Tight Binding Inhibitors of *N*-Acyl Amino Sugar and *N*-Acyl Amino Acid Deacetylases

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Enzymes within the amidohydrolase superfamily have been shown to catalyze the hydrolysis of amide and ester bonds to carbon and phosphorus centers in a wide range of substrates, including nucleic acids, carbohydrates, and amino acids.[1] The hydrolytic water molecule is activated in these enzymes by direct coordination to a mononuclear or binuclear metal center in the active site. A subset of the enzymes in this superfamily catalyzes the hydrolysis of acetyl groups from *N*-acyl amino acids or amino sugars. In an effort to more precisely define the role of the divalent cations in these systems, we have endeavored to design and synthesize structural mimics of the putative reaction intermediates. These compounds should prove useful in the elucidation of enzyme−substrate interactions and as lead compounds in drug discovery efforts.

The enzymatic hydrolysis of acetyl groups from *N*-acyl-substituted substrates may proceed via the direct attack on the amide bond by an activated water molecule. Compounds that mimic the structure and geometric constraints of the tetrahedral intermediate have proven to be useful probes of the reaction mechanism and tight binding enzyme inhibitors.[2] Phosphonamidates are particularly useful structural surrogates because of the tetrahedral geometry at the phosphorus center and the similarities in charge and bond lengths to those predicted to occur during the hydrolysis of amide bonds.[3,4]

Scheme 1. Reactions Catalyzed by NagA and DGD
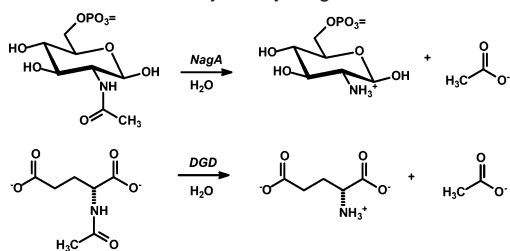
Two examples of deacetylases that belong to the amidohydrolase superfamily are *N*-acetyl-D-glucosamine-6-phosphate deacetylase (NagA) and *N*-acetyl-D-glutamate deacetylase (DGD). The reactions catalyzed by these two enzymes are presented in Scheme 1. NagA from *Escherichia coli* was purified to homogeneity after expression of the cloned gene (gi: 16128653) in *E. coli*. The purified protein was found to contain 1 equiv of Zn^{2+} that is likely bound to the M_β site. The values of k_{cat} and k_{cat}/K_m were determined to be 78 s^{-1} and 5.7×10^5 M^{-1} s^{-1}, respectively, at pH 7.5, 30 °C by following the loss of substrate at 215 nm. The gene for DGD was cloned from *Bordetella bronchiseptica* (gi: 33602261) and over-expressed in *E. coli*. The purified protein was assayed by following the formation of formate with formate dehydrogenase at 340 nm using *N*-formyl-D-glutamate as the substrate. The values of k_{cat} and k_{cat}/K_m were determined to be 400 s^{-1} and 1.7×10^6 M^{-1} s^{-1} at pH 7.6 and 30 °C. The enzyme was found to contain 2 equiv of Zn^{2+} per subunit, and thus the active site is likely to be binuclear

Scheme 2. Methyl Phosphonamidate Analogues of Intermediates
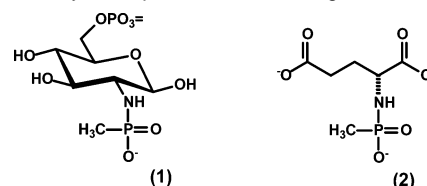
and similar to that determined previously for D-amino acid deacetylase from *Alcaligenes faecalis* DA1.[5]

Methyl phosphonamidate analogues of the tetrahedral intermediates for the reactions catalyzed by NagA and DGD were synthesized, and the structures (**1** and **2**) are presented in Scheme 2. These compounds are potent and time-dependent inhibitors of NagA and DGD. The inhibition constant for NagA was determined by incubating variable amounts of **1** with 10−45 nM of the enzyme for 30 min in 50 mM phosphate buffer, pH 7.5. The enzyme activity was determined using 0.5 mM *N*-acetyl-D-glucosamine-6-phosphate as the substrate. The residual activity was plotted versus the inhibitor concentration, and the results for 25 nM NagA are presented in Figure 1A. A fit of the data to eq 1 for the entire data set gave an average inhibition constant, K_i, of 34 ± 5 nM.[6] In eq 1, E_t is the total enzyme concentration, I is the inhibitor concentration, v_o is the activity of the enzyme in the absence of inhibitor, and v_i is the residual activity of the enzyme in the presence of inhibitor.
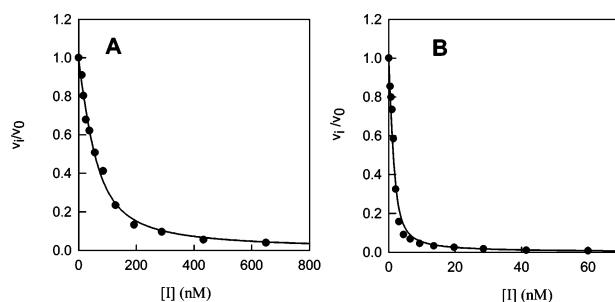

Figure 1. Inhibition of NagA (panel A) and DGD (panel B) by **1** and **2**, respectively. The kinetic data were fit to eq 1 to obtain a dissociation constant for **1** with NagA of 34 nM and 460 pM for the binding of **2** with DGD. Additional details are given in the text.

The time dependence to the onset of inhibition by **1** with NagA was determined by measuring the rate of bond cleavage as a function of time. In these experiments, 10 nM NagA was added to a solution of 0.5 mM substrate containing 0.4−2.4 μM **1**. The time courses for the change in the rate of substrate hydrolysis are shown in Figure 2A. The time courses were fit to eq 2 to yield the observed first-order rate constants for the onset of inhibition by **1** with NagA. In eq 2, P is the product concentration, v_r and v_s are the initial and final rates of product formation, and k_{obs} is the rate constant for the onset of the slower rate of product formation.[7] The apparent second-order rate constant for the formation of the inhibited form
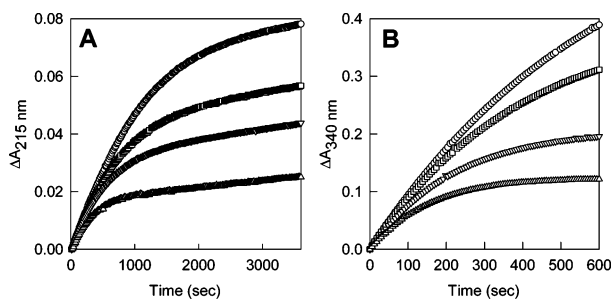
Figure 2. Time dependence for the onset of inhibition by **1** (panel A) with NagA and **2** (panel B) with DGD. In panel A, the concentrations of **1** are 0.40 μM (○); 0.80 μM (□); 1.6 μM (▽); 2.4 μM (△). In panel B, the concentrations of **2** are 15 nM (○); 25 nM (□); 50 nM (▽); 80 nM (△). The time courses were fit to eq 2 to obtain the first-order rate constants for the formation of the enzyme−inhibitor complex.

of NagA was determined by plotting k_{obs} versus the concentration of **1** as shown in Figure 3A. From the slope of this plot, the apparent second-order rate constant is 1.3×10^3 M^{-1} s^{-1}. Correction of the apparent second-order rate for the concentration of substrate according to eq 3 gave a value for k_1 of 6.1×10^3 M^{-1} s^{-1} (see Scheme 3).

The dissociation constant for the binding of **2** with DGD was determined by incubating 2.0 nM enzyme with 0.5−60 nM **2** for 75 min at pH 7.6. The residual enzyme activity was then determined with 3.0 mM *N*-formyl-D-glutamate as the substrate in 50 mM HEPES buffer, pH 7.6. The results are shown in Figure 1B and a fit of the data to eq 1 gives a K_i of 460 ± 70 pM. The time courses for the onset of inhibition by **2** were determined by measuring the rate of bond cleavage as a function of time. In these experiments, 1.0 nM DGD was added to a solution of 1.5 mM substrate containing between 10 and 300 nM of **2** at pH 7.6, and the formation of formate was determined as a function of time. The time courses were fit to eq 2 to yield the first-order rate constants for the onset of inhibition, and the results are presented in Figure 2B. The apparent first-order rate constants for the onset of inhibition were plotted versus the concentration of **2**, and the results are presented in Figure 3B.
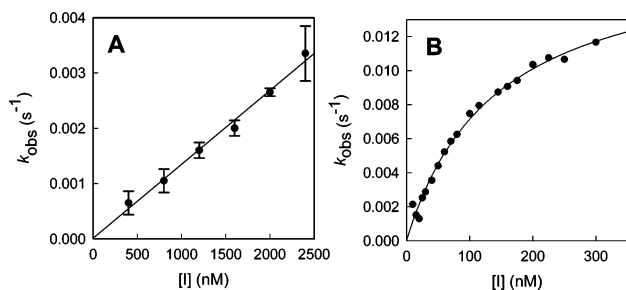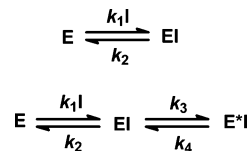

Figure 3. Effect of inhibitor concentration on the onset of inhibition by **1** (panel A) with NagA and **2** (panel B) with DGD. The data in panel B were fit to eq 4. Additional details are provided in the text.

The apparent rate constant for the slow onset of inhibition by **2** with DGD is constant at high levels of inhibitor. These results suggest that the initial binding event is followed by a subsequent transformation that enhances the tightness of the interaction between DGD and **2**, as shown in the second model that appears in Scheme 3. A fit of the data from Figure 3B to eq 4 gives values of $k_3 = 0.017$ s^{-1} and an apparent $k_2/k_1 = 140 \pm 20$ nM. The value of k_4 was undetermined. Correcting for the concentration of substrate gives a value for k_2/k_1 of 19 ± 3 nM. The second step in the binding of **2** with DGD may be the result of a protein conformational change that enhances the molecular interactions between the protein and transition state analogue. Bartlett and Marlowe have proposed for

similar instances that the slow step is due to the sluggish dissociation of a metal-bound water molecule that is necessary before the inhibitor can make optimal interactions within the active site.[3a] The pH dependence and solvent isotope effects for inhibition of stromelysin by a peptide phosphonamidate suggest that k_{on} is limited by ligand exchange for a zinc-bound water.[8] It is likely that the small value of k_1 for the binding of **1** with NagA is due to a similar phenomenon.

Scheme 3. Models for Binding of Enzyme Inhibitors

$$E \underset{k_2}{\overset{k_1I}{\rightleftharpoons}} EI$$

$$E \underset{k_2}{\overset{k_1I}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*I$$

The two phosphonamidate analogues synthesized for this investigation proved to be extraordinary potent inhibitors of two deacetylases belonging to the amidohydrolase superfamily. The inhibition constants for compounds **1** and **2** are 4×10^3- and 5×10^5-fold smaller than the respective Michaelis constants of their target enzyme. The K_i values for these compounds are among the lowest ever measured for the inhibition of deacetylases.[9]

$$v_i/v_o = ([E_t] - K_i - [I] + (([I] + K_i - [E_t])^2 + (4K_i[E_t]))^{1/2})/(2[E_t]) \quad (1)$$

$$P = v_s t + ((v_r - v_s)/k_{obs})(1 - e^{(-k_{obs}t)}) \quad (2)$$

$$k_1 = \text{app}k_{obs} (1 + [S]/[K_m]) \quad (3)$$

$$k_3 = (k_{obs} - k_4) (1 + (\text{app}(k_2/k_1)/[I])) \quad (4)$$

Supporting Information Available: The syntheses of compounds **1** and **2** are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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