Characterization of the Specificity of Arginine-Specific Gingipains from *Porphyromonas gingivalis* Reveals Active Site Differences between Different Forms of the Enzymes†

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ABSTRACT: *Porphyromonas gingivalis* is a pathogen associated with periodontal disease, and arginine-specific proteases (gingipains-R) from the bacterium are important virulence factors. The specificity of two forms of gingipain-R, HRgpA and RgpB, for substrate positions C-terminal to the cleavage site was analyzed, and notable differences were observed between the enzymes. Molecular modeling of the HRgpA catalytic domain, based on the structure of RgpB, revealed that there are four amino acid substitutions around the active site of HRgpA relative to RgpB that may explain their different specificity. Previously, differences in the ability of these two gingipain-R forms to cleave a number of proteins were attributed to additional adhesins on HRgpA mediating increased interaction with the substrates. Here, purified $RgpA_{cat}$, the catalytic domain of HRgpA, which like RgpB also lacks adhesin subunits, was used to show that the differences between HRgpA and RgpB are probably due to the amino acid substitutions at the active site. The kinetics of cleavage of fibrinogen, a typical protein substrate for the gingipain-R enzymes, which is bound by HRgpA but not $RgpA_{cat}$ or RgpB, were evaluated, and it was shown that there was no difference in the cleavage of the fibrinogen Aα-chain between the different enzyme forms. HRgpA degraded the fibrinogen Bβ-chain more efficiently, generating distinct cleavage products. This indicates that while the adhesin domain(s) play(s) a minor role in the cleavage of protein substrates, the major effect is still provided by the amino acid substitutions at the active site of *rgpA* gene products versus those of the *rgpB* gene.

Porphyromonas gingivalis is a major pathogen associated with the onset of adult periodontitis, one of the major causes of tooth loss today (*1*). Periodontitis results from chronic inflammation of the gingival and periodontal tissue and has recently been associated with cardiovascular disease and preterm delivery of low birth weight infants (*2*−*4*). The disease is characterized by massive accumulation of neutrophils, bleeding on probing, bone resorption, formation of periodontal pockets, and loss of tooth attachment. Approximately 15% of the population are known to suffer from the most severe forms of the disease, which, if left untreated, results not only in tooth loss but also in systemic complications (*2*, *5*).

P. gingivalis is a black pigmented, anaerobic, Gram-negative bacterium that produces a number of virulence factors, such as cysteine proteases, haemagglutinins, lipopolysaccharides, and fimbriae, which enable the bacterium to colonize periodontal pockets (*6*). The proteolytic enzymes of the bacteria have been shown to play an important role in the pathogenesis of periodontitis (*7*, *8*). The Arg-X-specific and Lys-X-specific proteases produced by the bacteria, referred to as gingipains-R and -K, comprise a major proportion of its total activity and are considered to be important virulence determinants (*7*). Recent studies have revealed that null mutants of *P. gingivalis* for gingipain-R enzymes showed a marked decrease in virulence in in vivo models, and immunization with peptides corresponding to the N-terminal sequence of the catalytic domain of gingipains-R has also been shown to protect against infection by the bacteria in mouse models (*9*), indicating the overall importance of these enzymes in the pathogenesis of the disease by the bacterium.

The gingipains-R produced by *P. gingivalis* cleave peptide bonds exclusively after arginyl residues (*10*) and are encoded by two genes, *rgpA* and *rgpB* (*11*). The major forms of gingipain-R derived from the *rgpA* gene are a 50 kDa catalytic domain ($RgpA_{cat}$)[1] and a 95 kDa high molecular mass, noncovalent complex of catalytic and hemagglutinin/

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adhesin domains (HRgpA) (*12*), the former responsible for proteolytic activity and the latter for adhesion to extracellular matrix proteins and red blood cells. In comparison to *rgpA*, the *rgpB* gene lacks a sequence encoding the hemagglutinin/adhesin domains, and its product occurs predominantly as the soluble 50 kDa gingipain-R (RgpB) (*13*). Gingipain-R enzymes have been shown to activate coagulation factors and degrade components of the complement pathway and several physiologically important proteins, contributing to the virulence of the pathogen (*14*−*19*). In addition to causing destruction to the tissue supporting the teeth, gingipain-R enzymes play a major role in the deregulation of the inflammatory response and disruption of host defense mechanisms.

Analysis of the recently solved structure of RgpB indicates that the molecule is composed of two distinct domains, an N-terminal catalytic domain with topological similarity to caspases and a C-terminal domain with an Ig-like structure (*20*). The Ig-like domain of RgpB is thought to be involved in helping it bind to protein substrates or dock to endogenous proteins, other bacteria, or host cell surfaces (*20*). The catalytic domain of RgpB is almost identical to that of HRgpA ($RgpA_{cat}$) at the primary structure level, indicating that previously noted differences between HRgpA and RgpB in their ability to cleave protein substrates (*14*) are most likely due to the additional hemagglutinin/adhesin domains in HRgpA. This study initially aimed to determine the specificity of the two proteases toward substrate residues in the P_2'/P_3' region. Notable differences in specificity between HRgpA and RgpB led us to investigate whether the additional hemagglutinin/adhesin domains of HRgpA influence substrate specificity. Determination of the specificity of these proteases will aid in the design of drugs to combat periodontal disease.

EXPERIMENTAL PROCEDURES

Materials. Bz-L-Arg-pNA, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and leupeptin were purchased from Sigma (Sydney, Australia). Z-L-Lys-pNA was from Novabiochem (Darmstadt, Germany). An extract from the medicinal leech (*Hirudo medicinalis*) was a kind gift from Dr. Christian Sommerhof (Ludwig Maximilian University, Munich, Germany). The extract at 0.1 mg/mL totally inhibited 0.4 nmol of α-thrombin amidolytic activity.

Fluorescence-Quenched Substrates. The fluorescence-quenched substrates were synthesized as described previously (*21*). Substrates were dissolved in DMF, and the concentration of the stock solution was determined spectrophotometrically, assuming an absorption coefficient of 10^4 M^{-1} cm^{-1} at 360 nm. Each substrate consisted of a 10-residue peptide with a 2-aminobenzoyl (Abz) group at the N-terminus and a penultimate 2,4-dinitrophenyl- (Dnp-) derivatized lysine. Peptides had the following general sequence: Abz-Val-Gly-Pro-Arg-Ser-X-X-Leu-Lys(Dnp)-Asp-OH, with the X denoting the variable amino acid positions at P_2' and P_3'. The P_2' specificity was determined using substrates with a leucine in the P_3' position, whereas the P_3' substrates contained a phenylalanine at the P_2' position.

Cultivation of the Bacteria and Purification of Enzymes. *P. gingivalis* strain HG66 cells used as a source of the enzymes in this study were grown as described previously (*22*). HRgpA and RgpB were purified from the HG66 strain culture fluid as described previously (*23*). Briefly, HRgpA was purified using gel filtration and arginine−Sepharose chromatography, while RgpB was purified using a combination of gel filtration and anion-exchange chromatography on Mono Q (*24*). $RgpA_{cat}$ was purified from the fractions containing RgpB that were eluted from a Sephadex G-150 gel filtration column (*25*). Briefly, fractions were pooled and loaded onto a DE-52 cellulose column, where $RgpA_{cat}$ was eluted in the void volume using 50 mM Bis-Tris and 1 mM $CaCl_2$, pH 6.5. $RgpA_{cat}$ was loaded on a Mono Q column, eluted with 1 M NaCl, 50 mM Bis-Tris, and 1 mM $CaCl_2$, pH 6.5, buffer, and purified to homogeneity.

The purity of enzymes in each batch was checked using SDS−PAGE. Both RgpB and $RgpA_{cat}$ migrated as a single band with mobility equivalent to a molecular mass of 48 kDa and homogeneity greater than 95% as determined using laser densitometry scanning of the gel. HRgpA resolves into four major bands and one minor band on SDS−PAGE (*23*). The identity of each protein band was confirmed by N-terminal sequence analysis as being derived from the RgpA polyprotein. Also, N-terminal sequence analysis was used to check for cross-contamination of RgpB and $RgpA_{cat}$ preparations. In each case, only one sequence was obtained, which differed at only residue 8, which was Glu and Gln in RgpB and $RgpA_{cat}$, respectively. Comparative analysis of amino acid derivative peaks of cycle 8 of the Edman degradation for the 48 kDa band seen for $RgpA_{cat}$ and RgpB clearly indicated that any cross-contamination was below 10% of the major form of the gingipain-R being analyzed.

Kinetic Studies. The k_{cat} and K_m values were measured at 37 °C using substrates at concentrations ranging from 1 to 50 μM, with a final concentration of active site titrated enzyme of 1.0 nM in 0.2 M Tris-HCl, 5 mM $CaCl_2$, 10 mM cysteine, and 0.1 M NaCl, pH 7.6. The assay was performed in a total volume of 200 μL in microplates and was carried out in triplicate. Enzyme solution (100 μL) was added to 100 μL of substrate solution, and the initial velocity was recorded (usually over 10 min) at seven different substrate concentrations at 330/420 nm on a fluorescent plate reader (Biolumin960, Molecular Dynamics). The initial velocities were plotted against substrate concentrations, and the lines were fitted to a single site binding equation using nonlinear regression analysis in the program GraphPad Prism. The K_m values were derived from the fitted line, while k_{cat} values were calculated from the V_{max} values by taking into account the amount of active enzyme used in the assays, the latter determined as described previously (*26*). Four substrates (P_2' His, P_2' Ser, P_3' Gly, and P_3' Ala), toward which HRgpA and RgpB displayed a significant difference in activity, were assayed as above with the three gingipain-R enzymes, HRgpA, RgpB, and $RgpA_{cat}$, on a fluorescent plate reader (Fluostar Galaxy, BMG Technologies).

[1] Abbreviations: $RgpA_{cat}$, catalytic domain derived from the *rgpA* gene; HRgpA, 95 kDa form of arginine-specific gingipain derived from the *rgpA* gene; RgpB, 50 kDa form of arginine-specific gingipain derived from the *rgpB* gene; Ig, immunoglobulin; Bz or Z, benzoxycarbonyl; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; DMF, dimethylformamide; Dnp, dinitrophenyl; Abz, *o*-aminobenzoic acid. The residues of a peptide substrate are designated P_n, ..., P_2, P_1, P_1', ..., P_n' and interact with corresponding subsites in the protease designated S_n, ..., S_2, S_1, S_1', ..., S_n'; cleavage occurs by definition between the P_1 and P_1' positions.

Modeling. The X-ray crystal structure of RgpB (PDB identifier 1CVR; *20*) was obtained from the Protein Data Bank (*27*). RgpB and RgpA are 90% identical, and the sequences were aligned using the alignment package available in Quanta (Accelrys Inc., San Diego). The molecular model of RgpA was built using the program Modeler (*28*) and the X-ray crystal structure of RgpB as a template. A Ramachrandran plot confirmed that all residues in the model of RgpA were in allowed conformations.

ELISA of Gingipain Binding to Extracellular Matrix Proteins. To test the binding of the gingipain-R enzymes to different extracellular proteins, proteins to be tested were coated onto Nunc maxisorp ELISA plates at a concentration of 1 μg/mL in phosphate-buffered saline (PBS) at 4 °C for 16 h. The wells were then blocked with 0.5% (w/v) BSA in PBS for 1 h at 37 °C. Every incubation was followed by three washes with 0.1% (v/v) Tween 20−PBS. Following blocking, the wells were incubated with serial dilutions of HRgpA, RgpB, and $RgpA_{cat}$, all previously inactivated with TLCK, starting from 50 μg/mL in BSA−PBS for 2 h. The wells were then incubated with chicken anti-gingipain-R (1 μg/mL) in BSA−PBS for 1 h, followed by incubation with anti-chicken IgY−horseradish peroxidase conjugate (1 μg/mL) in BSA−PBS for 1 h at 37 °C. The plate was then washed, and binding was detected by incubation with tetramethylbenzidine substrate solution. The reaction was terminated by adding 2 M H_2SO_4, and the product was read at 450 nm.

Analysis of Degradation of Proteins by SDS−PAGE. Protein degradation was carried out at 37 °C in 0.2 M Tris-HCl, 1 mM $CaCl_2$, and 10 mM cysteine buffer. Aliquots were taken at various time points, and enzyme activity was stopped by adding 1 mM TLCK. Aliquots containing the proteins and degradation products were electrophoresed on 10% Tris−Tricine (*29*) sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels and visualized by Coomassie blue R-250 staining. Fibrinogen cleaved by HRgpA was also blotted to a PVDF membrane following SDS−PAGE, and bands were excised for N-terminal sequencing as previously described (*23*).

Western Blot Analysis of the Degradation of Human Plasma Fibrinogen by Arg-Gingipains. Plasma was collected by mixing 9 mL of human blood with 1 mL of 3.2% (w/v) sodium citrate and centrifuging the mixture for 5 min at 2700 rpm. Plasma was depleted of albumin by mixing it with Cibacron Blue−Sepharose equilibrated in PBS using 2 mL of the chromatography matrix per 1.5 mL of plasma. After 30 min of gentle mixing at room temperature, the matrix was removed by centrifugation, and the supernatant was used in further experiments. Plasma prepared in this way (50 μL) was made up to 100 μL using a buffer containing either 20 mM cysteine and 0.1 M Tris, pH 8.0, or the same buffer supplemented with 1 mg/mL leech extract. To this mixture was then added 50 μL of activated gingipain-R to yield a final enzyme concentration of 10 nM. The plasma mix was incubated at 37 °C, and 20 μL aliquots were transferred at 5, 10, and 15 min into 80 μL of 5 mM TLCK in PBS to stop the reaction. Samples were boiled with SDS−PAGE reducing buffer for 10 min, electrophoresed on 10% Tris−Tricine (*29*) SDS−PAGE gels, and transferred onto nitrocellulose membrane overnight at 30 V. The membranes were blocked with 1% (w/v) low fat milk powder in Tris-buffered saline (TBS) overnight. The membranes were washed in Tween−TBS and incubated with goat anti-human fibrinogen diluted 1:5000 in 1% (w/v) bovine serum albumin in Tween−TBS for 1 h. The membranes were washed and incubated for 1 h with anti-goat alkaline phosphatase diluted 1:100000 in 1% (w/v) bovine serum albumin in Tween−TBS. Bound antibodies were detected using BCIP/NBT substrate.

RESULTS

The specificity of HRgpA and RgpB at the P_2' position was determined using fluorescence-quenched substrates with 18 different amino acids at this position, excluding substrates with an arginine or a cysteine residue. Substrates with a cysteine residue were not used, as oxidation of the cysteine residue would interfere with the activity of the enzymes. Similarly, cleavage of substrates containing an additional arginine residue would probably result in higher K_m and k_{cat}/K_m values due to secondary cleavage of the substrates. The specificity of the enzymes for the P_3' position was determined using substrates with 16 substitutions at this position, with cysteine, arginine, phenylalanine, and glutamate excluded. Each substrate contained the following sequence of amino acids, (Abz)-Val-Gly-Pro-Arg-Ser-Xaa-Leu-Leu-Lys(Dnp)-Asp-OH, where Xaa is representative of any one of the 18 amino acids examined at the P_2' position in this example. The Lys(Dnp) group quenches the fluorescence of the N-terminal Abz group in an uncleaved substrate by resonance energy transfer. Cleavage of the substrate relieves quenching, resulting in an increase in fluorescence proportional to the concentration of the released fluorophore fragment, thus allowing the determination of the initial velocities of the enzymes in relation to substrate concentration and hence the kinetic parameters, K_m, V_{max}, and k_{cat}. Values for k_{cat}/K_m were derived from the individual constants.

HRgpA Specificity at P_2' and P_3'. Phenylalanine is the most preferred amino acid at P_2' for cleavage by HRgpA, followed by leucine and tyrosine (Table 1). The k_{cat}/K_m value of the most preferred amino acid at P_2' for HRgpA, phenylalanine (12.5 $\mu M^{-1}\cdot s^{-1}$), was 11.4-fold greater than the k_{cat}/K_m value of the least preferred amino acid at P_2', which was proline (1.1 $\mu M^{-1}\cdot s^{-1}$). Leucine was the most preferred and isoleucine was the least preferred amino acid at the P_3' position (Table 2). The K_m values determined for HRgpA, which reflect the binding affinity of an enzyme for a substrate, indicate that HRgpA does not show an affinity for a particular group of amino acids (Tables 1 and 2). HRgpA had the greatest affinity for the substrate with a positively charged lysine or a polar uncharged asparagine residue at P_2', with K_m values of 15.1 and 15.6 μM, respectively. The K_m values for HRgpA at P_3' again reflect the lack of preference for any particular group of amino acids at this position; the K_m value for the substrate with a proline at P_3' (6.2 μM) was significantly lower than any other substrate (Table 2).

RgpB Specificity at P_2' and P_3'. Results obtained indicate that serine, a polar hydrophilic amino acid, was the most preferred residue at P_2' ($k_{cat}/K_m = 7.9\ \mu M^{-1}\cdot s^{-1}$) for RgpB (Table 1). As with HRgpA, proline ($k_{cat}/K_m = 0.5\ \mu M^{-1}\cdot s^{-1}$) was the least preferred amino acid at P_2' for RgpB. RgpB did not display a preference for any particular group of amino acids at P_3' (Table 2), with the k_{cat}/K_m value for lysine (3.8

Table 1: Substrate Specificity of HRgpA and RgpB at P2′ [a]

	HRgpA			RgpB		
substrate	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
GPR-S**F**L	20.5	25.6	12.5	24.5	8.6	3.5
GPR-S**L**L	26.2	28.1	10.7	21.2	6.1	2.8
GPR-S**Y**L	24.2	25	10.3	44.9	14.8	3.3
GPR-S**Q**L	26.4	25.7	9.7	42.4	9.5	2.2
GPR-S**G**L	23.4	21.8	9.3	34.7	11.2	3.2
GPR-S**W**L	21.3	18.9	8.8	nd	nd	nd
GPR-S**T**L	39.1	31.7	8.1	32.5	7.8	2.4
GPR-S**S**L	24.2	17.4	7.2	55.8	44.1	7.9
GPR-S**H**L	39.5	21.7	5.5	71.8	7.8	1.1
GPR-S**N**L	15.6	8.1	5.2	17.4	3.9	2.2
GPR-S**V**L	17.9	8.2	4.6	41.9	10.4	2.5
GPR-S**D**L	42.1	17.5	4.1	32.3	4.7	1.4
GPR-S**K**L	15.1	6.2	4.1	59.3	11.8	1.9
GPR-S**A**L	27.3	10.2	3.7	29.7	9.4	3.2
GPR-S**I**L	77.9	23.6	3.0	84.8	10.2	1.2
GPR-S**M**L	20.4	4.9	2.4	38.9	13.4	3.4
GPR-S**E**L	22.8	5.3	2.3	25.2	4.0	1.6
GPR-S**P**L	21.2	2.3	1.1	49.4	1.9	0.5
selectivity factor			11.4			16

[a] Units for K_m are expressed in μM, k_{cat} were expressed in $s^{-1} \times 10$, and k_{cat}/K_m were expressed in $\mu M^{-1} \cdot s^{-1}$. Assays were carried out in triplicate, and values for K_m and k_{cat} had a standard error of less than or equal to 10%. Only residues from P3 to P3′ are indicated, and amino acids unique to each substrate are in bold. nd = not determined.

Table 2: Substrate Specificity of HRgpA and RgpB at P_3' [a]

	HRgpA			RgpB		
substrate	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
GPR-S**F**L	20.5	25.6	12.5	24.5	8.6	3.5
GPR-SF**N**	23.1	27.0	11.7	46.5	12.5	2.7
GPR-SF**G**	27.8	2.9	10.4	53.3	18.8	3.5
GPR-SF**D**	17.8	15.4	8.4	41.5	11.5	2.7
GPR-SF**P**	6.2	4.3	6.9	18.7	5.5	2.9
GPR-SF**H**	35.6	24.7	6.9	47.9	14.4	3.0
GPR-SF**K**	20.9	13.2	6.3	12.3	4.7	3.8
GPR-SF**S**	19.4	11.9	6.1	19.7	4.8	2.4
GPR-SF**Y**	24.8	15.2	6.1	15.8	3.2	2.0
GPR-SF**A**	13.1	7.8	5.9	31.6	7.2	2.2
GPR-SF**T**	23.7	14.1	5.9	36.9	3.3	0.9
GPR-SF**W**	17.8	9.9	5.5	25.1	7.8	3.1
GPR-SF**V**	64.0	29.7	4.6	30.4	11.1	3.6
GPR-SF**Q**	31.8	10.7	3.3	27.0	7.5	2.7
GPR-SF**M**	19.1	5.3	2.7	27.1	2.7	0.9
GPR-SF**I**	32.5	6.4	1.9	60.6	10.6	1.7
selectivity factor			6.6			4.3

[a] Units for K_m are expressed in μM, k_{cat} were expressed in $s^{-1} \times 10^5$, and k_{cat}/K_m were expressed in $\mu M^{-1} \cdot s^{-1}$. Assays were carried out in triplicate, and values for K_m and k_{cat} had a standard error of less than or equal to 10%. Only residues from P_3 to P_3' are indicated, and amino acids unique to each substrate are in bold.

$\mu M^{-1} \cdot s^{-1}$), the most preferred amino acid at P_3', only 4.3-fold greater than the value for threonine (0.9 $\mu M^{-1} \cdot s^{-1}$), the least preferred amino acid at P_3'. The K_m values for RgpB indicate that it too does not show a preference toward a particular group of amino acids, although it has a broader range of K_m values, indicating a greater difference in its binding affinity toward substrates. RgpB had the highest affinity toward the substrate with an asparagine at the P_2' position (K_m = 17.4 μM). The substrate with a lysine at P_3' had a K_m value of 12.3 μM, which was the lowest value overall for RgpB.

Modeling of RgpA. RgpA and RgpB share 90% sequence identity, with the majority of the substitutions mapping to the Ig-like domain in the C-terminus. Only four substitutions

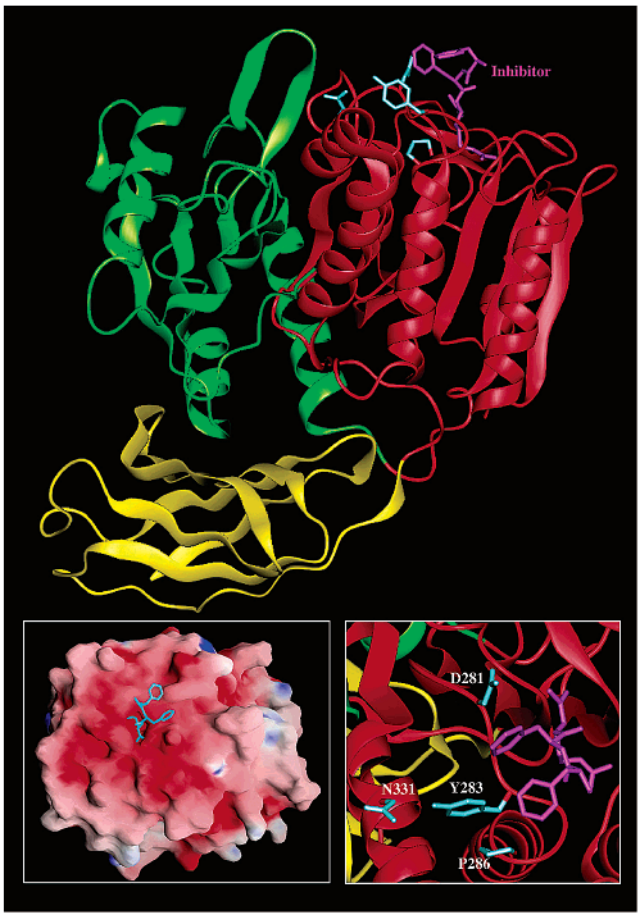

FIGURE 1: Crystal structure of RgpB (*20*). (A, top) Overall structure of RgpB demonstrating the two domains making up the overall catalytic domain in red and green. The Ig domain is in yellow. The inhibitor, D-Phe-Phe-Arg chloromethyl ketone, in the active site of RgpB is shown in purple, while substitutions in the active site of RgpB in relation to RgpA are shown in blue ball-and-stick representation. (B, bottom left) Electrostatic potential of the active site of RgpB (red equals electronegative, blue is electropositive, and white is neutral). (C, bottom right) Close-up of the active site of RgpB, showing the substitutions relative to RgpA in blue ball-and-stick representation. The figure was produced using Quanta (Accelrys Inc.) and GRASP.

(D281 → N, Y283 → S, P286 → S, and N331 → K) map to the protease domain, all of which map to the region surrounding the active site (see Figure 1).

Comparison of the Specificity of HRgpA, RgpB, and $RgpA_{cat}$ at P_2' and P_3'. $RgpA_{cat}$ was used to help to determine if the differences in specificity between HRgpA and RgpB were due to the additional adhesin domains in HRgpA or due to the four amino acid substitutions in its active site compared to RgpB. The substrates to be used were selected on the basis of results obtained previously. One substrate each from the P_2' and P_3' range, for which HRgpA and RgpB had a high and a low K_m value, respectively, was selected. Values for K_m were determined for HRgpA, RgpB, and $RgpA_{cat}$ toward substrates with a histidine or serine at the P_2' and a glycine or alanine at the P_3' position. The K_m values for HRgpA and $RgpA_{cat}$ were similar and differed from the values obtained for RgpB. Thus, for instance, the K_m value for RgpB toward the substrate with a serine at the P_2' position was 55 μM, whereas the values for HRgpA and $RgpA_{cat}$ were 24 and 30 μM, respectively (Figure 2). The K_m values for RgpB were nearly 2-fold higher than the values for HRgpA

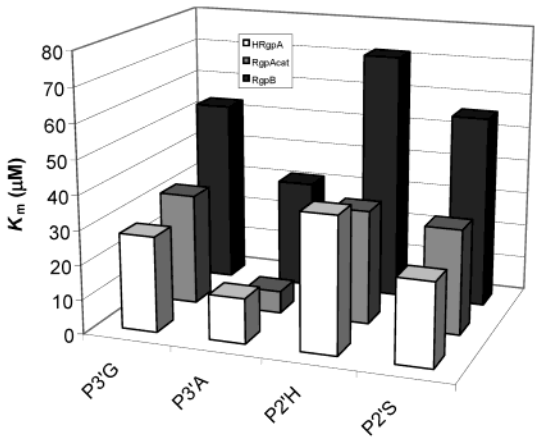

FIGURE 2: Comparison of the K_m values of HRgpA, $RgpA_{cat}$, and RgpB toward fluorescence-quenched substrates with a glycine or an alanine at the P_3' and a histidine or a serine at the P_2' position.

and $RgpA_{cat}$, indicating that RgpB had a weaker affinity toward the substrates in comparison to HRgpA and $RgpA_{cat}$.

Binding of Gingipains to Fibrinogen, Fibronectin, and Laminin. Results obtained in the specificity studies indicated that the adhesin domains are not responsible for the differences in specificity of HRgpA and RgpB toward peptide substrates. Since the binding activity of $RgpA_{cat}$ had not previously been characterized, experiments were carried out to determine if the enzyme adhered to fibrinogen, fibronectin, and laminin. HRgpA bound all three proteins, whereas $RgpA_{cat}$ and RgpB showed very little or no binding activity toward the proteins (less than 10% of the value for HRgpA) (results not shown). The lack of binding activity for RgpB and $RgpA_{cat}$ indicates that the gingipains require the additional adhesin domains found in HRgpA in order to bind proteins and that the catalytic or Ig-like domains of the smaller proteases do not possess any binding activity.

Degradation of Fibrinogen by the Gingipains. Specificity studies using peptide substrates indicated that HRgpA and $RgpA_{cat}$ are similar in their preferences for cleavage and differ from RgpB. We wanted to determine if this was also so for a protein substrate, such as fibrinogen, which is cleaved by all of the gingipain-R forms under study. Thus the pattern of cleavage of fibrinogen was used to evaluate whether the pattern of cleavage of the protein and/or rate of cleavage was different for the individual gingipain-R enzymes. We examined cleavage of the protein in its purified form and in plasma to evaluate whether there was a difference in the rate or position of cleavage in a complex milieu in comparison to the purified protein. Cleavage of the purified protein was followed directly using SDS−PAGE for the purified protein (Figure 3) and by western blotting for the plasma protein (Figure 4). It was apparent that all three forms of gingipain-R cleaved fibrinogen very similarly in terms of the kinetics of Aα-chain degradation, which was most sensitive to cleavage. The Aα-chain was cleaved at a number of positions, but for each enzyme isoform, a band of approximately 28 kDa appeared and grew stronger over the period of the incubation. N-Terminal sequencing of the approximately 28 kDa band revealed that it was derived from the N-terminal portion of the Aα-chain, having been cleaved at position 22 of the mature chain at the sequence VER↓HQS (arrow indicates cleavage position). In contrast to the situation with the Aα-chain, HRgpA was significantly more efficient at cleaving

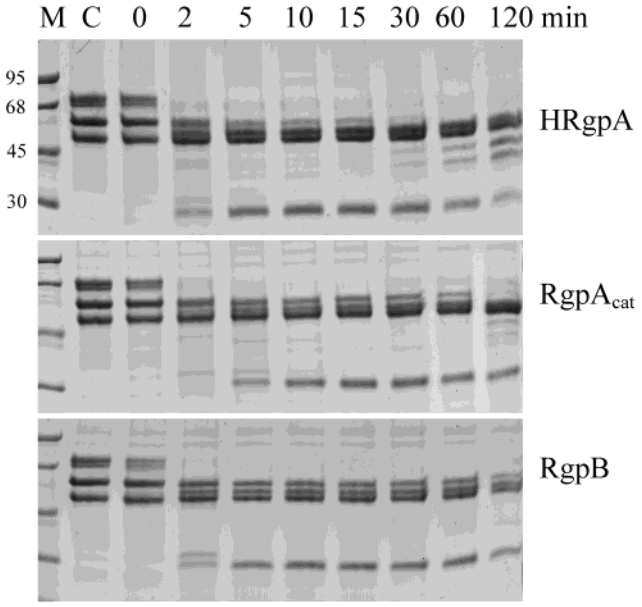

FIGURE 3: Degradation of fibrinogen by HRgpA, $RgpA_{cat}$, and RgpB at 0, 2, 5, 10, 15, 30, 60, and 120 min. A 10 nM concentration of enzyme was used for the assay. M designates the protein molecular mass markers (sizes indicated next to the markers for the HRgpA gel: 95, 68, 45, and 30 kDa), and C designates control protein not incubated with enzyme. 10 μg of protein was loaded per lane and electrophoresed on 10% Tris−Tricine SDS−PAGE gels.

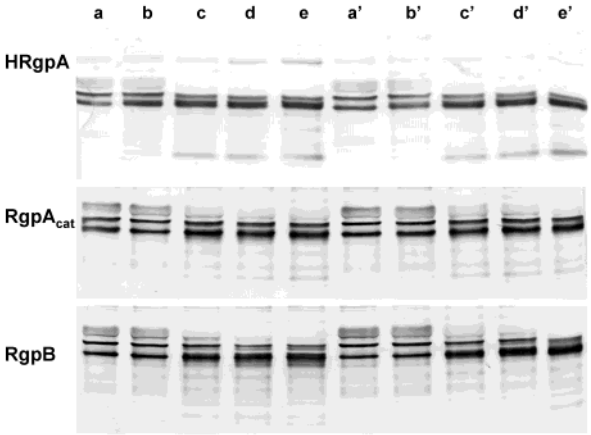

FIGURE 4: Cleavage of fibrinogen in plasma by gingipains. Samples of freshly collected human plasma depleted of albumin by absorption on the Cibacron Blue Sepharose alone (lanes a−e) or pretreated with a leech extract (lanes a′−e′) were incubated with 10 nM gingipain (each form) for 0 min (lanes b and b′), 5 min (lanes c and c′), 10 min (lanes d and d′), and 15 min (lanes e and e′). Lanes a and a′ are control plasma preincubated for 15 min in the absence of gingipain. The reaction was stopped using TLCK (4 mM), samples were boiled in reducing treatment buffer and electrophoresed on 10% Tricine SDS−PAGE gels, and then immunoblots were performed with fibrinogen being detected using a goat anti-human fibrinogen antibody.

the Bβ-chain than RgpB and $RgpA_{cat}$, leading to accumulation of a lower molecular mass cleavage product which migrated just above the γ-chain. N-Terminal sequencing of this band revealed that it was indeed derived from the Bβ-chain and resulted from two cleavages at positions 42 and 44 in the sequence GYR↓AR↓PAK (arrows indicate the position of cleavage).

The gingipain-R forms once again cleaved the Aα-chain of fibrinogen in plasma with similar kinetics, although only

HRgpA was able to generate the approximately 28 kDa band derived from cleavage of the Aα-chain in contrast to the situation with the pure protein (Figure 4). Once again, only HRgpA was able to cleave the Bβ-chain efficiently in the plasma context. The profile of cleavage of the α-chain and β-chain was the same in the absence or presence of leech extract (applied at a concentration that totally inhibited thrombin activity), indicating that cleavage of these chains was independent of thrombin. It should be noted that thrombin was apparently active in cleaving fibrinogen in the absence of leech extract to some extent, however, since a band corresponding to the γ-chain dimer was observed, which is not seen when the leech extract was present. This was only apparent for HRgpA treatment, indicating that HRgpA is more efficient at activating prothrombin than the other protease forms, as has been observed previously (*30*). Once activated, thrombin would activate factor XIII to cause dimerization of the γ-chains by transglutamination.

DISCUSSION

The initial focus of the present study was to map the preferences of the gingipain-R enzymes for prime site amino acid residues in peptide substrates. A previous study (*25*) examined the specificity for residues in the nonprime sites and found that the gingipains were relatively nonspecific for these residues and also that no marked difference in specificity could be found between different forms of the gingipains-R. Our investigation therefore examined whether this was the case for the prime sites of substrates.

The two gingipain-R forms initially analyzed, HRgpA and RgpB, were not very selective for substrate residues at the P_2' and P_3' sites; thus selectivity factors (or the ratio between top and bottom values for each kinetic term) ranged from 4.3 to 18.0. Both enzymes were most selective for substrate residues at the P_2' subsites, with RgpB somewhat more selective than HRgpA at P_2' and slightly less selective at P_3' (Tables 1 and 2). Overall, it appeared that the specificity constant for any given substrate, usually accepted to be the k_{cat}/K_m value, was mostly influenced by the k_{cat} value. In general, variation between K_m values was lower than for the k_{cat} values for any given subsite and enzyme. It was clear that HRgpA and RgpB were different in their specificity for different amino acids at the prime subsites. We therefore were interested to know the underlying reasons for this.

Initial analysis of the difference in the specificity of the prime subsites led us to hypothesize that it was the additional adhesin domains of HRgpA which might give rise to the differences in specificity. For this to be true, however, it would be necessary for the active sites of the proteases to be identical. We therefore modeled the catalytic domain of HRgpA ($RgpA_{cat}$) upon the crystal structure of RgpB to determine whether this was correct. The surface of the active site and its surroundings are relatively flat, except for the S_1 pocket, which is a fairly narrow slot, bordered by in-plane hydrogen bond acceptors and covered by a hydrophobic lid (Figure 1A). The active site is also characterized by a negative electrostatic potential, which may have an effect on the binding of substrates (Figure 1B). There are four amino acid substitutions, D281 → N, Y283 → S, P286 → S, and N331 → K, found in the active site of HRgpA compared to RgpB (Figure 1C). Apart from these four substitutions, the active sites of HRgpA and RgpB are identical. The amino acid substitutions could have a profound effect on the binding of substrates and subsequent cleavage, particularly P286S, since P285 and P286 form a double proline motif at the end of helix 9 in RgpB. These residues form one of the walls of the S_1 pocket, and substitution of P286 with a serine may thus alter the accessibility to the S_1 pocket. These substitutions might change direct interactions with prime site substrate residues, but it is more likely that they cause a general change in the conformation of the prime site binding sites of the active site, thus giving rise to the differences in specificity noted.

To resolve whether it was the contribution of the active site substitutions or the additional adhesin domains that determined the differences in specificity between HRgpA and RgpB, we purified the small quantity of $RgpA_{cat}$ to be found in culture supernatants of *P. gingivalis*. $RgpA_{cat}$ is the catalytic domain of RgpA, analogous to RgpB, except that the four amino acid substitutions would be present at the active site. We showed that $RgpA_{cat}$ was similar to HRgpA in terms of it kinetics of cleavage of four substrates containing P2′ or P3′ substitutions. We selected the substrates analyzed on the basis of their having large differences in terms of their kinetics of cleavage by HRgpA and RgpB. Thus it appears that it is most likely that it is the active site substitutions in HRgpA versus RgpB that dictate their differences in specificity for prime site residues and not the additional adhesin domains found in HRgpA.

What then is the function of the additional adhesin domains in terms of the specificity of the proteases for cleavage of peptide or protein substrates? Previous work has attributed quite large differences in the cleavage of protein substrates by HRgpA versus RgpB to the additional adhesin subunits on HRgpA interacting with the protein substrates. Our data would indicate that the adhesin domains do not influence cleavage of peptide substrates, but is this also true for protein substrates to which the adhesin domains of the gingipains might bind? We first needed to establish whether the catalytic domains of the gingipains could bind to proteins in the same way as HRgpA. Since the catalytic domains of the gingipains have an Ig-like domain, which might conceivably have adhesin activity, it was important to determine whether $RgpA_{cat}$ or RgpB could bind to proteins. We have previously shown that HRgpA binds to fibrinogen, fibronectin, and laminin, but RgpB does not (*24*). Work carried out here showed that $RgpA_{cat}$ also did not bind to these proteins. This strongly indicates that the additional adhesin subunits in HRgpA mediate binding to the tested proteins.

We then tested whether binding to a protein substrate by HRgpA influences cleavage. We hypothesized that the binding event might influence the position of cleavage within the substrate, and therefore the pattern of cleavage bands seen on SDS−PAGE, and/or the rate of cleavage of a protein substrate. To test whether the adhesins have an influence on protein substrate cleavage, we examined whether HRgpA, $RgpA_{cat}$, and RgpB differed in their cleavage of purified fibrinogen (Figure 3). HRgpA, $RgpA_{cat}$, and RgpB cleaved the Aα-chain of fibrinogen with very similar kinetics. The γ-chain of fibrinogen was essentially not degraded, but differences were observed for cleavage of the Bβ-chain between the gingipain-R forms. HRgpA cleaved the Bβ-chain most efficiently, followed by $RgpA_{cat}$ and then RgpB. This indicates that the additional adhesin domains of HRgpA may

position the enzyme advantageously for cleavage of the Bβ-chain relative to $RgpA_{cat}$. The greater efficiency of both RgpA forms also indicates that the active site differences found between RgpA and RgpB forms have a strong effect in this context. The position of cleavage of the fibrinogen Aα-chain and Bβ-chain would be predicted to preclude formation of the fibrinopeptides A and B, thus explaining why fibrinogen is rendered nonclottable by the gingipains-R and thus overall the anti-clotting effect that these enzymes have in plasma, despite their activation of procoagulant enzymes.

We also tested for cleavage of the protein in plasma in order to evaluate whether the adhesins served to target HRgpA to a substrate such as fibrinogen, resulting in faster cleavage (Figure 4). It was shown that cleavage profiles in plasma were similar to those with purified fibrinogen in that all gingipain-R forms cleaved the Aα-chain rapidly. However, there were some differences in the cleavage of the Bβ-chain, with only HRgpA cleaving this chain. This difference was not due to activation of the coagulation cascade (*15*, *30*, *31*), since the same cleavage pattern was observed in the presence of the leech extract containing hirudin and other very effective inhibitors of proteases from the coagulation cascade (*32*, *33*).

The results of this study strongly imply that the differences previously noted between different forms of gingipain-R in terms of their cleavage of isolated protein substrates are primarily due to differences at the active site of the enzymes, with the adhesin domains playing a minor role. However, cleavage in complex (patho)physiological fluids such as plasma may be affected by the presence of the competing proteins, since it has been noted that HRgpA is more efficient in a number of biological contexts than RgpB. This enhanced activity has often been attributed to the extra adhesin subunits found on HRgpA compared to RgpB, and indeed, we have shown here that fibrinogen in plasma is cleaved in a different way from purified fibrinogen. However, this difference is limited to the rate of β-chain degradation and accumulation of some cleavage products. Thus, it appears that the adhesin subunits of HRgpA play relatively little overall roles in determining the cleavage rate of peptide or protein substrates in a complex physiological environment.

P. gingivalis is one of the major pathogens associated with adult periodontitis. Since periodontitis is one of the main causes of tooth loss today, and more recently has been linked to cardiovascular diseases, factors contributing to the virulence of the bacterium are attractive targets for the design of drugs or vaccines against the disease (*2*, *3*). Gingipains-R are essential for the survival of *P. gingivalis* and responsible for various intrinsic and extrinsic factors associated with its virulence (*14*−*16*, *18*). Determination of the P_2' and P_3' specificity of the gingipain-R enzymes thus helps to define the properties of their active site and improve our understanding of the specificity of these enzymes, which can be used further to develop selective synthetic inhibitors to combat periodontal disease.

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