Chemistry 5.07
Problem Set #3 - Enzyme catalysis and kinetics

Problem 1.

Interleukin 1 β (IL-1 β) has been implicated in the pathogenesis of acute chronic inflammatory diseases including septic shock, rheumatoid arthritis etc. IL-1 β must be processed from a pro-peptide (pro-IL-1 β) form of 31 kDa to the active form of 17.5 kDa by a cysteine protease designated ICE, or interleukin converting enzyme. The cleavage of IL-1 β occurs between Asp116 and Ala117. ICE is a unique cysteine protease, but appears to use a similar mechanism to other cysteine proteases requiring a histidine and a cysteine for catalysis. To study the enzyme, Merck had to develop an assay. As you can imagine, assays with a protein such as IL-1 β is cumbersome, and thus they did a number of experiments using peptides to see if they could find an effective peptide analog(s) to report on the substrate specificity and cleavage efficiency. They initially tried to determine the binding specificity length on either side of the bond to be cleaved (Figure 1), as well as the optimized amino acid binding at each subsite (Figure 2). The cleaved bond is between P1 and P1' (see Figure 1A, right) with aspartate at P1 (see Figure 1B), very important for cleavage. The assay required monitoring the reaction by HPLC where the peptide substrates and products needed to be separated and quantitated as a function of time. This assay is very slow and not very sensitive.

A.

Amino-terminal truncations

Peptide	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	Relative V_{max}/K_m
1	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	1.00 ± 0.05
2		Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.64 ± 0.01
3			Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.29 ± 0.02
4				Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.12 ± 0.00
5			Ac-Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn		0.81 ± 0.01
6				Ac-Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn		< 0.005
7					His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn		< 0.005
8						Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn		< 0.005

Carboxy-terminal truncations

Peptide	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	Relative V_{max}/K_m
1	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	1.00 ± 0.05
9	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu		1.22 ± 0.16
10	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser			0.80 ± 0.16
11	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg				0.88 ± 0.04
12	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val					1.02 ± 0.09
13	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro						1.25 ± 0.15
14	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala							< 0.05
15	Asn	Glu	Ala	Tyr	Val	His	Asp-CO-NH_2								0.93 ± 0.03
16			Ac-Tyr	Val	His	Asp-NH-CH_3									12.00 ± 2.0
17			Ac-Tyr	Val	His	Asp-AMC									1.9 ± 0.1

B.

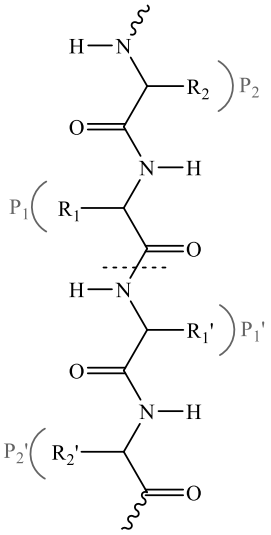
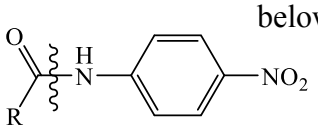


Figure 1 (previous page). Substrate specificity of Interleukin Converting Enzyme (ICE). A. Table of analogs of interleukin 1 β (IL-1β) used in assays to better understand the substrate specificity of ICE. B. Schematic of IL-1β with positions of P1, P2, P1', and P2' indicated and corresponding to amino acids listed in A.

Thornberry, Nancy A., Herbert G. Bull, Jimmy R. Calaycay, Kevin T. Chapman, Andrew D. Howard, Matthew J. Kostura, Douglas K. Miller et al. "A novel heterodimeric cysteine protease is required for interleukin-1βprocessing in monocytes." *Nature* 356, no. 6372 (1992): 768-774.

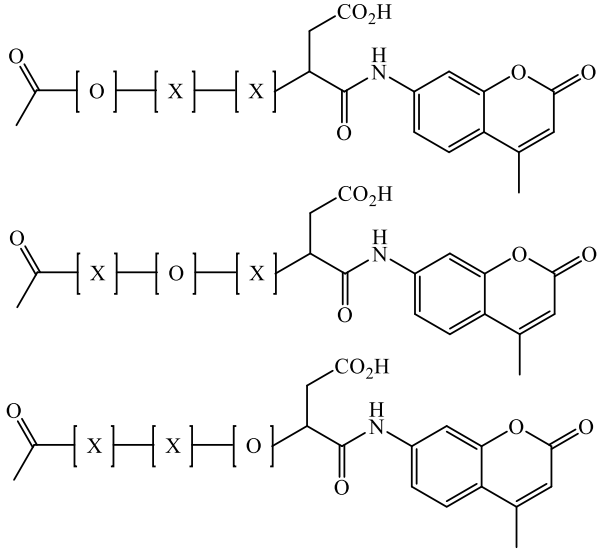
Taking advantage of information gained by the studies in Figure 1, they then turned to develop a more high throughput (HTP), sensitive, assay using coumarin analogs, which upon peptide bond hydrolysis, become highly fluorescent. Thus the reaction could be monitored continuously in a spectrofluorometer. Alternatively, they used p-nitroanilide in place of the coumarin (see structure below).



p-nitroanilide

Using this assay they looked further at the substrate specificity and the results are reported in Figure 2 (below).

A.



B.

Comparison of fluorogenic substrates for ICE	
Substrate	10^{-5} x k_{cat} / $K_{m'}$ (M^{-1} s^{-1})
Ac-WEHD-AMC	33.4 ± 0.3
Ac-WVHD-AMC	15.7 ± 0.1
Ac-YEHD-AMC	9.56 ± 0.20
Ac-WEAD-AMC	7.55 ± 0.07
Ac-YVHD-AMC	2.81 ± 0.14
Ac-WVAD-AMC	2.41 ± 0.60
Ac-YEAD-AMC	1.85 ± 0.07
Ac-YVAD-AMC	0.66 ± 0.14
pro-IL-1 β	1.5

Figure 2. Examining the substrate specificity of Interleukin Converting Enzyme (ICE) using a coumarin fluorescence assay. A. Chemical structures of fluorescent coumarin substrates. B. Results of ICE activity in coumarin fluorescence assays using the substrates shown in A.

Source: Rano, Thomas A., Tracy Timkey, Erin P. Peterson, Jennifer Rotonda, Donald W. Nicholson, Joseph W. Becker, Kevin T. Chapman, and Nancy A. Thornberry. "A combinatorial approach for determining protease specificities: application to interleukin-1β converting enzyme (ICE)." *Chemistry & biology* 4, no. 2 (1997): 149-155.

The mechanism of cysteine proteases is proposed to be similar to serine proteases that you have or will be discussing in recitation (Figure 3).

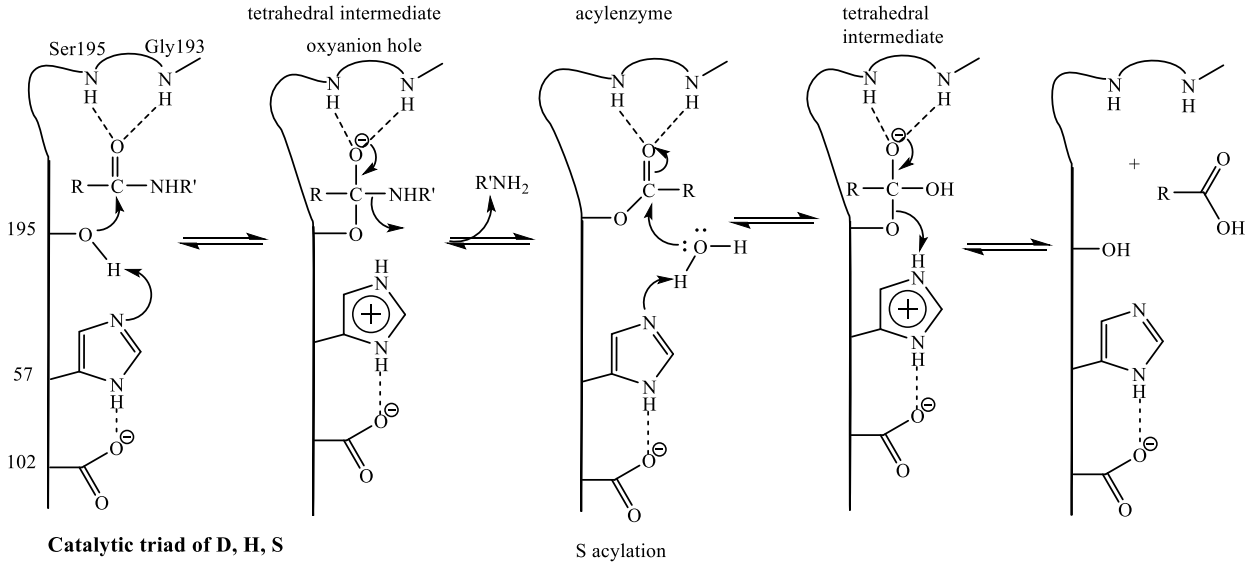


Figure 3. Schematic of a generic mechanism for serine proteases. These proteins have a catalytic triad, a coordinated catalytic pocket consisting of His at position 57, Ser at position 195 and Asp at position 102 of the polypeptide chain. Though distant in the primary structure of the protein, these residues are in close proximity in the quaternary structure as shown.

However with cysteine proteases we know now from crystallographic studies and studies with many covalent inhibitors of cysteine proteases, that there are only two amino acids that play an essential role in catalysis: a cysteine and a histidine.

Questions:

a. What does the data in Figure 1 tell you about the substrate binding site to ICE? How does the k_{cat}/K_m or (V_{max}/ K_m) for the real substrate pro-IL-1β meet your expectations relative to the other data? Explain why?

b. Explain why the investigators decided to use the substrate analogs shown in Figure 2 to continue their substrate specificity studies. Can you think of how a more informative assay could be carried out? How would the corresponding p-nitroanilide analogs function in the assay? What criteria would you use to determine if the coumarin or the nitroanilide would be best for high throughput assays?

c. Explain why k_{cat}/K_m is the kinetic parameter used in the studies described in Figure 1 and not k_{cat}.

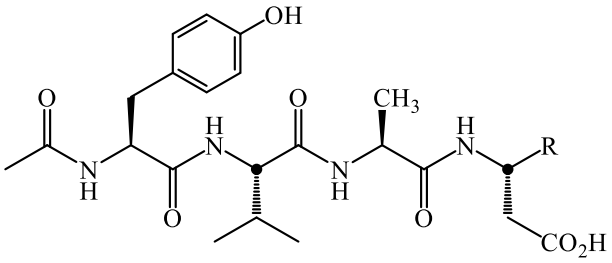
d. Using the mechanism shown in Figure 3 for serine proteases, draw a corresponding mechanism for cysteine proteases. Based on what you have learned about serine proteases and the chemical difficulties associated with catalysis, provide an explanation for why an aspartate residue is not found in the active sites of cysteine proteases.

e. Peptide bond hydrolysis catalyzed by the enzyme is still 10^{10} x that of the non-enzymatic reaction. Discuss the different mechanisms of catalysis and their contributions to this rate acceleration.

Problem 2.

As with serine proteases, a number of different successful approaches have been achieved to target cysteine proteases once their specificity is understood. Shown below are two distinct approaches to inhibit cysteine proteases in general. The data below is shown for inhibition of ICE. The first is peptide aldehyde analogs  where R in Figure 4 is replaced by an aldehyde (CHO). The second type of inhibitor is acyloxymethyl ketone analogs shown in Figure 5AB.

A.



B.

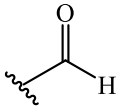
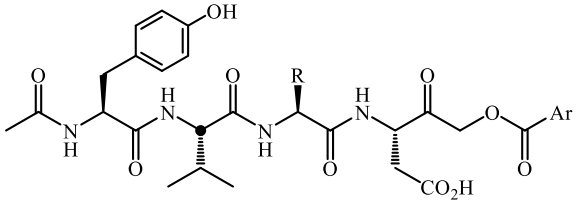
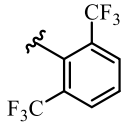
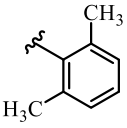
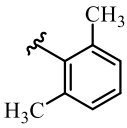
R	K_i (nM)
	0.76

Figure 4. Peptide aldehyde analogs of substrates for serine proteases. A. Chemical structure of a prototypical peptide aldehyde inhibitor with the 'R' group representing the variable side chain. B. Chemical structure of an R group for a peptide aldehyde inhibitor. In this case, the R group is an aldehyde and has an inhibition constant of 0.76 nM.

A.



B.

#	Ar	R
1		CH_3
2		CH_3
3		$(CH_2)_4$NH-biotin

C.

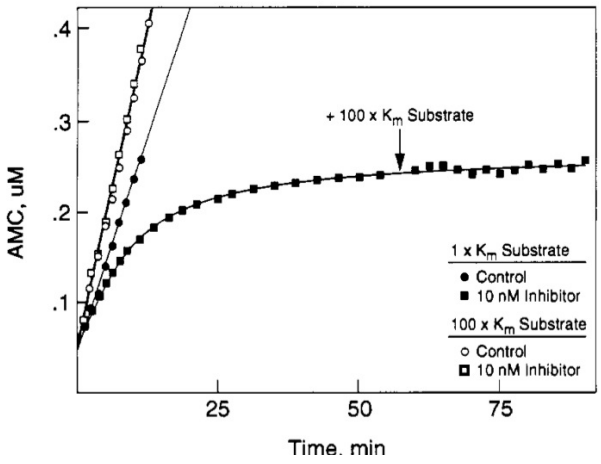


Figure 5. Inactivation of ICE by peptide (acyloxy) methyl (AMC) ketones. A. Chemical structure of a prototypical AMC ketone analogs. The variable side chain is represented by the 'R' group. B. Table of possible R groups of the peptide AMC ketone analogs. C. Graph of reaction rates in the presence or absence of the AMC analogs listed in B.

Questions:

a. Propose a mechanism of inhibition of ICE by the peptide aldehydes such as the one shown in Figure 4. This is a mechanism-based inhibitor. What type of inhibition would you expect to see using substrates in Figure 2 to analyze for inhibition? Show the equation and the kinetic reciprocal plots ($1/v$ vs $I/[S]$) with varying concentrations of substrate and inhibitor.

b. From a chemical perspective, provide two reasons why you might not want to use an aldehyde as a therapeutic.

c. The acyloxymethylketone analogs shown in Figure 5AB with the data shown in Figure 5C, have a mechanism of inhibition distinct from the peptide aldehydes. Propose a chemical mechanism for their inhibition of ICE. Provide an explanation for the data in Figure 5C.

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