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ICE Processing and Kinetic Mechanism

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Abstract Interleukin-1β converting enzyme (ICE) has been the focus of major scientific efforts to discover pharmaceutically effective inhibitors. Little is known about the rates of the individual steps in catalysis. We report here that the rates of the two individual chemical steps in catalysis (acylation and deacylation) are each partially rate-limiting. This keeps the overall rate of the reaction less than 3% of the rate of the reaction for papain with its optimized substrate.

Eight human ICE-like proteases have been published to date. They have levels of sequence identity that range from around 30% to greater than 50% throughout the full lengths of the proteins. This degree of relatedness increases when only the active domains are compared. This indicates that the greatest variability between family members occurs in their N-terminal prodomains. We propose several possibilities for the role for these prodomains in the regulation of enzyme processing. J. Cell. Biochem. 64:11–18. © 1997 Wiley-Liss, Inc.

Interleukin-1β converting enzyme (ICE) is the first of a growing family of novel cysteine proteases to be cloned [Cerretti et al., 1992; Thornberry et al., 1992]. Its capacity to cleave pro-IL-1β was first described in 1989 [Black et al., 1989; Kostura et al., 1989]. Based on the inhibition profile of the activity by class-specific inhibitors, ICE was classified as a cysteine protease [Black et al., 1989]. Its resistance to E-64 inhibition indicated that it was an unusual cysteine protease. Papain is typical of the class of cysteine proteases and shows sensitivity to E-64 as well as iodoacetic acid [Barrett et al., 1982]. ICE cleaves pro-IL-1β after Asp-27 and Asp-116 [Black et al., 1988; Howard et al., 1991]. Further characterization of this enzyme indicated its absolute requirement for Asp in the S1 position of the substrate, while small hydrophobic moieties are tolerated on the P1′ side of the enzyme [Sleath et al., 1990].

Reports of the cloning of ICE appeared in 1992 [Cerretti et al., 1992; Thornberry et al., 1992]. The protease was purified from lysates of THP-1 cells by a peptide aldehyde-affinity column [Thornberry et al., 1992]. The purified protease is a heterodimer with subunits of 20 and 10 kDa. Analysis of the cDNA indicated that both subunits were derived from a common precursor of 45 kDa. All the processing sites for the transition from precursor to the mature protease occur at Asp-X sites, indicating a possible autocatalytic activation of ICE. Selective labeling with iodoacetic acid revealed that the active site cysteine was Cys-285 [Thornberry et al., 1992].

The sequence of ICE did not show any significant homology with any known protein in the Genbank database [Cerretti et al., 1992; Thornberry et al., 1992]. The residues around the active site had limited homology with several serine proteases [Thornberry et al., 1992]. That ICE may resemble a serine protease more in catalytic mechanism came up again when a specific ICE inhibitor from cowpox virus showed homology to the serpin family [Ray et al., 1992]. Subsequent studies with the purified crmA gene product showed that it was a potent inhibitor of ICE with a K_i value of <4 pM [Komiyama et al., 1994]. One of the implications of this work is that the geometry of the active site of ICE maybe very similar to that of a serine protease [Komiyama et al., 1994].

The crystal structure showed that ICE had a tertiary structure of the form $(\alpha\beta)_2$ [Walker et al., 1994; Wilson et al., 1994]. The active site was composed of residues from both the p20 and p10 subunits, with Cys-285 covalently attached to an inhibitor. This confirmed this residue as the catalytic cysteine. The catalytic histidine was assigned to His-237 and the deep S1

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pocket comprised of arginine residues that bind the carboxylate of the side-chain Asp in the P1 position of the substrate [Walker et al., 1994; Wilson et al., 1994]. The overall fold of ICE was novel and did not show significant similarity to any of the known serine or cysteine proteases [Walker et al., 1994].

IL-1β is a potent proinflammatory mediator, whose production is tightly regulated [Buras et al., 1994; Godambe et al., 1994; Herzyk et al., 1992; Vannier et al., 1992]. There are several steps at which production of IL-1β is controlled, at the levels of both transcription and post-translational processing. Bacterial endotoxin dramatically upregulates the expression of pro-IL-1β message. Once the message is translated into protein, however, ICE is required to produce the active cytokine. There is a separation between the processing of pro-IL-1β and its export from the cell [Chin and Kostura, 1993]. A portion of the work presented in this report seeks to indicate that another point of regulation of IL-1β production is the rate at which ICE processes the precursor form of the cytokine.

IL-1β is not the only inflammatory mediator whose production is tightly regulated. The production of the prostagladins PGG_2 and PGH_2 is regulated at multiple points as well. The main enzyme responsible for the conversion of arachidonic acid to PGH_2 during an inflammatory condition is prostaglandin H synthase II (PGHS-II). The production of this enzyme is regulated at the transcriptional level, where IL-1 has been shown to potently induce its expression. Post-transcriptionally, IL-1 has also been shown to stabilize the message for PGHS-II [Ristimaki et al., 1994]. Finally, an effect of glutathione on PGHS-II activity has been demonstrated [Capdevila et al., 1995], showing evidence for the synthesis of inflammatory mediators being tightly regulated. The work presented in this report first analyzes the rate at which ICE processes its substrate and then examines potential roles for the N-terminal domains of the ICE family members.

Analysis of ICE Turnover Mechanism

Very little is known about the detailed catalytic mechanism of ICE. ICE has a low turnover rate for a synthetic substrate (Ac-YVAD-pNA, $k_{cat} = 1\ s^{-1}$), compared to papain (Z-Phe-Cit-pNA, $k_{cat} = 43\ s^{-1}$) [Gray et al., 1984]. The actual steps of the mechanism have not been dissected to identify the rate-limiting step in catalysis/proteolysis. The work presented here attempts to break the catalytic mechanism for ICE into its individual steps (Fig. 1) and assigns one as the rate-determining step in the process.

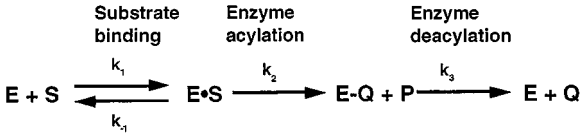

Fig. 1. Basic protease reaction mechanism.

The enzyme used in these studies was expressed in *Escherichia coli* from the full-length cDNA for ICE. The recombinant protein was soluble but its activity decayed rapidly in the lysis buffer ($t_{1/2} = 40$ min). When GSSG was added to the lysis buffer, enzymatic activity was reversibly inhibited. Modification of the enzyme in this manner, though, greatly increased its stability ($t_{1/2}$ for decay >20 h). This stabilization permitted purification of the enzyme to homogeneity by the methods described in the literature [Thornberry et al., 1992]. After elution from the affinity column in the presence of excess aldehyde inhibitor (Ac-YVAD-cho), the enzyme was reactivated with NH_2OH and protected with GSSG. The typical yield for this procedure was on the order of 0.3 mg of enzyme from 25 g of cell paste. On reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the protein was homogeneous, and the two bands of the heterodimer were observed. The molecular weights of these bands detected by mass spectroscopy agreed with the calculated molecular weights based on cDNA sequence [Cerretti et al., 1992; Thornberry et al., 1992].

Enzyme turnover studies were performed at 30°C with either of two synthetic substrates, Ac-YVAD-pNA or Ac-YVAD-mNA, with addition of 10 mM DTT to prevent air oxidation of the active site cysteine. All experiments were performed in a buffer composed of 100 mM HEPES, pH 7.5, 10% sucrose, and 0.1% CHAPS (HSC). K_{cat} values of 0.78 s^{-1} and 0.88 s^{-1} were obtained for the Ac-YVAD-pNA and Ac-YVAD-mNA substrates, respectively, indicating that the enzyme does not discriminate between these two leaving groups (Fig. 2). While there is a difference in the pK value for m-nitroaniline

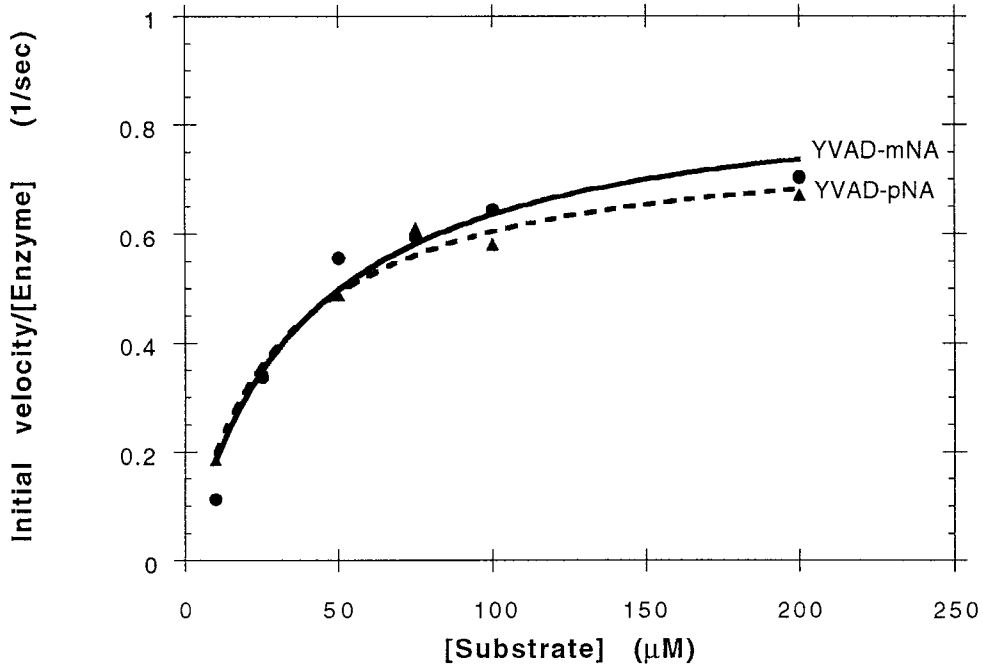

Fig. 2. Steady-state kinetic parameters for synthetic substrates. All experiments were performed in HSC buffer in the presence of 10 mM DTT. The data were fit by nonlinear least-squares to the Michaelis-Menten equation. Kinetic parameters for YVAD-pNA are K_m = 29 μM and k_{cat} = 0.78 s^{-1}; for YVAD-mNA, K_m = 38 μM and k_{cat} = 0.88 s^{-1}.

and p-nitroaniline (2.5 vs 1.0, respectively), this difference is not manifest in any change in the overall turnover of the enzyme. The k_{cat} value for the pNA substrate is over 50 times slower than the turnover number for papain with its optimized substrate (Z-phe-cit-pNA).

Proteolysis by a thiol protease can be broken into three steps (Fig 1). The first reversible substrate binding step is followed by two chemical steps: enzyme acylation followed by deacylation via hydrolysis of the enzyme-acyl intermediate. The overall turnover number for the enzyme is composed only of the rates from the two chemical steps. By performing the assay in the presence of hydroxylamine (a potent nucleophile), where the deacylation step is relatively fast, a value for the acylation rate of the enzyme can be determined [Hinkle and Kirsch, 1971] (Fig 3). By extrapolating to infinite NH_2OH concentration, the rate of enzyme acylation was determined to be 1.78 s^{-1}. Based on the mechanism in Figure 1, the turnover number for the enzyme is equal to the set of terms in equation 1:

$$k_{cat} = (k_2 \cdot k_3)/(k_2 + k_3) \quad (1)$$

The value of k_{cat} is 0.78 s^{-1} and for k_2 the value is determined to be 1.78 s^{-1}. The value for k_3 (the deacylation rate) is then calculated to be 1.39 s^{-1}. These calculations indicate that both chemical steps contribute to the overall slow rate of hydrolysis of YVAD-pNA by ICE when compared to the turnover number for papain with its optimal substrate.

The reason for the slow rate of catalysis by ICE is not immediately obvious based on the work presented here. At high NH_2OH concentrations, saturation kinetics are still observed. This would imply that binding of substrate to the enzyme is not rate-limiting. The three-dimensional structure of ICE has been determined by two independent groups [Walker et al., 1994; Wilson et al., 1994]. One suggestion was that the reason for the low turnover number was that ICE lacked the polarizing acidic residue in its active site analogous to Asp-158 in papain [Wilson et al., 1994]. In the case of cathespin B, however, the residue that hydrogen bonds with the active site histidine is an asparagine rather than aspartic acid [Jia et al., 1995]. It was proposed that this residue in cathespin B helps orient the histidine in the active site [Jia et al., 1995]. In the case of ICE, the active site histidine (His-237) forms a hydrogen bond between one of the imidazole nitrogens and the main chain carbonyl of Pro-177 [Walker et al., 1994].

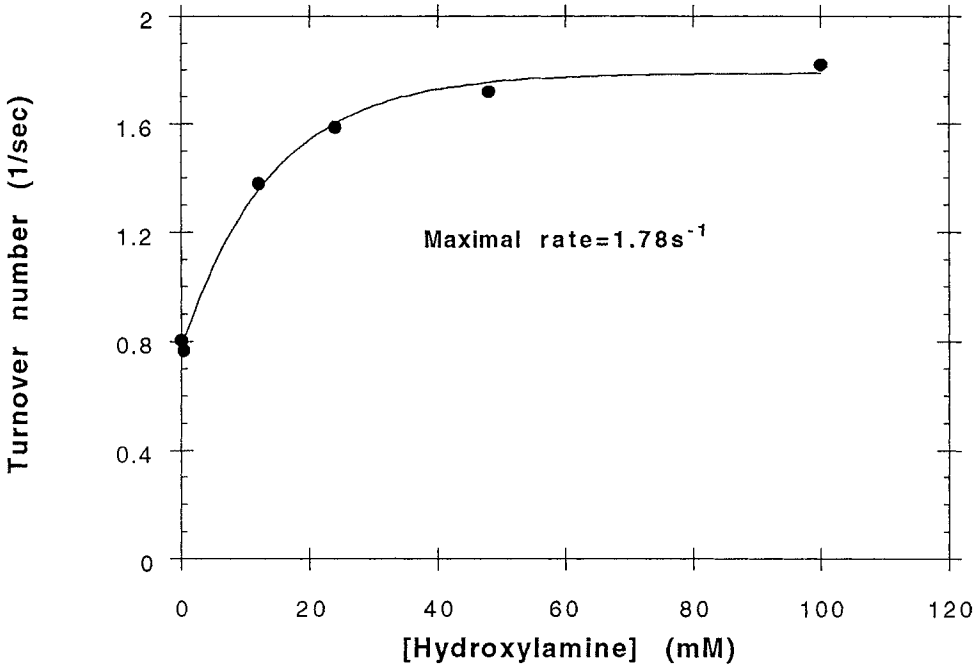

Fig. 3. Effect of hydroxylamine on ICE turnover. The substrate used in these experiments was YVAD-pNA.

In both published ICE structures, an inhibitor is bound in the active site, so the data are not available to determine whether Cys-285 and His-237 are within hydrogen-bonding distance in the unliganded enzyme and at the correct angle to form a hydrogen bond. This may effect the ability of the active site cysteine to attack the substrate scissile bond. Finally, both crystal structures show that ICE exists as an $(\alpha\beta)_2$ tetramer. Recent data indicate that the tetramer is the active species in vivo [Gu et al., 1995]. The association constant for heterodimer to tetramer is unknown, as well as whether the tetramer displays different kinetic behavior from that of the heterodimer. This is an active area of investigation in several laboratories in the field. Additionally, there are now a number of ICE family members that have been cloned and are in the process of being characterized. Several of these homologues appear to have identical amino acids in analogous positions to those that are implicated in catalysis by ICE [Fernandes-Alnemri et al., 1995; Kamens et al., 1995; Nicholson et al., 1995]. It will be interesting to see whether the turnover numbers for these enzymes are similar to ICE or are more comparable to the rates of catalysis of the papain family of cysteine proteases. For CPP32, the turnover number has been found to be about 15-fold higher than the turnover number for ICE (R. Talanian, unpublished observations).

Possible Role for the Prodomain of ICE

The various members of the ICE family of proteases tend to show high degrees of homology between the domains that make up the (putative in some cases) mature proteases. Broader divergence is seen in the sequences of their prodomains [Faucheu et al., 1995; Fernandes-Alnemri et al., 1995; Kamens et al., 1995; Munday et al., 1995; Nicholson et al., 1995]. The lengths of these prodomains vary greatly between the family members (Table I). The shortest ones belong to those family members that are most similar to CPP32 in sequence, while ICE has one of the longer prodomains (Fig 4). It is possible that these prodomains contain information that is required for the protein.

In the case of α-lytic protease, the 166-amino acid pro-region was found to accelerate the rate limiting step in the in vitro folding process by a factor of 10^7 [Baker et al., 1992b]. This prodomain was also found to be a potent inhibitor of the mature protease [Baker et al., 1992a]. While experiments such as these have not yet been tried with ICE, some information on the role of the N-terminal domain of ICE is available.

TABLE I. Lengths of ICE Family Member Prodomains

Protein	Prodomain length
ICE	119
Tx/ICE(relII)/Ich-2	104
CPP32/Yama	28
Ich-1	170
ICE(relIII)	~121
Mch-2	~30

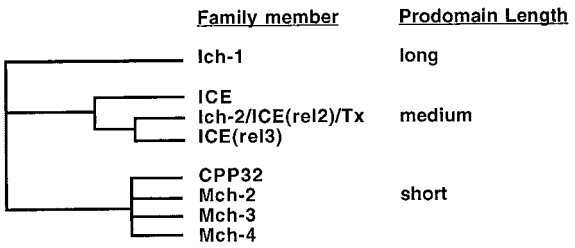

Fig. 4. ICE family tree.

There are two instances in which ICE has been refolded in vitro. In the first case [Walker et al., 1994], ICE was refolded from individual p20 and p10 subunits that had been expressed and purified independently. Active ICE was isolated but the efficiency of the refolding process in the absence of the N-terminal domain was not reported. ICE has also been refolded beginning with the full-length p45 precursor [Ramage et al., 1995]. In this instance, reduced thiol was found to be required for processing. The kinetics of processing indicated that the N-terminal domain of ICE was the last piece to be cleaved from the mature protease. Ramage et al. [1995] postulated that since the N-terminal domain is released last it may play a regulatory role in ICE maturation. Under the conditions of refolding, it can be observed from the data presented that all of the p45 can be processed to smaller pieces, though it is unclear as to the final yield of correctly processed material [Ramage et al., 1995]. Autoprocessing of ICE in the presence of the N-terminal domain, though, appears to rule out this domain as acting as an inhibitor of the enzyme. It would appear likely that the N-terminal domain does play a role in autoprocessing, but the definitive experiments in this area have not yet been done.

Another implication for the N-terminal domain of ICE is in the trafficking of ICE to the appropriate part of the cell. In this case, the N-terminal domain could function in one of two ways, either by preventing localization of immature p45 ICE or by directing p45 to its appropriate position in the cell where it can then be activated. Recent experiments [Singer et al., 1995] have localized active ICE to the extracellular side of the plasma membrane in monocytic cells. In contrast, p45 ICE is found on the intracellular side of the plasma membrane. The active ICE found extracellularly is not accessible to low concentrations of inhibitors placed in the culture media. This implies that either it is contained in some type of vesicle that renders it inaccessible, or that it is inactive once transported out of the cell. In either case, the fact that p45 is not found extracellularly implies that the N-terminal domain of ICE effects this process in some manner.

The proform of ICE (p45) can be seen in stimulated as well as unstimulated monocytes [Ayala et al., 1994]. Also, in human monocytes, expression of pro-IL-1β protein can be dissociated from its processing and secretion of the active cytokine [Chin and Kostura, 1993]. The processing and release of IL-1β are independent of new protein synthesis. These data indicate that p45 ICE and proIL-1β can coexist in the cell without processing of the cytokine. Activation of p45 ICE appears to occur after production of pro-IL-1β protein and does not require additional protein synthesis [Chin and Kostura, 1993].

The above experiments indicate that potential roles for the prodomain of ICE exist. Possible roles are its effect on processing of proICE to active ICE and a possible role in directing ICE to the appropriate site in the cell. If the primary structures of the ICE family are examined, it can be seen that the length of the prodomain on each enzyme varies considerably (Table I). The different family members can be grouped into subfamilies depending on their primary structure homologies of the mature domains. When this is done, it can be seen that the subfamilies group by the length of the prodomain, as well as by overall structural homology.

When immunoblots are probed with antibodies to ICE, the only species that is seen in lysates from stimulated or unstimulated cells is p45 [Ayala et al., 1994]. This finding is in contrast to what is seen for ICE-LAP-3, a family member that is closer to CPP32 in primary structure than to ICE. In Jurkat T-cells or BJAB B cells that have been induced to un-

dergo apoptosis, accumulation of the mature form of ICE-LAP-3 is observed. Prior to stimulation, only the proform is seen [Duan et al., 1996]. Nearly identical results are seen for CPP32 in cells that have been induced to undergo apoptosis [Schlegel et al., 1996].

Several possibilities could account for these observations. The first is that a natural inhibitor might exist in the cell that slows down or prevents processing. This inhibitor could be similar to the viral crmA protein or p35 from insect cells. Both proteins have been found to block ICE activity. To date, however, a mammalian inhibitor of ICE has not be found. Indeed, when active ICE is added to cytosolic extracts of human monocytes, no inhibition of enzyme activity can be observed [Ayala et al., 1994]. This would appear to argue against a natural inhibitor of ICE being present. This cannot be concluded for ICE-LAP-3, however, as the appropriate experiments have not been performed.

Another possibility is that processing of p45 down to the active domain is slow relative to the rate of protein turnover of the mature form. In the absence of stimuli, none of the mature form of ICE-LAP-3 is observed either [Duan et al., 1996]. Once cells are stimulated to undergo apoptosis, however, a dramatic increase in accumulation is seen for the mature form of ICE-LAP-3. These data might indicate that pro-ICE-LAP-3 is catalytically inactive and requires processing by another protease for activation. Once processed, the mature form of this protease is stable enough to accumulate in the cell. ICE, on the other hand, may not be processed by another protease and would be dependent on the low level of activity of p45 for processing. In apoptosis, then, it is possible that a proteolytic cascade exists that would activate the terminal event required for apoptosis. This would be similar to the coagulation cascade seen with the closely related family of serine proteases.

These are several possible explanations for the roles of the N-terminal domains in this group of proteases, but their precise roles are still under investigation. These investigations are hampered by not knowing the biological substrates for any of the family members with the exception of ICE. As the field matures, many of the types of experiments that have been done to examine the cellular localization of ICE, and its processed forms will most likely be used to address the processing mechanisms of the other members of the family.

The information presented here addresses the two different roles for the members of the ICE family and how those functions might be regulated. In the absence of an appropriate stimulus, mature ICE is being inactivated at least as fast as it is processed. This processing can be autocatalytic as long as it is slow. Once in the presence of substrate, the proICE continues to be processed at the same rate, but the rate of inactivation is slowed in the presence of substrate. With family members that are involved in apoptosis, sustained activity would be required once the cells have committed to die. The proteolytic step required for cell death should be completely shut off until it is needed. One method of doing this is to have the proform of the enzyme totally inactive and require processing by a second protease that may be in very low amounts in the cell. Overexpressed levels of ICE have been shown to cause apoptosis. Overexpression should cause increased amounts of the active enzyme, since autocatalysis is a zero-order process. The rate of enzyme inactivation need not change, but there is an increase in the total amount of active enzyme in the cell. Therefore, the two mechanisms of protein activation are different. In the absence of substrate, ICE degrades as it is made and the active form never accumulates to a significant degree. The apoptosis-related enzymes are totally turned off until the cascade of events that occurs during apoptosis amplifies the processing of the proform of the enzyme. The mature form is stable and is not required to be deactivated since the cell has already committed to die.

The whole picture of the biological role for the ICE-like proteases is barely sketched. It may turn out that the activation and processing of each family member is different. This would allow discrimination between the various family members when a particular biological response is required. As more of the natural substrates for the family members are described, the picture of their individual roles should become clearer.

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