IA₃, A YEAST PROTEINASE A INHIBITOR, IS INTRINSICALLY UNSTRUCTURED IN SOLUTION

Terry Green¹, Kyle Perry¹, Leif Smith¹, Lowri H. Phylip², Timothy M. Logan³, Stephen J. Hagen⁴, Ben M. Dunn¹, and Arthur S. Edison^{1,*}

¹Departments of Biochemistry & Molecular Biology and ⁴Physics, Box 100245, University of Florida, Gainesville, FL 32610-0245; ²School of Biosciences, Cardiff University, P. O. Box 911, Cardiff CF10 3US, Wales; ³Department of Chemistry and Program in Molecular Biophysics, Florida State University, Tallahassee, FL 32306

*art@ascaris.ufbi.ufl.edu

INTRODUCTION. IA₃ is a 68 amino acid yeast protein that inhibits yeast proteinase A (YprA) with a K_i of about 1 nM. No other aspartic proteinase has been found to be inhibited by IA₃, and at least 15 aspartic proteinases related to YprA cleave IA₃ as a substrate[1,2]. An X-RAY crystal structure has shown that the first 34 amino acids of IA₃ binds to YprA as an α -helix[3]. IA₃ does not crystallize in the absence of YprA, and we have now found by NMR and CD that IA₃ is unfolded in solution and undergoes a large structural transition upon binding to YprA.

METHODS. Recombinant IA₃ with a His-tag for purification was produced in *E. coli* with ¹⁵N or ¹⁵N/¹³C uniform isotopic enrichment. Triple resonance NMR was used to assign most of the backbone and side-chain resonances of free ¹⁵N/¹³C-labeled IA₃. Samples of ¹⁵N-labeled IA₃ were then added to natural abundance YprA and were titrated with YprA and TFE (trifluoroethanol). Circular dichroism (CD) was also used to monitor changes of IA₃ upon addition of TFE. The inhibition constants (K_i) of IA₃ for YprA were determined as a function of temperature for van't Hoff analysis.

RESULTS. The chemical shifts of free IA₃ were found to be essentially random-coil values. The CD spectrum of IA₃ was characteristic of a random-coil peptide but changed to α -helix upon addition of approximately 30% TFE. The ¹⁵N-HSQC NMR spectra of IA₃ were characteristic of random-coil structure, but the addition of YprA produced a large structural transition in IA₃ (Fig. 1). The IA₃ samples used for NMR analysis were active and inhibited YprA with a K_i of about 2 nM. ¹H-¹⁵N heteronuclear NOEs at 600 MHz of free IA₃ were very small or negative, consistent with the expected dynamics of an unfolded protein. A van't Hoff analysis of the temperature-dependence of the K_i of IA₃ for YprA was nonlinear, suggesting a rather complex coupled equilibria of protein folding and inhibition (Fig. 2).

FIGURE 1. ^{15}N -HSQC of free ^{15}N -labeled IA₃ (left) and ^{15}N -labeled IA₃ and unlabeled YprA (right). The spectrum on the right was obtained from the sample shown on the left by adding an approximately stoichiometric amount of unpurified YprA (Sigma).

FIGURE 2. van't Hoff plot of the inhibition of YprA by IA₃. A standard 2-state thermodynamic analysis of the data generates the fit shown, which indicates a positive van't Hoff enthalpy $\Delta H(T_0) = H_{\text{bound}}(T_0) - H_{\text{unbound}}(T_0) = 76 \pm 9 \text{ kJ/mol}$ and $\Delta C_p = -4.6 \pm 1.2 \text{ kJ/mol-K}$, at a reference temperature $T_0 = 25^\circ\text{C}$. These data and the simple model therefore suggest that IA₃ binds endothermically to YprA at room temperature, but with an overall heat capacity change that decreases with temperature, so that binding becomes exothermic near 40°C . The entropy of binding is not well determined in this type of analysis. However, this standard two-state thermodynamic model may be regarded as oversimplifying the actual binding, which entails both protein folding and ligand binding.

DISCUSSION. IA₃ has a large structural transition upon binding to YprA. NMR and CD data have shown that the fully active IA₃ is unstructured in solution, and X-RAY data have shown that the N-terminal half of IA₃ forms a near-perfect α -helix in complex with YprA. The precise mechanism of inhibition of YprA by IA₃ is still unclear. Specifically, we do not yet know whether YprA selects very small (currently undetectable) amounts of α -helical IA₃ for binding or whether YprA provides a template upon which IA₃ folds into a helix. More thermodynamic measurements are needed, but the van't Hoff analysis suggests that the folding and inhibition steps are coupled

together. To sort out the steps of folding and binding, we are currently designing major modifications to IA₃ to force it to become an α -helix in solution.

ACKNOWLEDGMENT. This work was supported by the University of Florida College of Medicine, the National High Magnetic Field Laboratory, the BBSRC (72/C13544 to LHP) and the NIH (1R01AI49063-01 to BMD). NMR data were collected at the Advanced Magnetic Resonance Imaging and Spectroscopy Facility at the McKnight Brain Institute of the University of Florida.

REFERENCES

1. Dreyer, T., Valler, M.J., Kay, J., Charlton, P., and Dunn, B.M. (1985) *Biochem. J.* 231, 777–779.
2. Phylip, L.H., Lees, W.E., Brownsey, B.G., Bur, D., Dunn, B.M., Winther, J.R., Gustchina, A., Li, M., Copeland, T., Wlodawer, A., and Kay, J. (2001) *J. Biol. Chem.* 276, 2023–2030.
3. Li, M., Phylip, L.H., Lees, W.E., Winther, J.R., Dunn, B.M., Wlodawer, A., Kay, J., and Gustchina, A. (2000) *Nat. Struct. Biol.* 7, 113–117.