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HYDROLYSIS OF ALANINE OLIGOPEPTIDES BY AN ENZYME LOCATED IN THE MEMBRANE OF *MYCOPLASMA LAIDLAWII*

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SUMMARY

Membranes obtained from *Mycoplasma laidlawii* and whole cells suspended in an isotonic salt solution cleave the N(α)-terminal L-alanine residue of oligoalanine peptides of the general formula Ala(L)–[Ala(L)–Ala(D)]$_n$, $n = 2,4,8$. The intact cells were found to be impermeable to L-alanine when suspended in an isotonic salt solution. Entry of L-alanine into the cells was observed only when suspended in the appropriate growth medium.

INTRODUCTION

Membrane-bound enzymes such as NADH oxidase, ATPase, and phosphatases, have been found in *Mycoplasma*[1]. *Mycoplasma fermentans* cells and membrane preparations were found to hydrolyze some peptides, as well as the β-naphthylamides of histidine, lysine and arginine[2].

In this communication we report on the hydrolytic activity associated with the cell membrane of *Mycoplasma laidlawii* toward alanine oligopeptides of the formula Ala(L)–[Ala(L)–Ala(D)]$_n$ ($n = 2,4,8$), as well as other peptides. All of the alanine oligopeptides were hydrolyzed by intact cells to yield L-alanine under the conditions at which peptide hydrolysis was tested. It is thus suggested that the enzyme (or enzymes) hydrolyzing the alanine oligopeptides are located externally in the *M. laidlawii* cell membrane.

EXPERIMENTAL

Synthesis of oligopeptides

L-Alanine oligopeptides of the structure (Ala)$_n$ ($n = 2,3,5,6$) were prepared by condensing benzyloxycarbonyl-L-alanine with the appropriate intermediate peptide *p*-nitrobenzyl ester, followed by removal of the blocking group with HBr[3]. Alanyl oligopeptides composed of L and D residues of the sequence L(LD)$_n$ ($n = 2,4,8$) were synthesized by coupling the hydroxysuccinimide ester of benzyloxycarbonyl-L-alanine with D-alanine or with the corresponding [Ala(L)–Ala(D)]$_n$ peptides[4]. To avoid race-

mization, care was taken to cool the reaction mixtures at the coupling step. The protecting group was removed by catalytic hydrogenation.

The purity of the alanine peptides containing up to three amino acid residues was checked by thin-layer chromatography on cellulose plates (Riedel-de Haen AG, Germany) using the solvent system *n*-butanol–acetic acid–water (4:1 1, v/v/v). Higher peptides were separated by high-voltage electrophoresis at pH 1.4 as described[3]. All the peptides used were chromatographically or electrophoretically pure and yielded the expected ratios of amino N to total N (Van Slyke method[5] and micro-Kjeldahl[6], respectively).

Growth conditions and preparation of cell suspensions

M. laidlawii (obtained from Dr S. Razin, The Hebrew University-Hadassah Medical School, Jerusalem) were grown statically in a modified Edward medium[7] containing 2% (v/v) PPLO serum fraction (Difco) The organisms were incubated for 16–18 h at 37 °C to yield a suspension containing about $2 \cdot 10^9$ cells per ml. The cells were sedimented at 12000 × *g* (Sorvall refrigerated centrifuge) for 10 min, washed once in a salt solution, 0.25 M in NaCl and 0.01 M in $MgCl_2$, and suspended in the same salt solution to a concentration of $2 \cdot 10^{10}$ cells/ml. Protein in cell suspensions was determined by the method of Lowry[8].

Determination of peptidase activity

The rate of β-naphthylamine release from L-alanyl β-naphthylamide (Mann Research Laboratory, N.Y.) by *M. laidlawii* cell suspension was determined fluorimetrically. β-Naphthylamine, when excited at 335 nm shows an intense emission at 410 nm[9]. The substrate L-alanyl β-naphthylamide does not fluoresce appreciably under these conditions. The rate of enzymic activity was followed at 410 nm using an Aminco–Keirs spectrofluorimeter (American Instruments Co.) equipped with a RCA IP 21 phototube and a Graphispot time recorder (Sephram, Paris). The standard reaction mixture (1 ml) placed in the thermostated cell (QS, 10.20 mm light path, Hellma, Germany) contained 2.8 μmoles of L-alanyl β-naphthylamide, 50 μmoles of pH 8.5 veronal buffer and 0.5 ml *M. laidlawii* suspension (0.3–0 6 mg cell protein corresponding to $0.5 \cdot 10^{10}$–$1.0 \cdot 10^{10}$ cells). The pH activity profile of the enzyme was determined in the following buffers 0.05 M potassium phosphate for pH 6.9–8.0, 0.05 M borate for pH 7 5–8.8; 0.05 M veronal for pH 8.1–8.55 and 0 05 M $NaHCO_3$/NaOH for pH 8.8–9 4. The intensity of β-naphthylamine emission was not affected appreciably by the different buffer ions or pH in the above range.

Hydrolysis of oligopeptides was carried out in 0 5 ml of 0.25 M NaCl solution (0.01 M in $MgCl_2$ and 0 05 M in veronal buffer, pH 8 3) The reaction mixture contained mycoplasma cells corresponding to approximately 0 2 mg protein and 50 mM peptide. After 30 min of incubation at 37 °C the mycoplasma was sedimented by centrifugation and the supernatant was analyzed for products of hydrolysis either by thin-layer chromatography as described above or on a Beckman Spinco 120 B amino acid analyser[10] Using the standard Dowex-50 column and the buffer systems· 0 2 M sodium citrate for pH 3 25 and pH 4 25 and 1.2 M sodium citrate–NaCl for pH 5.25 it was possible to separate the liberated alanine from the remaining non-hydrolyzed peptides [Ala–(L)–Ala(D)]$_n$. Control experiments revealed that only a negligible amount of free L-alanine was present in a salt suspension of mycoplasma.

Water volume of cells

Mycoplasma pellet (7000 × *g*, 15 min, Sorvall refrigerated centrifuge) was resuspended in fresh growth medium to a concentration of 10^{11} cells/ml. 1 ml of this suspension was added to 1 ml of a salt solution in $H_2{}^{18}O$ (135 mM in NaCl, 4 mM in KCl and 10 mM in phosphate buffer, pH 7.6), 94 % in ^{18}O. The cells were separated from the above suspension by the differential flotation method previously described[11], using polyethylene tubes of about 0.4 ml. Each tube contained 0.05 ml of oil (density 1.066 g/ml) on which 0.2 ml of the mycoplasma suspension was layered. The tubes were spun for 2.5 min at about 10000 × *g* in an Eppendorf Microcentrifuge. The tubes were then cut with a razor blade through the separating oil, as close to the pellet as possible. The pellet was then thoroughly resuspended in 0.10 ml of H_2O. A 40 μl sample of this suspension was withdrawn and analyzed for the isotopic ratio $^{18}O/^{16}O$ by mass spectrometry[12]. The water content of the pellet, V (ml), is calculated from the measured $^{18}O/^{16}O$ value of the resuspended pellet (R') and the $^{18}O/(^{18}O + {}^{16}O)$ value of the original suspension (R), using the following relation

$$\frac{RV}{RV + 0.1} = R'$$

where 0.1 ml is the volume of H_2O added to the pellet. The number of cells in the incubation suspension was determined in a bacteria counting chamber using phase contrast microscopy, as well as by colony count. The colonies were grown on agar plates for a week at 37 °C. Similar values were obtained by both methods. The water volume associated per single cell in the pellet was calculated to be 0.24 ± 0.02 μm^3. The deviation results from the variability obtained in four different experiments. The main error originated in the counting procedure of the cells.

The inulin volume of the pellet, which measures the extracellular water, was determined as follows. To the suspension of cells in growth medium diluted with $H_2{}^{18}O$ (94 % ^{18}O) in a ratio of 1:1 (v/v) and which contained $5 \cdot 10^{10}$ cells per ml, 15 μl of an aqueous solution of [^{14}C]inulin (174 mg/ml, spec. act. 180 μCi/ml, pH 7.6) was added. A 0.2-ml aliquot was withdrawn, and the cells were sedimented by the differential flotation method described above. The pellet was resuspended in 0.1 ml H_2O and the radioactivity of a 20-μl sample was determined in the Packard Tri-Carb liquid scintillation spectrometer using Bray's solution[13]. Radioactivity of 10 μl of the original suspension was also determined. The inulin volume was calculated from the obtained counting rates assuming that the concentration of inulin in the extracellular water of the pellet is the same as in the bulk of the solution. The inulin volume per cell in the pellet was found to be 0.14 μm^3. This gives a corrected water volume per *M. laidlawii* cell of 0.10 ± 0.02 μm^3.

Permeability to L-*alanine*

A suspension of *M. laidlawii* in salt solution (1 ml, $2 \cdot 10^{10}$–$3 \cdot 10^{10}$ cells, 250 mM NaCl, 10 mM $MgCl_2$ and 50 mM veronal at pH 8.5), or in fresh growth medium (pH 8), was incubated with 10 μl of an appropriate radioactive stock solution of L-alanine ($3.5 \cdot 10^{-3}$ M), inulin ($3.8 \cdot 10^{-2}$ M) or dextran ($1.7 \cdot 10^{-3}$ M) at 37 °C. Aliquots of 0.2 ml were withdrawn to 0.4-ml polyethylene tubes. Cells were separated by the differential flotation method described above. To determine the total amounts of alanine, inulin and dextran in the pellet it was resuspended in 0.1 ml distilled water, and the radio-

activity was determined in 50-μl samples The radioactive substances L-[^{14}C]alanine (spec. act. 14.2 mCi/mmole) and [^{14}C]dextran (molecular weight 28700; spec. act. 0.46 μCi/mg) were purchased from the Radiochemical Centre, Amersham, England. [^{14}C]Inulin (spec. act. 6.6 Ci/mole) and [^{3}H]inulin (spec. act. 250 μCi/ml, 1 15·10^{-3} M) were obtained from New England Nuclear, U.S A.

The relative permeability of L-alanine with respect to inulin was determined in a suspension containing both these substances (L-alanine was labeled with ^{14}C and inulin with ^{3}H) by a procedure similar to that given above.

RESULTS

Peptide hydrolysis by M. laidlawii

M. laidlawii cells were found to hydrolyze L-alanine β-naphthylamide with an apparent K_m of 6·10^{-4} M (pH 8.5). The initial rate of hydrolysis, under the conditions specified in Methods, was 0.9 $\pm$ 0.1 nmoles β-naphthylamine/min per mg cell protein. The optimal pH range for the amide hydrolysis is 8 to 9 as shown in the pH activity profile given in Fig. 1.

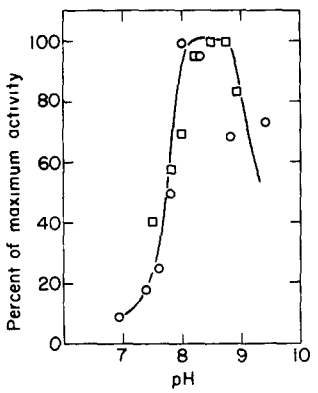

Fig 1 Hydrolysis of L-alanyl β-naphthylamide by *M laidlawii* cells at 25 °C, at different pH values Two sets of experiments are summarized The reaction mixture (1 ml) contained: 2 8 μmoles L-alanyl β-naphthylamide, 50 μmoles buffer (buffer composition is given in the experimental section) and about 5·10^{9} *M. laidlawii* cells (corresponding to about 0 3 mg cell protein) The initial rate determined fluorimetrically was 0 9 $\pm$ 0 1 nmoles β-naphthylamine liberated per min per mg cell protein, at 25 °C, pH 8 6 (□), 0 05 M boric acid–borax buffer, (○), 0 05 M phosphate, veronal or $NaHCO_3$–NaOH buffers

L-Alanine at a concentration of 4·10^{-2} M does not inhibit the hydrolysis of L-alanyl β-naphthylamide under the standard experimental conditions employed. The dipeptide L-Ala–D-Ala on the other hand was found to be an effective inhibitor of alanyl β-naphthylamide hydrolysis, 50 % inhibition being obtained at a concentration of dipeptide of 2.5·10^{-2} M.

Study of the hydrolysis by *M. laidlawii* cells of the following dipeptides (all amino acids in the L-configuration). His–Ala, Lys–Ala, Ala–Lys, Lys–Lys, Orn–Orn, revealed that all but Orn–Orn are quantitatively hydrolyzed to give the corresponding amino acids. Incubation of the cells with Ala–Gly–Gly, Ala–Orn–Orn, Lys–Orn–Orn, Gly–Phe–Ala, Ser–Gly–Gly, Orn–Orn–Ala, Orn–Orn–Lys, revealed that the first two

tripeptides yield alanine and the corresponding dipeptides; Lys–Orn–Orn gives lysine and Orn–Orn; Gly–Phe–Ala is only slightly attacked at the Gly–Phe peptide link, whereas the remaining tripeptides are resistant to attack by *M. laidlawii*. When the terminal amino group of the di- or tripeptides was blocked with an acetyl or a benzyloxycarbonyl group, none of the products obtained was hydrolyzed.

The above data suggest that an enzyme(s) of the aminopeptidase type are present in the membrane of *M. laidlawii*.

Incubation of cells with $(Ala)_n$ oligopeptides (containing 2,3,5,6 L-alanine residues) for 30 min, under the conditions specified in Experimental, resulted in complete hydrolysis of the peptides as revealed by the appearance on the thin layer chromatography plates of alanine exclusively.

Upon incubation of *M. laidlawii* cells with alanine oligopeptides of the general formula $Ala(L)–[Ala(L)–Ala(D)]_n$, the latter were hydrolyzed to yield L-alanine and the corresponding $[Ala(L)–Ala(D)]_n$ peptides. L-Alanine was separated from the $[Ala(L)–Ala(D)]_n$ peptides using the amino acid analyser column as described in Experimental. The results presented in Table I clearly demonstrate that a sequence of at least two N-terminal L-alanine residues is required for the cellular hydrolysis under investigation. The extent of hydrolysis did not exceed 60 % even on incubation of the suspension tested at 37 °C for 150 min. Only negligible amounts of free alanine were found in *M. laidlawii* suspensions devoid of substrate.

TABLE I

HYDROLYSIS OF VARIOUS $ALA(L)–[ALA(L)–ALA(D)]_n$ PEPTIDES BY *M. laidlawii* CELLS

The reaction mixture contained mycoplasma cells corresponding to approximately 0.2 mg protein and 25 μmoles peptide in 0.5 ml of a buffered salt solution (pH 8.3, 0.05 M veronal, 0.25 M NaCl, 0.01 M $MgCl_2$). Incubation was carried out at 37 °C for 30 min.

Alanine peptides		*mole L-alanine liberated per mole peptide*
Sequence	*Number of residues*	
LD	2	0.00
LLD	3	0.60
LDLD	4	0.10
LLDLD	5	0.56
LDLDLDLD	8	0.02
LLDLDLDLD	9	0.45

Location of the peptidase activity in the cell membrane

The enzymic activity was found to be part of the insoluble fraction of the broken cells. Cells were disrupted either by osmotic lysis or by sonication[14]. The insoluble membrane fragments (34000 × *g*, 30 min) were resuspended in the buffered salt solution (10 mM veronal, 50 mM NaCl, pH 8.5) and then peptidase activity was determined using β-naphthylamide as substrate. An enzymic activity corresponding to 60–70 % of that of the intact cells was found in the membrane preparations. Only a few percent of the initial activity could be detected in the supernatant.

Incubation of cells (1.5 mg protein), with 0.5 mg phospholipase C (Worthington Biochemical Corp., lot PHLC-OKA, crude preparation containing 0.1 % proteolytic

activity) in 0.4 ml buffered isotonic salt solution (0.05 M veronal buffer, pH 8.5) at 37 °C caused a marked decrease in peptidase activity. A residual activity corresponding to 20 % of that of the untreated cells was recorded after 15 min of incubation.

Permeability of M. laidlawii cells to L-*alanine*

The distribution of L-alanine and of high molecular weight solutes between the extra and intra cellular water volume was measured with cells suspended in isotonic salt solution as described in Experimental. The results summarized in Table II clearly show that under the conditions employed the cells are impermeable to alanine, as alanine is confined to the inulin volume of the pellet (trapped volume).

The data presented in Table III show that L-alanine penetrates cells suspended in the growth medium employed, in contrast to the above findings which refer to cells suspended in the salt solution. In the experiments summarized in the table the cells were incubated with L-[^{14}C]alanine and [^{3}H]inulin in isotonic salt solution or in the growth medium. The ratio of alanine to inulin in the cells pellet and in the supernatant was compared. In isotonic salt solution this ratio was found to be the same

TABLE II

THE DISTRIBUTION OF L-ALANINE AND HIGH MOLECULAR WEIGHT SOLUTES IN *M laidlawii* PELLET

Solute	*Concentration* $\times$ 10^4 *M*		*Relative solute concentration* **
	in supernatant	*in pellet**	
Dextran	0 17	0 10	0 60
Inulin	14 60	8 60	0 58
L-Alanine	0 35	0 22	0 63

* Concentration is per water content of the cells pellet, determined separately as described in the experimental section

** Solute concentration in the pellet was compared to that of the supernatant which was taken as 1 00

TABLE III

PERMEABILITY OF *M laidlawii* TO L-ALANINE

The incubation suspension contained *M laidlawii* cells (1 ml, $6 \cdot 10^{10}$) suspended either in pH 7 9 salt solution (50 mM potassium phosphate, 250 mM NaCl, 10 mM $MgCl_2$) or in fresh growth medium at pH 7 9 and the labeled stock solution (40 μl) The stock solution was composed of a mixture of [^{3}H]inulin (methoxy), $1\ 15 \cdot 10^{-3}$ M, specific activity 250 Ci/mole and L-[U-^{14}C]-alanine $3\ 5 \cdot 10^{-3}$ M, specific activity 14 2 Ci/mole at a ratio of 1 · 1 (v/v). Incubation was done at 37 °C without shaking 200-μl aliquots were withdrawn after 20 min and pellets were prepared for counting as described in the experimental section In each experiment the found ratio of L-alanine to inulin in the suspension is taken as 1.0

Incubation mixture	*Fraction analyzed*	L-*alanine* (*nmole*/μl)	*Inulin* (*nmole*/μl)	*Ratio* L-*alanine*/*inulin* (*normalized*)
Growth medium	Suspension	0 071	0 236	1 0
	Pellet	0 142 $\pm$ 0 011	0 267 $\pm$ 0 003	1 7
Salt solution	Suspension	0 074	0 233	1 0
	Pellet	0 083 $\pm$ 0 004	0 256 $\pm$ 0 008	1 0

in the pellet as in the incubation mixture over a time period of 2 min to 70 min. Thus alanine occupies that part of the pellet's volume which is accessible to inulin. On the other hand, cells suspended in their growth medium were permeable to alanine as shown by the higher ratio of alanine to inulin in the pellet. This ratio increased slowly from 1.2 after 2 min of incubation to 2.5 after 70 min. Calculations of alanine and inulin concentrations in the cell pellet after 20 min incubation are per total water volume of the pellet.

DISCUSSION

The evidence that the enzyme (or enzymes) of *M. laidlawii* which hydrolyzes N-terminal L-alanyl residues from the peptides listed in Table I is located in the cell membrane was demonstrated by showing that practically all of the enzymatic activity is confined to insoluble material obtained by several methods of cell disruption. Furthermore, the fact that phospholipase C inactivates the enzyme in the intact cell indicates that protein–lipid interaction is important for the activity or stability of the enzyme(s). The external location of the enzyme is concluded from the fact that the nonapeptide, Ala(L)–[Ala(L)–Ala(D)]$_4$, is cleaved by intact cells under conditions at which the cells are non-permeable to alanine.

The finding that *M. laidlawii* cells kept in salt solution are impermeable to low molecular weight compounds such as L-alanine is rather surprising. Even when the cells were suspended in the growth medium at 37 °C, the internal concentration of L-alanine was found to be rather low. It would seem, therefore, that the permeability of L-alanine into *M. laidlawii* is very slow and most probably an energy requiring process. Such low permeability of *Mycoplasma* to an amino acid is hard to reconcile with a "permease mechanism" for transport of amino acids described by Razin *et al.*[15]. It should, however, be stressed that the "permease effect" was observed at pH 5–6, whereas our measurements were carried out at pH 8–8.6 which correspond to the pH optimal for growth of *M. laidlawii*.

The presence of peptidase in the cell membrane of *Mycoplasma* is of special interest since various peptides are known to be growth promoting in this class of microorganisms[16].

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