Correlation of Binding-Loop Internal Dynamics with Stability and Function in Potato I Inhibitor Family

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The side-chain of Arg^{50} and Arg^{52} at positions P_6' and P_8', respectively, anchor the binding loop to the protein scaffold by means of hydrogen bonds in *Cucurbita maxima* trypsin inhibitor-V (CMTI-V), a potato I family member. Here, we have investigated the relative contributions of Arg^{50} and Arg^{52} to the binding-loop flexibility and stability by determining changes in structure, dynamics, and proteolytic stability as a consequence of individually mutating them into an alanine. We have compared chemical shift assignments of main-chain hydrogens and nitrogens, and ^{1}H-^{1}H inter-residue nuclear Overhauser effects (NOEs) for the two mutants with those of the wild-type protein. We have also measured NMR longitudinal and transverse relaxation rates and ^{15}N-^{1}H NOE enhancements for all backbone and side-chain NH groups and calculated the model-free parameters for R50A-rCMTI-V and R52A-rCMTI-V. The three-dimensional structures and backbone dynamics of the protein scaffold region remain very similar for both mutants, relative to the wild-type protein. The flexibility of the binding loop is increased in both R50A- and R52A-rCMTI-V. In R52A-rCMTI-V, the mean generalized order parameter,$<S_2>$, of the P_6-P_1 residues of the binding loop (39-44) decreases to 0.68 +/- 0.02 from 0.76 +/- 0.04 observed for the wild-type protein. However, in R50A-rCMTI-V, the flexibility of the whole binding loop increases, especially that of the P_1'-P_3' residues (45-47), whose $<S_2>$ value drops dramatically to 0.35 +/- 0.03 from 0.68 +/- 0.03 determined for rCMTI-V. More strikingly, S_2 values of side-chain N_eHs reveal that, in the R50A mutant, removal of the R50 hydrogen bond results in the loss of the R52 hydrogen bond too, whereas in R52A, the R50 hydrogen bond remains unaffected. Kinetic data on trypsin-catalyzed hydrolysis of the reactive-site peptide bond (P_1-P_1') suggest that the activation free energy barrier of the reaction at 25 ^{0}C is reduced by 2.1 kcal/mol for R50A-rCMTI-V and by 1.5 kcal/mol for R52A-rCMTI-V, relative to rCMTI-V. Collectively, the results suggest that although both the P_6' and P_8' anchors are required for optimal inhibitor function and stability in the potato I family, the former is essential for the existence of the latter and has greater influence on the binding-loop structure, dynamics, and stability.