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SYNTHESIS AND CLEAVAGE OF L-LACTYLPUROMYCIN AND OTHER LACTYL DERIVATIVES BY LEUCINE AMINOPEPTIDASE

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SUMMARY

Leucine aminopeptidase (L-leucine-peptide hydrolase, EC 3.4.1.1) from calf lens was found to catalyze the formation of L-lactylpuromycin from its components. Several dipeptides which are not cleaved by this enzyme can also accept a lactyl residue. In turn, lactylpuromycin and similar compounds are cleaved by leucine aminopeptidase although much slower than, *e.g.* leucine amide. The formation of L-lactylpuromycin in intact cells of *Bacillus subtilis* and yeast, previously reported from this laboratory, is probably explicably on the basis of these findings.

INTRODUCTION

During studies on the biosynthesis of puromycin derivatives in intact cells, we detected a new compound which was shown to be L-lactylpuromycin[1]. The formation of this substance was first observed by incubating intact cells of *Bacillus subtilis* or yeast with puromycin and radioactive lactate. Subsequent experiments have shown that this compound was also synthesized in the presence of cell-free extracts from these cells and of homogenates from porcine liver or kidney. As no dependence on ATP or any other energy source could be demonstrated, the formation of L-lactylpuromycin *via* a synthetase-type reaction seemed unlikely. Alternatively, we reasoned that our observation could be due to the reversal of a hydrolytic reaction with, depending on the conditions, up to a few percent of the acylpuromycin being present at equilibrium. Indeed, L-lactylpuromycin was found to be readily hydrolyzed by the extracts. A search for the enzyme(s) catalyzing these reactions was undertaken.

In this paper we present our results on the synthesis and cleavage of L-lactylpuromycin and some other lactyl derivatives by leucine aminopeptidase (L-leucine-peptide hydrolase, EC 3.4.1.1) from calf lens and pig kidney.

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MATERIALS AND METHODS

Materials

The following radioactive compounds were purchased from The Radiochemical Centre (England): DL-[1-^{14}C]lactate (26 Ci/mole), L-[U-^{14}C]lactate (56 Ci/mole), [1-^{14}C]-pyruvate (23.8 Ci/mole), [U-^{14}C]glycine (109 Ci/mole), [4,5-$^{3}H_2$]leucine (19 Ci/mole). Puromycin-dihydrochloride was obtained from Nutritional Biochemicals Corp. (U.S.A.). Amino acids and peptides were obtained from Sigma (St. Louis) or from Cyclo (Los Angeles). [^{14}C]lactyl puromycin was synthesized as described previously[1].

Synthesis of acyl derivatives

Using the method described by Anderson *et al.*[2], the hydroxysuccinimide esters of L-lactic acid and other acids used in this study were prepared. These esters were used for the acylation of puromycin, amino acids, amino acid esters and amides. For the acylation step, the hydroxysuccinimide ester dissolved in dioxane was added dropwise to the amino compound and the pH maintained at 8.0 with NaOH. The synthetic compounds were purified by preparative paper electrophoresis at pH 1.8 or 4.8.

Radioactive α-hydroxy acids were prepared from the corresponding amino acids by treatment with $NaNO_2$.

Enzymes

Leucine aminopeptidase was purified from calf lenses according to Hanson *et al.*[3]. The preparation was electrophoretically homogeneous and had a specific activity of 120 units/mg. Pig kidney aminopeptidases were commercial products from Seravac and Sigma. The latter, sold as Type IV, is the microsomal enzyme now designated as aminopeptidase M as first described by Wachsmuth *et al.*[5]. Activities were checked with L-leucine amide as substrate by following the decrease in $A_{238\ nm}$ (ref. 6). Alternatively, L-leucine-*p*-nitroanilide (Sigma) was used as substrate[7].

Assay systems

Synthesis of L-*lactylpuromycin.* The reaction mixture contained 2 μl 0.1 M $MnCl_2$, 2 μl [^{14}C]lactate (1 μCi), 10 μl puromycin (100 mg/ml, neutralized with NaOH to pH 6.5), 50 μl 0.1 M Tris–HCl buffer (pH 7.0) and 1 μl (5 μg) enzyme. At the end of the incubation at 37 °C, aliquots of 10 or 25 μl were directly applied onto Whatman 3 MM paper. The reaction mixture was separated by high-voltage electrophoresis at pH 1.8 (1 M acetic adjusted with formic acid; 60 V/cm, 1 h). The amount of radioactive lactylpuromycin was determined by counting the appropriate segments of the electropherogram in a liquid scintillation counter.

Hydrolysis of L-*lactylpuromycin.* Cleavage of this and other acylpuromycins was assayed as described in the legend to Table III. In experiments with [^{14}C]lactyl-puromycin, the amount of labelled lactate liberated through enzymatic hydrolysis was determined after separation by paper electrophoresis at pH 4.8 (1% pyridine–1% acetic acid, 40 V/cm, 1 h).

Formation of L-*lactyl peptides.* Electrophoretically purified L-[^{14}C]lactate was used for these experiments. The reaction mixtures were similar to the one used for assaying the synthesis of lactylpuromycin except that the puromycin was replaced

by different dipeptides. For each peptide, several different pH values were used to find optimal conditions for the synthesis of lactyl peptides. Upon electrophoresis at pH 4.8, lactyl peptides migrate about half-way between free lactate and the origin.

RESULTS

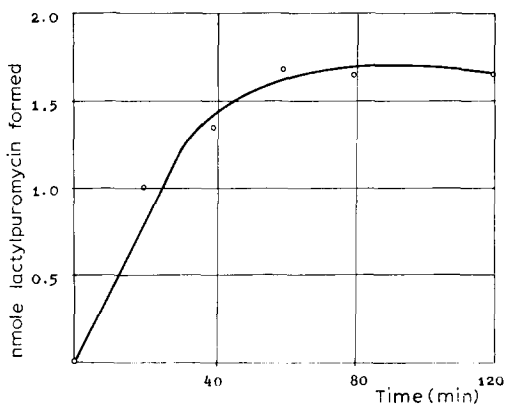
Formation of lactylpuromycin by calf lens leucine amino peptidase

Incubation of L-lactate and puromycin in the presence of leucine amino peptidase results in a slow formation of lactylpuromycin. In a typical experiment with 30 μg enzyme (Fig. 1) a plateau is reached after about 1 h, with approx. 10% of the total lactate now present as lactylpuromycin. The rate of the formation of lactylpuromycin is proportional to the amount of enzyme added. At constant enzyme and puromycin concentration, the amount of lactylpuromycin formed in a given period of time increases with the concentration of L-lactate. These data indicate that leucine amino peptidase from calf lens catalyzes the formation of L-lactylpuromycin from its components up to an equilibrium concentration.

In this reversed reaction leading to formation of a peptide bond, lens leucine amino peptidase shows a marked preference for the acid with a two-carbon side chain (lactate). Using the same conditions as in Fig. 1, formation of glycolyl- or L-α-hydroxyisocaproylpuromycin could not be detected.

Hydrolysis of lactylpuromycin

Synthetic L-lactylpuromycin was found to be hydrolyzed by calf lens leucine aminopeptidase. In a typical experiment, with 15 μg enzyme and a substrate concen-



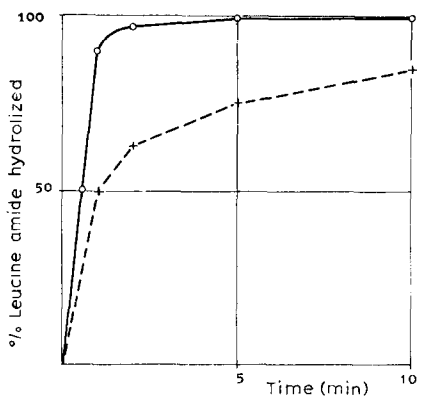


Fig. 1. Time course of L-lactylpuromycin formation. The reaction mixture contained 1.8 μmoles puromycin, 18 nmoles L-[^{14}C]lactate (1 μCi) and 30 μg calf lens leucine aminopeptidase in a final volume of 0.07 ml (0.01 M Tris–HCl, pH 8, containing 3 mM $MnCl_2$).

Fig. 2. Effect of L-lactylpuromycin on the hydrolysis of leucine amide. The incubation mixture contained 0.7 μmole L-leucine amide and 15 μg calf lens leucine amino peptidase in a total volume of 0.075 ml (buffer as in Fig. 1). The reaction was terminated by spotting aliquots onto sheets of Whatman 3 MM paper soaked with formic acid–acetic acid buffer, pH 1.8. Leucine amide and released leucine were separated by high-voltage electrophoresis. The separated substances were stained with ninhydrin and the eluted color quantitated[16]. A parallel incubation mixture contained, in addition, 0.5 μmole L-lactylpuromycin. The relative amounts of leucine amide cleaved in the absence (○—○) and presence of lactylpuromycin (+---+) are plotted.

tration of 6.6 mM lactylpuromycin a slow but significant hydrolysis of the substrate was observed. About 2 nmoles of lactate were liberated per min for at least up to 1 h. Again, higher enzyme to substrate ratios lead to an increase of the rate of hydrolysis. The cleavage rates of leucine amide, a typical substrate of leucine aminopeptidase, and lactylpuromycin were studied in the same incubation mixture and compared with the rates for the separated substrates. As shown in Fig. 2, the hydrolysis of leucine amide is slowed down in the presence of lactylpuromycin. It appears, therefore, likely that the same active site is required for the hydrolysis of these two substrates.

The Michaelis constant for lactylpuromycin was determined according to Lineweaver and Burk[8]. A value of $3 \cdot 10^{-2}$–$6 \cdot 10^{-2}$ mole/l was found, which is in the same order of magnitude as the value for leucine amide ($3.125 \cdot 10^{-2}$ mole/l, ref. 15).

Synthesis of other lactyl derivatives

Besides puromycin, it was tested whether other compounds with a free α-amino group serve as lactyl acceptors in the presence of leucine amino peptidase. No lactylation of free amino acids (glycine, tyrosine, leucine) could be observed. Several peptides refractory to cleavage by leucine amino peptidase were found to serve as lactyl acceptors in this system, but the amounts synthesized were lower than with puromycin (Table I).

TABLE I

SYNTHESIS OF L-LACTYL PEPTIDES BY LENS LEUCINE AMINO PEPTIDASE

The reaction mixtures contained in a final volume of 70 μl of Tris–HCl buffer (0.1 M, pH 7.2–8.0): 2 μl of 0.1 M $MnCl_2$, 1.5–2.0 μmoles peptide or puromycin (neutralized with NaOH), 37 nmoles L-[^{14}C]lactate (1 μCi), and 30 μg calf lens leucine aminopeptidase. The amount of L-lactyl peptides or L-lactylpuromycin formed after 20 min incubation at 37 °C were determined as described in Materials and Methods.

Peptide	*nmole lactyl peptide formed*	*Relative rate of formation*
Gly–D-Leu	0.38	38
L-Phe–L-Pro	0.13	13
Gly–L-Pro	0.03	3
D-Leu–Gly	0	0
Puromycin	1.0	100

Cleavage of lactyl compounds by lens leucine amino peptidase

A series of synthetic substrates containing *N*-lactyl bonds were tested as substrate of calf lens leucine amino peptidase. The results are summarized in Table II: Lactyl amino acids and lactyl amines were not hydrolyzed by the enzyme. Lactyl amino acid amides and lactyl peptides were found to be cleaved. The relative cleavage rates for the tested lactyl derivatives were all in the same order of magnitude and comparable to the hydrolysis rate of lactylpuromycin. As far as the structural requirements are concerned the following pattern emerges from these limited data: the L-lactyl residue has to be linked to an amino acid with a blocked carboxyl group in order to make the bond susceptible to hydrolysis by leucine amino peptidase.

TABLE II

HYDROLYSIS OF L-LACTYL DERIVATIVES OF THE TYPE L-LACTYL-X

1 μmole [^{14}C]lactate-labelled substrate (10 μCi/mmole) dissolved in 0.075 ml 0.1 M Tris–HCl, pH 8.5, containing 5 mM $MnCl_2$, was incubated with 5 μl (30 μg) enzyme. Aliquots of 0.025 ml were removed at zero time and after 30 and 60 min incubation at 37 °C. Liberated lactate was separated from undigested substrates by high-voltage paper electrophoresis at pH 4.8.

X	*μmole lactate liberated in 60 min*	*Relative cleavage rate*
Phenylalanyl amide	0.39	100
Tyr–Gly	0.27	69
Tyrosine amine	0.19	49
Leucine amide	0.12	31
Gly–Tyr	0.11	28
Tryptophane amide	0.094	24
Tyrosine	0	0
Leucine	0	0
Tyramine	0	0
Puromycin	0.26	66

Cleavage of other acylpuromycins by leucine amino peptidase

A series of acylpuromycins was synthesized and their rate of cleavage by leucine amino peptidase compared. Table III summarizes the results obtained. From the α-hydroxyacyl derivatives tested the C-3 compound (lactate) appears to be most susceptible to hydrolysis.

Comparison of different amino peptidases

Of the purified enzymes tested, only calf lens leucine amino peptidase and the soluble enzyme from pig kidney[9] catalyzed the formation and cleavage of lactylpuromycin. The microsomal enzyme from pig kidney[5] (Sigma Type IV, not inhibited by EDTA) was completely inactive in these tests. These findings are in agreement to similarities proposed earlier between soluble leucine aminopeptidase from pig kidney and calf lens[3].

TABLE III

RELATIVE CLEAVAGE RATE OF ACYLPUROMYCINS

The incubation mixtures contained 1 mg substrate (2 mg in case of D,L-α-hydroxybutyrylpuromycin) dissolved in 0.2 ml 0.05 m Tris–HCl, pH 8.6, containing 10% methanol and 4 mM $MnCl_2$. The reaction was started by the addition of 0.01 ml enzyme solution (30 μg). The reaction was terminated by spotting 0.05-ml aliquots onto Whatman 3 MM paper at zero time, and after 5 and 10 min incubation at 37 °C. Puromycin was separated from uncleaved substrates by high-voltage paper electrophoresis at pH 4.7 or 1.8. The released puromycin was located on the dried electropherograms by reaction with ninhydrin and quantitated after subsequent elution[16].

Acyl group	*μmole puromycin liberated in 10 min*	*Relative rate*
Glycolyl	0.14	42
L-Lactyl	0.33	100
D,L-α-Hydroxybutyryl	0.12	36
D-Lactyl	0	0
Pyruvyl	0	0
Bromopropionyl	0	0

DISCUSSION

Aminopeptidases hydrolyze proteins and peptides in a step-wise fashion starting at the N-terminus. Enzymes of this type are widely distributed in tissues of higher organisms. The so-called leucine aminopeptidase isolated from acetone powders of swine kidney has been investigated in detail[9]. A similar or identical enzyme present in bovine lens has been purified to homogeneity by Hanson and Hütter[3]. Although no detailed reaction mechanism can be formulated at present, it appears likely that the amino, rather than the ammonium form, of the substrate is susceptible to leucine aminopeptidase. In line with this notion are the high pH optimum (at about pH 9) and the observation of Hill and Smith[10] that certain α-hydroxy analogues of substrates do interact with the enzyme.

The ability of leucine amino peptidase to cleave certain substances with an α-hydroxy rather than an α-amino group is further substantiated in this paper. A series of compounds with an L-lactyl group linked to a peptide or an aminoacyl amide were found to be hydrolyzed. The influence of the type of α-hydroxy acid present in the substrate was studied with the corresponding acylpuromycins. L-Lactylpuromycin was found to be a better substrate than the corresponding glycolyl- or α-hydroxybutyryl derivative. In an experiment analogous to the above-mentioned work (ref. 10) the hydrolysis of L-leucine amide, which is an excellent substrate for leucine amino peptidase, was found to be retarded in the presence of a second substrate, L-lactylpuromycin. Competition for the same active site is the most likely explanation for this finding.

More surprising is the fact that the hydrolysis of some of the lactyl derivatives is reversible and that under suitable conditions, formation of an amide bond can be observed. Following our original findings on the synthesis of L-lactylpuromycin in intact cells[1], this reaction has been studied in detail with purified aminopeptidases from bovine lens and porcine kidney. Using labelled lactate, the formation of this compound is readily detectable, since under certain conditions, *i.e.* at high concentrations of puromycin, up to 10% of the total radioactivity is, at equilibrium, present as lactylpuromycin (see Fig. 1). Several peptides resistant to hydrolysis by leucine aminopeptidase were also found to serve as acceptors for L-lactate. Two basic structural requirements appear to be obligatory for a compound to serve as an acceptor for the "lactylation" by leucine amino peptidase: a L-amino acid with (a) a free α-amino group and (b) a carboxyl group blocked by a residue which is refractory to attack by the enzyme.

These observations demonstrating that a proteolytic enzyme catalyzes not only the hydrolysis but also, up to an equilibrium value, the synthesis of a substrate is not without precedence[11,12]. Such findings have generally been interpreted as evidence for the existence of enzyme bound intermediates in the catalytic process. Cleavage of L-lactylpuromycin could, in principle, proceed *via* two types of intermediates: (1) a lactyl group bound to an amino acid side chain; or (2) the puromycin moiety forming, with its α-amino group, an amide bond with a carboxyl group on the protein. Our results with aminopeptidases do not allow us to distinguish between these possibilities.

However, the acyl–enzyme intermediate appears to be the more likely candidate, since Hanson und Lasch[13] have demonstrated a transamidation reaction

catalyzed by leucine aminopeptidase. Starting with leucine amide as substrate, the formation of leucylleucyl amide could be demonstrated. Such a finding is more readily explicable by assuming an acyl–enzyme as intermediate. The formation of peptide bonds as occurring during synthesis of L-lactylpuromycin, could then be visualized to proceed *via* a lactyl–enzyme.

Finally, it should be mentioned that the microsomal aminopeptidase operates via a different mechanism[14]. This enzyme requires no metal for activity and kinetic experiments indicate, that contrary to leucine amino peptidase, the substrate has to be in the ammonium form. In accordance with this, we found that aminopeptidase M shows no activity towards L-lactyl derivatives. Also, synthesis of L-lactylpuromycin from its components could not be observed.

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