The action of a water-soluble carbodiimide on adenosine-5'-polyphosphates

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ABSTRACT

When aqueous solutions of adenosine-5'-mono-, di-, or triphosphates are treated with a water soluble carbodiimide the major product is the expected diadenosine-5'-5'-polyphosphate. The yields of these pyrophosphates are greatly increased in the presence of the Mg^{2+} ion. Adenosine-5'-tetraphosphate behaves differently. The major product is adenosine-5'-monophosphate. We believe that this hydrolysis occurs via a cyclic trimetaphosphate intermediate.

INTRODUCTION

When adenosine-5'-triphosphate is treated with dicyclohexylcarbodiimide in anhydrous pyridine, the β and γ phosphate groups are partially interchanged (1). It is believed that the intramolecular attack of the α -phosphate on the activated γ -phosphate gives an ester of the trimetaphosphate ion, which may then open by either of two equivalent paths (Figure 1).

We have used aqueous solutions of water-soluble carbodiimides as activating agents in a variety of reactions of nucleoside-5'-phosphates that involve the formation of phosphodiester (2), phosphoramidate (3) and pyrophosphate bonds (4). In this context, it seemed interesting to study the products formed when aqueous solutions of nucleoside-5'-oligophosphates are treated with a carbodiimide. Here we report on a number of these reactions, one of which appears to involve the elimination of three phosphate groups from adenosine-5'-tetraphosphate via a novel cyclic trimetaphosphate intermediate.

EXPERIMENTAL**Materials**

Adenosine-5'-mono-, di-, tri- and tetraphosphates (AMP, ADP, ATP and ATeP, respectively) (5) were obtained as sodium salts from Sigma Chemical Company, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (CDI) from JBL Scientific, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-

toluenesulfonate (morpho CDI) from Aldrich Chemical Co. Calf intestinal alkaline phosphatase and venom phosphodiesterase-I (*crotalus adamanteus*) were purchased from Boehringer Mannheim Biochemicals and Pharmacia P.-L. Biochemicals Inc., respectively.

Chromatography and Identification of Products

Paper chromatography was performed on Whatman 3MM paper by the descending technique. The following systems were used:

System I: 95% ethanol, 1 M ammonium acetate made up to 2×10^{-3} M in EDTA and brought to pH 5.0 with glacial acetic acid (7:3).

In order to get improved resolution of the various polyphosphates, we applied a solution of 0.25 M EDTA Na_4 to the origin of the chromatogram in the form of a band about 1 cm wide. The polyphosphate mixtures were applied to the dried band.

System II: n-propanol, concentrated ammonia and water (55:10:35).

System III: 0.03 M potassium phosphate (pH 7.1).

Paper electrophoresis was performed at 3000 volts using varsol as a coolant. Each UV absorbing spot was eluted from the chromatogram and the optical density was read at 259 nm against the eluate from the same area of a blank chromatogram. The yields are expressed as the percentage of the total absorbancy of all the material on the paper.

The identity of the reaction products was confirmed by digestion with alkaline phosphatase and venom phosphodiesterase-I. The adenosine-5'-oligophosphates were converted to adenosine on exhaustive degradation with

Figure 1: The mechanism proposed by Webb to explain the interchange of the β and γ phosphates of ATP that occurs in the presence of a carbodiimide (1).

alkaline phosphatase. Less complete degradation gave a series of compounds that co-chromatographed with the series of nucleoside 5'-oligophosphates shorter than the starting material.

The diadenosine-5'-5'-polyphosphates were unaffected by alkaline phosphatase, showing that they do not contain phosphomonoester or mono-esterified oligophosphate groups. Short digestions with phosphodiesterase-I gave approximately equal amounts of adenosine-5'-phosphate and a material which had the same mobility as the adenosine polyphosphate with a phosphate chain one shorter than that of the starting material (6). More extensive degradation with phosphodiesterase-I gave a series of products with the mobilities of shorter adenosine-5'-oligophosphates and, finally, adenosine-5'-monophosphate.

Enzyme Degradations

a) Calf intestinal alkaline phosphatase. An aliquot of the compound containing approximately 1 μ mole of adenosine was incubated at 37°C with 12U of

Table 1.

Chromatographic and electrophoretic mobilities. The R_f and R_m values of the various adenosine-5'-polyphosphates and adenosine-5',5'-polyphosphates are given relative to adenosine-5'-phosphate.

Compound	R_f in system		R_m in system
	I	II	III
AMP	1.00	1.00	1.00
ADP	0.57	0.90	1.17
ATP	0.34	0.89	1.01
ATeP	0.23	0.79	1.14
ADP-Urea	0.76	1.25	0.69
ADP-morpho Urea	0.82	1.25	0.79
p5A	0.14		
p7A	0.05		
Ap2A	0.53	1.14	
Ap3A	0.34	0.96	
Ap4A	0.21	0.82	
Ap5A	0.13	0.80	
Ap6A	0.09	0.71	
Ap8A	0.04	0.62	

Table 2.
Products from the reaction of CDI with adenosine-5'-polyphosphates.
For reaction conditions see text.

Nucleo- side	Time	Mg ²⁺	AMP	ADP- Urea	ADP	Ap2A	Ap3A	ATP	ATeP	Ap4A	Ap5A	Ap6A ^a	Ap8A ^b
AMP	2h	+	20.0			80.0							
		-	81.3			18.7							
	4h	+	15.1			84.7							
		-	80.6			19.4							
	6h	+	13.4			86.6							
		-	77.5			22.5							
	24h	+	12.3			87.6							
		-	75.9			24.1							
ADP	2h	+	-	9.2	33.1		5.2			52.5			
		-	9.8	8.6	52.2		2.2			27.2			
	4h	+	-	15.7	12.3		7.9			64.7			
		-	11.8	16.1	39.7		3.4			29.0			
	6h	+	-	17.3	7.5		9.9			65.3			
		-	12.7	19.0	31.1		4.0			33.2			
	24h	+	-	19.4	2.1		11.6			66.9			
		-	13.9	23.1	22.0		5.6			35.4			
	2h	+	-		3.5			4.0		2.9		89.6	
		-	-		21.1			45.9		7.1		25.8	
ATP	4h	+	-		2.5			2.3		4.7		90.5	
		-	1.8		22.5			41.1		6.7		27.9	
	6h	+	-		1.6			1.4		6.5		90.5	
		-	2.5		21.7			38.7		6.2		30.9	
	24h	+	-		-			-		9.5		90.5	
		-	2.8		21.9			36.9		6.1		32.3	
ATeP	2h	+	-		-			-	5.5		13.0		81.4
		-	38.2		3.9			20.3	17.5		7.9		12.2
	4h	+	-		-			-	-		17.1		82.9
		-	39.1		5.3			17.6	17.0		7.4		13.6
	6h	+	-		-			-	-		16.6		83.4
		-	39.9		5.0			15.3	16.1		8.3		14.4
	24h	+	-		-			-	-		15.6		84.4
		-	39.9		4.8			15.3	14.6		10.7		14.8

a) contained small amount of unresolved Ap5A

b) contained small amount of unresolved Ap7A

Figure 2: A scheme that accounts for all of the products formed by the action of a water-soluble carbodiimide on AMP, ADP, ATP and ATeP. a) $n = 0$; b) $n = 1$; c) $n = 2$; d) $n = 3$.

the enzyme in 20 μl of 0.05 M Tris buffer, pH 9.0. After 3 hr. incubation, an aliquot was withdrawn and examined by paper chromatography in System I.

b) Venom phosphodiesterase-I. Approximately 1 μmole of the compound was incubated at 37°C with 20 μl of 0.05 M Tris buffer, pH 9.0, and 0.2U of phosphodiesterase-I. Aliquots were removed after 5 min. in the case of Ap8A, 15 min. in the case of Ap5A or Ap6A, 30 min. in the case of Ap3A or Ap4A, and 50 min. in the case of Ap2A, and chromatographed directly in System I.

Preparation of samples

The reactions were carried out in Eppendorf tubes (1.5 mL). Aqueous solution containing ATeP, ATP, ADP or AMP (50 mM), Hepes (2 M), CDI (2.5 M) were thoroughly mixed with a vortex mixer and incubated at 37°C, pH 6.5. The total reaction volume was 100 μl . In another set of experiments the same concentrations of reagents were used and, in addition, 125 mM, 100 mM, 75 mM or 50 mM MgCl_2 for ATeP, ATP, ADP or AMP, respectively.

The pH was adjusted so that each mixture was at pH 6.5 at the reaction temperature. The pH of each mixture was measured at the beginning and the end of the experiment using a Microelectrodes, Inc. MI-410 micro combination pH probe and a Beckman Model 4500 digital pH meter. The pH was always found to remain unchanged throughout an experiment.

RESULTS AND DISCUSSION

The major products of the action of CDI on aqueous solutions of AMP, ADP and ATP are the anticipated diadenosine-5'-5'-polyphosphates, Ap2A, Ap4A and Ap6A respectively (Table 2). Side reactions lead to the hydrolysis of ATP to ADP and of ADP to AMP, and to the synthesis of Ap3A from ADP, and Ap4A or Ap5A from ATP. In addition, ADP yields a product with an R_f of 0.76 in System I. The major product from the reaction of ATeP is AMP. In addition ATeP gives some Ap8A, Ap5A and ATP. The yield of AMP obtained directly from ATeP can be increased to as much as 90% if the concentration of ATeP is decreased to 0.01 M and the concentration of CDI is 1.0 M. In the absence of CDI, the nucleoside-5'-polyphosphates are not significantly hydrolyzed in 24 hrs. under the same conditions.

The compound with an R_f of 0.76 formed by the action of CDI on ADP is unaffected by alkaline phosphatase but is degraded by venom phosphodiesterase-I to yield AMP. It seems plausible, therefore, that it is an adduct of CDI with the β phosphate of ADP. To test the hypothesis that an adduct is formed, we carried out a reaction using a different water-soluble

Figure 3: A proposed mechanism for the formation of AMP from ATeP in the presence of a water-soluble carbodiimide.

carbodiimide, morpho CDI, in place of CDI. We obtained a product with properties very similar to that previously obtained with CDI, but which could be resolved from it by paper chromatography or paper electrophoresis (Table 1). Analogy with the work of Khorana (7) suggests, but does not prove, that these compounds are N-phosphorylureas (I), formed by the rearrangement of the isoureas that result from the attack of ADP on the water-soluble carbodiimides.

I

The presence of divalent metal ions is known to facilitate reactions involving the nucleophilic attack of one phosphate anion on the activated derivative of another (8). It is not surprising, therefore, that the Mg^{2+} catalyzes the synthesis of diadenosine-5'-5'-pyrophosphates and leads to 80-90% yields in most cases (Table 2).

All of the observed products can be accounted for if we assume that the first product of the reaction is always the isourea formed by the attack of the terminal phosphate on the carbodiimide (Figure 2). The formation of pyrophosphates containing twice the number of phosphate groups present in the starting material must involve attack of an unactivated substrate on the activated substrate. Hydrolytic removal of a single phosphate group occurs by water attack on the penultimate phosphate. Attack on the terminal phosphate groups, which must be more extensive, would not be detected since it regenerates the starting material. Precedent for attack on the penultimate phosphate of a nucleoside-5'-polyphosphate is found in the work of Moffatt and his co-workers (9). The condensation of a molecule of the initial substrate with a molecule of its first hydrolysis product leads to the synthesis of Ap3A from ADP, etc.

The simplest explanation of the direct and efficient production of AMP from ATeP, is the participation of a cyclic covalent intermediate VII (Figure 3). This intermediate is analogous to that involved in the interchange of the β and γ phosphates of ATP (9) (Figure 1). It may reopen to give starting material, or eliminate inorganic trimetaphosphate to give AMP. The condensation of AMP with ATeP then leads to an appreciable yield of Ap5A.

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5. Abbreviations: AMP, ADP, ATP, and ATeP, p5A, p7A refer to the homologous series of adenosine-5'-mono-, di-, tri-, tetra-, penta-, and heptaphosphates. Ap2A, Ap3A, Ap4A, Ap5A, Ap6A and Ap8A refer to the homologous series of α, ω -di(adenosine-5')polyphosphates; e.g., Ap8A is p₁, p₈-di(adenosine-5')octaphosphate. ADP-Urea and ADP-morpho Urea refer to the adenosine-5'-diphosphoryl-(N-ethyl, N'-dimethylamino-propyl)-urea and adenosine-5'-diphosphoryl-[N-(2-morpholinoethyl), N'-cyclohexyl]-urea.
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