Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8

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Subsite interactions are considered to define the stringent specificity of proteases for their natural substrates. To probe this issue in the proteolytic pathways leading to apoptosis we have examined the P_4 , P_1 and P_1' subsite preferences of human caspases 1, 3, 6, 7 and 8, using internally quenched fluorescent peptide substrates containing *o*-aminobenzoyl (also known as anthranilic acid) and 3-nitro-tyrosine. Previous work has demonstrated the importance of the S_4 subsite in directing specificity within the caspase family. Here we demonstrate the influence of the S_1 and S_1' subsites that flank the scissile peptide bond. The S_1 subsite, the major specificity-determining site of the caspases, demonstrates tremendous selectivity, with a 20000-fold preference for cleaving substrates containing aspartic acid over glutamic acid at this position. Thus caspases are among the most selective of known endopeptidases. We find that the caspases show an unexpected degree of discrimination in the P_1' position,

with a general preference for small amino acid residues such as alanine, glycine and serine, with glycine being the preferred substituent. Large aromatic residues are also surprisingly well-tolerated, but charged residues are prohibited. While this describes the general order of P_1' subsite preferences within the caspase family, there are some differences in individual profiles, with caspase-3 being particularly promiscuous. Overall, the subsite preferences can be used to predict natural substrates, but in certain cases the cleavage site within a presumed natural substrate cannot be predicted by looking for the preferred peptide cleavage sites. In the latter case we conclude that second-site interactions may overcome otherwise sub-optimal cleavage sequences.

Key words: apoptosis, specificity, protease.

INTRODUCTION

Caspases drive the intracellular pathways leading to apoptosis and pro-inflammatory cytokine activation. Their enzymic properties are governed by a dominant specificity for substrates containing aspartic acid, and by the use of a cysteine side-chain for catalysing peptide-bond cleavage [1–3]. While the use of a cysteine side-chain as a nucleophile during peptide bond hydrolysis is common to several protease families, the primary specificity for aspartic acid turns out to be very rare among proteases. Currently the only other known mammalian protease with the same primary specificity is the serine protease granzyme B [4,5], which is a physiological caspase activator [6–9].

Using a combinatorial approach with positional scanning of synthetic tetrapeptidyl-aminomethyl coumarin derivatives, Thornberry and co-workers explored the S_2 – S_4 subsite occupancy (N-terminal to the scissile bond). Based on S_4 subsite preferences they subdivided the caspases into three substrate specificity groups: group I consisting of caspases 1, 4 and 5 with a preference for aromatic amino acid residues in the P_4 position; group II containing caspases 6, 8, 9 and 10 with a preference for branched apolar residues; and finally group III consisting of caspases 2, 3 and 7 with a preference for aspartic acid [5,10]. However, because the test substrates used in the seminal positional scanning approach were not extended in the primed-side (C-terminal to the scissile bond), it was not possible to determine the influence of prime-side binding. Consequently, insight into the P_1' preferences of apoptotic caspases is largely based on scattered observations from the cleavage of natural substrates (reviewed in [3,11]).

Despite success in defining substrate preferences for individual caspases, the various published positional scanning approaches [5,10,12] have reported only a few numerical specificity constants (k_{cat}/K_m values). This has made it difficult to predict whether the use of optimal synthetic substrates would be appropriate in diagnosing individual caspase activities in complex mixtures, such as in cells undergoing apoptosis. Therefore we have determined specificity constants, and, when possible, individual k_{cat} and K_m values for a nested set of substrates where P_4 is widely varied. We have also performed a systematic approach to determine the preference of caspases for aspartic acid, compared with other residues, in the P_1 position. In addition, we report distinct preferences for the P_1' subsite in caspases 1, 3, 6, 7 and 8 in detail, using peptide substrates based on intramolecular quenching.

Binding site nomenclature is in accordance with Schechter and Berger [13].

EXPERIMENTAL

Materials

Active caspases 3, 6, 7 and 8 were expressed in *Escherichia coli* and isolated in the fully active form as previously described [14]. Purified caspase-1 was kindly provided by Dr Nancy Thornberry (Department of Enzymology, Merck Research Laboratories, Rahway, NJ, U.S.A.). Purified bovine poly(ADP-ribose)-polymerase (PARP) was the gift of Dr Guy Poirier (Laval University Medical Research Center, Laval University, Quebec,

Abbreviations used: Abz, *o*-aminobenzoyl; AFC, 7-amino-4-trifluoromethyl coumarin; DCC, deleted in colorectal cancer; DTT, dithiothreitol; PARP, poly(ADP-ribose)polymerase; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

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Canada). Rabbit anti-PARP serum was obtained from Biomol (Plymouth Meeting, PA, U.S.A.).

The active concentrations of each purified enzyme were determined by titration using benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) [14]. Intramolecularly quenched substrates, based on the *o*-aminobenzoyl (Abz; also known as anthranilic acid)/nitro-tyrosine pair, were prepared by solid-phase synthesis as described previously [15,16]. The purity of the peptides was determined by HPLC, and peptides that were found to be less than 90% pure were purified by preparative HPLC as previously described [15–17]. The composition of the peptides was verified by matrix-assisted laser-desorption ionization–time-of-flight MS performed on a Finnigan MAT Lasermat 2000 mass analyzer. Preparative and analytical HPLC were performed using a Ranin or a Waters HPLC apparatus, equipped with a Vydac C₁₈ (4.6 mm × 250 mm, flow rate 1.5 ml/min) or a Nova-Pack C₁₈ (8 mm × 100 mm, flow rate 2.0 ml/min) reverse-phase column for analytical separations. For preparative purification a Vydac 10 µm C₁₈ reverse-phase column (25 mm × 250 mm, flow rate 45 ml/min) was used. Benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC), Z-IETD-AFC, Z-VEID-AFC, and Z-VAD-FMK were purchased from Enzyme System Products (Livermore, CA, U.S.A.). Dithiothreitol (DTT) was purchased from Diagnostic Chemicals Limited (Oxford, CT, U.S.A.). Sucrose was obtained from Mallinckrodt (Phillipsburg, NJ, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Determination of catalytic parameters

Hydrolysis of the peptide substrates was followed on a Molecular Devices/fmax plate reader at 37 °C. The substrates were dissolved in DMSO at concentrations between 2 and 10 mM. Portions (2 µl) of substrate were added to 98 µl portions of caspase buffer [10 mM Pipes, 0.1 M NaCl, 0.1 mM EDTA, 10 mM DTT, 10% (w/v) sucrose and 0.1% CHAPS, pH 7.2] containing the enzyme of interest [18] to give initial substrate concentrations (S_0) in the range of 3–50 µM. Cleavage of the substrate as a function of time was followed by monitoring the emission at 405 nm upon excitation at 320 nm, and the initial velocity (v_0) was determined from the linear portion of the progress curve. The substrate concentration was determined from the final fluorescence of totally hydrolysed substrate using Abz-Gly-NH₂ as a standard.

The individual catalytic parameters k_{cat} and K_m were determined by direct fitting of v_0 versus S_0 values to the Michaelis–Menten equation, using non-linear regression. When k_{cat} and K_m could not be determined directly, the k_{cat}/K_m ratio was determined from the relationship $v_0 = (k_{cat}/K_m)E_0S_0$, which is valid when $S_0 \ll K_m$. All determinations were performed using the DeltaGraph 4.05 or GraphPad Prism 2.0 software programs. Standard deviations of the reported values were below 10%.

RESULTS

Caspase substrate-binding subsites

As for most proteases, the substrate-binding cleft of caspases may be subdivided into a number of subsites [19–25], each consisting of residues important for the recognition of the substrate. These subsites are referred to as S_{1+n} to S_{1+n}' with the catalytic apparatus placed between the S_1 and S_1' position [13]. The corresponding substrate residues are referred to as P_{1+n} to P_{1+n}' with the bond between P_1 and P_1' being the scissile bond.

The substrates used in the present study are based on the principle of intramolecular quenching. They contain a fluorogenic

Table 1 P_4 specificity based on the substrate Abz-GXEVD-GVY(NO₂)D (k_{cat}/K_m in M⁻¹·s⁻¹)

Values for preferred P_4 residues are shown in bold; n.d. indicates that the k_{cat}/K_m value could not be measured, which in our assay system equated to a value of less than 1.0 M⁻¹·s⁻¹. Standard deviations of the reported values were below 10%.

P_4	Caspase-1	Caspase-3	Caspase-6	Caspase-7	Caspase-8
Alanine	1200	750	640	23	5100
Aspartic acid	1000	200 000	390	33 000	21 000
Glutamic acid	740	2100	540	120	5600
Phenylalanine	44 500	1400	160	40	8200
Glycine	340	220	40	10	460
Lysine	n.d.	30	n.d.	n.d.	n.d.
Leucine	6400	690	4500	10	83 000
Asparagine	370	1300	290	20	5900
Proline	630	200	260	2	4900
Glutamine	320	770	620	10	1600
Arginine	n.d.	130	n.d.	n.d.	n.d.
Serine	600	2700	1100	180	2300
Threonine	490	2800	11 000	150	4700
Valine	2200	1500	12 000	10	25 000
Tyrosine	75 000	1900	100	75	4900

group (Abz) and a quenching group (3-nitro-tyrosine) bracketing the substrate sequence [15,16]. These groups are positioned at opposite ends of the substrate and cleavage results in the separation of the two groups and increased fluorescence. This strategy allows the use of actual peptide-bond substrates rather than the activated amides found in substrates based on fluorogenic or chromogenic leaving groups. Thus the scissile bond is chemically identical to that found naturally in proteins. The substrates we decided to synthesize were based on the three frames: Abz-GXEVD-SVY(NO₂)D-OH that allows determination of the S_4 subsite preference, Abz-GDEVX-SVY(NO₂)D-OH that allows determination of the S_1 subsite preference, and Abz-GDEVX-VY(NO₂)D-OH that allows determination of the S_1' subsite preference. Single letter amino acid notation has been used, and in each case X stands for one of the following amino acid residues: A, D, E, F, G, K, L, N, P, Q, R, S, T, V or Y. For ease of synthesis the following amino acids were omitted from the scanning approach: C, H, I, M and W.

P_4 substrate preference

We first scanned the P_4 position to explore the preferences of caspases 1, 3, 6, 7 and 8. (Table 1). The P_4 specificities we found using the peptide substrates closely resembled those previously described [5,12,26]; caspase-1 preferred large aromatic residues in this position, caspases 3 and 7 preferred aspartic acid, and caspases 6 and 8 preferred branched aliphatic residues, although both aspartic acid and valine were well tolerated by caspase-8. While the earlier publications described the specificity of individual caspases, they did not give the actual catalytic parameters. Thus they did not permit comparisons between different substrates for a single enzyme, a goal that would be of great value in caspase research. However, this can simply be accomplished by determining the defining k_{cat}/K_m values of each enzyme for the individual substrates. Using these values we can estimate the contribution of individual caspases to the cleavage of a particular substrate, which is related by the equation $v = (E \cdot S \cdot k_{cat}) / (S + K_m)$, where the rate of hydrolysis at a given substrate concentration, S , is simply related to the k_{cat}/K_m value

and the concentration of each caspase, E . The cleavage rates are thus dominated not only by k_{cat}/K_m , but also by the absolute physiological caspase concentrations, which can be quite varied. Since caspase-3 is usually the most abundant caspase *in vivo* [27–29], almost all cleavage of synthetic DEVD-based substrates could be attributed to this enzyme. Thus, caspase-3 would also be very effective in cleaving the allegedly specific caspase-6 and -8 substrates that contain valine or leucine in P_4 . Factors such as cellular localization and specific second-site interactions will determine which caspases actually cleave natural substrates *in vivo*, and we expand on this issue in the Discussion section.

Another observation that can be made from our experiments is the rather broad specificity of caspase-8, which can quite efficiently cleave substrates with aspartic acid, valine or leucine in the P_4 position. Interestingly, the basis for the observed differences in k_{cat}/K_m could not be traced back to differences in K_m since the values observed for FEVD, VEVD and LEVD were 22 μM , 16 μM and 15 μM , respectively, compared to 23 μM for DEVD. Thus the basis for the discrimination between different P_4 substituents by caspase-8 was mainly due to differences in the k_{cat} values, presumably reflecting differences in the ability to stabilize the transition state of these different substrates. This was confirmed by the observation that tetrapeptide aldehyde inhibitors (transition-state analogues) show only limited differences in their K_i values for the inhibition of caspase-8 as a function of the P_4 substituent [30].

P_1 substrate preference

We tested a subset of substrates based on the DEVX-S frame with alanine, aspartic acid, asparagine, glutamic acid, glutamine and serine as P_1 substituents. We could detect no hydrolysis of the tested substrates by caspases 1, 6, 7 or 8, unless they contained aspartic acid in P_1 . Only the substrate containing a glutamic acid residue in P_1 was cleaved at any measurable rate by caspase-3, with a k_{cat}/K_m of 10 $\text{M}^{-1}\cdot\text{s}^{-1}$, which is 20000-fold lower than that observed with an aspartic acid residue in P_1 . The limit of detection in our assays was $k_{\text{cat}}/K_m > 1.0 \text{ M}^{-1}\cdot\text{s}^{-1}$, which meant that the aspartic acid/glutamic acid selectivity ratio at P_1 was greater than 1000 for caspase-1; 400 for caspase-6; 3000 for caspase-7; and 20000 for caspase-8.

We could not determine the actual cleavage site in the substrate owing to the extremely low amount of product. So there is a formal possibility that cleavage may have occurred between the aspartic acid and glutamic acid residues upstream of the designed site. Thus the actual preference for aspartic acid in P_1 could be even higher. However, all the other substrates tested should show similar parameters, so we feel this extremely unlikely. We conclude that the preference of caspases 1, 3, 6, 7 and 8 for aspartic acid in P_1 is extremely stringent, and that these caspases surpass most other protease families in their exquisite primary substrate selectivity. It should be emphasized, however, that the analysis is restricted to the human caspases, and cannot a priori be extended to more diverse homologues.

Since substrates without aspartic acid in P_1 were cleaved so poorly there was a formal possibility that they acted as competitive inhibitors. To check this, caspase-3 was incubated with various concentrations of Abz-GDEV $\underline{\text{D}}$ -SVY(NO $_2$)D-OH, Abz-GDEV $\underline{\text{E}}$ -SVY(NO $_2$)D-OH or Abz-GDEV $\underline{\text{A}}$ -SVY(NO $_2$)D-OH and assayed with acetyl-DEVD-*p*-nitroanilide. The ability of the glutamic acid substrate to act as a competitive substrate was decreased by at least 7-fold compared to the aspartic acid substrate, and the substitution of the P_1 aspartic acid residue for alanine completely abolished the ability to act as a competitive inhibitor or substrate (results not shown). Therefore the decrease

Table 2 P_1' specificity ordered by decreasing activity of caspase-3 against Abz-GDEV $\underline{\text{D}}$ -XVY(NO $_2$)D (k_{cat}/K_m in $\text{M}^{-1}\cdot\text{s}^{-1}$)

n.d. indicates that the k_{cat}/K_m value could not be measured, which in our assay system equated to a value of less than 1.0 $\text{M}^{-1}\cdot\text{s}^{-1}$. Standard deviations of the reported values were below 10%.

P_1'	Caspase-1	Caspase-3	Caspase-6	Caspase-7	Caspase-8
Glycine	2700	193 000	1100	55 100	38 900
Serine	1100	200 000	390	33 000	21 400
Alanine	290	157 000	290	25 900	6900
Tyrosine	210	63 500	220	14 500	2700
Phenylalanine	140	63 000	190	16 600	2400
Threonine	190	46 000	45	1900	720
Asparagine	110	23 500	40	11 700	2600
Arginine	25	14 000	30	620	130
Lysine	35	7900	25	320	90
Glutamine	n.d.	5900	5	330	60
Aspartic acid	n.d.	4200	10	210	50
Valine	65	3900	20	140	120
Leucine	20	3300	10	920	70
Glutamic acid	n.d.	2400	5	50	n.d.
Proline	n.d.	750	n.d.	n.d.	n.d.

in catalytic activity observed when substituting the P_1 aspartic acid residue for glutamic acid can be attributed to a decrease in both binding affinity and acylation rate. Consequently, the presence of aspartic acid in natural substrates is essential for recognition, as well as hydrolysis.

P_1' substrate preference

Natural caspase substrates have an abundance of glycine, serine, asparagine or alanine residues in the P_1' position (reviewed in [3,11]), and so it is tempting to assume that this may reflect a subsite preference. Therefore we evaluated the importance of the S_1' subsite in the determination of substrate specificity (Table 2). All caspases appeared to prefer small residues such as glycine, serine and alanine in the P_1' position although the aromatic residues phenylalanine and tyrosine were also surprisingly well-tolerated. The least-tolerated substituents were: charged residues, aspartic acid, glutamic acid, lysine and arginine; branched aliphatic residues, leucine and valine; and not unexpectedly proline. Occupancy of the S_1' subsite by these residues all resulted in a reduction in k_{cat}/K_m of at least 10-fold, but generally higher, and even up to 1000-fold in the case of glutamic acid or proline in P_1' . The order of S_1' preference resembles the findings of Talanian and co-workers who, using HPLC-based analysis of peptide hydrolysis, demonstrated that caspases 1 and 4 had significant degrees of selectivity in S_1' [12]. However, the degree of selectivity observed in the present study, especially for the apoptotic caspases 3, 7 and 8, is much higher than that previously found for caspases 1 and 4.

In the case of caspases 1 and 6 it was only possible to determine k_{cat}/K_m values since the substrates based on the DEV $\underline{\text{D}}$ -X frame, were not well tolerated by these proteases that prefer aromatic or aliphatic P_4 substituents. However, in the case of caspases 3, 7 and 8 the individual catalytic parameters could be determined for most of the substrates (Table 3). Thus the basis for the discrimination between different P_1' substituents by caspases is mainly due to differences in the k_{cat} values, reflecting differences in the ability to stabilize the transition state of these different substrates, akin to what we find for the P_4 position.

Table 3 Variation in catalytic parameters as a function of P₁'

n.d. indicates that the K_m value was outside the measurable range. Standard deviations of the reported values were below 10%.

P ₁ '	Caspase-3		Caspase-7		Caspase-8	
	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat} (s ⁻¹)	K_m (μM)
Alanine	1.4	9.2	0.30	12	0.16	23
Aspartic acid	0.15	36	0.003	16	n.d.	n.d.
Glutamic acid	0.10	42	0.001	18	n.d.	n.d.
Phenylalanine	0.79	13	0.29	17	0.1	42
Glycine	2.5	13	0.76	14	0.69	18
Lysine	0.42	53	0.02	74	n.d.	n.d.
Leucine	0.18	55	0.05	53	n.d.	n.d.
Asparagine	0.63	27	0.12	10	0.11	41
Proline	0.03	41	n.d.	n.d.	n.d.	n.d.
Glutamine	0.26	44	0.02	50	n.d.	n.d.
Arginine	0.34	24	0.01	20	n.d.	n.d.
Serine	1.4	6.8	0.21	6.4	0.28	13
Threonine	1.2	27	0.07	36	0.05	70
Valine	0.24	61	n.d.	n.d.	n.d.	n.d.
Tyrosine	1.1	17	0.31	22	0.17	61

Kinetics of PARP hydrolysis

To allow comparison with a natural protein substrate we determined the rate of PARP cleavage by caspase-3. Experiments were performed by incubating 440 nM purified bovine PARP for 30 min at 37 °C with a range of caspase-3 concentrations (between 67 pM and 440 nM) in caspase buffer. The reaction mixture was resolved by SDS/PAGE and cleavage was visualized by probing a Western blot with anti-PARP serum. Conversion (50 %) was observed at 3.62 nM caspase-3 and the apparent second-order rate constant determined from the relation $k = \ln 2 / E_0 t$. Interestingly, the k_{cat}/K_m of $1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ observed for PARP cleavage is significantly lower than the previously reported value of $5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ [31]. This difference cannot be accounted for by enzyme preparations since the caspase-3 used in the present study shows essentially the same catalytic activity on acetyl-DEVD-*p*-nitroanilide ($k_{cat}/K_m = 1.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$), as that used by Casciola-Rosen and colleagues ($k_{cat}/K_m = 1.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) [31]. The origin of the substrate is probably important since we used purified bovine PARP [32], whereas Casciola-Rosen and colleagues used *in vitro* translated material (which may have contained other caspases) as well as slightly different assay conditions.

DISCUSSION

The first step in proteolysis is to position the scissile peptide bond across the catalytic apparatus, and different protease families have developed distinct surface pockets for substrate binding in order to properly align the substrate with the catalytic apparatus. Thus the primary specificity of matrix metalloproteases is dominated by a hydrophobic S₁' pocket [33], members of the papain family have a dominant S₂ pocket [34], and serine proteases from both the subtilisin and chymotrypsin families have a dominant S₁ pocket [35]. Perhaps the closest similarity to the caspase solution for substrate binding is found in the subtilisins, which utilize a combination of S₁ and S₄ pockets to drive specificity [16]. The high selectivity observed with the caspases in both S₁ and S₄ is, however, unparalleled. The caspases are the most specific of the selective proteases described so far, and they have never been seen to cut more than just a few bonds

in their target protein [11]. What are the molecular determinants of this extreme specificity?

Granzyme B, the serine protease that shares the closest similarity in substrate preference with caspases, has a surprisingly low selectivity for aspartic acid of only 12-fold over glutamic acid [4]. However, this number may need to be revised upwards by an order of magnitude with the recent ability to produce recombinant granzyme B (C. Craik, personal communication). Similarly, trypsin-like serine proteases with a S₁ preference for basic side-chains have rather low selectivities. Trypsin shows an arginine/lysine selectivity ratio of approx. 7–12 [36], and even the very specific tissue plasminogen activator has an arginine/lysine selectivity ratio of only 85 [36]. Selectivity is quite high in enzymes such as bacterial glutamyl endopeptidases, which have glutamic acid/aspartic acid ratios between 315 and 5300 [17,37]. By comparison the aspartic acid/glutamic acid ratio in caspases is as high as 20000. The caspase S₁ pocket formed by the all-important Arg¹⁷⁹, Arg³⁴¹ and Gln²⁸³ accepts the aspartic acid side-chain of a substrate, but entry of even a closely related glutamic acid side-chain is evidently unfavourable for both binding and catalysis. This makes the primary specificity of the caspases much higher than for all other known protease families.

The major feature distinguishing the caspases from one another is the S₄ substrate preference, as previously demonstrated [5,12]. Clearly this preference enables one to characterize caspases in purified systems, but the use of supposedly selective substrates to discriminate between mixtures of caspases, for example in extracts of apoptotic cells, is complicated by at least two factors. First, some caspases (such as caspase-3) have higher absolute k_{cat}/K_m values in general, presumably due to a higher intrinsic catalytic efficiency. Second, concentrations of caspases in a biological milieu are quite varied, with executioners such as caspases 3, 6 and 7 predominating over initiators such as caspases 8 and 9 [27–29]. Therefore the activity of the most abundant and most active caspase will often determine the readout regardless of the substrate used. Consequently most investigators will be measuring the activity of caspase-3, owing to its inherently high abundance and catalytic efficiency, regardless of which substrate they choose to use for the studies.

In contrast to the S₄ subsite selectivity, which distinguishes individual caspases, the S₁' subsite can direct substrate selectivity, but does not discriminate between individual caspases. Thus the preference in this position is quite well-conserved among the different caspases. Both k_{cat} and K_m are influenced by P₁', indicating an effect on substrate binding as well as acylation. Conversely, there is no absolute requirement for occupancy of S₁', at least in caspase-1. This enzyme had originally been shown to cleave a simple substrate containing an amide at P₁', but not a peptide containing a carboxy group at this position [38], leading to the conclusion that occupancy of primed subsites was unimportant for catalysis. These apparently conflicting observations can be rationalized if one proposes that S₁' occupancy does not increase catalysis, but that certain residues are undesirable due to unproductive interactions with this region of the enzyme. This hypothesis is supported by examination of the caspase side-chains placed within the area that is most likely to interact with the P₁' residue (see Figure 1). Thus the most likely explanation for the preference at this position is discrimination against large bulky amino acid residues combined with a requirement for flexibility of the peptide backbone of the scissile bond.

On the basis of the subsite preferences it may appear relatively easy to predict, or understand, the likely *in vivo* substrates of caspases. For example, the cleavage sites within the human interleukin-1β precursor (FEAD-G and YVHD-A) match the observed subsite preference for caspase-1 based on the present

Figure 1 Graphic representation of the catalytic site of caspase-3 (1pau) [21] (green) in complex with the covalently bound inhibitor Ac-Asp-Glu-Val-Asp-aldehyde (blue)

The catalytic residues Cys²⁸⁵ and His²³⁷ are shown in orange. The atom colours are cyan for nitrogen, red for oxygen and yellow for sulphur. The residues P₄–P₁' (grey) of Eglin (1cse) [41] are docked to caspase-3 by superimposition of the C α atoms of the aldehyde inhibitor. The figure highlights residues that are placed in the vicinity of the putative S_i' binding site. Briefly, the residues at positions 176, 239, 240, 244, 288 and 338 are the most likely to interact with the P_i' side-chain. The acceptance of the large aromatic P_i' substituents is difficult to rationalize based on our current knowledge of the caspase structure, although the hydrophobic patch on the south rim of the active-site cleft (amino acid residues 176 and 244) may accommodate large aromatic residues. Indeed, in the case of caspase-3 one can even conjecture that the aromatic side-chain stacks in between the flexible side-chain of Met¹⁷⁶ and the aromatic Phe²⁴⁴. The discrimination against negatively-charged P_i' residues is much easier to envisage due to the presence of Glu²³⁹ and Glu²⁴⁰ in the vicinity of the putative S_i' pocket. The Figure was prepared using Molscript [42] and rendered using Raster3D [43]. 1pau and 1cse are the Brookhaven Protein Data Bank accession numbers for the three-dimensional structures used in this model.

peptide scanning approach. Similarly, we find that PARP is cleaved at the natural DEVD-G site by caspase-3 with a k_{cat}/K_m of $1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is very close to that of the corresponding peptide substrate Abz-GDEVD-GVY(NO₂)D-OH where we find a k_{cat}/K_m of $2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. In other cases the correlation is far from clear-cut. Many putative substrates have caspase cleavage sites that would not be considered likely candidates based on their amino acid sequence; for example p21 (DHVD-L), nuclear mitotic apparatus protein (DSLDD-L), PKC- θ (DEVD-K), Hsp90 (DEED-D), Bcl-2 (DAGD-V) and NF- κ B (VFDD-L) ([11] and references therein). However, the biological significance of these cleavage events, which are usually analysed by incubation using purified caspase in combination with *in vitro* translated natural and mutated cDNAs, is sometimes difficult to ascertain. As far as we are aware, only in the case of the putative tumour suppressor DCC ('deleted in colorectal cancer') has a non-optimal caspase cleavage site (LSVD-R) been demonstrated to be biologically relevant, since mutation of the aspartic acid residue abrogates the pro-apoptotic function of DCC [39]. Evidence that regions

other than the substrate-binding cleft influence the cleavage of natural substrates comes from the observation that auto-modification of PARP dramatically alters its susceptibility to proteolysis [40]. Thus caspase-7 is much more efficient with auto-modified PARP than caspase-3, despite its lower catalytic efficiency with DEVD-based synthetic substrates. Consequently, the possibility exists that second-site interactions are important for recruiting caspases to sub-optimal cleavage sites, or that the cleavage is intended to occur slowly at the intracellular concentration of the responsible caspase.

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