The observation that the nematode cell death effector gene product Ced-3 is homologous to human interleukin-1β-converting enzyme (caspase-1) has led to the discovery of at least nine other human caspases, many of which are implicated as mediators of apoptosis. Significant interest has been given to aspects of the cell biology and substrate specificity of this family of proteases; however, quantitative descriptions of their biochemical characteristics have lagged behind. We describe the influence of a number of environmental parameters, including pH, ionic strength, detergent, and specific ion concentrations, on the activity and stability of four caspases involved in death receptor-mediated apoptosis. Based on these observations, we recommend the following buffer as optimal for investigation of their characteristics in vitro: 20 mM piperazine-N,N′-bis-(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-(3-cholamidopropyl)-dimethylammonio-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2. Caspase activity is not affected by concentrations of Ca2+ below 100 mM, but is abolished by Zn2+ in the submicromolar range, a common characteristic of cysteine proteases. Optimal pH values vary from 6.8 for caspase-8 to 7.4 for caspase-3, and activity of all is relatively stable between 0 and 150 mM NaCl. Consequently, changes in the physiologic pH and ionic strength would not significantly alter the activity of the enzymes, inasmuch as all four caspases are optimally active within the range of these parameters found in the cytosol of living and dying human cells.

Apoptotic cell death is a process that enables metazoans to eliminate cells that are damaged, mislocated, or have become superfluous, and is characterized by controlled proteolysis of cellular components resulting from activation of an in-built program (2, 3). The signal for the execution of the cell may come from various stimuli: specific death receptor ligation (4), ionizing radiation (5), anti-neoplastic drugs (6), and growth factor withdrawal (7). However, despite the variety of death signals, the key features of execution appear to be quite similar; the death signal converges upon the activation of a number of proteases, which in turn cleave protein substrates (8, 9), thus giving rise to characteristic apoptotic morphology.

Since the discovery that "ced-3", a key effector gene of programmed cell death in C. elegans, exhibited homology with interleukin 1β-converting enzyme (ICE)1 or caspase-1, the involvement of proteolytic enzymes in apoptosis has been an issue of significant interest (10–12). This has resulted in the cloning of several mammalian genes encoding ICE/Ced-3 homologues, known commonly as caspases (13), several of which are important for promotion of the death pathway in mammals (reviewed in Ref. 9). However, with the notable exception of caspase-1 (14–16), little attention has been given to the key biochemical properties of these enzymes, which is important for understanding the effect of the intracellular environment on their activity. For example, changes in pH, redox potential, and Zn2+ concentration all have effects on apoptosis (17–21). In the present article, we present a characterization of some of the basic biochemical properties of four of the caspases. We have chosen to focus on those that play a central role in the apoptotic pathway initiated by ligation of the death receptors Fas and tumor necrosis receptor 1: caspase-3 (Yama/CPP32/apopain), caspase-6 (Mch2), caspase-7 (Lap3/Mch3/CMH1), and caspase-8 (FLICE/MACH) (22–26).

EXPERIMENTAL PROCEDURES

Materials—Active caspases-3, -6, -7, and -8 were expressed in Escherichia coli and isolated as described previously (22, 24, 27). The expression constructs for caspases-3, -6, and -7 contained a His6 tag at the C terminus of the full-length protein, while caspase-8 was constructed to have a His8 tag at the N terminus replacing residues 1–216 of the zymogen. The concentrations of the purified enzymes were determined from the absorbance at 280 nm based on the molar absorption coefficients for the caspases calculated from the Edelhoch relationship (28): caspase-3 (ε280 = 26000 M−1 cm−1), caspase-6 (ε280 = 26000 M−1 cm−1), caspase-7 (ε280 = 24510 M−1 cm−1), and caspase-8 (ε280 = 27380 M−1 cm−1). Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC) was purchased from Enzyme System Products.

DTT was from Diagnostic Chemicals Limited. Sucrose was from Mallinkrodt. All other chemicals were from Sigma. Z-DEVD-fluoromethyl ketone was the kind gift of Joe Krebs, IDUN Pharmaceuticals.

Determination of the pH Dependence of the Caspases—The pH dependence of the hydrolysis of the substrate Z-DEVD-AFC were evaluated in the pH range 5.5–10. The enzymatic reaction was carried out at 37 °C in the following buffers: 20 mM MES (pH 6.0–6.5), 20 mM HEPES (pH 6.2–7.3), 20 mM PIPES (pH 6.9–8.0), 20 mM CHES (pH 7.5–9.0) or 20 mM CHES (pH 8.0–10.0), containing NaCl, DTT (fresh), EDTA, CHAPS, and sucrose at optimized concentrations as described under “Results and Discussion.” For reasons discussed later, the optimal buffer used as a basis for further studies was 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2. The enzyme concentrations used were 1.2 nM (caspase-3), 18 nM (caspase-6), 5 nM (caspase-7), and 80 nM (caspase-8). The initial rates of enzymatic hydrolysis were measured by release of AFC from the substrate Z-DEVD-AFC using a Perkin-Elmer LS50B fluorimeter equipped with a thermostated plate reader. The pH dependences of the initial rates of hydrolysis for all four caspases were fitted to a bell shape described for two ionizing groups by the equation v = (limit × log(pH − pKα))/(log(2 × pH − pKα1 − pKα2) + log(pH − pKα2 + 1)) using Grafit 3.01 (29).

DISCUSSION

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1 The abbreviations used are: ICE, interleukin-1β-converting enzyme; Z, carbobenzoxy; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid; CHES, 2-(N-cyclohexylamino)ethanesulfonic acid; PIPES, piperazine-N,N′-bis-(2-ethanesulfonic acid); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; AFC, 7-amino-4-trifluoromethyl coumarin; PAGE, polyacrylamide gel electrophoresis.
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Biochemical Characteristics of Caspases-3, -6, -7, and -8

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**Biochemical Characteristics of Caspases-3, -6, -7, and -8**

**Determination of the Zn$^{2+}$ and Ca$^{2+}$ Ion Sensitivity of the Caspases—**

The sensitivity toward Zn$^{2+}$ and Ca$^{2+}$ was determined in optimal buffer (without EDTA), containing varying concentrations of ZnCl$_2$ or CaCl$_2$ as described above. A concentration of 20 mM $\beta$-mercaptoethanol was used to replace DTT because it does not chelate zinc to the same extent as DTT. The influence of the concentration of the reductant on the zinc sensitivity was exploited for caspase-3 using varying concentrations of $\beta$-mercaptoethanol. The inhibition of the caspases by ZnCl$_2$ was fitted to an equation describing simple competitive inhibition, $v = V_{max}/(1 + ([Zn]_{i}/K_i))$ using Grafit 3.01 (29).

**Determination of the Sensitivity of the Caspases to Ionic Strength—**

The sensitivity toward ionic strength was determined in optimal buffer containing varying concentrations of NaCl as described above.

**Determination of the In Vitro Stability of the Caspases—**

The stability of the four caspases was determined by incubating the enzymes in optimal buffer at 0 °C or 37 °C. At various time points, a sample was withdrawn and the activity was determined as described above.

**RESULTS AND DISCUSSION**

The Caspases—Heterologous expression of the caspases is required to obtain sufficient amounts of starting material for a rigorous characterization. Although the mechanism is not understood, when expressed in *E. coli*, these caspases spontaneously undergo what appears to be autoprocessing to yield the appropriate subunits characteristic of the active enzymes (Fig. 1). Processing at interdomain Asp residues was confirmed for all of the recombinant proteases by sequencing of the N termini of the two subunits (see Refs. 22 and 27 for further details). Note that both the large and small subunits migrate as homogenous bands in SDS-PAGE, with the exception of the large subunit of caspase-6, which, based on N-terminal sequencing, represents alternative cleavages at the C terminus of the large subunit. The N-terminal peptides of caspases-3, -6, and -7 were also removed during processing, as demonstrated to occur during Fas-mediated apoptosis in vivo (22). Caspase-8 could not be expressed as a full-length protein and thus was engineered to replace DTT because it does not chelate zinc to the same extent as DTT; in the case of caspase-6, there is a significant reduction in activity in the presence of NaCl, which will be discussed in detail below. However, 100 mM NaCl is required in the assay buffer to maintain a consistent ionic strength when varying pH. A relatively high concentration of DTT (10 mM) is required for full activity of the recombinant enzymes. They may be preactivated by DTT and the DTT removed by gel filtration; however, if neither reducing agent nor EDTA is present in the exchange buffer, the activity declines rapidly, presumably due to oxidation of the catalytic cysteine (data not shown). EDTA (1 mM) is incorporated into the assay buffer to avoid inactivation by trace metals.

**Effects of pH—** Only minor differences were observed in the pH profiles of the four caspases. The bell-shaped pH dependence signifies the existence of one active form of the enzyme with the increase in activity most likely due to the de-protonation of the catalytic Cys residue. In this respect, the caspases closely resemble other unrelated cysteine proteases in their activity pH profiles (30). Caspase-3 was found to be active over a broader pH range with an optimum slightly higher than the other three (see Fig. 2). Although we have analyzed the pH dependence of all four enzymes as a simple bell-shaped curve, there is a faster than expected drop-off in activity at low pH, most clearly observed with caspases-3 and -6. This indicates that more than one group is protonating, possibly another group on the enzyme, or the substrate carboxylate(s), inasmuch as the three-dimensional structure of caspases-1 and -3 demonstrates binding of unprotonated side-chains in its specificity pockets (31–33). The pH dependence of these caspases also indicates that they all are fully active within the pH range found in normal as well as apoptotic cells, designated by the shaded background in Fig. 2 (18, 21). It is possible that changes in pH during apoptosis may affect caspase activity indirectly by altering the structure of a particular set of natural substrates. However, this hypothetical event would change only the susceptibility of the substrate, not the activity of the caspases.

**Effects of Ionic Strength—** We used NaCl in the range 0–1 M in assay buffer to address the dependence of ionic strength on caspase activity. Differential effects were found depending on the enzyme, with caspases-3 and -8 having fairly flat profiles,

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Note that both the large and small subunits migrate as homogenous bands in SDS-PAGE, with the exception of the large subunit of caspase-6, which, based on N-terminal sequencing, represents alternative cleavages at the C terminus of the large subunit. The N-terminal peptides of caspases-3, -6, and -7 were also removed during processing, as demonstrated to occur during Fas-mediated apoptosis in vivo (22). Caspase-8 could not be expressed as a full-length protein and thus was engineered to replace DTT because it does not chelate zinc to the same extent as DTT; in the case of caspase-6, there is a significant reduction in activity in the presence of NaCl, which will be discussed in detail below. However, 100 mM NaCl is required in the assay buffer to maintain a consistent ionic strength when varying pH. A relatively high concentration of DTT (10 mM) is required for full activity of the recombinant enzymes. They may be preactivated by DTT and the DTT removed by gel filtration; however, if neither reducing agent nor EDTA is present in the exchange buffer, the activity declines rapidly, presumably due to oxidation of the catalytic cysteine (data not shown). EDTA (1 mM) is incorporated into the assay buffer to avoid inactivation by trace metals.

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**Effects of Ionic Strength—** We used NaCl in the range 0–1 M in assay buffer to address the dependence of ionic strength on caspase activity. Differential effects were found depending on the enzyme, with caspases-3 and -8 having fairly flat profiles,
whereas caspases-6 and -7 demonstrated maximal activity at 0.03 M and 0.25 M (Fig. 3). Although the activity of caspase-6 declined faster than the others as ionic strength increased, none of the enzymes demonstrated substantial adverse effects in the physiologic range of ionic strength (34), designated by the shaded background in Fig. 3. The apparent stability to substantial changes in ionic strength indicates that this would not be limiting during commitment to apoptosis.

Effects of Zn$^{2+}$—Several studies have reported that Zn$^{2+}$ inhibits apoptosis. Originally, this effect was believed to be due to the inhibition of nucleases; however, caspase-6 (17) and, more recently, caspase-3 (20) have been found to be inhibited completely by 2 mM Zn$^{2+}$. The influence of transition metal ions on the activity of cysteine proteases has been well established for a long time; for instance, members of the papain family are sensitive to Zn$^{2+}$, mercury, and various organomercurials (35, 36). Because DTT chelates Zn$^{2+}$, we compared caspases for sensitivity to this ion in the presence of 20 mM β-mercaptoethanol, which we determined to be the concentration of this reductant required for optimal activity of the recombinant enzymes (data not shown). Due to the inherent tendency of Zn$^{2+}$ to react with thiols, we can only obtain an apparent binding constant and, under these conditions, all the caspases are inhibited by small amounts of Zn$^{2+}$, although there are significant differences in the affinity (Fig. 4).

Caspase-6 is most readily inhibited by Zn$^{2+}$, completely inactivated by 0.1 mM, and caspase-3 is the least sensitive, requiring more than 1 mM for complete inactivation. To estimate the real binding affinity, we probed the influence of reductant on their activity in the physiologic range of ionic strength (34), designated by the shaded background in Fig. 3. The apparent stability to substantial changes in ionic strength indicates that this would not be limiting during commitment to apoptosis.

Effects of Zn$^{2+}$ and Ca$^{2+}$—Several studies have reported that Zn$^{2+}$ inhibits apoptosis. Originally, this effect was believed to be due to the inhibition of nucleases; however, caspase-6 (17) and, more recently, caspase-3 (20) have been found to be inhibited completely by 2 mM Zn$^{2+}$. The influence of transition metal ions on the activity of cysteine proteases has been well established for a long time; for instance, members of the papain family are sensitive to Zn$^{2+}$, mercury, and various organomercurials (35, 36). Because DTT chelates Zn$^{2+}$, we compared caspases for sensitivity to this ion in the presence of 20 mM β-mercaptoethanol, which we determined to be the concentration of this reductant required for optimal activity of the recombinant enzymes (data not shown). Due to the inherent tendency of Zn$^{2+}$ to react with thiols, we can only obtain an apparent binding constant and, under these conditions, all the caspases are inhibited by small amounts of Zn$^{2+}$, although there are significant differences in the affinity (Fig. 4). Caspase-6 is most readily inhibited by Zn$^{2+}$, completely inactivated by 0.1 mM, and caspase-3 is the least sensitive, requiring more than 1 mM for complete inactivation. To estimate the real binding affinity, we probed the influence of reductant on their activity in the physiologic range of ionic strength (34), designated by the shaded background in Fig. 3. The apparent stability to substantial changes in ionic strength indicates that this would not be limiting during commitment to apoptosis.

FIG. 2. The pH dependence of the four caspases for the hydrolysis of the synthetic peptide substrate Z-DEVD-AFC. The dependence was fitted to a bell shape characterized by the following pK_a values: pK_a = 6.4 and pK_a = 8.6 for caspase-3, pK_a = 6.9 and pK_a = 7.2 for caspase-6, pK_a = 6.5 and pK_a = 7.7 for caspase-7, and pK_a = 6.0 and pK_a = 7.7 for caspase-8. The shaded area illustrates the pH range found in normal and apoptotic cells, with the latter favoring lower pH (18, 21).

FIG. 3. NaCl dependence of caspases. Caspases-3 (■), -6 (□), -7 (▲), and -8 (●) were incubated under optimal buffer conditions, with the indicated concentration of NaCl, and initial rates of substrate hydrolysis determined. The rates of hydrolysis have been normalized to the rate of hydrolysis in the absence of NaCl. The shaded area illustrates the range of ionic strength normally found in the cytosol (34).

FIG. 4. Sensitivity of the four caspases to the presence of Zn$^{2+}$. Caspases were incubated under optimal buffer conditions, with DTT replaced by β-mercaptoethanol, at the indicated concentration of Zn$^{2+}$, and initial rates of substrate hydrolysis determined. The apparent binding constants for Zn$^{2+}$ to the individual caspases (K_{Zn,app}) are 8.8 μM for caspase-3, 0.3 μM for caspase-6, 1.7 μM for caspase-7, and 1.9 μM for caspase-8.

FIG. 5. Influence of the concentration of β-mercaptoethanol on the apparent binding constant for Zn$^{2+}$ to caspase-3. The influence of β-mercaptoethanol on K_{Zn,app} were investigated using the concentrations 0.25 mM (▲), 0.5 mM (■), 1 mM (▲), 2 mM (○), 4 mM (□), 8 mM (▲), 16 mM (●), and 32 mM (○).
the inhibition of caspase-3 by Zn²⁺. Not surprisingly, there was a significant influence of the concentration of β-mercaptoethanol on the $K_{\text{app}}$ giving rise to values converging on an approximate value of 0.15 μM (Fig. 5). From these results, it is quite evident that Zn²⁺ is a good inhibitor of the caspases, albeit very dependent on the thiol content, and therefore presumably the redox potential of the cell. The influence of Ca²⁺ was investigated in a similar manner and was found to have no effect on the activity of any of the caspases at concentrations up to 100 mM (data not shown). Thus, the reported role of Ca²⁺ in apoptosis (see, for example, Ref. 37) is unlikely to be due to any effect on the caspases.

**In Vitro Stability**—On the basis of the foregoing results, the optimal general caspase buffer was designated as 20 mM Pipes, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2. The stability of the four caspases was tested by incubating the enzymes at 0 °C or 37 °C in the optimal assay buffer and determining the activity at various times (Fig. 6). None of the caspases showed any decrease in activity at 0 °C over the 150-min period. Caspases-3 and -6 retained full activity for 150 min at 37 °C, whereas caspases-7 and -8 showed an appreciable decrease in activity. To verify that the decrease in activity observed at 37 °C with caspases-7 and -8 was not due to sample variation, the experiment was performed with two different preparations of these enzymes giving rise to almost identical results, reducing the probability that the decrease in activity is associated with sample variations. The reason for the decrease in activity is not clear; however, SDS-PAGE analysis of caspases incubated at 0 °C and 37 °C for 150 min does not reveal any indications of degradation (data not shown). Based on these observations, the most probable explanation is a conformational change, possibly due to slow dissociation of the subunits after dilution into assay buffer, as originally described for caspase-1 (11). This assumption is supported because the decrease in activity observed with caspase-8 appears to approach a level of approximately 60%, and remains there for an extended period of time. Whether such dissociation occurs in a cell under physiologic conditions remains an open question, but it is evident that none of the investigated caspases undergo autoactivation that will significantly affect their role in apoptosis. This is in contrast to caspase-1, which has been shown to inactivate spontaneously by autolytic degradation of its small subunit (38).

**Biologic Perspective**—The results demonstrate that all four caspases are optimally active under normal physiologic conditions. We have to activate the recombinant enzymes by adding thiols, presumably because of reversible modification of the catalytic cysteine during expression and purification. In _in vivo_, however, the glutathione balance would favor the reduced form, with the result that, once processed from their single chain zymogens, the caspases would be fully active. We do not rule out the possibility that natural caspase substrates are affected by changes in environmental parameters that would alter their susceptibility to specific proteolysis _in vivo_. In this context, caspase-1 was shown to exhibit a marked salt dependence due to effects of NaCl on the substrate pro-interleukin-1β, but not on a synthetic peptidyl substrate (39). However, changes in the pH and ionic strength of the cytosol would not significantly alter the activity of the enzymes themselves, inasmuch as all four caspases are optimally active within the range of these parameters found within cell cytosols, irrespective of their metabolic status.

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**REFERENCES**

1. Schechter, I., and Berger, A. (1967) _Biochem. Biophys. Res. Commun._ 27, 157–162
2. Ellis, H. M., and Horvitz, H. R. (1986) _Cell_ 44, 817–829
3. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D.,  and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 18530–18533
4. Tomlinson, I. R., and Harbor, Y. J. (1991) _Nature_ 353, 333–338
5. Datta, R., Kojima, H., Banach, D., Bump, N. J., Talanian, R. V., Alnemri, E. S., Weichselbaum, R. R., Wong, W. W., and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 1965–1969
6. Fearon, H. O., McCurrach, M. E., O’Neill, J. Z., Zhang, K., Lowe, S. W., and Lazebnik, Y. A. (1997) _Genes Dev._ 11, 1266–1276
7. Deckwerth, T. L., and Johnson, E. M., Jr. (1993) _Cell. Biol._ 123, 1207–1222
8. Martin, S. J., and Green, D. R. (1995) _Cell_ 82, 349–352
9. Chinnaiyan, A. M., and Dixit, V. M. (1996) _Curr. Biol._ 6, 555–562
10. Ceretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Huebner, K., and Black, R. A. (1992) _Science_ 256, 87–90
11. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D.,  and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 18530–18533
12. Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T.,  and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 18530–18533
13. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) _J. Biol. Chem._ 272, 18530–18533
14. Gottlieb, R. A., Nordberg, J., Skowronski, E., and Babior, B. M. (1996) _J. Biol. Chem._ 271, 1259–1267
15. Meisenholder, G. W., Martin, S. J., Green, D. R., Nordberg, J., Babior, B. M.,  and Gottlieb, R. A. (1996) _J. Biol. Chem._ 271, 1259–1267
16. Rano, T. A., Timkey, T., Peterson, E. P., Rotonda, J., Nicholson, D. W., Becker, J. W., Chapman, K. T., Howard, A. D., and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 144–159
17. Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T.,  and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 18530–18533
18. Gottlieb, R. A., Nordberg, J., Skowronski, E., and Babior, B. M. (1996) _Proc. Natl. Acad. Sci. U. S. A._ 93, 8385–8400
19. Elgueta, R. A., Brown, J., and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 18530–18533
20. Perry, D. K., Smyth, M. J., Merriam, J. E., March, C. J., and Hopp, T. P. (1995) _J. Biol. Chem._ 270, 5323–5326
21. Sneath, P. R., Hendrickson, R. C., Kronheim, S. R., March, C. J., and Black, R. A. (1990) _J. Biol. Chem._ 265, 14526–14528
22. Rano, T. A., Timkey, T., Peterson, E. P., Rotonda, J., Nicholson, D. W., Becker, J. W., Chapman, K. T., Howard, A. D., and Kufe, D. W. (1997) _J. Biol. Chem._ 272, 144–159
23. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., Moir, R. D., Goldman, R. D., Fiorier, G. G., Kaufman, S. H., and Earnshaw, W. C. (1996) _Proc. Natl. Acad. Sci. U. S. A._ 93, 8385–8400
24. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) _J. Biol. Chem._ 272, 18530–18533
25. Boldin, M. P., Gencharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) _Cell_ 85, 800–815
26. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O’Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Breit, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) _Cell_ 85, 817–827
27. Zhou, Q., Snips, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. S. (1997) _J. Biol. Chem._ 272, 7797–7800
28. Edelhoch, H. (1967) _Biochemistry_ 6, 1948–1954
29. Leatherbarrow, R. J. (1993) _Grafts_ Version 3.0, Erithacus Software Ltd., Staines, United Kingdom
30. Lewis, E. R., Johnson, F. A., and Shafer, J. A. (1981) _Biochemistry_ 20, 48–51
31. Wilson, K. P., Black, J. A. F., Thompson, J. A., Kim, E. E., Griffith, J. P., Navia,
32. Rotonda, J., Nicholson, D. W., Fazil, K. M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E. P., Raper, D. M., Ruel, R., Vaillancourt, J. P., Thornberry, N. A., and Becker, J. W. (1996) *Nat. Struct. Biol.* 3, 619–625
33. Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Hous, W., Mankovich, J. A., McGuiness, L., Orlewicz, E., Paskind, M., Pratt, C. A., Reis, P., Summani, A., Terranova, M., Welch, J. P., Xiong, L., and Muller, A. (1994) *Cell* 78, 343–352
34. Godt, R. E., and Maughan, D. W. (1988) *Am. J. Physiol.* 254, C591–C604
35. Suyterman, L. A., and Wijdenes, J. (1976) *Eur. J. Biochem.* 71, 383–391
36. Bond, J. S. (1989) in *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J., and Bond, J. S., eds) pp. 232–240, IRL Press, Oxford
37. Blank, X., Hughes, F. M., Jr., Huang, Y., Cidlowski, J. A., and Putney, J. W., Jr. (1997) *Am. J. Physiol.* 272, C1241–C1249
38. Talanian, R. V., Dang, L. C., Ferenz, C. R., Hackett, M. C., Mankovich, J. A., Welch, J. P., Wong, W. W., and Brady, K. D. (1996) *J. Biol. Chem.* 271, 21853–21858
39. Miller, D. (1996) in *Therapeutic Modulation of Cytokines* (Henderson, B., and Bodmer, M. W., eds) pp. 143–170, Springer Verlag, Berlin
40. Bury, A. (1981) *J. Chromatog.* 213, 491–500