Table I.	Free	Energies	of	Association	for	1b	and	Amides	in	C <sub>4</sub> D <sub>4</sub>
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substrate	binding energy, kcal/mol (enantiomer)	saturation achieved, %	enantioselection: $\Delta\Delta G$ , kcal/mol
MeNHCOMe	-3.17	65	
MeNHCOBn	-2.18	62	
BnNHCOH	-3.24	64	
BnNHCOMe	-2.84	48	
BnNHCOCF <sub>3</sub>	no complex observed		
BnNHCOEt	-2.33	67	
PhCHMeNHCOMe	-3.04 (S), $-2.62$ (R)	56 (S), 67 (R)	0.42
PhCHMeNHCOH	-3.18 (S), $-2.85$ (R)	57 (S), 48 (R)	0.33
PhCHMeNHCOEt	-1.80(S), -1.55(R)	56 (S), 45 (R)	0.25
1-NpCHMeNHCOMe	-2.56(S), -2.31(R)	57 $(S)$ , 51 $(R)$	0.25
BnOAlaNHCOMe	-2.29(S), -1.81(R)	64(S), 50(R)	0.48
MeOPGlyNHCOMe	-1.91(S), -2.06(R)	44 (S), 45 (R)	-0.15

namics<sup>5</sup> starting from the two conformations of 1 observed by X-ray crystallography. In these simulations, the benzyl groups of 1b were replaced by methyls. After energy minimizing using the OPLS/AMBER force field<sup>6</sup> with N-methylacetamide in the binding cavity, we carried out 250 ps of molecular dynamics at 300 K. The average potential energy stabilized within the first 50 ps. Simulated annealing to  $\sim$  50 K over 100 ps and energy minimizing gave the final conformers. The conformer of the complex derived from the 1b crystal structure was found to be more stable by 2.5 kcal/mol in steric energy. When the rigid rotor/harmonic oscillator approximation is used, it is also higher in entropy by 8.8 cal deg<sup>-1</sup> mol<sup>-1</sup> than the **1a**-derived complex and thus is 5.1 kcal/mol more stable in free energy at 300 K. Its stereostructure is shown below:



As revealed in the structure above, the atoms bearing hydrogens that display the described NOE signals are indeed close in space. Furthermore, the observed coupling constants for hydrogens of the diiodotyrosine  $\alpha$  and  $\beta$  carbons in the complex ( $J_{a,b} = 2.8$  and 9.2 Hz) are similar to those calculated by using Altona's equation<sup>7</sup> (1.4 and 9.8 Hz). If the 1/amide complex has the geometry shown, then we would expect selective binding with the amides of primary amines having nitrogen attached to a chiral center of the S configuration.8

As summarized in the table, we do indeed find enantioselective binding of 1b with certain chiral amides. Binding energies were measured by NMR titration, and error propagation analysis gives error limits of ±0.1 kcal/mol. While the chiral binding differences are not large, they lie well outside the error range of the measurements. Except for the acetamide of phenylglycine (PGly) methyl ester, which has substituents having similar steric demands,9 it is the S enantiomer that binds more tightly. Distinctions between amide enantiomers were also observed by <sup>1</sup>H NMR. With PhCHMeNHCHO, for example, signals from the two enantiomers for the chiral methine hydrogen and the formamide C-H and N-H separated by >0.1 ppm upon treatment with 1b.

It should be easy to design chiral hosts that bind enantiomeric guests with significantly different association energies because the thermodynamics of enantiomeric complexation are relatively

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simple. Enantiomeric guests have identical solvation energies. and differences in binding energies result exclusively from the relative stabilities of the complexes. In contrast, differences in the solvation energies of nonenantiomeric guests can have a major effect on selectivity.<sup>10</sup> Nevertheless, many previous reports of chiral hosts note little detectable difference in the energies of diastereomeric complexes. A likely explanation is that many different conformations of complexes are involved. In our host, cyclophane linkages, bridged macrocyclic structures, and  $C_2$ symmetry all operate to reduce but not eliminate conformational heterogeneity. Further rigidification is clearly desirable and should provide enhanced enantioselection.11

Supplementary Material Available: Stereopair plots of the X-ray structures of 1a and 1b (1 page). Ordering information is given on any current masthead page.

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## Proline Assignments and Identification of the Cis K116/P117 Peptide Bond in Liganded Staphylococcal Nuclease Using Isotope Edited 2D NMR Spectroscopy

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Proline is usually the most difficult type of amino acid residue to assign in a protein because the pyrrolidine ring lacks an amide proton, and therefore the essential sequential connectivities involving this proton are absent.<sup>1,2</sup> Although connectivities involving the proline  $\delta$ -protons can substitute for the lacking amide proton connectivities,<sup>1,3</sup> the  $\delta$ -protons are often difficult to identify because

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**Figure 1**. 500 MHz carbon-13 edited NOESY spectra<sup>13</sup> of the Nase/ pdTp/Ca<sup>2+</sup> complex labeled with (a) [2-<sup>13</sup>C]Pro, 37 °C, (b) [4-<sup>13</sup>C]Pro, 37 °C, and (c) [4-<sup>13</sup>C]Pro, 47 °C. The solution composition for all experiments reported herein was as follows: <sup>2</sup>H<sub>2</sub>O, 99.99%; NaBO<sub>3</sub>, 50 mM; NaCl, 100 mM; Nase, 1.5 mM; pdTp, 5 mM; CaCl<sub>2</sub>, 10 mM; pD = 7.4. AM 500 spectrometer settings were as follows: 90° pulse, 30  $\mu$ s; recycle delay, 1.8 s; spectral window, 7400 Hz in F<sub>2</sub> and 3000 Hz in F<sub>1</sub>; mixing time, 0.15 s; 128–192 scans per t<sub>1</sub> value; 200 t<sub>1</sub> values, using time proportional phase increments. Chemical shifts are relative to HDO at 4.66 and 4.57 ppm at 37 and 47 °C, respectively. The labeled proteins were prepared as reported.<sup>4</sup>

their chemical shifts fall in a crowded region of the spectrum containing numerous  $\alpha$ - and  $\beta$ -protons. Also, in larger proteins such as Staphylococcal nuclease, Nase, the short  $T_2$  values of the motionally restricted proline protons make it difficult to link the  $\delta$ -protons to the other ring protons using RELAY COSY or HOHAHA experiments. For these reasons only three of the seven proline  $\alpha$ -protons in Nase were assigned,<sup>4</sup> whereas ca. 90% of the non-proline backbone protons were assigned.<sup>4</sup> Herein we report sequential assignments of all proline residues in Nase using isotope edited 2D NMR spectroscopy together with previously reported<sup>4</sup> non-proline  $\alpha$ -proton assignments.

The chemical shifts of the proline  $\alpha$ - and  $\gamma$ -protons and carbons are obtained from the <sup>13</sup>C edited NOESY spectra, Figure 1a-c, and the <sup>1</sup>H/<sup>13</sup>C heteronuclear shift correlation spectra, HMQC, Figure S1a,b (Supplementary Material). With the exception of P3', which resides in the disordered leader sequence, all proline spin systems are identified by comparison of these spectra. These identifications are confirmed by sequential NOE connectivities between  $\alpha$ -protons of residue i and the  $\delta$ -protons of proline i+1,  $d_{\alpha\delta}$ , and intraresidue proline  $\alpha$ - $\beta$ - $\gamma$  connectivities observed in natural abundance NOESY and HOHAHA spectra, respectively. The latter spectrum and the HMQC spectra yield assignments of the P3'  $\alpha$ -,  $\beta$ -, and  $\gamma$ -protons.

Because of the long mixing time used in Figure 1b,c, one observes not only strong geminal and vicinal connectivities involving the proline  $\gamma$ -protons (as expected these connectivities are also strong when the mixing time is 50 ms, Figure S2 (Supplementary



Figure 2. 500 MHz HOHAHA spectra of the deuteriated Nase/ pdTp/Ca<sup>2+</sup> complex labeled with (a) protiated Lys and Ile and (b) protiated Leu. The connectivities within the boxes in (a) must be due to Lys or Ile because these connectivities are absent in (b). These connectivities are assigned to Lys because all five sets of  $\alpha$ - $\beta$ - $\gamma$  methyl Ile connectivities have been assigned elsewhere in the spectrum. The samples of deuteriated Nase were prepared as reported,<sup>4</sup> and, as noted previously, Ala residues were ca. 50% protiated in the deuteriated Nase samples, explaining the appearance of the strong Ala connectivities in the spectra. NT 500 spectrometer settings were as follows: 90° pulse, 32  $\mu$ s; recycle delay, 2 s; spectral window, 5400 Hz in F<sub>2</sub> and 5000 Hz in F<sub>1</sub>; WALTZ mixing time, 30 ms; 64 scans per t<sub>1</sub>; 250 t<sub>1</sub> values. Chemical shifts are relative to HDO at 4.66 ppm, 37 °C.

Material)) but also connectivities to the proline  $\alpha$ -protons and the  $\alpha$ -protons of residues that precede prolines in the sequence. These longer range connectivities arise, in part, from magnetization transfer from the proline  $\gamma$ -protons to the proline  $\beta$ - and  $\delta$ -protons and then to the proline and non-proline  $\alpha$ -protons, respectively. Because the  $\alpha$ -protons of E10, E30, T41, and H46 have been assigned,<sup>4</sup> d<sub> $\alpha\gamma$ </sub> connectivities observed in Figure 1b,c provide  $\alpha$ proton assignments of P11, P31, P42, and P47.

Because the K116-P117  $d_{\alpha\gamma}$  is not observed in Figure 1b,c, we approach the Prol17 assignment by analysis of the <sup>13</sup>C edited NOESY spectrum of Nase labeled with [2-13C]Pro. This spectrum, Figure 1a, shows that the Pro  $\alpha$ -proton at 4.64 ppm has strong connectivities to protons at 4.02 and 1.77 ppm and a weak connectivity to a proton at 1.33 ppm. These three protons are assigned to the  $\alpha$ - and  $\beta$ -protons of a Lys residue because the HOHAHA spectrum of deuteriated Nase containing protiated Lys, Figure 2a, shows connectivities at 4.02, 1.77 and 4.02, 1.33 ppm, whereas these connectivities are absent in the HOHAHA spectrum of Nase containing deuteriated Lys, Figure 2b. Because Nase contains a single Lys-Pro bond we assign the 4.64, 4.02; 4.64, 1.77; and 4.64, 1.33 ppm connectivities observed in Figure 1a to  $d_{\alpha\alpha}(116,117)$ ,  $d_{\beta\alpha}(116,117)$ , and  $d_{\beta'\alpha}(116,117)$ , respectively. Note that this assignment of the P117  $\alpha$ -proton places the P117  $\gamma$ protons at 1.35 and 2.02 ppm. These assignments together with the HMQC spectrum of [4-13C]Pro labeled Nase, Figure S1b (Supplementary Material), assign the most upfield Pro  $\gamma$ -carbon to P117, in agreement with the assignment obtained in nonliganded Nase by site-directed mutagenesis.<sup>5</sup> We note that the intense K116-P117  $d_{\alpha\alpha}$  connectivity shows that the Lys-Pro peptide bond of the liganded protein is cis in solution, as in the crystal structure,<sup>6,7</sup> and in agreement with the model of Dobson and Fox.<sup>8,9</sup>

The assignment of the remaining proline, P56, follows by elimination. The Gly55  $\alpha$ -proton assignments<sup>4</sup> show that their chemical shifts overlap those of the various Pro  $\delta$ -protons.

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Therefore the connectivities involving the Gly55  $\alpha$ -protons and the P56  $\gamma$ -protons are obscured by the intense  $\beta$ -proton connectivities in Figure 1b,c.

The long-range connectivities in Figure 1, that link Pro residues to remote residues in the sequence, are consistent with reported<sup>4</sup> Nase assignments and the distances calculated from the crystal structure.<sup> $\bar{7}$ </sup> These results are further evidence that there is a close correspondence between the solution and crystal structure of the ternary complex throughout most of the protein, as concluded previously.4,10,11

The isotope editing approach described herein should be a generally useful method of assigning proline spin systems in moderate size proteins. Labeling with  $[3,5-{}^{13}C_2]$  proline should improve the efficiency of the method, because all relevant information could be obtained by using one double-labeled sample. The double-labeled proline could be synthesized by following the scheme used to obtain [4-13C]proline.12

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Registry No. Nase, 9013-53-0; Pro, 147-85-3.

Supplementary Material Available: Spectra (500 MHz <sup>1</sup>H/<sup>13</sup>C HMQC) of Nase labeled with [2-13C]Pro and [4-13C]Pro and spectrum (500 MHz <sup>13</sup>C edited NOESY) of Nase labeled with [4-13C]Pro (3 pages). Ordering information is given on any current masthead page.

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## **Observation and Sequence Assignment of a Cis Prolyl** Peptide Bond in Unliganded Staphylococcal Nuclease

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Cis-trans isomerization of X-prolyl peptide bonds is now considered as both a major determinant of the rate of protein folding and an explanation for the existence of multiple folded forms of proteins in solution.<sup>1-3</sup> Indirect evidence for these effects of prolyl peptide bonds has been recently obtained by examining the properties of mutant proteins in which specific prolyl residues have been replaced by site-directed mutagenesis techniques. For



Figure 1. <sup>1</sup>H-<sup>13</sup>C heteronuclear chemical shift correlation spectra of wild-type SNase (left panel) and its P117G mutant (right panel) labeled with [4-13C]proline. The data were obtained in proton detection experiments of <sup>1</sup>H-<sup>13</sup>C chemical shift correlations at pH 5.5 and 40 °C.<sup>17</sup> Assignments are made in the text.

example, by investigating substitutions for specific proline residues, Richards and co-workers have deduced that isomerization of a single prolyl peptide bond is the rate-determining step in the folding of thioredoxin from *Escherichia coli*.<sup>4</sup> Also, Dobson and Fox have implicated cis and trans isomers of a single prolyl peptide bond in staphylococcal nuclease (SNase) as the explanation for the coexistence of multiple folded forms of the protein.5-7 However, the conclusions reached in these and all other solution studies of isomerization of prolyl peptide bonds in proteins have not been based on the observation of cis prolyl peptide bonds but on the effects of this putative isomerization on biophysical properties such as the rates of folding and the <sup>1</sup>H NMR spectra of the resolved histidine H, resonances. In this communication we report observation and assignment of selected <sup>1</sup>H and <sup>13</sup>C NMR resonances of a single proline cis X-prolyl peptide bond in SNase.

A number of studies of the resonances of the resolved H, protons of the four histidine residues in SNase have suggested the presence of an equilibrium mixture of two monomeric folded forms since two resonances are detected for each of these protons.<sup>5-10</sup> Investigation of the temperature dependence of these resonances reveals that the multiplicity is preserved in the unfolded form. In the presence of the active site ligands  $Ca^{2+}$  and thymidine 3',5'-bisphosphate (pdTp), the multiplicity of resonances in the folded state is eliminated. Crystallographic studies of SNase complexed with the active-site ligands have revealed that the peptide bond between Lys 116 and Pro 117 is cis whereas the remaining six X-prolyl peptide bonds are trans.<sup>11</sup> The <sup>1</sup>H NMR spectrum of the aromatic region of the site-directed mutant in which Pro 117 is replaced with a glycine residue (P117G) reveals only a single resonance for each  $H_{\epsilon}$  proton. The simplest explanation for these observations is that the two folded forms of unliganded SNase in solution as detected by <sup>1</sup>H NMR spectroscopy can be associated with a major cis and a minor trans isomer of the Lys<sub>116</sub>-Pro<sub>117</sub> peptide bond. However, the <sup>1</sup>H NMR spectrum of P117G differs from that of the wild-type enzyme,

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