An aminopeptidase nutritionally important to *Fusobacterium nucleatum*

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The properties of an aminopeptidase (AP) from *Fusobacterium nucleatum* were studied in view of the fact that this organism, along with other Gram-negative anaerobes involved in periodontal diseases, survives in the subgingival environment by obtaining energy via the fermentation of a small number of peptide-derived amino acids. The AP was found to be cell-associated and was isolated from disrupted chemostat-grown cells. It was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, two column chromatographic steps and IEF. The enzyme was found to have a molecular mass of 54 kDa, a pI of 5.1, a pH optimum between 7.5 and 8.0 and, using Leu-Ala as substrate, it gave K_m and V_{max} values of 0.66 mM and 0.12 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. No complete homology was found between the N-terminal sequence of the first 20 amino acids (MDXKXYVDLKERFLRYVKFN...) and any other published sequence, but residues 8–20 gave a 62% match with residues 9–21 of an AP from *Haemophilus influenzae*. The enzyme was inactivated by chelating agents, bestatin, *p*-hydroxymercuribenzoate and some heavy metals. Cobalt ions restored EDTA-inactivated activity but did not reverse inhibition by 1,10-phenanthroline. In addition, bestatin and 1,10-phenanthroline had an inhibitory effect on the batch growth of *F. nucleatum* in a complex medium in which peptidase activities would be nutritionally essential. No such inhibition was observed in a chemically defined medium in which growth was not dependent upon peptidase activities. The peptidase described in this paper therefore appears to be a cobalt-activated metallo-AP which, together with other peptidases, is considered to be important in the survival of *F. nucleatum* in the subgingival environment of the mouth.

Keywords: *Fusobacterium nucleatum*, oral cavity, aminopeptidase, nutritional role

INTRODUCTION

Fusobacterium nucleatum has been isolated from both active and inactive periodontitis sites in the human mouth (e.g. Dzink *et al.*, 1988) and is one of the most frequently detected cultivable bacteria in subgingival dental plaque (Moore *et al.*, 1991; Moore & Moore, 1994). Its taxonomy, biology and possible role in periodontal diseases have recently been reviewed by Bolstad *et al.* (1996).

In a previous study, we showed that *F. nucleatum* can obtain energy via the fermentation of simple sugars such as glucose, or of amino acids such as glutamate, histidine, serine and lysine (Rogers *et al.*, 1991). Such metabolic versatility may well explain its ubiquity in the mouth.

Contrary to earlier studies (Gharbia & Shah, 1988; Gharbia *et al.*, 1989) we also showed that peptides were not essential for the growth of *F. nucleatum* strains since they grew in a peptide-free chemically defined medium (Rogers *et al.*, 1992). However, they were unable to grow on casein or albumin as a sole source of nitrogen and energy, primarily because they lack endopeptidase activities (Rogers *et al.*, 1992). On the other hand, an organism such as *Porphyromonas gingivalis*, often found together with *F. nucleatum* in diseased sites

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Abbreviation: AP, aminopeptidase.

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(Moore, 1987), does possess such activities (Minhas & Greenman, 1989; van Winkelhoff *et al.*, 1988). The resulting peptides would be potential carbon and energy sources provided that they contained residues such as glutamate, histidine, serine and lysine and that *F. nucleatum* possessed the relevant exopeptidase activities. In a follow-up study (Rogers *et al.*, 1992), we showed that (a) resting cells cleaved a wide range of peptides containing up to six residues and that the four key energy-yielding amino acids were rapidly taken up after release from a peptide and (b) the glutamate-rich hydrophilic fraction of a commercial peptone promoted growth and peptides of molecular mass <1 kDa were rapidly utilized by growing cells. It is thus clear that the utilization of these small peptides would require exopeptidase activities to provide free amino acid residues such as glutamate, histidine, serine and lysine and that such activities could be crucial for the continued presence of *F. nucleatum* in the mouth, particularly in the subgingival environment.

In the present study, we report on the isolation, purification and properties of an aminopeptidase (AP) from *F. nucleatum*.

METHODS

Micro-organism. *Fusobacterium nucleatum* ATCC 10953 was maintained in a locally prepared cooked meat medium containing 0.3% glucose, and in glycerol broth stored at -80°C .

Growth conditions. The organism was grown under continuous culture conditions as described previously (Rogers *et al.*, 1991). Briefly, it was grown in BM medium (Shah *et al.*, 1976) at 37°C , pH 7.4 and a dilution rate of $D = 0.1\text{ h}^{-1}$ under an atmosphere of N_2/CO_2 (95:5).

Preparation of cell-free extracts. The culture overflow was collected at 4°C and the cells were harvested by centrifugation at 6000 g for 15 min at 4°C . Deposited cells were resuspended in saline to 1/10th of their original volume and disrupted using a French pressure cell. After centrifugation at 10000 g to remove unbroken cells, the supernatant was lyophilized and the powder stored at 4°C in a desiccator. To determine the cellular location of the peptidase, a small aliquot of the 10000 g supernatant was centrifuged at 100000 g to sediment cell envelope material. These various fractions were then tested for (amino) peptidase activity (see below).

AP assay. During purification, enzyme activity was monitored by a modification of the L-amino-acid oxidase method of Lewis & Harris (1967), using the tripeptide Leu-Gly-Gly as the substrate. Briefly, about 1 μl test sample was loaded onto a cellulose acetate strip and laid in a staining solution (containing 5 mg substrate ml^{-1} ; 4 mg o-dianisidine dihydrochloride ml^{-1} ; 1 mg L-amino-acid oxidase ml^{-1} ; 1 mg peroxidase ml^{-1} ; and 100 μM MgCl_2 in 100 mM Tris/HCl buffer, pH 8.0) for 2–3 min. The strip was then removed, lightly blotted, placed between two pieces of plastic film and incubated at 37°C . Peptidase activity was shown by the appearance of a brown stain after about 30 min incubation. The method was made semi-quantitative by testing serial double dilutions of a sample and visually estimating the end point. For example, if a sample gave a visible reaction when

diluted 1/8, but not 1/16, then it was deemed to contain 8 units (U) of activity; i.e. the reciprocal of the end point.

Protein assay. Protein was determined spectrophotometrically by the BCA reagent (Pierce Chemical) with BSA as standard.

Enzyme purification

$(\text{NH}_4)_2\text{SO}_4$ fractionation. To approximately 4 g freeze-dried cell extract (see above) was added 340 ml 40 mM Tris/HCl buffer, pH 7.4, and the mixture was stirred at 4°C for 30 min. Protamine sulphate to 0.4% (w/v) was then added and stirring was continued for a further 60 min. The (inactive) proteins precipitating at 60% and 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ at 4°C were sequentially removed by centrifugation (5000 g, 30 min, 4°C) and the peptidase was recovered by precipitation at 80% saturation. The precipitate was dissolved in 3 ml distilled water.

Column chromatography. The chromatographic purification of this crude enzyme preparation was accomplished using Bio-Rad Econo-Pac columns. In the first step, the aqueous preparation was applied to an Econo-Pac HIC (hydrophobic interaction chromatography) column equilibrated with 100 mM sodium phosphate buffer, pH 6.8, containing 1.5 M $(\text{NH}_4)_2\text{SO}_4$. The enzyme was eluted with a reverse linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (1.5–0 M) at a flow rate of 1 ml min^{-1} . The active fractions were pooled, concentrated [using a Centriprep filtration unit (exclusion limit 30 kDa; Amicon)] and equilibrated with 1.5 M $(\text{NH}_4)_2\text{SO}_4$ in 100 mM sodium phosphate buffer, pH 6.8.

In the second step, the concentrated fraction was applied to an Econo-Pac Q, IEC (ion exchange chromatography) column equilibrated with 50 mM Tris/HCl buffer, pH 8.0. The enzyme was eluted with a linear gradient of NaCl (0–1 M) in 50 mM Tris/HCl, pH 8.0, at a flow rate of 1 ml min^{-1} . Active fractions were pooled, concentrated using the Centriprep unit as described above and desalted against MilliQ water prior to freeze-drying; they were stored desiccated at -80°C . It was subsequently found that this material gradually lost activity after storage at -80°C but that this could be avoided by addition of 1 mM EDTA to the pooled active fractions obtained following ion exchange chromatography. Prior to freeze-drying and storage, these fractions were desalted against MilliQ water.

IEF. Pre-cast gels, pH range 3–10, were obtained from Novex; they were 1.0 mm thick and contained 5% acrylamide with 2% ampholytes. Gels were run on a 'Mighty Small II' dual-cooled vertical slab gel electrophoresis unit (Hoeffer). The anode buffer was 10 mM phosphoric acid and the cathode buffer consisted of 20 mM each of the free bases of lysine and arginine. Lyophilized preparation (see above) was dissolved in double-strength sample buffer (Novex) at a concentration of 100 $\mu\text{g protein ml}^{-1}$. Samples (25 μl) were loaded into two lanes of the gel, which was then electrophoresed at 100 V for the first hour, 200 V for the second hour and finally at 500 V for 30 min. After electrophoresis, the gel was blotted onto Problott (Applied Biosystems), using a Hoeffer TE42 Transfer Electrophoresis Unit, over 2 h using 250 mA constant current. The membrane was then washed and cut to separate the two lanes. One lane was stained for AP activity as described above; the other lane was stained with Coomassie blue (0.025% Coomassie blue R-250, 40% methanol, 10% acetic acid) for 1 min before destaining with 10% acetic acid in 50% methanol. The band having AP activity was identified by matching the two halves. It was excised from the Coomassie-blue-stained half of the membrane, air-dried and the N-

terminal sequence was determined using a Hewlett Packard G1000A Protein Sequencer with an on-line HP1090A HPLC system. A search of protein sequences was conducted using the database of the European Bioinformatics Institute, BLITZ service, which uses a MPsrch program from the University of Edinburgh, UK.

pI and molecular mass determination. The pI of the enzyme was determined using the IEF technique described above with an additional lane containing reference compounds within the pI range of 4.45–9.6 (Bio-Rad). Proteins were fixed with 20% TCA and visualized by staining the slab gel with Coomassie blue (see above), followed by destaining in several changes of 10% acetic acid in 40% ethanol.

Molecular mass determination was carried out by Western blotting the IEF gel onto cellulose nitrate membrane using a Tris/glycine/methanol buffer. The band showing AP activity was excised and the protein was eluted by immersion in approximately 1 ml 50% acetonitrile and then lyophilized. Samples were then redissolved in MilliQ water and the molecular mass was determined by SDS-PAGE techniques (Laemmli, 1970), using 12% acrylamide with stacking gels of 4% acrylamide. Broad-range molecular mass markers ranging in mass from 14.3 kDa to 170 kDa (Boehringer) were also run.

Enzyme reactivation with various cations. As described above, the stability of the enzyme activity was preserved by treatment with 1 mM EDTA, prior to freeze-drying. Accordingly, prior to characterization of the enzyme, reactivation by various metal ions, at final concentrations of 10 μ M, 0.1 mM and 1.0 mM, respectively, was studied. Briefly, 25 μ l of appropriately diluted enzyme in 30 mM Tris/HCl, pH 7.5, was pre-incubated for 10 min at 37 °C with 25 μ l of the test metal ion. The substrate, 25 μ l 3 mM Leu-Gly-Gly in the above buffer, was then added and the mixture was incubated for a further 20 min. The reaction was stopped by the addition of 75 μ l ice-cold 1,10-phenanthroline (final concentration, 1 mM). The release of leucine, as a measure of enzyme activity, was determined by a modification of the cadmium–ninhydrin method of Doi *et al.* (1981). Co^{2+} was the most effective in enzyme reactivation, the optimum concentration being 0.1 mM. Mn^{2+} was less effective while Ca^{2+} and Mg^{2+} were completely ineffective. Accordingly, all further experiments were conducted in the presence of Co^{2+} at a final concentration of 0.1 mM. Inhibition by 1,10-phenanthroline was found to be completely irreversible.

Effect of pH and buffer strength. Activity against Leu-Gly-Gly as a function of pH was tested using 100 mM phosphate buffer at pH values of 5.5, 6.5 and 7.5 and Tris/HCl buffer at pH values of 7.5, 8.0, 8.5, 9.0 and 9.5. Briefly, an appropriate concentration of purified enzyme in distilled water was pre-incubated for 10 min at 37 °C with buffer containing Co^{2+} at a final concentration of 0.1 mM. The substrate Leu-Gly-Gly was added to give a final concentration of 3 mM. After incubation at 37 °C for 20 min, peptidase activity was assayed by measuring the amount of free leucine released by the cadmium–ninhydrin method (see above). The effect of buffer strength was determined by testing, at strengths of 100, 60 and 30 mM, each of the following buffers: phosphate at pH 7.5 and Tris/HCl at pH values of 7.5 and 8.0. The protocol described above was used.

Substrate specificity. The spectrum of peptidase activity was investigated using a number of unsubstituted peptides, ranging from di- to heptapeptides (see Table 2). Based on prior results, these experiments were conducted at pH 8.0 using Tris/HCl buffer at a final concentration of 10 mM. The reaction mixture

consisted of 100 μ l 30 mM Tris/HCl, pH 8.0, containing 0.3 mM Co^{2+} , 100 μ l appropriately diluted enzyme solution in distilled water and 100 μ l peptide in distilled water. It was incubated at 37 °C for 2, 5, 10 and 20 min in the case of di- and tripeptides and for 30, 45 and 60 min in the case of larger peptides. At the appropriate times, 60 μ l of the reaction mixture was removed and added to 60 μ l ice-cold 1,10-phenanthroline to stop the reaction. After equilibration at room temperature, free amino acid and peptide levels in the samples were determined by a previously described reverse-phase HPLC (RP-HPLC) technique (Rogers *et al.*, 1991). From such data, rates of peptide breakdown could be determined.

Kinetic parameters. The apparent K_m and V_{max} values for the peptidase were determined using the substrates Leu-Gly-Gly and Leu-Ala. Rates of breakdown were determined using four different substrate concentrations ranging from 0.5 to 5.0 mM. The protocol used was that described above where the release of leucine from each substrate was assayed by the RP-HPLC technique. The K_m and V_{max} values were computed from the slope and intercept of the regression line of Lineweaver–Burk plots.

Effect of inhibitors and metal ions on peptidase activity. Experiments were performed using a variety of enzyme inhibitors and metal ions, usually at 1 mM final concentration. Reaction mixtures containing 25 μ l appropriately diluted enzyme solution in 30 mM Tris/HCl buffer, pH 8.0, were pre-incubated with 25 μ l inhibitor or metal ion for 10 min at 37 °C prior to the addition of 25 μ l of the substrate Arg-Gly-Gly (3 mM in 0.3 mM Co^{2+}). After incubation for a further 20 min, the reaction was stopped by the addition of 75 μ l ice-cold 1,10-phenanthroline (final concentration, 1 mM). Enzyme activity was measured, as described above, by the RP-HPLC technique. Inhibition or stimulation of enzyme activity was expressed as a percentage of the activity without modifiers.

Effect of various protease inhibitors on the growth of *F. nucleatum* in defined and complex media. If peptidase activity is important to the survival and growth of the organism, then appropriate inhibitors added to a complex medium lacking the relevant energy-yielding amino acids (glutamate, histidine, serine and lysine) in free form might be expected to reduce or even prevent growth. In contrast, such growth inhibition should not occur when the growth medium is chemically defined. Accordingly, a 1.75% (w/v) brain heart infusion broth (BHI; Oxoid), to which was added 0.5 g cysteine l^{-1} , was inoculated with an exponential phase culture grown in the same medium. An identical broth containing the appropriate test inhibitor (see Table 3) was also inoculated. This medium had previously been shown to contain levels of glucose and the four energy-yielding amino acids, in free form, well below those supporting visible batch culture growth. A chemically defined broth medium (CDM) devoid of fermentable carbohydrate was used in parallel with the BHI broth. It contained 20 mM each of glutamate, histidine, serine and lysine (Rogers *et al.*, 1991, 1992) and both control and inhibitor-containing broths were inoculated with an exponential phase CDM-grown culture. All inoculated broths were incubated anaerobically at 37 °C and the OD_{560} was measured (Spectronic 20; Bausch & Lomb) at intervals of about 3 h for 21 h.

RESULTS

Peptidase activity could not be detected in either culture supernatants or cytoplasmic contents and was associated with the cell envelope fraction.

Table 1. Purification of *F. nucleatum* AP

Data are means of three separate experiments; differences did not exceed about 10% between experiments.

Fraction	Volume (ml)	Total activity (U)*	Total protein (mg)	Specific activity [U (mg protein) ⁻¹]	Purification factor (-fold)	Yield (%)
Crude extract (powder)	340	5400	2250.0	2.4	1.0	100.00
(NH ₄) ₂ SO ₄ fractionation	44	1840	88.6	20.8	8.7	3.90
Hydrophobic interaction chromatography	26	1656	12.0	138.0	58.0	0.50
Ion exchange chromatography	2	512	2.0	256.0	107.0	0.09

* One unit of AP activity is defined as the reciprocal of the double dilution end point giving a positive L-amino-acid oxidase reaction when incubated in the presence of Leu-Gly-Gly (see text).

Table 2. Relative rates of breakdown of various peptides by an *F. nucleatum* peptidase

Data represent the means of three separate experiments in which differences did not exceed 10%. Rate of breakdown using Leu-Gly-Gly as substrate was taken as 1. This was equivalent to a rate of 0.09 $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

Peptide	Rate	Peptide	Rate
Leu-Ala	24.4	Val-Leu-Ser	0.8
Arg-Gly	15.7	His-Gly-Gly	0.3
Phe-Ala	14.5	Ser-Gly-Gly	0.3
Ser-Ala	8.3	Pro-Gly-Gly	0.2
Glu-Ala	8.2	Glu-Gly-Phe	0.1
Lys-Ala	6.9	Bz-Gly-Arg	0.0
Arg-Ala	5.8	Met-Arg-Phe-Ala	0.0
Pro-Ala	4.3	Leu-Trp-Met-Arg	0.0
Gly-Arg	2.4	Gly-Lys-Arg-Trp	0.0
His-Ala	0.7	Tyr-Glu-Glu-Trp	0.0
Gly-Gly-Arg	1.5	Gly-Gly-Lys-Ala-Ala	0.1
Arg-Gly-Gly	1.4	Tyr-Gly-Gly-Phe-Leu	0.0
Gly-Ser-Phe	1.3	Leu-Trp-Met-Arg-Phe	0.0
Ala-His-Lys	1.2	Gly-Lys-Arg-Trp-Gly	0.0
Lys-Gly-Gly	1.1	Leu-Trp-Met-Arg-Phe-Ala	0.0
Leu-Gly-Gly	1.0		

Enzyme purification

An AP was partially purified by the methods described above and the results of each step of a typical purification protocol are summarized in Table 1. In the (NH₄)₂SO₄ fractionation steps, two-thirds of the activity was lost, mostly at the 70% saturation level. However, it resulted in a nine-fold purification factor. The two column chromatography steps ultimately resulted in a purification factor of 107. When run on a 12% acrylamide SDS-PAGE gel (4% stacking gel) the material obtained after ion exchange chromatography showed two well-separated bands of 64.9 and 54 kDa with a minor band of 21.2 kDa. This partially purified

enzyme preparation was used in the subsequent characterization tests.

Properties of the peptidase

pI and molecular mass. IEF showed that the enzyme had a pI of pH 5.1. Elution of the active band from the Western blot and subsequent SDS-PAGE revealed that it had a molecular mass of 54 kDa.

Effect of pH on enzyme activity. The optimum pH for peptidase activity was shown to be between 7.5 and 8.0 in the presence of 30 mM Tris/HCl.

Substrate specificity. The partially purified enzyme hydrolysed a variety of unsubstituted di- and tripeptides but did not attack larger peptides or the N-terminally blocked dipeptide Bz-Gly-Arg (Table 2). The dipeptides were broken down more rapidly than the tripeptides, which were attacked N-terminally. In addition, the enzyme failed to hydrolyse the β -naphthylamide or *p*-nitroanilide derivatives of a variety of amino acids.

Effects of inhibitors and metal ions. The chelating agents EDTA and 1,10-phenanthroline totally inhibited enzyme activity, the latter being effective at 0.1 mM. As noted above, EDTA-inactivated activity could be restored most effectively by 0.1 mM Co²⁺, less so by Mn²⁺ and not at all by Ca²⁺ and Mg²⁺. However, as also noted above, inhibition by 1,10-phenanthroline was irreversible. Amastatin and bestatin, each at a final concentration of 0.1 mM, also totally inhibited enzyme activity. Iodoacetate and *p*-hydroxymercuribenzoate showed at least 90% inhibition while leupeptin, PMSF and 2-mercaptoethanol showed only 35, 26 and 13% inhibition, respectively. Of the divalent cations, Zn²⁺, Hg²⁺ and Ni²⁺ reduced activity by 93, 82 and 43%, respectively; Mg²⁺ and Mn²⁺ were slightly stimulatory.

Kinetic properties. Using the substrates Leu-Ala and Leu-Gly-Gly, the respective K_m values were 0.66 and 4.0 mM; the respective V_{max} values were 0.12 and 0.09 $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

Table 3. Effect of various protease inhibitors on the batch culture growth of *F. nucleatum* ATCC 10953

Experiments were performed on three separate occasions; differences did not exceed 10% between experiments. Broth cultures were incubated anaerobically at 37 °C in either a chemically defined medium (CDM) or BHI, both containing the inhibitor as indicated. Optical density readings were taken at about 3 h intervals up to 21 h. Figures in parentheses represent the change in OD₅₆₀ expressed as a percentage of the change in OD₅₆₀ in the control.

Inhibitor	Change in OD ₅₆₀			
	CDM		BHI	
	0-9 h	0-21 h	0-9 h	0-21 h
Bestatin, 0.4 mM	0.25 (90)	0.41 (89)	0.20 (50)	0.20 (29)
Iodoacetate, 0.2 mM	0.05 (14)	0.05 (11)	0.06 (15)	0.14 (21)
1,10-Phenanthroline, 0.1 mM	0.27 (97)	0.43 (94)	0.12 (30)	0.10 (14)
None (control)	0.28	0.46	0.40	0.70

N-terminal sequence of the enzyme. The first 20 amino acid residues were determined to be MDXKXYVDLKERFLRYVKFN. As can be seen, residues 3 and 5 could not be identified. Complete N-terminal sequence homology could not be identified through the MPsrch protein-protein database search but a 62% match was found between residues 8-20 of the enzyme and residues 9-21 of *Haemophilus influenzae* strain Rd peptidase T (pep T) (EC 3.4.11. -).

Effect of various protease inhibitors on the batch culture growth of *F. nucleatum*. Most evident was the fact that iodoacetate caused significant depression of CDM-based growth while the remaining inhibitors had little or no effect (Table 3). However, BHI-based growth was markedly reduced in the presence of bestatin (an AP inhibitor) and 1,10-phenanthroline (a metalloprotease inhibitor), as well as by iodoacetate, which probably has wider inhibitory properties against other metabolic (enzymic) functions.

DISCUSSION

The finding that the peptidase activity appeared to be associated with the cell envelope fraction is in agreement with our earlier studies on the processing of small peptides by resting cells of *F. nucleatum* (Rogers *et al.*, 1992) and the findings of Bakken *et al.* (1989), who suggested that small peptides may be cleaved external to the inner membrane of this organism. The APs, particularly those of *Escherichia coli* and the lactic acid bacteria, are usually cytoplasmic but this does not exclude the possibility that some may be associated with the cell envelope (Gonzales & Robert-Baudouy, 1996). Indeed, for those peptidases involved in bacterial nutrition, location close to membrane systems involved in the transport of exogenous peptides would facilitate reaction with relevant substrates as soon as they enter the cell (Gonzales & Robert-Baudouy, 1996). It is also worth noting that the apparent location of enzyme

activities can be affected by growth rate (Minhas & Greenman, 1989; Spratt *et al.*, 1995).

Since the optimal pH for activity of the peptidase and the pH of periodontal pockets and subgingival plaque are within the range 7.5-8.0 (Bickel & Cimasoni, 1986; Eggert *et al.*, 1991), the peptidase, if produced *in vivo*, would be active against natural substrates, particularly those in gingival crevicular fluid.

The finding that the peptidase N-terminally attacked a variety of small peptides and lacked both endo- and carboxypeptidase activities indicates that it is an AP of broad substrate specificity. Based on their catalytic mechanisms (sensitivity to various inhibitors), bacterial APs can be divided into three main groups, the largest of which is the metallo-APs. The activity of these enzymes is regulated by the presence of divalent metallic cations and they are inhibited by chelating agents and by bestatin and amastatin. While Zn²⁺ is the most frequently associated cation, there is a group of Co²⁺-requiring metallo-APs (Gonzales & Robert-Baudouy, 1996) which includes a methionine AP from *E. coli* (Roderick & Matthews, 1993), a dipeptidase from *Lactobacillus sake* (Montel *et al.*, 1995) and an AP from *Streptococcus mitis* (Andersson *et al.*, 1992). Based upon its substrate specificity and sensitivity to various inhibitors, especially metal ion chelators, the *F. nucleatum* AP could therefore be categorized as a Co²⁺-activated metallo-AP. Like most enzymes of this type (e.g. Montel *et al.*, 1995), the sensitivity of the AP to *p*-hydroxymercuribenzoate and iodoacetate indicated that thiol groups are involved in catalysis. Also, the ability of Mn²⁺, albeit less than that of Co²⁺, to restore the activities of EDTA-inactivated enzyme is consistent with the behaviour of other metallo-APs (e.g. Montel *et al.*, 1995).

In terms of its enzymic properties, the AP displayed Michaelian saturation kinetics with regard to the substrates tested. The values obtained indicate a mod-

erate or weak substrate affinity, common for bacterial APs. The low pI value and alkaline pH optimum are also typical (Gonzales & Robert-Baudouy, 1996).

The use of a wide variety of peptides as nutrients is among the important physiological roles of bacterial APs (Lazdunski, 1989). For example, the proteolytic system of organisms such as *Lactococcus lactis* is of importance to the dairy industry and has been well characterized. Peptide breakdown by these organisms is accomplished by the combined action of a number of peptidases, often with overlapping and/or complementary functions (Tan *et al.*, 1993). *Salmonella typhimurium* also possesses a number of different peptidases displaying broad specificities and overlapping activities. With the exception of a methionine-specific AP that removes N-terminal Met residues from proteins (Miller *et al.*, 1989), none of them is essential for the cells' physiology and survival (Lazdunski, 1989). Allowing for the fact that different strains were used, it appears that *F. nucleatum* may contain more than one peptidase, since our previous studies (Rogers *et al.*, 1992) showed that resting cells could attack peptides containing up to five residues while the AP described here could not. The co-operative effects of such peptidases would be vital to the growth and survival, in the subgingival environment, of potential pathogens such as *F. nucleatum*. The notion that peptidase activities are of nutritional importance is supported by the present findings that both bestatin and 1,10-phenanthroline inactivated the isolated metallo-AP and that these inhibitors also markedly affected the organism's growth in BHI but not CDM. The ability of these two compounds to inhibit the peptidase activities and growth of Gram-negative anaerobes has previously been reported (Grenier, 1992; Grenier & Michaud, 1994; Wallace & McKain, 1996). Growth in BHI but not CDM would be reliant on peptides to provide the necessary energy-yielding amino acids; this, in turn, would be dependent upon functional (amino) peptidase activities. Metabolizable peptides could be provided from the degradation of proteins and oligopeptides via the trypsin-like protease (endopeptidase) activities of organisms such as *Capnocytophaga gingivalis* (Spratt *et al.*, 1995) and *P. gingivalis* (Marsh *et al.*, 1994; Pike *et al.*, 1994). Among the key determinants of the microbial ecology, and thus pathogenic potential, of organisms in periodontal pockets may be the efficiency of peptide utilization among the various species (Tang-Larsen *et al.*, 1995).

Finally, we have also isolated, purified and characterized a Co^{2+} -activated metallo-AP from *F. nucleatum* ATCC 25586. Its characteristics are almost identical to those described here for strain ATCC 10953.

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