosphodiesterase.

In order to evaluate the contribution of the steric and charge factors to the above mentioned resistance of Compound II we investigated the rate of venom phosphodiesterase hydrolysis of Compound II and unmodified UpA together with two model derivatives of Compound II:

1. Uridylyl(3′ — 5′)N^6-(*N*-threonylcarbonyl)adenosine methyl ester (Compound III) had no negative charge and steric requirements of the side chain similar to those of I.
2. Uridylyl(3′ — 5′)N^6-[*N*-(*O*-benzyl)threonylcarbonyl] adenosine benzyl ester (Compound IV) also had no negative charge. The presence of two additional bulky groups increased the steric hindrance in comparison with that of Compound II.

Compound II was prepared by chemical synthesis [13]. 2′3′-*O*-Isopropylidene-N^6-[*N*-(*O*-benzyl)threonlycarbonyl] adenosine benzyl ester was condensed with 5′-*O*-monomethoxytrityl-2′-*O*-acetyluridine 3′-phosphate and the phosphate function of the dimer was protected with 2-cyanoethanol. Step by step deprotection in the following order: acid hydrolysis, alkaline hydrolysis. hydrogenolysis produced dinucleoside monophosphate identical in all respects with the compound isolated from tRNA [7]. Compound IV was the product of partial removal of the protection of completely blocked dinucleoside monophosphate [13].

Compound III was obtained analogously to Compound II starting with 2,′3′-*O*-isopropylidene-N^6-[*N*-(*O*-benzyl)threonylcarbonyl] adenosine methyl ester.

The homogeneity of Compound III was confirmed on paper chromatography in four solvent systems. R_F values were as follows: Whatman No. 1 paper, descending development R_F = 0.72 in isopropanol/conc. ammonia/water (7:1:2, v/v); 0.61 in ethanol/1 M ammonium acetate (7:3, v/v); 0.54 in *n*-butanol/acetic acid/water (5:3:2, v/v); 0.69 in isopropanol/1% $(NH_4)_2SO_4$ (2:1, v/v).

Digestion with pancreatic ribonuclease. 0.2 mg Compound III was dissolved in 20 μl water and 50 μl 0.13 M Tris buffer (pH 8) and ribonuclease (bovine pancreas, Reanal product, 1 mg/20 μl water) was added and the mixture incubated at 37°C. After 48 h, the substrate was completely digested. Paper chromatography in isopropanol/conc. ammonia/water (7:1:2, v/v) showed only spots moving like Compound I methyl ester (R_F 0.80) and uridine 3′-phosphate (R_F 0.18).

UpA was obtained by enzymatic synthesis.

Two sets of experiments were performed. In the first, the progress of snake venom hydrolysis of Compounds II—IV and UpA as a function of time was followed at pH 8. This is the highest pH value at which the ester groups were stable under the digestion conditions applied. In the second one, the pH dependence of hydrolysis was tested. The results are given in Figs. 2 and 3.

Fig. 2 demonstrates that, after 24 h, the hydrolysis of methyl ester II was almost complete (approx. 97%), whereas only approx. 28% unblocked Compound II was cleaved after this time. Therefore of two compounds with similar steric hindrance, the one bearing negative charge is distinctly more resistant to phosphodiesterase cleavage. The effect of the steric factor on the overall slowing down of the cleavage of the internucleotide bond in Compound II is also clearly pronounced. Unmodified dinucleoside monophosphate (UpA) needs only 3.5 h to be more than 95% digested.

Calculation of the ratios of hydrolysis rates at various stages of digestion for the pertinent pairs of dinucleoside monophosphates makes possible comparison of the importance of various influences on resistance (Table I).

It turns out that, although the effect of the negative charge prevails, its order is the same as that of the steric factor. Schweizer et al. [1] noted, that only a small dimunition of the rate of hydrolysis of the internucleotide bond of modified dinucleoside monophosphate, adenyl(3′—5′)N^6-(Δ^2-isopen-

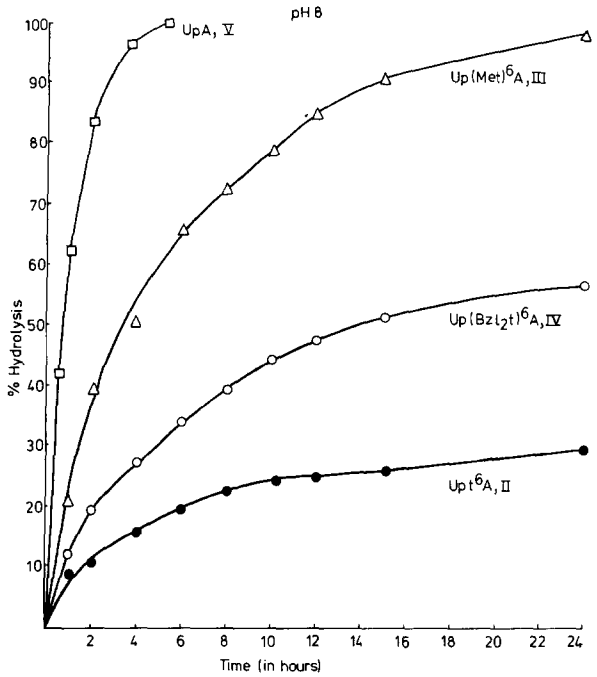


Fig. 2. Time course of hydrolysis by venom phosphodiesterase of Upt^{6}A (II, ●——●), its methyl ester (III, △——△) dibenzyl derivative (IV, ○——○) and unmodified UpA (V, □——□) at pH 8. The reaction mixtures were made up in 2 ml vials, at 0° C from 100 μl of 0.13 M succinic acid/Tris buffer, 40 μl water solution of compounds II—V containing approx. 1.04 μmol (0.60—0.96 mg) of the substrates, 10 μl of 0.13 M $MgCl_2$ and 100 μl of Worthington snake venom diesterase solution containing 46 μg of enzyme of potency 1.36 assayed according to [15]. The enzyme was pretreated to inactivate 5′-nucleotidase [16]. The mixtures were incubated for 24 h in a 37 ± 0.2° C water bath, returned to the ice bath, applied to silica gel plates (Merck 60 F_{254}, 5 cm X 10 cm X 0.25 mm) and developed in an *n*-butanol/water solution (86:14, v/v). The intensity of spots was measured on a Vitatron TLD 100 densitometer with a 254 nm filter. Per cent hydrolysis was calculated from the ratio of the amount of uridine formed to the sum of unhydrolyzed substrate and the respective 5′-phosphate.

TABLE I

THE FACTORS CONTRIBUTING TO THE RESISTANCE OF COMPOUND II TOWARDS SNAKE VENOM DIESTERASE

Calculated from the data given in Fig. 2 as the ratio of % hydrolysis of less resistant species to % hydrolysis of more resistant one.

Pair of dinucleoside monophosphates compared*	Kind of effect traced	Inhibition factor at various reaction stages after		
		1	2	4 h
UpA/Upt^{6}A	Summary — negative charge and steric	9.4	7.5	6.1
Up(Met)^{6}A/Upt^{6}A	Negative charge	3.5	3.4	3.1
UpA/Up(Met)^{6}A	Steric	2.7	2.2	1.9
Up(Met)^{6}A/Up(Bzl$_2$t)^{6}A	Additional steric	1.9	2.1	1.9

*For explanation of abbreviations see Fig. 1.

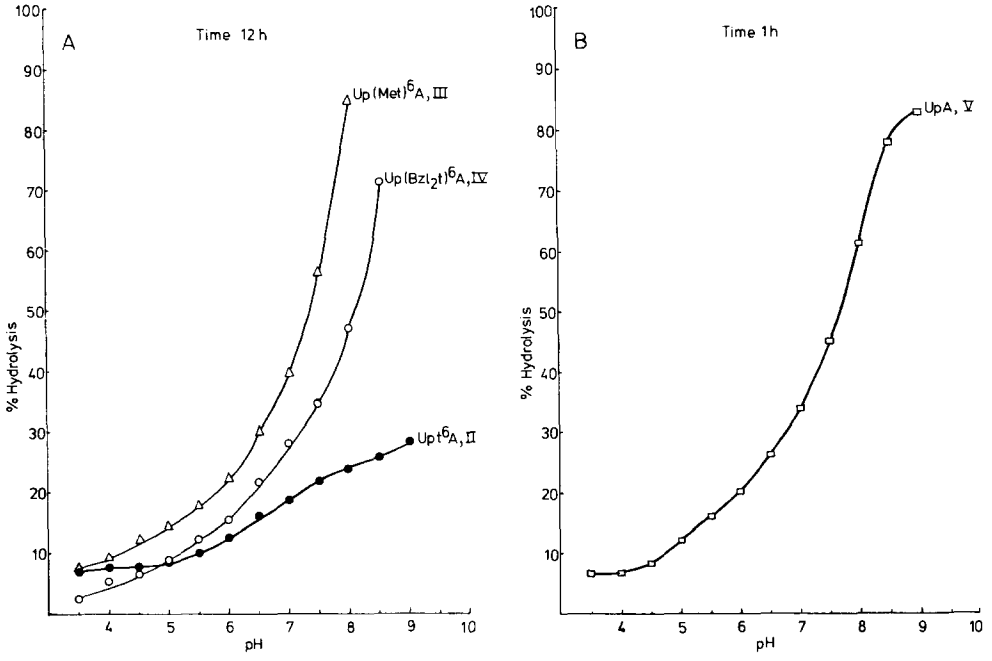


Fig. 3. Effect of pH on the hydrolysis by venom phosphodiesterase of: A. Upt^{6}A (II, ●——●) its methyl ester (III, △——△) and dibenzyl derivative (IV, ○——○). Reaction time 12 h. B. UpA (V, □——□). Reaction time 1 h. The procedure was essentially the same as described under Fig. 2. 0.13 M succinic acid and 0.13 M Tris base were mixed up to give buffers of pH 3.5—9 with 0.5 pH unit intervals. The amounts of reagents were half of these used in rate determination at pH 8.

tenyl)adenosine occurred, compared with unsubstituted ApA. The distinct inhibitory effect of the *N*-threonylcarbonyl substituent can be explained in terms of the rigid conformation of Compound I, due to the intramolecular bifurcated hydrogen bond of the hydrogen H(N-11) to nitrogen N-1 and oxygen O-14, similar to that found for solid state from X-ray diffraction data [16]. The isoprene side chain of N^6-(Δ^2-isopentenyl)adenosine may have a higher freedom of rotation and therefore be able to assure better access into the enzyme active site (Fig. 4).

The fact that the inhibiting factor of two additional bulky benzyl groups is not higher than that of Compound I side chain itself may also support the assumption of the rigid conformation of the latter.

The pH dependence data (Fig. 3) provide further information about the role of negative charge in the resistance of Upt^{6}A to phosphodiesterase cleavage. Response to pH during hydrolysis of esters III and IV is similar to that of unmodified dimer UpA, although its hydrolysis proceeds at a faster rate. The unblocked Compound II has a different pH dependence profile. At pH 3.5, it is cleaved as fast as its methyl ester and distinctly faster than the dibenzyl derivative. The steric effect prevailed at this pH value, because half of Compound II molecules were already in a nonionized form (p*K*a of carboxyl group of Compound I is 3.8 [17]). Unfortunately, pH regions lower than 3.5 are not accessible to this type of investigation because phosphodiesterase loses its activity almost completely.

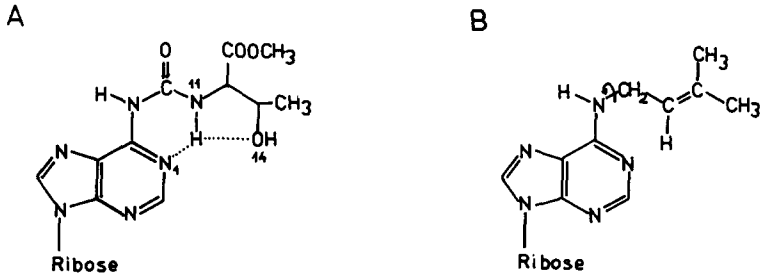


Fig. 4. Rigid conformation of N^6-(*N*-threonylcarbonyl)adenosine methyl ester (A) and flexible conformation of N^6-(Δ^2-isopentenyl)adenosine (B).

The observations presented above, together with the data of Richards and Laskowski [11], lead to the following, more general conclusion: The degree of resistance of oligonucleotide to snake venom phosphodiesterase induced by a single negative charge at the 3′ end of an oligonucleotide is of an order of magnitude comparable to that introduced by a bulky side chain of aglycon at this position.

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References

1 Schweizer, M.P., Thedford, R. and Slama, J. (1971) Biochim. Biophys. Acta 232, 217—226
2 Rogg, H., Wehrli, W. and Staehelin, M. (1971) Methods Enzymol. 20, 121
3 Holy, A. and Sorm, F. (1969) Coll. Czech. Chem. Commun. 34, 3383—3401
4 Holy, A. (1973) Coll. Czech. Chem. Commun. 38, 100—114
5 Sequin, U. (1974) Helv. Chim. Acta 57, 68—81
6 Ogilvie, K.K. and Hruska, F.K. (1976) Biochem. Biophys. Res. Commun. 68, 375—378
7 Strikaitis, M. and Schweizer, M.P. (1975) J. Carbohydr. Nucleosides, Nucleotides 2, 433—444
8 Cunningham, R.S. and Gray, M.W. (1974) Biochemistry 13, 543—553
9 Barrel, B.G. and Clark B.F.C. (1974) Handbook of Nucleic Acid Sequences, Joynson-Bruvvers, Ltd., Oxford
10 Barciszewski, J. and Rafalski, A.J. (1978) Atlas of Transfer Ribonucleic Acids and Modified Nucleosides, PWN, Warsaw, in the press
11 Richards, G.M. and Laskowski, Sr., M. (1969) Biochemistry 8, 1786—1795
12 Holy, A. (1972) Coll. Czech. Chem. Commun. 37, 1555—1576
13 Boryski, J. and Golankiewicz, B. (1978) Bull. Acad. Polon. Sci., Ser. Sci. Chim. 26, 21—31
14 Worthington Enzyme Manual (1972) Worthington Biochemical Corporation, p. 79, Freehold, N.J.
15 Sulkowski, E. and Laskowski, Sr., M. (1971) Biochim. Biophys. Acta 240, 443—447
16 Parthasarathy, R., Ohrt, J.M. and Chheda, G.B. (1974) Biochem. Biophys. Res. Commun. 60, 211—218
17 Adamiak, R.W. and Wiewiórowski, M. (1975) Bull. Acad. Polon. Sci., Ser. Sci. Chim. 23, 241—253