THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 49, Issue of December 4, pp. 32608–32613, 1998
Printed in U.S.A.

Inhibition of Human Caspases by Peptide-based and Macromolecular Inhibitors*

(Received for publication, April 22, 1998, and in revised form, September 17, 1998)

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Studies with peptide-based and macromolecular inhibitors of the caspase family of cysteine proteases have helped to define a central role for these enzymes in inflammation and mammalian apoptosis. A clear interpretation of these studies has been compromised by an incomplete understanding of the selectivity of these molecules. Here we describe the selectivity of several peptide-based inhibitors and the coxpox serpin CrmA against 10 human caspases. The peptide aldehydes that were examined (Ac-WEHD-CHO, Ac-DEVD-CHO, Ac-YVAD-CHO, *t*-butoxycarbonyl-IETD-CHO, and *t*-butoxycarbonyl-AEVD-CHO) included several that contain the optimal tetrapeptide recognition motif for various caspases. These aldehydes display a wide range of selectivities and potencies against these enzymes, with dissociation constants ranging from 75 pM to >10 μM. The halomethyl ketone benzyloxycarbonyl-VAD fluoromethyl ketone is a broad specificity irreversible caspase inhibitor, with second-order inactivation rates that range from 2.9×10^2 M^{-1} s^{-1} for caspase-2 to 2.8×10^5 M^{-1} s^{-1} for caspase-1. The results obtained with peptide-based inhibitors are in accord with those predicted from the substrate specificity studies described earlier. The cowpox serpin CrmA is a potent (K_i < 20 nM) and selective inhibitor of Group I caspases (caspase-1, -4, and -5) and most Group III caspases (caspase-8, -9, and -10), suggesting that this virus facilitates infection through inhibition of both apoptosis and the host inflammatory response.

Members of the caspase family of cysteine proteases, which at present includes 11 homologues of human origin, are important mediators of both inflammation, where they are involved in the production of several inflammatory cytokines, and apoptosis, where they participate in signaling and effector pathways (for review, see Ref. 1). The evidence for the central role of these enzymes in both of these biological processes was initially obtained using potent peptide-based and macromolecular inhibitors. For example, the finding that Ac-YVAD-CHO, a potent inhibitor of caspase-1, prevented the release of interleukin-1β (IL-1β)[1] from monocytes, suggested that this enzyme was, in fact, the pro-IL-1β-processing enzyme (2). This was later confirmed with the description of caspase-1-deficient mice, which are defective in the production of this cytokine (3, 4). Similarly, the observation that apoptosis could be attenuated by the cowpox serpin CrmA, also known to be a potent caspase-1 inhibitor, provided the first compelling evidence that caspases play an important role in mammalian cell death (5). This has recently been confirmed by several studies, including the description of caspase-3-deficient mice, which have a striking defect in the programmed cell deaths that occur during neuronal development (6).

Studies using these and other caspase inhibitors continue to be an important component of the repertoire of scientists investigating these complex biological processes in whole cells and *in vivo*. Regarding the latter, there are numerous recent reports that some caspase inhibitors are efficacious in several different animal disease models. For example, both reversible and irreversible peptide-based inhibitors have been reported to be effective in animal models of stroke, myocardial ischemia/reperfusion injury, liver disease, and traumatic brain injury (7–12). Unfortunately, little information has been available on the selectivity of these inhibitors for caspase family members.

The most distinguishing catalytic features of caspases are their stringent specificity for Asp in the S_1 subsite and a requirement for at least four amino acids to the left of the cleavage site (2). The three-dimensional crystal structures of caspase-1 and caspase-3 suggest that these enzymes employ a typical cysteine protease mechanism, involving a catalytic diad of Cys and His (13–15). This information has led to the identification of several classes of peptide-based inhibitors using strategies that have proven successful for inhibition of other cysteine proteases (for review, see Ref. 16). Examples of reversible inhibitors include aldehydes, nitriles, and ketones. Among the irreversible caspase inhibitors that have been described are diazomethyl ketones, acyloxymethyl ketones, and halomethyl ketones. The most potent of these compounds contains a tetrapeptide recognition motif that is optimal for the enzyme of interest.

In this regard, we have recently described a combinatorial approach that has been used to determine the tetrapeptide substrate specificity of 10 human caspases (17). The results indicate that, although all of these enzymes have a common stringent requirement for Asp in the P_1 position, their extended specificities are distinct. Specifically, the results divide these enzymes into three major groups and identify P_4 as the single most important determinant of specificity among caspases. Group I enzymes (caspase-1, -4, and -5) prefer the sequence WEHD, but are rather promiscuous compared with other

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[1] The abbreviations used are: IL-1β, interleukin-1β; Z, benzyloxycarbonyl; FMK, fluoromethyl ketone; AMC, aminomethylcoumarin; Boc, *t*-butoxycarbonyl; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

caspases. The optimal tetrapeptide recognition motif for Group II enzymes (caspase-2, -3, and -7) is DE*X*D, and these enzymes are highly selective, with a near absolute specificity for Asp in P_4. Group III caspases (caspase-6, -8, -9, and -10) prefer the sequence (LV)E*X*D. These results, together with those from several independent studies, suggest that Group I caspases function primarily as inflammatory mediators, whereas Group II and Group III caspases play important roles in the effector (Group II) and signaling (Group III) events during apoptosis.

An intimate understanding of the individual substrate specificities of the caspases permits the design of potent peptide-based inhibitors. In this study, we have investigated the potency and selectivity of several peptide aldehyde inhibitors, the fluoromethyl ketone Z-VAD-FMK and the cowpox serpin CrmA. The results are in accord with predictions from the earlier substrate specificity results and facilitate the interpretations of previous studies that have employed some of these compounds in whole cells and *in vivo* models of disease.

EXPERIMENTAL PROCEDURES

Materials—Ac-YVAD-CHO and Ac-DEVD-CHO were purchased from the Peptide Institute (Osaka, Japan). Ac-WEHD-CHO was prepared as described (18). The tetrapeptide aldehydes were reconstituted in Me_2SO as 10 mM stock solutions; subsequent dilutions were made in distilled water and kept at −20 °C. The P_1 Asp methyl ester of Z-VAD-FMK (methyl ester) was a generous gift from Enzyme Systems (Dublin, CA). To generate the free acid, this compound was incubated in a 50% methanol and 10% triethylamine aqueous solution at room temperature for 5 min. Under these conditions, the half-life of the free acid is ~50 min. Ac-YVAD-AMC, Ac-DEVD-AMC, and Ac-YVAD-CHO were obtained from the Peptide Institute. Ac-WEHD-AMC and Ac-VEHD-AMC were prepared as described previously (18).

Synthesis of N-Boc-IETD-CHO and N-Boc-AEVD-CHO—Commercially available *N*-Boc-*O*-benzylthreonine was first treated with mesyl chloride and allyl alcohol in the presence of pyridine to afford the corresponding allyl ester in 50% yield. Treatment of this intermediate with trifluoroacetic acid followed by coupling with *N*-Boc-glutamic acid benzyl ester using hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride provided the protected dipeptide in 90% yield. The *N*-Boc deprotection-coupling sequence was repeated using *N*-Boc-isoleucine, and *N*-Boc-IET allyl ester was obtained in 76% yield. The allyl group was then removed by treatment with tetrakis(triphenylphosphine)-palladium and pyrrolidine to provide *N*-Boc-IET-COOH in 88% yield. *N*-Allyloxycarbonylaspartic acid benzyl acetal was prepared from allyloxycarbonylaspartic acid *tert*-butyl ester in 34% overall yield using the following sequence. First, the carboxylic acid was activated with isobutyl chloroformate and *N*-methylmorpholine and selectively reduced by sodium borohydride. Then, oxidation was carried out using oxalyl chloride and Me_2SO, followed by treatment of the resulting aldehyde with benzyl alcohol and tosic acid. The tripeptide *N*-Boc-IET-COOH was then coupled with *N*-allyloxycarbonylaspartic acid benzyl acetal in the presence of bis(triphenylphosphine)-palladium chloride, tributyltin hydride, hydroxybenzotriazole, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and the title product *N*-Boc-IETD-CHO was obtained in 64% overall yield by treatment with hydrogen and 10% palladium hydroxide on carbon. The same synthetic sequence applies to the synthesis of Boc-AEVD-CHO. In this case, alanine was used instead of isoleucine, and valine instead of threonine.

Preparation of Recombinant CrmA—An *Escherichia coli* expression vector containing CrmA, which contains an N-terminal His tag, was obtained from Long Quan and Guy Salvesen (Burnham Institute). Cells from an overnight culture were diluted 15-fold and grown in LB medium at 37 °C to A_{600} = 0.7. The cells were harvested by centrifugation at 3500 × *g*, resuspended in fresh LB medium (19), and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. The cells were harvested by centrifugation and washed with 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM EDTA. Pellets were resuspended in 20 mM Tris, pH 8.0, and 150 mM NaCl using a Dounce homogenizer and lysed using a French press. The soluble proteins were obtained by centrifugation at 120,000 × *g* for 45 min at 4 °C. The supernatant was then loaded on a freshly prepared Talon column (2.5 × 8 cm, CLONTECH), and the column was washed using the resuspension buffer. The protein was eluted using 100 mM imidazole. Fractions containing protein were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4, 5 mM DTT, and 300 mM NaCl. This protein was maintained at 4 °C without loss of activity for at least 1 year.

Preparation of Recombinant Caspases—The method used for production of caspase-1, -2, -3, -4, -5, -7, -8, and -9 involves folding of active enzymes from their constituent large and small subunits, which are expressed separately in *E. coli*. Subunits were engineered for expression as described previously (17).[2] The selections of subunit boundaries were based on a comparison of the proenzyme sequences with the sequences of those caspase subunits that have been purified from natural sources (caspase-1 and caspase-3). Consequently, it is possible that, in some cases, the enzyme produced using this method is not identical to that which exists in nature.

Exponentially growing *E. coli* BL21(DE3) pLysS cells carrying the respective expression plasmid were cultured in M9 medium at 37 °C with overnight induction using 1 mM isopropyl-β-D-thiogalactopyranoside for expression of the recombinant proteins. Under these conditions, the subunit protein was localized in the inclusion body fraction, where it generally constituted >90% of the total protein. To obtain active enzyme, the individual subunits from purified inclusion bodies were solubilized in 6 M guanidine HCl and then rapidly diluted to a final concentration of 100 μg/ml at room temperature under conditions determined to be optimal for each enzyme. Caspase-1, -4, and -5 were folded in 100 mM HEPES, 10% sucrose, 0.25–1% Triton X-100, and 10 mM DTT, pH 7.5, in the presence of the peptide aldehyde inhibitor Ac-YVAD-CHO (10 μM). Caspase-2 was folded in 100 mM HEPES, 20% sucrose, and 10 mM DTT, pH 7.5. Caspase-3 was folded in 100 mM Tris, 10% sucrose, 0.1% CHAPS, 150 mM NaCl, and 10 mM DTT, pH 8.0. Caspase-7, -8, and -9 were folded in 100 mM HEPES, 10% sucrose, 0.1% CHAPS, and 10 mM DTT, pH 7.5. Folding was achieved by overnight incubation at room temperature. Following folding, active enzyme was separated from inactive subunit protein by ion-exchange chromatography. With the exception of caspase-5, which was purified by cation-exchange chromatography using a 1-ml Hi-Trap SP column (Amersham Pharmacia Biotech), the enzymes were purified by anion-exchange chromatography using a 1-ml Hi-Trap Q column (Amersham Pharmacia Biotech). Caspase-1 was purified to homogeneity by affinity chromatography as reported previously (18).

Active recombinant caspase-6 and caspase-10 were instead prepared by expressing a construct encoding the entire proenzyme, minus the N-terminal peptide, in *E. coli* under conditions where a portion of the protein produced is cytosolic and undergoes self-maturation. Caspase-6 was engineered as a Met-Ser-Phe^{25}–Asn^{293} N-terminal domainless construct, and caspase-10 as a Met-Leu-Lys^{221}–Ile^{479} N-terminal domainless construct. Exponentially growing *E. coli* BL21(DE3) pLysS cells carrying the respective expression plasmid were cultured in LB medium at 25 °C with a 3-h incubation using 1 mM isopropyl-β-D-thiogalactopyranoside for expression of the recombinant proteins. The *E. coli* cells were harvested; washed in phosphate-buffered saline; and broken by a French press in 25 mM HEPES, 10% sucrose, 0.1% CHAPS, and 4 mM DTT, pH 7.5, in the presence of protease inhibitors (Sigma P2714), taking care to keep the solutions on ice. The suspension was centrifuged at 100,000 × *g* for 1 h. The enzyme in the resulting supernatant was subsequently partially purified by anion-exchange chromatography (Hi-Trap Q columns).

Enzymatic Assays—The activity of each enzyme was measured using continuous fluorometric assays analogous to those previously described for caspase-1 and caspase-3 (2, 20). In each case, a substrate with the general structure Ac-*X*E*X*D-AMC was employed, incorporating a peptide that is identical or similar to the optimal tetrapeptide recognition motif for each enzyme (17): caspase-1, -4, and -5 (Ac-WEHD-AMC); caspase-2, -3, -7, and -8 (Ac-DEVD-AMC); and caspase-6, -9, and -10 (Ac-VEHD-AMC). Briefly, appropriate dilutions of enzyme were added to reaction mixtures containing substrate (at a concentration ≤ K_m) and various concentrations of the inhibitor of interest in a final reaction volume of 100 μl. Liberation of AMC was monitored continuously at room temperature using a Tecan Fluostar 96-well plate reader (black plates from Dynatech Laboratories Inc.) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Unless otherwise indicated, all experiments were carried out at room temperature under standard reaction conditions defined as 0.1 M HEPES, 10% sucrose, 0.1% CHAPS, and 10 mM DTT, pH 7.5, at 25 °C.

Data Analysis—All kinetic constants were computed by direct fits of

[2] The small subunit of caspase-8 was engineered for expression as the Met-Ser^{375}–Asp^{479} small subunit. During folding and purification, this subunit was autoproteolytically cleaved at Asp^{384}-Leu^{385} to generate a subunit with a molecular mass of 10,880.4 Da.

TABLE I
Inhibition of caspases by peptide aldehydes

Enzyme	Aldehyde K_i				
	WEHD	YVAD	DEVD	IETD	AEVD
			nM		
Group I					
Caspase-1	0.056	0.76	18	<6	<12
Caspase-4	97	362	132	400	375
Caspase-5	43	163	205	223	438
Group II					
Caspase-3	1960	>10,000	0.23	195	42
Caspase-7	>10,000	>10,000	1.6	3280	425
Caspase-2	>10,000	>10,000	1710	9400	>10,000
Group III					
Caspase-6	3090	>10,000	31	5.6	52
Caspase-8	21.1	352	0.92	1.05	1.6
Caspase-9	508	970	60	108	48
Caspase-10	330	408	12	27	320

the data to the appropriate equation using a nonlinear least-squares analysis computer program (NLIN) developed in this laboratory. For the reversible inhibitors (peptide aldehydes and CrmA), in cases where no time-dependent inhibition was observed, reaction rates were fit by nonlinear regression to the Michaelis-Menten equation for competitive inhibition to obtain a value for the dissociation constant K_i. In cases where inhibition was time-dependent, K_i was instead calculated from the steady-state velocities or from the rate constants for association (k_{on}) and dissociation (k_{off}) of enzyme-inhibitor complexes according to the equations developed by Morrison and Walsh (21) for analysis of slow and tight-binding inhibitors. The second-order inactivation rates (k) for the irreversible caspase inhibitor Z-VAD-FMK was measured as described previously (22). The errors in reproducing the rate and dissociation constants were never >10%.

RESULTS

Inhibition of Caspases by Tetrapeptide Aldehydes—The selection of tetrapeptide aldehydes for this study was based on an understanding of the known substrate specificity of these enzymes (17). The aldehydes Ac-WEHD-CHO and Ac-DEVD-CHO contain the optimal tetrapeptide recognition motif for Group I and II caspases, respectively. The peptide in Boc-IETD-CHO resembles the preferred sequences for Group III caspases. This is also the sequence found at the site of cleavage of the caspase-3 proenzyme, a likely endogenous substrate for these enzymes. Ac-YVAD-CHO was selected because it was formerly the most potent reversible caspase inhibitor known and, as a consequence, has been used extensively as a biological tool. Finally, Boc-AEVD-CHO was included as it was anticipated to be a broad specificity caspase inhibitor, based again on the known amino acid preferences of these enzymes.

All of these aldehydes were evaluated for inhibition against 10 human caspases, and the relevant kinetic parameters were determined. In every case, the observed inhibition was competitive and reversible. Potent inhibition was generally observed to be time-dependent, reflecting the low aldehyde concentrations employed and relatively small association rate constants for these inhibitors (0.08×10^5 M^{-1} s^{-1} –4×10^5 M^{-1} s^{-1}). The results, summarized in Table I and shown graphically in Fig. 1, are in accord with the known substrate specificity of these enzymes and their catalytic efficiency for cleavage of tetrapeptide substrates. Group I caspases are relatively promiscuous; although Trp is preferred in aldehyde inhibitors, other amino acids are also well tolerated, such that all of the aldehydes tested had K_i values <1 μM. Among these enzymes, the dissociation constants for caspase-4 and caspase-5 are significantly higher (average of 340-fold) than those for caspase-1, despite the fact that WEHD is the preferred tetrapeptide recognition sequence for all of these enzymes. This is consistent with the finding that caspase-4 and caspase-5 have k_{cat}/K_m values for cleavage of their preferred substrate, Ac-WEHD-AMC, that are ~100-fold lower than that for caspase-1 under these conditions[3] and the fact that aldehyde inhibitors of caspases are known to form a thiohemiacetal with the catalytic cysteine.

The only potent aldehyde inhibitor of Group II caspases is Ac-DEVD-CHO. This observation reflects the stringent specificity of these enzymes for Asp in their S_4 subsites. As is observed with Group I caspases, the observed potency correlates with the catalytic efficiency of these enzymes for cleavage of Ac-DEVD-AMC. For example, Ac-DEVD-CHO is a potent inhibitor of caspase-3 (K_i = 230 pM), a highly efficient catalyst with a k_{cat}/K_m value of 1.6×10^6 M^{-1} s^{-1}. In contrast, caspase-2 cleaves the tetrapeptide substrate poorly (k_{cat}/K_m = 24 M^{-1} s^{-1}) and is only weakly inhibited by this aldehyde (K_i = 1.7 μM).

Group III caspases are broadly inhibited by Boc-IETD-CHO, Ac-DEVD-CHO, and Boc-AEVD-CHO, with K_i values ranging from ~1 to 300 nM. As anticipated from the substrate specificity studies, Ac-WEHD-CHO and Ac-YVAD-CHO are relatively poor inhibitors of this group of enzymes, consistent with the finding that hydrophobic amino acids in P_4 are generally not well tolerated by members of this group and, in the case of Ac-YVAD-CHO, reflecting this group's stringent specificity for Glu in S_3.

Inhibition by Z-VAD-FMK—The results obtained with the fluoromethyl ketone inhibitor Z-VAD-FMK are summarized in Table II. It was found to be a competitive and irreversible inhibitor of all 10 caspases. It is a broad specificity inhibitor, with second-order inactivation rates (k) that vary from 2.9×10^2 M^{-1} s^{-1} for caspase-2 to 2.8×10^5 M^{-1} s^{-1} for caspase-1. These rate constants indicate that the half-life for irreversible inhibition at 1 μM inhibitor is ≤40 min for all of these enzymes.

Inhibition by CrmA—The selectivity of CrmA for the caspases is summarized in Table III and shown in Fig. 2. It is a highly selective inhibitor of Group I and III caspases (with the exception of caspase-6), with dissociation constants ranging from 10 pM for caspase-1 to 17 nM for caspase-10 [4] and association rate constants of ~0.1×10^6 M^{-1} s^{-1} –20×10^6 M^{-1} s^{-1}. In contrast, the K_i for Group II caspases and caspase-6 is ≥1000 nM. These results are in accord with those previously published for a subset of these caspases (24, 25). Inhibition of caspases by CrmA, a member of the serpin superfamily of protease inhibitors, presumably requires recognition of the sequence LAVD in its reactive site loop (26), explaining why Group II caspases, with their stringent S_4 Asp specificity, are so poorly inhibited by this protein.

DISCUSSION

We have previously used a combinatorial approach to obtain an intimate understanding of the tetrapeptide substrate specificity of 10 human caspases (17). We have now extended this work to determine the selectivities of CrmA and several small molecule inhibitors, including Z-VAD-FMK and peptide aldehydes that contain the optimal tetrapeptide recognition motif, for each group of caspases.

The cowpox serpin CrmA was initially identified as an inhibitor of the host inflammatory response to viral infection (26). It was later found to be a potent inhibitor of caspase-1, which is involved in the production of IL-1β in monocytes, explaining the observed inhibition of inflammation that is observed with this protein (27). Since the subsequent finding that caspases are involved not only in inflammation, but also in mammalian apoptosis, several investigators have shown that overexpression of CrmA attenuates apoptosis in at least some cell-based models, leading to the suggestion that this may be another

[3] M. Garcia-Calvo, unpublished data.

[4] The results obtained with caspase-10 differ from previously reported results that claim that CrmA is a relatively poor inhibitor (23). Although the reason for this discrepancy has not been rigorously established, it is possible that the concentration of enzyme employed in the previous study precluded detection of potent inhibition.

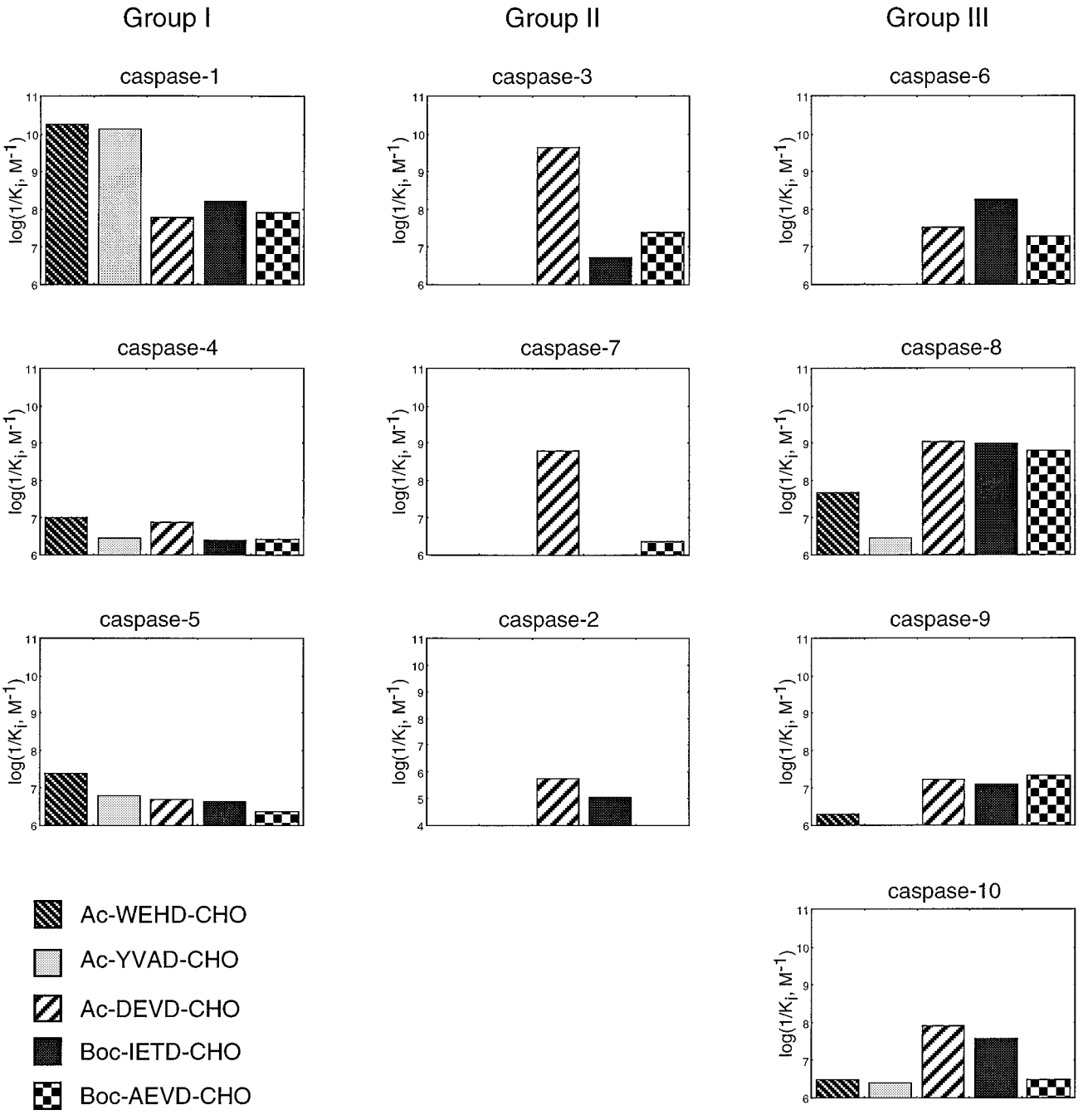

FIG. 1. **Inhibition of caspases by tetrapeptide aldehydes.** Dissociation constants (K_i) were determined for inhibition of caspases by five tetrapeptide aldehyde inhibitors. Here the results are plotted as the log of the inverse of the K_i values. Except in the case of caspase-2, the values on the *y axis* translate to dissociation constants that range from 1 μM to 10 pM. In the case of caspase-2, because all of the inhibitors tested had K_i values >1 μM, the scale is instead from 100 μM to 10 pM. The results obtained are consistent with the reported specificity of these enzymes. Group I caspases, which are relatively promiscuous, are broadly inhibited by all of the aldehydes tested. In contrast, the only potent inhibitor of Group II caspases, which are highly specific for Asp in P_4, is Ac-DEVD-CHO. Group III caspases prefer those aldehydes containing relatively small amino acids in P_4 and Glu in P_3, reflecting their stringent S_3 subsite specificity.

mechanism by which the virus circumvents the host responses to infection (5, 28–30).

Previous studies with a subset of caspases have shown that CrmA is a potent inhibitor of those caspases involved in the generation of pro-inflammatory cytokines (caspase-1, -4, and -5) (24, 25) and caspase-8 (24), a key mediator of Fas-induced apoptosis (31, 32). These findings, together with the results presented here, definitively establish that cowpox virus facilitates infection through both inhibition of inflammation and apoptosis. In mammalian cell death, caspases are involved in both effector processes (where they are responsible for cleavage of key homeostatic and structural proteins) and signaling events (where they are responsible for the activation of effector caspases) (for review, see Ref. 1). A comparison of the sequences of the sites that are cleaved in these endogenous substrates with the optimal tetrapeptide specificity of the caspases suggests that Group II caspases are involved primary in effector events, whereas Group III enzymes function as upstream activators (17). The inhibition profile obtained with CrmA suggests that, in apoptosis, this serpin functions exclusively to inhibit activator caspases. In this regard, the lack of inhibition of caspase-6 (a Group III caspase) by CrmA is notable. This

TABLE II
Inhibition of caspases by Z-VAD-FMK

Enzyme	k	$t_{1/2}$ at 1 μM
	$M^{-1}\ s^{-1}$	s
Group I		
Caspase-1	280,000	2.5
Caspase-4	5500	130
Caspase-5	130,000	5.3
Group II		
Caspase-3	16,000	43
Caspase-7	18,000	39
Caspase-2	290	2400
Group III		
Caspase-6	7,100	98
Caspase-8	280,000	2.5
Caspase-9	180,000	3.9
Caspase-10	ND[a]	ND

[a] ND, not determined.

TABLE III
Inhibition of caspases by CrmA

Enzyme	K_i	$10^6 \times k_{on}$
	nM	$M^{-1}\ s^{-1}$
Group I		
Caspase-1	0.01	17
Caspase-4	1.1	>10
Caspase-5	<0.1	1.5
Group II		
Caspase-3	1600	ND[a]
Caspase-7	>10,000	ND
Caspase-2	>10,000	ND
Group III		
Caspase-6	1300	ND
Caspase-8	<0.34	0.3
Caspase-9	<2.3	0.14
Caspase-10	17	0.17

[a] ND, not determined.

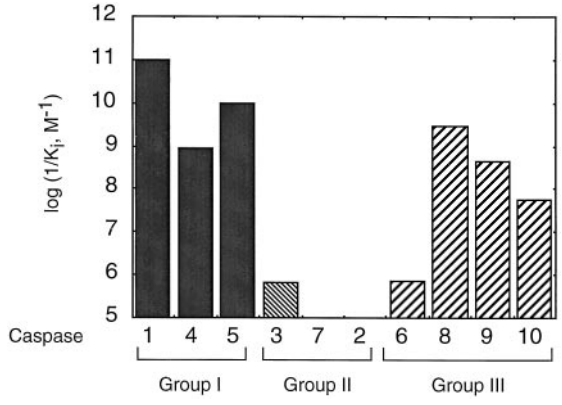

FIG. 2. **Inhibition of caspases by CrmA.** Dissociation constants (K_i) and association rate constants (k_{on}) were determined for the inhibition of caspases by CrmA. Here the results are shown as the log of the inverse of K_i values. All of the Group I caspases are potently inhibited by CrmA, with values for K_i <1 nM and association rate constants $>10^6$ $M^{-1}\ s^{-1}$. In contrast, CrmA is a poor inhibitor of all Group II caspases ($K_i > 1$ μM). Regarding Group III caspases, CrmA is a potent inhibitor of caspase-8, -9, and -10, but is a relatively poor inhibitor of caspase-6.

result, together with the fact that caspase-6 has a short N-terminal domain (in common with effector caspases) and the finding that caspase-6 cleaves lamin A *in vitro* (33, 34), suggests that this caspase is primarily involved in the effector phase of apoptosis.

Because of the relatively stringent selectivity of CrmA, it is a useful tool for identifying the involvement of particular caspases in models of apoptosis. For example, there are many examples of models of apoptosis that are CrmA-insensitive, suggesting either that caspase-8, -9, and -10 are not involved or that they are in a conformation or location in which they are inaccessible to the serpin. In particular, the finding that CrmA is a potent inhibitor of caspase-9, together with the observations that this serpin is ineffective in several models of apoptosis, suggests that the recently described caspase-3 activation mechanism employing a complex of this caspase, Apaf-1, dATP, and cytochrome *c* (35) may not be involved in these models. In this regard, it is important to point out that there is some evidence that the active form of caspase-9 in cells retains its N-terminal domain upon activation and that this is not the form of the enzyme used in this study (see "Experimental Procedures").

In contrast to the importance of CrmA as a probe of biological function for various caspases in whole cells, the peptide-based inhibitors employed in this study clearly have more limited utility. None of these compounds have been found to have good efficacy ($IC_{50} < 1$ μM) in whole cell models of apoptosis, presumably because of poor cell penetration (for examples, see Refs. 20, 29, 36, and 37). To date, virtually nothing has been reported regarding the membrane permeability of any of these inhibitors. In addition, the studies presented here show that many of these compounds are relatively broad specificity inhibitors. Accordingly, it is clear that although peptide-based caspase inhibitors may be used to incriminate caspases, in a general sense, in whole cell models of apoptosis and in animal models of disease, none of these compounds can be used to implicate a particular caspase. On the other hand, the unique and characteristic inhibition profile of each caspase is expected to be useful for identifying caspases in cell extracts and in *in vitro* models of apoptosis, where membrane permeability is not an issue.

The efficacy of inhibitors such as Z-VAD-FMK in clinical models of disease may be a consequence of their ability to inhibit multiple caspases. For example, this compound and a closely related inhibitor, Z-VAD-(2, 6-dichlorobenzoyloxopentanoic acid, have been shown to attenuate ischemic brain damage in rodents (9, 10). IL-1β-mediated inflammation and apoptosis have both been implicated in the damage that occurs during ischemia, and it is likely that the beneficial effects of these compounds are due to inhibition of both processes. Indeed, decreased levels of IL-1β were observed in animals treated with Z-VAD-FMK in a model of ischemic brain injury (10). Notwithstanding, it is important to note that the attenuation of damage by this compound might also be a consequence of inhibition of enzymes other than caspases, given the intrinsic reactivity of the halomethyl ketone moiety.

The results presented here suggest that the intimate understanding of caspase substrate specificities reported earlier (17) should be useful in attempts to design selective inhibitors of individual caspases or groups of caspases. Selective inhibition is expected to be of paramount importance for the treatment of chronic diseases in which caspases are potential targets, given that members of this family have distinct biological functions in inflammation and apoptosis. For example, caspases involved in cytokine production (*e.g.* caspase-1) are considered putative targets for the treatment of a number of chronic inflammatory diseases (*e.g.* rheumatoid arthritis). Clearly, for treatment of these disorders, selective inhibition of these enzymes will be required to ensure that there is no adverse effect on the apoptosis that is necessary for proper tissue function and maintenance.

Acknowledgments—We gratefully acknowledge Michel Gallant and Yves Gareau for help in preparing some of the peptide aldehydes described in this study. We also thank Long Quan and Guy Salvesen for providing an expression vector containing the CrmA construct and Greg Cuca (Merck) for efforts in expression and purification of the recombinant CrmA protein.

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J. Biol. Chem. 1998, 273:32608-32613.
doi: 10.1074/jbc.273.49.32608