Coupling between trans/cis proline isomerization and protein stability in staphylococcal nuclease

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Abstract
The nuclease A produced by two strains of Staphylococcus aureus, which have different stabilities, differ only in the identity of the single amino acid at residue 124. The nuclease from the Foggi strain of S. aureus (by convention nuclease WT), which contains His124, is 1.9 kcal·mol⁻¹ less stable (at pH 5.5 and 20 °C) than the nuclease from the V8 strain (by convention nuclease H124L), which contains Leu124. In addition, the population of the trans conformer at the Lys116-Pro117 peptide bond, as observed by NMR spectroscopy, is different for the two variants: about 15% for nuclease WT and 9% for nuclease H124L. In order to improve our understanding of the origin of these differences, we compared the properties of WT and H124L with those of the H124A and H124I variants. We discovered a correlation between effects of different residues at this position on protein stability and on stabilization of the cis configurational isomers at Pro117. In terms of free energy, approximately 17% of the increase in protein stability manifests itself as stabilization of the cis configuration of the Lys116-Pro117 peptide bond. This result implies that the differences in stability arise mainly from structural differences between the cis configurational isomers at Pro117 of the different variants at residue 124. We solved the X-ray structure of the cis form of the most stable variant, H124L, and compared it with the published high-resolution X-ray structure of the cis form of the least stable variant, WT (Hynes TR, Fox RO, 1991, Proteins Struct Funct Genet 10:92-105). The two structures are identical within experimental error, except for the side chain at residue 124, which is exposed in the models of both variants. Thus, the increased stability and changes in the trans/cis equilibrium of the Lys116-Pro117 peptide bond observed in H124L relative to WT are due to subtle structural changes that are not observed by current structural determination techniques. Residue 124 is located in a helix. However, the stability changes are too large and follow the wrong order of stability to be explained simply by differences in helical propensity. A second site of conformational heterogeneity in native nuclease is found at the His46-Pro77 peptide bond, which is approximately 80% trans in both WT and H124L. Because proline to glycine substitutions at either residue 47 or 117 remove the structural heterogeneity at that position and increase protein stability, we determined the X-ray structures of H124L+P117G and H124L+P47G+P117G and the kinetic parameters of H124L, H124L+P47G, H124L+P117G, and H124L+P47G+P117G. The individual P117G and P47G mutations cause decreases in nuclease activity, with k₄₅⁰ affected more than k₅₅⁰, and their effects are additive. The P117G mutation in nuclease H124L leads to the same local conformational rearrangement described for the P117G mutant of WT (Hynes TR, Hodel A, Fox RO, 1994, Biochemistry 33:5021-5030). In both P117G mutants, the loop formed by residues 112-117 is located closer to the adjacent loop formed by residues 77-85, and residues 115-118 adopt a type I′ β-turn conformation with the Lys116-Gly117 peptide bond in the trans configuration, as compared with the parent protein in which these residues have a type VI β-turn conformation with the Lys116-Pro117 peptide bond in the cis configuration. Addition of the P47G mutation appears not to cause any additional structural changes. However, the electron density for part of the loop containing this peptide bond was not strong enough to be interpreted.

Keywords: enzyme activity; global protein stability; guanidinium chloride denaturation; helix stability; NMR spectroscopy; proline isomerization; staphylococcal nuclease; X-ray crystallography

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Structural fluctuations in proteins occur on different time scales. Among the slowest are those that arise from Xxx-Pro peptide bond \textit{trans/cis} isomerizations, with typical time constants in the range of ten to several hundred seconds. The role of Xxx-Pro peptide bond isomerizations in the kinetics of protein folding has been investigated thoroughly, and it is now widely accepted that such isomerizations are often the reason for slow steps in protein folding reactions (Kim & Baldwin, 1982, 1990). Similarly, there is substantial evidence that this isomerization can play an important role in protein function (Brown et al., 1977; Marsh et al., 1979; Dunker, 1982; Brandl & Deber, 1986; Touchette et al., 1986; Langsetmo et al., 1989). Therefore, determining the detailed structural and thermodynamic consequences of proline \textit{trans/cis} isomerizations, as well as protein structural properties that influence \textit{trans/cis} equilibria at Xxx-Pro peptide bonds, is of great interest to investigators. Proline residues are often found in loops and turns of proteins. The limited backbone flexibility of proline probably restrains the conformation and flexibility of a loop. Thus far, with systems exhibiting Xxx-Pro heterogeneities, protein structure determination methods have not provided full structures of both conformers: one with a trans-proline and the other with a cis-proline peptide bond. However, in several cases, it has been possible to characterize structural differences by NMR spectroscopy (Fox et al., 1986; Evans et al., 1987, 1989; Higgins et al., 1988; Chazin et al., 1989; Loh et al., 1991). Such phenomena have been studied most extensively in staphylococcal nuclease, where measurable populations of both the cis and trans configurations have been detected at the Lys116-Pro117 and His46-Pro47 peptide bonds (Evans et al., 1987; Loh et al., 1991). The relative intensities of doubled resonances assigned to the $^1$H$^+$ protons of histidines 8, 121, and 124 and the doubled resonances assigned to the $^1$H$^+$ protons of histidine 46 provide an easy measure of the \textit{trans/cis} equilibria of the Lys116-Pro117 and the His46-Pro47 peptide bonds, respectively. In nuclease WT, approximately 90% of the molecules have a cis Lys116-Pro117 peptide bond and approximately 20% have a cis His46-Pro47 peptide bond.

Recently, the conformation of the loop formed by residues 112–117 in nuclease, containing Pro117, has been analyzed in detail. A model peptide for this region, designed to approximate the unfolded state, showed the \textit{trans/cis} equilibrium to be 24 (Raleigh et al., 1992). Thus, noncovalent intramolecular forces serve to perturb the Lys116-Pro117 peptide bond equilibrium from predominantly \textit{trans} in the unfolded protein to predominantly \textit{cis} in the folded wild-type protein. Analysis of the structure suggested that these noncovalent interactions consist of hydrogen bonds on the C-terminal side and hydrogen bonding and packing interactions on the N-terminal side of the 112–117 loop (Hinck, 1993; Hodel et al., 1993, 1994). They anchor this loop to the body of the protein and act to stabilize the cis configuration of the Lys116-Pro117 peptide bond (Hodel et al., 1993, 1994): the anchoring is thought to impose greater strain on the backbone of the loop in molecules with a \textit{trans} Lys116-Pro117 peptide bond than in those with the cis configuration (Hodel et al., 1993).

Nuclease has been isolated from both the Foggi and V8 strains of \textit{Staphylococcus aureus} (Cusumano et al., 1968; Cone et al., 1970). These two nuclease differ at residue 124 in the third helix of the nuclease structure. Whereas the Foggi strain nuclease (WT) contains a histidine, the V8 strain nuclease (H124L) contains a leucine at this position. Depending on solution conditions, H124L is between 1.2 and 1.9 kcal-mol$^{-1}$ more stable than WT (Shortle, 1986; Tanaka et al., 1993). Also, the population with a \textit{trans} Lys116–Pro117 peptide bond is approximately a factor of two smaller in H124L than in WT (Alexandrescu et al., 1990; Hinck, 1993). In order to investigate the structural origins of these differences, we solved the X-ray structure of H124L and compared it with the refined X-ray structure of WT (Hynes & Fox, 1991). The results showed that the structures are identical, at the limit of resolution, except at residue 124, the side chain of which is exposed in both. These observations suggest that the H124L mutation causes subtle changes in tertiary interactions that influence the conformation of loop residues 112–117 and result in a more stable protein. In order to improve our understanding of these changes, we also examined the protein stabilities and \textit{trans/cis} ratios of the Lys116–Pro117 peptide bond in the H124L and H124A nuclease variants. They are intermediate between the values for histidine and leucine.

In order to further analyze factors determining the conformation of the $\beta$-turns containing Pro47 and Pro117, we solved the X-ray structures of H124L + P117G and H124L + P47G + P117G. The Pro to Gly mutations force the peptide bond into the \textit{trans} configuration, thus eliminating the \textit{trans/cis} peptide bond heterogeneity.

In a separate study (Hinck et al., 1996), we introduced pairs of cysteines into nuclease as a means of altering folded-state strain, according to whether the engineered disulfide is oxidized or reduced. This system also exhibited coupling between protein stability and the fractional \textit{cis} configuration at the Lys116–Pro117 peptide bond.

\section*{Results}

\subsection*{Peptide bond \textit{trans/cis} ratios}

As mentioned in the Introduction, the $^1$H$^+$ resonances of His8, His121, and His124 in the NMR spectrum of nuclease report on the Lys116–Pro117 peptide bond \textit{trans/cis} equilibrium (Evans et al., 1987; Alexandrescu et al., 1988), and the $^1$H$^+$ resonances of His46 report on the His46–Pro47 peptide bond \textit{trans/cis} equilibrium (Loh et al., 1991). Figure 1 compares the histidine $^1$H$^+$ region of the H NMR spectra of WT, H124L, and H124L + P117G. Comparison of the ratio of the areas of the peaks assigned to the \textit{trans} and \textit{cis} forms shows a greater \textit{trans} population in WT compared with H124L. As expected, H124L + P117G exhibits only one peptide bond configuration at Lys116–Pro117, but maintains heterogeneity at the His46–Pro47 peptide bond. The equilibrium constants for the Lys116–Pro117 peptide bond \textit{trans/cis} equilibrium of WT, H124A, H124L, and H124L are shown in Table 1. The free energy difference between the folded protein with the \textit{trans} and with the \textit{cis} Lys116–Pro117 peptide bond was calculated from the NMR data (Fig. 2) for each of the variants at residue 124.

\subsection*{Protein stabilities}

The stabilities of WT, H124A, H124L, H124L + P117G, H124L + P47G, and H124L + P47G + P117G were determined by guanidinium chloride (GdmCl) denaturation. The results are summarized in Table 1. The stability of the H124 variants increases in the order WT < H124A < H124L < H124L. The free energy difference between unfolded and folded protein, $\Delta G_c$, correlates with the relative free energy difference between molecules with the \textit{trans} and \textit{cis} configuration of the Lys116–Pro117 peptide bond, $\Delta G_{\text{trans/cis}}$ (Fig. 2).

At 20°C and pH 5.5, the P117G and P47G mutations increase the protein stability by 1.5 and 0.9 kcal-mol$^{-1}$, respectively. On
The fit of the data yielded a molecular weight close to that of the nuclease monomer. From the plot of absorbance versus radius from the center of rotation, it was estimated that the highest protein concentration in the cell was approximately 0.4 mM. No multimeric species appeared to be present at this concentration.

**Enzyme activity**

The enzyme activities of H124L, H124L+P47G, H124L+P117G, and H124L+P47G+P117G were analyzed at pH 9.5 (the pH optimum of WT nuclease activity) in the presence of Ca²⁺, using thymidine 3'-phosphate 5'-(p-nitrophenyl phosphate) as a substrate. The observed values of $k_{cat}$, $K_m$, and $k_{cat}/K_m$ are summarized in Table 2. The P117G and P47G mutations each cause a small (seventhfold) decrease in nuclease activity; each mutation has a small effect on $k_{cat}$ as well as on $K_m$. These changes appear to be additive, leading to a decrease in activity of the double proline mutant (P47G + P117G) by a factor of about 50.

**Crystal structures**

We determined high-resolution X-ray structural models for nuclease H124L, H124L+P117G, and H124L+P47G+P117G. Final refinement statistics and a description of the crystals are shown in Table 3.

Superposition of the X-ray structure of H124L with that of WT (1STN; Hynes & Fox, 1991) resulted in an RMS deviation (RMSD) of 0.16 Å between Cα carbons. These structures are essentially identical. The backbone conformation around residue 124 is not affected by the mutation, and the side chain of this residue is solvent exposed and has the same orientation in both variants (Kinemage 1), i.e., the χ1 angles of this residue in the two structures are nearly identical. As in the structure of WT, the loop residues 42–53 have higher B-factors than the rest of the protein, suggesting greater flexibility for this part of the protein. Nevertheless, following refinement of the structure of H124L, this loop exhibited the same conformation in H124L as in WT.

Nuclease H124L+P117G shares the same overall fold with WT and H124L. However, the structures of H124L+P117G and H124L differ in conformation near the mutation site. The positions of residues 112–117 with respect to the rest of the protein are different in the two variants; in H124L+P117G, these residues are closer to the loop formed by adjacent residues 77–85 (Kinemage 2). Residues 115–118, which form a type VIα β-turn in H124L and WT, adopt a type I' β-turn in H124L+P117G, with the Lys116–Gly117 peptide bond in the trans configuration. The side chains of Tyr115 and Lys116 are oriented differently in the two variants: in H124L, they point away from the rest of the protein and into solution; in H124L+P117G (Kinemage 2), they point into the active site, as described by Hynes et al. (1994) for P117G. Although residues 112–114 are located differently in the structures of H124L+P117G and P117G, they adopt the same extended conformation as in H124L and WT. Similarly, although the conformation of the loop formed by residues 77–85 is the same in all four structures, it is closer to the loop formed by residues 112–117 in H124L+P117G and P117G than in WT and H124L. This shift is maximal for Gly116, whose backbone position has moved by 0.9 Å (Kinemage 2). A water molecule appears to be involved in stabilizing this new local arrangement. It forms a bridge by hydrogen bonding to the Tyr115 side chain hydroxyl group on one loop and

**Sedimentation equilibrium**

As a control for possible aggregation of the nuclease variants studied, sedimentation equilibrium data were collected. For each of three different centrifugation speeds, the plot of the natural log of the absorbance versus the squared radius yielded straight lines for the three measured variants (WT, H124A, and H124I), suggesting that only one molecular species was present in solution. The fit of the data yielded a molecular weight close to that of the
and unfolded form of the protein... kinetic equilibrium between the... side chain of Glu46 and the backbone carbonyl oxygen of Thr32 on the other loop (Kinemage 2; Fig. 3); the water oxygen is in the same plane as the tyrosine ring, and the angle between the tyrosine C=O bond and the water oxygen is 120 degrees, as is expected for this kind of hydrogen bond (Ippolito et al., 1990).

Table 1. Thermodynamic parameters for guanidinium chloride denaturation of nuclease variants of staphylococcal nuclease and experimental values for the trans/cis ratios of the Lys124-Pro117 peptide bond

<table>
<thead>
<tr>
<th>Nuclease variant</th>
<th>$C_m$ (M)$^a$</th>
<th>$m$ (kcal·mol$^{-1}$·M$^{-1}$)$^b$</th>
<th>$\Delta G^o_w$ (kcal·mol$^{-1}$)$^d$</th>
<th>Trans/cis at residues 116-117</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.77 ± 0.05</td>
<td>5.6 ± 0.3</td>
<td>4.3 ± 0.2</td>
<td>0.173 ± 0.002$^c$</td>
</tr>
<tr>
<td>H124A</td>
<td>0.94 ± 0.06</td>
<td>5.3 ± 0.3</td>
<td>5.0 ± 0.2</td>
<td>0.136 ± 0.004$^c$</td>
</tr>
<tr>
<td>H124L</td>
<td>0.98 ± 0.01</td>
<td>5.91 ± 0.05</td>
<td>5.8 ± 0.1</td>
<td>0.124 ± 0.004$^c$</td>
</tr>
<tr>
<td>H124L</td>
<td>1.08 ± 0.03</td>
<td>5.8 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>0.097 ± 0.009$^c$</td>
</tr>
<tr>
<td>P117G</td>
<td>0.97</td>
<td>6.08</td>
<td>5.9 ± 0.2</td>
<td>0.06  $^c$</td>
</tr>
<tr>
<td>H124L+P117G</td>
<td>1.29 ± 0.03</td>
<td>5.9 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>0.06  $^c$</td>
</tr>
<tr>
<td>H124L+P47G</td>
<td>1.21 ± 0.02</td>
<td>5.88 ± 0.03</td>
<td>7.1 ± 0.1</td>
<td>0.06  $^c$</td>
</tr>
<tr>
<td>H124L+P47G+P117G</td>
<td>1.41 ± 0.01</td>
<td>5.28 ± 0.05</td>
<td>7.4 ± 0.1</td>
<td>0.06  $^c$</td>
</tr>
</tbody>
</table>

$^a$ Experimental conditions were 206 mM sodium acetate, pH 5.5, 20 °C. The results represent averages of three experiments for each variant. Indicated errors for $\Delta G^o_w$ and $m$ represent one standard deviation from the mean.

$^b$ $C_m$ represents the [GdmCl] at which half of the protein is denatured.

$^c$ $m$ is the slope of the graph of $\Delta G^o_w$ versus [GdmCl].

$^d$ $\Delta G^o_w$ is the extrapolated stability at 0 M GdmCl.

Trans/cis ratios of the Lys124-Pro117 peptide bond were obtained from the ratio of the areas under the histidine $^1$H$^+$ proton resonances of His121.

The $C_m$ and $\Delta G^o_w$ values for P117G are from Hynes et al. (1994). The $m$-value for this variant was calculated from those values.

The Lys124-Pro117 peptide bond is fully trans.

Table 2. Kinetic parameters for the hydrolysis of thymidine 3'-phosphate 5'(p-nitrophenyl phosphate) catalyzed by H124L and mutant staphylococcal nuclease

<table>
<thead>
<tr>
<th>Nuclease variant</th>
<th>$k_m$ (mM)$^a$</th>
<th>$k_{cat}$ (min$^{-1}$)$^b$</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$·min$^{-1}$)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H124L</td>
<td>0.074 ± 0.003</td>
<td>17 ± 3</td>
<td>226 ± 37</td>
</tr>
<tr>
<td>H124L+P47G</td>
<td>0.104 ± 0.007</td>
<td>3.4 ± 0.1</td>
<td>33 ± 2.5</td>
</tr>
<tr>
<td>H124L+P117G</td>
<td>0.120 ± 0.007</td>
<td>3.9 ± 0.1</td>
<td>32 ± 2.0</td>
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<tr>
<td>H124L+P47G+P117G</td>
<td>0.30 ± 0.02</td>
<td>1.3 ± 0.1</td>
<td>4.4 ± 0.4</td>
</tr>
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</table>

$^a$ Assay conditions were 0.1 M CHES, 0.2 M KCl, 0.1 M CaCl$_2$, pH 9.5, 26 °C. Indicated errors represent the estimated uncertainties in the fit of the data to Michaelis–Menten kinetics.

In the structure of H124L+P117G, the electron density is weak for the flexible loop formed by residues 42–53 and is discontinuous for residues 45–50. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. The other residues in this loop were not included in the model. The other residues in this loop and residues 45–50. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. The other residues in this loop refined to a solvent-excluded volume. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. The other residues in this loop refined to a solvent-excluded volume. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume. Because no electron density was seen for these six residues throughout the refinement, they were not included in the model. The other residues in this loop refined to a solvent-excluded volume.
Table 3. Final refinement statistics and parameters

<table>
<thead>
<tr>
<th></th>
<th>H124L</th>
<th>H124L+P117G</th>
<th>H124L+P47G+P117G</th>
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<tr>
<td>Protein residues included in the refinement</td>
<td>6-141</td>
<td>6-44, 51-141</td>
<td>6-44, 51-141</td>
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<tr>
<td>Number of non-hydrogen protein atoms</td>
<td>1,090</td>
<td>1,039</td>
<td>1,039</td>
</tr>
<tr>
<td>Number of water molecules</td>
<td>69</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>Crystallographic parameters:</td>
<td></td>
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<tr>
<td>Space group</td>
<td>P4₁</td>
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<td>P4₁</td>
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<td>Cell parameters (Å):</td>
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<tr>
<td>a, b</td>
<td>47.74</td>
<td>49.01</td>
<td>49.18</td>
</tr>
<tr>
<td>c</td>
<td>63.71</td>
<td>63.66</td>
<td>63.51</td>
</tr>
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<td>Resolution range (Å)</td>
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<td>6.0–1.95</td>
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<td>36,225</td>
<td>30,773</td>
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<td>Number of unique reflections (</td>
<td>F</td>
<td>&gt; 0.0σ)</td>
<td>15,999</td>
</tr>
<tr>
<td>Number of unique reflections (</td>
<td>F</td>
<td>&gt; 2.0σ)</td>
<td>13,478</td>
</tr>
<tr>
<td>% Completeness of unique reflections (Å)</td>
<td>98.8 (±1.7)</td>
<td>98.9 (±1.95)</td>
<td>94.5 (±1.95)</td>
</tr>
<tr>
<td>% Completeness in highest resolution shell (Å)</td>
<td>97.6 (1.75–1.7)</td>
<td>97.1 (2–1.95)</td>
<td>90.0 (2–1.95)</td>
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<tr>
<td>Rmerge (%)</td>
<td>5.90</td>
<td>6.20</td>
<td>4.74</td>
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<tr>
<td>R-factor for</td>
<td>F</td>
<td>&gt; 2.0σ (%)</td>
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<td>Free R-factor for</td>
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<td>&gt; 2.0σ (%)</td>
<td>22.4</td>
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<td>Mean B-factor (Å²):</td>
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<td>Protein backbone atoms</td>
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<td>Water atoms</td>
<td>38.2</td>
<td>41.5</td>
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<tr>
<td>Bond lengths (Å)</td>
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<td>0.011</td>
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<tr>
<td>Bond angles (degrees)</td>
<td>1.693</td>
<td>1.632</td>
<td>1.602</td>
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<td>Dihedral angles (degrees)</td>
<td>22.866</td>
<td>22.730</td>
<td>24.112</td>
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<tr>
<td>Improper angles (degrees)</td>
<td>1.225</td>
<td>1.218</td>
<td>1.380</td>
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</table>

*Rmerge(|F|) = \|∑ₜSₜ(h,k,l)|Fᵢ(h,k,l) - |Fᵢ(h,k,l)|/∑ₜSₜ(h,k,l) \| × 100%.

(Weber & Drickamer, 1983; Silva & Weber, 1993; Gross & Jaenicke, 1994; Prehoda & Markley, 1996). The size of ΔV_unfolding is determined by differences in the solvent-excluded volume and by volume differences due to differences in protein-solvent interactions between the native and the denatured states. The P117G mutation in nuclease is at the surface of the protein and does not disrupt hydrophobic interactions. Thus, it seems reasonable that any changes in ΔV_unfolding are due to changes in the solvent-excluded volume. Initial results (Royer et al., 1993) suggested that proline to glycine mutations make the protein more compact, i.e., that they cause a decrease in the solvent-excluded volume of the native state of nuclease. However, more recent experimental data (Vidugiris et al., 1996) indicated that the ΔV_unfolding values for H124L and H124L+P117G are identical within experimental error. We calculated the solvent-excluded volumes of H124L and H124L+P117G in the native (X-ray structures) and unfolded (modeled by extended chain) states using the PQMS program by Connolly (1985) (Table 4). The calculated native-state volume of

![Fig. 3. Stereo representation of the electron density surrounding residues 115-116 and 80-82 in staphylococcal nuclease mutant H124L+P117G. The figure shows an SA-omit Fᵢ – Fₑ map (Hodel et al., 1992) calculated using the final refined model with residues 115-116, 80-82, and the water molecule omitted. The map was contoured at 1.8σ.](image-url)
tion are equivalent for the two variants and because the error in these calculations are consistent with the more recent experimental Juy et al., 1983; Raleigh et al., 1992). The size of this population Xxx-Pro peptide bonds in polypeptides are unique because the Discussion

line compared with a glycine. Assuming that the effects of solvation are equivalent for the two variants and because the error in these calculations is estimated to be approximately 20 mL·mol⁻¹, these calculations are consistent with the more recent experimental results that the P117G mutation does not cause significant changes in ΔV° unfolding.

H124L+P117G is approximately 48 mL·mol⁻¹ smaller than that of H124L. Similarly, the denatured-state volume of H124L+P117G is approximately 20 mL·mol⁻¹ smaller than that of H124L, as expected from the three additional carbon atoms present in a proline compared with a glycine. Assuming that the effects of solvation are equivalent for the two variants and because the error in these calculations is estimated to be approximately 20 mL·mol⁻¹, these calculations are consistent with the more recent experimental results that the P117G mutation does not cause significant changes in ΔV° unfolding.

Discussion

Xxx–Pro peptide bonds in polypeptides are unique because the energy of the cis configuration of these bonds is closer to that of the trans than in other peptide bond linkages. In random coil polypeptides, the population of cis Xxx–Pro bonds varies between 5% and 30% (Brandts et al., 1975; Grathwohl & Wüthrich, 1976; Juy et al., 1983; Raleigh et al., 1992). The size of this population is influenced mainly by the identity of neighboring residues. In highly structured proteins, however, the protein fold can have a significant impact on the population, often leading to a predominant cis configuration in type VI reverse turns. In this article, we attempt to further analyze factors involved in stabilizing particular conformations in proline-containing loops of nuclease and to determine their relationship to the global protein stability.

From extensive studies of the Lys¹¹⁶–Pro¹¹⁷ peptide bond in nuclease (Alexandrescu et al., 1989, 1990; Hinck, 1993; Hodel et al., 1993, 1994, 1995), a generalization has emerged concerning the dependence of the configuration of the 116–117 peptide bond on solution conditions or residue substitutions at positions external to the 112–117 loop: if the change destabilizes the protein, it destabilizes the cis configuration of the Lys¹¹⁶–Pro¹¹⁷ peptide bond; conversely, if it stabilizes the protein, it stabilizes the cis configuration. Mutations at Lys¹¹⁶ do not follow these rules; in fact, the relationship between the stability of the cis configuration and the global protein stability appears to be reversed. For example, K116G has a trans Gly¹¹⁶–Pro¹¹⁷ peptide bond, yet it is more stable than WT (Hodel et al., 1993). An explanation for this is that mutations that introduce flexibility at residue 116 reduce the strain otherwise present when the Pro¹¹⁷ peptide bond is trans.

It is easy to rationalize how mutations at sites near Pro¹¹⁷ affect the configuration of its peptide bond. However, it is more difficult to explain the influence of mutations farther away in the protein structure, particularly at residues whose side chains are not involved in tertiary interactions. Their effect on the trans/cis ratio of the Lys¹¹⁶–Pro¹¹⁷ peptide bond must be a manifestation of the cooperativity in the protein fold.

His¹²⁴ is located on the solvent-exposed side of the helix adjacent to the loop containing the Lys¹¹⁶–Pro¹¹⁷ peptide bond and is not involved in tertiary interactions. Mutation of this residue to Ala, Ile, and Leu stabilizes the cis configuration of the Lys¹¹⁶–Pro¹¹⁷ peptide bond, following the expected correlation with protein stability (Fig. 2), the more stabilizing mutation favoring the cis configuration more. The slope of the correlation indicates that the energy required for the perturbation of the trans/cis ratio at the Lys¹¹⁶–Pro¹¹⁷ peptide bond represents approximately 17% of the overall observed stabilization energy of each of the substitutions at residue 124. In order to investigate these effects, it is important to determine whether they may arise from protein aggregation. Because protein stability appears to increase as a function of the hydrophobicity of residue 124, the increase in stability might be due to differential dimerization of the protein. Our sedimentation equilibrium results, however, show no evidence for dimerization. Moreover, the decrease in protein stability at higher protein concentrations, observed by calorimetry (Tanaka et al., 1993), has been explained by dimerization in the denatured state rather than in the native state. Thus, dimerization effects of residue 124 mutations probably are destabilizing rather than stabilizing. The calorimetric results indicate that the unfolded-state dimer is more stable for nuclease H124L than for WT, suggesting that the more hydrophobic residue 124 mutations favor denatured state dimerization.

Although the cis and trans configurational states in the folded protein are at equilibrium with those of the unfolded protein, a perturbation of the trans/cis ratio in the folded state can only be explained by relative changes in chemical potentials within the folded state. A relative increase in the population of the cis state can be explained by stabilization of the folded structure with a cis Lys¹¹⁶–Pro¹¹⁷ peptide bond, a destabilization of the folded structure with a trans peptide bond, or both. Because destabilization of the trans state in the folded protein would lead to overall destabilization of the protein, which is not observed, the major effect must be on the chemical potential of the cis state. Because this is the state observed in the X-ray structures, it was hoped that comparison of the structures of WT and H124L would reveal a mechanism for stabilization. For example, some hydrogen bond that is important in stabilizing the cis configuration might be present in H124L but not in WT. However, the crystal and solution (Wang J, Truckses DM, Dzukula Z, Zolnai Z, Markley JL, in prep.) structures of nuclease H124L do not show any such obvious structural difference from the X-ray structure of WT (Hynes & Fox, 1991). Because no differences in the structural models were observed, the shift in the trans/cis ratio of the Lys¹¹⁶–Pro¹¹⁷ peptide bond must be caused by subtle structural changes that cannot be detected by current structure determination techniques.

The model of Hodel et al. (1993) attributes the higher population of the cis species at the 116–117 peptide bond to strain induced in the loop containing Pro¹¹⁷ when the peptide bond is trans, by its anchorage to the rest of the protein. If, as is suggested here, the energy of the trans configuration is unaffected by mutations that alter the trans/cis ratio, then the strain (higher energy) induced in the loop must be offset equally by favorable interactions (lower energy) in the rest of the protein. Thus, the important factor in determining the position of the trans/cis ratio is the differential energy of the variant when the peptide bond is cis. Variants with a lower trans/cis ratio are ones characterized by more favorable interactions in the cis configuration that lower its energy (Alexandrescu et al., 1988).

Considering the molecular origin of the thermodynamic stability increase due to the mutations might provide further insight into the

<table>
<thead>
<tr>
<th>Nuclease variant</th>
<th>Solvent-excluded volume (mL·mol⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>H124L (native)</td>
<td>9,909</td>
</tr>
<tr>
<td>H124L (denatured)</td>
<td>7,622</td>
</tr>
<tr>
<td>H124L+P117G (native)</td>
<td>9,861</td>
</tr>
<tr>
<td>H124L+P117G (denatured)</td>
<td>7,603</td>
</tr>
</tbody>
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structural differences that lead to the changes in the trans/cis equilibrium. Residue 124 is located in a helix. Previous investigations of the effects on protein stability of amino acid substitutions in helical regions have revealed several relevant factors: the helical propensity of the amino acid, the position within the helix (center or near the ends), and/or interactions of the amino acid with other residues within the helix and/or with the rest of the protein (Hori-vitz et al., 1992; Blaber et al., 1993, 1994; Pinker et al., 1993).

In our case, residue 124 is in the fourth position of helix 3 formed by residues 121–135; i.e., it is located at the N-terminal end of the helix. The histidine has a positive charge (at the pH of the measurements) that probably interacts unfavorably with the helix dipole at this position, leading to destabilization of the helix. This unfavorable interaction is eliminated with leucine at this position. Support for this idea comes from the observation that the H124L mutation stabilizes the protein more at pH 5.0 than at pH 7.0 (1.9 kcal-mol\(^{-1}\) compared to 1.3 kcal-mol\(^{-1}\)) (Tanaka et al., 1993). Another consideration is a possible interaction between His\(^{124}\) and another charged side chain. The nearest charge-bearing residue is His\(^{125}\). However, the side chains of these two histidines are more than 6 Å apart, and each is surrounded by solvent. Also the pK\(_a\) of His\(^{125}\) is not higher in H124L than in WT (Alexandrescu et al., 1988), as would be expected if there was a charge-charge interaction between these two histidines. Thus, an unfavorable interaction between these two residues seems unlikely.

A final consideration is the relative helical propensity of the amino acid at residue 124. Model studies suggest the following values for global protein stabilization due to mutation of an intra-helical histidine (Blaber et al., 1993): His \(\rightarrow\) Ile, 0.27 kcal-mol\(^{-1}\); His \(\rightarrow\) Leu, 0.35 kcal mol\(^{-1}\); and His \(\rightarrow\) Ala, 0.39 kcal-mol\(^{-1}\). Helical propensities determined from host-guest model studies agree with this order of stabilization (O'Neil & DeGrado, 1990; Lyu et al., 1990), but the values are somewhat different: His \(\rightarrow\) Ile, 0.17 kcal-mol\(^{-1}\); His \(\rightarrow\) Leu, 0.54 kcal-mol\(^{-1}\); and His \(\rightarrow\) Ala, 0.71 kcal-mol\(^{-1}\). Our studies show that these replacements at residue 124 in nuclease result in larger stabilizations than predicted from the model studies, especially for His \(\rightarrow\) Ile and His \(\rightarrow\) Leu. Also, the order of stability is different than expected from helical propensity values. Therefore, simple helical propensity of the mutated residue does not adequately explain the observed changes in global protein stability.

Unfolding of nuclease WT has been shown to be a three-state process under conditions of low pH (pH 4.1) and high salt concentration (>100 mM NaCl) (Carra et al., 1994; Carra & Privalov, 1995). Our stability measurements were performed in 200 mM sodium acetate, at pH 5.5. It seems unlikely that our GdmCl titration studies are monitoring only partial unfolding rather than the full denaturation transition because the stability values determined here are in agreement with those obtained under different solution conditions and monitored by different probes (Shortle & Meeker, 1986). Also, the order of stability is different than expected from helical propensity values. Therefore, simple helical propensity of the mutated residue does not adequately explain the observed changes in global protein stability.

Sedimentation equilibrium experiments with nuclease WT, H124A, and H124I were performed using a Beckman Optima XL-A analytical ultracentrifuge. Experimental conditions were the same as for the stability measurements, except that protein concentrations were 0.2 mg/mL and no GdmCl was used. Equilibrium distributions at rotation speeds of 25,000, 33,000, and 37,000 rpm were examined by detecting absorbance at 280 nm. The natural log of the absorbance was plotted versus the radius square and fitted to \(\ln A = \ln A_0 + r^2 \times \text{constant}\), where \(A_0\) is the absorbance, \(r\) is the radius from the center of rotation.

Crystallography

Crystals of all nuclease variants were obtained using a modification of the conditions and method described by Loll and Lattman (1989). Lyophilized protein was dissolved in filtered 10.5 mM potassium phosphate buffer, pH 8.15; the protein concentration was 20 mg/mL. Freshly distilled, filtered 2-methyl-2,4-pentanediol (MPD) (Eastman Kodak Company) was added slowly with mixing to the protein solution, to a final concentration of 17% (w/w). This solution was allowed to sit at 4 °C for at least 24 h and then was centrifuged to eliminate precipitated protein. Vapor diffusion trays were set up with the wells containing solutions ranging between 20–30% MPD (w/w) in 10.5 mM potassium phosphate, pH 8.15. Crystals of the space group P4\(_1\) grew within one to two weeks (Table 3). X-ray diffraction intensities were recorded on a Rigaku
R-AXIS II C area detector and reduced using the R-AXIS data processing software. A single crystal was used for each of the three described data sets. Further details of the data collection and reduction are described in Table 3.

Refinement of the nuclease variant structures was performed with X-PLOR versions 3.0 and 3.1 (Brünger, 1992). A round of refinement typically consisted of positional refinement, simulated annealing, repeated positional refinement, individual atomic B-factor refinement, and manual fitting of the model into electron density maps. Whereas the models of the H124L and the H124L+P117G variants were fitted using 2F ‑ F' and F ‑ F' maps generated by X-PLOR, most maps of the H124L+P47G+P117G variant were generated by the holographic method (Somoza et al., 1995). X-PLOR maps were used only for the final rounds of refinement of this mutant. Model changes were carried out with the PS FRODO graphics display. Following initial rigid-body refinement, each structure was refined with approximately 9% of the data omitted for calculation of the free R-factor (Brünger, 1993). Only reflections greater than 2σ(Fo) were included in the refinement. Water molecules were added into positive, spherical F ‑ F' density and no 2F ‑ F maps were included in the refinement. As refinement progressed, residues 45–50, 124, and without water molecules were added into the model if their B-factors remained below 60 Å² and if they were within reasonable hydrogen bonding distance of a hydrogen bond donor or acceptor. Because the crystals of all three variants diffracted more strongly along the c* axis than along the a* and b* axes, the structure factors Fo in each case were scaled with an optimized overall anisotropic B-factor. Details of the refinement of each structure are described below, and the RMSDs from ideal geometry as well as the R-factors of the final models are reported in Table 3.

The structure of the H124L+P47G+P117G mutant was solved by molecular replacement and refined using the uncomplexed WT nuclease crystal structure (Hynes & Fox, 1991) as a starting model (Somoza et al., 1995). During initial refinement, residues surrounding the mutations (residues 45–50, 116–118, and 123–125) were omitted. As refinement progressed, residues 116–118 and 123–125 were added into the electron density. Residues 115–118 initially were refined in a type II' β-turn conformation. However, F ‑ F' density and no 2F ‑ F maps were included in the refinement. Water molecules were added into positive, spherical F ‑ F' density in each model and were kept in the model if their B-factors remained below 60 Å² and if they were within reasonable hydrogen bonding distance of a hydrogen bond donor or acceptor. Because the crystals of all three variants diffracted more strongly along the c* axis than along the a* and b* axes, the structure factors Fo in each case were scaled with an optimized overall anisotropic B-factor. Details of the refinement of each structure are described below, and the RMSDs from ideal geometry as well as the R-factors of the final models are reported in Table 3.

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The structure of the H124L+P47G+P117G model, little discontinuous electron density was visible for these residues throughout the refinement. The structure of the H124L+P47G+P117G without residues 45–50 and without water molecules was used as a starting model for the refinement of the H124L+P117G structure. The initial R-factor, which was 25.2% (data between 6.0 and 1.95 Å), dropped to 22.4% after the first round of refinement; at this stage the free R-factor was 25.4% (data between 6.0 and 2.0 Å) (fig. 1). A 180 degree rotation, yielded by a rotation search, resulted in an R-factor of 25.4% (data between 6.0 and 2.0 Å resolution) after further adjustment of the orientation with rigid body refinement. Residues 45–50 and 123–125 were excluded during the first round of refinement, which lowered the R-factor to 23.4% and resulted in a free R-factor of 27.9% (data between 6.0 and 1.7 Å resolution). Water molecules were incorporated during the following rounds of refinement, and a leucine was fitted into F ‑ F' density at residue 124, after which residues 123–125 were included in the refinement. Fairly clear F ‑ F' density was also present for residues 45–50. Thus, these residues were included in the model.

The coordinates of the structures of H124L, H124L+P117G, and H124L+P47G+P117G have been submitted for deposit at the Protein Data Bank (Brookhaven); ID codes 1SNQ, 1SNP, and 1SNQ.

Molecular volume calculation

The molecular solvent-excluded volumes of the H124L and the H124L+P117G X-ray structures were calculated using the PQMS program (Connolly, 1985, 1993). In this calculation, the molecule is modeled as a static collection of hard spheres that completely exclude a spherical probe representing the solvent. A probe radius of 1.4 Å was used. The solvent-excluded volumes of the same proteins in the unfolded state were calculated similarly. For this purpose, the protein chain was unfolded by setting all φ and ψ angles to 180 degrees. Errors in these calculations are estimated to be 20 mL-mol⁻¹.

NMR spectroscopy

Protein dissolved in 99.9% D₂O was heated to 50 °C for approximately 40 min to exchange amide protons with deuterium. Then sodium acetate-d₃ dissolved in D₂O, pH 5.5, was added to a final buffer concentration of 200 mM. The sample was lyophilized, redissolved in 100% D₂O, and the pH was adjusted to 5.3 (incorrect for deuterium isotope effects). The sample was centrifuged to eliminate any precipitant and transferred to an NMR tube. All samples contained approximately 1 mM EDTA to chelate any left-over metal ions in solution. Protein concentrations were between 2 and 2.5 mM. One-dimensional 1H NMR spectra were recorded on a 500 MHz Bruker spectrometer with a DMX console. The spectral width was approximately 6,099 Hz, with a data size of 8,192 total points. The number of averaged transients was 4,096 or 8,192 (eight initial “dummy scans” were discarded). NMR spectra were processed with the Felix computer program (BIOSYM/Molecular Simulations, Inc., San Diego, California). No apodization was applied, and the frequency spectra were baseline corrected with a second-order polynomial using a five-point interval. The line-fitting routine in Felix was used to obtain accurate integrals of the histidine 1H resonances. They were fitted to a Lorentzian lineshape using a simulated annealing optimization method. Each spectrum was recorded three times, and each of these spectra was fitted three times in order to obtain an estimate of the uncertainty in the measurement.

Enzyme activity

The nuclease activities of the proline mutants were measured using the enzyme assay described by Grissom and Markley (1989). The initial rate of cleavage of p-nitrophenyl phosphate (PNP) from thymidine 3'-phosphate 5'-PNP was monitored at 330 nm with a Hewlett Packard 8452A diode array spectrophotometer. The extinction coefficient of PNP was assumed to be 9.4 M⁻¹ cm⁻¹ (Grissom & Markley, 1989). Assay conditions were 100 mM CHES, 200 mM KCl, and 100 mM CuCl₂ at pH 9.5 and 28 °C. The same substrate stock solution was used for all assays, and the experi-
ments were performed on the same day in order to minimize differences due to substrate degradation.

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