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UNUSUAL PATTERN OF NUCLEOSIDE POLYPHOSPHATE HYDROLYSIS BY THE ACID PHOSPHATASE (OPTIMUM pH = 2.5) OF *ESCHERICHIA COLI*

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SUMMARY : An acid phosphatase with an optimum pH of 2.5, was partially extracted by a single wash of whole cells of *E. coli* by 1 mM EDTA 50 mM Tris buffer pH 7.8. Its enrichment coefficient in this extract was about 100. Ribonucleoside polyphosphates were hydrolyzed by the enzyme at very different rates according both to the nature of the base and the position of the phosphate group. UTP and ppGpp were the most sensitive. The significance of these differences are briefly discussed.

INTRODUCTION

The existence in *Escherichia coli* K 12 of a phosphatase activity displaying a maximal efficiency at pH 2.5 has been reported by ROGERS and REITHEL (1) who have clearly separated this activity from a number of other acid phosphatases and determined a Km of 1.3 mM for the synthetic substrate paranitrophenylphosphate (PNPP).

According to these authors, most phosphate esters, including sugar monophosphates, phosphorylethanolamine, phosphoserine, 3' or 5' AMP, were hydrolyzed with an efficiency five to one hundred times lower than for PNPP. Similar conclusions were drawn by HAFKENSCHIED (2) who also observed that ATP and ADP were poor substrates compared to PNPP and concluded that the enzyme could not be an ATPase. Only were fructose 1.6 diphosphate and 2.3 diphosphoglyceric acid reported to be degraded with an efficiency approaching that of PNPP.

No data are presently available concerning the rate of dephosphorylation of the main ribonucleoside polyphosphates by this enzyme. We demonstrated here that, in addition to crude acellular extracts, a 1 mM EDTA wash of intact cells contained an acid phosphatase with optimum at pH 2.5 corresponding to that previously described by the authors mentioned above.

This activity was the cause of a rapid breakdown of UTP, GTP and ppGpp in 1 M formic acid supernatants of crude extracts. The efficiency of the enzyme on ATP, CTP, or all nucleoside diphosphates, was much lower, while nucleoside monophosphates remained practically unaffected. Particular attention was given to the mode of attack of the regulatory nucleotide

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ppGpp and the structure of the products sequentially released has been established. The significance of such differences in susceptibility among nucleoside polyphosphates is briefly discussed.

MATERIAL AND METHODS

The enzymatic preparation used throughout this study was a hundred fold purified fraction obtained by a single wash of whole cells of *E. coli* strain SBS 10 270 (F⁻met,arg A) by 50 mM Tris HCl buffer (pH 7.8) containing 1 mM EDTA. This fraction was extensively dialyzed in 50 mM Tris HCl buffer pH 7.8 and concentrated a hundred fold through an UM_{10} Amicon membrane. The assays were performed at 37°C in a volume of 50 µl containing 10 µl of enzymatic extract (120 µg of proteins) and made 0.2 mM in the nucleotide studied and 0.3 M in formic acid (final pH 2.5). The radioactive nucleotides used, labelled with ^{32}P in the 5' α position, were obtained from the Radiochemical Centre, Amersham. ppGpp was prepared *in vitro*, according to a procedure described by BLOCK and HASELTINE (3, 4). Ribosomes were extracted from cultures of strain CR 341 (thr, leu, met, thy A, thi, lacy) grown in ML Broth containing 250 µg/ml of thymine, and harvested in mid-exponential phase. ppGpp was labelled in three different positions : 8 (^{14}C), 5' ($\alpha^{32}P$) and 3' ($\beta^{32}P$) using respectively 8 (^{14}C) GTP, 5' ($\alpha^{32}P$) GTP as acceptor of pyrophosphate and ($\gamma^{32}P$) ATP as donor in the third case (5). The labelled ppGpp was purified on polyethylene-imine cellulose plates (Macherey Nagel MN 300 PEI) developed with 1.5 M LiCl : 2 M HCOOH. It was eluted with 2.5 M LiCl : 1 M HCOOH and precipitated from the concentrated eluate with absolute ethanol at 0°C in presence of carrier ppGpp. The chromatographic analysis of nucleotides and of degradation products was made on PEI cellulose plates developed with 0.5 M or 1.5 M KH_2PO_4. Two microliter samples were rapidly spotted on the chromatogram and immediately dried at room temperature. The radioactive spots, located by autoradiography, were cut and counted by liquid scintillation. The unknown products obtained from ppGpp were resolved in a two dimensional chromatographic system with 1.5 M LiCl:2 M HCOOH in the first dimension and 1.5 M KH_2PO_4 in the second dimension. The two isogenic spoT and spoT⁺ strains used, were selected among pyr E⁺ transductants of strain Gy. 2812 (spoT, pyr E) kindly supplied by Dr. DEVORET (Gif-sur-Yvette). The genotype of SBS 10 518 was thi_1 arg G_6 thy A_{37} rbs_1 mal A_1 rps L_8 rps L_{17} λ^R λ^- glt S_7 gad S_1 gad R_2 spoT and that of SBS 10 519 was the same except spoT. The pyr E donor was strain SBS 10 110, a galU derivatives of strain CR 341 (6).

RESULTS

The presence in acellular extracts of *E. coli* K 12 of a heat labile, nondialysable, SDS sensitive and ammonium sulphate precipitable activity, able to catalyze the breakdown of certain nucleotides at 0°C and in 1 M formic acid (pH 2.0), was noticed in the course of experiments on guanosine nucleotide metabolism. Moreover, the degradation of labelled GTP could barely be prevented by the addition of a five fold excess of ATP (data not shown). This difference in hydrolytic efficiency led us to investigate the susceptibility of various nucleoside polyphosphates as substrates for this enzymatic activity and to compare them to that of paranitrophenyl

TABLE I - Enrichment in acid phosphatase activity of some acellular fractions -

Fractions	Total activity (nmole PNPP hydrolyzed) per minute	Activity %	Enzymatic specific activity (nmole PNPP hydrolyzed per minute per mg of protein)
- Crude	290	100	7.3
- 1 M formic acid supernatant of the crude sonicate	288	99.3	86
- 1 mM EDTA wash of whole cells	35	12	690

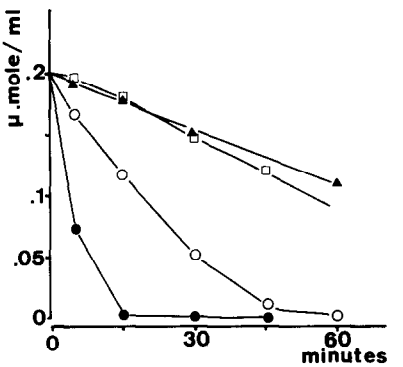

Figure 1 : Comparaison of the susceptibility of the four ribonucleoside triphosphates to hydrolysis by the acid phosphatase.
The initial concentration of nucleotides was 0.2 mM. The reaction was made at 37°C and at pH 2.5 as described in Material and Methods. The symbols are (—▲—) ATP ; (—□—) CTP ; (—○—) GTP and (—●—) UTP.

phosphate (PNPP), apparently the most efficient substrate described in the literature for the only acid phosphatase able to function at this very low pH (1, 2).

Primitively and in order to avoid the inconvenient of spotting on chromatograms samples containing too many proteins, 1 M formic acid supernatants of crude extracts were used as source of enzyme. A better enrichment in activity, however, was obtained in one-step by washing whole cells by Tris HCl buffer pH 7.8 containing 1 mM EDTA. As seen in Table I, an appreciable fraction of the total enzyme was extracted with the EDTA, which displayed a specific activity about 100 times higher than in the crude extract. This EDTA extract was used in all experiments reported here. The complete purification and the characterization of this acid phosphomonoesterase have now been achieved, and will be reported in details elsewhere (*).

Among the four ribonucleoside triphosphate, UTP was found to be the most rapidly degraded by the acid phosphatase of *E. coli* at pH 2.5. CTP and ATP were hydrolyzed at the same slow rate and GTP had an intermediate sensitivity (figure 1). This hydrolysis of nucleoside triphosphate yielded the corresponding nucleoside diphosphate and in some cases the reaction proceeded to the stage of monophosphate. Within each base family however, the γ phosphate residue was removed much more efficiently than the β phosphate of the corresponding diphosphonucleoside (figure 2). This behaviour which was very different from that of alkaline phosphatase for ins-

(*) E. DASSA and P.L. BOQUET, submitted for publication.

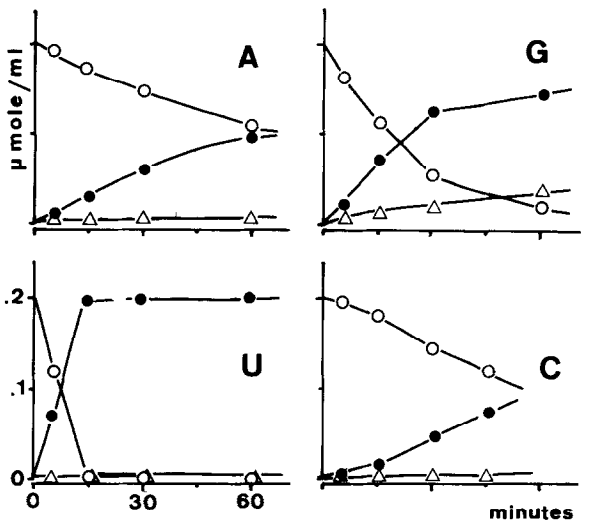

Figure 2 : Analysis of the products of [α^{32}P] labelled nucleoside triphosphates degradation.

The conditions are identical to those of figure 1. A, G, U and C refer to the corresponding nucleoside family. Open circles are for nucleoside triphosphates, closed circles are for nucleoside diphosphates and triangles for nucleoside monophosphates.

Ordinate and abscissa are the same in all graphs.

tance (7) was not due to the inhibitory effect of the free phosphate released. Indeed, the degradation of nucleoside diphosphates proceeded at least ten times more slowly than for the corresponding triphosphates and, under the same conditions, the hydrolysis of nucleoside monophosphates could not be detected (figure 3a). A comparison of the initial velocities of nucleoside hydrolysis with that of the synthetic substrate PNPP at the same concentration is given in Table II. The rate of hydrolysis of the β phosphate was apparently not related to that of the γ phosphate in the homologous triphosphate as shown with UTP.

Among different guanosine nucleoside polyphosphates tested, the regulatory compound ppGpp was more susceptible than GDP and even more than GTP (figure 3b). The reaction yielded sequentially two products (refered to as A and B) which migrated respectively in the chromatographic system used as a triphosphorylated and a diphosphorylated guanosine. They were shown to be different from GTP and GDP in a two dimensional system (figure 4).

The structure of these compounds was determined by submitting double labelled ppGpp to the action of the phosphatase. The degradation products A and B released from a mixture of 8 (^{14}C) ppGpp and 5' (α^{32}P)ppGpp

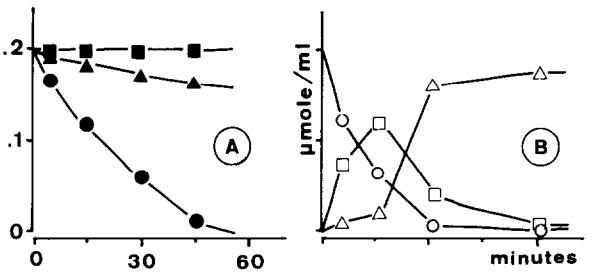

Figure 3 : Compared sensitivity of different guanosine nucleoside polyphosphates.

a) $\alpha^{32}P$ labelled GTP (—●—), GDP (—▲—) and GMP (—■—) were submitted to the action of the phosphatase under conditions identical to those of figure 1 and 2.

b) $\alpha^{32}P$ labelled ppGpp (see Material and Methods) at an initial concentration of 0.2 mM was degraded with the enzyme in the same conditions as in figure 1, 2 and 3a (—○—), and the concentration of the degradation products A (—□—) and B (—△—) was measured.

Ordinate and abscissa are the same in both graphs.

TABLE II - Initial velocities for the hydrolysis of nucleoside polyphosphates and PNPP at 0.2 mM. The amount of enzyme in the assays was the same as in all experiments (The letters in parentheses refer to the position of the phosphate group hydrolyzed).

Substrate	Initial rate nmole/min/ml	Relative rate PNPP = 1.00
PNPP	14	1.00
ATP (γ)	2.2	0.16
ADP (β)	< 0.1	< 0.10
AMP	< 0.01	-
GTP (γ)	6.7	0.48
GDP (β)	0.66	0.05
GMP	0.01	-
CTP (γ)	2.17	0.15
CDP (β)	< 0.01	-
CMP	< 0.01	-
UTP (γ)	25.0	1.76
UDP (β)	< 0.01	-
UMP	< 0.01	-
ppGpp (5'β)	16.7	1.17
pGpp (3'β)	5.0	0.35
pGp	< 0.01	-

displayed a constant $^{14}C/^{32}P$ ratio, indicating that they both corresponded to guanosine 5' α phosphate derivatives. In experiments with a mixture of 3' $(\beta^{32}P)$ ppGpp and 8 (^{14}C) ppGpp the 3' β phosphate residue was lost in compound B (Table III). Since A and B were released sequentially, their

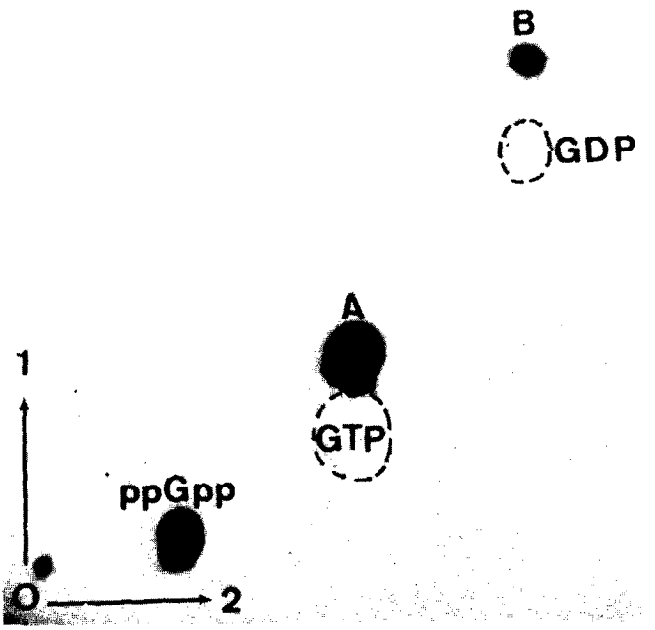

Figure 4 : Two dimension chromatography analysis of the degradation products A and B obtained from [α^{32}P] labelled ppGpp.
The conditions of the reaction were those of figure 3b. The sample was withdrawn 15 minutes after the beginning of the reaction. Cold nucleotides were co-chromatographed as references.

TABLE III - Results of the chromatographic analysis of the degradation products obtained from double-labelled ppGpp.

Rf	8 (^{14}C) ppGpp + 5' (α^{32}P) ppGpp		Products	8 (^{14}C) ppGpp + 3' (β^{32}P) ppGpp	
	^{14}C / ^{32}P	Interpretation		^{14}C / ^{32}P	Interpretation
0.79	-	-	PO_4^{--}	0.2	^{32}P alone
0.45	1.3	1	B	5.8	^{14}C alone
0.29	0.8	1	A	1.2	1
0.17	1.0	1	ppGpp	1.0	1

structure was very likely pGpp and pGp respectively. This was confirmed by an analysis of the labelled products released from A and B in presence of alkaline phosphatase and of snake venom phosphodiesterase (data not shown).

TABLE IV - Repartition of the acid phosphatase among some fractions obtained from isogenic spoT / spoT$^+$ strains.

	Enzymatic specific activity (nmoles PNPP hydrolyzed per minute per mg of protein)	
	SBS 10 519 (SpoT)$^+$	SBS 10 518 (SpoT)
- Crude sonicate	7.59	5.43
- 1 M formic acid supernatant of the crude extract	88.7	99.7
- Formic acid pellet of the crude extract	6.29	1.16
- 1 mM EDTA wash	693	332

The acid phosphatase, however, was apparently not involved in the spoT gene product mediated ppGpp degradation : the total amount of acid phosphatase was identical in wild type spoT$^+$ and in spoT isogenic strains of *E. coli* (Table IV).

DISCUSSION

The existence, in the periplasmic space or at least at the periphery of the cell,(as suggested by the extraction procedure) of a phosphatase with an optimal pH of 2.5 was puzzling and raised the question of the possible presence in the neighbourhood of the cell envelope of a microenvironment where such pH conditions might be satisfied.

HAFKENSCHIED (2) stated that among the sugar phosphates, alcohol phosphates and adenosine nucleotides tested, none of them exceed the efficiency of PNPP. The present results indicate that ppGpp was degraded as rapidly as PNPP and that UTP was a better substrate.

Contrary to the alkaline phosphatase which sequentially hydrolyzed all the phosphate residues of nucleotides (7) the acid phosphatase displayed considerable differences according to the position of these residues. This result may either indicate that energy rich phosphate bonds were cleaved preferentially or that the nucleoside molecule caused a steric inhibition in its immediate vicinity.

The great difference between the rate of hydrolysis of paranitrophenylphosphate and that of all nucleoside monophosphates or of sugar mono-

phosphates (1, 2) apparently favors the second hypothesis if one considers the small size of the nitrophenyl residue. The difference in efficiency between the hydrolysis of ATP (or CTP) and that of UTP (or GTP), however, seems difficult to explain only in terms of free access to the γ phosphate group.

The increase in the sensitivity of splitting of the 5' β bond in the regulatory molecule ppGpp, compared to that of GDP, and the sequential attack of the enzyme in the 5' and in the 3' position may help to evaluate the contribution of both the nucleotide moiety and the energetical state of phosphate residues.

Although this mode of ppGpp hydrolysis was shown to be unrelated to the spoT mediated pathway of degradation (see Table IV), the possibility that the acid phosphatase might play a role in ppGpp metabolism could not be completely ruled out.

In conclusion, it appears that in addition to the physiological problems evoked by this unusual optimal pH, this enzyme may serve as an interesting analytical tool for the study of nucleoside polyphosphate structure.

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