Evidence for a Tetrahedral Intermediate Complex during Serpin-Proteinase Interactions*

(Received for publication, May 2, 1991)
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Proteinase inhibitors in the serpin family form complexes with serine proteinases by interactions between the \( \gamma \)-OH group at serine 195 of the enzyme and a specific peptide bond within the reactive site loop of the inhibitor. However, the type of complex formed (i.e. Michaelis, acyl, or tetrahedral) is unknown. Until now, \(^{13}C\) NMR spectroscopy studies have only been useful in examining complexes formed with either peptidolabeled or small protein inhibitors, where \(^{13}C\)-labeled amino acids can be inserted semi-synthetically. Recombinant DNA technology has, however, made it possible to specifically enrich larger proteins with \(^{13}C\).

In the case of serpins we have examined the structure of the complex formed between human \( \alpha_{1}-\)proteinase inhibitor uniformly labeled with \(^{13}C\) methionine and porcine pancreatic elastase. \(^{13}C\) NMR spectroscopic studies revealed a large upfield chemical shift of the carbonyl signal of Met-358 upon complex formation suggesting for the first time that a tetrahedral adduct is formed between a serpin inhibitor and a serine proteinase.

All small proteinase inhibitors (e.g. the Kunitz pancreatic trypsin inhibitor) interact with serine proteinases by the same mechanism (1). This involves formation of a tight complex as the proteinase attempts to cleave a peptide bond (\( PI \square PI' \)) representing the reactive site of the inhibitor. The overall mechanism by which such an enzyme-inhibitor interaction occurs can be written as follows,

\[
E + I = L + C = X = L* = E + I^* \quad (1)
\]

where \( E \) is free enzyme, \( I \) and \( I^* \) are virgin and modified inhibitors, respectively, and \( X \) is a loose, noncovalent complex of enzyme and virgin or modified inhibitor, respectively. \( X \) is a relatively long lived intermediate, and \( C \) is the stable enzyme-inhibitor complex. How large inhibitors, such as those which are members of the serpin family, interact with their target proteinases has not yet been established, although it is assumed that they follow the same reaction path.

Even with small proteinase inhibitors there is still some question as to the nature of the transition state (C) between the intermediate non-covalent Michaelis complexes (L and \( L^* \)). X-ray crystallographic studies of the pancreatic trypsin inhibitor-trypsin complex suggest that the reactive site peptide bond is not fully trigonal but distorted halfway to tetrahedral (2-4). Indeed, \(^{13}C\) NMR spectroscopy of the soybean trypsin inhibitor-trypsin complex, with \(^{13}C\) introduced into the \( P \) residue semi-synthetically (5), provided no evidence for a tetrahedral complex since a large upfield shift of the carbonyl resonance of the \( P \) residue which would be predictive of a tetrahedral complex was not observed.

In comparison with small proteinase inhibitors the direct labeling of the reactive site of serpins by chemical synthesis is not feasible, since it has already been shown (6) that cleavage of the \( P \square P' \) peptide bond in \( \alpha_{1}-\)PI, a member of this family, results in separation of the 2 residues by 68 A, strongly supportive of a major conformational change between the native (I) and post-complex serpin (I*). However, cDNA clones of both normal human \( \alpha_{1}-\)PI and site-specific mutants, constructed in yeast plasmids, functionally expressed, and purified to homogeneity, have recently become available (7, 8). In order to examine the mechanism by which serpin proteinase inhibitors interact with their target proteinases we took advantage of this expression system and utilized a growth medium enriched with methionine containing \(^{13}C\) in the carbonyl group in order to uniformly label all 8 methionine residues of \( \alpha_{1}-\)PI (9), including the \( P \)-Met-358. As a control we used a cell line infected with an \( \alpha_{1}-\)PI cDNA variant coding for Val-358 in the \( P \) position so that only the other 7 methionine residues were labeled with \(^{13}C\). This variant has previously been shown to be as efficient in its rate of inactivation of porcine pancreatic elastase (PPE) as the wild-type \( \alpha_{1}-\)PI (8). A comparison was then made of the \(^{13}C\) NMR spectra of each purified inhibitor both before and after the addition of PPE (\( I/E \) ratio of 1:0.7).

MATERIALS AND METHODS

 Yeast cells, with expression plasmids for Met-358 \( \alpha_{1}-\)PI and Val-358 \( \alpha_{1}-\)PI, were grown at 30 \( \degree\)C in 0.5% yeast extract, 0.5% Tryptone media containing 50 mg/liter \([^{13}C]\) methionine. The \( \alpha_{1}-\)PI was extracted by crushing the cells in a French press, followed by centrifugation at 48,200 \( \times \) g for 30 min. Purification of the two inhibitors was then carried out as previously described (8).

 Samples to be examined by \(^{13}C\) NMR spectroscopy contained \( \alpha_{1}-\)PI (1.4 mM, 40% active) with or without added PPE (0.4 mM, 100% active) in 0.1 mM sodium phosphate buffer, \( pH \) 6.5 (50 \( \mu \)l) and 50 \( \mu \)l of \( D_2O \). The molar ratio of inhibitor enzyme was 1:3.7 based on the activity of each protein. Spectra were recorded at 62.9 MHz on a Bruker AM-250 spectrometer, interfaced with an Aspect 3000 computer and equipped with a 5-mm double frequency \( \mathrm{H}, \mathrm{C} \)-probe. Probe temperature was set at 4 \( \degree\)C; spectral width, 20,000 \( \mathrm{Hz} \); 16,000 data points; 30,000 scans per spectrum (24-h accumulation); relaxation delay, 2 s; \( \mathrm{H} \) decoupling by WALTZ-16; line broadening, 16 Hz. Chemical shifts are referenced to the methyl signal of internal disocyanidyl suberate (0 ppm).

RESULTS AND DISCUSSION

The spectra collected of \([^{13}C]\) methionine-labeled Met-358 \( \alpha_{1}-\)PI with and without PPE are shown in Fig. 1. In order to slow down the rate of the reaction and thus maximize the
The possibility of capturing the transition state, inhibitor and proteinase (12) and is readily detectable after SDS-gel electrophoresis at pH 6.5. A comparison of Fig. 1a versus 1b shows that the spectrum of the Met-358 α1-PI-elastase complex exhibits a more intense signal at 134 ppm. The difference spectrum between Fig. 1b and 1a (Fig. 2a) indicates that the increase in intensity of the peak at 134 ppm is accompanied by a decrease in the intensity of the carbonyl resonance at 176 ppm after complex formation. The difference spectrum of [1-13C]methionine Met-358 α1-PI versus the [1-13C]methionine Val-358 mutant (Fig. 2b) indicates an increase in the carbonyl peak at 176 ppm, due to the extra methionine in the former protein. Therefore, the increased signal at 134 ppm (Figs. 1b and 2a) represents the 13C label of Met-358 which has shifted upfield upon complex formation by about 42 ppm from the carbonyl resonance region (170-180 ppm). This shift agrees with that predicted when a tetrahedral complex is formed (5, 10, 11).

A spectrum taken after the 13C-labeled complex was boiled with 1% SDS (Fig. 1c) showed that the additional peak at 134 ppm had disappeared, presumably because the reaction between inhibitor and proteinase was driven completely and irreversibly to a stable, artificially formed acyl enzyme covalent complex. This type of proteinase-proteinase inhibitor intermediate is characteristic of serpin-proteinase interactions (12) and is readily detectable after SDS-gel electrophoresis. The difference spectrum of the [1-13C]methionine Val-358 mutant and its complex with PPE (Fig. 2c) was essentially flat, as expected, since Val-358 α1-PI would not be labeled with [13C]Met in the P, position and, therefore, incapable of signaling for the formation of a tetrahedral adduct.

Surprisingly, the peak found at 134 ppm was measurable even after several weeks of incubation of complexes at 4 °C followed by several days at room temperature. This suggests that the tetrahedral adduct may be much more stable than previously thought. Gel electrophoresis in a nondenaturing system of the samples kept at 4 °C (not shown) revealed that the normal complex between α1-PI and elastase had disappeared and been replaced by a band migrating with an intermediate mobility between native inhibitor and complex. This intermediate band has been previously detected during long incubations (24 h) of α1-PI and trypsin when an excess of proteinase over inhibitor was present (13). It is likely that a similar situation has developed during the long term incubations followed in these experiments since the mixture of α1-PI and PPE, which initially had a slight excess of inhibitor to proteinase (1:0.7), exhibited about 30% of the original proteinase activity after incubation at 4 °C for 6 weeks.

The data presented here suggest that at 4 °C and pH 6.5 the interaction between α1-PI and PPE leading to the formation of free, modified inhibitor (α1-PI*) proceeds very slowly. It is likely that during short incubation times (<24 h) the tetrahedral intermediate is in equilibrium with the Michaelis complex, since it has been previously shown, using α2-antiplasmin-trypsin complexes (14), that serpin-proteinase complex formation is reversible. However, during longer incubation times (6 weeks) the ultimate conversion of I to I* results in the consumption of excess inhibitor and the formation of free enzyme (30%). The latter may now utilize the complex as substrate, degrading it to a form with intermediate mobility during gel electrophoresis without destroying its tetrahedral adduct structure.

From these data we conclude that a tetrahedral complex is
formed during the interaction of α1-PI and PPE which slowly dissociates to give an unstable acylated intermediate that is hydrolyzed to I* and E. In the presence of denaturing agents, low or high pH, or nucleophiles, the stable tetrahedral complex is "pushed" toward an acyl-enzyme complex. If denaturing agents are utilized this latter complex is stabilized (e.g. SDS-stable serpin-proteinase complexes); however, in the presence of nucleophiles hydrolysis is accelerated. Whether stable tetrahedral complex formation is representative of all serpin-proteinase interactions remains to be determined since kₙ and kₜ, and complex stability differ dramatically between a group of inhibitors and a given enzyme (e.g. α2-antiplasmin and plasmin versus α1-PI and plasmin (15)) as well as between a single inhibitor and a variety of enzymes (e.g. α1-PI and trypsin versus α1-PI and human neutrophil elastase (16)). However, it is clear that the types of interactions described in this report are considerably different from those found during the interactions of small inhibitors with serine proteinases. Indeed, the stable tetrahedral complex detected in these experiments may very likely represent a combination of C and X noted for small inhibitors (Equation 1) such that the reaction mechanism would have to be modified for serpins, as follows.

\[ E + I + L + C \rightleftharpoons L^* \rightleftharpoons E + I^* \]  

In this case L continues to represent a loose, noncovalent complex of enzyme and virgin inhibitor, C is a long lived, tetrahedral intermediate detected for the first time in the current study, and L* is a cleaved form of the complex which cannot be converted back to C.

Acknowledgments—We thank Dr. Ian Bathurst of Chiron Corp., Emoryville, CA for the gift of yeast containing the plasmids for high level expression of Met-358 α1-PI and Val-358 α1-PI and Dr. Anne-Marie Strang for NMR sample preparation. We also thank Dr. Jan Potempa for helpful discussions during the preparation of this manuscript.

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