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Haloacetamidine-Based Inactivators of Protein Arginine Deiminase 4 (PAD4): Evidence that General Acid Catalysis Promotes Efficient Inactivation

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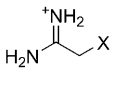
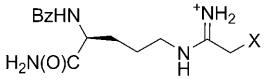
Dysregulated protein arginine deiminase (PAD) activity, particularly PAD4, has been suggested to play a role in the onset and progression of numerous human diseases, including rheumatoid arthritis (RA). Given the potential role of PAD4 in RA, we set out to develop inhibitors/inactivators that could be used to modulate PAD activity and disease progression. This effort led to the discovery of two mechanism-based inactivators, denoted F- and Cl-amidine, that inactivate PAD4 by the covalent modification of an active-site cysteine that is critical for catalysis. To gain further insights into the mechanism of inactivation by these compounds, the effect of pH on the rates of inactivation was determined. These results, combined with the results of solvent isotope effect and proton inventory studies, strongly suggest that the inactivation of PAD4 by F- and Cl-amidine proceeds by a multistep mechanism that involves the protonation and stabilization of the tetrahedral intermediate formed upon nucleophilic attack by the active-site cysteine, that is, Cys645. Stabilization of this intermediate would help to drive the halide-displacement reaction, which results in the formation of a three-membered sulfonium ring that ultimately collapses to form the inactivated enzyme. This finding—that protonation of the tetrahedral intermediate is important for enzyme inactivation—also suggests that, during catalysis, protonation of the analogous intermediate is required for efficient substrate turnover.

In nature, a myriad of post-translational modifications are found in proteins. These modifications, and the requisite modifying enzymes, can have far-reaching effects on living systems. Within the family of protein-modifying enzymes are the protein arginine deiminases (PADs). These enzymes catalyze the hydrolysis of arginine residues to form citrulline.[1–3] Much effort from our laboratory has been focused on gaining insight into the mechanism of the PADs, and, in particular, PAD4.[3–5] Our interest in PAD4, and the PADs in general, was piqued as evidence emerged that linked dysregulated PAD activity to the increased incidence and severity of rheumatoid arthritis (RA).[2,3,6] This disease, which afflicts nearly 1% of the population, is an autoimmune disorder that appears to be triggered, at least in part, in response to aberrant citrullination, a result of dysregulated PAD activity. Based on this apparent causal relationship, we set out to develop inhibitors/inactivators that could be used to modulate PAD activity and disease progression. This effort led to the discovery of two mechanism-based inactivators, denoted F- and Cl-amidine, which are the most potent PAD inhibitors described to date.[7,8] At the same time, Fast and colleagues reported that 2-chloroacetamidine, that is, the warhead in Cl-amidine, also inactivates PAD4 and other members of the guanidinium-modifying family of enzymes.[9]

Much work has been done to characterize the mechanism of inactivation, including dialysis experiments to verify irreversible inhibition. Additionally, analysis of the kinetics of inactivation demonstrated that F- and Cl-amidine possess k_{inact}/K_I values of 3000 and 13000 $M^{-1}min^{-1}$, respectively.[7,8] Subsequent crystallographic data confirmed that inactivation was due to the covalent modification of an active-site cysteine (Cys645) that is critical for catalysis—this residue promotes arginine deimination by covalent catalysis according to a mechanism that is analogous to that of the Cys proteases.[5] While this finding conclusively demonstrated the mode of inactivation–alkylation of the thiolate, the precise mechanism of inactivation has yet to be established.



CA; X = Cl
FA; X = F



Cl-amidine: X = Cl
F-amidine: X = F

Although unclear, the mechanism of inactivation presumably proceeds through one of at least two routes: direct displacement of the halogen through an S_N2 mechanism or initial attack on the iminium carbon, followed by displacement of the halide to form a sulfonium ring, and ending with concomitant re-formation of the imine and opening of the sulfonium ring (Scheme 1). While the former possibility is the more intuitive, the latter is analogous to the mechanism by which the fluoromethylketones inactivate the cysteine proteases,[10–12] and therefore warranted further investigation.

We began our investigations by examining the influence of pH on k_{inact}/K_I, that is, the second-order rate constant of enzyme inactivation. These studies were pursued because pH rate profiles can suggest the identity of catalytically important functional groups, in the inactivator or free enzyme, up to and including the first irreversible step of the reaction. For these studies, k_{inact}/K_I values were determined for both Cl- and F-ami-

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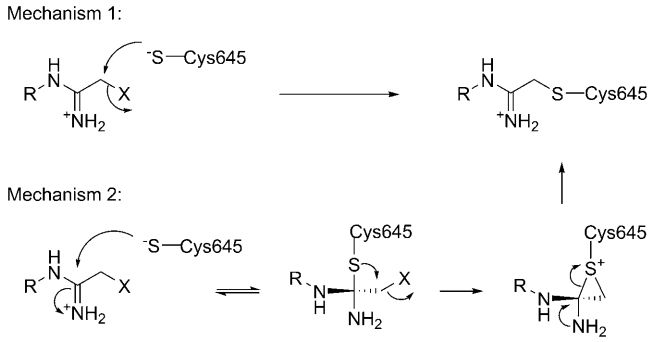


Scheme 1. Structures of compounds described in this communication and proposed mechanisms of PAD4 inactivation by Cl- or F-amidine.

dine over a pH range of 6.0–8.5. Interestingly, the rates of inactivation increased until ~pH 8.1, after which point further increases in alkalinity resulted in a dramatic drop in the rates of inactivation (Figure 1 A and B).

Fits of the F-amidine versus pH rate data give estimates of the pK_a values for the ascending and descending limbs of 7.05 and 8.05, respectively, with a pH-independent value of 1425 $M^{-1}min^{-1}$. Interestingly, these values are in reasonable agreement with those obtained for substrate turnover (i.e., 7.3 and 8.2) and, as such, they likely correspond to the pK_a values of His471 and Cys645, respectively. Given that His471 is thought to act as a general acid in the acylation step of the reaction, and that this drop in reactivity mimics the pH profile for substrate turnover,[5] these data suggest that general acid catalysis also contributes to enzyme inactivation by F-amidine. Although the data for Cl-amidine fit poorly to canonical pH versus rate equations, the trends of the pH rate profiles are similar (i.e., decreased inactivation at pH values >8), thereby suggesting that general acid catalysis also plays a role in the inactivation of PAD4 by this compound.

To gain further insight into this process, the effect of pH on PAD4 inactivation by 2-fluoroacetamidine (FA)—the fluoroacetamidine warhead alone—was examined. As with Cl- and F-amidine, the rates of inactivation are roughly bell-shaped (Figure 1 C). In contrast, the rate at which 2-chloroacetamidine (CA) inactivates PAD4 does not decrease with increasing alkalinity (Figure 1 D).[5] This break from the trend suggests that CA inactivates PAD4 through a mechanism that is different from the mechanism by which F-amidine, Cl-amidine, and FA inactivate the enzyme. Given that chloride is an inherently better leaving group than fluoride, the most reasonable assumption is that inactivation by CA can proceed through the direct S_N2 displacement of chloride, that is, mechanism 1 in Scheme 1. Based on the fact that general acid catalysis is unlikely to enhance the rate of this reaction, this assumption is not only plausible, but likely.

The break in the trend for CA also argues, albeit indirectly, that PAD4 inactivation by Cl-amidine, F-amidine, and FA proceeds through the proposed multistep mechanism, that is, mechanism 2. This argument, however, does not explain why the chloride of Cl-amidine is not directly displaced. The simplest and most reasonable hypothesis to explain this discrepancy is that CA, lacking any peripheral structural elements, has far fewer limitations on its orientation within the active site. As such, this molecule can inactivate PAD4 by a direct S_N2 displacement that is solely dependent on the concentration of the thiolate. By contrast, the "backbone" amide groups of Cl-amidine are instrumental in binding,[4,7,8,13] and therefore restrict conformational freedom, and lead to a mode of binding that is not conducive to an S_N2 displacement.

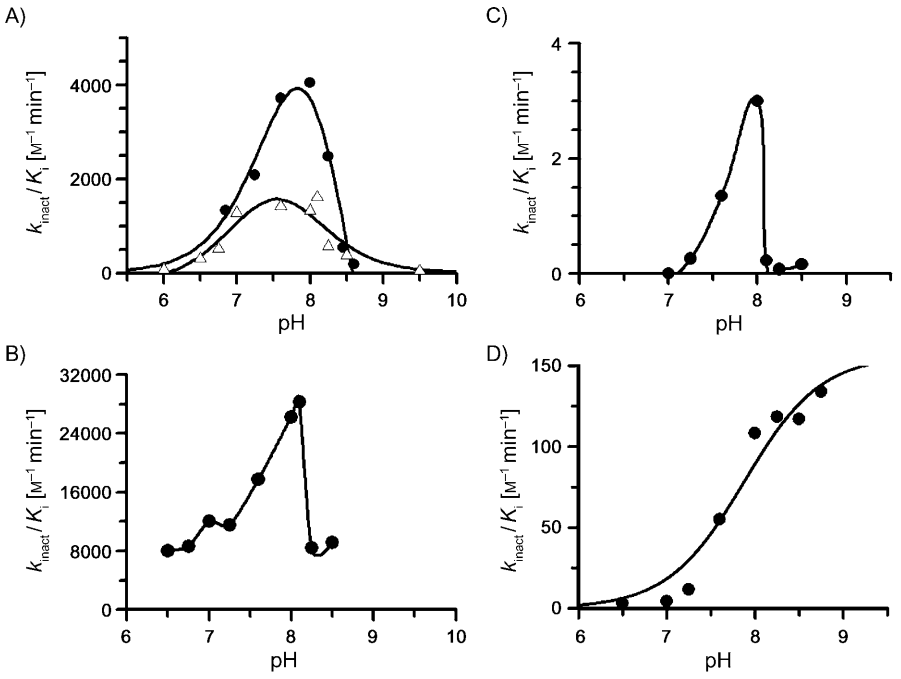


Figure 1. Inactivation pH profiles of Cys645 by using A) F-amidine, B) Cl-amidine, C) 2-fluoroacetamidine, and D) 2-chloroacetamidine. △: D_2O, ●: H_2O.

While this hypothesis rationalizes the observed differences in the kinetics of inactivation, it does not directly explain the observed decrease in inactivation rate at higher pH. Although we have suggested that this drop in reactivity suggests a role for general acid catalysis, alternative explanations for the observed drop in reactivity include deprotonation of the warhead and or decomposition of the inhibitors.

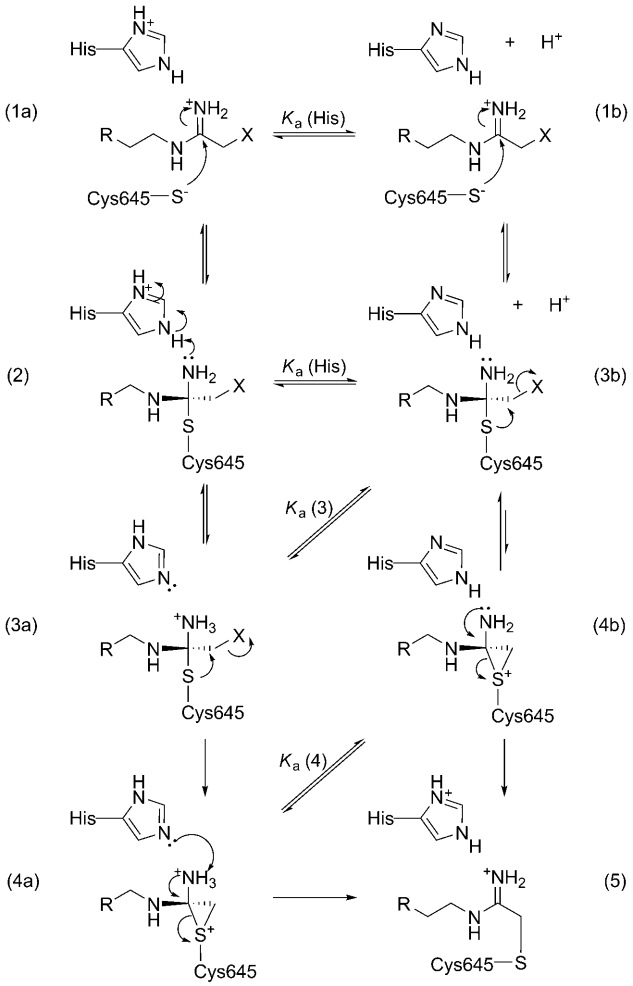
Warhead deprotonation would be expected to decrease the rate of inactivation because the positive charge of the protonated arginine side chain is crucial for substrate binding and catalysis.[4] To investigate this possibility, the pK_a of the fluoroacetamidine warhead in

F-amidine was determined by NMR. By monitoring the chemical shifts of the protons that are in closest proximity to the amidine, that is, the protons adjacent to the fluoride and the protons on the δ-carbon, the pK_a of the warhead was found to be 10.2 (Figure 2). Virtually identical results were obtained by fluorine NMR (Figure S1 in the Supporting Information). While this pK_a is significantly lower than that of arginine (pK_a = 12.5), it is high enough to ensure complete protonation in the pH regime of our experiments.

As described above, the dramatic loss in inactivator potency could be due to inhibitor decomposition at higher pH values. To test this possibility, samples of the inhibitors were subjected to reaction conditions in the absence of enzyme, and subsequently analyzed by analytical HPLC. Essentially no decomposition was observed (Figure S2). This possibility was further ruled out by a slight modification to our assay procedure. Typically, the inactivator was incubated in assay buffer for 10 min prior to the addition of PAD4 to initiate the inactivation reaction. Decreasing this time to 2 min did not affect the rate of inactivation (data not shown).

Having ruled out inactivator deprotonation and decomposition as reasons for the sharp decline in inactivation rates above pH 8.0, the most reasonable explanation for the observed effect on k_{inact}/K_I, which reports on ionizable groups in the free enzyme, is the presence of a protonated active-site residue that is important for binding and orienting the warhead for nucleophilic attack by the active-site thiol. If, as we expect (see below), this group represents the imidazolium form of His471, it could polarize the acetamidine, fix the charge on one of the two NH_2 groups, and thereby promote reaction with the active-site thiolate.

To gain further insights into this process, k_{inact} values were determined for F- and Cl-amidine as a function of pH. These experiments were performed to identify catalytically important functional groups in the enzyme·inactivator complex. Additionally, we reasoned that for mechanism 1, the simple S_N2 mechanism, the plots of k_{inact} versus pH should rise with increasing pH and plateau at a limiting value that is dependent on the concentration of the active-site thiolate. In contrast, for the more elaborate mechanism depicted in Scheme 2, we reasoned that if general acid catalysis plays a role in promoting enzyme inactivation after the inactivator has bound the enzyme, then k_{inact} would rise to a limiting value and then fall as the pH is increased. Note that k_{inact} values were not determined for FA and CA because the high K_I values for these compounds made such a determination impractical.



Scheme 2. Proposed general acid-catalyzed mechanism of inactivation.

Although these data fit poorly to canonical pH versus rate equations, the results of these studies demonstrated that the plots of k_{inact} versus pH plots are also bell-shaped (Figure 3), with the groups contributing to the overall shape of the curve possessing an average pK_a of 7.5. As described above, these results further discount the simple S_N2 mechanism. Additionally, the drop in reactivity at higher pH values indicates that general acid catalysis promotes enzyme inactivation after the formation of the initial enzyme·inactivator complex. These results also indicate that the decrease in k_{inact} is the major contributor to the observed effect on k_{inact}/K_I, thus indirectly arguing against a pH effect on inactivator binding.

The observed effect on k_{inact} is again consistent with a role for general acid catalysis in enzyme inactivation, specifically in the enzyme·inactivator complex. Based on the proposed mechanism of catalysis (Figure S3), the most likely residue is His471, which serves to protonate the departing ammonia through

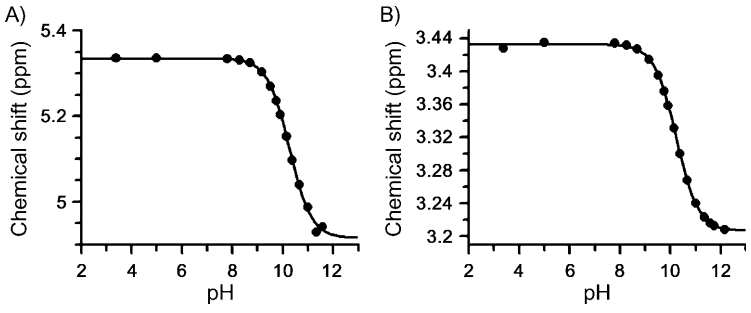


Figure 2. NMR titration curves: A) protons adjacent to the fluoride, B) protons on the δ-carbon.

the course of catalysis. Although we and others have suggested that, for substrate turnover, proton donation occurs during the collapse of the tetrahedral intermediate,[1,14] it is equally plausible that protonation of the intermediate occurs during the formation of this intermediate in either in a stepwise fashion or concomitant with the change in hybridization that occurs upon thiolate attack on the guanidinium carbon. If either of the latter possibilities is correct, the above-described inactivation data would imply a role for this residue in stabilizing the tetrahedral intermediate formed during the inactivation reaction (Scheme 2).

The pK_a of His471 has been assigned as 7.3;[5] thus at higher pH, this residue is unprotonated and cannot donate a proton to stabilize the tetrahedral intermediate formed by thiolate attack on the iminium carbon (3b; Scheme 2). Without this stabilization, the equilibrium is greatly shifted towards form 1b. In contrast, by stabilizing the tetrahedral intermediate, the displacement of the halide (3a) would be favored. The protonation state of the intermediate (3) is also likely influenced by the increasing pH. Based on model compounds, the pK_a of this intermediate is expected to be 2 units lower than that of the parent compound. Thus, at pH > 8, a similar decline in reactivity would be expected. These overlapping effects, that is, loss of stabilization from the protonated histidine (2) and deprotonation of intermediate (3), might account for the sharp declines in the rates of inactivation that are observed above pH 8.

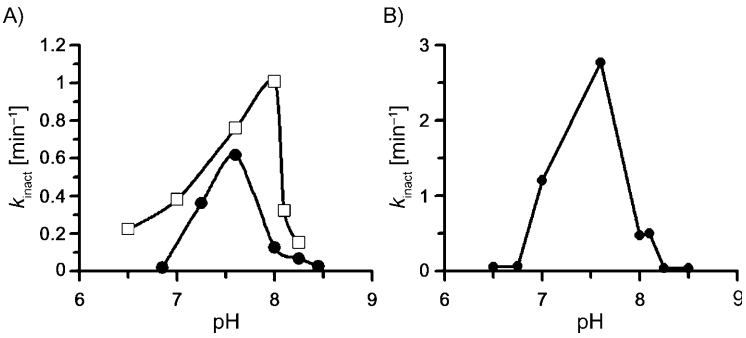


Figure 3. Rates of PAD4 inactivation by A) F-amidine in D_2O (□) and H_2O (●) and B) Cl-amidine in H_2O.

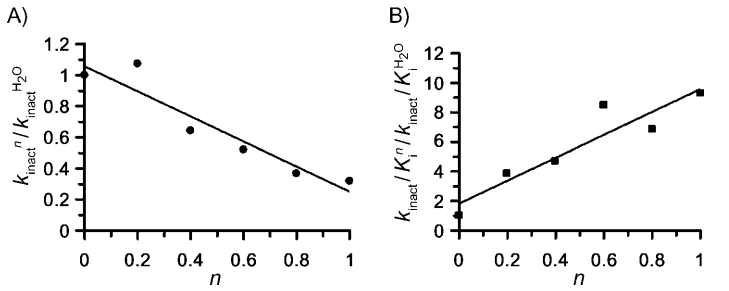


Figure 4. Protonic inventories obtained for the F-amidine-induced inactivation of PAD4: A) k_{inact} and B) k_{inact}/K_I. The term *n* refers to the fraction of D_2O in the reaction. Data were obtained at pL = 7.6.

Further evidence to support the proposed mechanism of inactivation comes from measurements of the deuterium solvent isotope effect (SIE). For these studies, we focused our efforts on F-amidine, as opposed to CA or Cl-amidine, because this compound can presumably only inactivate the enzyme through the second mechanism, and the K_I values are low enough to facilitate the measurement of k_{inact} values.

The average of two independent experiments indicates that the SIE on k_{inact} is normal ($k_{inact}^{H}/k_{inact}^{D} = 2.17 \pm 0.94$; Figures 3 and 4), whereas for k_{inact}/K_I, the SIE was inverted over the entire pL range studied [$(k_{inact}/K_I^{H})/(k_{inact}/K_I^{D}) = 0.24 \pm 0.14$; at the pL optimum (Figures 1 and 4)]. The normal SIE on k_{inact} is suggestive of a proton in flight in the transition state. Such an effect is consistent with the proposed mechanism of inactivation, wherein proton donation to the tetrahedral intermediate would be expected to yield a normal isotope effect. Consistent with this possibility is the fact that proton inventory analyses performed at the pL optimum are linear ($r^2 = 0.947$); this is consistent with a single hydrogenic site in the isotope-sensitive step of enzyme inactivation (Figure 4).

The large and inverse SIE observed for k_{inact}/K_I is consistent with the inverse SIE obtained for substrate turnover;[5] this further suggests that similarities exist between the mechanism of substrate turnover and enzyme inactivation. As previously described for substrate turnover,[5] this large inverse SIE could be explained by pre-equilibrium ionization of the thiol to form the thiolate. Consistent with this possibility is the fact that, while the displacement of the pK_a is expected to be ~0.4–0.6 pH units higher in D_2O than in H_2O, the observed difference (~0.2) is smaller; such smaller differences are highly suggestive of ionization of a thiol to the thiolate. Also consistent with a single ionization of an active site residue is the fact that the proton inventory for k_{inact}/K_I is linear (Figure 4B; $r^2 = 0.931$).

In conclusion, the data strongly suggest that the inactivation of PAD4 by F- and Cl-amidine proceeds by the multistep mechanism, that is, mechanism 2. Our data further suggest that protonation of the tetrahedral intermediate (3; Scheme 2) helps to drive the halide-displacement to form the three-membered sulfonium ring (4), which ultimately collapses to form the inactivated enzyme (5). This finding—that protonation of the tetrahedral intermediate is important for enzyme inactivation—might also suggest that, during catalysis, protonation of the analogous intermediate is required for efficient substrate turnover.

Experimental Section

Chemicals: Dithiothreitol (DTT), benzoyl L-arginine ethyl ester (BAEE), and tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma–Aldrich, F- and Cl-amidine were synthesized as previously described,[7,8] 2-chloroacetamidine was obtained from Oakwood Products (Columbia, SC), and protein arginine deiminase 4 (PAD4) was purified as previously described.[5]

Inactivation kinetics: Inactivation experiments with F-amidine, Cl-amidine, and 2-fluoroacetamidine were performed and data were

fit to equations as previously described.[5] The inactivation of PAD4 by 2-chloroacetamidine has been described previously.[5] The inactivation rates obtained for F-amidine, Cl-amidine, and 2-fluoroacetamidine from this analysis were plotted against pH. Given that the dramatic decrease in activity at pH values >8 precluded fitting of the data to standard equations, the curves for 2-fluoroacetamidine and Cl-amidine were simply generated by using the line function in GraFit (version 5.0.11).[15] The fit for the F-amidine data was determined graphically.

Solvent isotope effects: These experiments were performed as previously described,[5] except that the reaction was carried out in $>95\%$ D_2O. Data were fit to equations described previously.[5]

Proton inventory studies: Briefly, the experiments were performed as described previously for the inactivation kinetics experiments.[5] The only difference was that inactivation reactions were performed in the presence of various D_2O concentrations (0–95%). The data collected were processed by using the methods described above. k_{inact} and k_{inact}/K_I values were obtained and plotted as a ratio of D_2O to H_2O as a function of D_2O concentration (n). The resulting plot was fit by using the line function in GraFit (version 5.0.1.1).[15]

Synthesis of 2-fluoroacetamidine: Ammonia (1.6 mL, 2 M in MeOH) was added to a solution of 2-fluoro-ethylthioimidate (250 mg, 1.6 mmol) in dry ethanol (10 mL) at 0 °C. The reaction mixture was allowed to warm to RT. After 16 h of stirring, the volatiles were removed under reduced pressure to afford a white powder that contained both the title compound and ammonium chloride. The final composition of this mixture was determined by NMR using an internal standard. ^{1}H NMR (300 MHz, D_2O): $\delta=5.19$ (d, $J_{H-F}=44.7$ Hz, 2H); ^{13}C NMR (100 MHz, D_2O): $\delta=166.12$ (d, $J_{C-F}=19.9$ Hz), 77.49 (d, $J_{C-F}=178.3$ Hz); ^{19}F NMR (379 MHz, D_2O): $\delta=-233.67$ (t, $J_{H-F}=44.8$ Hz); HRMS-ESI ($C_2H_6FN_2^+$): calcd 77.0515, obsd 77.0526.

NMR titration: NMR titrations were conducted on a 400 MHz spectrometer in H_2O (5% D_2O). Proton chemical shifts were measured relative to 4,4-dimethyl-4-silapentane-1-sulfonic acid, and fluoride chemical shifts were relative to trifluoroacetic acid (TFA; internal tube). Upon dissolution of F-amidine, the pH was measured by using a micro-pH meter, and the first spectra were taken. For subsequent measurements, the sample was transferred to a clean vial, where the pH was adjusted with NaOH (1 M) before being transferred back to the NMR tube. Changes in chemical shift values were plotted against pH. pK_a values were determined by fitting the data to Equation (1):

$$y = \frac{(\mathrm{Lim1} + \mathrm{Lim2})10^{\mathrm{pH}-\mathrm{p}K_a}}{10^{\mathrm{pH}-\mathrm{p}K_a} + 1} \quad (1)$$

using GraFit (version 5.0.11).[15] Lim1 is the minimum rate, and Lim2 is the maximum rate.

Decomposition experiments: To investigate the effect of the reactions conditions on Cl-amidine and F-amidine, these compounds were subjected to the assay conditions in the absence of enzyme. Briefly, each inhibitor was dissolved in assay buffers (pH 6.5, 7.0, 7.5, 8.0, and 8.5) and incubated at 37 °C. After 30 min, the samples were flash frozen in liquid nitrogen. Each sample was analyzed by reversed-phase (C18) analytical HPLC. The C18 column was pre-equilibrated with ddH_2O/0.05% TFA, and the reaction components were separated by using a linear gradient of acetonitrile/0.05% TFA.

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