Engineering Alternative β-Turn Types in Staphylococcal Nuclease‡

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ABSTRACT: We have refined the crystal structures of three point mutants of staphylococcal nuclease designed to favor alternative β-turn types. Single amino acid substitutions were made in a type VI_a β-turn (residues 115–118; Tyr-Lys-Pro-Asn) containing a *cis* Lys 116–Pro 117 peptide bond. The mutations result in two new backbone conformations, a type I β-turn for P117T and a type I′ β-turn for P117G and P117A. The P117G and P117A structures exhibit a dramatic difference in backbone conformation in the region of the mutation compared to the nuclease A structure such that the side chain of Lys 116 is reoriented to point into the nucleotide binding pocket. The distinct conformation observed for the nuclease A, P117G, and P117T β-turn sequences agrees with correlations between β-turn type and sequence identified from protein crystal structures. The P117A turn conformation provides an exception to these correlations. The results demonstrate that single residue changes can significantly alter backbone conformation, illustrating the process by which diversity in the structure of the protein surface can evolve on a conserved structural core, and suggest protein engineering applications in which the positioning as well as the identity of side chains can be modified to design new enzyme functions. Nuclease variants at the type VI_a β-turn site also allow the relationship between the amino acid sequence and β-turn conformation to be examined in the context of an identical protein fold in crystallographic detail.

Four-residue β-turns are discrete elements of surface structure with backbone conformations determined primarily by local sequence in combination with the adjacent protein topology (Rose et al., 1985). This has been shown experimentally in studies where β-turn and loop sequences transferred from one protein to a suitable host protein retain their structural (Hynes et al., 1989) and functional characteristics (Verhoeyen et al., 1988; Murray et al., 1988). A connection between local sequence and backbone conformation has been established in surveys of protein crystal structures which report significant correlations between β-turn type and the sequence in the second and third positions of the β-turn (Wilmot & Thornton, 1988). In contrast, certain β-turn types predominate in structurally homologous turn sites, indicating the potential influence of turn site geometry on β-turn conformation and the potential need to match particular β-turn types with specific contexts (Sibanda & Thornton, 1988).

Of the 20 naturally occurring amino acids, glycine and proline have the greatest influence on local backbone conformation as they differ significantly in the sterically allowed ranges of the ϕ and ψ backbone dihedral angles (Figure 1). Each β-turn type has unique sequence preferences for glycine and proline residues which can be visualized when the backbone dihedral angles (ϕ, ψ) of residues in the second and third β-turn positions are superimposed on sterically allowed regions (Figure 1). Type I β-turns tolerate all amino acids in both the second and third positions of the turn. Type II β-turns favor Gly in the third position, while Gly is favored in the

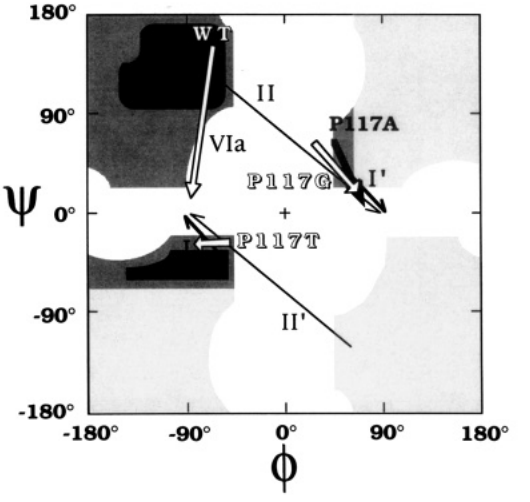

FIGURE 1: Backbone conformations of ideal and experimentally observed β-turns. The Ramachandran plot illustrates the allowed dihedral space of a polypeptide backbone. All shaded areas represent the sterically allowed range of Gly. Due to contacts between the β-carbon and adjacent backbone atoms, non-Gly residues are found in the medium- and dark-shaded areas (Richardson, 1981). Pro is further restricted by the side-chain ring to have a ϕ value close to −65°. The geometry of a β-turn is uniquely defined by the ϕ and ψ angles of the residues in the second and third position of the four-residue turn. β-Turn geometries can thus be represented by an arrow on the Ramachandran plot: the tail of the arrow originates at the ϕ, ψ angles of the second residue of the turn, and the arrow ends at the ϕ, ψ angles of the third residue of the turn. Ideal geometries for type I and II and mirror image I′ and II′ β-turns (Wilmot & Thornton, 1988) are represented by single-headed arrows as labeled. Open arrows correspond to the crystallographically determined conformations of the type VI_a β-turn of nuclease (WT) (residues 115–118; Tyr-Lys-Pro-Asn), the type I β-turn of P117T, and the type I′ β-turn of P117G. The thick black arrow corresponds to the crystallographically determined conformations of the type I′ β-turn of P117A.

‡ The crystallographic coordinates have been deposited in the Brookhaven Protein Data Bank under the following file names: P117G, 1SYC; P117A, 1SYG; P117T, 1SYE; P117G complex, 1SYD; P117T complex, 1SYF.

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second position of type II′ β-turns. Type I′ β-turns favor Gly in the third position while the second position is restricted to Gly or a side chain with an unbranched β-carbon due to the restricted left-handed helix geometry. Proline occurs frequently in the second position of type I and II β-turns (Wilmot & Thornton, 1988) and is present in the third position of a type VI_a β-turn, contributing to the stabilization of the *cis* peptide bond conformation.

The peptide unit preceding a proline residue can more readily adopt the *cis* conformation due to a reduced energy difference between the *cis* and *trans* isomers compared to other amino acid residues (Ramachandran & Mitra, 1976). The *cis* peptide bond is energetically unfavorable and thus type VI_a β-turns are strained regions of protein molecules under stress imposed by the remainder of the protein. Previous work in this laboratory suggests that the stress is most likely imposed by restriction on the loop length and conformational restrictions imposed by the position of the ends (Hodel *et al.*, 1993).

The restrictions listed above are not absolute, and residues are found outside of their expected conformational ranges at low frequency (Nemethy & Scheraga, 1977). Further progress in our understanding of backbone conformation requires the independent evaluation of the effects of local sequence and turn site geometry. As each β-turn in the structural data base differs in sequence as well as topological environment, it is necessary to engineer point mutations in a constant protein context to examine specifically the contribution of local sequence to the folded backbone conformation.

Staphylococcal nuclease adopts two folded conformations in solution differing in the *cis/trans* conformation of the Lys 116–Pro 117 peptide bond (Fox et al., 1986; Evans et al., 1987, 1989). The crystal structures of nuclease and the nuclease–Ca^{2+}–pdTp[1] complex clearly reveal that the major conformation of residues 115–118 is a type VI_a β-turn with a *cis* Lys 116–Pro 117 peptide bond (Loll & Lattman, 1989; Hynes & Fox, 1991). This β-turn element was considered a favorable site at which to examine the relationship between amino acid sequence and β-turn conformation in a constant protein context, due to the existence of conformational alternatives in the wild-type protein. The *cis* configuration of a peptide bond between two residues, X–Y, is thought to be much less favorable when residue Y is not a proline. The free energy difference between the nuclease Lys 116-Pro 117 *cis* and *trans* conformations is modest (1.1 kcal/mol) (Evans et al., 1989). Although the free energy difference between the *cis* and *trans* conformations has not been established for peptide bonds that do not involve proline residues, it is believed to be larger than that for proline-containing peptides, and thus substitutions for Pro 117 were expected to favor a *trans* peptide bond. Herein we report the crystal structures of Pro 117 → Thr, Pro 117 → Ala, and Pro 117 → Gly. We reasoned that if the resulting nuclease variants are stably folded and the 115–118 region is ordered, the P117T and P117A segments would be consistent with a type I β-turn, while the P117G segment could adopt a type I, I′, or II β-turn conformation as explained above.

MATERIALS AND METHODS

Preparation of the Nuclease Variants. The nuclease A gene was subcloned into M13mp18 to produce single-stranded template DNA. The P117T and P117G mutants were prepared by primer-directed mutagenesis using synthetic oligonucleotides (Zoller & Smith, 1983). Plaques were screened by differential hybridization using 5-^{32}P-labeled oligonucleotides and sequenced to verify the mutation. The P117A mutant was prepared by PCR mutagenesis using synthetic oligonucleotides (Higuchi, 1990) and was sequenced to verify the mutation. The mutant genes were subcloned into the plasmid pAS1 and expressed in *Escherichia coli*, and protein was prepared as described (Evans et al., 1989).

Crystallization and X-ray Intensity Data Collection. Nucleases P117T, P117A, and P117G were crystallized from low-salt buffer (10.5 mM potassium phosphate, pH 8.15, 2 mg/mL of protein) using 25% 2-methyl-2,4-pentanediol (MPD) as a precipitant (Arnone et al., 1969), yielding bipyramidal crystals in space group $P4_1$. The Ca^{2+}–pdTp complex crystals were grown under the same conditions but with the addition of Ca^{2+} and pdTp at the concentrations shown in Table 1. Unit cell dimensions were within 1% of the structure of nuclease alone or complexed with pdTp and Ca^{2+} (Hynes & Fox, 1991; Loll & Lattman, 1989). A single crystal was used in each of the five data sets described in Table 1. X-ray diffraction intensities were measured using an area detector system and Cu Kα radiation from a rotating anode generator. Redundant symmetry-related reflections were averaged to give reasonable *R*-merge statistics. The effective resolution of a data set is best defined by D2, the highest resolution shell with a mean intensity greater than or equal to $2\sigma(I)$. D2 varied from 1.7- to 2.0-Å resolution for the five data sets. Details of the data collection and reduction are given in Table 1.

Crystallographic Refinement. The refinement of the P117T and P117G structures reported herein was carried out with PROLSQ (Hendrickson, 1985). Model building was facilitated by FRODO (Jones, 1985). A segment containing the site of substitution (residues 115–118) was omitted from the starting model in each refinement. All crystals displayed anisotropic diffraction intensities as had been observed for nuclease A (Hynes & Fox, 1991). Following initial cycles of scale, positional, and *B*-factor refinement a local scale factor was calculated (Matthews & Czerwinski, 1975) and applied to the observed structure factors (F_o) relative to the calculated structure factor amplitudes (F_c), as previously described for nuclease A (Hynes & Fox, 1991). The local scale factor was calculated as $\sum(F_c)/\sum(F_o)$, summing over a $5 \times 5 \times 5$ reflection box centered on the reflection in question which was excluded from the summation to prevent bias. Reflections with fewer than 40 values in the summation were removed from the data set. This method preserves the overall *B*-factor while minimizing systematic variation in the observed intensities. The structure for residues 115–118, initially removed from the model, was clear for the two complex structures and was included in an early stage of the refinement. Corrections were made to other parts of the protein chain and refined. Water molecules were added to the model when observed in difference maps within hydrogen-bonding distance from polar protein atoms. The water molecules were refined at full occupancy and kept in the model if their *B*-factors remained below 60 Å^2. The P117T and P117G protein structures were refined in a similar fashion; however, the conformation of residues 115–118, deleted from the initial model, was not interpretable early in the refinement. Errors in the remainder of the protein model and the water model were refined before the conformation of residues 115–118 became clear. This segment was then added to each protein model and refined. Only data with $F_o > 2\sigma(F_o)$ were included in the final stages of refinement. The final structures were refined to high resolution with reasonable *R*-factors and errors in the covalent geometry as shown in Table 1.

[1] Abbreviation: pdTp, thymidine 3′,5′-diphosphate.

Table 1: Crystallographic and Geometric Parameters at the Conclusion of Refinement

	P117G complex	P117T complex	P117G	P117T	P117A
a, b (Å)	48.6	48.3	48.9	48.4	48.4
c (Å)	63.5	63.1	63.7	62.9	63.4
[pdTp] (μM)	620	620	0	0	0
[$CaCl_2$] (μM)	155	310	0	0	0
temperature (°C)	5	5	25	25	5
resolution range (Å)	8.0–1.7	8.0–1.8	8.0–1.8	8.0–1.8	6.0–1.9
total measurements	108 292	99 105	33 206	70 066	87 597
no. of unique reflections	17 294	13 515	20 238	13 919	10 281
% unique reflections (resolution, Å)	96 (1.67)	100 (1.8)	59 (1.7)	99 (1.8)	99 (1.9)
D2[a]	1.7	1.9	1.75	2.0	1.9
R_{sym}[b]	4.5	7.2	1.3[c]	6.5	7.0
R-factor[d]	18.4	16.8	18.2	18.2	19.4
no. of protein atoms	1089	1092	1089	1092	1090
no. of water molecules	83	89	69	55	51
no. of total atoms	1172	1181	1158	1147	1141
mean B-factor	22.9	23.6	23.9	28.3	36.2
main-chain atoms	17.9	18.6	19.8	23.9	33.1
side-chain atoms	25.7	26.3	26.4	31.5	40.1
backbone atoms (residues 115–118)	31.5	27.1	18.3	40.9	34.3
side-chain atoms (residues 115–118)	46.9	39.4	26.0	55.7	52.3
water	36.2	37.1	36.0	39.0	47.0
deviations from ideal geometry					
bond lengths (Å)	0.016	0.015	0.016	0.015	0.006
bond angles (Å, deg)[e]	0.033	0.034	0.035	0.036	1.5[e]

[a] D2 represents the resolution shell where the $\langle I \rangle = 2\sigma$. [b] Merging R-factor for symmetry-related refletions is defined as $\Sigma|\langle I \rangle - I|/\Sigma\langle I \rangle$. [c] The low R_{sym} value for P117G is due to the fact that the data set had very low redundancy at high resolution as reflected by the low completeness value. Therefore, the multiple measurements that went into the R_{sym} calculation are primarily medium- to low-resolution reflections which are more accurately measured leading to the low R_{sym}. [d] Crystallographic R-factor is defined as $\Sigma|F_o - F_c|/\Sigma F_o$. [e] Bond angle deviation is in degrees for P117A (XPLOR refined) and in 1–3 distances for all others (PROLSQ refined).

The refinement of P117A was carried out through the program XPLOR (Brünger et al., 1987), and model building was facilitated by FRODO. The structure of uncomplexed nuclease A (Hynes & Fox, 1991) with residues 112–118 was used as a starting model. This model was refined to the data first through multiple cycles of B-factor and positional refinement and then through the simulated annealing omit procedure (Hodel et al., 1992). The segment of residues 112–118 were manually built into the density calculated from the refined, edited structure. After some refinement, the observed structure factors were modified by local scaling as described above. The structure was then further refined, and water molecules were added. Final refinement parameters are shown in Table 1.

Thermodynamic Stability Determinations. The thermodynamic stability of nuclease and its variants was measured by guanidine hydrochloride (GuHCl) denaturation, monitoring the fluorescence of the single tryptophan at residue 140. Protein concentrations were 6 μM (0.1 mg/mL) in a buffer of 200 mM sodium acetate, pH 5.5. The excitation wavelength was 300 nm, and emission was monitored at 340 nm using an SLM Model 8000 fluorometer. Extrapolation to zero denaturant concentration provides an estimate of ΔG_D in the absence of denaturant (Pace, 1975).

RESULTS

Thermodynamic Stability. Nucleases P117T, P117A, and P117G fold into stable, enzymatically active molecules which bind the nucleotide inhibitor pdTp and Ca^{2+}. The concentration of GuHCl which produced half-unfolding (C_m) for nuclease is C_m = 0.74 M. Under these conditions the free energy of folding was ΔG_D = 4.2 kcal/mol. The thermodynamic stability of P117T (ΔG_D = 4.2 kcal/mol, C_m = 0.75 M) is very similar to that of nuclease ($\Delta\Delta G_D$ = –0.02 kcal/mol). The stability of P117A is slightly higher than that of wild type (ΔG_D = 4.8 kcal/mol, C_m = 0.80 M) while the stability of P117G (ΔG_D = 5.9 kcal/mol, C_m = 0.97 M) is significantly increased compared to wild type ($\Delta\Delta G_D$ = 1.7 kcal/mol) (Figure 2).

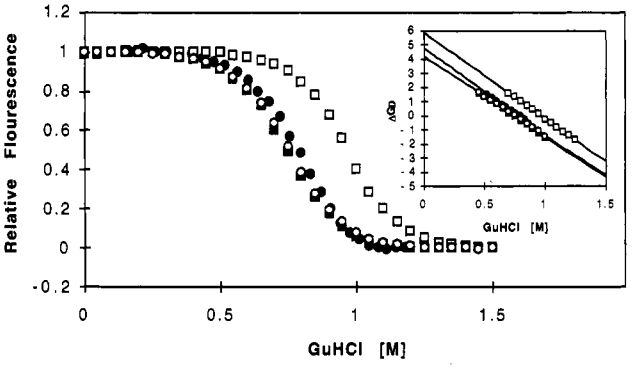

FIGURE 2: Thermodynamic stability of nuclease (filled squares), P117T (open circles), P117A (filled circles), and P117G (open squares) measured by guanidine hydrochloride (GuHCl) denaturation. Measurements were monitored by Trp 140 fluorescence. The inset shows the Gibbs free energy of denaturation (ΔG_D) as a function of GuHCl concentration. Extrapolation to zero denaturant concentration provides an estimate of ΔG_D in the absence of denaturant (Pace, 1975). Uncertainty in the slope of this extrapolation leads to errors of 0.2 kcal/mol in ΔG_D.

P117T–Ca^{2+}–pdTp Refined Structure. The refined crystal structure of P117T is similar to that of nuclease A; however, residues 115–118 (Tyr-Lys-Thr-Asn) adopt a type I β-turn conformation with clear electron density (Figure 3A). The P117T β-turn occupies the same overall position as the type VI_a β-turn in nuclease and differs primarily in the conformation of the peptide unit preceding Thr 117 (Figure 3C). The peptide unit is *trans* with the carbonyl oxygen reoriented by 180° relative to the *cis* peptide in the nuclease structure. Smaller changes occur in the position of the peptide groups preceding Lys 117 and Asn 118 as well as the side chain of Lys 117. The backbone atoms of the P117T β-turn do not interact with surrounding protein or ligand atoms. The pdTp and Ca^{2+} ligands are clearly represented in the electron density. When

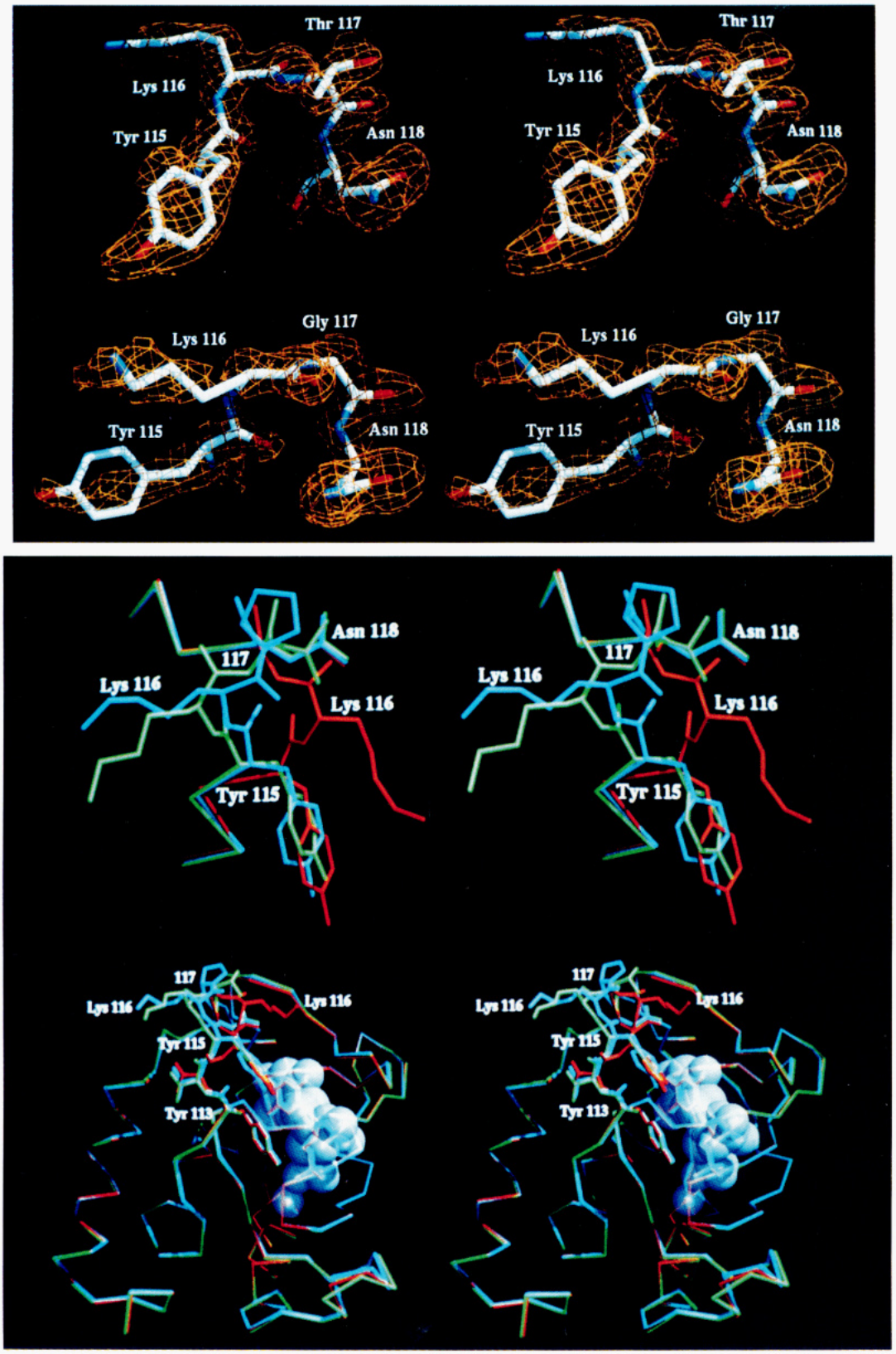

FIGURE 3: Crystal structures of the P117T– and P117G–pdTp-Ca^{2+} complexes and superpositions with the nuclease complex (Loll & Lattman, 1989). From top to bottom: (A) Structure of the P117T type I β-turn (residues 115–118; Tyr-Lys-Thr-Asn) with a 1.8-Å $F_o - F_c$ residue-deleted electron density map, contoured at 2.5σ (σ is the root-mean-square difference electron density in the unit cell). The four β-turn residues were omitted from the $F_o - F_c$ Fourier map calculation in order to remove the bias of phase information contributed by the model. (B) Structure of the P117G type I′ β-turn (residues 115–118; Tyr-Lys-Gly-Asn) with a 1.7-Å $F_o - F_c$ residue-deleted electron density map, contoured at 2σ. The four β-turn residues were omitted from the $F_o - F_c$ Fourier map calculation. (C) Backbone atoms of the nuclease type VI_a β-turn (blue), the P117T type I β-turn (green), and the P117G type I′ β-turn (red) from residues 112–121. All atoms are shown for residues 115–118. The superposition was made by matching the backbone atoms outside of the β-turn region. The average temperature factors of the backbone atoms of the four β-turn residues of nuclease, P117T, and P117G are 23.0, 26.4, and 31.6 Å^2, respectively. (D) Superimposed α-carbon tracings of nuclease (blue), P117T (green), and P117G (red). The bound nucleotide (pdTp) and Ca^{2+} (shaded spheres) are shown along with all atoms of residues 113–118 (Tyr-Val-Tyr-Lys-Xxx-Asn). Excluding the β-turn region and a highly mobile loop (residues 43–55), the rms deviations between backbone atoms comparing nuclease with P117T and P117G are 0.20 and 0.19 Å, respectively.

the backbone atoms of the two mutants outside the altered turn region are matched to the structure of the nuclease–pdTp–Ca^{2+} complex (Loll & Lattman, 1989), the ligand positions superimpose precisely (Figure 3D). In the nuclease A and P117T structures, the interaction of the β-turn with bound ligands is limited to the side chain of Tyr 115 in the first position of the β-turn, which packs on the nucleotide base of the pdTp inhibitor. Final refinement parameters for all structures appear in Table 1.

P117G–Ca^{2+}–pdTp Refined Structure. The refined crystal structure of P117G is again similar to nuclease A; however, in this case residues 115–118 (Tyr-Lys-Gly-Asn) are found in a type I′ β-turn conformation with weak electron density characteristic of increased mobility (Figure 3B). The peptide unit preceding Gly 117 is *trans* and is reoriented by 180° relative to the P117T structure. The β-turn has shifted considerably in position relative to both nuclease A and P117T (Figure 3C), moving close to a loop including Gln 80 which runs along the opposite side of the nucleotide binding pocket (Figure 3D). The carbonyl oxygen of Lys 116 forms a close contact (3.5 Å) with the backbone nitrogen of Gln 80. The combination of the change in β-turn type and orientation has positioned the side chain of Lys 116 so that it now points into the nucleotide binding pocket (Figure 3D). The side chain of Lys 116 is modeled into weak electron density (Figure 3B) but does not appear to interact directly with the bound nucleotide. Refinement parameters for all structures appear in Table 1.

Refined Structures of P117T and P117G. The binding of pdTp and Ca^{2+} to nuclease A induces a conformational change in residues 113–118 such that Tyr 113 and Tyr 115 move into the binding pocket and pack with the base. In the absence of nucleotide Tyr 113 swings out of the binding pocket and packs against Tyr 115 (Hynes & Fox, 1991). The backbone ϕ, ψ angles of residues 112–115 correspond to those of an extended β-sheet, while with bound nucleotide Tyr 113 adopts an α_L conformation. Given the proximity of these tyrosines to the altered β-turn site, it was of interest to examine the structures of P117T, P117A, and P117G in the absence of bound pdTp and Ca^{2+}.

In the absence of pdTp and Ca^{2+} residues 115–118 of P117T again adopt a type I β-turn conformation, but with a different orientation relative to that seen for the P117T complex (Figure 4). Tyr 115 occupies part of the nucleotide binding pocket, overlapping a portion of the space filled by the pdTp base in the structure of the P117T complex. In P117T the backbone and side chains of Tyr 113 and Val 114 have rotated approximately 180° about the axis of the backbone path relative to the complex structure. Tyr 113 has rotated out of the binding pocket, maintaining the α_L backbone conformation observed in the complex. The Tyr 113 side-chain conformation is stabilized to some extent through hydrophobic packing with Lys 116. The rotation of Val 114 has repositioned the side chain so that it now points toward the binding pocket.

Residues 115–118 of P117G adopt a type I′ β-turn conformation as seen in the P117G–Ca^{2+}–pdTp complex, but again with a difference in orientation with respect to the remainder of the protein (Figure 4). As was seen in the P117T structure, Tyr 115 is observed in the nucleotide binding pocket although it overlaps the nucleotide base position to a lesser extent. Tyr 115 packs against the side chain of Lys 116 which also extends into the binding pocket. The difference in the backbone conformation of Tyr 113 and Val 114 for P117G and its complex is similar to that seen in the nuclease A structure. Tyr 113 adopts an α_L conformation in the P117G complex, while in the absence of ligands the ϕ, ψ angles of Tyr 113 correspond to those of an extended β-strand. Therefore, the transformation between the P117G and P117G complex structures involves switching between discrete minima of backbone conformation. The side chain of Tyr 113 in P117G differs in orientation from that seen in the nuclease A structure, possibly due a lack of packing interactions with Tyr 115.

Table 2: rms Deviations (Å) between Backbone Atoms

nuclease complex and P117T complex	0.16
nuclease complex and P117G complex	0.14
nuclease and P117T	0.20
nuclease and P117G	0.24

Structure of Unliganded P117A. Contrary to expectation, residues 115–118 in P117A adopt a type I′ β-turn conformation nearly identical to the conformation found in P117G. The conformation of the β-turn is clearly defined by the electron density (Figure 5). Many small changes in the backbone conformation of residues 112–117, relative to that of P117G, allow the ϕ angle of Ala 117 to relax into the α_L region of ϕ–ψ space (Figure 1). However, Ala 117 is still somewhat strained with the β-carbon in close contact with the carbonyl oxygens of residue 116 (2.8 Å) and residue 117 (2.9 Å) (Figure 5). Any β-branched residue, such as threonine, would be strictly prohibited from this conformation due to steric hindrance with the adjacent peptide groups.

Localization of the Effects. The observed differences between nuclease, P117T, P117A, and P117G whether liganded or not are largely restricted to the conformational changes in residues 113–118 as described above and a poorly modeled flexible loop covering residues 41–55. The rms deviations between the backbone atoms, excluding these regions, are shown in Table 2. The ends of the loop containing the mutation (residues 112–117) are anchored by Val 111, which makes several intramolecular packing contacts, and the side chain of Asn 118, which is rigidly fixed by two hydrogen bonds to the backbone of a nearby loop (residues 79 and 80). Both of these residues remain in the same position in all models of nuclease A, P117T, P117A, and P117G.

Three Variations on Induced Fit. Nuclease A shows a modest induced fit of the active site loops upon binding ligands. Tyr 115, the first residue of the β-turn, forms one part of the nucleotide binding pocket. The conformation of residues 113–118 is distinct in the nuclease A, P117G, and P117T structures. On binding pdTp and Ca^{2+}, this polypeptide is restructured in a different way in each of these proteins to produce a common mode of interaction between pdTp and Tyr 133 and Tyr 115 (Figure 4). Despite the added conformational restrictions placed on this segment by the bound ligands, the β-turn types are preserved, and only their orientations are modified.

Variation in β-Turn Mobility. Examination of the backbone temperature factors of nuclease A, P117T, and P117G in the region of the 115–118 β-turn provides some insight into the degree to which single-residue changes can alter local flexibility. In addition, the linkage of the β-turn to Tyr 113 and Tyr 115 reveals the differential impact of ligand binding on β-turn dynamics. The structures of P117G, P117T, and their complexes were refined with identical restraints and had similar average backbone atom *B*-factors (Table 1). The backbone temperature factors of residues 110–120 of nuclease, P117T, and P117G in the presence and absence of bound pdTp and Ca^{2+} are shown in Figure 6. The β-turn conformations reported herein were all clearly observed in residue-deleted difference and $2F_o - F_c$ maps, despite the high *B*-factor values obtained from the refinement for some residues (Figure

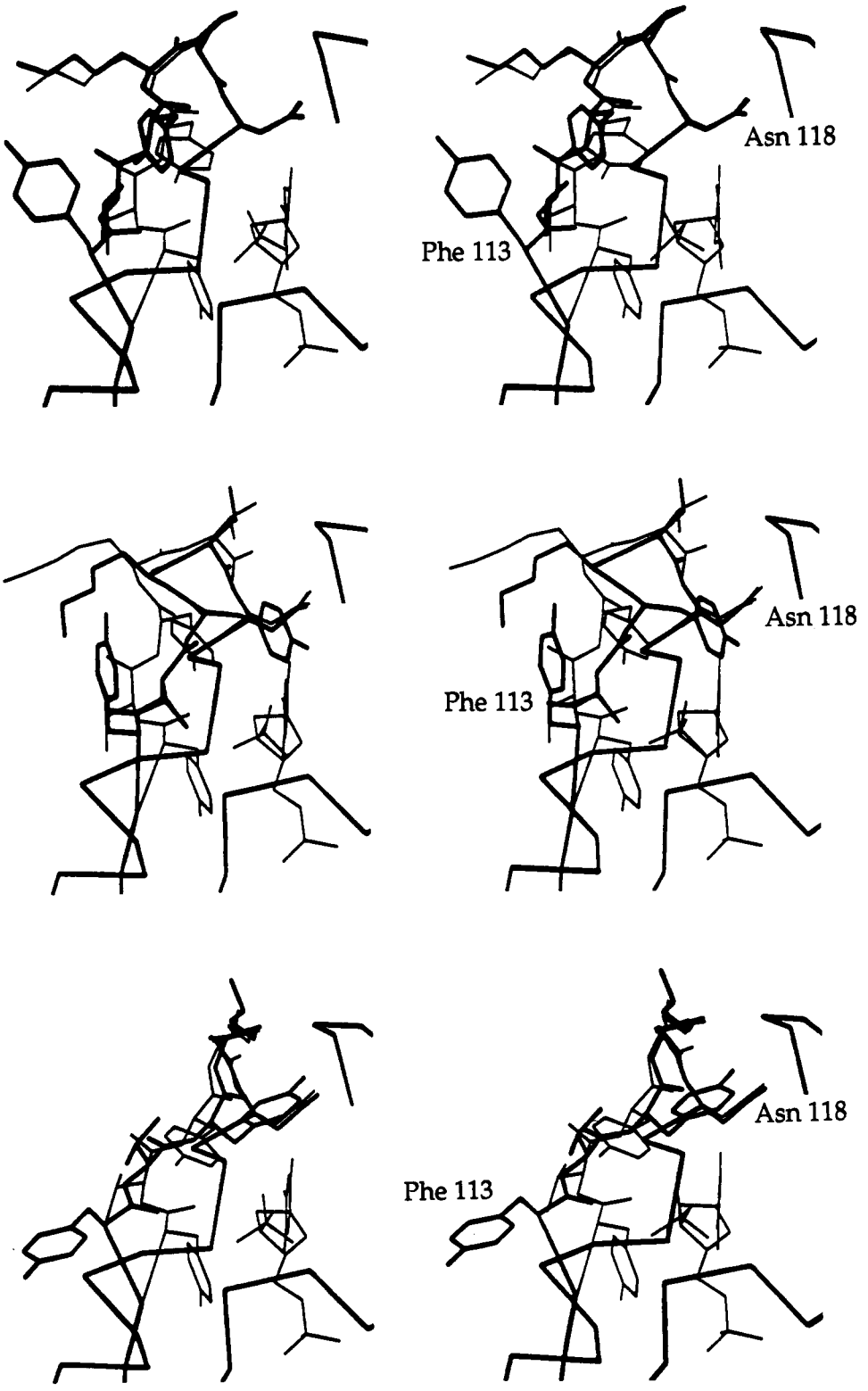

FIGURE 4: Superposition of nuclease A and the P117T and P117G variants with their respective Ca^{2+}–pdTp complex structures. The liganded and unliganded structures were aligned excluding residues 113–118 and 41–51, which clearly varied between the pairs of structures. All atoms are shown for residues 113–118 (Tyr-Val-Tyr-Lys-Pro-Asn for nuclease A), and an α-carbon trace is shown for the remainder of the protein. The structure of the protein alone is shown in heavy lines while the nuclease–Ca^{2+}–pdTp complex is shown in thin lines. Tyr 113 is below Tyr 115 in each panel. From top to bottom: (a) nuclease A, (b) P117T, and (c) P117G.

6). The smooth change in *B*-factor with residue number reflects in part the restraints imposed on the variation in *B*-factor for bonded pairs of atoms. Likewise, rotational disorder of side chains about χ_1, resulting in high *B*-factors, will also tend to increase the backbone *B*-factors due to the bonded pair restraints of the *B*-factors. The temperature factors for this region of nuclease A are largely unchanged by ligand binding. In P117T the β-turn temperature factors are similar to those of nuclease in the presence of nucleotide but increase dramatically in the absence of ligands. P117G exhibits the opposite behavior with elevated temperature factors in the presence of ligand and temperature factors that are actually lower than nuclease in its absence. These differences are significant when compared with the small

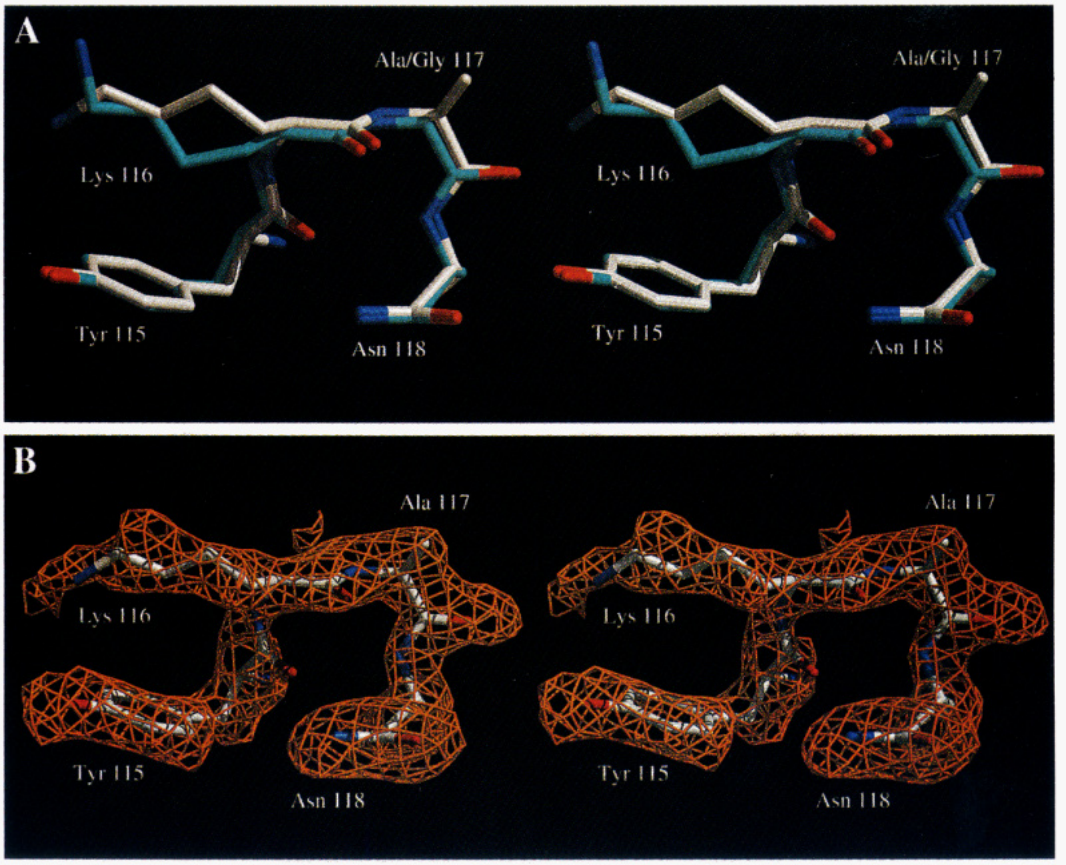

FIGURE 5: (A) Superposition of the type I′ β-turns (residues 115–118) of P117A (white) and P117G (cyan). (B) Structure of the P117A type I′ β-turn (residues 115–118; Tyr-Lys-Ala-Asn) with a 1.9-Å $F_o - F_c$ residue-deleted electron density map, contoured at 2.0σ (σ is the root-mean-square difference electron density in the unit cell). Residues 112–118 were omitted from the $F_o - F_c$ Fourier map calculation in order to remove the bias of phase information contributed by the model.

variation in overall *B*-factor (Table 1). Such examples of diversity in mobility in the same protein context due to point mutations provide a data base for dynamics simulations aimed toward understanding the determinants of protein flexibility.

Sequence Dependence of β-Turn Conformation. An example of the roles played by local sequence and turn site geometry in the determination of a β-turn conformation is seen in the unliganded P117G structure. The sequence of the β-turn covering residues 115–118 (Tyr-Lys-Gly-Asn) is effectively identical to that of a second β-turn covering residues 27–30 (Tyr-Lys-Gly-Gln). Both β-turns adopt a type I′ conformation. Superposition of the β-turns reveals a close match between backbone as well as side-chain atoms (Figure 7). The geometry of the backbone strands leading into and away from the β-turns (Figure 7) as well as the surrounding protein environment is different. Here we see differing turn site geometries which determine the location of the β-turn while the detailed conformation or β-turn type appears to be largely dependent on local sequence.

DISCUSSION

The empirical rules for β-turn type based on the presence of glycine and proline residues in the second and third β-turn positions are satisfied by the β-turn types observed for nuclease A, P117T, and P117G but not for P117A (Figure 1). The challenge in predicting the outcome of such mutations results from overlap in the conformational ranges of Gly and Pro with the remaining amino acids. For this reason, sequences including Gly or Pro in the second or third β-turn position are generally compatible with more than one β-turn type. The Lys-Pro sequence of nuclease is consistent with the type VI_a β-turn, which strongly favors Pro for the *cis* peptide conformation but could also adopt a type I β-turn conformation. The Lys-Gly sequence of P117G is compatible with the observed type I′ β-turn as well as β-turn types I and II. The type I β-turn was the expected ordered conformation for P117T as it is the only β-turn type consistent with the Lys-Thr sequence in the second and third β-turn positions (Figure 1), although modest displacement from other ideal β-turn geometries might be tolerated. The type I β-turn was also the expected ordered conformation for P117A; however, this variant adopted the type I′ β-turn in a conformation nearly identical to that of P117G.

The hypothesis presented below provides one explanation for the conformations observed for nuclease A and the P117T, P117A, and P117G variants. The modest stress imposed by nuclease A on the 115–118 segment, to favor the strained *cis* Lys116–Pro117 peptide bond in nuclease A, is not sufficient to overcome the increased free energy difference between *cis* and *trans* isomers expected for non-prolyl peptide bonds at this site in the P117T, P117A and P117G variants, resulting in the observed *trans* peptide bonds. The P117T variant adopts a type I β-turn conformation, consistent with the empirical rules described above and demonstrating that the conformation is accessible in this protein context. Although residues 115–118 appear to be in a relaxed conformation, the ϕ, ψ angles of residues 112–114 enter less favorable regions of the Ramachandran diagram, indicating that they may be strained in order to connect the anchored ends of the loop. The P117T type I β-turn conformation may in fact represent that of the 115–118 segment in the minor conformer of nuclease A with a *trans* K116–P117 peptide bond. The comparable stability

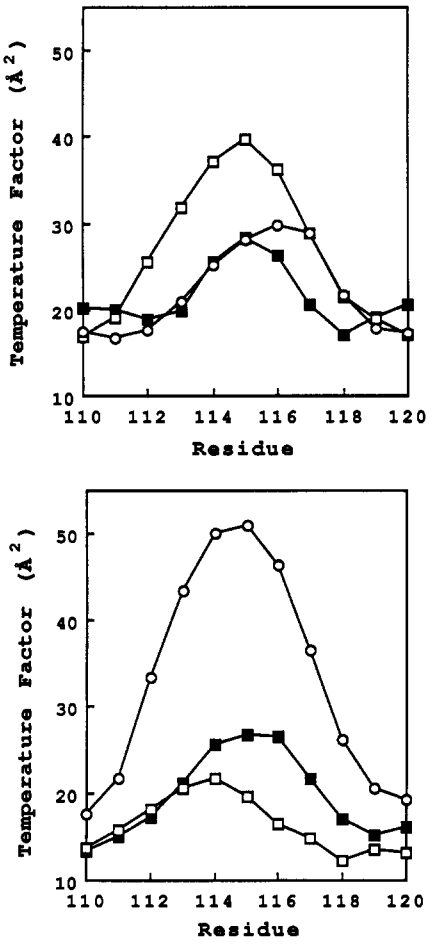

FIGURE 6: Mean backbone *B*-factors in the β-turn region (residues 110–120) for nuclease A (filled squares), P117T (open circles), and P117G (open squares) presented for (a, top) the Ca^{2+}–pdTp complex and (b, bottom) the protein alone.

of nuclease and P117T is consistent with this hypothesis. Nuclease must impose a stress on the 112–118 polypeptide segment to favor the *cis* peptide bond. The remainder of the protein must be displaced from its minimum energy conformation by the 115–118 segment with a *trans* K116–P117 peptide bond. The remainder of the protein must relax toward this free energy minimum by imposing the energetically unfavorable *cis* K116–P117 peptide bond. Thus both nuclease and P117T must be similarly displaced from the minimum free energy conformation of the remainder of the protein, due to the unfavorable conformational space available to the 112–118-residue segment.

The sequence for residues 115–118 of the P117G variant (Tyr-Lys-Gly-Asn) is compatible with type I, I′, and II β-turns. The observation of a type I′ β-turn conformation for this segment of P117G while a type I β-turn conformation is present in P117T suggests that removal of the side-chain atoms of residue 117 removes unfavorable van der Waals interactions between that side chain and the remainder of the segment backbone due to the conformational restraints imposed by the remainder of the protein. The removal of the residue 117 side chain thus allows the segment to adopt a conformation more compatible with the minimum free energy conformation for the remainder of the protein. A favorable energy of interaction within the segment or between the segment and the remainder of the protein could also explain the conformational variation observed; however, the paucity of interactions suggests such favorable interaction terms must play a minor role.

The 115–118 residue segment of P117A adopts a type I′ β-turn even though this turn type is somewhat strained with a non-glycine residue in the third position. This suggests that the conformation of the 112–118 residue segment with a type I turn, such as that seen in P117T, has more strain than the strain induced by the close contact between the Ala 117 β-carbon and Lys 116 carbonyl oxygen in the type I′ turn. Although alanine can be accommodated in the third position of a type I′ turn, threonine is more stringently forbidden from this conformation.

The P117G and P117A structures indicate that in the specific context of nuclease the type I′ β-turn conformation is lower in free energy than the alternative type I or type II β-turn geometries. This is supported by the observed increase in the stabilities of P117A and P117G, which can be attributed to a decrease in the free energy of the folded form. Increased stability results from a decrease in the free energy of the folded form and/or an increase in the free energy of the unfolded form. The effect of replacing the conformationally restricted

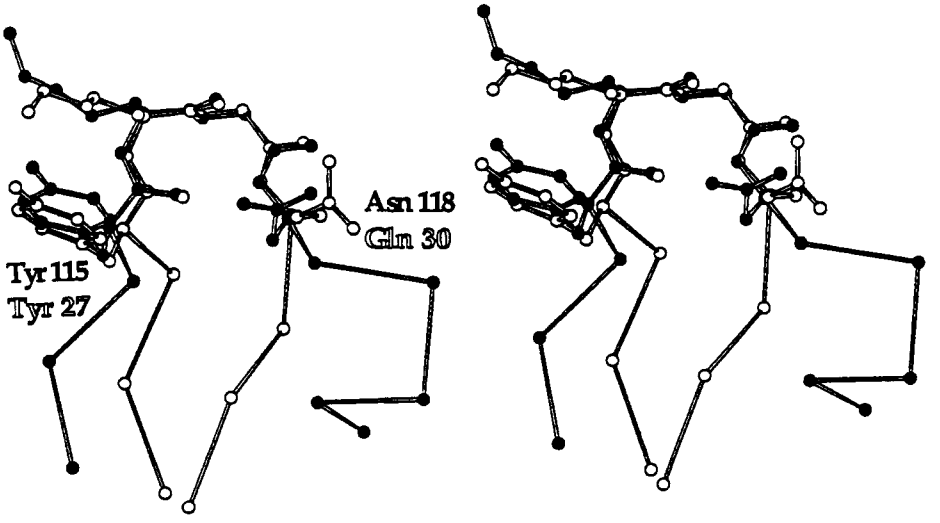

FIGURE 7: Superposition of residues 27–30 (Tyr-Lys-Gly-Gln; open atoms) with residues 115–118 (Tyr-Lys-Gly-Asn; filled atoms) from the P117G structure. All atoms are shown for the type I′ β-turn residues illustrating the close correspondence between the backbone atoms and the similar confomations of the side chains. The rms deviation between backbone atoms of the four β-turn residues is 0.52 Å. The α-carbon positions of the backbone segments leading into and away from the β-turns are shown to indicate the different topologies of the two β-turn sites.

proline with the less restricted alanine or glycine residue on the unfolded state would be to increase the entropy of unfolding (Matthews et al., 1987). This would decrease the free energy of the unfolded form and thereby reduce the overall stability. Therefore, the measured increase in the stabilities of P117A and P117G must be due to a decrease in the free energy of the folded form that more than compensates for the entropic contribution to the unfolded form.

By the same argument, the strain of placing an alanine in the third position of a type I′ turn is reflected in the difference in the stabilities of P117A and P117G. The change in the entropy of the unfolded state for an alanine residue relative to a glycine residue is estimated to be −2.4 cal/deg mol (Nemethy et al., 1966). This increase in entropy would result in an increase in the stability of P117A compared to P117G of approximately 0.7 kcal/mol at 298 K. However, P117G is 1.8 kcal/mol more stable than P117A at 298 K by GuHCl denaturation. Thus the strain of placing an alanine in the third position of a type I′ turn can be crudely estimated to be 2.5 kcal/mol.

From crystallographic (Hynes & Fox, 1991) and NMR (Evans et al., 1987) analyses it has been established that in the absence of bound nucleotide and Ca^{2+} there is a minor folded form of nuclease in which the peptide bond preceding Pro 117 is *trans* and is likely to adopt a type I β-turn conformation. The *cis* and *trans* forms interconvert in the folded state (Fox et al., 1986) and shift to a fully *cis* conformation in the presence of bound nucleotide. Thus both of the possible β-turn conformations for this sequence exist, and the preference for the type VI_a over the proposed type I β-turn is slight. This preference is clearly influenced by the specific context, as shown by the effect of the bound nucleotide which packs against the side chain of Tyr 115 in the first position of the type VI_a β-turn. In addition, while Pro is common in the second position of type I β-turns, it is rarely found in the third position due to steric restrictions (Wilmot & Thornton, 1988; MacArthur & Thornton, 1991).

The conformation of β-turns can be altered by point mutations and changes in turn length (Hynes et al., 1989), to generate alternative backbone geometries which could provide an added dimension to the design of functional modifications to proteins. By identifying the correct sequences, it may be possible to generate an ensemble of backbone conformations in a β-turn site of interest. The alternate backbone conformations could then be evaluated for use in the design of functional modifications, keeping in mind that most β-turn positions tolerate a range of side chains. For example, the type I′ backbone conformation of P117G and P117A should tolerate substitutions at residue 116. The side chain of residue 116 in these variants has been completely reoriented to point directly into the nucleotide binding pocket and side-chain variation at this position could modify substrate binding specificity.

The only method currently available to construct three-dimensional models from primary sequence data is to build upon the known crystal structure of a protein with which it shares sequence and therefore structural homology (Blundell et al., 1989). The primary limitation of this approach, beyond the need for a homologous protein structure, is the ability to accurately model β-turns and loops at the protein surface. Conformational variation in β-turns within families of homologous proteins such as aspartic (Sibanda et al., 1989) and serine proteases is extensive and has been shown to contribute to functional diversity (Craik et al., 1983). β-Turn modeling procedures have been successful in a number of cases (Fine et al., 1986; Chothia et al., 1986; Moult & James, 1986) but encounter situations analogous to nuclease and P117G, where there are multiple conformational solutions. Resolution of these cases requires improved methods which correctly weigh the relative importance of local sequence and turn site geometry constraints. Point mutations which shift the backbone conformation between distinct conformational minima allow the relationship between the amino acid sequence and backbone conformation to be examined in the context of an identical protein fold in crystallographic detail. The coordinates of the P117T, P117A, and P117G nuclease variants and their complexes reported herein have been deposited in the Brookhaven Protein Data Bank.

ADDED IN PROOF

Replacement of a *cis*-proline with alanine in human carbonic anhydrase II (P202A) results in a highly destabilized protein where the *cis* peptide bond conformation is maintained (Tweedy et al., 1993).

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