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Native Chemical Ligation in Covalent Caspase Inhibition by p35

Brief Communication

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Summary

Wide-spectrum caspase inhibition by the baculoviral p35 protein was previously shown to be a consequence of covalent inhibition in which a thioester bond is stably formed between the cleavage residue Asp87 of p35 and the active site Cys360′ of caspase-8. Here we show that the N-terminal fragment of cleaved p35 (p35-N) is a circular peptide when dissociated from the caspase. Biochemical and crystallographic data suggest that p35-N circularization results from the trapping of a native chemical ligation intermediate in the p35/caspase complex, in which the N-terminal Cys2 of p35 attacks the Asp87-Cys360′ thioester to form an equilibrium between Asp87-Cys2 and Asp87-Cys360′. This provides a crucial covalent interaction for keeping the N terminus of p35 bound in the caspase active site, which explains the absolute requirement of Cys2 for caspase inhibition. Participation of native chemical ligation in caspase inhibition by p35 illustrates an unusual mechanism of protease inhibition.

Introduction

Caspases are central effectors of apoptotic cell death. The p35 protein from baculoviruses is an effective caspase inhibitor that prevents host cell death and contributes to the pathogenesis of the viral infection [1–4]. Unlike many protein caspase inhibitors, p35 is a wide-spectrum caspase inhibitor against most, if not all, groups of caspases and blocks apoptosis induced by numerous stimuli and in diverse organisms [3, 5, 6]. These studies have demonstrated that p35 can rescue cells from apoptosis and enable them to revive their normal cellular functions, thereby establishing its promise for controlling apoptosis in degenerative diseases.

The p35 protein contains a solvent-exposed and highly protruding reactive site loop (RSL) that harbors a caspase recognition sequence (DQMD^{87}) [7], and caspase inhibition by p35 correlates with the cleavage at Asp87 by the caspase [1–3]. Our crystal structure of the postcleavage complex between p35 and caspase-8 (see Figure S1A in the Supplemental Data available with this article online) revealed that caspase-8 is inhibited by p35 via a covalent thioester linkage between the active site Cys360′ of caspase-8 (prime [′] is used to denote residues in caspase-8) and the cleavage residue Asp87 of p35 [8]. During normal substrate cleavage, a water molecule is activated by the active site His317′ of caspase-8 to quickly hydrolyze the thioester intermediate. In the p35/caspase-8 complex, the thioester bond is preserved by the N terminus of p35, which interacts with the caspase active site to exclude solvent. Prior to caspase cleavage, the N terminus of p35 is buried in the core of the structure and its repositioning into the caspase active site is realized through a series of dramatic postcleavage conformational changes [8, 9] (Figures S1B and S1C). Structure-based mutational studies have shown that the two N-terminal residues of p35 in direct contact with the caspase, Cys2 and Val3, are critical for caspase inhibition by p35 [8, 10, 11].

The covalent interaction between p35 and the caspase provides a framework for understanding the efficient and wide-spectrum inhibition of caspases by p35, in which the substrate recognition sequence in the RSL acts as the "bait" for a caspase and the N terminus is required to stabilize the complex and "kill" the caspase. However, we were intrigued by a number of unexplained observations. Specifically, residue Cys2 of p35, which is the N-terminal residue in the mature protein, has to be a Cys, as C2G, C2A, and the conserved isosteric C2S mutations all abolish its inhibitory activity [8, 10, 11]. In contrast, a conservative mutation V3I remains active [10]. In addition, the required interaction between the N terminus of p35 and the caspase buries an exceptionally small surface area (~140 Å^2/partner), which is paradoxical to the apparent strength of the inhibition. Finally, the electron density map is continuous from Asp87 to Cys2, which is inconsistent with absence of a covalent bond between the two residues in the original structure.

Here we show that although the N terminus of p35 is not blocked before complex formation with caspase-8, the postcleavage N-terminal fragment of p35 (p35-N, residues 2–87) becomes completely resistant to N-terminal sequencing and therefore appears "blocked." We demonstrate that this resistance to sequencing occurs because p35-N dissociates from the caspase as a circular peptide. This circularization is highly suggestive of a native chemical ligation reaction [12] in which the free N-terminal Cys2 attacks the thioester bond at Asp87 (Asp87-Cys360′), leading to reversible trans-thioesterification followed by a spontaneous irreversible S⇒N acyl shift and the formation of a peptide bond between Asp87 and Cys2. However, in the native p35/caspase-8 complex, both biochemical and structural observations clearly indicate a covalent linkage between p35 and its target caspase, and therefore do not support p35-N existing as an already circularized peptide in the complex. Instead, the data suggest that the native p35/caspase-8 complex traps an intermediate of the native chemical ligation reaction, in which reversible trans-thioesterification leads to an equilibrium between formation and breakage of two thioester bonds, Asp87-Cys2 and Asp87-Cys360′. Importantly, in this complex, the N-terminal amino group is sterically hindered from attacking the Asp87-Cys2 thioester,

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preventing completion of the chemical ligation reaction. Upon denaturation, however, the thioester becomes accessible to the Cys2 amino group and rearranges to form the circular peptide. This revised model of caspase inhibition by p35 reveals that the N terminus of p35 utilizes a covalent mechanism to remain in the caspase active site and contributes to caspase inhibition. It explains each of the intriguing and unexplained observations described above and provides additional novel insights into caspase inhibition by p35 as well as protease inhibition in general.

Results

p35 Becomes Resistant to N-Terminal Edman Sequencing after Complex Formation with a Caspase

Ever since the original report on the caspase inhibitory activity of p35, it has been noted that p35 could not undergo N-terminal Edman sequencing [1, 3, 8, 11]. Although these experiments were performed on the p35-N fragment after p35 forms a complex with and gets cleaved by a target caspase, it was assumed that the N terminus of full-length p35 is also blocked, for example, by acetylation.

Our site-directed mutational studies showed that p35 mutations such as C2S completely abolished the inhibitory activity of p35 against caspase-3 or caspase-8 and prevented the formation of a covalent complex with either of the target caspases [10]. Because the N terminus of p35 directly contacts the caspase active site and N-terminal modifications often vary with the nature of the N-terminal residue, we performed N-terminal sequencing on the C2S mutant to determine whether its N terminus is still blocked and whether the mutational effect may be due to a change of N-terminal modification. To our surprise, the C2S mutant of full-length p35 could be N-terminally sequenced (Table 1), indicating that the N terminus is not blocked. In addition, to our further surprise, even wild-type full-length p35 could be N-terminally sequenced, demonstrating the absence of covalent modification that prevents Edman degradation. Therefore, the earlier assumption that the N terminus of p35 is modified is incorrect. Rather, it is the p35-N fragment generated after complex formation with a caspase that is resistant to N-terminal sequencing (Table 1). This resistance is not simply due to caspase cleavage, because the C2S mutant of p35 can still be cleaved by caspase-8 but its p35-N fragment is not resistant to Edman sequencing.

Table 1. Edman Sequencing of p35 and p35-N

p35 or Its Fragment p35-N	Edman Sequencing
p35	
Wild-type	Sensitive
C2S	Sensitive
p35-N	
Wild-type	Resistant
C2S	Sensitive
Wild-type, 10 mM DTT	Resistant
Wild-type, 100 mM DTT	Resistant
Wild-type, 10 mM cysteine	Resistant
Wild-type, 100 mM cysteine	Resistant
p35-N with CNBr Treatment	
Fragment D87–V7	Present
Fragment N36–P42	Present
Fragment M46–P52	Present
Fragment F47–I53	Present

Protein expression and purification were performed as described previously [8, 10]. All Edman sequencing experiments were performed on an Applied Biosystems Procise 494/HT protein sequencer (Foster City, CA), with cyanogen bromide (CNBr) digestion performed similarly as described previously [16].

Native Chemical Ligation Chemistry in Caspase Inhibition by p35: The p35-N Fragment Is a Circular Peptide

The resistance of p35-N, but not p35, to Edman sequencing is intriguing. Because the thiol side chain of the N-terminal Cys2 residue is absolutely required for caspase inhibition by p35, we contemplated the possibility that the thiol group may participate in a chemical reaction that is required for both the stabilization of the postcleavage p35/caspase complex and the modification of the p35 N terminus.

In the p35/caspase-8 complex, the cleavage residue Asp87 of p35 is linked to the active site Cys360′ of caspase-8 via a thioester bond, which is protected from hydrolysis due to elimination of solvent accessibility by the N terminus of p35 [8]. The repositioning of the N terminus of p35 into the active site could allow the thiol side chain of Cys2 to attack the thioester bond in the same fashion that would occur in native chemical ligation [12, 13]. Two steps are involved in native chemical ligation. First, the thiol of an N-terminal Cys residue attacks a C-terminal thioester, leading to a reversible trans-thioesterification. Second, the free amino terminus attacks the thioester to induce an irreversible S⇒N acyl shift, leading to formation of a peptide bond between the C-terminal residue of the thioester and the attacking N-terminal Cys. For the p35-N fragment, such a reaction would produce a circular peptide by linking Asp87 and Cys2 via a peptide bond and would explain the consequent resistance of p35-N to Edman sequencing.

To determine whether wild-type p35-N is circular, we first attempted mass spectrometry analysis of tryptic fragments of p35-N. Although the masses of the observed fragments are consistent with the sequence of p35-N, a crucial anticipated fragment, corresponding either to a linear p35-N (residues Cys2–Arg20) or to a circular p35-N (residues Asp81–Arg20), could not be recovered. We therefore took an alternative approach using cyanogen bromide (CNBr) digest and Edman N-terminal sequencing. We subjected the p35/caspase-8 complex to SDS-PAGE followed by blotting onto a PVDF membrane (Figure 1A). The protein band corresponding to p35-N was excised and submitted for N-terminal sequencing, with and without pretreatment by CNBr that cleaves proteins after Met residues (Figure 1B).

As expected, N-terminal sequencing of p35-N without CNBr treatment did not yield any amino acids (Figure 1C). With CNBr treatment, if p35-N is a circular peptide, a unique sequence of D87-C2-V3-I4-F5-P6-V7 should be revealed. Indeed, this sequence was clearly present in the N-terminal sequencing reaction of CNBr-treated p35-N, in addition to the expected N termini from other regions of p35-N (Figures 1D–1F; Table 1). This

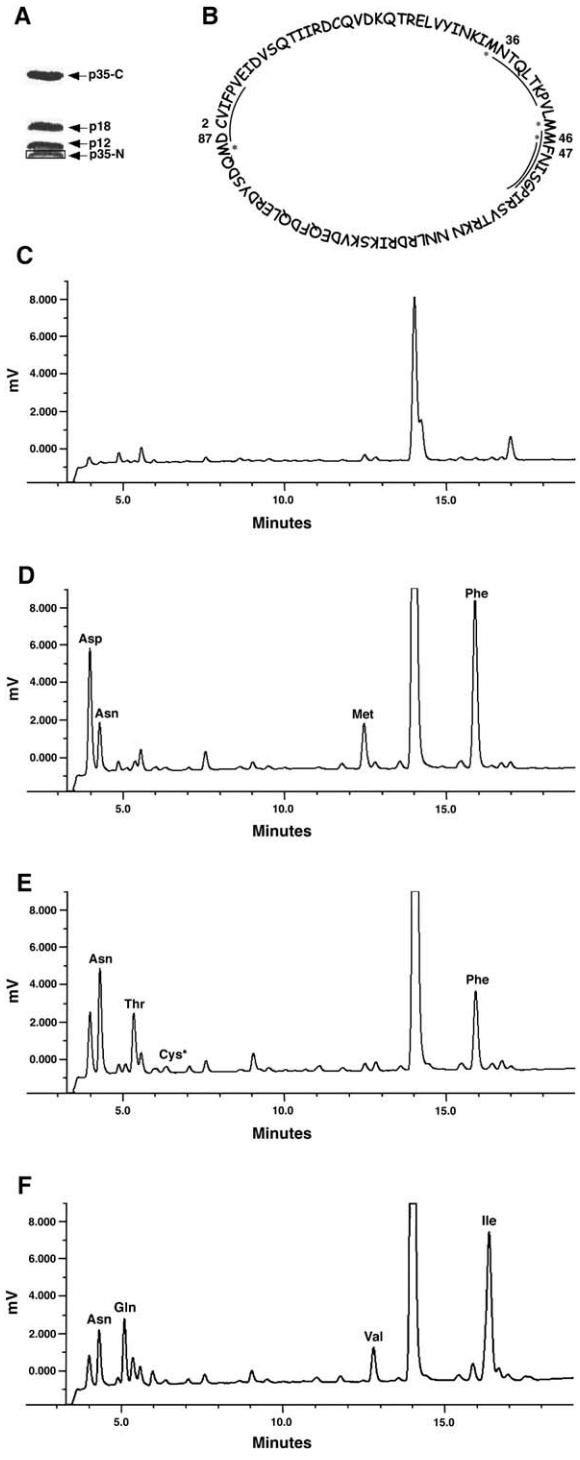

Figure 1. Edman Sequencing of CNBr-Treated p35-N

(A) SDS-PAGE of p35/caspase-8 complex, showing the band corresponding to p35-N. p35-C is the C-terminal fragment of cleaved p35, and p18 and p12 are the large and small subunits of caspase-8, respectively.

(B) Sequence of p35-N (residues 2–87) and expected CNBr treatment fragments if p35-N is circular. Residue numbers for selected residues are labeled.

(C) Chromatogram for the first cycle of Edman sequencing of p35-N.

(D) Chromatogram for the first cycle of Edman sequencing of CNBr-treated p35-N. Asp87 will only be present if p35-N is circular because otherwise it would have been released during CNBr digest.

(E) Chromatogram for the second cycle of Edman sequencing of CNBr-treated p35-N, showing the presence of an acrylamide derivative of Cys (labeled as Cys*).

(F) Chromatogram for the third cycle of Edman sequencing of CNBr-treated p35-N.

In (D)–(F), amino acid residues in each of the four expected CNBr-derived fragments are labeled.

demonstrates that p35-N is circular, linked via a peptide bond between Asp87 and Cys2.

Although p35-N is circular upon dissociation from the caspase, the native p35/caspase-8 complex appears to trap an intermediate in the first step of native chemical ligation, which leads to p35-N circularization upon complex denaturation and dissociation. In such an intermediate, the thiol group of Cys2 would react with the Asp87-Cys360′ thioester to establish an equilibrium between the Asp87-Cys2 and Asp87-Cys360′ thioesters. Notably, in native chemical ligation reactions, formation and breakage of the equivalent thioesters are reversible.

To exclude the possibility that generation of the circular peptide takes place in solution after denaturation via an attack of Cys2 on the Asp87-Cys360′ thioester, we used high concentrations of free thiols to compete with the reaction. We treated the p35/caspase-8 complex with 10 mM or 100 mM dithiothreitol (DTT) or cysteine in SDS buffer, boiled the samples for 10 min, separated the samples on an SDS-PAGE, and blotted the samples to a PVDF membrane. The p35-N bands remained resistant to Edman sequencing even at 100 mM DTT or cysteine (Table 1), showing that the circularization was not inhibited by free thiols. This suggests that the Asp87-Cys2 thioester intermediate is already formed in the native p35/caspase-8 complex, which is trapped because the acyl shift required for peptide bond formation in the second step of the reaction is prevented, as described below.

Continuous Electron Density Supports Covalent Interaction between the p35 N Terminus and Residue Asp87 in the Native p35/Caspase-8 Complex

The crystal structure of the p35/caspase-8 complex [8] supports the presence of a covalent interaction between the p35 N terminus and the p35 cleavage residue Asp87, as would be expected in a trapped thioester intermediate state of native chemical ligation. The p35/caspase-8 structure was originally determined by molecular replacement using uncleaved p35 and caspase-8 as models [7, 8]. Based on the initial electron density map, calculated without the region of the RSL around the cleavage residue Asp87 (Δ81–95) and with the N-terminal residues still buried in the core of p35, and a simulated annealing omit electron density map, Asp87 is clearly connected to both Cys360′ of caspase-8 and Cys2 at the N terminus of p35. Although the continuity of the electron density at 3.0 Å resolution does not unambiguously demonstrate the covalent interaction, the combined biochemical and crystallographic data compellingly indicate the existence of a thioester bond between Asp87 and Cys2. Indeed, the electron density can be easily modeled as the equilibrium between Asp87-Cys2 and Asp87-Cys360′ thioester bonds (Figures 2A and 2B).

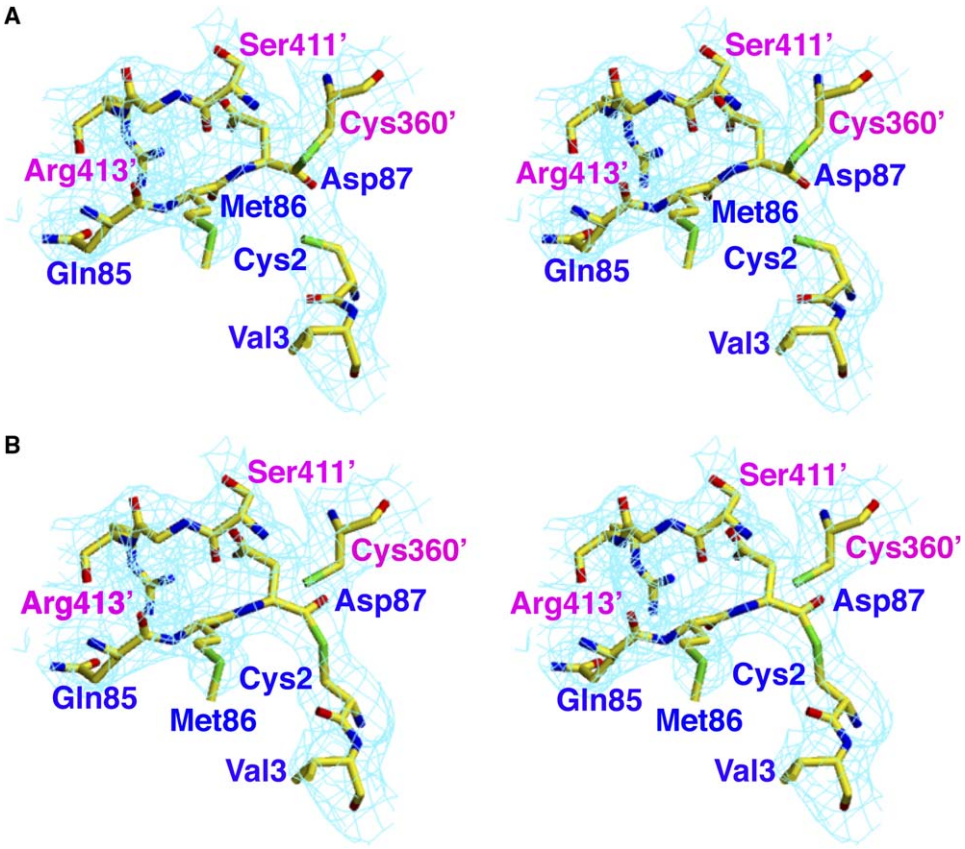

Figure 2. Stereo Diagrams of Modeled Reversible Trans-Thioesterification, the Native Chemical Ligation Intermediate Trapped in the p35/Caspase-8 Complex

The thioesters, Asp87-Cys360′ (A) and Asp87-Cys2 (B), are shown in stick models with several adjacent residues and superimposed with the simulated annealing omit map contoured at 1σ. Selective residues are labeled in magenta and blue for caspase-8 and p35, respectively. Note the continuity of the electron density from Asp87 to Cys2 of its N terminus and to Cys360′ of caspase-8.

The Native p35/Caspase-8 Complex Prevents Irreversible Acyl Shift and Completion of the Native Chemical Ligation Reaction

Analysis of the crystal structure of the p35/caspase-8 complex reveals that once attacked by the thiol of Cys2, the carbonyl carbon of Asp87 is no longer accessible to the free amino group of Cys2. This makes the free amino group sterically unable to attack the Asp87-Cys2 thioester, much in the same way that water is unable to attack the Asp87-Cys360′ bond. Because attack by the free amino group induces the S⇒N acyl shift, which is absolutely required for the second step of the chemical ligation reaction, steric hindrance of such an attack explains why circularization of p35-N cannot proceed within the complex, and only occurs upon complex denaturation.

Discussion

The discovery that p35-N is a circular peptide reveals the involvement of native chemical ligation chemistry in caspase inhibition by p35. In this model, three chemical steps are involved in the formation and dissociation of the p35/caspase complex (Figure 3). First, a target caspase recognizes the substrate sequence in the RSL of p35. The active site Cys of the caspase (Cys360′ in caspase-8) forms a thioester intermediate with the cleavage residue Asp87. The portion of the RSL beyond Asp87 dissociates from the caspase, leading to dramatic conformational changes in p35. Second, as a result of the p35 conformational change, the previously buried p35 N terminus is liberated and finds its way into the active site of the caspase. There, a native chemical ligation reaction involving the Asp87-Cys360′ thioester and the N-terminal Cys2 is trapped at the thioester equilibrium intermediate step. At this point, Asp87 is inaccessible to the free amino group of p35-N, preventing p35-N circularization. The Asp87-Cys2 thioester provides a crucial covalent interaction for keeping the N terminus of p35 in the caspase active site. Finally, upon denaturation of the p35/caspase complex, the free amino group of Cys2 is able to participate in the S⇒N shift required for peptide bond formation between Asp87 and Cys2. This model explains each of the three previously unexplained intriguing observations on caspase inhibition by p35: (1) the absolute requirement for Cys2, (2) the continuous electron density from Asp87 to Cys2, and (3) the exceptionally small surface area of interaction at the p35 N terminus.

Previously we, as well as others, have shown that under mild SDS treatment in the absence of reducing agent and at lower pH, p35-N is present both as an SDS-stable adduct with the large subunit of the caspase and as a dissociated peptide [8, 11]. It is likely that in these

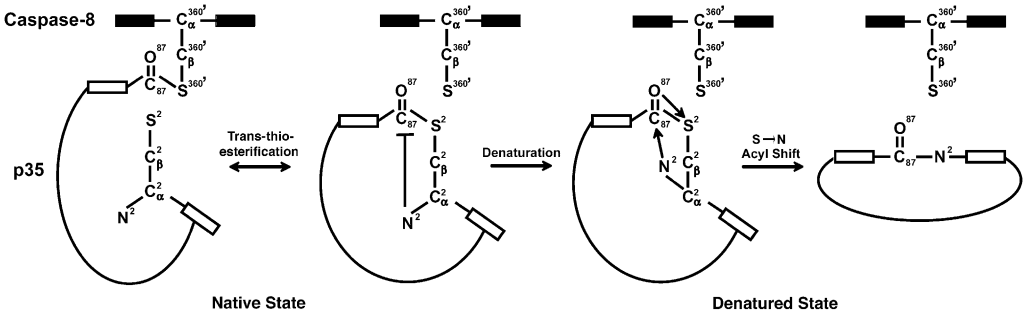

Figure 3. Native Chemical Ligation in the Native and Denatured States of the p35/Caspase-8 Complex

Residue numbers are labeled to the top right or bottom right of each atom, and hydrogen atoms are omitted from the diagram. Caspase-8 is shown by filled rectangles, while p35 is shown by open rectangles. Curved lines represent the protein sequences between Cys2 and Asp87 in p35-N. In the native state, the free amino group at the N terminus of p35-N is inaccessible to Asp87.

SDS-PAGE samples, the partition between the adduct and the isolated p35-N is reflective of the native thioester equilibrium between the p35-N/caspase thioester and the p35-N circular thioester that rearranges to form a circular peptide. Consistent with this observation, the p35-N band in these mildly treated samples is also resistant to Edman sequencing and is circular (data not shown).

If in the p35/caspase complex the thioesters between Asp87 and Cys360′ or Cys2 are in equilibrium, why is it that the complex does not fall apart when the Asp87-Cys360′ bond breaks transiently and why does the N terminus not leave the active site when the Asp87-Cys2 bond breaks transiently? Presumably the fast rate of the reversible chemical reaction disallows dissociation of either the N terminus or the caspase recognition sequence of p35 from the caspase active site. These p35 segments are temporarily held in place, albeit weakly, by noncovalent interactions. However, the inhibitory reaction does exhibit leakage, in which dissociation of a fraction of the complexes may occur stochastically [3].

Because the Asp87-Cys2 and Asp87-Cys360′ thioesters interconvert through a tetrahedral intermediate, is it possible that this is the trapped state in the p35/caspase-8 complex? Our experimental data do not distinguish this possibility from the thioester equilibrium because both would have been consistent with the continuity of the electron density. The choice of thioester equilibrium over tetrahedral intermediate is based primarily on what we know about protein structure and function. Proteins do not appear to have the ability to stabilize tetrahedral intermediates of any kind, and a stable tetrahedral intermediate has never been observed in any crystal structure. The closest example might be the cryogenic trapping of a transient tetrahedral intermediate in the hydrolysis of the acyl-enzyme complex of elastase with β-casomorphin-7, in which the tetrahedral state lasts less than 2 min after initiation of the reaction [14].

Significance

Native chemical ligation chemistry is now frequently used in vitro as a semisynthetic technique to ligate peptide segments containing fluorophores, stable isotopes, or unnatural amino acids to recombinantly expressed proteins via a peptide bond [12, 13]. In vivo, native chemical ligation is utilized as part of the chemistry of posttranslational protein splicing, a natural process identified in many organisms [15]. In protein splicing, an intein in a polypeptide chain is excised and two exteins upstream and downstream of the intein are joined [15]. The revelation of a novel role for native chemical ligation in caspase inhibition by p35 further demonstrates the power of this chemistry and adds to our understanding of the wide repertoire of modes of protease inhibition.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.chembiol.com/cgi/content/full/13/2/117/DC1/.

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Accession Number

The p35/caspase-8 complex structure has been deposited in the Protein Data Bank under ID code 1FUN.