DEGRADATION OF PYRIMIDINE NUCLEOTIDES BY ENZYME SYSTEMS OF *STREPTOMYCES*

II. PYRIMIDINE 5'-NUCLEOTIDE PHOSPHORIBO (DEOXYRIBO) HYDROLASE OF *STREPTOMYCES VIRGINIAE*

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A novel enzyme, pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase, which hydrolyzes the N-glycosidic linkage in pyrimidine 5'-nucleotides to pyrimidine bases and pentose-5-phosphates, was extracted and purified to about 31-fold from the cells of *Streptomyces virginiae* and its properties were examined.

The purified enzyme hydrolyzed 5'-UMP, 5'-CMP, 5'-deoxyUMP, 5'-deoxyTMP and 5'-deoxyCMP. Purine 5'-nucleotides were degraded by the crude extracts, but this activity was removed by the purification. Nucleosides and 3'-nucleotides of purines and pyrimidines were never cleaved even by the crude extracts. 2'-O-methyl-5'-UMP was also resistant to the enzyme reaction.

The K_m value for 5'-UMP was 6.95×10^{-3} M. Co-factors of low molecular weight were unnecessary for the reaction. Phosphate, pyrophosphate and ATP had little stimulatory effect on the reaction. The enzyme was relatively heat stable between pH 5.5 and 8.8. The activity remained unaffected after heating the enzyme solution at 65° for 45 min. Ca^{++} had a marked stabilizing effect on the enzyme.

This report is concerned with partial purification and properties of a pyrimidine 5'-nucleotide-degrading enzyme from *Streptomyces virginiae* which shall be called pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase.

Distribution of the enzyme, products of the reaction and some conditions of 5'-UMP degradation have been reported in the preceding paper (1).

MATERIALS AND METHODS

Reagents. Ribose-1-phosphate was prepared from uridine by uridine phosphorylase of *Pseudomonas putrefaciens* IFO 3910 and its cyclohexylamine salt was obtained according to the method of PLESNER and KLENOW (2). 5-Phosphoribosyl-1-pyrophosphate was purchased from Sigma Chemical Company.

Ba-salt of ribose-5-phosphate was obtained from Nutritional Biochemicals Corp. 2'-O-methyl-5'-UMP was supplied by Dr. Honjo of our laboratories. Sources of other reagents have been reported elsewhere (3).

Enzyme extraction. *Streptomyces virginiae* IFO 3729 was cultured and crude extracts were prepared as described previously (1).

EDTA-treatment. The crude extracts were mixed with EDTA of a final concentration of 5 mM in a dialyzing tube. The mixture was stood for 10 min at 4°, then the tube was dialyzed against running desalted water for 2 hr at 24° followed by dialysis against several changes of distilled water at 4° for 18 hr. This dialyzed solution was designated as "EDTA-treated enzyme".

Assay of enzyme activity. The assay is based on an increase of molecular extinction coefficient at 290 m μ in an alkaline pH upon conversion of 5'-UMP to uracil (1). The test system contained 20 μ moles of 5'-UMP, 100 μ moles of acetate buffer (pH 6.0) and 2.5 to 12.5 units of the enzyme in a total volume of 1.0 ml. The mixture was incubated at 55° for 1 hr and the reaction was stopped by heating the mixture in a boiling water bath for 5 min. This solution was diluted in 0.2 N NaOH and the optical density at 290 m μ was measured with a Beckman DU spectrophotometer. The blank tests were run without 5'-UMP, which was added after the reaction was stopped.

A unit of the enzyme was defined as the amount causing the formation of 1 μ mole uracil in 1 hr. The specific activity was defined as units of enzyme per mg of protein.

Under the conditions of the assay, the rate of uracil formation was proportional to the amount of enzyme.

Other determinations. Pentose was estimated by the orcinol reaction (4) using ribose-5-phosphate as standard. Reducing sugar was determined by Somogyi's method (5) using D-ribose and 2-deoxy-D-ribose as standards. Protein and phosphate were estimated as previously described (6). Formation of cytosine from 5'-CMP was determined by a decrease (4.50×10^3) of molecular extinction coefficient at 250 m μ in 0.2 N NaOH.

Paper electrophoresis of nucleic acid components were carried out as described previously (1).

RESULTS

Purification of enzyme

Cells of *Streptomyces virginiae* were harvested from 1,630 ml of 4-day culture broth and suspended in 320 ml of distilled water and sonicated at 10 kc. for 10 min. The sonicate was centrifuged at $37,000 \times g$ for 1 hr. To 300 ml of the supernatant fluid, 6 ml of 100 mM CaCl₂ and 6 ml of 1 M acetate buffer (pH 6.0) were added. Then the mixture was heated at 55° for 1 hr and centrifuged at $27,000 \times g$ for 1 hr. Twenty ml of 10% solution of streptomycin sulfate was added to 300 ml of the supernatant fluid. Massive precipi-

Table 1. Summary of the purification of the enzyme.

Fraction	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units per mg protein)	Total activity (units)	Recovery (%)
Crude extracts	300	5.75	39.0	6.78	11 700	100.0
Heat treated	310	4.55	35.2	7.74	10 900	93.1
Streptomycin treated	330	—	28.8	—	9 500	81.2
Ammonium sulfate fraction	11.1	22.0	908.0	41.3	10 080	86.1
Sephadex fraction	20.0	0.7	147.0	210.0	2 940	25.1

Fig. 1. Time course of 5'-UMP degradation.

The assay system described in text containing 11.9 units of purified enzyme was incubated at 55°.

- Formation of uracil —△— Formation of pentose
 —▽— Formation of reducing sugar —○— Formation of inorganic phosphate

Fig. 2. Effect of pH on the degradation of pyrimidine 5'-nucleotides.

One ml of the reaction mixture containing 500 μmoles of acetate buffer (—●—▲—) or Tris buffer (—○—△—), 1 μmole of CaCl_2 , 10 μmoles of 5'-UMP, 5'-CMP, 5'-deoxyUMP or 5'-deoxyCMP and 2.9 units of the purified enzyme (0.014 mg as protein) was incubated at 55° for 1 hr.

(A) Pyrimidine 5'-ribonucleotides

—●—○— 5'-UMP —▲—△— 5'-CMP

(B) Pyrimidine 5'-deoxyribonucleotides

—●—○— 5'-deoxyUMP —▲—△— 5'-deoxyCMP

Fig. 3. Effect of substrate concentration on the rate of degradation.

One ml of the reaction mixture containing 200 μmoles of acetate buffer (pH 6.0), 1 μmole of CaCl_2 , 11.7 units of purified enzyme (0.08 mg as protein) and 5'-UMP as indicated was incubated at 55° for 30 min.

Table 2. Effect of phosphate, pyrophosphate and ATP.

One ml of the reaction mixture containing 20 μ moles of 5'-UMP, 100 μ moles of acetate buffer (pH 6.0), 1 μ mole of CaCl_2 , additions as indicated and 5.78 units of enzyme (0.04 mg as protein) was incubated for 1 hr at 55°.

Addition	Concn. (mM)	Uracil formed (μ moles per ml)
None		6.50
KH_2PO_4	0.1	6.68
	0.5	6.75
	1.0	6.55
	2.5	6.80
	5.0	6.50
Na-pyrophosphate	0.1	6.75
	0.5	7.12
	1.0	6.80
	2.5	3.85
	5.0	0.32
ATP	0.1	6.50
	0.5	6.80
	1.0	6.50
	2.5	6.75
	5.0	6.68

tates formed were centrifuged off and ammonium sulfate fractionation was carried out with the supernatant solution. The precipitates formed between ammonium sulfate saturation of 35 and 55% were collected and dissolved in 10.1 ml of 20 mM acetate buffer (pH 6.0). Then this solution was loaded on a 2×42 cm column of Sephadex G-100 and the column was washed with 20 mM acetate buffer (pH 6.0). The enzyme was eluted following the main peak of protein. The filtered fractions with high specific activity were pooled and designated as "purified enzyme" (Table 1).

5'-UMP degradation by purified enzyme

Fig. 1 illustrates the time course of the degradation of 5'-UMP by the purified enzyme. The formation of uracil, ribose-5-phosphate and reducing sugar corresponded in amount to each other. The formation of inorganic phosphate was negligible.

Effect of pH

Optimum pH for the degradation of every pyrimidine 5'-nucleotide was about 7 (Fig. 2). However, the shapes of the pH-activity curves were not exactly the same. 5'-CMP was degraded in a broader pH range as compared with other nucleotides.

Table 3. Effect of various enzyme inhibitors and heavy metal ions.

One ml of the reaction mixture containing 20 μ moles of 5'-UMP, 100 μ moles of acetate buffer (pH 6.0), additions as indicated and 5.2 units of enzyme (0.6 mg as protein) was incubated at 55° for 80 min.

Addition	Concentration	
	1 mM	10 mM
	per cent inhibition	
None	0	0
NaF	-1.2	-0.8
<i>p</i> -chloromercuribenzoate	7.5	95.1
NaN ₃	-6.5	1.6
KCN	5.9	65.9
Na ₂ HAsO ₄	2.5	10.0
Na ₂ HPO ₄	-0.5	15.6
CH ₃ COOAg	89.5	100.0
HgCl ₂	39.4	92.7
CuCl ₂	83.6	94.2
ZnCl ₂	92.7	100.0
CdCl ₂	84.3	100.0
CrCl ₃	-1.2	45.1
EDTA	92.9	94.8

Effect of substrate concentration

The K_m value for 5'-UMP calculated from LINEWEAVER and BURK plot (7) was about 6.95×10^{-3} M (Fig. 3).

Effect of phosphates

Even by the use of "purified enzyme" preparation, the reaction proceeded without the addition of any co-factors. The activity was also unaffected by the removal of low molecular weight compounds by dialysis.

Addition of phosphate, pyrophosphate and ATP to the reaction mixture did not show any appreciable stimulation (Table 2). Pyrophosphate was inhibitory at high concentrations.

Stability of enzyme

The purified enzyme was stable at low temperatures at pH 6.0. After 1 week at 5° or 2 months at -4°, no loss of activity was noted. The activity in the crude extracts remained unchanged in buffers between pH 5.5 and 8.8 at 65° for 1 hr, and even at $75^\circ \pm 3^\circ$ the loss of the activity was within 10%. However, the activity was completely lost within 2 min in a boiling water bath. The activity was unstable at pH 4.0 above 37°.

Table 4. Recovery of EDTA-inhibition by metal ions.

One ml of the reaction mixture containing 20 μ moles of 5'-UMP, 100 μ moles of acetate buffer (pH 6.0), 5 μ moles of metal salts as indicated and the EDTA-treated enzyme (0.38 mg as protein) was incubated at 55° for 80 min.

Addition	Uracil formed (μ moles per ml)
None ^a	5.61 ^a
None	0.42
KCl	0.53
MgCl ₂	2.67
CaCl ₂	3.87
BaCl ₂	3.11
AlCl ₃	2.63
SnCl ₂	2.82
MnCl ₂	2.23
FeCl ₂	1.91
FeCl ₃	1.89
CoCl ₂	1.08
NiCl ₂	0.69
CuCl ₂	0.04
ZnSO ₄	0.11

^a The reaction was carried out using the enzyme without EDTA treatment.

Fig. 4. The effect of Ca⁺⁺, Mg⁺⁺ and Ba⁺⁺ at various concentrations.

One ml of the reaction mixture containing 20 μ moles of 5'-UMP, 100 μ moles of acetate buffer (pH 6.0) and metal ions as indicated and 0.38 mg as protein of the EDTA-treated enzyme was incubated for 90 min at 55°.

Fig. 5. Effect of Ca⁺⁺ on the stability of enzyme.

The EDTA-treated enzyme was incubated with and without added 0.5 mM CaCl₂ at (A) 22° and (B) 65° for 45 minutes in 10 mM buffers of varying pH. Buffers used were acetate buffer (below pH 6.0) and Tris acetate buffer (above pH 6.6). The remaining activity was examined in the assay system containing 5mM CaCl₂.

—○— with CaCl₂

—●— without CaCl₂

Effect of some enzyme inhibitors and metal ions

The presence of SH-residue in the enzyme is suggested by the inhibition of *p*-chloromercuribenzoate and the requirement of metal ions is supposed by the inhibition of EDTA and KCN. Some heavy metal ions were inhibitory for the degradation. (Table 3).

To find out the required metal ions, the reaction was carried out using the EDTA-treated enzyme with and without the presence of metal ions. As indicated in Table 4, the activity lost during the EDTA-treatment was restored by a variety of metal ions, such as Mg⁺⁺, Ca⁺⁺, Ba⁺⁺, Al⁺⁺⁺, Sn⁺⁺, Mn⁺⁺, Fe⁺⁺, Fe⁺⁺⁺ and Co⁺⁺. Then the effect of Mg⁺⁺, Ca⁺⁺ and Ba⁺⁺ was examined at various concentrations (Fig. 4). Of these three metal ions, Ca⁺⁺ was found to be most effective in recovering the activity, especially at very low concentrations.

Fig. 6. Effect of Ca^{++} on the reaction at various temperatures.

See text for the reaction system. Amount of enzyme used was 6.25 units (corresponding to 0.75 mg protein) per ml of the reaction mixture.

--○-- 1 hr incubation —○— 2 hr incubation
 ○ with 1 mM CaCl_2 ● without CaCl_2

Effect of Ca^{++}

The thermostability of the EDTA-treated enzyme was tested with and without the addition of Ca^{++} (Fig. 5). In the absence of Ca^{++} , the activity was significantly lost even at 22° at pH range lower and higher than 6.0 and 6.5. However, the enzyme became very stable with added Ca^{++} , especially at pH 8.0. At this pH, the activity remained unchanged by the treatment at 65° for 45 min. Moreover, it is noteworthy that the activity was increased by the exposure of the EDTA-treated enzyme to Ca^{++} at 22°.

On the other hand, the effect of Ca^{++} on the reaction rate of the EDTA-treated enzyme was examined. As depicted in Fig. 6, the rate of the reaction was unaffected by Ca^{++} at temperatures below 47°. The reaction proceeded even at such high temperature as 75° when Ca^{++} was added.

These data suggest that Ca^{++} takes part in the stability of the enzyme activity rather than in the reaction itself, though the latter possibility has not entirely been excluded.

Substrate specificity

The substrate specificity was studied with three enzyme preparations: (A) crude extracts of 1-day culture cells, (B) crude extracts of 4-day culture cells and (C) purified enzyme from (B). The rate of the hydrolysis of the N-glycosidic linkage was determined by the appearance of reducing activity.

Table 5. The substrate specificity of the enzyme.

Various nucleotides and nucleosides were incubated with (A) enzyme extracted from 1-day culture cells, (B) crude extracts shown in Table 1 and with (C) purified enzyme. Five units of the enzyme was put in 1 ml of the reaction mixture and incubated at 55° for 2 hr.

The rate of the reaction was estimated spectrophotometrically (S) or by the appearance of reducing activity (R). Relative rates based on 5'-UMP are also indicated.

Substrate	Concn. tested (μ moles per ml)	Assay	Enzyme preparation					
			A		B		C	
			Hydrolysis (μ moles per ml)	Relative rate	Hydrolysis (μ moles per ml)	Relative rate	Hydrolysis (μ moles per ml)	Relative rate
5'-UMP	16.8	S	8.48		8.74		9.11	
		R	8.60	100	8.56	100	9.56	100
5'-CMP	16.8	S	5.36		6.63		7.76	
		R	5.25	73	6.21	73	7.36	77
5'-deoxyUMP	17.9	R	6.99	81	6.80	79	7.11	74
5'-deoxyCMP	16.6	R	4.97	58	4.84	57	6.06	64
5'-deoxyTMP	14.9	R	6.52	76	6.25	73	7.06	74
5'-AMP	16.8	R	— ^b	—	0.06	—	—	—
5'-GMP	16.0	R	1.00	12	1.59	19	0	0
5'-IMP	16.0	R	0.75	9	0.70	8	0	0
5'-XMP	18.4	R	—	—	1.59	—	—	—
5'-deoxyAMP	17.3	R	—	—	1.01	—	—	—
5'-deoxyGMP	16.6	R	—	—	1.70	—	—	—
3'-UMP	20.0	S·R	—	—	0	0	0	0
UDP ^a	16.0	S	—	—	—	—	0.5 ^a	0
UTP	16.0	S	—	—	—	—	0	0
Uridine	16.0	S·R	—	—	0	—	0	—
2'-O-methyl-5'-UMP	5.0	S·R	—	—	0	—	—	—

^a Small amount of 5'-UMP was contaminated in the UDP preparation.

^b Not tested.

For 5'-UMP and 5'-CMP, the assay was also performed by spectrophotometric method.

As indicated in Table 5, pyrimidine 5'-nucleotides were strongly degraded. Although purine 5'-nucleotides were hydrolyzed by the crude extracts, they were not hydrolyzed by the purified enzyme. This finding suggests the presence of another enzyme which specifically attacks purine 5'-nucleotides.

By paper electrophoresis, the degradation of both purine and pyrimidine 5'-nucleotides was found to result in the formation of their corresponding bases.

In any case, the formation of nucleosides was never detected.

Uridine and 3'-UMP, as well as nucleosides and 3'-nucleotides of other bases, were completely resistant to the reaction. When 2'-hydroxy residue of 5'-UMP was methylated, i.e. 2'-O-methyl-5'-UMP, the compound became inert as substrate. UDP and UTP were not degraded.

The substrate specificity of the enzyme extracted from *Streptomyces hygroscopicus* was analogous to that of the enzyme from *Streptomyces virginiae*.

Examination of reverse reaction

The reaction mixture containing 2 μ moles of uracil, 10 μ moles of ribose-5-phosphate, 100 μ moles of acetate buffer (pH 6.0) or Tris buffer (pH 8.0), 1 μ mole of CaCl_2 , 2 μ moles of MgCl_2 and 24 units of the purified enzyme in a total volume of 1 ml was incubated at 37°. The reaction was stopped at 5 min intervals by heating the solution in a boiling water bath for 5 min. The formation of 5'-UMP from uracil was estimated spectrophotometrically by the decrease of optical density at 290 m μ . No shift of optical density occurred during the reaction period of 1 hr.

The reaction was carried out also using 5-phosphoribosyl-1-pyrophosphate and ribose-1-phosphate. However, the formation of 5'-UMP or uridine was never detected in any case.

DISCUSSION

This enzyme, "pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase", hydrolyzes the N-glycosidic linkage of pyrimidine 5'-nucleotides (Scheme 1).

Scheme 1. Pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase.

The reverse reaction has not been demonstrated.

Since the relative rates of the hydrolysis of 5'-UMP, 5'-CMP, 5'-deoxyUMP, 5'-deoxyCMP and 5'-deoxyTMP (relative values based on the hydrolysis of 5'-UMP=100, Table 5) by the crude enzyme preparation extracted from 1-day culture cells, 4-day culture cells, and by the 31-fold purified enzyme were almost the same, a single enzyme was presumed to be responsible for the degradation of different pyrimidine 5'-nucleotides. This assumption will be

verified by further purification of the enzyme.

Co-factors of low molecular weight, including phosphate, pyrophosphate and ATP, are unnecessary for the reaction. Therefore the involvement of phosphorolytic or pyrophosphorolytic process seems improbable and the N-glycosidic linkage in pyrimidine 5'-nucleotides is supposed to be cleaved in a hydrolytic manner.

This enzyme seems to be a kind of metal enzyme. Like α -amylases from various sources, this enzyme is markedly stabilized by the presence of Ca^{++} .

As compared with the hitherto known pathways of the degradation of pyrimidine nucleotides (Scheme 2, right half), the pathway afforded by the present enzyme (Scheme 2, left half) is quite different.

Scheme 2. Degradation pathways of pyrimidine nucleotides.

Though it is not described in detail in the present report, another enzyme, which catalyzes the hydrolysis of purine 5'-nucleotides to purine bases and pentose phosphates, is found in cell-free extracts of *Streptomyces*. Studies on this enzyme are in progress.

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