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A Catalytic Mechanism for Caspase-1 and for Bimodal Inhibition of Caspase-1 by Activated Aspartic Ketones

Kenneth D. Brady,[a,*] David A. Giegel,[b] Christine Grinnell,[a] Elizabeth Lunney,[b] Robert V. Talanian,[a] Winnie Wong[a] and Nigel Walker[c,†]

[a]*BASF Bioresearch Corporation, 100 Research Drive, Worcester, Massachusetts 01605-4314, USA*
[b]*Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105, USA*
[c]*BASF Aktiengesellschaft, D-67056 Ludwigshafen, Germany*

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Abstract—We have evaluated 619 aspartic ketones with 9 different types of prime-side groups (acyloxymethyl, aryloxymethyl, arylthiomethyl, alkylthiomethyl, acylamino-oxymethyl, sulfonylaminomethyl, α-ketoamide, α-(1-phenyl-3-trifluoromethyl-pyrazol-5-yl)oxymethyl (PTP), and aliphatic ketones) as inhibitors of caspase-1. The inhibitory behaviors could be classified as reversible, inactivating, or bimodal (i.e. reversible inhibition followed by slow inactivation) based on the kinetically observed formation of reversible thiohemiketal complexes and conversion to an irreversible thioether adduct, and the mechanism of any given ketone was only poorly predictable on the basis of leaving group structure and chemistry. Among 201 bimodal inhibitors, the rate of conversion of the reversible thiohemiketal complex to the inactive thioether (k_i) was strictly first-order, consistent with direct conversion of the thiohemiketal to the thioether with no intermediate collapse to free ketone and thiolate. We have examined 22 crystallographic structures of caspase-1 complexed as a thiohemiketal with the inhibitors from 8 different ketone classes, and found the Cys285S–C–$C_{\alpha'}$-leaving group dihedral angle to be near either to 60° or to 180°. Only the 180° conformation was permissive for SN_2 displacement of the leaving group and, furthermore, positioned His237Nδ to stabilize developing charge on the leaving group. Among these structures and 19 additional complexes, all showed a strong interaction between His237Nδ and the ketone or thiohemiketal oxygen. We therefore propose a proteolytic mechanism for caspase-1 involving polarization of the scissile carbonyl by the His237 imidazolium group. During thiohemiketal/thioether conversion (but probably not during peptide hydrolysis), the leaving group is stabilized by the His237 imidazolium.

Introduction

As inhibitors of cysteine proteases, many activated ketones[1] offer low or moderate chemical reactivity and high biochemical specificity, making them promising candidates as therapeutically useful inhibitors of pathological proteolytic events. As inhibitors of cathepsins B,[2–5] S and L,[6] a series of benzoic acid acyloxymethyl ketones showed a strong correlation between the second-order inactivation rate and leaving-group pK_a.[3] Furthermore, inactivation rates of inhibitors possessing leaving groups with $pK_a > 4$ were generally quite slow. In the case of caspase-1, however, no such correlation was observable for inhibitors possessing leaving groups spanning the range $0.6 < pK_a < 7.2$.[7] Remarkably, several aryloxymethyl ketones with leaving groups of $pK_a \gg 5$ were fast, potent inactivators of caspase-1.[7,8] This phenomenon was exploited by researchers at Sterling-Winthrop to develop several alternative leaving-group functionalities.[9–11]

Our laboratory has studied a variety of activated ketones as inhibitors of caspase-1.[12] In many cases, we have observed clear differentiation of a reversible phase of inhibition (i.e. formation of a reversible thiohemiketal adduct; see Scheme 1B) and a slow irreversible inactivation of the enzyme at a rate that saturates with increasing inhibitor concentration (i.e. formation of an irreversible thioether adduct), a behavior which we refer to as 'bimodal'. This enables us to look for biochemical and structural factors which independently affect the first-order inactivation rate (k_i, Scheme 1B) and thiohemiketal complex stability (K_i; see eq (1)). Additionally, we have acquired crystallographic structural data for some of these compounds complexed as thiohemiketals

Abbreviations: Amc: 7-amino-4-methyl coumarin; AOMK: acyloxymethyl ketone; BSA: bovine serum albumin; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; HEPES: hydroxyethylpiperazineethane sulfonic acid; N-His (D381E) ICE: caspase-1 modified with an N-terminal $(His)_6$ tag and an Asp381- > Glu point mutation[16]; *p*-NA, *para*-nitroanilide; rfu, relative fluorescence units; PTP, α-(1-phenyl-3-trifluoromethyl-pyrazol-5-yl)oxymethyl.

*Corresponding author. Tel: +1 508-849-2621; fax: +1 508-754-7784; e-mail: bradykd@basf.com

† Current address: Tularik Incorporated, Two Corporate Drive, South San Francisco, CA 94080, USA.

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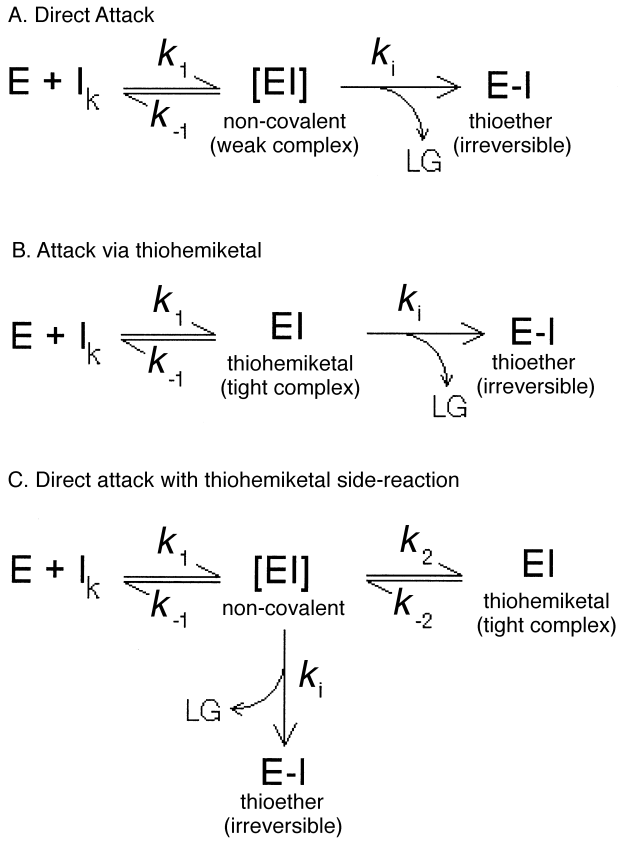


Scheme 1. Three possible mechanisms for the inhibition of caspase-1 by activated ketones.

or thioethers with caspase-1, including numerous compounds with potential 'leaving groups', allowing us to examine bonding geometry in the thiohemiketal complex which might affect the rate of progression to the irreversible thioether adduct.

In this report, we examine the kinetic properties of the reaction of caspase-1 with a great variety of activated ketone inhibitors, and show that for bimodal inhibitors the irreversible conversion of the thiohemiketal complex to a thioether adduct is strictly first-order and occurs with a narrow range of rates which are independent of the leaving group pK_a. Comparison of 22 structures of caspase-1 complexed as a thiohemiketal with some of these inhibitors offers a rationale for the pK_a independence and suggests a catalytic mechanism for the actions of caspase-1 on its peptide substrates and on these ketone inhibitors.

Results

Nature of the compounds

All of the compounds described in this study possess the aspartic acid ketone functionalities shown in Table 1. The compounds were all peptides or peptide mimetics ranging in size from mono- to tetrapeptide. The intent of this study is to provide a statistical examination of the kinetic and mechanistic properties of the inhibitors; more detailed investigations of individual compounds will be published elsewhere.

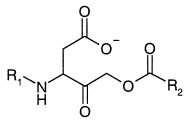
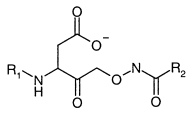
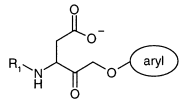
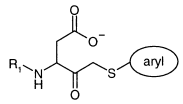
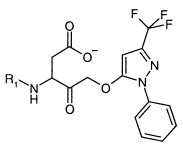
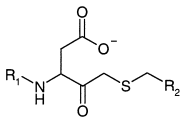
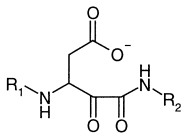
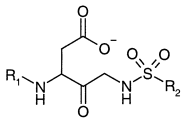
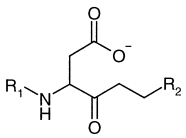
Possible inhibitory mechanisms

Schemes 1A–C depict three mechanisms by which inhibition by activated ketones might occur, and kinetic derivations for these schemes are given in the Experimental. Predicted kinetic behaviors based on these derivations are summarized in Table 2. Several of these predictions rest on the assumption that the non-covalent complex of Scheme 1A and C (i.e. a complex analogous to the Michaelis complex of enzyme and substrate) is much weaker than the covalent thiohemiketal complexes of Scheme 1B and C. As an example, we have found that the tetrapeptide Ac-YVA-NH-$(CH_2)_2$-COOH inhibits caspase-1 with $K_i = 200\,\mu M$. In contrast, Ac-YVAD-CHO,[13] which differs only by the presence of an aldehyde functionality, inhibits caspase-1 with $K_i = 0.7\,nM$, an increase of nearly 300,000-fold over the non-covalent peptide. Similarly, since mono- and dipeptides do not serve as substrates for caspase-1,[13] binding of these is presumably extremely weak. In contrast, we observe numerous mono- and dipeptide ketones with K_i in the range of 0.1–50 μM (e.g. see Figs 1 and 2), leading us to believe that the profound increase in affinity upon formation of a thiohemiketal or thiohemiacetal applies to shorter peptides as well.

Among these alternative mechanisms, bimodal inhibition occurs only when the second term of eq (2) is small relative to the first (i.e. $k_i \ll k_{-1}$, Scheme 1B). Under this condition, the faster reversible inhibition reaction is easily separated from the slower irreversible reaction, and K_i (eq (2)) can be evaluated either from the inhibition of the initial rate of enzymatic substrate hydrolysis, or from the saturation of k_{obs} with increasing inhibitor concentration. Figure 1 shows an example of these two evaluations for one acyloxymethyl ketone. While K_i determined by either of these approaches should be identical, eq (2) predicts that for a bimodal inhibitor, K_i will be uncorrelated with the saturating inactivation rate k_i. These properties are illustrated in Figure 2(A) and (B). The importance of the reaction time scale is illustrated by Figure 2(B), where an 'IC_{50}' evaluated following a long incubation of enzyme and inhibitor does correlate with k_i. The longer incubation allows the slow inactivation to contribute to the relative potency, IC_{50}.

The distinctions between bimodal inhibition and inactivation as outlined in Table 2 are relative–bimodal or inactivating kinetic behaviors are observable depending on the relative magnitudes of k_{-1}/k_1 and k_i. The more important underlying question is whether or not the irreversible attack by Cys 285 on $C_{\alpha'}$ occurs directly from the thiohemiketal (Scheme 1B), or whether this attack occurs only from a non-covalent enzyme/ketone complex (Scheme 1A or C). If one accepts the premise that the non-covalent complex of these peptide-like inhibitors with caspase-1 is profoundly weaker than the thiohemiketal complex, then for inhibitors which display bimodal kinetics, the irreversible attack at $C_{\alpha'}$ must occur from the thiohemiketal, as the alternative

Table 1. Reversible, bimodal and inactivating behavior among ketone classes

Structure	Class	Kinetic profile[a]	No. observed[b]	k_i ($s^{-1} \times 10^{-3}$)
	Acyloxymethyl ketone[3]	r b i	118 (39%) 166 (55%) 17 (6%)	< 0.05 1.86 ± 3.5 > 20
	Acylamino-oxymethyl ketone[1]	r b	14 (67%) 7 (33%)	< 0.05 1.98 ± 1.62
	Aryloxymethyl ketone[1]	r b i	7 (29%) 16 (67%) 1 (4%)	 1.42 ± 1.4 > 20
	Arylthiomethyl ketone[1]	r b	27 (90%) 3 (10%)	< 0.05 1.1 ± 1.2
	PTP ketone[9]	b i	4 (13%) 27 (87%)	6.8 ± 3.0 > 20
	Alkylthiomethyl ketone[1]	r	60 (100%)	
	α-Ketoamide[19]	r	46 (100%)	
	Sulfonylaminomethyl ketone[c,1]	r b	63 (93%) 5 (7%)	 0.99 ± 0.99
	Ketone (aliphatic)[20]	r	38 (100%)	
	Total		619	

[a]r: reversible (no detectable inactivation during 90 min of observation); b: bimodal kinetics; i: inactivating kinetics. See Table 2.
[b]Percentages (in parentheses) are the percentage of each chemical class displaying the given kinetic profile.
[c]Prepared from the bromomethylketone and Boc-sulfonamide using *tert*-butoxide as base.

mechanism (Scheme 1C) would generate a radically different kinetic profile (see eq (3)).

Krantz et al. observed that the second-order rate of inactivation (k_{on}) of cathepsin B by acyloxymethyl ketones can increase dramatically when the peptide portion of the inhibitor increases in length and specificity.[3] In the case of bimodal inhibitors of caspase-1, k_{on} (which is equal to k_i/K_i when $[I] \ll K_i$) also increases dramatically with improvements in the peptidic (or mimetic) portion of the molecule (data not shown). Figure 2(B) indicates that for these inhibitors the

Table 2. Predicted kinetic behaviors for Schemes 1A–C

Mechanism	$K_{i,eq}$[1]	$\Delta K_{i,kin}$[2]	Kinetic profile	Reversible phase observed?
Scheme 1A	Large	Small	Slow inactivating	No
Scheme 1A	Large	Large	Fast inactivating	No
Scheme 1B	Small	Small	Bimodal	Yes
Scheme 1B	Small	Large	Fast inactivating	Yes (short-lived)
Scheme 1C	–	–	Self competing	Yes. Inactivation rate is very small and independent of [I]

[1] $K_{i,eq} = k_{-1}/k_1$ (eq (2)).
[2] $\Delta K_{i,kin} = k_i/k_1$ (eq (2)).

increase in k_{on} results almost entirely from decreases in K_i. This independence of k_i and K_i suggests that the thiohemiketal/thioether conversion (i.e. the second half of Scheme 1B) involves only a small core of active-site residues which, while catalytically important, do not significantly affect the relative K_i of these ketone inhibitors.

Distribution of k_i

Table 1 and Figure 3 describe 619 inhibitors from nine subclasses of ketones which differ in the nature of the prime-side group. Three classes (aliphatic ketones, thiomethyl ketones, and α-ketoamides) were uniformly reversible. Three classes (acylamino-oxymethyl ketones, arylthiomethyl ketones, and sulfonylaminomethyl ketones) showed either reversible or bimodal kinetics. All three kinetic behaviors were observed among the acyloxy- and aryloxy-methyl ketones, while the PTP ketones were predominantly inactivators of caspase-1.

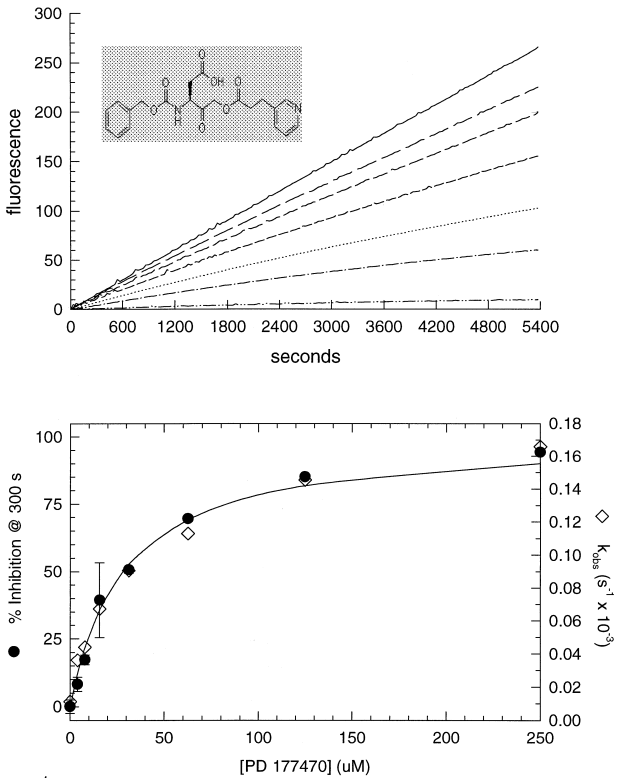


Figure 1. Evaluation of K_i by two techniques. Top: progress curves for the acyloxymethyl ketone PD177470 over the concentration range 0–250 μM demonstrate rapid inhibition followed by very slow inactivation. Bottom: analysis of the initial velocities provides $K_i = 9.0\,\mu M$. Analysis of k_{obs} provides $K_i = 14\,\mu M$ and $K_i = 1.7\times10^{-4}\,s^{-1}$.

Figure 3 shows the distribution of k_i observed among all of the bimodal compounds. Modeled as a log-normal distribution, we find a mean of $0.0010\,s^{-1}$ and a standard deviation of 2.9-fold. Consistent with Table 1, the different leaving group classes are evenly distributed across the observed range. There is likely to be measurement bias against low values of k_i, since values below 2×10^{-4} are easily missed if the data quality is sub-optimal. Values in the range 0.005–0.015 s^{-1} are, in theory, not difficult to measure, though eq (2) indicates that as k_i increases, the kinetic factor $\Delta K_{i,kin}$ also increases, so that it will become increasingly difficult to achieve saturation. It is also possible that the chemical nature of the thiohemiketal/thioether conversion constrains this rate to the observed range of values, independently of the nature of the leaving group or the binding affinity of the peptide.

Specific classes of activated ketones—the PTP ketones and the dichlorobenzoyloxmethyl ketones—consistently behaved as inactivators. As indicated in Table 2, fast inactivating kinetics can be observed for either Scheme 1A or B provided that k_i is sufficiently fast. In previous studies, a very short-lived reversible phase was observed for one PTP ketone,[12] suggesting that this class might inactivate via a thiohemiketal adduct. Alternatively, it is possible that some of these compounds are not disposed to form the thiohemiketal complex. Upon attack by Cys285, the carbonyl carbon must convert from sp2 to sp3 hybridization and change geometry from planar to tetrahedral, forcing a reorientation of the leaving group in the binding cavity. Since these leaving groups are bulky systems, they are perhaps not readily accommodated by the enzyme binding site after this change in carbon geometry. This barrier might then promote direct attack by the sulfur on the α-carbon (Scheme 1B). Unfortunately, the groups which leave most readily are unobservable by standard crystallography. Careful modelling, rapid mix kinetics, and/or time-resolved crystallography could perhaps distinguish between these inactivation mechanisms.

Structural determinants of bimodal inhibition

We have obtained crystallographic structures representing most of the bimodal inhibitor classes (Table 1) in complex with caspase-1 by using compounds which are either reversible or are extremely slow in the irreversible step. From 22 of these structures, we have measured several atomic distances and dihedral angles which we expect to be critical for the thiohemiketal/thioether conversion (Figs 4 and 5). First, the S-C-$C_{\alpha'}$-LG dihedral angle (see Fig. 6) must be near to 180° for efficient SN_2 displacement at the $C_{\alpha'}$ carbon.[14] Figure 4(A) shows that this angle is always near to either 60° or 180°. The α-ketoamides ($n = 3$) and the alkylthiomethyl ketones ($n = 5$) all adopted configurations near to 60°. Four of 5 of the acyloxymethyl ketones bound in the 180°

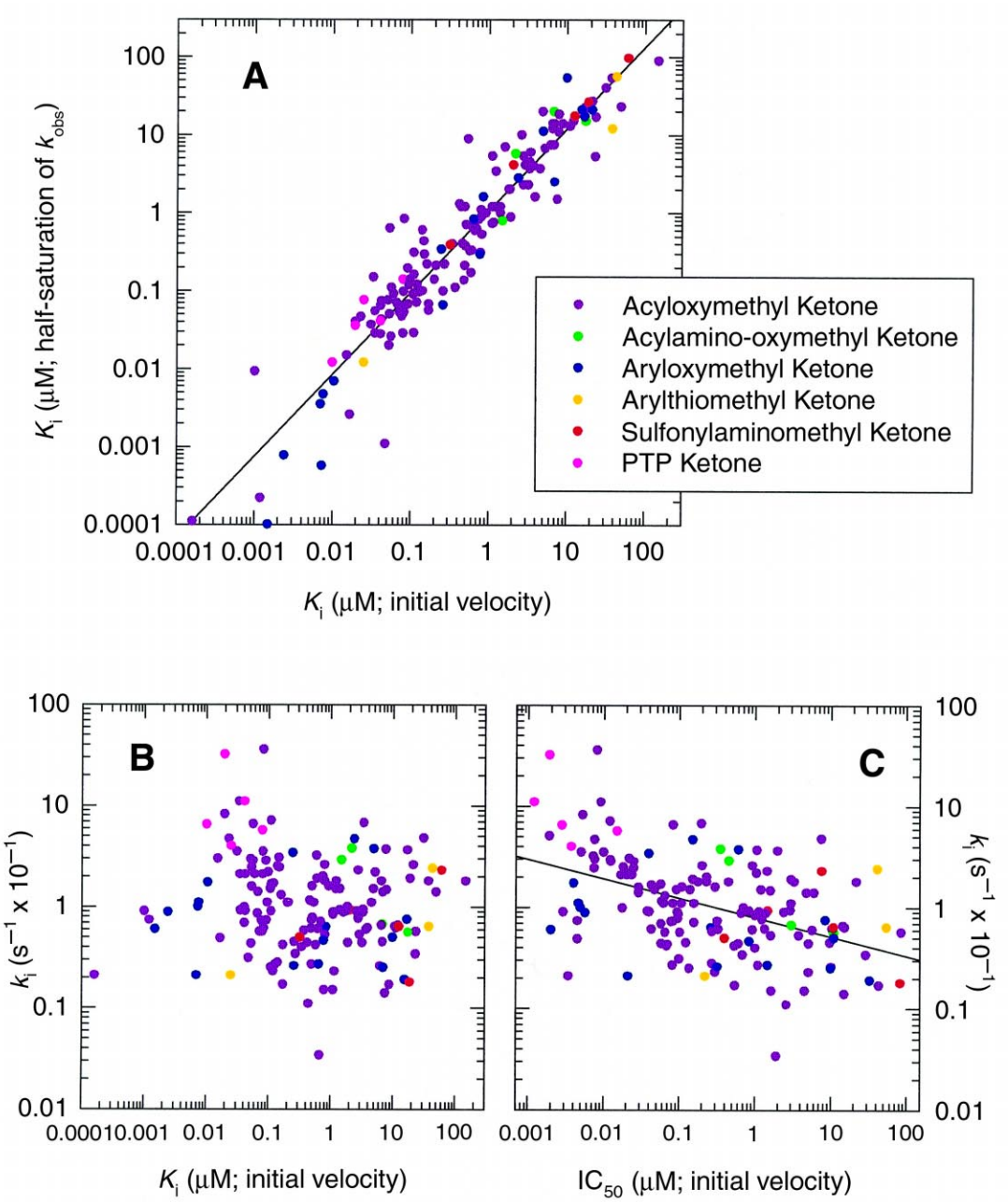


Figure 2. (A) K_i evaluated from inhibition of initial reaction velocities and K_i evaluated from the concentration at which k_{obs} was half-maximal are essentially identical (slope = 1.04; $r^2 = 0.892$). (B) k_i is uncorrelated with K_i ($r^2 = 0.012$, $p < 0.8$) but (C) is strongly correlated with IC_{50} ($r^2 = 0.215$, $p < 0.005$).

conformation, while 3/4 of the sulfonylaminomethyl ketones adopted the 60° conformation. Thus, we believe that certain classes of ketones may be predisposed toward one conformation or the other, but specific interactions in the P′ binding site may override this tendency.

The S-$C_{\alpha'}$ distance is also critical for direct thiohemiketal/thioether conversion, since this defines the probability of vibrational overlap of the atomic orbitals required to initiate SN_2 displacement. While this distance is highly constrained by the tetrahedral geometry of the thiohemiketal carbon, it is possible that it may be distorted by certain groups bound to the P′ binding pocket. Given the resolution of our structures, we cannot be certain that the observed variation in the S-$C_{\alpha'}$ distance is significant.

When the thiohemiketal adopts the 180° conformation necessary for SN_2 displacement, it coincidentally positions the leaving group atom in the proximity of His237Nδ (Fig. 4(C)). The distance (3.04 ± 0.24 Å) and Nδ-H-LG angle (121 ± 23°) are strongly suggestive of hydrogen bonding.

We have additionally examined 19 crystallographic structures of caspase-1 complexed with either ketone inhibitors from which a leaving group has been irreversibly displaced (17 structures) or with aldehyde inhibitors

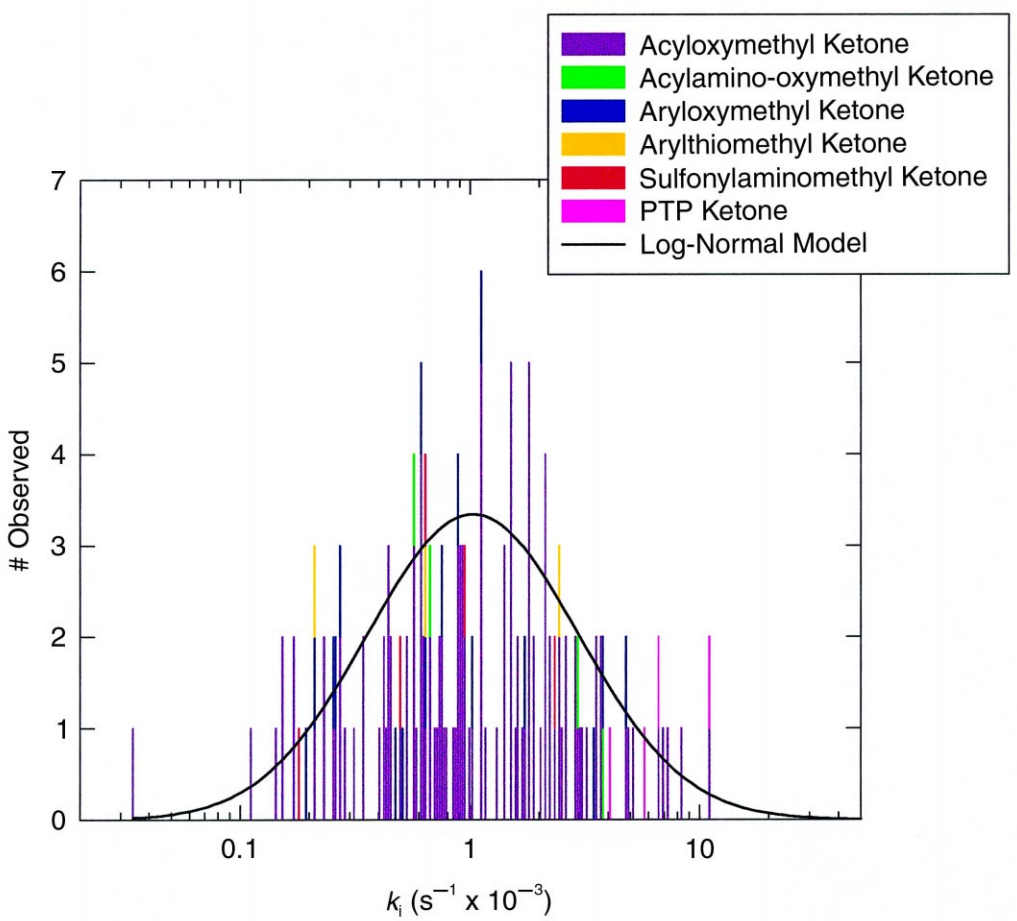


Figure 3. A frequency histogram of observed k_i values shows a mean of $1.03\times10^{-3}\,s^{-1}$, a standard deviation of 2.9-fold and a median value of $9.3\times10^{-4}\,s^{-1}$. Except for PTP ketones, all leaving-group classes were distributed across the observed range.

(2 structures). Almost all structures indicate a strong interaction between His237Nδ and the ketone/thiohemiketal oxygen (Fig. 5). The Nδ-H-O angle ($151\pm9°$) is probably indicative of hydrogen bonding, and would certainly be suitable for stabilization of negative charge which might develop during catalysis. Given the consistency of this observation, we propose Scheme 2 as a catalytic mechanism for caspase-1. Although it has not yet been demonstrated for any caspase, we propose by analogy to papain[15] that caspase-1 exists as a thiolate/imidazolium ion-pair. Upon binding of substrate, the imidazolium functions to polarize the carbonyl of the scissile amide bond, enabling subsequent attack by the thiolate anion. In the proposed tetrahedral intermediate, negative charge on the oxygen is balanced by the positive imidazolium ion. We considered that the imidazolium might also transfer a proton to the amine nitrogen of the leaving peptide, but the position of $C_{\alpha'}$ atom in our structures suggests otherwise. However, in many structures which have adopted the 180° S-C-$C_{\alpha'}$-LG dihedral angle, we observe a water molecule held in place via a hydrogen bond to Gly238 which might serve this role.

Curiously, the imidazolium ring is in position to donate a proton to the leaving group of those activated ketones in the 180° conformation, and we propose Scheme 3 as the mechanism for conversion of the thiohemiketal to the thioether. For those complexes which adopt an S-C-$C_{\alpha'}$-LG dihedral angle near to 180°, this mechanism requires only the chance alignment of the imidazolium ring and all atoms connected to the thiohemiketal carbon. As an intramolecular reaction, we believe it could generate the rather narrow range of rates observed for k_i (Fig. 3). Finally, the strong general acid catalysis could explain the remarkable indifference of the reaction to the leaving group pK_a.

Discussion

While caspase-1 is only a modestly active protease on its best known peptide substrate (Ac-YVAD-Amc; $k_{cat}/K_M = 5\times10^4\ M^{-1}\,s^{-1}$), its ability to displace a wide variety of activating groups from aspartic ketone inhibitors is remarkable. We have observed bimodal inhibitory kinetics for leaving-group classes with pK_a as high as 10 (e.g. sulfonylaminomethyl ketones, Table 1). Both the second-order inactivation rate[7] and the first-order rate of thiohemiketal/thioether conversion (Fig. 2) are independent of the leaving-group pK_a. Furthermore, for most classes of leaving-groups, we have found examples of both reversible and bimodal behaviors (Table 1). If the inactivating step of Scheme 1B is independent of

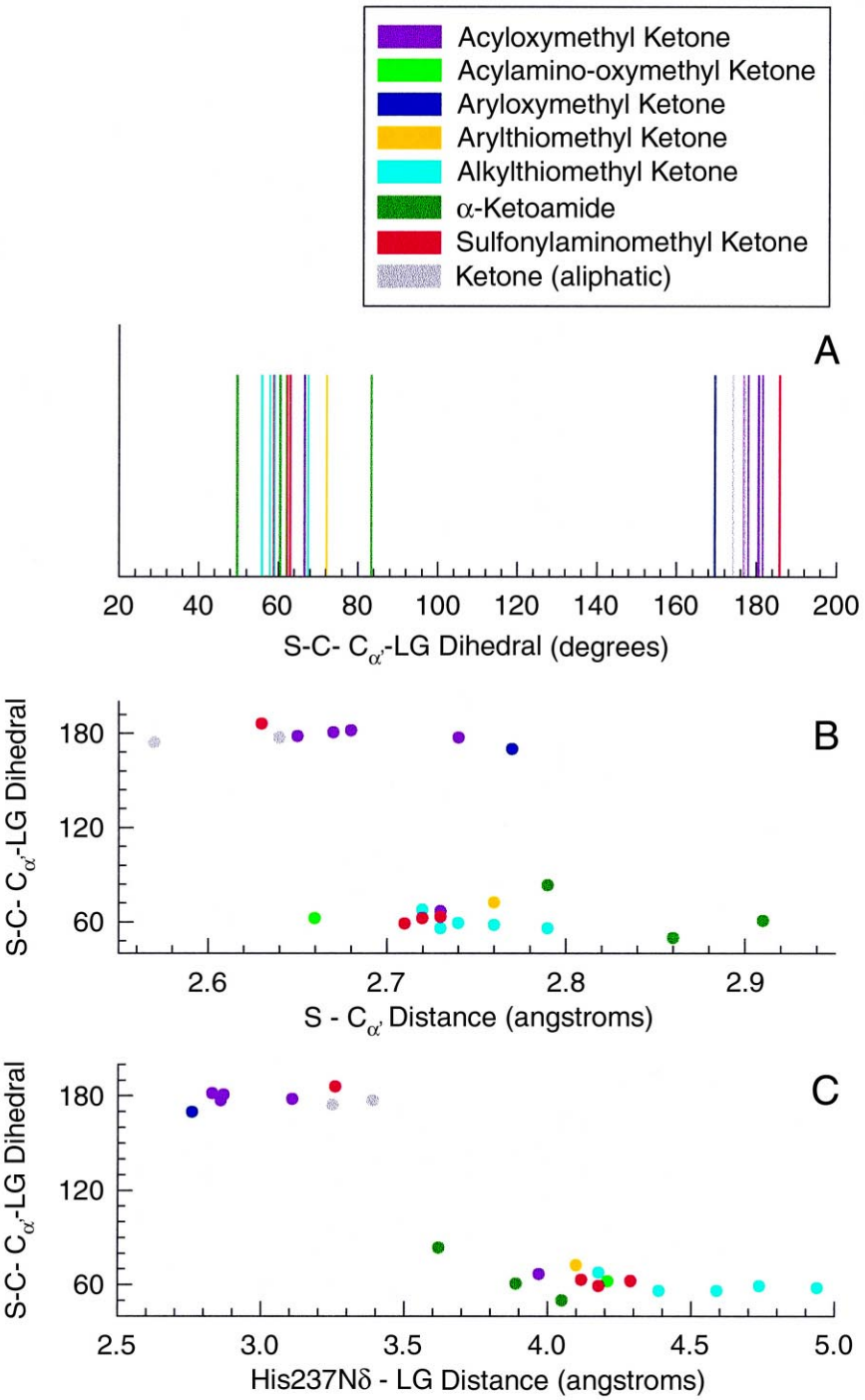


Figure 4. Crystallographically-observed active-site bonding angles and distances. A: distribution of the C_β-S-C-$C_{\alpha'}$ dihedral angle (Scheme 2). This angle takes on values near to 60° or 180°. An angle near to 180° is required for attack by 285S on the $C_{\alpha'}$ carbon. Among compounds for which 3 or more structures are available, some are found exclusively in the 60° orientation (alkylthiomethyl ketones, α-ketoamides) and some in both (acyloxymethyl ketones and sulfonylaminomethyl ketones). B: distribution of the Cys285S-$C_{\alpha'}$ distance as a function of the C_β-S-C-$C_{\alpha'}$ dihedral angle. C: distribution of the His237Nδ-LG distance as a function of the C_β-S-C-$C_{\alpha'}$ dihedral angle.

leaving-group pK_a, what factors do determine the fate of the thiohemiketal complex?

Among 22 crystallographic structures of caspase-1 complexed as a thiohemiketal with aspartic ketones, we have observed two distinct modes of binding which differ in the S-C-$C_{\alpha'}$-LG dihedral angle (Fig. 6). Fourteen of the complexes adopted a dihedral angle near to 60°, from which conformation SN_2 displacement is highly unlikely. Alkylthiomethyl ketones (5 structures, Fig. 4) and α-ketoamides (3 structures) all adopted this conformation, consistent with their uniformly reversible behavior (Table 1). Eight of the complexes adopted a dihedral angle near to 180°, a conformation permissive for SN_2 displacement. Furthermore, this conformation placed His237Nδ in a position which may stabilize

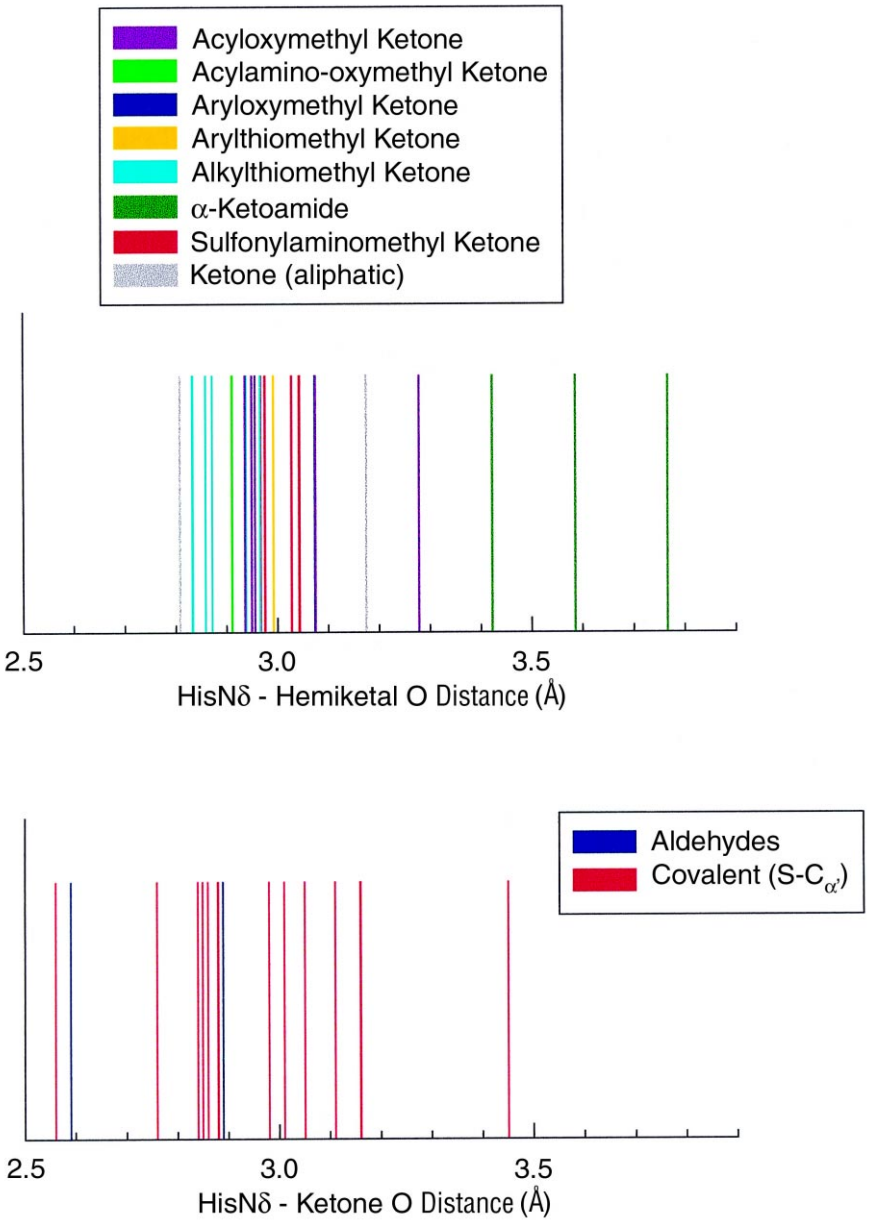


Figure 5. Crystallographically-observed interactions between His237Nδ and the ketone/thiohemiketal oxygen. Among the thiohemiketal complexes, this distance was 3.07 ± 0.25 Å. This distance was longest for the α-ketoamides, which may be distorted by the constrained, planar geometry of the ketoamide functionality. For the complexes lacking a prime-side group, this distance was 2.78 ± 0.14 (aldehydes) or 2.95 ± 0.22 (thioethers attached via $C_{\alpha'}$).

charge which develops on the leaving group during the displacement reaction (Fig. 4). Examples of acyloxymethyl and sulfonylaminomethyl ketones were found in both the 60° or 180° conformations, consistent with varied kinetic behavior of these two classes (Table 1). Curiously, the aliphatic ketones seem to prefer the 180° conformation (Fig. 4), and the uniformly reversible behavior of this ketone class (Table 1) must reflect the extreme pK_a of a carbanion 'leaving-group.'

In addition to these structures with intact prime-side groups, we have examined 2 aldehyde structures and 17 structures from which a leaving group has been displaced and Cys285 is covalently attached to $C_{\alpha'}$. In all complexes except for the α-ketoamides, a strong interaction is observed between His237Nδ and the ketone or thiohemiketal oxygen (Fig. 5). For most of the complexes, the interatomic distance is close (< 3.0 Å), and we believe that the His237 imidazole nitrogen must be protonated in order to allow this interaction. This interaction is so consistent that we further propose that it serves during peptide hydrolysis to polarize the scissile carbonyl (Scheme 2). The final kinetic hurdle to overcome during peptide hydrolysis, stabilization of negative charge on the amine leaving group, is not explained by our data, as we can only infer from our thiohemiketal complexes what might occur for a bound peptide. The position of $C_{\alpha'}$ in our structures suggests that His237 is very poorly positioned to donate a proton to a leaving amino group. A water molecule held in place

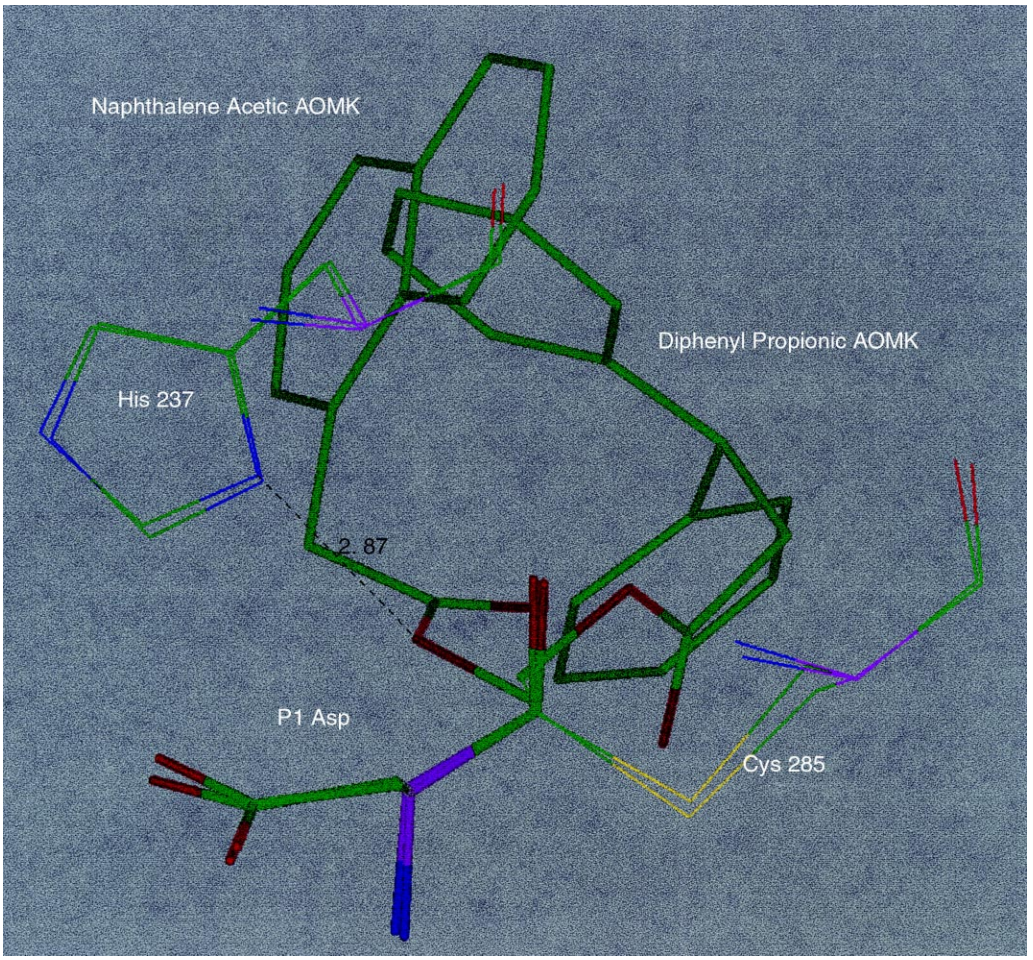
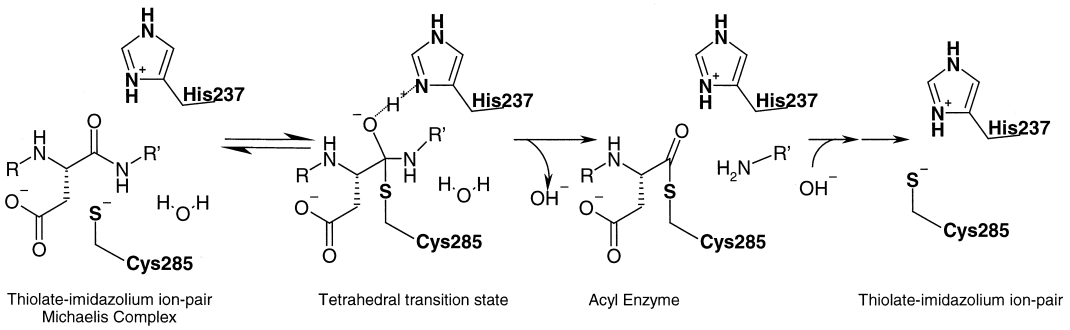


Figure 6. Superimposed crystallographic structure of two acyloxymethyl ketones complexed as thiohemiketals with caspase-1. One inhibitor, Z-Asp-(CH_2)-O-CO-CH_2-1-$C_{10}H_7$ ('Naphthalene Acetic AOMK') adopts a −177.5° dihedral angle. The other inhibitor, Z-Asp-(CH_2)-O-CO-CH_2-$CH=(C_6H_5)_2$ ('Diphenyl Propionic AOMK') adopts a 66.6° dihedral angle. For clarity, inhibitor bonds are drawn wide, the Z groups are omitted, and only residues His237 and Cys285 of caspase-1 are shown. The view is down the C–$C_{\alpha'}$ bond.

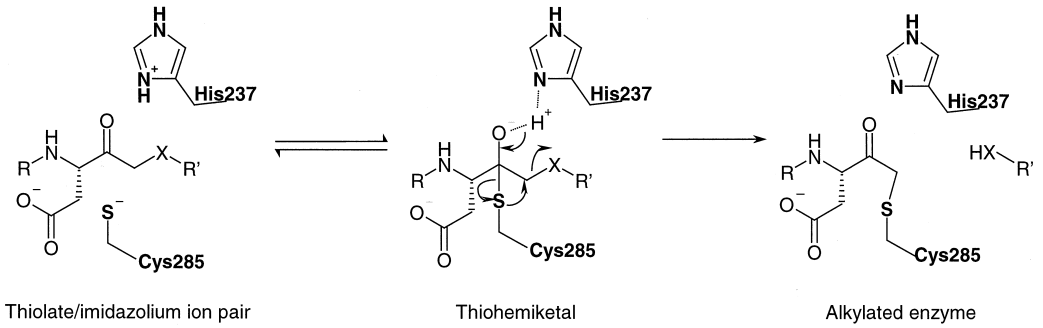


Scheme 2. Proposed catalytic mechanism of caspase-1.

via a hydrogen bond to Gly238 could possibly serve as a proton donor.

Conclusions

Activated ketones react with caspase-1 with one of three kinetic profiles (reversible, bimodal, or inactivating), and the action of any given ketone is only poorly predictable on the basis of leaving group structure and chemistry. We have identified two distinct binding modes for potential leaving groups in the P′ subsites of caspase-1. Only one of these modes is permissive for SN_2 displacement and is able to provide catalytic stabilization of negative charge on the leaving group. The different leaving group classes may be predisposed toward one binding mode or the other, but specific interactions in the P′ binding sites can override this tendency.



Scheme 3. Proposed catalytic mechanism of activated ketone inhibitors proceeding via a thiohemiketal complex.

Experimental

N-His (D381E) ICE was prepared as described previously.[16] K_i and k_{on} assays were performed and analyzed as described previously.[12] Compounds were diluted over a relevant concentration range onto a microtitre plate in triplicate. Enzyme was activated in HGDE buffer (100 mM HEPES pH 7.5, 0.5 mM EDTA, 5 mM DTT, 20% glycerol, 0.1% BSA) containing 15 μM Ac-YVAD-Amc for 30 s, then added to the plate. Monitoring of the enzymatic reaction (384 nm excitation, 460 nm emission) was begun immediately and continued for 5400 s. For each compound, curves were obtained for 7 compound concentrations and vehicle controls. Curves were analyzed as described previously.

K_i was evaluated by two methods. For most bimodal and reversible compounds, onset of the reversible phase of inhibition by most compounds was rapid while irreversible inactivation was much slower, so that inhibition of the enzymatic reaction during the first 300 s provided a good approximation of K_i. Alternatively, according to Scheme 1B, the rate of formation of the irreversible adduct is proportional to the amount of (reversible) thiohemiketal present, plots of k_{obs} versus [I] should show saturation kinetics, and the inhibitor concentration at which the inactivation rate is half-maximal (corrected for competitive effects of substrate) should be equal to K_i. An example of the application of these two techniques is shown in Figure 1.

Our IC_{50} assay differed importantly from the K_i assay by including a 60-min preincubation of the enzyme with inhibitors, thereby enhancing the relative potency of time-dependent inhibitors. Diluted inhibitor stocks were prepared by twofold serial dilution from a primary stock whose concentration was selected to achieve approximately 95% inhibition in the most concentrated well. Aliquots of each dilution were transferred to a microtitre plate in triplicate. Caspase-1 was diluted to approximately 24 nM in HGE buffer (100 mM HEPES pH 7.5, 0.5 mM EDTA, 20% glycerol, 0.1% BSA), and activated by adding dithiothreitol (DTT) to a final concentration of 5 mM. The activated enzyme was then aliquoted into wells containing inhibitor or vehicle, and the plate was pre-incubated for 60 min at ambient temperature. Substrate (Ac-YVAD-p-NA) was added to each well to a final concentration of 50 μM, and plates were incubated at 25°C. Beginning 5 min after addition of substrate, absorbance (405 nm) of wells was monitored for 60 min, and activity was calculated as the mean rate of change in absorbance during this interval. IC_{50} was calculated as the inhibitor concentration which caused a 50% reduction in reaction rate.

X-ray crystallography

Caspase-1 was crystallized and analyzed as described previously.[17] All crystalline complexes belonged to the $P4_32_12$ space group, unit cell dimensions were essentially constant, and crystal contacts which might distort the bound ligand were never observed. The resolution of these structures varied between 2.2–2.6 Å. Further details of methods and crystallographic structures will be published elsewhere.

Statistical methods

Confidence limits for linear regressions were evaluated using standard *t*-statistics.[18]

Synthesis

Synthetic methods for preparing the compounds used in this study were generally similar to those referenced in Table 1. Detailed descriptions of the synthesis, structures, and properties of individual compounds will be published elsewhere.

Kinetic derivations for Scheme 1

Although Scheme 1A and B are mechanistically distinct (i.e. 1A assumes direct attack of the thiolate on $C_{\alpha'}$; 1B assumes that this attack occurs from the thiohemiketal; see Scheme 3), they are kinetically differentiable only by affinity of the non-covalent E.I complex relative to the covalent E–I complex. Over a short time interval during steady state reaction, the net rate of formation of EI is nearly zero:

$$\frac{d[\mathrm{EI}]}{dt} = k_1[\mathrm{E}][\mathrm{I}] - (k_i + k_{-1})[\mathrm{EI}] \approx 0$$

Since the total amount of enzyme present is conserved ($E_0 = E + EI + E - I$), prior to significant irreversible formation of E–I, the amount of enzyme present as EI is:

$$[EI] = [E_t]\frac{[I]}{[I] + K_i} \quad (1)$$

where

$$K_i = \frac{k_{-1}}{k_1} + \frac{k_i}{k_1} = K_{i,eq} + \Delta K_{i,kin}. \quad (2)$$

K_i is thus determined by two factors, one describing the thermodynamic stability of the EI complex ($K_{i,eq}$), and a kinetic correction factor ($\Delta K_{i,kin}$).

Scheme 1C presents a third possibility, in which a thiohemiketal may form, but for which attack at $C_{\alpha'}$ occurs only from an intermediate non-covalent complex. Similar analysis of this mechanism leads to the rather complicated expression:

$$[E.I] = \frac{E_t[I]}{K_{i,1} + \frac{k_2}{k_1} + \frac{k_i}{k_1} + [I]\left(1 + \frac{k_2}{k_{-2}}\right)} \text{ where } K_{i,1} = k_{-1}/k_1.$$

If the thiohemiketal is of much higher affinity than the non-covalent complex, then the denominator is dominated by the k_2/k_{-2} term, and the expression simplifies to:

$$[E.I] \approx \frac{[E_t]}{F} \quad (3)$$

where F is a very large number representing the fold increase in affinity of the thiohemiketal relative to the non-covalent E.I complex. Thus, the steady-state rate of inactivation for Scheme 1C (i.e. $k_i[E.I]$) is very slow, and is largely independent of inhibitor concentration, and this mechanism has the peculiar property of being self-competing. If k_i is significant relative to k_{-1} and k_2, one might observe a burst of inactivation during the approach to steady-state.

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