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Cysteine 351 is an Essential Nucleophile in Catalysis by *Porphyromonas gingivalis* Peptidylarginine Deiminase[★]

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Abstract

Peptidylarginine deiminase (PAD), which catalyzes the deimination of the guanidino group from peptidylarginine residues, belongs to a superfamily of guanidino-group modifying enzymes that have been shown to produce an S-alkylthiouronium ion intermediate during catalysis. Thiol-directed reagents iodoacetamide and iodoacetate inactivate recombinant PAD, and substrate protects the enzyme from inactivation. Activity measurements together with peptide mapping by mass spectrometry of PAD modified in the absence and presence of substrate demonstrated that cysteine-351 is modified by iodoacetamide. The pK_a value of the cysteine residue, 7.7 ± 0.2 as determined by iodoacetamide modification, agrees well with a critical pK value identified in pH rate studies. The role of cysteine-351 in catalysis was tested by site-directed mutagenesis in which the cysteine was replaced with serine to eliminate the proposed nucleophilic interaction. Binding studies carried out using fluorescence spectrometry established the structural integrity of the C351S PAD. However, the C351S PAD variant was catalytically inactive, exhibiting <0.01% wild-type activity. These results indicate that Cys 351 is a nucleophile that initiates the enzymatic reaction.

Keywords

Peptidylarginine deiminase; *Porphyromonas gingivalis*; chemical modification; site-directed mutagenesis; cysteine

1. Introduction

Peptidylarginine deiminase (PAD1) catalyzes the deimination of the guanidino group from peptidylarginine residues of various peptides to produce peptidylcitrulline and ammonia. The peptidylarginine deiminase from *Porphyromonas gingivalis* is also able to catalyze the deimination of free L-arginine, but has primarily peptidylarginine specificity [1,2]. PAD is a virulence factor responsible for the survival of *P. gingivalis* in the gum by the production of

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ammonia [3,4], which effectively controls the local pH surrounding the pathogen. Although PAD is an attractive drug target, attempts to design inhibitors have been hampered because of the lack of knowledge about the catalytic mechanism of the enzyme.

P. gingivalis PAD, arginine deiminase (ADI), L-arginine: glycine amidinotransferase (AT), N^ω, N^ω-dimethylarginine dimethylamino hydrolase (DDAH), agmatine deiminase (AIH), mammalian peptidylarginine deiminase 4 (PAD4), and arginine succinyltransferase (AstB) have been proposed to constitute a novel superfamily of guanidino modifying enzymes [5]. The classification and characterization of the superfamily were based on sequence comparisons as well as structure and domain architecture. A bioinformatics approach, which includes FUGUE, a fold recognition program [6], was used to propose that the core domain structure adopts a common (α/β propeller) fold that is similar for all the members of the superfamily. These enzymes use similar substrates, among which arginine is the most common, and those which have been studied share similar catalytic mechanisms, despite a lack of significant amino acid sequence similarity [5,7].

The members of the superfamily catalyze a variety of reactions involving the guanidino group of arginine residues. PAD4 and ADI, like PAD, deiminate the guanidino group of arginine, giving rise to peptidyl citrulline/citrulline and ammonia as final products [8,9]. DDAH catalyzes the hydrolysis of N^ω-alkylated arginines to produce citrulline and the corresponding alkylamine [10]. AstB is thought to use the same catalytic mechanism as ADI but it carries the reaction further by removing a second NH₃ from the guanidino group, releasing CO₂ and producing ornithine and 2 moles of NH₃. In contrast, AT transfers the terminal amidino group from arginine to glycine, forming ornithine and guanidinoacetate [11].

Structures for all members of this superfamily, except for *P. gingivalis* PAD, have been determined. For mammalian PAD4, AT, DDAH and ADI, structural data suggest a nucleophilic attack by the thiol group of a cysteine residue on the guanidinium carbon of the arginine substrate [5]. AT was the first member of this superfamily for which structural analysis showed nucleophilic attack by the thiol group of the conserved Cys [12]. For ADI, the function of Cys in nucleophilic catalysis has been demonstrated by transient kinetic studies, such as intermediate trapping and rapid quench techniques, and by structural studies [8,13]. Finally, for DDAH, structural studies and mass spectrometry were used to demonstrate a covalent adduct between an active site cysteine residue and the substrate, and to provide the identity of the cysteine nucleophile [14,15].

According to the Conserved Domain Database for protein classification, the active site of PAD, predicted by alignment with related enzymes, contains amino acids Asp 130, Asp 187, His 236, Asp 238 and Cys 351 [16]. Using a proposed six-step mechanism of the catalytic reaction of arginine deiminase (ADI) [8] as a model, we hypothesize that Cys 351 of PAD initiates catalysis by nucleophilic attack on the guanidino group of a peptidylarginine substrate (Figure 1), and that the remaining active site residues mediate multiple proton transfers.

¹The abbreviations used are: AIH, agmatine deiminase; AstB, arginine succinyltransferase; ADI, arginine deiminase; AT, L-arginine:glycine amidinotransferase; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CAPSO, 3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic; CHES, 2-(N-cyclohexylamino)-ethanesulfuric acid; BISTRIS, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; BSA, bovine serum albumin; DNase I, Deoxyribonuclease I; DDAH, N^ω, N^ω-dimethylarginine dimethylamino hydrolase; DTT, dithiothreitol; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); IPTG, isopropyl β -D-thiogalactopyranoside; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propane sulfonic acid; PMSE, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; NaBA, N- α -benzoyl arginine; and PAD4, mammalian peptidylarginine deiminase 4.

We have previously reported the expression and characterization of a truncated form of *P. gingivalis* PAD in *Escherichia coli*, which like the native secreted enzyme form, lacks the first N-terminal 43 amino acids, but contains N-terminal poly-His and Xpress epitope tags [2]. In the present study a combination of chemical modification, mass spectrometry, and site-directed mutagenesis has identified Cys 351 as the active site nucleophile of the PAD from *P. gingivalis*.

2. Experimental

2.1 Materials

Integrated DNA Technologies, Inc. prepared the oligonucleotide primers for site-specific mutagenesis. TOP10F' competent cells and the *E. coli* BL21(DE3)pLysS competent cells were from Invitrogen. The *Pfu* polymerase was purchased from Stratagene and restriction enzymes were from New England Biolabs. The DNA miniprep kit was purchased from Qiagen. LB broth, IPTG and CHES were purchased from Research Products International Corp. Dithiothreitol (DTT), L-arginine, N- α -benzoyl arginine (N α BA), iodoacetamide, iodoacetic acid, NADPH, α -ketoglutarate, 5'-GDP, glutamate dehydrogenase, and L-canavanine were purchased from Sigma. All other reagents were of the highest quality commercially available.

2.2 Site-directed mutagenesis of truncated PAD

An oligonucleotide, 5'-GGAACAGATGCCCTGCATAGCCGTACTCACGAGGTAGCGG-3', that contained a mutant codon (*italics*) was designed to replace Cys at position 351 of PAD with Ser. The truncated PAD cDNA in a pCRT7/NT TOPO vector (mPADpCRT7/NT TOPO) previously prepared in this lab was used as the template [2]. PCR conditions were as described in the manufacturer's protocol for *Pfu* polymerase using 200 ng of DNA template and 125 ng of each primer, with the following temperature program: 94 °C for 5 min, followed by 16 cycles of 94 °C for 30 sec, 55 °C for 1 min, 68 °C for 13 min, followed by 15 min at 68 °C. The mutated plasmids obtained from PCR were transformed into TOP10F' cells for plasmid amplification. Candidate constructs for the mutant were then confirmed at the DNA Sequencing Facility at the University of Michigan.

For wild type and C351S PAD, the expression plasmids were first transformed into BL21(DE3)pLysS. The cells were grown in LB media supplemented with 34 μ g/mL chloramphenicol and 100 μ g/mL ampicillin. Protein expression was induced by the addition of IPTG to 0.5 mM, followed by incubation for 6 hours at 37 °C. The cells were harvested by centrifugation at 4200 rpm (H-6000 6 liter rotor, Sorvall RC-3B) at 4 °C for 25 min and frozen at -80 °C until purification. The mutant protein was purified using the same protocol as for wild-type enzyme [21].

2.3 Enzyme activity assays

Enzymatic activity was determined by measuring product formation using a colorimetric assay that detects formation of the ureido group of citrulline, adapted for use in microcentrifuge tubes as described previously [2]. Briefly, the reaction was carried out in 50 mM CHES/HCl, pH 9.5 containing 10 mM DTT, 200 μ M N- α -benzoyl-L-arginine (N α BA) (K_m of 15 μ M and k_{cat} of 0.124 sec⁻¹ [2]), and 50 μ g protein in a total volume of 200 μ L. The period of incubation was 10 min at 37 °C. A 400 μ L portion of freshly prepared color developing reagent containing 1 volume of Solution A (80 mM diacetyl monoxime and 2 mM thiosemicarbazide) and 3 volumes Solution B (3 M phosphoric acid, 6 M sulfuric acid, 2 mM ammonium iron (III) disulfate) was used to quench the reaction. The samples were incubated at 95 °C for 15 min, and cooled for 3 min on the bench top. The absorbance of the

samples was then measured at 540 nm and compared to a standard curve generated with various concentrations of N- α -benzoyl citrulline ethyl ester.

To determine the steady state kinetic parameters of the mutant C351S PAD, the standard colorimetric citrulline assay was altered to increase sensitivity. The assays were performed with a 100-fold increase in protein (5 mg), and the period of incubation was extended to 1 hour at 37 °C. Ammonia production by the C351S variant was determined using a modified version [2] of the spectrophotometric assay described by Özer [17] that couples the production of NH₃ by PAD to the reaction catalyzed by glutamate dehydrogenase. The reaction was monitored by following the decrease in absorbance at 340 nm as NADPH is converted to NADP⁺. The assay conditions were as follows: 45 mM CHES/HCl pH 9.5, 5 mM DTT, 10 mM α -ketoglutarate, 0.15 mM NADPH, 1 mM 5'-GDP, 14 units/mL glutamate dehydrogenase, 5 mg PAD, and 200 μ M NaBA in a total volume of 1 mL. The reaction was initiated with addition of PAD and was allowed run for 1 h. Controls were carried out to measure ammonia contamination in each reagent used.

2.4 Inactivation of PAD by thiol-specific reagents

The thiol-directed reagents iodoacetamide and iodoacetic acid were tested as potential inactivators of PAD. Inactivation reactions contained 50 mM TAPS/HCl pH 8.0, and iodoacetamide at concentrations of 0.1, 0.25, 0.5, 1, 5, or 10 mM, or iodoacetic acid at concentrations of 0.5, 1, 5, 10 or 20 mM. The inactivation reactions were initiated by addition of PAD to a final concentration of 1 mg/mL at 37 °C, and 20 μ L samples were periodically diluted 15-fold into the standard assay for activity determination. This dilution and the presence of 20 mM DTT in the assay effectively stopped further inactivation of the enzyme by the thiol-specific reagents during the assay. Pseudo first-order rate constants were obtained from plots of log (% activity remaining) *versus* time.

To determine whether inactivation of PAD by iodoacetamide occurs as a result of active site modification, substrate protection experiments were carried out. For these experiments, reaction mixtures contained 50 mM TAPS/HCl pH 8.0, 800 μ M NaBA (~ 50 times K_m, to maintain substrate saturation of the active site throughout the modification reaction), and 100 μ g PAD in a total volume of 105 μ L at 37 °C. The inactivation reactions were initiated by addition of iodoacetamide to a final concentration of 5 mM, and 20 μ L aliquots were removed at various times for assay. As a control, PAD was incubated in buffer with the same concentration of substrate, but in the absence of iodoacetamide.

2.5 Stoichiometry of PAD modification by mass spectrometry

Mass spectrometry was performed using matrix assisted laser desorption ionization time of flight (MALDI/TOF). Three samples were prepared with 100 μ g of PAD in 50 mM TAPS/HCl pH 8.0: the control sample, the modified sample containing 5 mM iodoacetamide, and the substrate protection sample, containing 5 mM iodoacetamide and 800 μ M NaBA. The samples were incubated for 10 min at room temperature and then quenched with 20 mM DTT. For the substrate protection sample the reaction was carried out for 10 min, during which additions of NaBA were made at 0 and 5 min to give a final concentration of 400 μ M at each addition. The final activity of the modified sample was approximately 1% of that for the control sample, while the substrate-protected sample retained 94% of the control activity. A portion of each sample (15 μ g) was then subjected to SDS-PAGE, stained with Coomassie blue, and de-stained with H₂O. The bands corresponding to the modified and protected proteins were excised and analyzed at the Proteomics Facility at Temple University School of Medicine. Protein fingerprinting analysis was done using AutoFlex MALDI/TOF/TOF from Bruker Dalton Inc., which uses Swiss-Prot database for peptide mass fingerprinting.

Linear mode MALDI/TOF was used to determine the molecular mass of the enzyme modified in the presence and absence of substrate. For this analysis the samples were prepared in solution as described above. The enzyme (1 mg/mL) was transferred from the storage buffer to 0.1% trifluoroacetic acid (TFA) solution by column centrifugation (Sephadex G-50, equilibrated with 0.1% TFA) to remove salts before mass spectrometry analysis [18]. A 4 μ L sample of the enzyme was mixed with 4 μ L of matrix composed of 10 mg of 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), 50% acetonitrile and 0.05% TFA. A 4 μ L portion of the matrix-PAD mixture was transferred to an aluminum plate, air-dried and analyzed by ionizing the samples with a laser using 30 laser hits per sample. The AutoFlex MALDI/TOF program was used to determine the mass of the protein; the molecular standard controls for mass spectrometry were 100 pmoles of bovine serum albumin (66430.09 Da) and 100 pmoles of aldolase (39212.28 Da) (Sigma, MSCAL1).

2.6 pH profile of the PAD reaction

The steady state kinetic parameters for PAD were determined using 0-250 μ M NaBA as substrate over a pH range of 6.5-10 with a mixed buffer system comprised of 50 mM each of MES, MOPS, CAPS, HEPES, CAPSO, BISTRIS, and CHES that guarantees constant ionic strength. The pH of the mixed buffer system was adjusted by the addition of HCl. Product detection was carried out using the standard colorimetric citrulline assay [2]. Each assay was performed in triplicate and the results obtained were fit directly to the Michaelis-Menten equation. The k_{cat}/K_m and k_{cat} values obtained from the Michaelis-Menten equation were plotted as a function of pH. The bell shape curves were fit to equation 1 for a two-pKa model according to Segel where y was substituted by either k_{cat}/K_m or k_{cat} [19].

$$\text{Log}(y_{\text{obs}}) = \log \left[y_{\text{min}} + \frac{(y_{\text{max}} - y_{\text{min}})}{(1 + 10^{(\text{pKa}1 - \text{pH})} + 10^{(\text{pH} - \text{pKa}2)})} \right] \quad (1)$$

2.7 pH dependence of PAD inactivation by iodoacetamide

The pKa of the active site cysteine residue was determined from an analysis of the pH-dependence of iodoacetamide inactivation. Inactivation reactions were carried out over a pH range from 6.0 to 10.0 using the 50 mM mixed buffer system and 1 mM iodoacetamide. The inactivation reactions were initiated by addition of 100 μ g PAD, and aliquots were periodically removed and diluted 10-fold into the standard assay with 20 mM DTT for activity determination. The slopes of the plots of log (% activity remaining) as a function of time for each pH value were used to calculate the k_{obs} . The pKa of the cysteine residue was determined by a fit of the data to equation 2.

$$k_{\text{inact(obs)}} = \frac{(k_{\text{inact(max)}})}{(1 + 10^{(\text{pKa} - \text{pH})})} \quad (2)$$

2.8 Binding of L-canavanine monitored by fluorescence spectrometry

Changes in endogenous tryptophan fluorescence were used to monitor binding of L-canavanine to PAD. For these experiments, the samples contained 25 μ g PAD in 2.0 mL of 50 mM CHES/NaOH pH 9.5 (PAD final concentration 0.2 μ M). The tryptophan residues were excited at 295 nm and the emission intensity was read from 320 nm to 480 nm [20]. The sample was titrated with L-canavanine over the range of 2.5 μ M to 30 mM, and the shift of emission wavelength and the decrease of intensity at 333 nm were recorded. The dilution-corrected decrease in fluorescence intensity at the emission maximum of 333 nm, ΔF , was

used to calculate the dissociation constant of L- canavanine using equation 3; where $\Delta F_{\max}$ is the maximal change in fluorescence and $[I]$ is the concentration of L-canavanine.

$$\Delta F = \frac{\Delta F_{\max} [I]}{K_d + [I]} \quad (3)$$

Free tryptophan was used in place of PAD as a control to ensure that any change in emission intensity or wavelength shift was due to the change in protein structure and not due to an interaction of the inhibitor with the tryptophan.

3. Results

3.1 Inactivation of PAD by iodoacetamide and iodoacetic acid

Truncated PAD, isolated as described in Rodríguez et al. [2], was tested for inactivation by iodoacetamide and iodoacetic acid. At pH 8.0, PAD exhibited pseudo first-order loss of activity in the presence of 0.1-10 mM iodoacetamide (Figure 2A) or 0.5-20 mM iodoacetic acid (Figure 2C). The second-order rate constants of $0.035 \text{ mM}^{-1} \text{ min}^{-1}$ and $0.0059 \text{ mM}^{-1} \text{ min}^{-1}$ were calculated from replots of k_{obs} versus iodoacetamide or iodoacetic acid concentration, respectively (Figures 2B and 2D).

3.2 Substrate protection against iodoacetamide inactivation

Inactivation of PAD by iodoacetamide and iodoacetic acid may be due to modification of active site residues, or modification of other more distant residues, resulting in a conformational change that is accompanied by a loss of activity. To discriminate between these possibilities, the effect of substrate on the rate of inactivation by iodoacetamide was explored. When substrate (800 μM NaBA) was present in the inactivation reaction, PAD retained 88% activity after a 4 min incubation, compared to only 32% residual activity under the same reaction conditions in the absence of substrate (data not shown). Overall, this result suggested that there is a catalytically important cysteine residue in the active site.

3.3 Stoichiometry of PAD modification by mass spectrometry

To determine the stoichiometry of cysteine modification, MALDI/TOF analysis was carried out on iodoacetamide-treated PAD. The samples analyzed included PAD modified in the presence and absence of NaBA, with approximately 94% and 1% of activity remaining, respectively. Non-modified PAD was used as control. MALDI/TOF analysis (Figure 3), shows that the non-modified PAD sample exhibited one major peak at $60886 \pm 10 \text{ Da}$ (Figure 3A), which is similar to the calculated mass of truncated PAD with its poly-His and Xpress epitope tags (60884.24 Da). The spectrum of the iodoacetamide-modified PAD sample showed a major peak at $61058 \pm 10 \text{ Da}$ (Figure 3B). This represents a 172 Da increase in mass compared to the non-modified enzyme, and is consistent with the modification of three cysteine residues by iodoacetamide (an increase of 58 Da is expected for each cysteine residue modified). The spectrum in Figure 3C showed a major peak at $60993 \pm 10 \text{ Da}$, a 107 Da increase in mass for PAD modification in the presence of NaBA. This result demonstrates that in the presence of substrate approximately two cysteine residues (1.8) are modified by the reagent, and one cysteine residue is protected from modification. Since the enzyme retained 94% activity in the presence of substrate, the simplest explanation is that modification of the other two cysteine residues does not affect PAD activity.

To identify which of the modified cysteine residues was protected by the substrate, MALDI/TOF/TOF was performed on trypsin-digested PAD that had been modified by

iodoacetamide in the presence or absence of NαBA. Non-modified PAD was used as control. The MALDI/TOF/TOF analysis of the control showed four peptides that accounted for six of the eight cysteine residues in PAD. Even though only 51% of the peptides were ionized by the laser during MALDI/TOF/TOF, two of the three iodoacetamide-modified cysteine residues were identified in the analysis. The sample of iodoacetamide-modified PAD showed that the peptide consisting of amino acids 100-122 (NTVITQYTQSGVNLSNCDFFIAK) had a mass of 2585.05 Da, which is approximately 58 Da higher than the calculated mass (2529.85). Only Cys 117 of this peptide could be modified by iodoacetamide. A second peptide consisting of amino acids 337-352 (GASGTPWLGTDLHCR) had a mass of 1698.7 Da, which is approximately 58 Da higher than the calculated mass of this peptide (1641.82 Da); this peptide has only Cys 351 as a viable candidate for iodoacetamide modification under the modification reaction conditions (pH 8.0)[18]. When the substrate is present in the modification reaction, peptide 337-352 showed a mass of 1641.82 Da, indicating that it was no longer modified by iodoacetamide. These results indicate that the cysteine protected by the NαBA is Cys 351, and provide evidence that Cys 351 is essential for catalysis.

3.4 pH profile

The plot of $\log(k_{\text{cat}}/K_m)$ vs. pH is bell-shaped with pKa values of 7.9 ± 0.2 and 8.6 ± 0.4 for the ascending and descending limbs, respectively (Figure 4A). The plot of $\log(k_{\text{cat}})$ vs. pH is also bell-shaped with two apparent pKa values of 7.7 ± 0.1 and 8.3 ± 0.6 for the ascending and descending limbs, respectively (Figure 4B). These pKa are most likely showing the protonation state of a cysteine and a histidine in the active site of the enzyme, however, assignment of these pK values to either residue based on this data alone is problematic.

3.5 pH dependence of PAD inactivation by iodoacetamide

The pH dependence of the inactivation of PAD by 1 mM iodoacetamide was determined and fit to equation 2. The results of these experiments showed a pKa value of 7.7 ± 0.2 (Figure 5), in good agreement with the pKa of the ascending limb of the bell-shape curve in plots of $\log(k_{\text{cat}}/K_m)$ and $\log k_{\text{cat}}$ vs. pH.

3.6 Expression and characterization of C351S PAD

Site directed mutagenesis to generate the C351S variant of PAD was performed using the cDNA for the truncated PAD [2] as template. Sequencing verified that no other mutations were introduced. The C351S variant was purified using the same protocol as for wild-type PAD. After purification, the mutant appeared to be approximately 85% pure on a Coomassie stained SDS-PAGE (data not shown).

The C351S variant of PAD was evaluated for catalytic activity using NαBA as assay conditions of increased enzyme and time of incubation as described in section 2.3, using either the colorimetric citrulline assay or the coupled ammonia assay [2]. These results indicate that the mutant enzyme has less than 0.01% activity of the wild-type PAD.

3.7 Ligand binding studies

L-canavanine has been shown to be a slow substrate for ADI; a stabilized S-alkylthiuronium intermediate is produced that is very slowly hydrolyzed to release product [21]. We have tested L-canavanine as a substrate/inhibitor of PAD and found that L-canavanine is a suicide substrate, $K_i = 4.19$ mM [22]. The binding constant of L-canavanine for PAD was determined by fluorescence titration. The decrease in fluorescence intensity at 333 nm was used to determine a dissociation constant of 5.6 ± 1 mM for L-canavanine, which is similar to the K_m for arginine (1.2 mM) (data not shown). The dissociation constant for L-

canavanine binding to the C351S mutant PAD was also determined to test whether the lack of activity for this mutant protein resulted from the inability of the enzyme to bind substrate. The binding constant for L-canavanine binding to the C531S variant was 4.9 ± 0.7 mM (data not shown), a value comparable to that for L-canavanine binding to the wild-type truncated PAD.

4. Discussion

PAD has been assigned to a superfamily of guanidino modifying enzymes [5]. Members of this superfamily have been proposed to have similar catalytic mechanisms in which the active site cysteine acts as a catalytic nucleophile that initiates catalysis and forms a covalent S-alkylthiuronium intermediate. All the members of the superfamily except for *Porphyromonas gingivalis* PAD have been shown to have a Cys as the catalytic nucleophile and three of the enzymes in this superfamily, AT, DDAH and ADI, have been shown to produce a covalent intermediate in their reaction mechanism [12,15,21]. The active site of PAD predicted by alignment with related enzymes suggests a catalytic role for Cys 351. We hypothesized that Cys 351 makes a nucleophilic attack on the guanidino group of a peptidylarginine substrate that initiates the enzyme reaction and that additional active site residues mediate multiple proton transfers.

The results of the enzyme modification and substrate protection experiments with iodoacetamide and iodoacetic acid indicate that a modified residue, most likely a cysteine residue, is critical for catalysis. This conclusion is also supported by the inhibitory metal-binding of Zn^{++} and Cd^{++} to the enzyme [2]. Comparison of the second-order rate constants describing inactivation by iodoacetamide and iodoacetic acid shows that the neutral iodoacetamide is more effective than the negatively charged iodoacetic acid in modification of the active site thiol. This difference in reactivity may result from anticipated electrostatic repulsion between iodoacetic acid and the negatively charged amino acid residues (Asp 130, Asp 187, and Asp 238) that are predicted to be found at the active site of the enzyme [16]. Similar results have been reported for mammalian PAD4 [23], where the uncharged reagent (iodoacetamide) was a better modifier than the negatively charged reagent (iodoacetic acid). Mass spectrometry analysis of iodoacetamide-modified PAD in the absence and presence of substrate indicated that there were three residues modified by iodoacetamide, but only one cysteine residue was protected by substrate. The MALDI/TOF/TOF analysis of tryptic peptides showed that the residue modified by iodoacetamide and protected by substrate was Cys 351; this result is consistent with the proposed role of this residue as the catalytic nucleophile.

To further verify the importance of Cys 351 in the enzyme catalytic mechanism, site directed mutagenesis was utilized to generate the C351S PAD variant. The mutant enzyme was expressed and purified following the same protocol employed for wild-type truncated PAD. To test for proper enzyme folding, binding studies were carried out by fluorescence spectrometry analysis. There are six tryptophans in PAD, which were excited at 295 nm. Binding studies indicated that the C351S mutant enzyme was still capable of proper folding because it bound L-canavanine with similar affinity and showed similar fluorescence change as the wild-type truncated PAD. The mutant enzyme had less than 0.01% activity of the wild-type PAD when assayed with either the colorimetric citrulline assay or the coupled spectrophotometric ammonia assay. Additionally, these results suggest that the serine side chain does not act as a nucleophile, indicating that Cys 351 is essential for catalysis.

pH rate studies were performed to identify ionizations that were critical for catalytic activity. The plot of $\log(k_{cat}/K_m)$ versus pH using NaBA as a substrate indicates that the ionization states of 2 residues on the free enzyme are critical determinants of catalytic activity. The

ascending limb of the plot shows a pK value of 7.9, while the descending limb shows a pK of 8.6. Neither of these pK values is significantly perturbed upon binding of substrate (log k_{cat} versus pH), indicating that a substrate-assisted deprotonation mechanism does not apply for this enzyme. This result is in sharp contrast those obtained for DDAH [23], another member of the GME superfamily. For DDAH, the plot of log (k_{cat}/K_m) for the substrate N^ω-methyl-L-arginine identified apparent pK values of 7.9 and 9.3 (corrected pK values of 8.5 and 8.7), while in the plot of log k_{cat} versus pH the apparent pK of the ascending limb had decreased to 6.1. Direct measurement of the pK of the active site cysteine in the free enzyme by UV-Vis difference spectroscopy gave a value of 8.9, and the pH dependence of iodoacetic acid-modification yielded a value of 8.7 for this pK. Finally, the results of UV-Vis difference spectroscopy for the DDAH-citrulline and DDAH-lysine complexes showed that in the presence of the cationic ligand, the cysteine was deprotonated. Collectively, these results support a substrate-assisted deprotonation mechanism for DDAH [23].

The simplest interpretation of the pH data for *P. gingivalis* PAD is that the ascending limb represents the ionization of the active site thiol, while the descending limb represents deprotonation of another group, possibly histidine. However, such an interpretation is complicated by the possibility of a reverse protonation mechanism, as has been postulated for PAD4 [24]. This enzyme, which displays corrected pK values of 7.3 and 8.2 in plots of log k_{cat}/K_m versus pH, shows a pK of 8.3 for inactivation by iodoacetamide, in good agreement the value obtained from the descending limb of the plot. Thus the results for PAD4 are consistent with a reverse protonation scheme, with the pK of the active site cysteine and histidine residues being 8.2 and 7.3, respectively.

To address the possibility that the PAD from *P. gingivalis* might employ a similar reverse protonation mechanism, the pH dependence of inactivation by iodoacetamide was determined. The resultant pK value determined from plots of $k_{\text{inact(obs)}}$ versus pH is 7.7, in good agreement with the pK values determined from the ascending limbs of both Log (k_{cat}/K_m) and log k_{cat} versus pH plots. Thus, the ascending limb observed in the pH rate profiles for PAD corresponds to ionization of Cys 351.

Although the members of the guanidino modifying enzyme superfamily have in common a general catalytic mechanism involving covalent catalysis by an active site cysteine residue and also share some overlap in substrate specificity, notable differences exist in kinetic and active site properties. For example DDAH employs substrate-assisted deprotonation of the active site cysteine [23], while PAD4 employs a reverse protonation mechanism [24]. Furthermore, members of the superfamily differ in their requirements for cofactors. PAD4 requires Ca²⁺ for optimal activity [25,26], ADI is activated by Mg²⁺ and K⁺ [21,27], while the PAD from *P. gingivalis* shows no metal dependence of enzyme activity [2].

In summary, the present results provide strong evidence that Cys 351 serves as a catalytic nucleophile in the reaction catalyzed by PAD from *Porphyromonas gingivalis*. Further studies are required to test for a proposed covalent intermediate in the catalytic mechanism and to investigate the possible involvement of a His residue (His 236?) that may be necessary for catalysis. These and future studies will bring us closer to the design of inhibitors that can target *P. gingivalis* PAD.

Research Highlights

- Peptidylarginine deiminase from *Porphyromonas gingivalis* is inactivated by thiol-directed reagents
- Cysteine 351 is the amino acid residue critical for catalysis

- The C351S variant has no detectable enzyme activity

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Figure 1.

Proposed mechanism for *P. gingivalis* PAD. Nucleophilic attack by a Cys residue initiates the catalytic reaction, forming a tetrahedral intermediate. Upon release of ammonia, a thiuronium ion intermediate is formed, which is released as peptidylcitrulline following hydrolysis with water.

Fig.2A

FIG.2B

FIG.2C

FIG.2D

Figure 2.

Inactivation of PAD by iodoacetamide and iodoacetic acid at pH 8 and 37 °C. (A) Pseudo-first order inactivation of PAD by various concentrations of iodoacetamide: 0 (■), 0.1 mM (Δ), 0.25 mM (*), 0.5 mM (○), 1 mM (●), 5 mM (□) and 10 mM (+). (B) Replot of k_{obs} versus [iodoacetamide] to determine second-order rate constant for the inactivation process. (C) Pseudo first-order inactivation of PAD by various concentrations of iodoacetic acid: 0 (□), 0.5 mM (*), 1 mM (Δ), 5 mM (◆), 10 mM (●) and 20 mM (○). (D) Replot of k_{obs} versus [iodoacetic acid] to determine second-order rate constant for the inactivation process.

Figure 3.

MALDI/TOF analysis of PAD and iodoacetamide-treated PAD. Spectrum A shows the peak of the non-modified PAD at 60886 Da. Spectrum B shows the peak for PAD treated with 5 mM iodoacetamide occurring at a m/z that is 172 Da higher than that of the control. Spectrum C shows the peak for PAD treated with 5 mM iodoacetamide in the presence of 800 μ M NaBA at 60986 Da, at a m/z 107 Da higher than that for the control.

FIG.4A

FIG.4B

Figure 4. pH profile of PAD. (A) Plot of $\log(k_{\text{cat}}/K_m)$ as a function of pH. (B) Plot of $\log k_{\text{cat}}$ as a function of pH. The solid line describes the fit of the data from three experiments to Equation 1.

Figure 5. pKa profile for modification of Cys351 by iodoacetamide. Pseudo first-order rate constants were plotted as a function of pH. The solid line describes the fit of the data from five experiments to Equation 2. Error bars indicate the mean $\pm$ S.D.