Inactivation of Subtilisin Carlsberg by *N*-((*tert*-Butoxycarbonyl)alanylprolylphenylalanyl)-*O*-benzoylhydroxylamine: Formation of a Covalent Enzyme–Inhibitor Linkage in the Form of a Carbamate Derivative[†,‡]

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ABSTRACT: The mechanism of inactivation of serine proteases by *N*-peptidyl-*O*-aroylhydroxylamines was studied by X-ray crystallography. Cocrystals of subtilisin Carlsberg inactivated with *N*-((*tert*-butoxycarbonyl)alanylprolylphenylalanyl)-*O*-nitrobenzoylhydroxylamine were grown, and diffraction data to 1.8-Å resolution were obtained. The resulting electron density maps clearly reveal that the γ-oxygen of the catalytic serine forms a carbamate derivative with the inhibitor. The peptide part of the inhibitor does not form the usual antiparallel β-sheet in the P binding cleft but protrudes out of the active site and is stabilized by a network of water molecules. These results, combined with kinetic characterization reported previously [Demuth, H.-U., Schoenlein, C., & Barth, A. (1989b) *Biochim. Biophys. Acta 996*, 19–22; Schmidt, C., Schmidt, R., & Demuth, H.-U. (1990) *Peptides* (Giralt, E., & Andreu, D., Eds.) ESCOM Science Publishers B.V., Amsterdam] support the existence of at least one intermediate between the formation of the Michaelis complex and the final product. We suggest a mechanism for the inactivation of subtilisin Carlsberg by *N*-((*tert*-butoxycarbonyl)alanylprolylphenylalanyl)-*O*-benzoylhydroxylamine whereby a negatively charged Michaelis complex undergoes a Lossen rearrangement giving rise to an isocyanate intermediate that reacts with the side chain of the active site serine.

N-Peptidyl-*O*-aroylhydroxylamines[1] are potent inhibitors for members of the serine protease as well as cysteine protease classes of enzymes. Demuth and co-workers (1988) introduced them as inhibitors for the serine protease dipeptidyl peptidase IV and showed that these compounds are mechanism-based inhibitors of serine proteases of different structural families (Demuth et al., 1989a), including subtilisin and thermitase from the subtilisin family and elastase from the chymotrypsin family. The tertiary structure of dipeptidyl peptidase IV is not yet known, but it is considered to be a member of an independently evolved family of serine proteases, which includes proline specific endopeptidase, on the basis of their primary structures (Rawlings et al., 1991; Polgar, 1992). Further investigations have shown that peptidyl hydroxylamine derivatives also inactivate cysteine proteases in a mechanism-based manner (Smith et al., 1988; Broemme et al., 1989; Robinson et al., 1991; Demuth et al., 1991).

It may not be too surprising that both serine and cysteine proteases are inactivated by the same type of compound, since they appear to catalyze the hydrolysis of peptide bonds by a more or less similar mechanism involving stabilization of an acyl or thioacyl derivative, derived from a tetrahedral intermediate during substrate turnover. Both types of protease form this intermediate through nucleophilic attack of the γ-oxygen of serine (γ-sulfur of cysteine) at the carboxyl carbon of the scissile peptide bond. The loss of the hydroxyl (sulfhydryl) proton is facilitated by transfer to the nitrogen $N_{\epsilon 2}$ of a histidine imidazole in concomitant (serine proteases) or subsequent (cysteine proteases) steps. Three-dimensional structures of members of both classes have identified a so-called oxyanion hole by which the developing negative charge on the carboxyl oxygen of the scissile peptide bond is stabilized in the transition state. Furthermore, it had already been observed that proteases of both classes are inhibited by the same types of compounds, e.g., chymotrypsin, subtilisin, and papain are all irreversibly inactivated by peptidyl chloromethyl ketones. However, differences in the active site and the nucleophilicity of the serine and cysteine heteroatoms cause the final inactivated complexes to be different. The inhibitor is covalently bound to the catalytic histidine of chymotrypsin (Schoellmann & Shaw, 1963) and subtilisin BPN′ (Robertus et al., 1972), whereas it is covalently linked to the active site cysteine of papain (Drenth et al., 1972).

The mechanism-based inactivation of enzymes requires that the inactivator[1] be a relatively inert compound in solution that is transformed in the active site of the target enzyme into a highly reactive species, which then reacts irreversibly with the enzyme itself (Abeles, 1983). It is the general assumption that the pathway leading to the actual inactivating species is very closely related to the mechanism of substrate turnover. Examples of this type of mechanism-based inactivation include the inactivation of α-chymotrypsin by 3-benzyl-6-chloro-2-pyrone (Ringe et al., 1986), α-chymotrypsin by 3-benzyl-*N*-((methylsulfonyl)oxy)succinimide (Groutas et al., 1989), and

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[‡] The coordinates of the subtilisin–inactivator complex are available from the Brookhaven Protein Data Bank under reference number 1SCN.

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[1] We refer in this article to the *N*-peptidyl-*O*-aroylhydroxylamines as "inactivator" because they form a stable covalent bond with the enzyme. We use the term inhibitor in the generic sense and, in particular, for eglin c, which is a competitive inhibitor of subtilisin.

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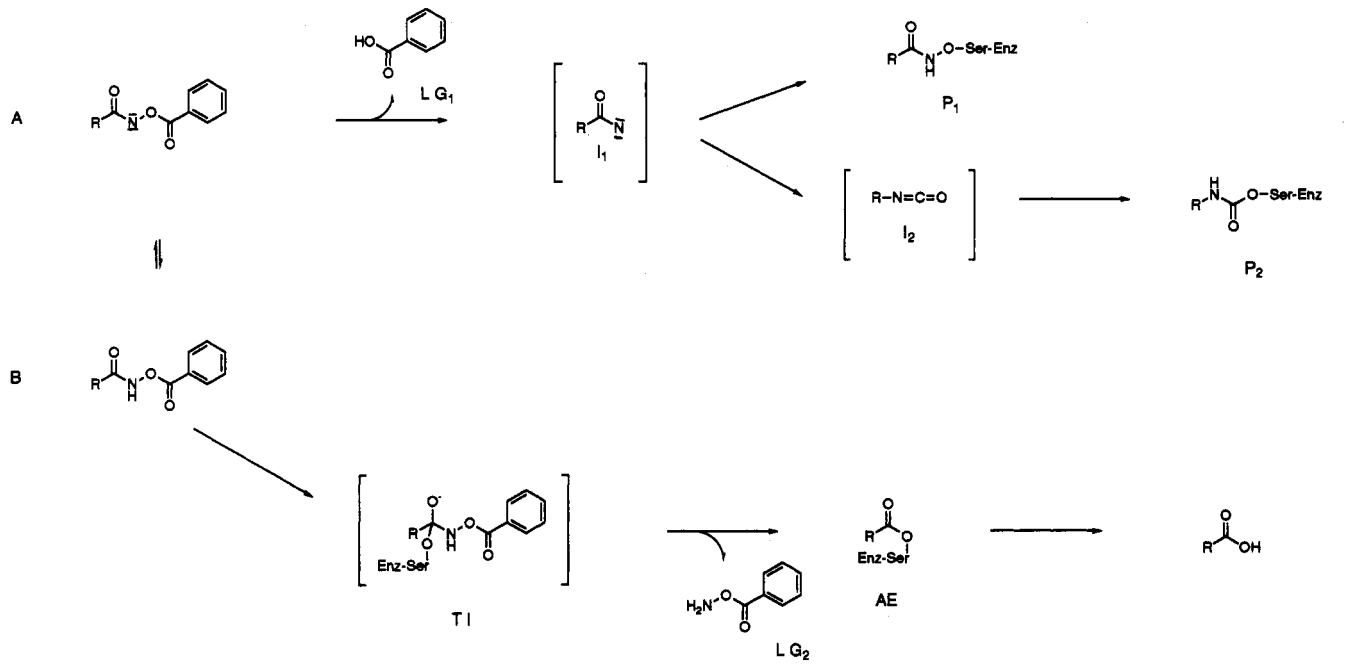


FIGURE 1: Suggested mechanisms for the inactivation of serine proteases by *N*-peptidyl-*O*-aroylhydroxylamines and enzyme-catalyzed turnover of the *N*-peptidyl-*O*-aroylhydroxylamine without inactivation of the protease.

human leukocyte elastase by cephalosporin derivatives (Knight et al., 1992). However, there is no guarantee that the catalytic apparatus is used in the same fashion as for real substrates. The kinetic characteristics that classify an inhibitor as a suicide substrate do not require that analogous intermediates are formed as for substrate hydrolysis, that is, in the case of these proteases, that the equivalent of an acylenzyme or of a tetrahedral intermediate are formed in the process of inactivation. This does not exclude, however, the possibility that such analogous intermediates can be formed between the enzyme and the compound, which then break down without causing inactivation. Consequently, the ratio of hydrolysis (k_{cat}) to inactivation (k_{inact}) has been used as a measure of the efficiency of an inactivator (Westkaemper & Abeles, 1983). For instance, dipeptidylpeptidase IV has a k_{cat}/k_{inact} ratio in the range of 10^4–10^6 for the reaction with *N*-peptidyl-*O*-aroylhydroxylamines, which indicates that inactivation is relatively inefficient, compared to subtilisin for which this ratio for the same compounds is in the range of 10–200 (Demuth et al., 1988).

In order to determine the mechanism of inactivation of the serine proteases by *N*-peptidyl-*O*-aroylhydroxylamines, it is imperative to establish the chemical structure of the product. Subtilisin forms an irreversibly inactivated adduct with this type of compound. Mechanistic characterization so far indicates the formation of an enzyme–inactivator complex which breaks down to form an irreversible enzyme adduct (Demuth et al., 1989b). Neither the nature of the inactivated complex nor the adduct itself have been established, including the identity of the residue(s) forming the adduct.

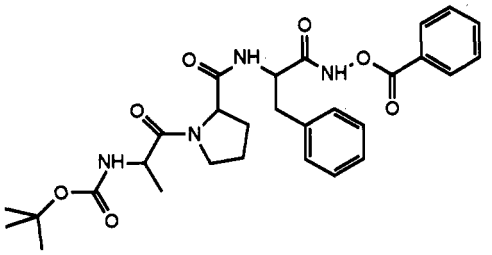
A number of possible mechanisms have been proposed for the reactions of these inactivators with serine and cysteine proteases, accounting for two isomeric complexes as the possible adducts (Demuth et al., 1988) (Figure 1). In one case, displacement of the leaving group by the γ-oxygen of serine leads to a hydroxylamine derivative (Figure 1, P_1). In the other case α-elimination of the leaving group is followed by a Lossen-type rearrangement of the atoms of the hydroxylamine functional group and covalent linkage to the γ-oxygen of serine to form a carbamate derivative (Figure 1, P_2). There is some evidence for the first mechanism from an NMR study of the inactivation of papain by these compounds (Robinson et al., 1991). The results of this study indicate that the sulfur of the active site cysteine is covalently linked to the hydroxylamine nitrogen of the inhibitor, thus forming a thiohydroxylamine adduct. The leaving group of the inactivator is replaced by the enzyme, and no rearrangement of the atoms of the hydroxylamine functional group takes place (Robinson et al., 1991; Menard et al., 1991). Such a study has not yet been carried out for any of the serine proteases. However, a mass spectroscopic study with subtilisin Carlsberg inactivated by Boc-Ala-Pro-Phe-NHO-Bz[2] shows that the leaving group benzoic acid is not part of the inactivated complex, but that the remaining portion of the inactivator—including the hydroxylamine nitrogen—is contained in the final adduct (H.-U. Demuth, manuscript in preparation).

The purpose of this study is to establish the final product of inactivation of subtilisin by a *N*-peptidyl-*O*-aroylhydroxylamine and thereby determine the mechanism by which this reaction occurs. Solution studies have not established with which residue of the enzyme the inactivator forms a covalent linkage. When we started this study, we did not exclude the possibility that a residue other than serine is involved in the formation of the covalent bond. Therefore, we determined the structure of the final complex of subtilisin Carlsberg inactivated by *N*-((*tert*-butoxycarbonyl)alanylprolylphenyl)-*O*-benzoate hydroxylamine by X-ray crystallography. The resulting electron density maps reveal unambiguously that the covalent linkage forms only with the side chain oxygen of the active site serine and as a carbamate.

MATERIALS AND METHODS

The inactivator Boc-Ala-Pro-Phe-NHO-Bz (Figure 2A) was synthesized and characterized in earlier studies (Demuth et al., 1989b). Freeze-dried subtilisin Carlsberg was purchased from Sigma and used without further purification.

[2] Abbreviations: Boc-Ala-Pro-Phe-NHO-Bz = *N*-((*tert*-butoxycarbonyl)alanylprolylphenylalanyl)-*O*-benzoylhydroxylamine.



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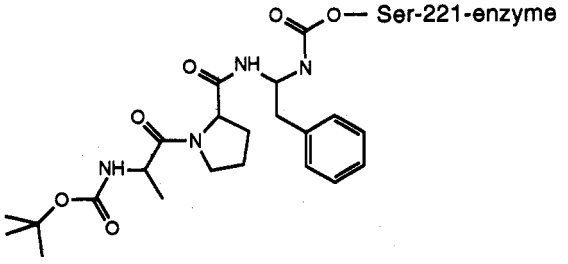

B

FIGURE 2: Chemical structures of the inactivator *N*-((*tert*-butoxycarbonyl)alanylprolylphenylalanyl)-*O*-benzoylhydroxylamine (A) and of the enzyme adduct, (*tert*-butoxycarbonyl)alanylprolyl-*N*-(1-amino-2-phenyl)ethylcarbamoyl ester of the catalytic Ser-221 (B).

Subtilisin Carlsberg was completely inactivated by incubation with the inactivator for 90 min at room temperature in 0.1 M Hepes buffer, pH 7.5, being 10% (v/v) in DMF and 0.01 M in inactivator and containing 0.62 mg/mL enzyme. The buffer was exchanged for crystallization buffer (20 mM sodium cacodylate, pH 5.6, 18% sodium sulfate) and the protein concentrated to 5 mg/mL by centrifugation with a Centricon 10 filter unit. The protein concentration was determined assuming $E_{1\%}^{280} = 0.96$ for subtilisin. The inactivation was verified by an activity assay at pH 8.6 with succinylalanylalanylprolylphenylalanyl-4-nitroanilide as substrate. The assay solution contained 1 μmol/mL substrate and 0.055 μg/mL enzyme in 0.1 M Tris-HCl buffer, pH 8.6. No residual activity for the inactivated enzyme sample was detected (level of detection: 0.5%).

Crystals were grown by batch precipitation (Petsko & Tsernoglou, 1976). Needles appeared after 10–14 days and grew to 0.02 × 0.05 × 1.5 mm³. The crystals belong to space group $P2_12_12_1$ with unit cell dimensions of 76.5, 55.4, and 53.4 Å (Table 1). Data were collected to 1.8-Å resolution at 4 °C from two crystals on a Xentronics area detector using Nickel-filtered Cu Kα radiation from an Elliott rotating anode generator (25 kV, 30 mA) and reduced with the program XDS (Kabsch, 1988). The two data sets were scaled together and merged by the program XSCALE (Kabsch, 1991), retaining all data. The overall R_{merge} of the scaled and merged data set is 11.5% on intensity. The overall completeness of the data set to 1.9 Å is 87.6% with additional data in the shell between 1.9- and 1.8-Å resolution (37.4% completeness).

Refinement was carried out with the program package TNT (Tronrud et al., 1987). The initial phases were calculated from coordinates of native subtilisin Carlsberg refined at 2.5-Å resolution (Neidhard et al., 1988). This model contained no water molecules and one calcium ion. Application of these phases to the data from 20- to 3-Å resolution resulted in an initial *R* factor of 38%. Ten sessions of iterative rebuilding and refining were performed so that all together 93 cycles of TNT were used. The curvature method was used for the first nine sessions; the conjugate gradient method was applied for the last session. In the first session the data from 20- to 3-Å resolution were used for positional refinement with an overall *B* factor of 9 Å², resulting in an overall *R* factor of 27.0%. A difference Fourier electron density map with coefficients $2F_o - F_c$ was calculated to which the model of the protein was fitted. There was electron density for the inactivator in the active site that clearly indicated a covalent linkage of the inactivator to the γ-oxygen of Ser-221 and that also showed the position of the phenyl ring of phenylalanine. Twenty water molecules were inserted by the program Waterhunter (Sugio, unpublished), and the resolution range extended to 2 Å resulting in an *R* factor of 24.8% after further refinement. For the third session, 40 further water molecules were selected, and all the data to 1.8-Å resolution were included. The overall *R* factor dropped to 22.0%. The extra density in the active site was clear and a model of the inactivator was built into it.

Table 1: Data Collection and Refinement Statistics for the Determination of the Structure of Subtilisin Carlsberg Inactivated with Boc-Ala-Pro-Phe-NHO-Bz

Data Collection	
space group	$P2_12_12_1$
unit cell dimensions (Å)	$a = 76.5$, $b = 55.4$, $c = 53.4$
number of crystals	2
molecules per asymmetric unit	1
data collection device	Siemens area detector
temperature during data collection	4 °C
number of compared reflections	38 539
number of unique reflections	17 548
resolution	1.9 Å
R_{merge}	11.5% (I)
completeness	87.6%
Refinement	
resolution range	20.0–1.8 Å
final *R* factor	19.1%
number of water molecules in final structure	142
number of cations in final structure (Ca^{2+}/Na^{2+})	2/1
restraints (*s* applied) rms observed	
bond lengths (0.02) (Å)	0.01
bond angles (3.0) (deg)	3.2
planar groups (0.02) (Å)	0.02

To distinguish between the two proposed inactivated complexes, that is, between a carbamate and a hydroxylamine derivative, we built the inactivator into the density manually as either isomer. The model of a carbamate derivative fits the electron density much better than that of an hydroxylamine derivative. The subsequent refinement of both models discriminated decisively between the two isomers by the following method. The standard geometric restraints (Table 2) for the carbamate and hydroxylamine linkages were derived from standard bond lengths and angles for small molecules (CRC Handbook of Chemistry and Physics, 1970/71). The geometric restraint on planarity of the carboxyl carbon of the carbamate was set very loose so that it would not be forced into an sp^2 conformation by this restraint. The model of this isomer accounted for all of the electron density, and the atoms were well centered. The observed data refined the carboxyl carbon of the carbamate linkage into the sp^2 conformation. In contrast, retaining the trigonal-planar geometry with appropriate bond lengths and angles during the refinement gave a model for the hydroxylamine linkage that does not fit

Table 2: Standard Geometric Restraints for Special Linkages in Refinement with TNT

		Carbamate Linkage			
bond length	Ser-221 C	Ser-221 O_γ			1.36 Å
bond length	Ape-450 C	Ape-450 O			1.23 Å
bond length	Ape-450 C	Ape-450 N_{hy}			1.32 Å
bond length	Ape-450 N_{hy}	Ape-450 C_1			1.47 Å
bond angle	Ape-450 O	Ape-450 C	Ape-450 N_{hy}		123°
bond angle	Ape-450 O	Ape-450 C	Ser-221 O_γ		124°
bond angle	Ape-450 N_{hy}	Ape-450 C	Ser-221 O_γ		113°
bond angle	Ape-450 C	Ser-221 O_γ	Ser-221 C_β		120°
bond angle	Ape-450 C	Ape-450 N_{hy}	Ape-450 C_1		113°
bond angle	Ape-450 N	Ape-450 C_1	Ape-450 N_{hy}		112°
bond angle	Ape-450 N_{hy}	Ape-450 C_1	Ape-450 C_β		111°
planar group[a]	Ape-450 O	Ape-450 C	Ape-450 N_{hy}	Ser-221 O_γ	4 Å
		Hydroxylamine Linkage			
bond length	Phe-450 C	Phe-450 N_{hy}			1.33 Å
bond length	Phe-450 N_{hy}	Ser-221 O_γ			1.46 Å
bond angle	Phe-450 O	Phe-450 C	Phe-450 N_{hy}		120°
bond angle	Phe-450 O	Phe-450 C	Phe-450 C_α		120°
bond angle	Phe-450 N_{hy}	Phe-450 C	Phe-450 C_α		120°
bond angle	Phe-450 C	Phe-450 N_{hy}	Ser-221 O_γ		120°
bond angle	Phe-450 N_{hy}	Phe-450 O	Phe-450 C_β		110°
planar group	Phe-450 N_{hy}	Phe-450 O	Phe-450 C	Phe-450 C_α	4 Å

[a] This restraint was kept very loose to not force the carboxyl carbon atom in a sp^2 conformation.

the observed electron density.

During the last seven sessions of iterative refining and manual rebuilding of the carbamate model, additional water molecules were selected and two further putative cation binding sites were identified. The geometry of the protein and the inactivator was constrained so that the root-mean-square deviation was equal to the applied sigma of the various geometric restraints. The overall final R factor is 19.1%, calculated with a model that included the protein, three metal ions (two calcium and one sodium ion), 142 water molecules, and the atoms of the (*tert*-butoxycarbonyl)alanylprolylphenylalanyl group and the hydroxylamine nitrogen of the inactivator. The *tert*-butoxycarbonyl group is too disordered to allow it to be positioned precisely. The model does not contain any atoms for the leaving group, nor is there any density to indicate the presence of such a group.

In order to confirm the results of the refinement, omit-maps were calculated: the atoms of the inactivator were taken out of the model, and three cycles of the conjugate gradient method of TNT were applied. Difference Fourier electron density maps with coefficients $F_o - F_c$ and $2F_o - F_c$ revealed the same isomer with the same conformation for the inactivator as modeled before. In order to verify the second and third metal ion binding sites additional, omit-maps were calculated. The metal ion in question and all residues, including water molecules, that had atoms within 5 Å were deleted from the model. Thus residues Ala-37–Leu-42 and two water molecules in the case of the second metal ion binding site and residues Ala-169–Ala-176, Glu-195–Glu-197, and three water molecules around the third metal ion binding site were deleted. A random error was introduced into the coordinates by the program Shakeup (Lavie, unpublished) and the omit-model rerefined. Subsequent difference Fourier electron density maps with coefficients $F_o - F_c$ were calculated with each truncated model. These maps confirmed unambiguously the previously obtained results. The final refined bond lengths and angles for the carbamate group of the inactivator are given in Table 3. Metal ion to ligand distances for the three metal ion binding sites are given in Table 4.

Table 3: Final Refined Bond Lengths and Bond Angles of the Covalent Linkage between Inactivator and Subtilisin Carlsberg (Carbamate Derivative)

	Bond Lengths		
Ser-221 O_γ	Ape-450 C		1.45 Å
Ape-450 C	Ape-450 O		1.23 Å
Ape-450 C	Ape-450 N_{hy}		1.42 Å
Ape-450 N_{hy}	Ape-450 C_1		1.59 Å
	Bond Angles		
Ser-221 C_β	Ser-221 O_γ	Ape-450 C	128°
Ser-221 O_γ	Ape-450 C	Ape-450 O	116°
Ser-221 O_γ	Ape-450 C	Ape-450 N_{hy}	120°
Ape-450 C	Ape-450 N_{hy}	Ape-450 C_1	117°

Table 4: Ligation Distances in Metal Ion Binding Sites in Subtilisin Carlsberg, Inactivated and Native Enzyme

site	ligand	distance (Å)
number 1	Gln-2 $O_{\epsilon 2}$	2.4
inactivated and native	Asp $O_{\delta 1}$	2.6
enzyme	Asp $O_{\delta 2}$	2.6
	Leu-75 O	2.4
	Thr-79 O	2.4
	Val-81 O	2.4
number 2	Ala-37 O	2.7
inactivated and native	His-39 O	2.5
enzyme	Leu-42 O	2.8
	Wat-572 O	3.1
	Wat-637 O	3.2
	Wat-640 O	3.3
number 3	Val-174 O	3.1
inactivated enzyme	Glu-195 O	2.8
	Glu-197 $O_{\epsilon 2}$	2.9
	Wat-673 O	3.4
number 3	Ala-169 O	2.4
native enzyme	Tyr-171 O	2.6
	Val-174 O	2.3
	Wat-450 O	2.3
	Wat-571 O	2.3

RESULTS

Subtilisin Carlsberg Inactivated by Boc-Ala-Pro-Phe-NHO-Bz. The model for the inactivated structure contains, besides the coordinates for the protein, those for the atoms of the (*tert*-butoxycarbonyl)alanylprolylphenylalanyl moiety of the inactivator and for the hydroxylamine nitrogen inserted between the α-carbon and carbonyl carbon of what was the phenylalanine. In addition there are three metal ions and 142 water molecules. There are no significant changes in the

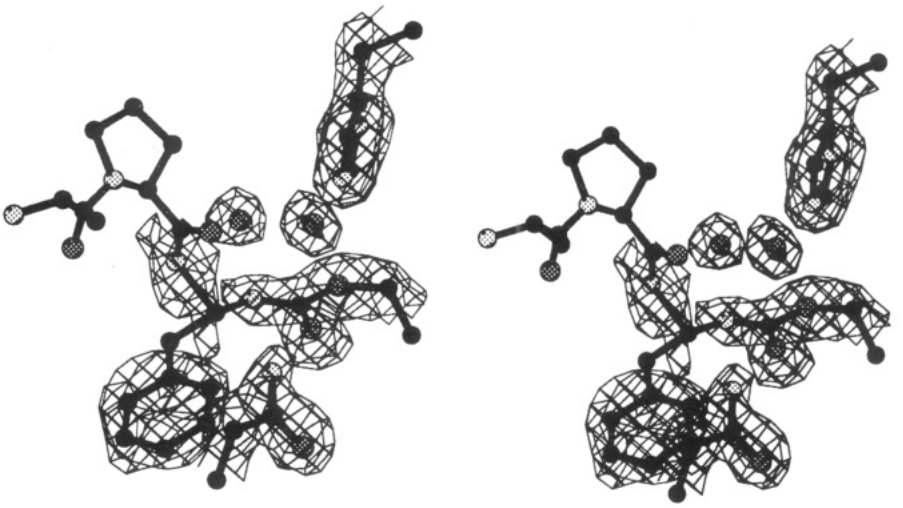

FIGURE 3: Stereoview of a difference Fourier electron density map with coefficients $2F_o - F_c$, calculated with all data, displayed at the 0.6σ level. Electron density is displayed only for the covalent linkage, the former phenylalanine of the inactivator, two water molecules, and the active site serine and histidine. The final model of the enzyme adduct is superimposed with carbon atoms represented in black, oxygen atoms in dark gray, and nitrogen atoms in light gray. The electron density clearly shows the conformation of the carbamate isomer. The poor density at the α- and β-carbons of the former phenylalanine is probably caused by the unusual binding of the inactivator which allows considerable flexibility for the atoms of the inactivator in this region.

structure of the protein model as compared to the model for native subtilisin in aqueous medium refined to 2.1-Å resolution (Fitzpatrick et al., 1993). The average α-carbon backbone root mean square deviation between these two models is 0.127 Å. Some side chains on the enzyme surface have different conformations, and two versions of the third metal ion binding site exist.

Final Adduct between the Inactivator and the Protease: Covalent Linkage. The electron density clearly shows that the inhibitor is covalently linked to the γ-oxygen of Ser-221 of the catalytic triad (Figure 3). There is no density whatsoever to indicate the presence of the leaving group of the inactivator. The electron density has been interpreted in terms of a covalent bond formed from the γ-oxygen of Ser-221 with an atom that is in a plane with two further atoms. This means that four atoms—one of which is the γ-oxygen of Ser-221—form a trigonal-planar structure. We interpret this density as a carbamate (Figure 2B) arising from the carboxyl carbon and carboxyl oxygen of the phenylalanine and the nitrogen (N_{hy}) of the former hydroxylamine function of the inhibitor (Figure 2A). This interpretation requires that the hydroxylamine nitrogen and the carboxyl carbon and the carboxyl oxygen of the former phenylalanine have rearranged so that the hydroxylamine nitrogen is now covalently bound between the carboxyl carbon and α-carbon of the former phenylalanine. Consequently, the phenylalanine is transformed into an *N*-(1-amino-2-phenyl)ethyl carbamate (referred to as Ape-450) (Figure 2B). The bond lengths and angles of this carbamate refined to the values given in Table 3 and are in good agreement with these values for small molecules. In addition, the electron density map clearly display the sp^2 conformation of the carboxyl carbon of the carbamate.

Noncovalent Interactions of the Inactivator with the Protease. The phenyl ring of Ape-450 is found in the P_1 binding pocket. It has displaced three water molecules previously observed in the native structure of subtilisin Carlsberg. A fourth water molecule which was found in the oxyanion hole has been displaced by the carboxyl oxygen of the carbamate linkage. This oxygen is pointing into the oxyanion hole within hydrogen-bonding distance (2.5 Å) of the side chain nitrogen $N_{\delta 2}$ of Asn-155 and the amide nitrogen of residue Ser-221 (2.9 Å) (Table 3 and Figure 4). The water molecule found at the bottom of the P_1 binding pocket is still observed within hydrogen-bonding distances of the carbonyl oxygens of residues Ala-152 (2.8 Å) and Leu-126 (2.8 Å) and the amide nitrogen of residue Ala-169 (3.2 Å).

The remaining peptide backbone of the inactivator does not form any interactions with the protein itself. The backbone is held in the active site by a series of interactions mediated by water molecules (Table 5 and Figure 4). The first water molecule (Wat-662) is within hydrogen-bonding distance to the carboxyl oxygen of the carbamate (3.0 Å), to the carboxyl oxygen of the prolyl residue of the inactivator (2.9 Å), and to the imidazole nitrogen $N_{\epsilon 2}$ of His-64 (2.8 Å). In addition, it is 2.8 Å away from the carboxyl carbon of the carbamate in a position as if it were poised to attack the carboxyl carbon of the acylenzyme. There is a water molecule in the same position in the native structure of subtilisin Carlsberg determined at 2.1-Å resolution (Fitzpatrick et al., 1993). A second water molecule (Wat-850) is at a distance of 3.0 Å from the carboxyl oxygen of the prolyl residue of the inactivator. Thus, this carboxyl oxygen is held in place by hydrogen bonds coming from two water molecules. The second water molecule also shares a hydrogen bond (3.2 Å) with the nitrogen $N_{\delta 2}$ of Asn-155. Finally, a water molecule (Wat-659) simultaneously interacts with the N_{hy} nitrogen of the carbamate (2.9 Å) and the carbonyl oxygen of Ser-125 (2.6 Å) and is at one end of a hydrogen-bonding network involving a total of four water molecules and various protein residues. The second water molecule of this chain (Wat-802) is at a distance of 3.0 Å from the first water molecule (Wat-659) and shares a further hydrogen bond (2.9 Å) with the backbone oxygen of Gly-100. The third water molecule (Wat-861) follows the second water molecule at a distance of 2.9 Å and has only one other hydrogen bond (3.0 Å) to the fourth water molecule (Wat-862) of this chain. The end of this chain is fixed by a hydrogen bond (3.2 Å) between the fourth water molecule and the nitrogen $N_{\delta 2}$ of the side chain of Asn-62. The position of this side chain is stabilized by another interaction with a further water molecule (Wat-588) at a distance of 3.0 Å to the γ-oxygen 1 of Asn-62. Finally, Wat-588 also hydrogen bonds to the hydroxyl group of the Tyr-209 side chain (3.0 Å).

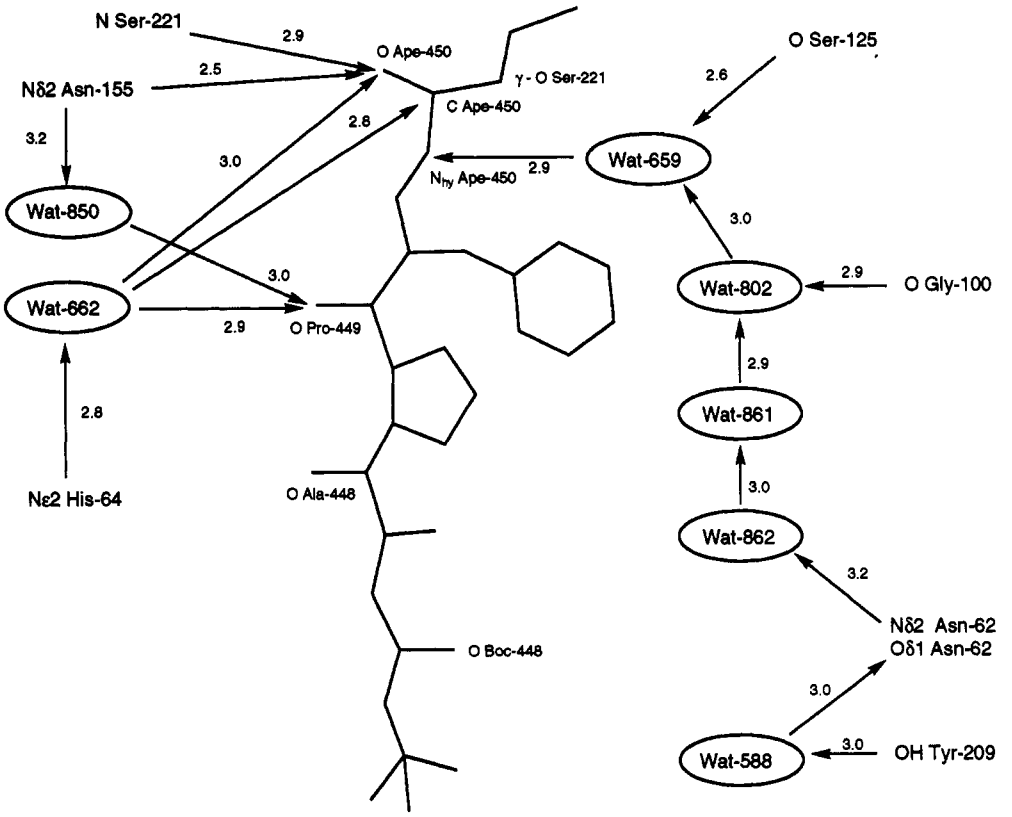

FIGURE 4: Hydrogen-bonding network between the covalently bound inactivator and the protease. It is left to speculation whether this hydrogen-bonding network already exists in the Michaelis complex or is formed either during or after the reaction.

Table 5: Hydrogen Bonds Stabilizing the Conformation of the Inactivator in the Active Site of Subtilisin Carlsberg (See Figure 2)

enzyme/water molecule	inactivator/water molecules	distance (Å)
oxyanion hole		
Ser-221 N	Ape-450 O	2.9
Asn-155 $N_{\delta 2}$	Ape-450 O	2.5
Wat-850	Asn-155 $N_{\delta 2}$	3.2
water network		
Wat-850 O	Pro-449 O	3.0
Wat-662 O	Pro-449 O	2.9
Wat-662 O	Ape-450 O	3.0
Wat-662 O	Ape-450 C	2.8
Wat-662 O	His-64 $N_{\epsilon 2}$	2.8
Wat-659 O	Ape-450 N_{hy}	2.9
Wat-659 O	Ser-125 O	2.6
Wat-659 O	Wat-802 O	3.0
Wat-802 O	Gly-100 O	2.9
Wat-802 O	Wat-861 O	2.9
Wat-861 O	Wat-862 O	3.0
Wat-862 O	Asn-62 $N_{\delta 2}$	3.2
Asn-62 $O_{\delta 1}$	Wat-588 O	3.0
Wat-588 O	Tyr-209 OH	3.0

Comparison of the Position of the Inactivator to That of the Loop of Eglin c Bound to Subtilisin Carlsberg. The positions of the inactivator residues were compared to those of residues Leu-I 45 to Val-I 43 of eglin c bound in the active site region of subtilisin Carlsberg (McPhalen & James, 1988). This comparison shows that these residues bind in a very different mode (Figure 5). Both backbones run in the same general direction from P_1 to P_3 but diverge considerably as the distance from the active site serine increases. The main chain of the residues of eglin c that bind in the P binding cleft of subtilisin form an antiparallel β-sheet with residues Ser-125–Gly-127 and Gly-100–Gly-102 of the enzyme. The inactivator, on the other hand, does not interact with these residues directly, only through the network of water molecules described above. It almost looks as though the inactivator would not remain bound in the absence of a covalent linkage. Table 6 lists the deviations between selected atoms of the inactivator and the analogous atoms of eglin c as an illustration. Carbon C_1 (the α-carbon of the former phenylalanine of the inactivator) of Ape-450 is located much closer to the P_1 pocket than the α-carbon of Leu-I 45 so that the phenyl ring of the *N*-(1-amino-2-phenyl)ethyl carbamate is pushed deeper into this hydrophobic pocket than the side chain of Leu-I 45. Due to the additional nitrogen atom N_{hy} in the final complex, the peptide part of the inactivator is not able to form the usual antiparallel β-sheet with residues Ser-125–Gly-127 and Gly-100–Gly-102, as postulated for the enzyme–substrate complexes and the acyl–enzyme intermediates, and as observed for the eglin c-subtilisin Carlsberg complex (McPhalen & James, 1988). Furthermore, the "backbone equivalent" of Leu-I 45, N_{hy}-C_1-NH of Ape-450, has rotated around an axis defined by atoms C_1–C_2 of this residue (these are the α- and β-carbons of the former phenylalanine) so that the (*tert*-butoxycarbonyl)alanylprolyl moiety of the inactivator is protruding out of the active site into solvent. Consequently the nitrogen atom N_{hy} of the carbamate is 2.6 Å away from the position of the carboxyl carbon of Leu-I 45. The distance between the positions of the carboxyl carbon of the carbamate and of the carboxyl carbon of Leu-I 45 is 1.9 Å.

Metal Ion Binding Sites. The structural role of the first metal ion binding site in the subtilisins has been very well established. The enzyme is known to contain a calcium ion which is always copurified and is associated with the structural integrity of the enzyme (Drenth et al., 1972; Bott et al., 1988). Two further metal ion binding sites have been reported for both subtilisins, Carlsberg and Novo (Drenth et. al., 1972; McPhalen & James, 1988). The second metal ion binding site is formed by a loop on the enzyme surface near residue

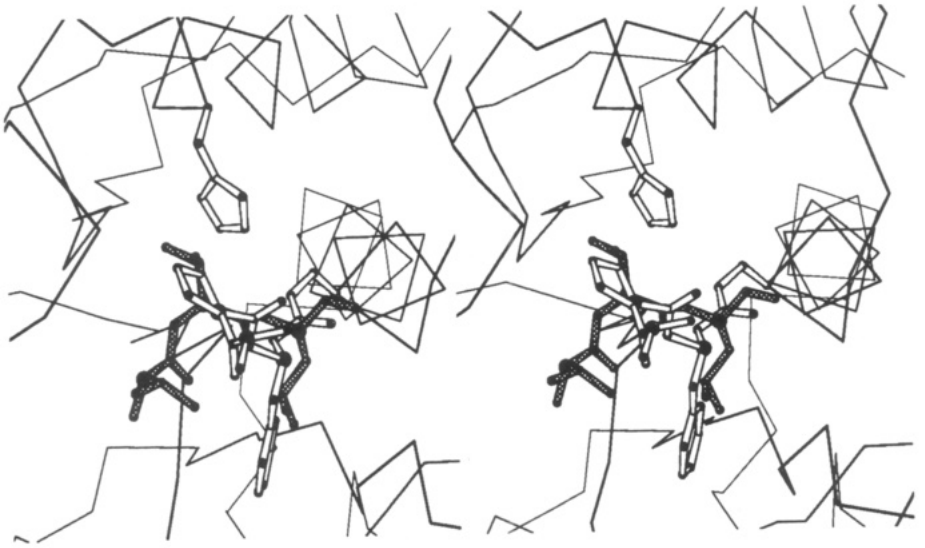

FIGURE 5: Comparison of the positions of Leu-45 to Val-43 of eglin c with those of the residues of the hydroxylamine inhibitor in the active site of subtilisin Carlsberg. A stereoview of the α-carbon backbone model of subtilisin with the side chains of histidine and serine of the catalytic triad with the complete model of residues Leu-I 45 to Val-I 43 of eglin c and the hydroxylamine inactivator superimposed is shown. No water molecules are shown. The bonds of the eglin c fragment are shaded in gray, the bonds of the inactivator and the catalytic serine and histidine are white, the α-carbon atoms of both the inactivator molecule, and the eglin c fragments are represented by larger black spheres.

Table 6: Comparison of the Position of α-Carbon Atoms of the Loop of Eglin c to Equivalent Carbon Atoms of the Hydroxylamine Inactivator in the Active Site of Subtilisin Carlsberg[a]

hydroxylamine inactivator	eglin c inhibitor	distance (Å)
Ape-450 C_1	Leu-I 45 C_α	1.4
Pro-449 C_α	Thr-I 44 C_α	3.0
Ala-448 C_α	Val-I 43 C_α	6.5

[a] The α-carbon backbone model of the subtilisin Carlsberg molecule, determined in complex with eglin c, was superimposed on the α-carbon backbone model of the subtilisin Carlsberg molecule, determined as the adduct with the hydroxylamine inhibitor, to obtain the rotation and translation matrix by the program HYDRA (root mean square deviation: 0.5 Å^2) (R. Hubbard, unpublished results). This matrix was then applied to the complete model of subtilisin Carlsberg complexed with eglin c. The positions of residues Leu-I 45 to Val-I 43 of eglin c were subsequently compared with the positions of residues Ala-448 to Ape-450 of the hydroxylamine inhibitor using the program FRODO (Jones, 1978, 1985).

His-39. This site is reported both for subtilisin Carlsberg and subtilisin Novo and is in the same position in both. The backbone oxygens of residues Ala-37, His-39, and Leu-42 and three water molecules coordinate this metal ion. The ligands are arranged in a trigonal bipyramid with a mean distance to the central atom of 2.9 Å in the structure described here (Table 4). The protein part of this metal ion binding site is the same for the inactivated structure reported here and for the native structure, although the liganded water molecules observed here could not be seen in the electron density calculated for the native structure. The B factors of the calcium ions in both models, and the water molecules liganded to it in the model for the inactivated protease, refined to relatively high values, while keeping the occupancies of all atoms at one. In the inactivated structure, we interpret this result as a higher occupancy for the metal ion and a slightly lower disorder in the positions of the associated water molecules. The difference Fourier electron density map calculated with coefficients $F_o - F_c$ displayed no significant peaks when the ion was modeled and refined as a calcium ion. The mean distance of the metal ion to the backbone oxygens is 2.6 Å and to the three water molecules 3.2 Å. These distances support that the metal ion is a calcium ion, because the coordination number of calcium is preferably six or seven with a mean ligand to calcium ion distance of 2.4 Å (Bott et al., 1988), and the error in the coordinates of the water molecules is probably higher than that of the backbone atoms.

The third metal ion binding site is found in a cavity on the surface close to residue Val-174. This metal ion binding site is interesting as two different configurations are reported for subtilisin Carlsberg and subtilisin Novo (Drenth et al., 1972; McPhalen & James, 1988). In the version observed in this structure the metal ion is coordinated by the backbone oxygens of Val-174, Asn-195, the side chain oxygen $O_{\epsilon 2}$ of the carboxyl group of Glu-197, and a water molecule. These four ligands are arranged in a tetrahedral geometry. The mean ligand to metal ion distance of the protein carboxyl oxygens is 2.9 Å; the water molecule as the fourth ligand is 3.4 Å away from the central ion. In this model, the set of ligands is different than those observed for this metal ion binding site in native subtilisin Carlsberg but the same as reported for subtilisin Novo (McPhalen & James, 1988). The comparison of the position of this metal ion in the model of native subtilisin Carlsberg (Fitzpatrick et al., 1993) and the structure described here shows that the metal ion has shifted by 2.2 Å. In the native subtilisin structure, the backbone oxygens of Ala-169, Tyr-171, and Val-174, as well as two water molecules, bind to the metal ion. The geometry of the complex is a distorted bipyramid with a mean distance of 2.4 Å for the ligands to the central atom (Table 4). The axial ligands are the carboxyl oxygen of Val-174 and a water molecule. The angle between this water molecule, the metal ion, and the carboxyl oxygen of Val-174 is approximately 138°. It is quite surprising to find different geometries in the inactivated and the native structure because both proteins were crystallized under the same conditions. In order to ensure that no model bias was influencing our results, we calculated omit-maps for this region for both structures (Fitzpatrick et al., 1993). The difference Fourier electron density maps clearly indicate that this site exists in two different geometries in subtilisin Carlsberg and that no model bias influenced the original findings.

There are two arguments against modeling a calcium ion in site three. Calcium ions are typically coordinated by six or seven ligands with a mean distance of 2.4 Å for the ligand to the metal atom (Bott et al., 1988; Betzel et al., 1992). However, our data did not indicate that there are any further

water molecules to bring the coordination number of ligands to six or seven. Furthermore, the geometry of the binding site does not leave space for further ligands. Additionally, there was always a strong peak of negative electron density in a difference Fourier electron density map calculated with coefficients $F_o - F_c$ when the ion was modeled as calcium. Calculating the same map with a much weaker scatterer in the model showed no significant residual electron density peaks for the metal ion. This supports the hypothesis that this site is a general cation binding site (Drenth et al., 1972). Sodium ions are probably bound in the structures that are discussed here, because the crystallization medium contained 18% sodium sulfate and 20 mM sodium cacodylate. Their ligation number is known to be 4–9 (Martin, 1984) and the mean ligand to metal ion distance is expected to be longer (Bott et al., 1988). However, it can not be excluded that a calcium ion with unusual coordination geometry and low occupancy is the metal ion in this site.

DISCUSSION

The purpose of this study is to clarify the mechanism of inactivation of serine proteases by *N*-peptidyl-*O*-aroylhydroxylamines by determining the chemical nature of the final inactivated adduct between the inhibitor and subtilisin Carlsberg. The electron density map of the crystallographically determined three-dimensional structure clearly reveals that the formation of a covalent bond to the γ-oxygen of the serine of the catalytic triad is responsible for the inactivation. The inhibition is irreversible due to formation of a carbamate derivative as the final product after rearrangement of the inactivator.

A central characteristic of mechanism-based inactivation is the formation of the actual inactivating species in the active site of the target enzyme. This distinguishes suicide substrates from precursors that form an enzyme-inactivating molecule in solution. Therefore, it is important to identify the reactions of a compound that occur in solution in the absence of enzyme, under enzyme assay conditions, from those that occur in the presence of enzyme. Specifically, can the products of nonenzymatic degradation in solution account for the irreversible inhibition of the enzyme? Up until now only two types of reactions have been observed for *N*,*O*-acylhydroxylamines in solution, these being hydrolysis and Lossen rearrangement. Their significance for the inactivation of subtilisin Carlsberg by Boc-Ala-Pro-Phe-NHO-Bz is discussed briefly.

N-Acylhydroxamic acids and their *O*-acyl derivatives are known to undergo acid- or based-catalyzed hydrolysis in aqueous solution to form hydroxylamine and to release one or two acid anions (Bauer & Exner, 1974). The spontaneous decomposition of a series of *N*-peptidyl-*O*-aroylhydroxylamines in aqueous solution has been studied to determine whether peptide derivatives undergo the same types of reactions. Only *N*-peptidylhydroxamic acids and benzoic acids were detected by thin layer chromatography (Demuth et al., 1987, 1988), mass spectroscopy, and NMR as the products of this degradation (H.-U. Demuth and Schierhorn, unpublished results), indicating that the *O*-acyl group is hydrolyzed preferentially under these conditions. Kinetic studies of this degradation showed no significant dependence on pH in the range from 5 to 8.

N,*O*-Diacylhydroxamic acids are also known to undergo Lossen rearrangements in solution (Bauer & Exner, 1974). The leaving group, here the benzoic acid anion, is released at the same time as the atoms of the *N*-acylhydroxylamine functional group rearrange and form the corresponding isocyanate. This compound then reacts with water to decompose to the amine and CO_2. However, for the rearrangement to occur, special conditions such as heat or the presence of strong base are required (Bauer & Exner, 1974). Thus, a Lossen rearrangement of the *N*-peptidyl-*O*-aroylhydroxylamine does not take place to any significant extent in solution under the conditions of the enzymatic inactivation. Consequently, none of the compounds that can form under enzyme assay conditions can give rise to the observed inactivated adduct, and the result of this structure determination confirms that Boc-Ala-Pro-Phe-NHO-Bz is a true mechanism-based inactivator of subtilisin.

The mechanism of inactivation, as characterized by the formation of an enzyme–inhibitor complex which breaks down to form an irreversible enzyme adduct, was established for this reaction by kinetic studies (Demuth et al., 1989b). It is known that the rate of the reaction is influenced by the electron-withdrawing ability of substituents in the 4-position of the aromatic ring of the leaving group indicating that loss of the *O*-acyl group is at least partially rate-determining (Schmidt et al., 1990).The inactivation of subtilisin Carlsberg is time-dependent, irreversible, and shows no lag-time, thus indicating that the inactivating species does not leave the active site to any significant extent before the final reaction occurs (Demuth et al., 1989b). The determination of the carbamate derivative as the final product requires that the atoms of the *N*-acylhydroxylamine functional group rearrange, thus strongly suggesting the formation of an additional intermediate (E·I*) which is the actual inactivating species as shown in the following scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E{\cdot}I^* \xrightarrow{k_3} E\text{–}I^*$$
$$\downarrow$$
$$LG_1$$

This extra intermediate (E·I*) (either a carbonyl nitrene I_1 or an isocyanate I_2; see Figure 1) arises after formation of the Michaelis complex (E·I) (compare Figure 1A) between enzyme and inactivator and expulsion of the leaving group (L G_1). If this intermediate is not postulated, the release of the leaving group, rearrangement, and reaction with the enzyme must occur in one step.

The precise mechanistic pathway leading to the inactivated subtilisin complex is still unclear. Two fundamentally different reactions between the enzyme and the inactivator can be envisioned (Figure 1), one leading to turnover of the inactivator and one leading to inactivation of the protease. In the first of these, the active site residues are involved in the course of the reaction right after the formation of the Michaelis complex. Thus, binding of the uncharged inactivator molecule (B) in the active site is followed by attack of the γ-oxygen of Ser-221 on the carboxyl carbon of the *N*-acylhydroxylamine function as for amide hydrolysis. The tetrahedral intermediate (T I) breaks down to form the acyl–enzyme (AE) and to release the leaving group (L G_2), which then decomposes in solution to ammonia and benzoic acid. No inactivation occurs by this process. This mechanism was proposed for the reaction between dipeptidyl peptidase IV and diacylhydroxylamines for which enzyme-catalyzed turnover of the inactivator is observed to a significant extent (Demuth et al., 1989a). The key intermediate for such turnover is postulated to be the tetrahedral intermediate. This intermediate was also assumed to be the key intermediate for the inactivation of the cysteine proteases cathepsin B (Smith et al., 1988) and papain by *N*-peptidyl-*O*-acyl hydroxamates (Robinson et al., 1991).

The second reaction differs from the first in that the catalytic triad is *not* involved in the step immediately following formation of the Michaelis complex. The active site of the enzyme only provides the environment that facilitates the formation of the highly reactive species that causes the inactivation. One such pathway consists of the binding of the deprotonated, negatively charged inhibitor (A) to the active site. Subsequently a carbonyl nitrene (I_1) forms by cleavage of the N–O bond, releasing benzoic acid ($L G_1$). This carbonyl nitrene (I_1) can explain the formation of a thiohydroxylamine derivative in the inactivation of papain by *N*-peptidyl-*O*-acyl hydroxamates (Robinson et al., 1991), by reacting directly with a nucleophile in the active site of the protease to form the hydroxylamine derivative. Alternatively, this very reactive, electron depleted nitrogen species (I_1) can also rearrange to an isocyanate (I_2), which reacts with the γ-oxygen of Ser-221 to form the carbamate (P_2).

Thus the lifetime of the nitrene and the nucleophilicity of the attacking group determine which product will form. In the papain case, the catalytic residues Cys-25 and His-159 form a thiolate–imidazolium ion pair (Polgar, 1973, 1974). The thiolate anion is therefore available for reaction with the nitrene. In the subtilisin case, the attacking group is the serine hydroxyl which is considerably less nucleophilic than a thiolate anion, presumably giving the nitrene time to rearrange before the final reaction occurs.

The formation of an *N*-peptidyl isocyanate (I_2) as an intermediate is a reasonable assumption. Serine proteases can be inactivated by treatment with alkyl isocyanates (Brown & Wold, 1973a). Both chymotrypsin and elastase were inactivated by butyl isocyanate and subsequent hydrolysis showed that the major amino acid derivative formed is *O*-(butylcarbamoyl)serine (Brown & Wold, 1973b). This reaction is analogous to that between subtilisin and the *N*-peptidyl-*O*-aroylhydroxylamine in that a carbamate derivative is the final product.

The observed final adduct can only be explained as the result of a Lossen-type rearrangement of the hydroxylamine derivative that must have occurred in the active site and could be catalyzed by the enzyme. Such a rearrangement, leading directly to a covalent bond between the inactivator and the active site serine, has never been observed. A similar reaction has been observed as a further step in the inactivation of chymotrypsin by 3-benzyl-*N*-((methylsulfonyl)oxy)succinimide (Groutas et al., 1989). This mechanism-based inactivation is initiated by the attack of the catalytic serine on the carboxyl carbon C_2 of the succinimide ring. The succinimide ring is opened by the same mechanism as that by which amide hydrolysis would proceed. Thus, an acyl–enzyme is formed and an *N*-acyl-*O*-methylsulfonylhydroxylamine is generated which undergoes a Lossen rearrangement, releasing a methyl sulfonate ion and generating an isocyanate. The other end of the inactivator remains bound on the enzyme as an acyl derivative. In the second inactivating step, the *imidazole* attacks the isocyanate as a nucleophile forming a second covalent bond to the inactivator (Groutas et al., 1989).

Although the mechanism of inactivation of chymotrypsin by the succinimide derivative involves a Lossen rearrangement (Groutas et al., 1989), as we have postulated for the inactivation of subtilisin by *N*-peptidyl-*O*-aroylhydroxylamines, and also gives rise to a covalently bound inactivator–enzyme adduct, the observed final products are completely different. The intermediary isocyanate that arises by the reaction of the succinimide with the protease forms a covalent bond with the histidine of the catalytic triad, in contrast to the hydroxylamine inactivator, which is covalently linked to the catalytic serine. The fundamental differences in the mechanisms described and proposed here concern the role of the catalytic triad. In the mechanism of inactivation of the protease by the succinimide, the catalytic residues generate the inactivating intermediate. In the mechanism of inactivation of the protease proposed here, the active site initially provides only the appropriate environment for the generation of the inactivating species, and the catalytic residues are not necessarily involved in the reaction until after this step.

CONCLUSIONS

This study establishes that *N*-((*tert*-butoxycarbonyl)-alanylprolylphenylalanyl)-*O*-benzoylhydroxylamine inactivates subtilisin Carlsberg by the formation of a carbamate derivative. The result reveals an unusual mode of binding for the peptide part of the inactivator in the final adduct. It is left to speculation whether the inactivator already assumes this position in the Michaelis complex or whether it initially forms the usual β-sheet in the substrate binding cleft and then is released.

The structure of the product and the kinetic characterization of the reaction support the existence of an additional intermediate after formation of the Michaelis complex and before formation of the carbamate derivative in the reaction of subtilisin with *N*-((*tert*-butoxycarbonyl)alanylprolylphenylalanyl)-*O*-benzoylhydroxylamine. We suggest that this intermediate arises from a Lossen-type rearrangement. However, the catalytic apparatus does not seem to be involved in the formation of the inactivating species, only in the capture of that species by the enzyme, resulting in an inactive adduct.

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