

ESI-MS of Serine Protease-Inhibitor Complexes: A Site-Directed Mutagenesis Study

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ESI-MS has been used to study a variety of protein-protein interactions. However, there remains a question about the relationship between the interactions of proteins in solution and that which is observed in the gas phase [1]. We have prepared a number of mutants of the serine protease inhibitor eglin c and examined their interaction with serine proteases in both the solution and the gas phase. Shown below is the data for the interaction with bovine alpha chymotrypsin. Eglin c is a small (70 amino acid), stable protein that inhibits a number of different serine proteases. The change in the eglin c variants is extremely small (one to two amino acids) and results in solution dissociation constants (K_d) ranging from 10^{-12} M to 10^{-6} M (Table 1). This is a system in which the structure has undergone minimal change while producing a substantial difference in solution binding affinities, thus providing a good model for examining the differences between solution and gas phase interactions.

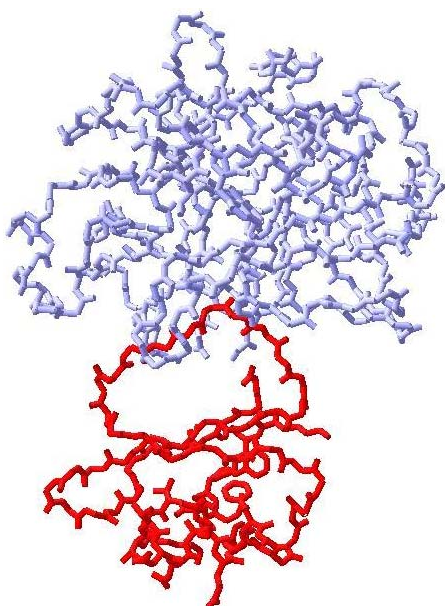


Figure 1. (left) Crystal structure of the complex between chymotrypsin and eglin c [2].

Table 1. Experimentally determined dissociation constants for eglin c variants with chymotrypsin.

Eglin c Variant	K_d (M)
Wild-type	3.9×10^{-12}
L45I	1.7×10^{-9}
L45A	7.1×10^{-9}
L45F	1.6×10^{-12}
L45AL47G	1.0×10^{-6}
L45FL47A	2.7×10^{-12}

-Gly-Ser-Pro-Val-Thr-**Leu**₄₅-Asp-**Leu**₄₇-Arg-Tyr-

Figure 2. Binding region of eglin c.

Several methods were used to examine eglin c - chymotrypsin complexes in the gas phase: ESI-MS, alteration of the interface conditions (*i.e.*, cone voltage), ESI-MS/MS, and competition binding experiments. In general, there was a positive correlation between the relative binding order observed in the gas phase and the binding constants obtained in solution. However, ESI-MS results could not be directly used to calculate quantitative K_d s.

Complexes of chymotrypsin and all of the studied eglin c mutants were observed in the gas phase at a 1:1 molar ratio of enzyme:inhibitor ($10 \mu\text{M}$ each). The amount of free eglin c corresponded qualitatively with the solution binding constants and, in all cases, very small amounts of complex dissociated chymotrypsin were detected. For A45G47 eglin c, unbound chymotrypsin was readily apparent at the concentrations used, positively correlating with a solution K_d of 10^{-6} M. For competitive binding experiments, equimolar concentrations of chymotrypsin and two different eglin c variants were mixed. The relative amounts of free eglin observed and the mass of the complex were consistent with solution experiments.

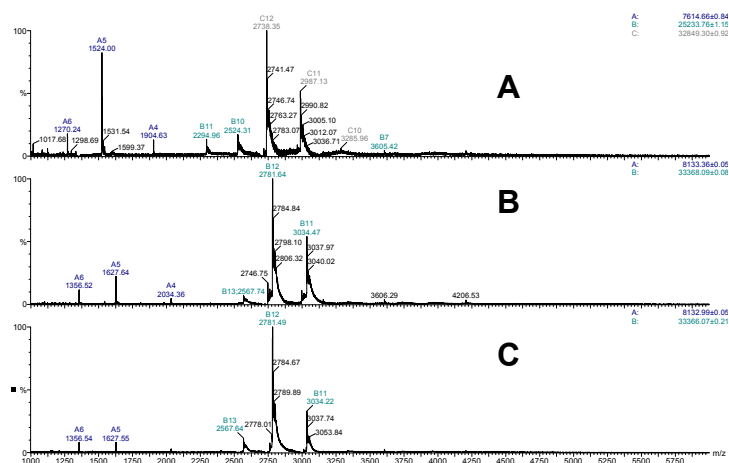


Figure 3. ESI-MS spectra of chymotrypsin with A) A45G47 eglin c, B) I45 eglin c, and C) wild-type eglin c. The +11, +12, and +13 charge states are observed for the complexes. A45G47 4-70 is observed due to cleavage of the N-terminal three amino acids by chymotrypsin.

The stability of the chymotrypsin-eglin c complexes was examined by ESI-MS-MS experiments of the +12 charge state of the complex (Figure 4). Dissociation curves were obtained based on normalized intensities of the parent and product ions (Figure 5). No correlation between solution binding data and the CID experiments was observed. Within the series of mutants studied, there was no apparent difference in the complex stability in the gas phase even though there were substantial differences observed by solution kinetics.

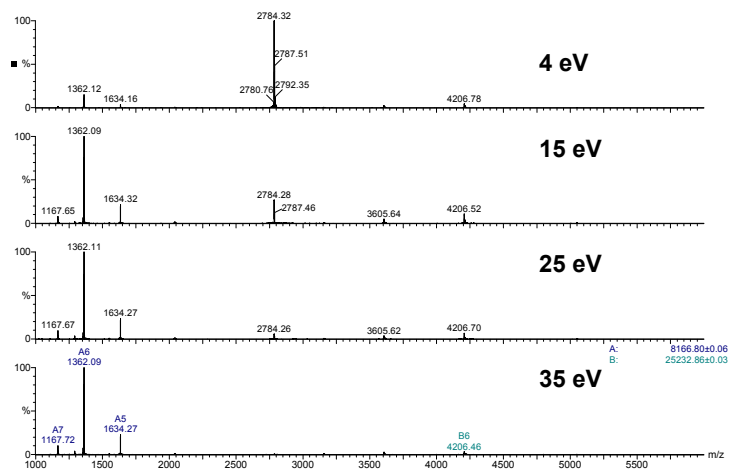


Figure 4. ESI-MS of the +12 charge state of F45 eglin c (m/z 2784.4).

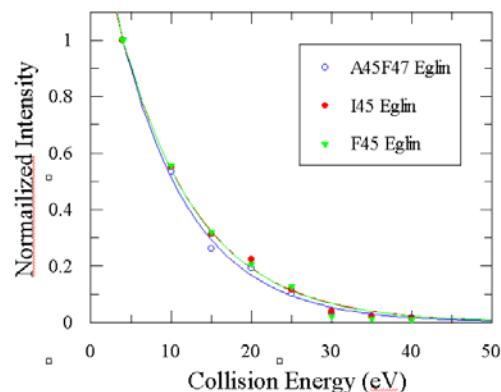


Figure 5. Dissociation curves for the ESI-MS-MS of chymotrypsin-eglin c complexes.

These data indicate that direct measurement of the complexes between chymotrypsin and the serine protease inhibitor eglin c by ESI-MS is a useful tool for determining relative solution dissociation constants for simple binding (1:1 molar ratio of enzyme and inhibitor) and competitive binding (more than one inhibitor) experiments. Examination of gas phase stability by CID is not indicative of the solution behaviour and does not reflect the specificity or selectivity of chymotrypsin for these eglin variants. Thus, caution must be exercised when determining the best method to examine non-covalent protein-protein interactions in the gas phase.

1. Loo, J. A., *Mass Spec. Rev.*, **16** (1997) 1.
2. Frigerio, F., Coda, A., Pugliese, L., Lionetti, C., Menegatti, E., Amiconi, G., Schnebli, H. P., Ascenzi, P., Bolognesi, M., *J. Mol. Biol.*, **225** (1992) 107.