Proteases:
References: Hartly and Kilby *Biochem. J.* 56, 208 (1954)

Carter and Wells (triple mutant of subtilisn) *Nature* 332, 564 (1988).

Hedstrom et al. (redesigning trypsin) *Science* 255, 1249-56 (1992).

Frey et al. (low barrier H bonds) *Science* 264, 1927-30; Biochemistry (1997)36, 4576.

Abeles and coworkers (trifluoroketones as probes of acidity of His) *Biochemistry* 29, 7600-7607 and 7608-17.

Scott, I and Rich, D (NMR to probe tetrahedral intermediates *J. Am. Chem. Soc.* 105, 6324 (1983; 1685 (1983); 104, 6811, 3535 (1982).

Abeles (mechanism based inhibitors) *C and E News* 1983 61, 48.

Walsh (mechanism based inhibitors) *Tetrahedron* 1982, 38 371.

Morrison and Walsh (slow binding inhibitors) *Advances in Enzymology* 61, 202 (1988).

In the following pages, overviews of the mechanism of serine, aspartate and metallo proteases (proteinases) is presented. In addition the active sites of a variety of proteases that are drug targets are presented.

Trifluorketones with a serine protease
A Merck inhibitor with the HIV aspartate protease
A Merck inhibitor with a cysteine protease.

Frey's Model

Proc. Natl. Acad. Sci. USA 95 (19

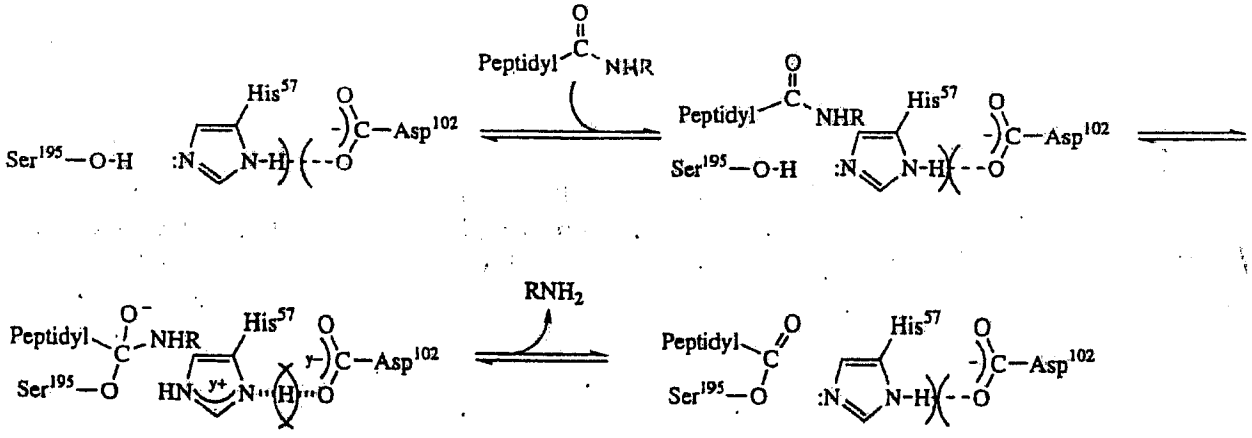


FIG. 5. The postulated LBHB-facilitated mechanism for the acylation of chymotrypsin by a peptide substrate.

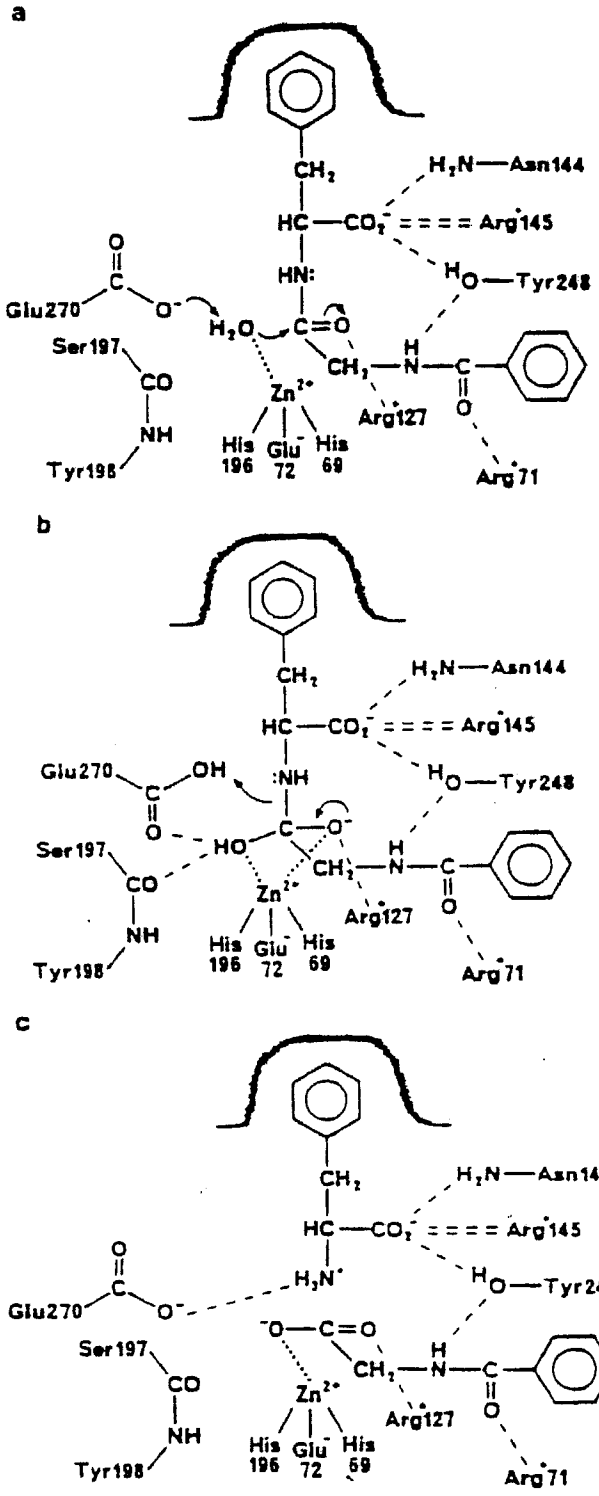


Figure 10. Proposed mechanism for CPA-catalyzed proteolysis: (a) the precatalytic Michaelis complex with the substrate carbonyl hydrogen bonded to Arg-127 allows for nucleophilic attack by a water molecule promoted by zinc and assisted by Glu-270; (b) the stabilized tetrahedral intermediate collapses with required proton donation by Glu-270; (c) the final product complex, after a second and final proton transfer mediated by Glu-270—unfavorable electrostatic interactions between Glu-270 and the ionized product carboxylate may facilitate product release.

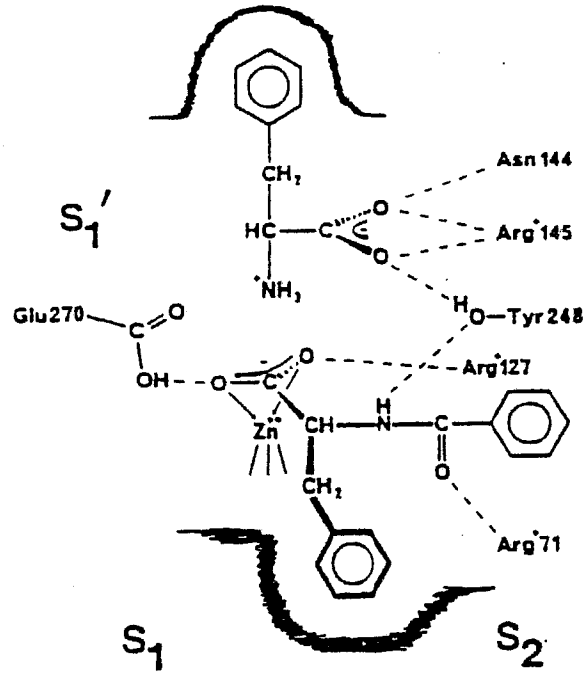
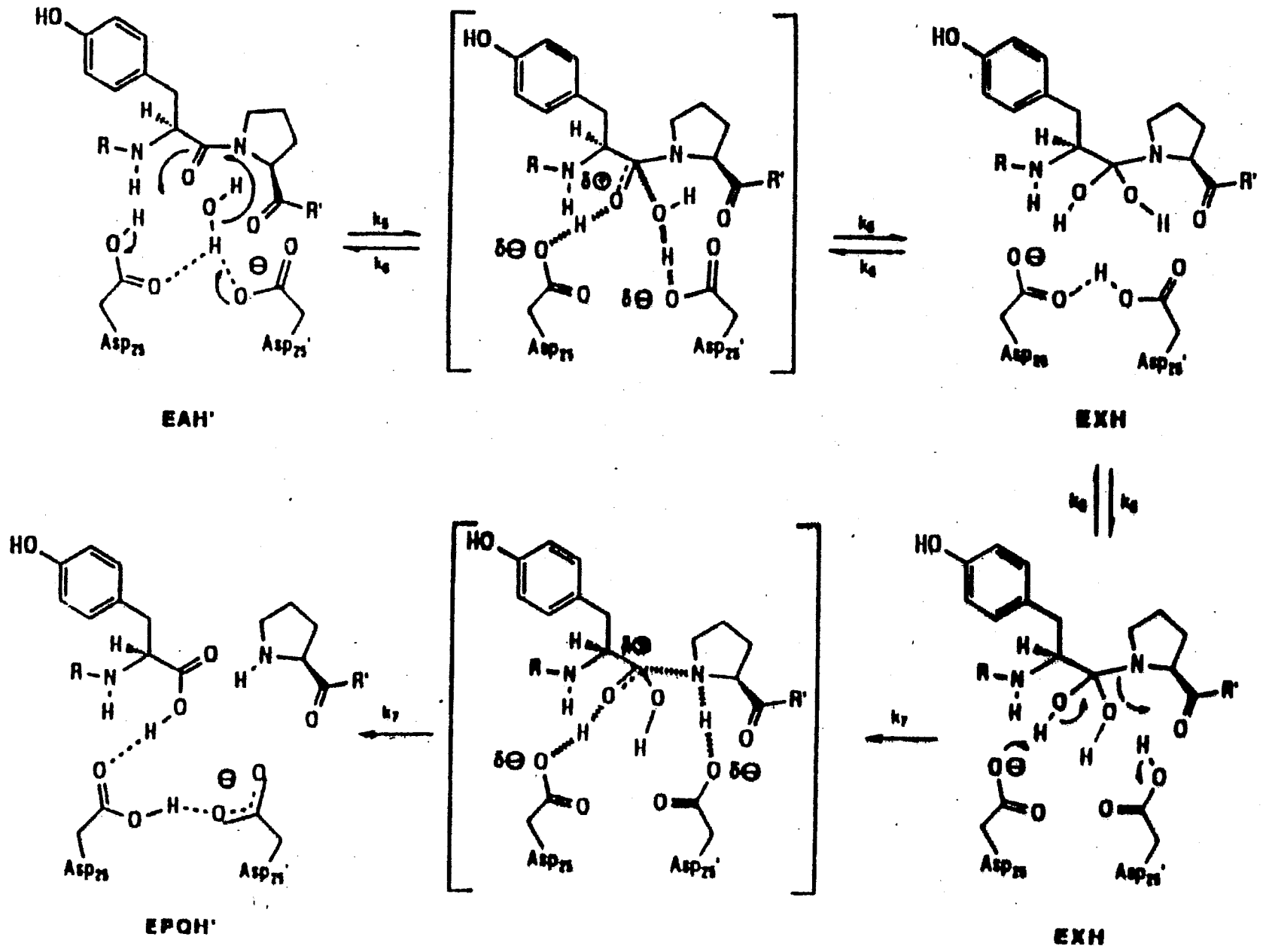


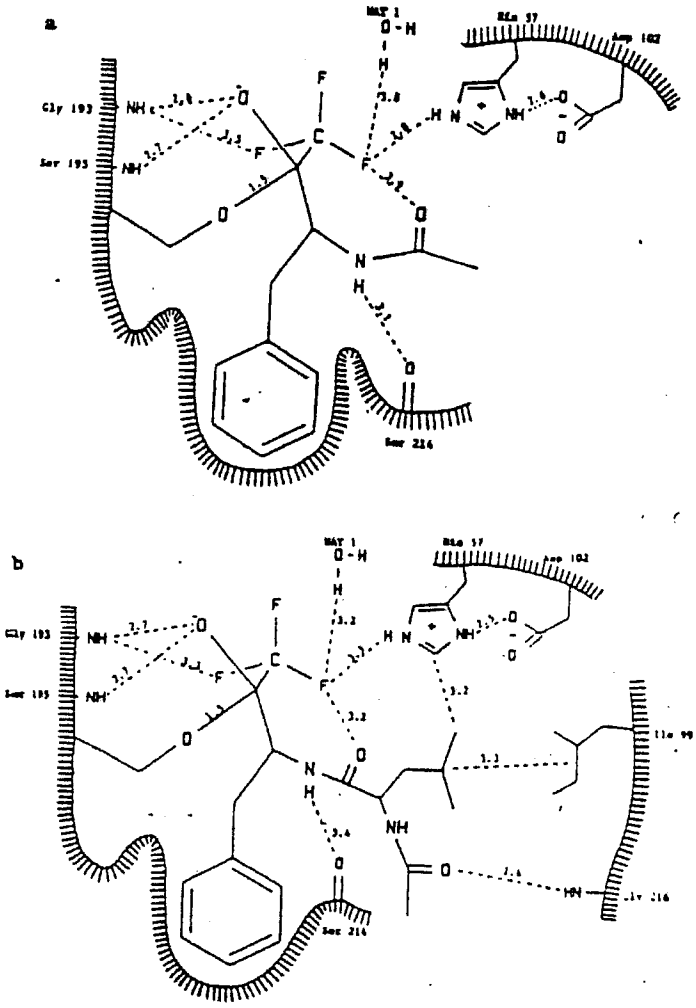
Figure 9. CPA crystals soaked in a buffer solution containing 0.1 M benzoyl-L-phenylalanine show enzymatic activity during the time frame of the X-ray diffraction experiment. A schematic view of the resulting enzyme–substrate–product complex is presented; note that this complex also represents an enzyme–products complex for the hydrolysis of *N*-benzoyl-L-phenylalanyl-L-phenylalanine.

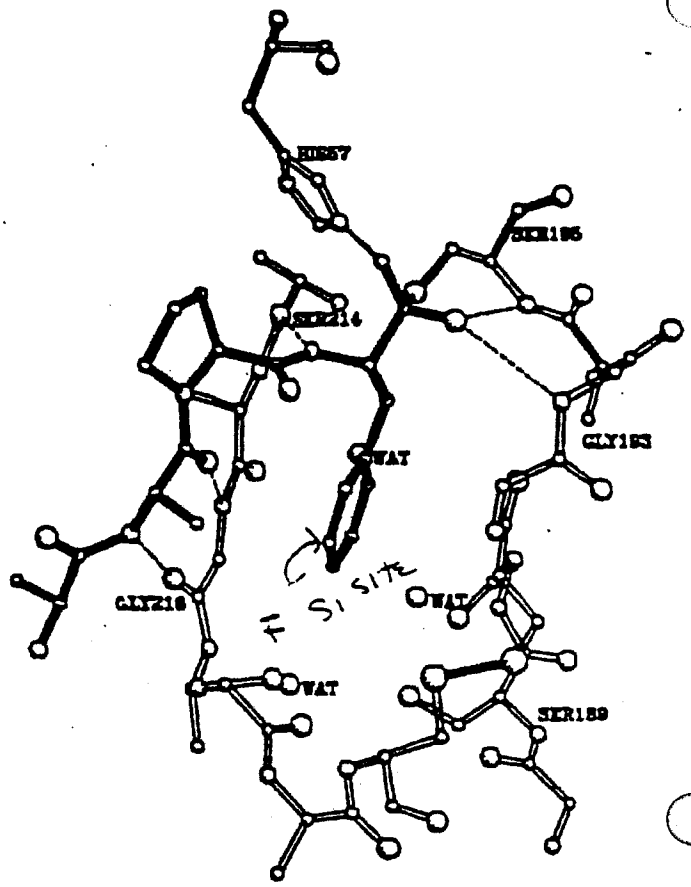
HIV-1 Protease 2. Chemical Mechanism



Structure of Chymotrypsin–Inhibitor Complexes

Biochem **29**: 7608-17 (1990)
Biochem **36**: 4576 (1997)

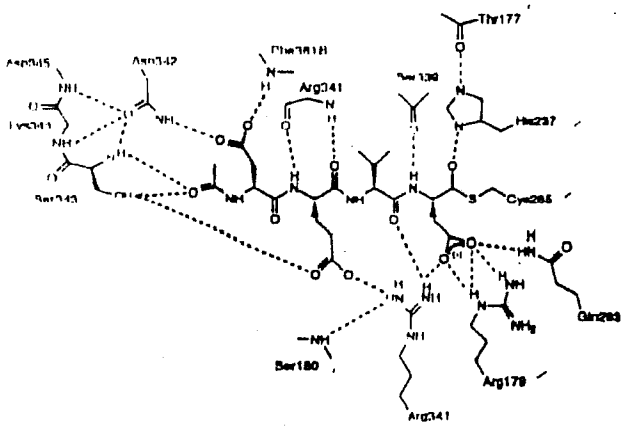




Inhibitor: Protein Polar Interactions

Merck Inhibitors of Cysteine Proteases

Caspase-3:DEVD



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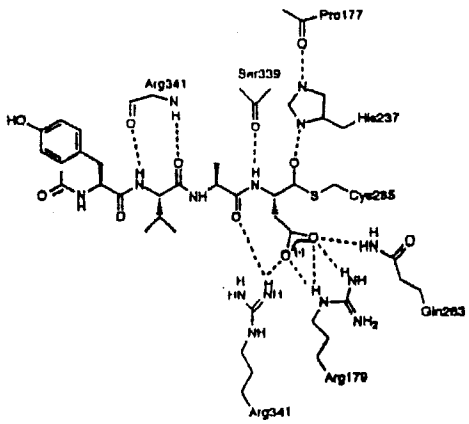
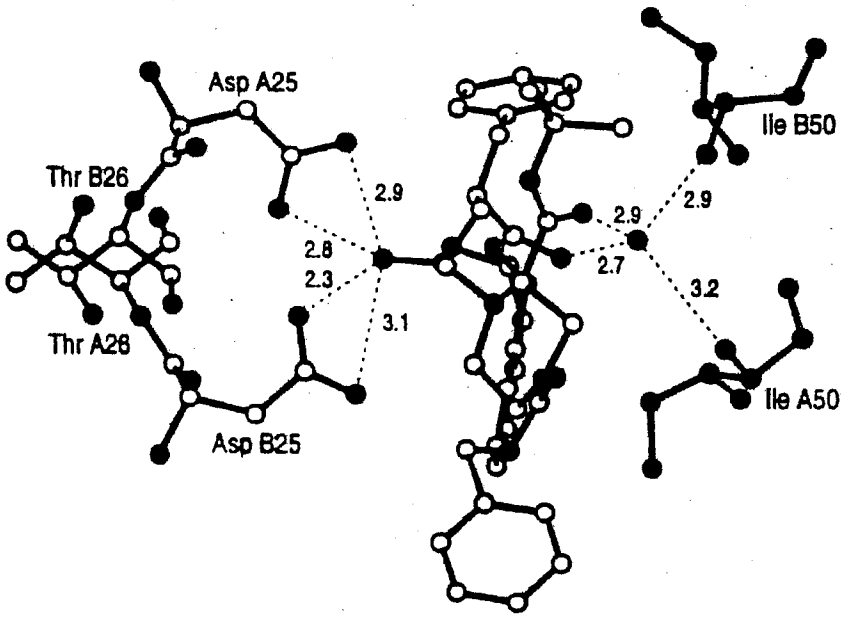
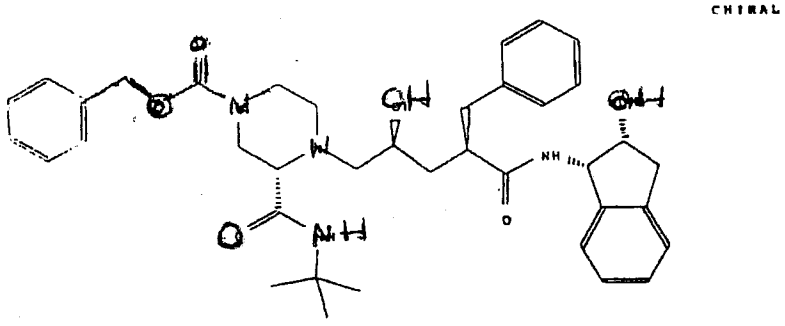


HIV-1 Protease Complexes

L-732,747 - Potential Hydrogen Bonds (Side)



Inhibitor bound to HIV protease



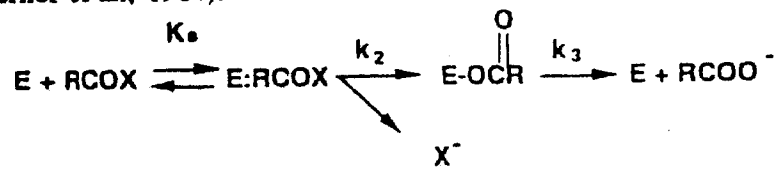
CHIRAL

COMPOUND NOT NAMED

Table 1: Kinetic Parameters of Hybrid Trypsins[a]

	K_s (M)	k_2 (s^{-1})	k_3 (s^{-1})
trypsin			
Ac-Phe-NH_2	1×10^{-3}	2.2×10^{-4}	30
suc-AAPF-*p*NA	>0.25	>0.2	36
Z-GPR-AMC	8.6×10^{-5}	319	101.5
D189S			
Ac-Phe-NH_2	0.17	0.018	39
suc-AAPF-*p*NA	0.015	0.29	33
Z-GPR-AMC	$>10^4$	>1.1	126.2
Tr→Ch[S1+L1+L2]			
Ac-Phe-NH_2	0.065	0.018	33
suc-AAPF-*p*NA	0.011	20	37
Tr→Ch[S1+L1+L2+Y172W]			
Ac-Phe-NH_2	>0.1	>0.08	38
suc-AAPF-*p*NA	5.0×10^{-4}	41	63
chymotrypsin			
Ac-Phe-NH_2	0.023	0.43	60
suc-AAPF-*p*NA	1.5×10^{-3}	850	52

[a] Mechanistic kinetic parameters for trypsin, chymotrypsin, D189S, and the hybrid trypsins Tr→Ch[S1+L1+L2] and Tr→Ch[S1+L1+L2+Y172W]. Data are taken from Hedstrom et al. (1994a) and Perona et al. (1994). K_s, k_2, and k_3 represent measures of substrate binding affinity, acylation rate, and deacylation rate, respectively. They are derived from steady-state kinetic constants obtained on equivalent pairs of amide and ester substrates, *suc*-AAPF-*p*NA and the ester substrate *suc*-AAPF-SBzl, Ac-Phe-NH_2 and the ester Ac-F-*p*NP (Hedstrom et al., 1992, 1994a), and Z-GPR-AMC and the ester Z-GPR-SBzl (Perona et al., 1994), according to the standard serine protease mechanism (Zerner et al., 1964):



For reactions in which acylation is the rate-determining step, $K_m = K_s$ and $k_{cat} = k_2$. If deacylation is rate-determining, then $K_m = K_s[k_{cat,ester}/(k_2 + k_{cat,ester})]$ and $k_{cat} = k_2 k_{cat,ester}/(k_2 + k_{cat,ester})$.

Enzyme	Kcat (sec^{-1})	Km(μM)	Kcat/Km	Kcat(WT)/Kcat(mutant)
WT	5.9×10^{-1}	220	2.5×10^{5}	1
S221A	3.4×10^{-5}	420	8.2×10^{-2}	5.8×10^{-7}
D32A	2.3×10^{-3}	480	4.7	3.8×10^{-5}
H64A	3.8×10^{-5}	390	9.6×10^{-2}	6.4×10^{-7}
triple mutant	3×10^{-5}	230	1.3×10^{-1}	5.1×10^{-7}

I. substrate N-succinyl-A-A-P-F-NH-⟨⟩-NO_2

II. substrate F-A-X-F-NH-⟨⟩-NO_2

	WT		mutant	
substrate P_2	Kcat (s^{-1})	Km (μM)	Kcat (s^{-1})	Km (μM)
A	8.1	10	8.1×10^{-6}	32
Q	7.0	39	3×10^{-5}	150
H	4.6	23	1.6×10^{-2}	380

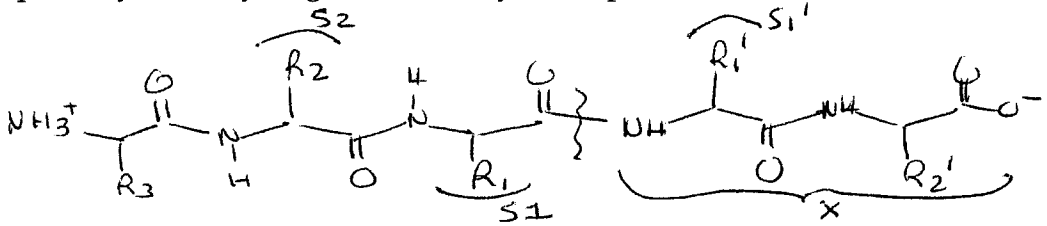
There exist four major classes of proteases:

Type	Function
1. serine (S, H, D)	digestion, immune response, blood coagulation, fibrinolysis, reproduction
(T, K)	proteosome
(S, K)	
2. cysteine (C, H)	common cold-rhinovirus protease
	caspases (apoptosis)
3. metallo proteases (Zn^{2+})	matrix metallo proteases (tissue
(E, D, H ligands)	invasion and inflammation), ACE
4. aspartate (2 Ds)	HIV protease, renin

Many additional types of proteases have been discovered in the past few years that do not exactly fit into the above paradigm. There are proteases that cut membrane proteins within the membrane that play a major role in generating the β amyloid protein associated with Alzheimers disease and a protein controlling cholesterol homeostasis that upon cleavage within the golgi membrane releases a soluble transcription factor. There are also Fe^{2+}, Ca^{++} and Co^{2+} proteases that have recently been characterized.

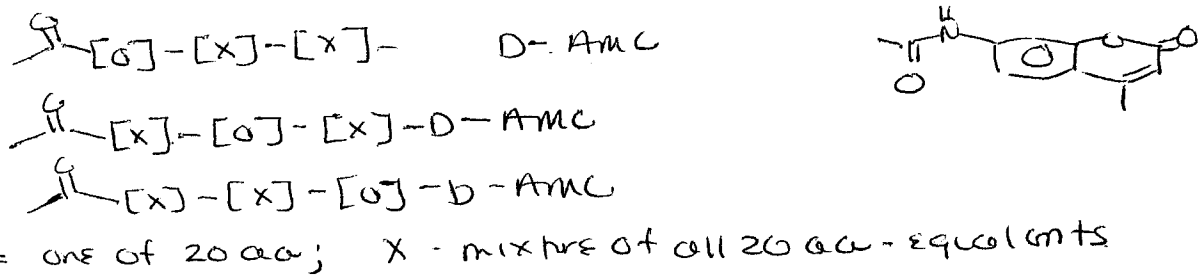
Proteases play a major role in regulation of metabolic pathways. They provide yet another example of post-translational modification that can either activate or inactivate a process in an irreversible fashion. In vivo most proteins are inhibited in a proenzyme or zymogen form or by small protein inhibitors.

Specificity

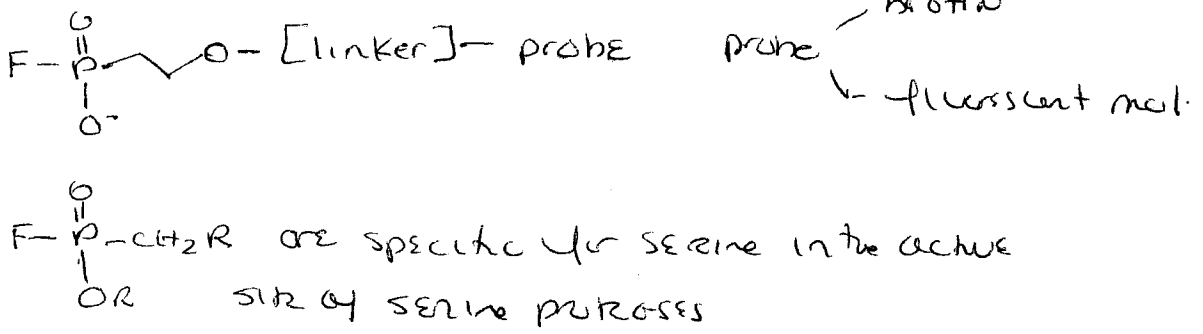


In the old days the substrate specificity was determined by synthesizing one molecule at a time and comparing the compounds by determining their k_{cat}/K_ms. Now combinatorial libraries are generated and fluorescence methods are used to monitor 10^5 reactions simultaneously in 360 well plate formats. These methods allow

one to look at S1-S4 and S1'-S4' sites simultaneously. Often some prejudice can be built into your library if you know what the actual substrate for the protein is and or if you know the structure of the protein. Different approaches to screen combinatorial libraries for caspases (C, H) are reported in [J. Biol.Chem. 272, 17907-11 (1997) or for collagenases [J. Biol. Chem 277, 8366-8371 (2002)]. A strategy for screening caspases is shown below where a very prejudiced library is used. Ac - X - X - X - D - [AMC]



Strategies for identifying proteases in vivo. [Cravatt Biochemistry (2001) 40, 4005-15; PNAS 99, 10335-40 (2002); PNAS 99, 11139-41 (2002)] As noted above proteases are often present in zymogen form. They need to be proteolytically processed to become active. A method has recently been developed taking advantage of our wealth of knowledge about serine proteases to specifically label the active sites of all serine proteases. Since the labeling occurs in the active site and is chemistry dependent, one only observes the active form of the protein. The strategy is shown below.



Summary of specificity rules for chymotrypsin.

The P1 site is aromatic or hydrophobic (W,F, Y, I, L); this amino acid must be in the L configuration; The X can be a wide range of leaving groups (NH-aminoacid, NH_2NH_2, NHOH, Cl, SR, OH). P1' and P2' have largely been ignored. A classic experiment by Hedstrom and Rutter showed that two loops outside the active site (185-188) and (221-225 play a key role in specificity. Also G216 makes two key H bonding interactions with the

P3 site. The second loop determines that the glycine is appropriately positioned for these interactions. The picture in most introductory text books of the importance of the S1 site is way too simple. Understanding specificity is essential to study mechanism.
As you will see over and over again, one needs to perturb the system to study it and knowing the flexibility within your substrate is an essential first step as you will see with chymotrypsin.

Recently an entire issue of Chemical Reviews was on all classes of proteases
Chemical Review 102 December 2002. Hedstrom has written an excellent review on serine proteases 102, 4501-4523.

Kinetics References:
King Altman *J. Physical Chemistry* 60, 1375 (1956); Mahler and Cordes, *Biological Chemistry* p. 282.

Net Rate constants Method W.W. Cleland, *Biochemistry* 14, 3220 (1975)

For Branched Reactions S. Cha *J. Biol Chem.* 243, 820 (1968)

Good Reviews on Kinetics: *Investigations of Rates and Mechanisms of Reactions* Volume 6 part 1 John Wiley and Sons 1986 Enzyme Kinetics as a Tool for Determination of Enzyme Mechanisms W. W. Cleleand Chapter 12, 791-820

The Kinetics of Enzyme Catalyzed Reactions with two or more substrates or products Nomenclature and Rate Equations *Biochem and Biophys Acta* 67, 104-137 (1963)

Transient Kinetics K. A. Johnson *The Enzymes* 20, 1-62 (1992)

The Enzymes 3rd Edition, volume 2 (1970) is in paperback and is devoted to enzyme kinetics

Advances in Enzymology 45, 273 (1977)

Coupled Assays *Anal. Biochemistry* 99, 142-145 (1979)

High throughput screening: *Curr. Opin Biotech.* 1998 9, 624-31.
Curr. Opin. Chem. Biol. 1998 5, 597-603.
Curr Opin. Chem. Biol., 1997 3 384-391

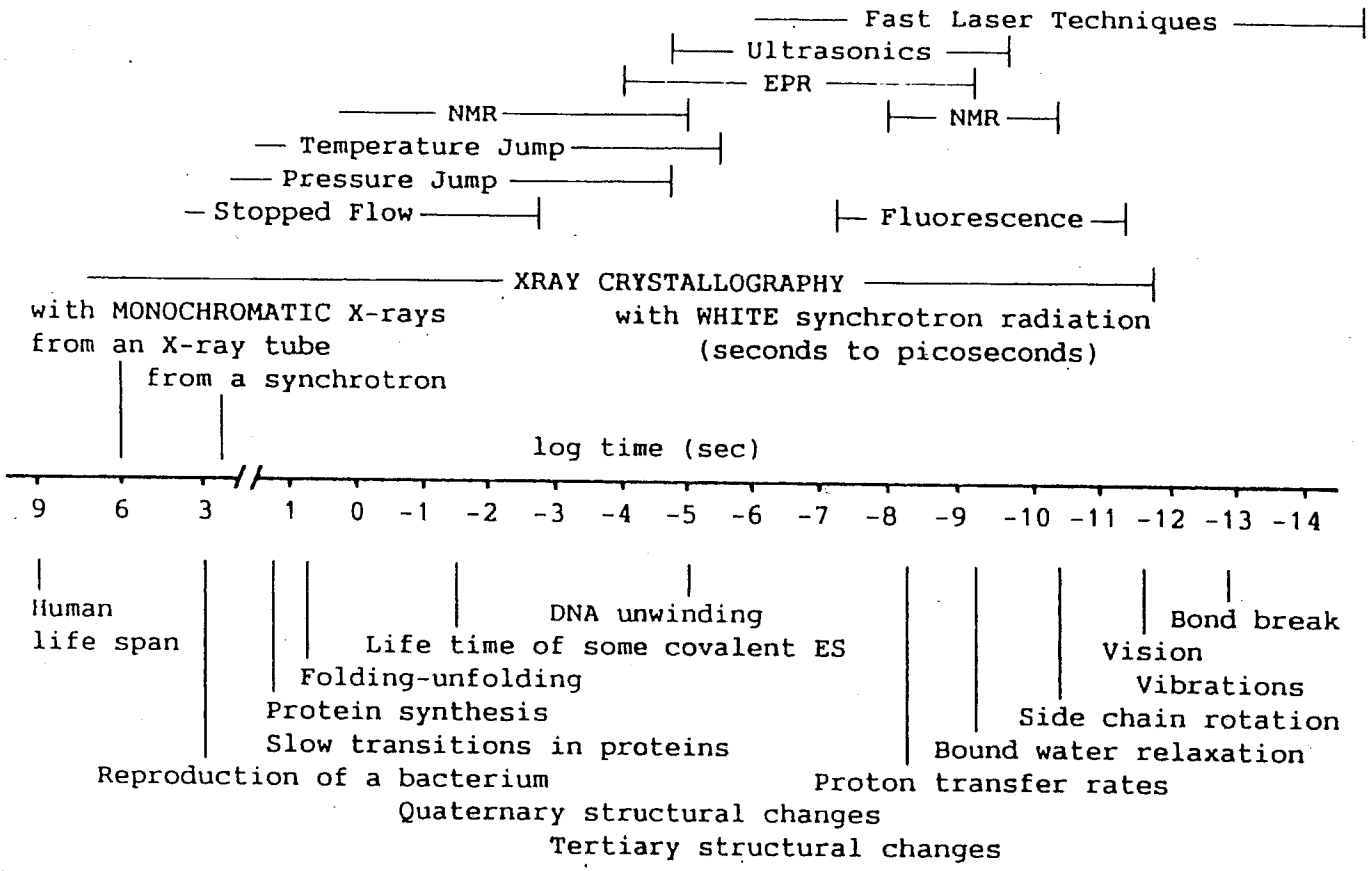


FIGURE 1: Approximate time scales for biological events (lower lines) and for physical methods for monitoring these (upper lines).

From Ken Johnson's Review in the Enzymes 22 1992 p. 17

Let us look at two simple cases which will give you a feeling for complexity of the analysis:

$$E + S \xrightarrow{k_1} ES \qquad d(E)/dT = k_1[E][S]$$

We set up the experiment such that [S]>>[E], that is we will have a pseudo first order reaction. The observed kinetics, if you can follow a way to monitor substrate (S) binding should be fit to a single exponetial. Integration of the above equation gives:

$$[E] = [E]_0 e^{-K1[S]t} \qquad [ES] = [E_0](1 - e^{-K1[S]t})$$

$k_{obs} = k_1[S]$, since we are under pseudo first order conditions the [S] does not change. If the [E] is approximately equal to the [S], then both concentrations vary as a function of time and the differential equations cannot be solved explicitly.

Now let us go to a more realistic, but also very simple case:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

Again let us assume that [S] is >> [E]. Solutions are given by

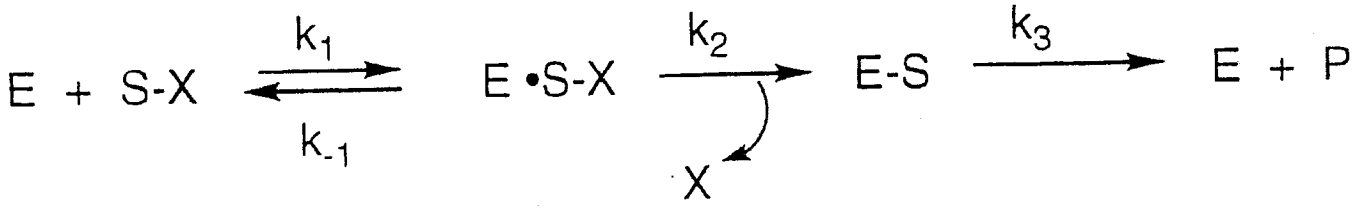
$$[ES]/[E_0] = \underbrace{\frac{K_1[S]}{K_1[S] + 1}}_{\text{amplitude}} (1 - e^{-Kobs[S]t})$$

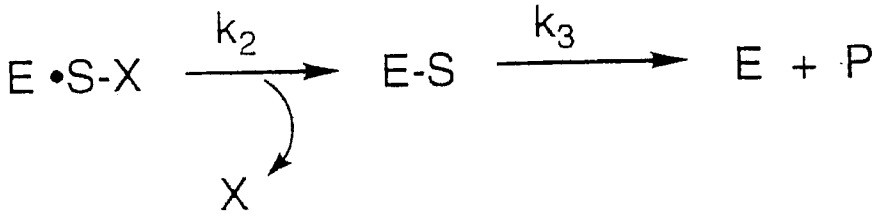
$$k_{obs} = k_1[S] + k_{-1}$$

The suprise is that kobs is equal to the sum of the forward and reverse rate constants. This is because the back reaction reduces the amplitude and therefore shortens the time to equilibrium.

In the best of all worlds, by measuring kobs as a function of [S] one could obtain the rate constants for binding and dissociation.

In the case of chymotrypsin we have a more complex situation that has been simplified.





$$[X]/E_0 = A_0 \left(1 - e^{-K_{obs}\, t}\right) + k_{cat}t$$

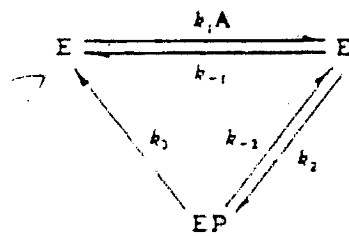
$$k_{obs} = k_2 + k_3 \qquad A_0 = \text{amplitude} = \left(\frac{k_2}{k_2 + k_3}\right)^2$$

CONTEMPORARY ENZYME KINETICS AND MECHANISM
Edited by Daniel L. Purich (1983)

The use of the determinant method for complex enzyme mechanisms is time-consuming because of the stepwise expansion and the large number of positive and negative terms that must be canceled. It is quite useful, however, in computer-assisted derivation of rate equations (cf. Chapter [5] by Fromm, in Volume 63).

The King and Altman Method

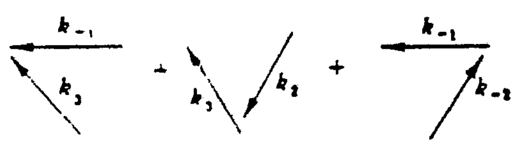
King and Altman[4] developed a schematic approach for deriving steady-state rate equations, which has contributed to the advance of enzyme kinetics. The first step of this method is to draw an *enclosed* geometric figure with each enzyme form as one of the corners. Equation (5), for instance, can be rewritten as:



The second step is to draw all the possible patterns that connect all the enzyme species without forming a loop. For a mechanism with n enzyme species, or a figure with n corners, each pattern should contain $n - 1$ lines. The number of valid patterns for any single-loop mechanism is equal to the number of enzyme forms. Thus, there are three patterns for the triangle shown above:

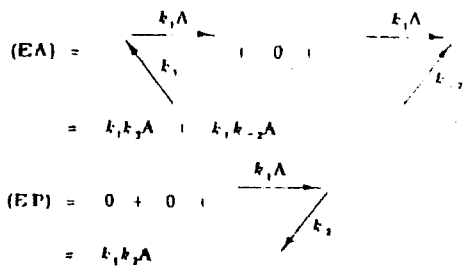
The determinant for a given enzyme species is obtained as the summation of the product of the rate constants and concentration factors associated with all the branches in the patterns *leading toward* this particular enzyme species. The same patterns are used for each species, albeit the direction in which they are read will vary. However, when an irreversible step is present, e.g., the EP → E step, some patterns become invalid for certain enzyme forms.

(E) =



$$= k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2}$$

[4] E. L. King and C. Altman, *J. Phys. Chem.* **60**, 1375 (1956).

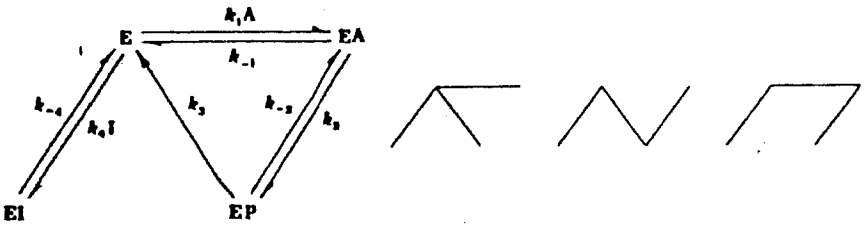


The rate equation is obtained as

$$\frac{v}{E_0} = \frac{k_3(EP)}{(E) + (EA) + (EP)}$$

where (E), (EA), and (EP) are the determinants for E, EA, and EP, respectively.

The presence of an enzyme intermediate(s) that is not part of a loop will not affect the number of King–Altman patterns. For instance, the addition of a competitive inhibitor, I, to the above system will result in the same number of patterns.



The additional E ⇌ EI branch is present in *all* the diagrams. Thus, in calculating the number of valid King–Altman patterns, only the *closed loops* need be considered. The determinants of E, EA, EAB, and EI can be obtained by the method just described:

$$(E) = k_{-4}(k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2})$$
$$(EA) = k_{-4}(k_1k_3A + k_1k_{-2}A)$$
$$(EP) = k_{-4}(k_1k_2A)$$
$$(EI) = k_4I(k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2})$$

It is more convenient, however, to treat this case by first considering only the loop portion (ignoring the additional E ⇌ EI step for the time being).

$$(E) = k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2}$$
$$(EA) = k_1(k_{-2} + k_3)A$$
$$(EP) = k_1k_2A$$

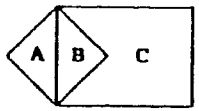
The determinant for EI is then obtained as

$$\begin{aligned}(EI) &= (E)k_4I/k_{-4}\\ &= (k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2})k_4I/k_{-4}\\ &= (k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2})I/K_I\end{aligned}$$

where $K_I = k_{-4}/k_4$.

The King–Altman method is most convenient for single-loop mechanisms. In practice, there is no need to write down the patterns. One can use an object, say, a paper clip, to block one branch of the loop, write down the appropriate term for each enzyme species, then repeat the process until every branch in the loop has been blocked once.

For more complex mechanisms having alternative pathways that form several closed loops, the precise number of valid King–Altman patterns must be calculated to avoid omission of terms. To illustrate the various situations that may occur in such calculation, let us consider Scheme 1.



Scheme 1

The total number of patterns with $n - 1$ lines is given by the equation

$$\frac{m!}{(n-1)!(m-n+1)!}$$

where m = the number of lines in the complete geometric figure. In the above scheme, $m = 8$ and $n = 6$, and the total number of patterns with 5 lines is

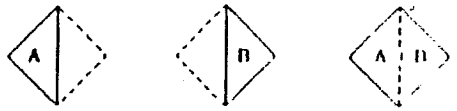
$$\frac{8!}{5!\,3!} = \frac{(8 \times 7 \times 6 \times 5 \times 4 \times 3 \times 2)}{(5 \times 4 \times 3 \times 2)(3 \times 2)} = 56$$

This number, however, includes patterns that contain the following loops, which must be subtracted from the total:

The number of patterns for a loop with r lines is given by

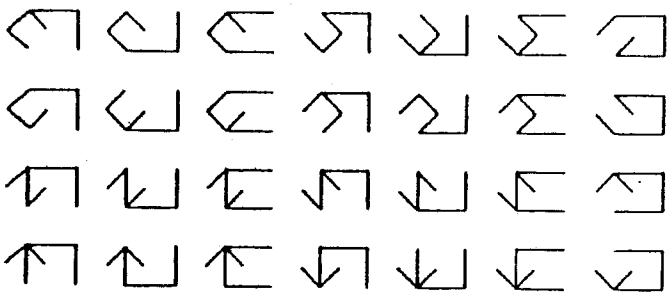
$$\frac{(m-r)!}{(n-1-r)!(m-n+1)!}$$

According to this equation, for loops A and B, $r = 3$, we have 10 patterns each; for loops A + B and B + C, $r = 4$, we have 4 patterns each; and for loops C and A + B + C, $r = 5$ (note that $0! = 1$), we have 1 pattern each. The total number of loop-containing patterns to be subtracted is 30. One of the patterns, however, occurred three times in the above calculations, but should be discarded only once. This pattern involves both loop A and loop B (solid lines indicate the loop that gives rise to this pattern).



Thus, the total of loop-containing patterns is 28, and the total number of valid patterns is $56 - 28 = 28$.

The 28 5-lined patterns are shown below.



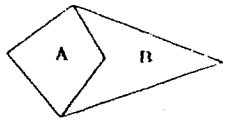
The conventional way of computing the valid King–Altman patterns is rather tedious. A set of formulas developed by the author allows the calculation of the desired number in a very short time. Each of these formulas is applicable to a particular geometric arrangement. For any figure consisting of three subfigures arrayed in sequence like the one shown in Scheme 1, the general formula for calculating the exact number of the valid King–Altman pattern, π, is

$$\pi = a \cdot b \cdot c - (l_{AB}^2 \cdot c + l_{BC}^2 \cdot a)$$

where a, b, and c = the number of lines in subfigures A, B, and C; l_{AB} and l_{BC} = the number of lines in the common boundaries between A and B, and B and C, respectively. For $a = 3$, $b = 3$, $c = 5$, $l_{AB} = 1$, and $l_{BC} = 2$ (Scheme 1), we have

$$\begin{aligned}\pi &= 3 \times 3 \times 5 - (1^2 \times 5 + 2^2 \times 3) \\ &= 45 - (5 + 12) \\ &= 28\end{aligned}$$

In the case of two subfigures A and B sharing a common boundary as shown in Scheme 2



Scheme 2

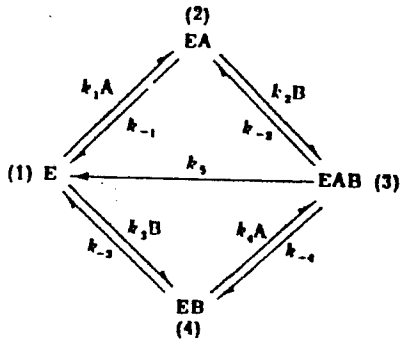
the formula is given by

$$\begin{aligned}\pi &= a \cdot b - l_{AB}^2 \\ &= 4 \times 4 - 2^2 \\ &= 12\end{aligned}$$

Formulas for calculation of up to four subfigures in every possible geometric arrangement have been established.

The Method of Volkenstein and Goldstein

Volkenstein and Goldstein[2] have applied the theory of graphs to the derivation of rate equations. Their approach has three main features: the use of an auxiliary "node," the "compression" of a path into a point, and the addition of parallel branches. These can be best explained by an example (Scheme 3).

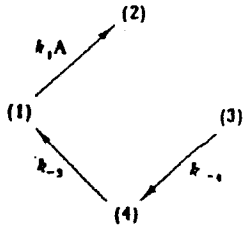


Scheme 3

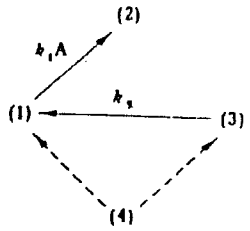
Each enzyme-containing species is assigned a number and referred to as a node.

Suppose we want to calculate the determinant for EA (node 2). First, we choose another node, say node 3, as the auxiliary node (a reference

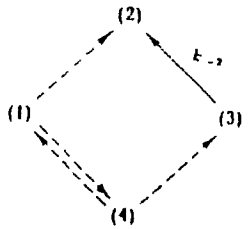
starting point). The choice of the auxiliary node is arbitrary; it will not affect the outcome of the derivation, but may affect the amount of work involved. All the possible pathways (flow patterns) leading from (3) to (2) are then written (marked by solid branches).



Path 3412

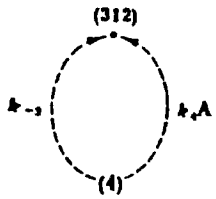


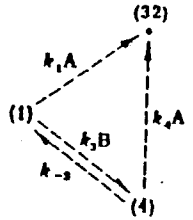
Path 312



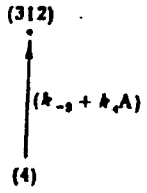
Path 32

The nodes not included in the pathways retain the branches leading *away* from them (dashed branches). Since path 3412 flows through all the nodes, it is one of the terms of the determinant with a path value of $k_1k_{-3}k_{-4}A$. Path 312 ($=k_1k_5A$) and path 32 ($=k_{-2}$) are now compressed into points.

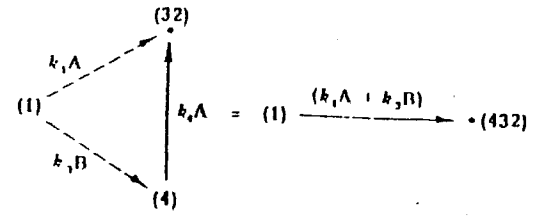




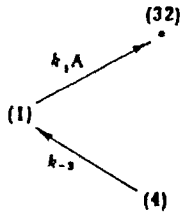
The two parallel branches leading from (4) to the compressed point (312) can be added together to yield



and the expression for this part is $(P312)(k_{-3} + k_4A) = k_1k_5A(k_{-3} + k_4A)$. The part containing point (32) can be treated by selecting a secondary auxiliary node, say node (4), and repeating the procedure described at the onset.



and



The contribution from this part is $(P432)(k_1A + k_3B) = k_{-2}k_4A(k_1A + k_3B)$ and $(P32)(k_1k_{-3}A) = k_1k_{-2}k_{-3}A$. Adding the terms together, we obtain the determinant for EA

$$\begin{aligned}(EA) &= k_1k_{-3}k_{-4}A + k_1k_5A(k_{-3} + k_4A) + k_{-2}k_4A(k_1A + k_3B) \\ &\quad + k_1k_{-2}k_{-3}A \\ &= k_1k_{-3}(k_{-2} + k_{-4} + k_5)A + k_{-2}k_3k_4AB + k_1k_4(k_{-2} + k_5)A^2\end{aligned}$$

The determinants for E, EB, and EAB can be obtained in a similar fashion. The complete rate equation is given by

$$\frac{v}{E_0} = \frac{k_5(EAB)}{(E) + (EA) + (EB) + (EAB)}$$

Rate equations for more complex mechanisms can be derived by repeating the procedure described above as many times as necessary. The choice of the auxiliary point becomes important for reaction schemes containing several loops. The process is analogous to deciding which row (equation) should be omitted from the matrix in the determinant method. In general, one should choose, by inspection of the geometric structure of the mechanism, a node such that, if one removes from the figure the auxiliary node and the node whose determinant is desired, the remaining nodes do not form a closed loop. In addition, one should select a node situated in a symmetrical position with respect to the desired node. For instance, node (4) is a better choice as an auxiliary node for the calculation of the determinant for node (2). Node (3) was chosen for the sole purpose of illustrating the use of secondary auxiliary nodes.

Cleland's Rules:

1. C, competitive inhibition: In a plot of 1/V vs 1/S (varying [I], where I is an inhibitor), an inhibitor effects the slope when I and the variable substrate either compete directly for the same form of the enzyme or react with forms separated from each other by reversible steps. In either case, one can effect the concentration of enzyme available by displacement of the equilibria. The variable substrate can overcome the inhibition.

2. UC, uncompetitive inhibition: An inhibitor, I, effects the intercepts of a plot of 1/V vs 1/S, when it combines with a form of the enzyme that differs from that responsible for combination with the variable S. The inhibitor lowers the total amount of the enzyme available for distribution among normal enzyme forms in a manner that cannot be overcome by saturation with substrate.

3. NC, noncompetitive inhibition: If the inhibitor combines with more than one form of the E (or is reversibly connected), the resulting pattern is the sum of the different inhibitors produced singly. In a 1/V vs 1/S, both the slopes and intercepts are effected.

Comparison of Different Symbolisms of Bisubstrate Kinetics

Alberty (1956)

$$\frac{V_f}{v_f} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{AB}}{AB}$$

$K_A = \frac{\phi_1}{\phi_0}$ $K_B = \frac{\phi_2}{\phi_0}$ $K_{AB} = \frac{\phi_{12}}{\phi_0}$ $V_f = \frac{e}{\phi_0}$

Dalziel (1957)

$$\frac{e}{v_f} = \phi_0 + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2} + \frac{\phi_{12}}{S_1 S_2}$$

Bloomfield et al. (1963)

$$\frac{V_{AB}}{v_f} = 1 + \frac{K_{AB}}{K_B}\frac{1}{A} + \frac{K_{AB}}{K_A}\frac{1}{B} + \frac{K_{AB}}{AB}$$

$K_A = \frac{\phi_2}{\phi_{12}}$ $K_B = \frac{\phi_1}{\phi_{12}}$ $K_{AB} = \frac{\phi_{12}}{\phi_0}$ $V_f = \frac{e}{\phi_0}$

Cleland (1963)

$$\frac{V_1}{v_1} = 1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{AB}$$

$K_a = \frac{\phi_1}{\phi_0}$ $K_b = \frac{\phi_2}{\phi_0}$ $K_{ia} = \frac{\phi_{12}}{\phi_2}$ $V_1 = \frac{e}{\phi_0}$

Our designation	*Meaning*	*Dalziel*	*Alberty*	*Bloomfield et al.*	*Cleland*	*Enzyme Commission*
K_a	Limiting Michaelis constant for *A*	$\frac{\phi_1}{\phi_0}$	K_A	$\frac{K_{AB}}{K_B}$	K_a	K_m^A
K_b	Limiting Michaelis constant for *B*	$\frac{\phi_2}{\phi_0}$	K_B	$\frac{K_{AB}}{K_A}$	K_b	K_m^B
K_{ia}	Dissociation constant for *A*	$\frac{\phi_{12}}{\phi_2}$	$\frac{K_{AB}}{K_B}$	K_A	K_{ia}	K_s^A
V_1	Limiting maximum velocity	$\frac{e}{\phi_0}$	V_f	V_{AB}	V_1	V
$\frac{V_1}{e_0}$	Turnover number	$\frac{1}{\phi_0}$	$\frac{V_1}{E_0}$	$\frac{V_{AB}}{E_0}$	$\frac{V_1}{E_t}$	None

In a similar vein, one could say that the competitive inhibition of A by Q would turn into a noncompetitive inhibition if an AQ term (a Q/B term in reciprocal form) were present.

Our next point for consideration is an examination of the reasons for the various terms being present. One can see rather easily why Q would be a competitive inhibitor of A in an ordered bireactant mechanism. Both Q and A compete for the same enzyme form, E. For this reason, saturation by A can completely overcome the inhibition by Q. No effect on the apparent V_1 or $1/V_1$ occurs, although the slope term (apparent K/V) is increased by the Q. Alternately, Q does not appear in any term in the rate equation which is not an interaction term with A.

Uncompetitive inhibition is at first a bit more difficult to grasp intuitively in relation to a realistic mechanism. One prerequisite for uncompetitive inhibition is that the inhibitor must combine with an enzyme form different from the one to which the variable substrate combines since combination with the same form produces competitive inhibition. A fixed inhibitor term arises in this manner. However, our example of P as an inhibitor of A gave noncompetitive inhibition unless B was saturating. Although P combines with EQ and A with E, we get the uncompetitive inhibition only if B is infinite, leaving no reversible step or series of steps to connect the two enzyme forms involved. Saturation by B interrupts the reversible series of steps linking E with EQ to give pure uncompetitive inhibition by eliminating the interaction term and leaving only the fixed inhibitor term.

The distinctive features of noncompetitive inhibition are that the inhibitor always combines with a different enzyme form than the one with which the variable substrate combines. However, the two forms are always linked by reversible steps, providing an interaction term in addition to the fixed inhibitor term.

PREDICTING INHIBITION PATTERNS

Cleland (2) has developed these ideas by formulating a set of rules for predicting the type of inhibition to be expected from an examination of steady-state mechanisms containing no random sequences unless they are in rapid equilibrium. (Enzyme forms in rapid equilibrium are to be considered as one single enzyme form for these purposes.) Inasmuch as the cases covered by these rules represent the bulk of commonly treated mechanisms, this approach is a powerful tool in scanning large numbers of possible mechanisms. If kinetic constants are to be calculated from the inhibition data or if nonrapid equilibrium random sequences are involved, one must derive the full rate equation.

These rules, broadly stated, are as follows:

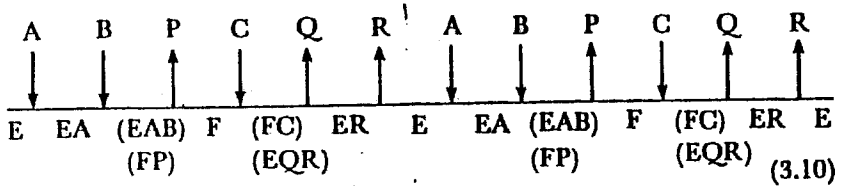
Rule 1 The ordinate intercept of a reciprocal plot is affected by a substance which associates reversibly with an enzyme form other than the one with which the variable substrate combines.

Rule 2 The slope of a reciprocal plot is affected by a substance which associates with an enzyme form that is the same as, or is at least connected by a series of reversible steps to, the enzyme form with which the variable substrate combines.

Thus, in Rule 1, the effect of an inhibitor cannot be completely eliminated by saturation if it does not bind to the same enzyme form as does the variable substrate. Rule 2 then adds that the effect of inhibitor cannot be even partially eliminated by saturation if the two enzyme forms involved are not in equilibrium by means of reversible steps connecting them. If only Rule 1 applies, uncompetitive inhibition is observed. If only Rule 2 is applicable, competitive inhibition is seen. Noncompetitive inhibition results from invocation of both rules. The definition of irreversible steps remains the same as in the preceding chapter, namely, release of product at zero free concentration of product and saturation by substrate.

Note 1 An inhibitor cannot bind to an enzyme form whose steady-state level is already zero because of saturation by a substrate.

Let us analyze the case of product inhibition for the Bi Uni Uni Bi Ping Pong mechanism, which we have written as two full sequences to help the analysis by reminding ourselves of the continuity of the pattern.



(3.10)

Inhibitor	Variable substrate	Saturating substrate(s)	Results
P	A	—	Intercept or I effect occurs since P reacts with F and A with E, which are different enzyme forms. Slope or S effect occurs since E and F are reversibly connected in the presence of P by the sequence E, EA, EAB-FP, and F. (Noncompetitive.)

Inhibitor	Variable substrate	Saturating substrate(s)	Results
P	A	B	I effect is not influenced. S effect is abolished because the reversible sequence from E to F is blocked by saturating levels of B as the steady-state level of EA falls to zero. (Uncompetitive.)
P	A	C	Saturating levels of C reduce the steady-state level of F nearly to zero; hence P cannot bind to F. (No inhibition.)
P	B	—	I effect occurs because P and B react with different enzyme forms. The E, EAB-FP, and F sequence is reversible, producing an S effect. (Noncompetitive.)
P	B	A	No change occurs from above. (Noncompetitive.)
P	B	C	Steady-state level of F is zero. (No inhibition.)
P	C	—	Only S effect occurs since P and C both combine with the same form F. (Competitive.)
P	C	A	(Competitive.)
P	C	B	(Competitive.)
Q	A	—	I effect occurs because Q and A react with different enzyme forms. No S effect occurs because the sequence from ER to E is blocked by the release of R, which is absent from the system, and the sequence from E to ER is blocked by the release of P. (Uncompetitive.)
Q	A	B	(Uncompetitive.)
Q	A	C	(Uncompetitive.)
Q	B	—	Q and B react with different enzyme forms—I effect. Both the EA-ER and the ER-EA sequences are blocked by P and R release, producing no S effect. (Uncompetitive.)
Q	B	A	(Uncompetitive.)
Q	B	C	(Uncompetitive.)
Q	C	—	Q and C react with different enzyme forms which are reversibly connected. (Noncompetitive.)

Inhibitor	Variable substrate	Saturating substrate(s)	Results
Q	C	A	(Noncompetitive.)
Q	C	B	(Noncompetitive.)
R	A	—	R and A both combine with E. (Competitive.)
R	A	B	(Competitive.)
R	A	C	(Competitive.)
R	B	—	R and B combine with different forms which are reversibly connected. (Noncompetitive.)
R	B	A	The steady-state level of E is reduced to zero. (No inhibition.)
R	B	C	(Noncompetitive.)
R	C	—	The sequence from F to E and from E to F are blocked by Q and P release. (Uncompetitive.)
R	C	A	Reduces steady-state level of E to zero. (No inhibition.)
R	C	B	(Uncompetitive.)

In this example thus far, we have been dealing only with inhibition by products combining solely with the enzyme form with which they are associated during the normal reaction sequence. If we expand to include dead-end inhibition, we must add a note to the rules we have been using. This applies to products which combine with enzyme forms in addition to their normal forms.

Note 2 A dead-end inhibitor, since it is not a normal part of the sequence, cannot serve to reverse the reaction sequence. Hence reversible sequences must be sought with the variable substrate by starting with the site of inhibition and going forward to the point of substrate addition.

We thus consider the mechanism of Eq. (3.10) again, with our inhibitor I able to bind various enzyme forms.

Enzyme form	Variable substrate	Saturating substrate	Results
E	A	—	I and A react only with E. (Competitive.)
E	A	B or C	(Competitive.)

Enzyme form	Variable substrate	Saturating substrate	Results
E	B	—	I and B react with different enzyme forms linked by the reversible sequence EI-E-EA. (Noncompetitive.)
E	B	A	Steady-state level of E is reduced to zero. (No inhibition.)
E	B	C	(Noncompetitive.)
E	C	—	I and B react with different enzyme forms not linked by reversible steps. (Uncompetitive.)
E	C	A	Steady-state level of E is zero. (No inhibition.)
E	C	B	(Uncompetitive.)
EA	A	—	The sequence from EA to E is not reversible. See Note 2. (Uncompetitive.)
EA	A	B	Steady-state level of EA is zero. (No inhibition.)
EA	A	C	(Uncompetitive.)
EA	B	—	Both B and I react with EA. (Competitive.)
EA	B	A	(Competitive.)
EA	B	C	(Competitive.)
EA	C	—	The sequence of F to EA is not reversible. (Uncompetitive.)
EA	C	A	(Uncompetitive.)
EA	C	B	(No inhibition.)
EAB-FP, FC-EQR	A, B, or C	None, A, B, or C	No inhibitor reacts with any of these enzyme forms because the binding sites are filled.
ER	A, B, or C	None, A, B, or C	No reversible sequence exists. (Uncompetitive.)
F	A	—	No reversible sequence exists. (Uncompetitive.)
F	A	B	(Uncompetitive.)
F	A	C	(No inhibition.)
F	B	—	No reversible sequence exists. (Uncompetitive.)
F	B	A	(Uncompetitive.)
		C	(No inhibition.)
F	C	None, A, or B	Both I and C combine only with F. (Competitive.)

Using this technique, one can examine the proposal by Fromm (3) that certain classes of dead-end inhibitors be used to elucidate the normal mechanism. The principle involved requires the use of substrate analogs which will give competitive inhibition with the substrate for which it is an analog. Since such behavior means that the analog is binding to the same enzyme form as that to which the analogous substrate is binding, one should be able to examine the type of inhibition resulting when the other substrates are the variable substrates and learn something about the mechanism.

Although a large number of the inhibition patterns predicted here for dead-end inhibition are uncompetitive, in practice one finds noncompetitive inhibition occurring much more frequently as a result of the inhibitor combining with more than one enzyme form. In addition, the inhibitor must be adsorbed in such a manner as to prevent completely substrate addition or product release if the predicted pattern is to be found. If alternate reaction sequences are established, a much more complex pattern will likely emerge, and these must be treated in a different manner (2).

Multiple combinations by inhibitor are cases that can also be treated by Cleland's rules. A dead-end inhibitor reacting with more than one enzyme form will introduce $1 + I/K_I$ terms into different parts of the denominator of the rate equation. Under such conditions the inhibitor terms will add but not multiply. Thus a slope effect and an intercept effect will combine to give noncompetitive inhibition. However, two slope effects, for example, could combine in such a manner as to still give a linear replot of slope versus inhibitor concentration. If the two effects multiplied, a parabolic replot would result.

Another type of multiple combinations by inhibitor can occur if a product inhibitor combines with an enzyme form in addition to the one with which it normally combines to give dead-end inhibition as well as product inhibition. Rule 3 can be used to predict the type of inhibition that would result.

Rule 3 a. If the inhibitor combines with more than one enzyme form, determine the slope and intercept effects for each form according to Rules 1 and 2.

b. Multiple intercept and slope effects will be multiplied if the two enzyme forms inhibited are connected by a reversible series of steps, and they will be summed if not so connected. (Notes 2 to 4, below, are all applicable in ascertaining whether the connection is reversible or not.)

By means of Rule 3, we can now predict whether the replots are going to be linear or parabolic. (Hyperbolic and 2/1 replots come about by

means of alternate reaction sequences.) Notes 3 and 4 are useful hints to apply to these cases:

Note 3 The variable substrate acts as an irreversible step in Rule 3 only for intercept effects because, at the ordinate intercept, the variable substrate is infinitely high in concentration.

Note 4 If the inhibitor reacts with one enzyme form to yield two or more different complexes, the inhibition is still linear even though one of the complexes is a normal part of the reaction sequence (e.g., if a product combines in a dead-end manner with the same enzyme form as it normally combines, no nonlinear terms are introduced).

We may look at some examples of this type of multiple inhibition by continuing to use Eq. (3.10) as the basic mechanism for our consideration.

Inhibitor	Variable substrate	Saturating substrate	Dead-end enzyme	Results
P	A	—	E	Noncompetitive plus competitive with E and F reversibly linked. (S-parabolic I-linear noncompetitive.)
P	A	B	E	(Noncompetitive.)
P	A	—	EA	Noncompetitive plus uncompetitive with EA and F reversibly linked. (S-linear I-parabolic noncompetitive.)
P	A	B	EA	(Uncompetitive.)
P	A	—	F	Noncompetitive plus uncompetitive but with the same enzyme form. See Note 4 (Noncompetitive).
P	A	B	F	(Uncompetitive.)
P	A	—	ER	Noncompetitive plus uncompetitive with no reversible connection. (Noncompetitive.)
P	B	—	E	Noncompetitive plus noncompetitive with Note 3 applicable. (S-parabolic I-linear noncompetitive.)

Inhibitor	Variable substrate	Saturating substrate	Dead-end enzyme	Results
P	B	A or C	E	(Noncompetitive.)
P	B	—	EA	Noncompetitive plus competitive. (S-parabolic I-linear noncompetitive.)
P	B	C	EA	(Competitive.)
P	B	—	F	Note 4. (Noncompetitive.)
P	B	C	F	(No inhibition.)
P	B	—	ER	Noncompetitive plus uncompetitive with no reversible connection. (Noncompetitive.)
P	C	—	E and EA	Competitive plus noncompetitive with a reversible connection. (S-parabolic I-linear noncompetitive.)
P	C	—	F	Note 4. (Competitive.)
P	C	—	ER	Competitive plus uncompetitive. (Noncompetitive.)

Obviously, only a limited number of examples have been shown here. However, this method is so simple to use that one is able to quickly scan large numbers of possible mechanisms and to discard those models that are inconsistent with the data.

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