Biochemical characterization of the arginine-specific proteases of *Porphyromonas gingivalis* W50 suggests a common precursor

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Extracellular proteases of *Porphyromonas gingivalis* specific for arginyl peptide bonds are considered to be important virulence factors in periodontal disease. In order to determine the number, inter-relationship and kinetic properties of these proteases, extracellular enzymes with this peptide-bond specificity were purified and characterized from *P. gingivalis* W50. Three forms, which we denote RI, RI-A and RI-B, accounted for all of the activity in the supernatant. All three enzymes contain an α chain of ~ 54 kDa with the same N-terminal amino acid sequence. RI is a heterodimer of non-covalently linked α and β chains which migrate to the same position on SDS/PAGE but which can be resolved by 8 M urea/PAGE. RI-A and RI-B are both monomeric, but the molecular mass of RI-B (70–80 kDa) is significantly increased due to post-translational modification with lipopolysaccharide. All forms show absolute specificity for peptide bonds with Arg in the P1 position and are also capable of hydrolysing N-terminal Arg and C-terminal Arg–Arg peptide bonds. Thus they show limited amino- and carboxy-peptidase activity. For the hydrolysis of *N*³-benzoyl-L-Arg-*p*-nitroanilide, the pH optimum is 8.0 at 30 °C. The $V_{\max}$ for all three enzymes is controlled

by ionization of two residues with apparent pK_a s at 30 °C of 6.5 ± 0.05 and 9.7 ± 0.05 , and ΔH values of ~ 29 kJ/mol and ~ 24 kJ/mol in the enzyme–substrate complex. By analogy with papain, the pK_a of 6.5 could be ascribed to a Cys and the pK_a of 9.7 to a His residue. E-64 [L-*trans*-epoxysuccinyl-leucylamide-4-(4-guanidino)butane] is a competitive inhibitor of RI, RI-A and RI-B. Based on physical properties and kinetic behaviour, RI-A appears to be analogous to gingipain from *P. gingivalis* HG66. However the α/β structure of RI differs significantly from that of the high-molecular-mass multimeric complex of gingipain containing four haemagglutinins described by others. Since the genes for RI and high-molecular-mass gingipain are identical, the data indicate that an alternative processing pathway is involved in the formation of RI from the initial precursor. Furthermore, the identical N-termini and enzymic properties of the catalytic component of RI, RI-A and RI-B suggest that the maturation pathway of the RI precursor may also give rise to RI-A and RI-B. The physiological functions of these isoforms and their role in the disease process may become more apparent through examination of their interactions with host proteins.

INTRODUCTION

Porphyromonas gingivalis, a Gram-negative, black-pigmented anaerobe present in the microflora of sub-gingival plaques of patients with progressive periodontitis, is strongly implicated in the aetiology of this disease [1,2]. Among the many virulence factors produced by this organism, the proteolytic enzymes are of particular importance. Several physiologically important proteins, including collagens [3,4], fibrin and fibrinogen [5], fibronectin [6,7], plasma protease inhibitors [8], immunoglobulins [9–11] and complement factors [10,12,13], have been shown to be degraded by proteases from *P. gingivalis*. It is therefore plausible that these proteases may play a major role in evading host defence mechanisms, in addition to causing destruction of the tissues supporting the teeth, and thus function as important virulence factors. However, the relationships of these proteolytic activities to the periodontal disease process is relatively unknown. This is largely due to the heterogeneities in the numbers, molecular masses and specificities of the various proteases of this organism that have been reported in the literature. They can be broadly classified as collagenases [4,14,15], Gly–Pro dipeptidase [16], and alkaline proteases specific for Lys [17,18] and Arg [18–22] peptide bonds.

Biochemical investigations of the Arg-X proteases (those acting on Arg–Xaa peptide bonds) have led to conflicting views on the numbers, molecular masses and peptide-bond specificities of these enzymes (reviewed by Potempa et al. [23]). Active-site labelling of the extracellular Arg-X proteases using radioiodinated [24] or biotinylated [25] arginyl chloromethyl ketone inhibitors has clearly demonstrated the presence of two catalytic polypeptides (~ 50 kDa and 70–80 kDa), although lower-molecular-mass forms have also been reported [21]. Chen et al. [20] purified and characterized an Arg-specific 50 kDa thiol protease, gingipain, from the culture supernatants of *P. gingivalis* HG66, and Pike et al. [18] characterized a high-molecular-mass gingipain (HMG) (95 kDa) which contained the gingipain monomer in association with three different sets of lower-molecular-mass proteins. Furthermore, Ciborowski et al. [22] have isolated a high-molecular-mass enzyme (120–180 kDa) which contains both Arg- and Lys-specific protease activity. It would therefore appear that a number of different forms of Arg-specific protease may be produced by *P. gingivalis*, but their total number, inter-relationships and comparative kinetics remain uncertain.

The present paper describes the purification and properties of three forms of proteases, specific for Arg peptide bonds, which

Abbreviations used: Arg-X protease, protease acting on Arg–Xaa peptide bonds; D,L/L-BRpnA, *N*³-benzoyl-D,L/L-Arg-*p*-nitroanilide; DTT, dithiothreitol; Caps, 3-(cyclohexylamino)-1-propanesulphonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid; E-64, L-*trans*-epoxysuccinyl-leucylamide-4-(4-guanidino)butane; HMG, high-molecular-mass gingipain; MALDI-ToF, matrix-assisted laser-desorption ionization time of flight; RpnA, Arg-*p*-nitroanilide.

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account for all the extracellular Arg-X protease activity from *P. gingivalis* W50 but which differ in several respects from the enzymes described above. Studies of the cloning, sequencing and expression of these enzymes are described in Aduse-Opoku et al. [26]. The data form the basis for the determination of the physiologically relevant target substrates and inhibitors of these enzymes and their role in the periodontal disease process.

EXPERIMENTAL

Materials

DEAE-Sephacel, Sephacryl S-200 HR and Sephacryl S-300 HR were obtained from Pharmacia Biotech (St. Albans, Herts., U.K.). Urea (Enzyme grade) and guanidine hydrochloride were purchased from Gibco-BRL (Life Technologies Ltd., Paisley, Scotland, U.K.). L-Arg conjugated to 4% beaded agarose, *N*-benzoyl-D,L-Arg-*p*-nitroanilide (D,L-BRpNA) and other peptide substrates were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or from Bachem Feinchemikalien AG (CH-4416 Bubendorf, Switzerland). Zwittergent 3-14 detergent was purchased from Calbiochem Novabiochem UK Ltd. (Nottingham, U.K.). All other chemicals and reagents were the purest grades available and were from BDH Chemicals (Poole, Dorset, U.K.) or Sigma.

Bacterial strains and growth conditions

Porphyromonas gingivalis W50 was obtained from Professor P. Marsh (PH LS, Porton Down, Wilts., U.K.). The bacterium was maintained on blood agar plates [2.5% (v/v) horse blood (TCS, Buckingham, U.K.) was added to sterile 4% blood agar base No.2 (Oxoid Ltd., Basingstoke, Hants., U.K.) in distilled water] at 37 °C in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, W. Yorkshire, U.K.) equilibrated in 80% nitrogen/10% hydrogen/10% carbon dioxide. *P. gingivalis* W50 was grown anaerobically at 37 °C in liquid medium containing 3.7% (w/v) brain/heart infusion (BHI) (Oxoid) containing 0.0005% (w/v) haemin. Incubation in the anaerobic cabinet was allowed to proceed for 6 days.

Arg-X protease assay

The standard assay was performed in cuvettes of 1 cm light-path at 30 °C containing the enzyme sample (0.3–0.5 µg) in 1 ml of 0.5 mM D,L-BRpNA/10 mM L-Cys/10 mM CaCl₂/100 mM Tris/HCl buffer, pH 8.0, and the reaction was monitored by the increase in absorbance at 405 nm due to *p*-nitroaniline (molar absorption coefficient 8800 M⁻¹·cm⁻¹ at 405 nm) in a Kontron Uvicon 930 Spectrophotometer (Kontron Instruments Ltd., Watford, Herts., U.K.) fitted with a B. Braun Thermoregulator to maintain the temperature at 30 °C. One unit of protease catalyses the formation of 1 µmol of *p*-nitroaniline/min in this assay system. Some assays were also performed in the presence of 10 mM dithiothreitol (DTT). D,L-BRpNA was routinely used in all assays, except in the determinations of *K_m* for BRpNA and *K_i*s of competitive inhibitors, when the L-form was used.

Buffers used for kinetic analyses were Caps [3-(cyclohexylamino)-1-propanesulphonic acid]; Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid], Hepes, Mes, Mops and Tris/HCl. Unless otherwise specified, all buffers were adjusted to an ionic strength of 12 mM by addition of NaCl. pH adjustments were made at room temperature; the actual pH at the temperature of the experiment was calculated from heats of ionization of the buffers: ΔpH/°C = −0.011 for Mes, −0.013 for Mops, −0.014 for Hepes, −0.028 for Tris/HCl [27], −0.011 for Ches and −0.012 for Caps [28].

Enzyme purification

Cells from 2 litres of a 6-day culture of *P. gingivalis* W50 were spun down (10000 g, 60 min), and solid ammonium sulphate (enzyme grade) was added to the supernatant to 85% saturation to effect complete precipitation of Arg-X proteases. The precipitated protein was separated by centrifugation (10000 g, 60 min) and the dark-brown pellet was suspended in 70–80 ml of 50 mM sodium acetate buffer, pH 5.3, containing 0.0055% Zwittergent. The insoluble material was separated by centrifugation. The pellet was stored at 4 °C (see below). All further steps were performed at 4 °C unless otherwise indicated.

The soluble fraction (80 ml) was subjected to gel filtration on a column of Sephacryl S-200 HR (1900 ml) equilibrated in 50 mM sodium acetate buffer, pH 5.3, containing 0.0055% Zwittergent. Usually, two peaks of Arg-X protease activity (fractions SI and SII) were obtained. Each fraction was adjusted to pH 6.0 by the addition of solid sodium acetate and applied consecutively to a column of Arg-agarose (25 ml) equilibrated in 0.2 M NaCl/10 mM CaCl₂/0.0055% Zwittergent/25 mM Mops, pH 6.0. The column effluent was collected in each case (SIU and SIU). Elution was performed first with 0.3 M L-Lys in buffer (to elute any Lys-X protease) followed by 0.025 M L-Arg hydrochloride adjusted to pH 6.0 in buffer. Arg-X protease purified by this procedure was referred to as RI. Enzyme fractions judged to be pure by constant specific activity and SDS/PAGE were combined and dialysed against 25 mM sodium acetate buffer, pH 5.3, and 0.0055% Zwittergent, and stored at 4 °C.

Purification of RI-A

Further purification of fractions SIU and SIU was achieved after dialysis against 50 mM sodium acetate buffer, pH 5.3, and 0.0055% Zwittergent, by ion-exchange chromatography on DEAE-Sephacel equilibrated in the same solution. Enzyme was eluted by a series of linear gradients of sodium acetate to a final concentration of 500 mM. Enzyme fractions judged to be pure by constant specific activity and SDS/PAGE were combined and subjected to dialysis against 50 mM acetate buffer, pH 5.3, and 0.0055% Zwittergent, and stored at 4 °C. This Arg-X protease was referred to as RI-A (see below). The protein was essentially pure at this stage.

Purification of RI-B

The pellet was solubilized by the addition of 20 ml of aqueous 1% (w/v) Zwittergent followed by 40 ml of distilled water and 60 ml of 100 mM sodium acetate buffer, pH 5.3. It was then stirred gently at 4 °C for 1–2 h. Any insoluble material was removed by centrifugation and the supernatant was used as the source of enzyme RI-B. The procedure for the purification of RI-B was similar to that used for RI-A, except that 0.05% Zwittergent was included in the affinity and DEAE-Sephacel chromatography buffers. Ion-exchange chromatography was performed at 22 °C, as the detergent was liable to crystallize at lower temperatures. Enzyme fractions judged to be pure by specific activity and SDS/PAGE were combined and dialysed against 25 mM sodium acetate buffer, pH 5.3, and 0.011% (w/v) Zwittergent, and stored at 4 °C.

Determination of protein concentrations

Protein concentrations were determined by measurement of absorbance at 280 nm and 260 nm [28]. Protein concentration (mg/ml) was calculated according to the formula $1.55A_{280} - 0.76A_{260}$.

Gel electrophoresis

PAGE in the presence of SDS [29] was carried out at 10 °C in 12.5 % (w/v) polyacrylamide slab gels (10 cm × 7 cm × 0.15 cm). Samples of protease (10–20 µg) were treated with 50 µl of leupeptin (1 mM) at 25 °C for 20 min and heated at 100 °C for 5 min, followed by dialysis against 0.011 % (w/v) Zwittergent/50 mM ammonium bicarbonate. In some cases the protease solutions were very dilute, and to ensure total inhibition the samples were routinely heated. The dialysate was dried *in vacuo* before being treated with SDS sample buffer.

Gels were stained for the presence of carbohydrate by the procedure of Glossman and Neville [30]. PAGE in the presence of 8 M urea at pH 8.8 in 7.5 % polyacrylamide gels was carried out according to Marshall and Inglis [31].

N-terminal sequencing of proteins

Arg-X proteases were subjected to N-terminal sequencing either from solution or after SDS/PAGE after transfer to an Immobilon membrane (Millipore). Sequencing was performed at the Protein Sequencing Facility, Department of Biochemistry, University of Cambridge, Cambridge, U.K., the Haemostasis Research Group, Royal Postgraduate Medical School, London, U.K., or The Institute of Cancer Research, London, U.K.

Enzyme kinetics

Values of $V_{\max}$ and apparent K_m were derived by non-linear regression analysis of the data using the ENZFITTER Data Analysis Program (Biosoft, Cambridge, U.K.) with 2.5–250 µM L-BRpNA at 30.0 ± 0.1 °C. To determine K_i values of inhibitors using Dixon plots [32], three or four concentrations of L-BRpNA and five different concentrations of inhibitor {0–20 µM E-64 [L-*trans*-epoxysuccinyl-leucylamido-4-(4-guanidino)butane]; 0–450 µM benzamide; 0–50 µM methylguanidine; 0–100 mM L-Arg} were used. To determine the enthalpy of ionization, measurements were made in the temperature range 25–40 °C.

The reversibility of the effect of pH on rate over the assay periods was checked by incubating the enzymes at pH values between 5 and 11 and re-assaying them after re-adjusting the pH to 8.0. A possible dependence of reaction rate on the concentration and type of buffer was eliminated by varying these, while keeping the ionic strength constant. Acetate, Mes, Mops, Hepes, Tris, Ches and Caps did not show any interference. For simple measurements of maximum velocities of Arg-X enzymes, 0.25 mM L-BRpNA was used in the assays, which is at least 20-fold in excess of the K_m at pH > 7.5.

Experiments to examine the effects of Gly and Gly-Gly on enzyme activity were usually performed in 100 mM Tris/HCl/10 mM L-Cys/10 mM CaCl₂, pH 8.0 (at 30 °C).

Thermal inactivation experiments were performed in acetate buffers (pH 4.5–6.0) and Tris/HCl buffers (pH 7.5–9.0). Buffer (0.1 M) containing the desired additives was incubated in stoppered cryotubes at the appropriate temperature in a water-bath for a minimum of 1 h. A small volume of concentrated protein solution was added to the buffer to give a final concentration of 50 µg/ml. After thorough mixing, 10 or 20 µl aliquots were withdrawn at various times and assayed immediately at pH 8.0, 30 °C.

Enzyme specificity

RI and RI-A were dialysed exhaustively against 50 mM sodium acetate buffer, pH 5.3, to remove detergent prior to use in these experiments. Substrates (0.1 mg) were dissolved in 200 µl of

0.01 M CaCl₂/0.01 M β-mercaptoethanol/0.1 M Tris/HCl, pH 8.0, divided into two 90 µl portions and treated with RI and RI-A respectively at an enzyme/substrate ratio of 1:500 (w/w) at 22 °C. Aliquots (5 µl) were removed after 5, 10, 30, 60 and 120 min and added to 5 µl of 10 % trifluoroacetic acid to stop the reaction. These were further diluted into distilled water as required (usually 1:50) for MALDI-ToF (matrix-assisted laser-desorption ionization time of flight) mass spectrometric analysis. α-Cyano-4-hydroxycinnamic acid at 10 mg/ml in 70 % acetonitrile/30 % water was used as the matrix. A 0.5 µl sample was mixed with 0.5 µl of α-cyano-4-hydroxycinnamic acid and left to air-dry prior to analysis using a Finnigan MAT Laser Desorption Mass Spectrometer (Thermo BioAnalysis Ltd., Hemel Hempstead, Herts., U.K.).

RESULTS

Purification of Arg-X proteases from *P. gingivalis* W50

Table 1 summarizes the results of a typical purification from 2 litres of 6-day culture supernatant. Approx. 40 % of the total Arg-X activity was solubilized in aqueous buffer (soluble fraction; S) from the ammonium sulphate precipitation, and contained both RI and RI-A. The residual activity (pellet fraction; P), which probably represents the extracellular membrane vesicles produced by this organism, required detergent solubilization [0.05 % (w/v) Zwittergent 3-14 (Calbiochem)] and contained RI and RI-B.

Gel filtration of fraction S achieved partial separation of RI (predominantly in fraction SI) from RI-A (in fraction SII). Although RI has a higher molecular mass than RI-A, fraction SI contained a significant amount of RI-A activity, albeit of considerably lower specific activity. Selective purification of these two forms was then achieved by affinity chromatography on Arg-agarose columns, to which only RI binds under the conditions used. Elution with 0.3 M L-Lys followed by 0.025 M L-Arg achieved further purification of RI from contaminating Lys-X enzyme(s). Ion-exchange chromatography on DEAE-Sephacel of the activity not bound on the affinity column yielded RI-A. RI-B was separated from RI in the solubilized pellet fraction using the same affinity and ion-exchange chromatographic steps, except for the inclusion of 0.05 % detergent at each stage. The following yields were obtained: 12 % for RI, 18 % for RI-A and 3 % for RI-B. The lower yield of RI-B reflects the requirement for several ion-exchange chromatography steps in order to obtain enzyme of high specific activity. The specific activities were 11 units/mg, 17 units/mg and 17 units/mg for RI, RI-A and RI-B respectively with D,L-BRpNA as substrate at 30 °C, based on protein measured by absorbance at 280 nm and 260 nm.

Molecular mass and subunit structure

The apparent subunit size of RI-A and RI was 53 ± 1 kDa on reducing SDS/PAGE (Figure 1); RI-B appeared as a 'fuzzy' band of subunit size 70–80 kDa. SDS/PAGE under non-reducing conditions did not yield higher-molecular-mass bands for RI and RI-B. Only RI-B gave a positive carbohydrate stain (results not shown). Furthermore, only RI-B was strongly bound to a lipopolysaccharide binding matrix (Detoxi-gel; Pierce Chemical Co.). Binding to this gel was not abolished by treatment with 8 M urea or 10 % (v/v) formic acid, suggesting that the lipopolysaccharide was covalently attached to the protein.

The molecular sizes obtained by gel filtration were ~ 110 kDa for RI, ~ 55 kDa for RI-A and ~ 100 kDa for RI-B. Together with the SDS/PAGE analyses, these data suggested that RI

Table 1 Purification and yield of the three forms of Arg-X protease from *P. gingivalis* W50

For experimental details, see the text. The values are from 2 litres of culture supernatant. Fractions were assayed using 0.25 mM L-BRpNA containing 10 mM L-Cys/10 mM CaCl₂/100 mM Tris/HCl buffer, pH 8.0, at 30 °C.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)		Purification (fold)
				Step	Total	
Culture supernatant	852	30000	0.028	100	100	1
85% Ammonium sulphate	845	1500	0.56	100	100	20
Soluble fraction (S)	320	450	0.71	38	38	25.4
Gel filtration						
Fraction SI	153	281	0.54	48	18	19.3
Fraction SII	144	39	3.69	45	17	131.8
Affinity chromatography; fractions SI and SII (bound): RI	45	4.2	10.71	15	12	382.5
DEAE-Sepacel chromatography of (unbound) SI and SII: R-IA	150	8.8	17.0	47	18	608.6
Pellet fraction (P)	465	696	0.67	55	55	23.9
Affinity chromatography						
Fraction P (bound): RI	32	2.9	11	7	4	394
Fraction PU (unbound)	182	280	0.65	39	22	23.2
DEAE-Sepacel chromatography of PU: R-IB	27	1.6	16.9	6	3	603

Figure 1 PAGE of Arg-X proteases

Pure RI, RI-A and RI-B were treated with 1 mM leupeptin to inhibit protease activity and subjected to SDS/PAGE in 12.5% acrylamide gels (**a**). The molecular masses of marker proteins are indicated alongside the gel. Lane 1, marker proteins; lane 2, RI; lane 3, RI-A; lane 4, RI-B. RI was also subjected to 8 M urea/PAGE in 7.5% gels (**b**). Gels were stained for protein with Coomassie Blue.

is a dimer of similarly size subunits, RI-A is a monomer and RI-B a single subunit with covalent modifications. This was examined by N-terminal sequence analysis of the three enzymes, giving the following sequences: RI, YTPVEEKQNGRMIVIVAKKY... and SGQAEIVLEAHDVWNDGSGY...; RI-A and RI-B, YTPVEEKQNGRMIVIVAKKY....

RI-A and RI-B had identical N-terminal sequences over the first 20 cycles. However, analysis of RI gave two strong signals for each cycle which were present in stoichiometric amounts. One of these sequences was identical to that obtained for RI-A and RI-B. The dimeric nature of RI was then confirmed by 8 M urea/PAGE (Figure 1), which resolved the two chains. N-terminal sequence analysis of these chains demonstrated that the faster-migrating band had the N-terminal sequence Ser-Gly-Gln-Ala-Glu..., while the slower-migrating band had the N-terminal sequence common to RI-A and RI-B.

Enzymic properties

Maximum enzyme activity for all three proteases was obtained in the presence of 10 mM L-Cys and 20 μ M–10 mM Ca²⁺ at pH 8.0. While no amidase activity was detected in the absence of reducing agents, there was no lag period in attainment of maximal activity following introduction of the enzymes into the assay solution. Hence if reduction of thiol groups in the protein is essential for activity, this process occurs instantaneously.

RI is significantly less stable than the other two forms in the absence of Ca²⁺ at pH values of > 7.0 at 30 °C. For example, the half-life for the activity of RI is 20 min at 30 °C, pH 8.0, in the absence of Ca²⁺ and β -mercaptoethanol, whereas under similar conditions both RI-A and RI-B have half-lives of approx. 10 h. Similarly, in the presence of 10 mM β -mercaptoethanol, RI is at least 20-fold less stable than either RI-A or RI-B ($t_{1/2}$ of 30 min compared with 8.5 h). At pH values of < 6, however, all three enzymes are stable regardless of the presence/absence of Ca²⁺. All three enzymes are routinely stored at 4 °C at pH 5.3 in the absence of Ca²⁺ and reducing agents, and have retained full activity for over 1 year.

Linear Arrhenius plots for activity against 0.25 mM L-BRpNA in 10 mM DTT/10 mM CaCl₂/100 mM Hepes buffer, pH 8.0, over the temperature range 25–50 °C were obtained for all three enzymes. In all cases, the gradient corresponds to an activation energy of 28 kJ/mol.

Steady-state kinetics and substrate specificity

The chromogenic substrates Gly-Arg-*p*-nitroanilide, carbo-benzoyl-Lys-Arg-*p*-nitroanilide, *N*-acetyl-Arg-*p*-nitroanilide, Pro-Phe-Arg-*p*-nitroanilide, *N*-benzoyl-Arg-*p*-nitroanilide and Arg-*p*-nitroanilide (RpNA) were tested in the standard assay system. For all three enzymes, Pro-Phe-Arg-*p*-nitroanilide appeared to be the best substrate tested, giving the highest value for V_{max}/K_m (3.28 units/mg per μ M, compared with 1.83 units/mg per μ M for BRpNA in the case of RI). However, BRpNA was the substrate of choice because of its ease of availability and low cost. For all substrates tested, K_m values followed the pattern RI-B > RI-A > RI. RpNA (500 μ M) was

Figure 2 pH-dependence of $V_{\max}$, K_m and $V_{\max}/K_m$ for hydrolysis of L-BRpNA by RI-A

The enzyme was assayed at 30 °C against L-BRpNA in 0.1 M buffer containing 10 mM DTT and 10 mM CaCl_2 . (a) Assays were performed with 0.25 mM L-BRpNA, and values for $V_{\max}$ are fitted to theoretical two-ionization curves for groups of pK_a 6.46 ± 0.05 and 9.87 ± 0.05 . (b) Assays were performed with 1.25–250 μM L-BRpNA, and K_m values were obtained by non-linear regression analysis of the data. The points are fitted to a theoretical single-ionization curve for a group with a pK_a of 9.8 ± 0.3 . (c) The data from (a) and (b) are fitted to theoretical two-ionization curves for groups of pK_a 6.27 ± 0.1 and 8.73 ± 0.1 .

hydrolysed very slowly by all three enzymes (5% of values obtained for BRpNA), suggesting that they possess aminopeptidase activity. High concentrations of RpNA (250 μM) inhibited the rate of hydrolysis of BRpNA (125 μM) by ~25%.

RI and RI-A were found to cleave specifically at the C-terminal side of L-Arg residues in melittin, glucagon and insulin B chain (oxidized), as indicated in bold in the following sequences: melittin, G¹IGAVLKVL¹⁰TGLPALISW²⁰**KRKR**QQ; glucagon, H¹SQGTFTSDY¹⁰SKYLD²⁰SRRAQ²⁰DFVQW-

LMNT; insulin B chain, F¹VNQHL¹⁰CGSH¹⁰LVEALYL¹⁰VCG-²⁰ERGFF²⁰YTPKA³⁰.

MALDI-ToF analyses of digests at different time points showed that, with melittin, cleavage occurred first at Arg²⁴, followed by a second cleavage at Arg²². In the case of glucagon, four peptides (residues 1–17, 18–29, 1–18 and 19–29) were observed very early in the digestion, indicating cleavage at Arg¹⁷–Arg¹⁸ and Arg¹⁸–Ala¹⁹. However, peptide 1–18 was absent at later time points, suggesting cleavage at the C-terminal Arg¹⁷–Arg¹⁸ bond to generate the final product [glucagon(1–17)].

The hydrolysis of C-terminal Arg–Arg and N-terminal Arg–Xaa peptide bonds was examined using three other peptides: bradykinin analogue, M¹KRSR⁵GPSPR¹⁰R; bovine adrenal medulla peptide BAM-12p, R¹VGRP⁵E; H 1-7 (calf thymus histone H1; residues 34–40), R¹RKAS⁵GP. In the case of the bradykinin analogue, peptides comprising residues 1–3, 4–11, 1–5 and 6–11 were found very early in the digestion (5 min incubation). However, peptides 4–10 and 6–10 appeared in later time points (30 min incubation), confirming that both RI and RI-A are capable of hydrolysing C-terminal Arg–Arg peptide bonds. In the case of BAM-12p, peptides 1–4, 5–6 and 2–6 were found very early in the digestion, showing that these enzymes are capable of hydrolysing N-terminal Arg–Val and also internal Arg–Pro peptide bonds. Finally, in the case of H 1-7, only peptides 2–7 and 3–7 were found early in the digestion, suggesting that Arg–Arg–Xaa peptide bonds at the N-terminus can be cleaved consecutively by RI and RI-A. Thus RI and RI-A show aminopeptidase activity when Arg is the N-terminal amino acid.

Effect of pH on enzyme activity

pH-activity curves were determined against 0.5 mM D,L-BRpNA in the presence of 10 mM L-Cys or 10 mM DTT/10 mM CaCl_2 in a series of buffers over the pH range 5.5–10.6 at 30 °C. The pH optimum for all three enzymes is in the range pH 7.9–8.1, and the $V_{\max}$ depends on two ionizing groups with pK_a values of 6.50 ± 0.05 and 9.7 ± 0.05 . Only the data for RI-A are shown (Figure 2, top panel).

To determine the enthalpy of ionization of the groups that control $V_{\max}$, assays exactly analogous to those described above (Figure 2, top panel) were performed at various temperatures and at various pH values. van't Hoff plots of pK_1 versus $1/T$ and pK_2 versus $1/T$ for RI-A gave enthalpies of ionization of 29.3 ± 3 kJ/mol (6.99 kcal/mol) and 24.2 ± 3 kJ/mol (5.76 kcal/mol) for pK_1 and pK_2 respectively.

pH-dependence of K_m and $V_{\max}/K_m$

Figure 2 (middle panel) shows the values of K_m for hydrolysis of L-BRpNA by RI-A obtained from a series of assays at 30 °C at various substrate concentrations over a range of pH values in the presence of DTT as reducing agent. The K_m for BRpNA increases at alkaline pH, and the theoretical single ionization curve fitted to the data is for a single ionization constant of pK_a 9.8 ± 0.3 . From these data, values of $V_{\max}/K_m$ can be derived, as shown in Figure 2 (bottom panel). The data fit the theoretical curves for two ionizing groups with pK_a values of 6.27 ± 0.1 and 8.73 ± 0.1 .

The amidolytic activities of RI, RI-A and RI-B increased between 1.5- and 2-fold in the presence of Gly (100 mM), and 5–6-fold in the presence of Gly–Gly (100 mM). However, since the $V_{\max}/K_m$ ratio was unaffected, stimulation by these compounds *in vivo*, perhaps derived from collagen breakdown, would only be relevant in the presence of excess substrate.

Figure 3 Competitive inhibition of RI-A by E-64

A range of L-BRpNA concentrations (5–50 μM ; s) was assayed at 30 °C in the presence of 0–20 μM E-64 ($[I]$). The Dixon plots show competitive inhibition.

Inhibition by E-64, iodoacetate and iodoacetamide

Proteases were incubated in 0.1 M Tris/10 mM Ca^{2+} /10 mM DTT or β -mercaptoethanol, pH 8.1, for 30 min at 22 °C with 1 mM E-64, and aliquots (10 or 20 μl) were withdrawn at regular time intervals over a period of 3 h and assayed against L-BRpNA (250 μM) at 30 °C, such that the final concentration of E-64 in the assay solution was 10–20 μM . There was no loss of enzyme activity during this period. However, if E-64 was incorporated into the assay buffer at a concentration of 0.1 mM, there was a large decrease in enzyme activity which could be reversed by a high concentration (500 μM) of substrate. These data indicate that E-64 is not an irreversible inhibitor of these enzymes, but a

Figure 4 Effects of iodoacetate and iodoacetamide on RI-A activity

Portions of RI-A (0.05 mg/ml; 100 μl) in duplicate were mixed with 200 μl of 12.5 mM CaCl_2 /125 mM Tris/HCl buffer, pH 8.0, and also with buffer containing 15 mM β -mercaptoethanol at 30 °C for 20 min. To one tube from each set was added 200 μl of iodoacetate or iodoacetamide (25 mM in 12.5 mM CaCl_2 /125 mM Tris/HCl buffer, pH 8.0) previously incubated at 30 °C, and aliquots (40 μl) were withdrawn at different times and added to 0.8 ml of assay buffer (12.5 mM L-Cys/12.5 mM CaCl_2 /125 mM Tris/HCl buffer, pH 8.0, 30 °C). The enzyme activity remaining was measured by the addition of 0.2 ml of 2.5 mM D,L-BRpNA and monitoring the increase in absorbance at 405 nm; the data are shown as first-order plots. ●, iodoacetate; ▲, iodoacetamide; ○, β -mercaptoethanol + iodoacetate; △, β -mercaptoethanol + iodoacetamide.

competitive inhibitor. Dixon plots for the inhibition of RI-A by E-64 are shown in Figure 3, and the K_i values for E-64, methylguanidine, benzamidine and L-arginine are given in Table 2.

Iodoacetate and iodoacetamide at a concentration of 5 mM in the assay buffer decreased the activity of all three enzymes to

Table 2 Kinetic constants for Arg-X protease activity at 30 °C

Kinetic constants were measured as described in the Experimental section. The pH at which measurements were made is given, where relevant. Enthalpies of ionization of groups controlling V_{max} (kJ/mol) are given in parentheses. n.d., not determined.

Parameter	pH	RI	RI-A	RI-B
K_m (μM)	8.0	6.1	10 (46*)	13.8
V_{max} (units/mg)	8.0	11	17 (85*)	17
V_{max}/K_m (units/mg per μM)	8.0	1.83	1.7 (1.85*)	1.23
pK values controlling:				
K_m (± 0.3)		9.75	9.80	9.84
V_{max} (± 0.05)		6.5 and 9.84 (33.5 and 24.7 kJ/mol)	6.55 and 9.73 (29.3 and 24.2 kJ/mol)	6.52 and 9.68 (37.8 and 24.6 kJ/mol)
V_{max}/K_m (± 0.1)		6.30 and 8.60	6.27 and 8.73	6.20 and 8.75
Competitive inhibitors (K_i ; μM)				
E-64	6.4	1.5	2.1	2.2
	7.2	1.5	2.3	2.1
	8.0	1.5	2.3	1.9
	8.0	90	112	112
Benzamidine	6.4	14	14	14
	7.3	12	14	14
	8.0	12	12	14
	8.8	n.d.	15	n.d.
	10.0	n.d.	15	n.d.
L-Arginine	8.0	5000	7500	8000

* Measured in the presence of 50 mM Gly–Gly.

~ 57% and ~ 17% respectively, probably due to a decrease in the effective concentration of Cys in the assay buffer. Incubating the enzymes with 10 mM iodoacetate or iodoacetamide in pH 8.0 buffer containing 10 mM Ca^{2+} at 30 °C for 30 min had no effect on enzyme activity. However, on treating the enzymes with 10 mM β -mercaptoethanol at 30 °C for 20 min prior to incubating with alkylating agents, there was complete irreversible inactivation of the enzymes. Figure 4 shows the pseudo-first-order plots for one such inactivation with a large excess of alkylating agent, the exact concentration of which is difficult to gauge because of the presence of β -mercaptoethanol in the reaction mixture. In order to determine whether an active-site residue was being modified, the experiment was conducted in the presence of the competitive inhibitor methylguanidine (350 μM). Identical pseudo-first-order rate constants were obtained, indicating that methylguanidine did not protect against irreversible inactivation by iodoacetate or iodoacetamide.

DISCUSSION

Structure

Active-site labelling of *P. gingivalis* culture supernatants [24] demonstrated the presence of just two catalytic chains of different molecular mass, and this has been confirmed in the present study: in RI and RI-A the catalytic polypeptide has a molecular mass of ~ 54 kDa, whereas RI-B, a form probably associated with vesicles, has a molecular mass of 70–80 kDa due to post-translational modification. We have therefore been unable to substantiate other reports which describe the purification of an approx. 50 kDa Arg-specific protease from this organism. Given that the N-terminal amino acid sequence of at least one of these lower-molecular-mass enzymes [33] is the same as that of the α chain of RI-A, it is possible that either they represent C-terminally nicked versions of the α chain generated during purification procedures or that they are the product of a truncated form of the same gene. RI-A appears to be analogous to gingipain from *P. gingivalis* HG66 [20] on the basis of molecular mass, N-terminal sequence and stimulation by Gly–Gly. The relationship between RI and HMG [18] is less clear. RI is composed of two chains, α and β , of very similar molecular mass, and earlier

work demonstrated that the β chain represents an adhesin/haemagglutinin [34]. Conversely, HMG is composed of the gingipain subunit and four haemagglutinating polypeptide chains of sizes 44, 27, 17 and 15 kDa. While we cannot exclude the possibility that there may be significant differences between *P. gingivalis* strains, we have also purified RI from strain 381 and found it to be identical to RI from W50.

Scheme 1 describes the organization of the *prpRI* (protease poly-protein for RI) product and the putative processing sites for the release of the α and β chains of RI [26]. The alternative processing sites proposed by Pavloff et al. [35] to generate HMG are also shown. The PrpRI α and β chains are adjacent to one another, flanked by large N- and C-terminal extensions (the pro- and γ regions). The N-terminal sequences of the purified enzymes suggest that the processing site between the pro- and α regions is Arg²²⁷–Tyr²²⁸, and that between the α and β chains is Arg⁷¹⁹–Ser⁷²⁰. The processing site between the β and γ regions of the precursor has been assigned to Arg¹²⁶²–Phe¹²⁶³, which would maintain an arginyl peptide bond processing specificity. Assuming this cleavage site, the molecular mass of the β component calculated from the coding sequence [26] would be 58.1 kDa, which is only slightly higher than the size estimated by SDS/PAGE. Polypeptides derived from the γ region at the C-terminus of PrpRI were not present in any of the enzyme preparations described in the present study. However, it is not unprecedented for C-terminal domains of microbial protease precursors to be cleaved and released from the mature enzyme following the export and maturation process, as in the IgA1 proteases of *Neisseria gonorrhoeae* [36].

Apart from the gross structural differences between RI and RI-A/RI-B, the principal distinguishing characteristic observed in these studies was the low stability of RI at high pH in the absence of Ca^{2+} . The basis for the difference in half-lives is unknown, but may reflect an increased instability of the α component when associated with the β component in RI.

Steady-state kinetics and inhibition by E-64, iodoacetate and iodoacetamide

No significant differences were found with respect to the kinetic properties of RI, RI-A and RI-B. The V_{max} of all three forms

Scheme 1 PrpRI translation product and the generation of three isoforms of arginine-specific proteases

PrpRI is divided into four regions: a propeptide (pro), a catalytic domain (α), an adhesin domain (β) and a C-terminal domain (γ). Vertical arrows above PrpRI indicate the positions of the proteolytic processing sites required to generate the heterodimeric RI. The peptide bonds at these sites and resulting free N-termini are bracketed below PrpRI. (Note that the sequence at the junction of the β and γ regions is italicized to indicate that this processing site is an assumption based on the molecular size of the free β component.) The open arrowhead marks the putative signal peptidase cleavage site. Filled arrowheads mark the positions of alternative processing sites proposed by Pavloff et al. [35] which generate a multimeric, non-stoichiometric complex of polypeptides derived from the C-terminus in association with the catalytic domain, referred to as HMG. PrpRI numbering is used throughout. The monomeric RI-A and RI-B may be derived either by processing of PrpRI or by truncated transcription of the gene. The LPS annotation to RI-B denotes the covalent attachment of lipopolysaccharide to this form.

depended on two ionizing groups with pK_a values of 6.50 ± 0.05 and 9.70 ± 0.05 , and the K_m depended on a single ionizing group with a pK_a of 9.8 ± 0.3 . These values and the corresponding enthalpies of ionization (29.3 ± 3 and 24.2 ± 3 kJ/mol) are consistent with the ionization of an imidazole group of a His residue and/or the thiol group of a Cys residue.

The pK_a values of His and Cys residues in model compounds and proteins [37–39] would suggest that the residue of pK_a 6.5 is a His and that the group of pK_a 9.7 is a thiol. However, comparison of these data with equivalent studies on papain gives a different interpretation. In papain, the overall activity is governed by groups of pK_a 4.2 and 8.5 [40,41]. Based on the reaction of Cys²⁵ with several haloacetamides [39,42,43] over a wide pH range, it was suggested that Cys²⁵ and His¹⁵⁹ exist as an ion pair. This was also confirmed by fluorescence [44,45] and absorption data [46,47]. Thus, in free papain, the thiol group has a pK_a of 4.2 and the imidazole a pK_a of 8.6, whereas in the thiolester intermediate the imidazole has a pK_a of 4.1. Extending this argument to the present work suggests that the pK_a of 6.5 is due to a Cys residue and the pK_a of 9.7 is due to a His in the active site of all three proteases.

E-64 is classified as an effective irreversible inhibitor of Cys proteases that does not affect Cys residues in other proteins [48]. However, it has been suggested that E-64 is only a competitive inhibitor of a Cys protease purified from *P. gingivalis* W83 [49]. It was of interest, therefore, to determine whether the nature of the inhibition by this compound differentiated the enzymes purified in the present study. The inhibition of all three proteases by E-64 was competitive, with K_i values between $1.9 \mu\text{M}$ and $2.5 \mu\text{M}$ at pH 8.0 and 30°C , which are substantially lower than those previously reported. Hence the RI proteases of *P. gingivalis* are atypical Cys enzymes in terms of their interaction with this inhibitor. Iodoacetate and iodoacetamide had no effect on enzyme activity unless they were pre-reduced. These observations may imply that, following enzyme purification, the thiol group in the active site of these enzymes is in an oxidized form or that the thiol/imidazole group(s) are not available to react with these inhibitors prior to reduction. These data appear to contrast with the findings of other workers, although full experimental details were not always given [20,21]. The competitive inhibitor methylguanidine did not protect the reduced proteases from inactivation by iodoacetate or iodoacetamide. It is possible that, because of its small size, methylguanidine may occupy only the S1 pocket of these enzymes and therefore not block the thiol group in the active site. However, we cannot exclude the possibility that these alkylating agents inactivate by modifying a non-active-site residue. Further description of the interaction of these enzymes with irreversible inhibitors will be presented in a separate study.

Derivation of RI, RI-A and RI-B

No significant differences in the catalytic component of RI, RI-A and RI-B were detected by N-terminal sequencing or analysis of enzymic properties, suggesting that all three forms may be derived from the same precursor. During the cloning of *prpRI*, a second genomic locus was observed which hybridized to DNA probes to the coding region of the RI α component [26]. Furthermore, insertional inactivation of two genes was required to abolish all the cell-bound and extracellular Arg-X protease activity of *P. gingivalis* A.T.C.C. 33277 [50]. It is therefore possible that the monomeric forms, RI-A and RI-B, are derived from a second gene with high sequence identity to the α coding region of *prpRI*. In order to resolve these possibilities, a *prpRI*-isogenic mutant of strain W50 has been constructed in this laboratory and the production of RI, RI-A and RI-B examined

[51]. These studies demonstrated the loss of all three enzymes from the *prpRI*[−] strain, and provide further evidence that RI, RI-A and RI-B are derived from the same gene.

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