

Two Conformational States of Turkey Ovomucoid Third Domain at Low pH: Three-Dimensional Structures, Internal Dynamics, and Interconversion Kinetics and Thermodynamics^{†,‡}

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ABSTRACT: Turkey ovomucoid third domain (OMTKY3) is shown to exist at low pH as two distinctly folded, interconverting conformations. Activation parameters were determined for the transition, and these were of the type reported previously for cis/trans isomerizations of prolyl peptide bonds. Multidimensional, multinuclear NMR spectroscopy was used to determine the three-dimensional structure of each of the two states of P₅-Pro¹⁴Asp OMTKY3 at pH 2.5 and 25 °C, under conditions where the two states have equal populations with interchange rates of 0.25 s⁻¹. The results showed that the two states differ by cis/trans isomerization of the P₈-Tyr¹¹–P₇-Pro¹² peptide bond, which is cis in the conformer dominant at neutral pH and trans in the conformer appearing at low pH. The major structural differences were found to be in the region of the reactive site loop. The core of the protein, including the antiparallel β -sheet and a α -helix, is preserved in both structures. The state with the cis peptide bond is similar to previously reported structures of OMTKY3 determined by NMR spectroscopy and X-ray crystallography. The cis-to-trans transition results in the relocation of the aromatic ring of P₈-Tyr¹¹, disrupts many interactions between the α -helix and the reactive-site loop, and leads to more open spacing between this loop and the α -helix. In addition, the configurations of two of the three disulfide bonds, P₁₁–Cys⁸–P₂₀′–Cys³⁸, and P₃–Cys¹⁶–P₁₇′–Cys³⁵, are altered such that the C α –C α distances for each disulfide bridge are longer by ~ 1 Å in the trans state than in the cis. Mutations at P₁-Leu¹⁸, P₆-Lys¹³, and P₅-Pro¹⁴ influence the position of the cis \rightleftharpoons trans equilibrium. In P₁-Leu¹⁸Xxx OMTKY3 mutants, the trans state is more favored by P₁-Gly¹⁸ than by Ala¹⁸ or Leu¹⁸; in P₆-Lys¹³Xxx OMTKY3 mutants, the trans state is more favored by P₆-Glu¹³ and P₆-Asp¹³ than Lys¹³ or His¹³. Stabilization of the trans state in P₅-Pro¹⁴Xxx OMTKY3 mutants follows the series Xxx = Gly > Asp > Glu > Ala \sim His > Pro. In comparing the state with the trans peptide bond to that with the cis, the pK_a values of P₁₂-Asp⁷ and P₁′-Glu¹⁹ are higher and those of P₉-Glu¹⁰ and P₂₅′-Glu⁴³ are lower. The pK_a values of other titrating groups in the molecule are similar in both conformational states. These pK_a changes underlie the pH dependence of the conformational equilibrium and can be explained in part by observed structural differences. ¹⁵N transverse relaxation results indicate that residues P₆-Lys¹³–P₃–Cys¹⁶ in the trans state undergo a dynamic process on the microsecond–millisecond time scale not present in the cis state.

Turkey ovomucoid third domain, OMTKY3 (Figure 1), and bovine pancreatic trypsin inhibitor, BPTI, are the two most- and best-studied, small molecular weight, standard-mechanism (2), canonical (3) protein inhibitors of serine proteinases. OMTKY3 was first isolated as a 56-amino acid limited-proteolysis fragment from whole ovomucoid, the second most abundant (after ovalbumin) protein from egg white (4). The protein is monomeric and stable over a broad pH range (pH 1–12) (5). Two types of conformational

heterogeneity have been observed in OMTKY3 at low pH. The first is the low pH transition (pH_{mid} 2.8) observed initially by optical spectroscopy in whole chicken ovomucoid (6); this “Donovan transition” has been assumed to be triggered by deprotonation of an acidic group in the protein.

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[‡] Structures have been deposited at the Protein Data Bank (trans state at pH 2.5, PDB code 1M8C; cis state at pH 2.5, PDB code 1M8B) and NMR data at the BioMagResBank (trans state at pH 2.5, bmr 5473; cis state at pH 2.5, bmr 5472).

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¹ Nomenclature: OMTKY3 stands for the third domain of the proteinase inhibitor (ovomucoid) from turkey (*Meleagris gallopavo*), Swiss-Prot P01004. Residues in OMTKY3 are denoted as P_i-Xxx_j, where P denotes the Schechter–Berger notation for proteinase inhibitors (*I*) with *i* values for residues to the N-terminal side of the cleavage site and *j*′ values for those to the C-terminal side, Xxx is the three-letter amino acid code, and *j* is the residue number according to 56-residue OMTKY3 domain (starting with the sequence L¹AAVS⁵...). OMTKY3 mutants are also described by dual notation, e.g., P₅-Pro¹⁴Asp for substitution of P₅-Pro¹⁴ by Asp. C denotes the state of OMTKY3 with the cis P₈-Tyr¹¹–P₇-Pro¹² peptide bond, and T denotes the state with the trans peptide bond. Abbreviations: DTT, dithiothreitol; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; *E. coli*, *Escherichia coli*; GdmCl, guanidinium chloride; OMTKY3(6–56), OMTKY3 consisting of residues 6–56; rmsd, root-mean-squared deviation; SNase, staphylococcal nuclease; TPPI, time-proportional phase incrementation.

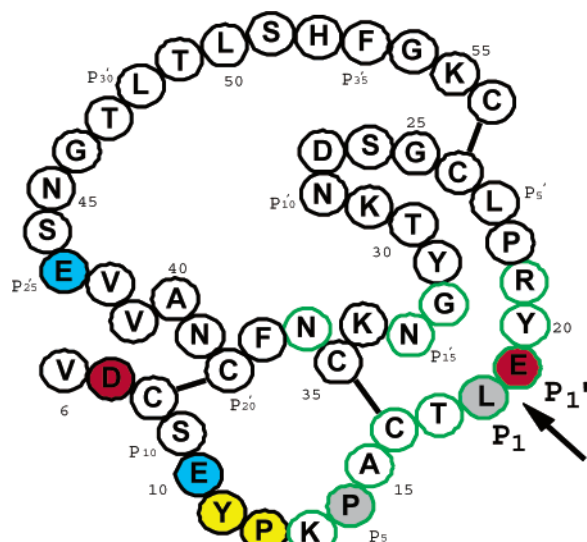


FIGURE 1: Primary structure of turkey ovomucoid third domain, OMTKY3. Residues in the consensus region of contact with serine proteinases are outlined in green. The arrow indicates the reactive-site peptide bond. The conformational transition studied here is linked to cis/trans isomerism about the peptide bond joining residues P₈-Tyr¹¹ and P₇-Pro¹² (highlighted in yellow). This bond normally is cis, but when P₅-Pro¹⁴ is mutated to other residues or when P₁-Leu¹⁸ is mutated to Gly or His, the trans configuration becomes more highly populated. Residues colored red and blue are ones whose pK_a is shifted up or down, respectively, as the result of the cis → trans transition. The conformational heterogeneity is abolished by mutation of P₇-Pro¹² to Gly.

The second is a transition observable by NMR spectroscopy at low pH, which leads to doubling of peaks (7). Because this transition affects the local environment of residues in the consensus contact region with serine proteinases (residues outlined in green in Figure 1), the structural consequences of this transition and its dependence on the identity of residues in the contact region are of considerable interest. We report here that the transition involves cis ⇌ trans isomerization of the P₈-Tyr¹¹–P₇-Pro¹² peptide bond, rearrangement of two of the three disulfide bridges in the molecule, and enhanced internal dynamics of residues P₆-Lys¹³–P₃-Cys¹⁶. We use the notation *C* for the state shown to contain the cis P₈-Tyr¹¹–P₇-Pro¹² peptide bond and *T* for the state shown to contain the trans peptide bond. Mutations of residues in the region of OMTKY3 that contacts serine proteinases were found to affect the equilibrium between *C* and *T*. Three-dimensional structures of the individual *C* and *T* states have been determined under conditions where they are equally populated. The dependence of the equilibrium on pH can be explained by structural differences that change pK_a values of individual residues. These results complement the large database of sequence structure–function relationships in avian ovomucoid third domains (8).

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Variants of OMTKY3(6–56) with P₅ residue substitutions (Ala, Asp, Gly, Glu, and His) were prepared at Purdue University according to published procedures (9). OMTKY3 variants produced at Wisconsin, which consisted of residues 1–56, were produced as described elsewhere (10, 11).

NMR Sample Preparation. Samples used for NMR pH titration studies were prepared by dissolving protein previ-

ously lyophilized from water in one of the two solvents described below to give a final protein concentration of approximately 2–3 mM. The two solvents, which differed only in the content of D₂O (90% H₂O/10% D₂O or 100% D₂O); each contained 100 mM potassium chloride, 0.02% sodium azide, 2% (v/v) of a proteinase inhibitor cocktail (Product no. P 2714, Sigma Chemical Co., Saint Louis, MO, dissolved in 1 mL), and 1 mM DSS as an internal chemical shift reference. The pH of the sample was measured at room temperature, both before and after the NMR data were collected; if these measurements differed by more than 0.10 pH unit, the spectrum was not used. The glass electrode (Biological Combination Electrode, Beckman, Fullerton, CA) was calibrated with commercial standard buffers at pH 1.0, 4.0, and 7.0 (Fisher Scientific, Fair Lawn, NJ).

NMR Spectroscopy. NMR data were collected at the National Magnetic Resonance Facility at Madison (NMRFAM) on Bruker DMX 600 and DMX 750 spectrometers equipped with three-axis gradients triple-resonance probes. Unless otherwise indicated, all data sets were collected at sample pH 2.5 and temperature 25 °C. [¹H–¹H]-TOCSY spectra used to follow the pH dependence of proton chemical shifts were acquired with mixing times of 50 ms; the TPPI mode was used for quadrature detection of the indirect dimension. Data sets collected for determining sequence-specific backbone and side chain assignments consisted of 2D ¹H NOESY, 2D ¹H_z ¹⁵N_z-2 spin order exchange experiments, 3D HNCA, 3D HNCOC, 3D HNCACB, 3D CCONH, 3D HNCO, 3D HCCH–COSY, 3D H(CCO)NH, and 3D ¹⁵N TOCSY-HSQC. Distance constraints were based on four additional nuclear Overhauser enhancement (NOE) data sets, each collected with the mixing time *t*_m = 125 ms: 3D ¹⁵N NOESY, 3D ¹³C NOESY with ¹³C carrier frequency in the aliphatic region, 2D ¹H NOESY in 90% H₂O/10% D₂O, and 2D ¹H NOESY in 100% D₂O. Care was taken to discriminate NOE cross-peaks from cross-peaks originating from direct chemical exchange or spin diffusion mediated by chemical exchange (12). An HNCOC experiment with long-range coherence transfer was performed to obtain the ¹³J_{NC'} connectivities for detection of hydrogen bonds (13). ¹⁵N transverse (*R*₂) relaxation data (14) were collected with the Bruker DMX750 spectrometer using relaxation delays of 0, 16, 32, 64, 96, 144, 192, and 240 ms.

NMR spectra were processed with either XWINNMR3.2 (Bruker, Billerica, MA) or FELIX95 (Molecular Simulations Inc., San Diego, CA) software. All ¹H dimensions were referenced directly to internal DSS, and ¹³C and ¹⁵N dimensions were referenced indirectly to DSS (15). Sparky software (<http://www.cgl.ucsf.edu/home/sparky>) was used in assigning and analyzing NMR spectra. The parameter-fitting module of Sparky was used to determine ¹⁵N *R*₂ relaxation parameters by fitting peak intensities determined at different relaxation delays.

Structural Calculation and Structural Analysis. The simulated annealing protocol of the torsion angle dynamics program DYANA (16) was used in calculating all structures. Peak lists from the three aforementioned NOESY spectra, containing a total of 2 614 NOE cross-peaks for the *C* state and 2 505 NOE cross-peaks for the *T* state, were generated by manual peak picking with the program Sparky. NOE intensities were converted into upper distance bounds with the CALIBA function of DYANA. Additional NOE con-

straints were added in each preliminary round of calculations, and restraints that were consistently violated were rechecked by examining the spectra and removed if assignments proved to be incorrect. Constraints representing nine hydrogen bonds deduced from $^3J_{\text{NC}}$ HNCOC data were included in the input data for both the *C* and *T* states (see Table S1 of the Supporting Information). Additional hydrogen bonds (4 for the *C* state and 3 for the *T* state) were deduced from the titration shifts of amide protons (17); constraints representing these were included in the input data for the respective structure determinations. Constraints representing ranges of ϕ and ψ dihedral angles (54 for the *C* state and 48 for the *T* state), generated from $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, $^{13}\text{C}'$, and ^{15}N secondary shifts by the program TALOS (18), were included in later rounds of refinement. TALOS constraints were used only when nine out of 10 predicted values were clustered. In addition, six upper-bound and six lower-bound distance constraints were introduced to represent the three disulfide bridges: (P₁₁-Cys⁸-P₂₀'-Cys³⁸, P₃-Cys¹⁶-P₁₇'-Cys³⁵, and P₆'-Cys²⁴-P₃₉'-Cys⁵⁶). For reasons discussed below, the P₈-Tyr¹¹-P₇-Pro¹² peptide bond was set to *cis* in the *C* state and *trans* in the *T* state. For structural analysis and evaluation, 20 conformers with the lowest target function values were selected from the final 50 structures calculated. The mean structure, calculated with MOLMOL software (19) from the ensemble of 20 best structures, was minimized in DYANA using 300 steps of variable target function minimization.

Kinetics and Thermodynamics of the Interconversion. [^1H - ^{15}N]-two-spin-order exchange experiments (20) were carried out with samples at pH 2.5 at five temperatures (25, 30, 35, 40, and 45 °C) to determine the temperature dependence of the equilibrium. A procedure proposed by Otting et al. (20) was used to measure the exchange rate constants. [^1H - ^{15}N]-two-spin-order exchange spectra were recorded at different temperatures under a range of mixing times: at 25 °C with $\tau_{\text{mix}} = 80, 100, 120, 140$, and 160 ms; at 30 °C with $\tau_{\text{mix}} = 30, 40, 50, 60, 70$, and 90 ms; at 35 °C with $\tau_{\text{mix}} = 40, 60, 80$, and 90 ms; at 40 °C with $\tau_{\text{mix}} = 20, 30, 40, 60$, and 80 ms; and at 45 °C with $\tau_{\text{mix}} = 30$ and 50 ms. The exchange rate constants were determined from the initial buildup rates of the exchange cross-peaks in the difference spectra, $\Delta I/\Delta\tau_{\text{mix}}$, and the intensities of the direct correlation peaks at zero mixing time, I_0 . At zero mixing time:

$$k = \Delta I/(I_0\Delta\tau_{\text{mix}}) \quad (1)$$

Instead of recording a separate experiment with zero mixing time, I_0 was obtained by extrapolation from the data recorded at short mixing times. Activation free energies ΔG^\ddagger were determined from measurement of the exchange rate constant k at various temperatures T , according to the Eyring relation:

$$\frac{\Delta G^\ddagger}{T} = -R\left(\ln k + \ln \frac{h}{\kappa k_B T}\right) \quad (2)$$

where k_B denotes the Boltzmann constant, h the Planck constant, and κ the transmission coefficient, which was assumed to be unity over the entire temperature range investigated. $\Delta G^\ddagger/T$ for the whole molecule was measured by averaging values determined from a number of individual

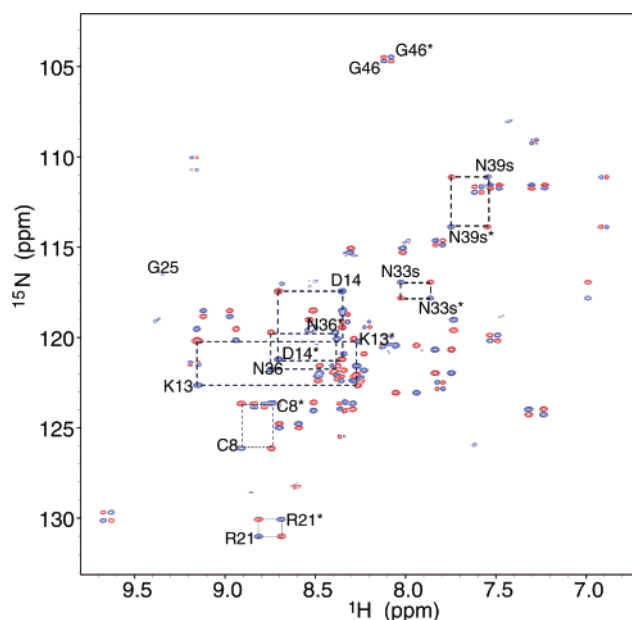


FIGURE 2: [^1H - ^{15}N]-two-spin-order-exchange difference spectra of P₅-Pro¹⁴Asp OMTKY3 at pH 2.5. The direct peaks (blue) for the *C* and *T* states are connected to one another by exchange cross-peaks (red). Resonances from the *T* form are identified by an asterisk; those from the *C* form have no designator. An “s” following N33 or N39 indicates that the marked peaks are from the side chain $^1\text{H}^\alpha$ atoms.

peaks. The activation enthalpies ΔH^\ddagger and activation entropies ΔS^\ddagger were determined from eq 3:

$$\frac{\Delta G^\ddagger}{T} = \frac{\Delta H^\ddagger}{T} - \Delta S^\ddagger$$

RESULTS

From analysis of [^1H - ^1H]-TOCSY spectra of several OMTKY3 variants, P₅-Pro¹⁴Asp OMTKY3 was chosen for structural studies because the *C* and *T* states of the protein were found to be stable and equally populated at pH 2.5.

Resonance Assignments for P₅-Pro¹⁴Asp OMTKY3. [^1H - ^{15}N]-two-spin-order-exchange experiments were used to cross-assign ^1H and ^{15}N resonances from individual atoms in two states of the protein. Blue contours (Figure 2) are direct cross-peaks observed in a normal [^1H - ^{15}N]-HSQC spectrum. Red contours are exchange cross-peaks, which have negative intensities relative to the direct cross-peaks; they connect pairs of direct cross-peaks (one from each of the two states) into a characteristic rectangular pattern. Exchange peaks were observed for a number of backbone amides plus the side chain amides of P₁₅'-Asn³³, P₁₈'-Asn³⁶, and P₂₁'-Asn³⁹. (The $^1\text{H}^{\beta 2/\beta 3}$ signals of P₁₅'-Asn³³ are outside the spectral region shown in Figure 2.) Direct cross-peaks from residues unaffected by the conformational change, e.g., P₇'-Gly²⁵ and P₈'-Ser²⁶, are greatly suppressed in the difference spectrum. Suppression of direct cross-peaks in the difference spectrum helped to resolve exchange peaks with small chemical shift differences, for example, those from P₂₈'-Gly⁴⁶ (Figure 2). The results of this experiment clearly demonstrate that the peak doubling arises from a dynamic conformational equilibrium, rather than from a covalent change such as deamidation. The assigned [^1H , ^{15}N]-HSQC spectrum of P₅-Pro¹⁴Asp OMTKY3 at pH 2.5 is provided in the Supporting Information (Figure S1).

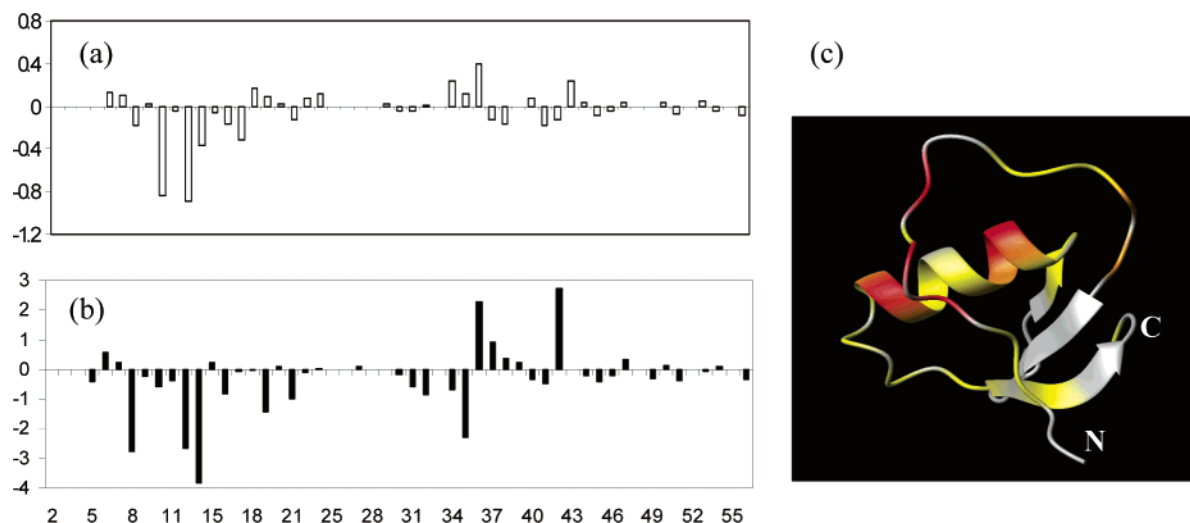


FIGURE 3: Chemical shift differences between the *C* (cis $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond) and *T* (trans $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond) conformers of $P_5\text{-Pro}^{14}\text{Asp}$ OMTKY3 derived from the $[\text{H}-^{15}\text{N}]$ -HSQC spectrum at pH 2.5: (a) differences in ^1H chemical shifts, (b) differences in ^{15}N chemical shifts, (c) weighted average chemical shift differences for ^1H and ^{15}N mapped onto the solution structure of OMTKY3 (32), with residues having $\Delta_{\text{avg}}(\text{NH})$ of less than 0.1 ppm in gray, $\Delta_{\text{avg}}(\text{NH})$ between 0.1 and 0.3 ppm in yellow, $\Delta_{\text{avg}}(\text{NH})$ between 0.3 and 0.5 ppm in orange, and $\Delta_{\text{avg}}(\text{NH}) > 0.5$ ppm in red. See the text for the definition of $\Delta_{\text{avg}}(\text{NH})$. The N and C termini are indicated.

Assignments of the backbone $^1\text{H}^N$, $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, ^{13}C , and ^{15}N resonances for the two states were completed by analyzing HNCA, HNCOC, HNCO, H(CCO)NH, and $[\text{H}, ^{15}\text{N}]$ -HSQC data from double-labeled samples at pH 2.5. HNCACB, CCONH, ^{15}N TOCSY-HSQC, and HCCH-COSY data were used in assigning side chain ^1H and ^{13}C resonances. Side chain $^{13}\text{C}'$ signals from Asn residues were assigned by reference to HNCO data collected with the dephasing and rephrasing times optimized for coherence transfer between the protons and nitrogen of each side-chain amide. The aromatic side chain ^1H resonances were assigned from the 2D NOESY spectrum of a sample in D_2O , on the basis of observed NOE cross-peaks between the aromatic protons and the aliphatic protons in the same residue. Resonances were assigned to specific states on the basis of observed scalar connectivities and NOE connectivities. A $[\text{H}, ^{15}\text{N}]$ -HSQC spectrum of a sample at pH 4.0 was collected to confirm the identities of specific resonances in the *C* state, which is more highly populated at this pH. Assignments for the *C* state were consistent, for the most part, with those reported earlier for OMTKY3 under different conditions of pH and temperature (21–23). The assigned chemical shifts have been deposited at BMRB and are also available from the Supporting Information (Tables S2 and S3).

Evidence that the *C* and *T* States Differ by Prolyl Peptide Bond Isomerization. Multiple lines of evidence led to the conclusion that the two states differ by the configuration of the $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond. The fact that the residues with the largest chemical shift deviations are adjacent to $P_7\text{-Pro}^{12}$ (Figure 3) provided the first suggestion that this residue might be involved. This hypothesis was strengthened by results of NMR investigations of the double mutant ($P_7\text{-Gly}^{12}$, $P_5\text{-Asp}^{14}$) OMTKY3, in which the proline presumed to undergo cis/trans isomerization had been mutated to glycine. Residues of wild-type OMTKY3 (or of single P_5 -variants) that gave rise to doubled peaks showed single peaks in $[\text{H}-^1\text{H}]\text{-TOCSY}$ of ($P_7\text{-Gly}^{12}$, $P_5\text{-Asp}^{14}$) OMTKY3 at pH 2.3 (see Figure S2 of the Supporting Information).

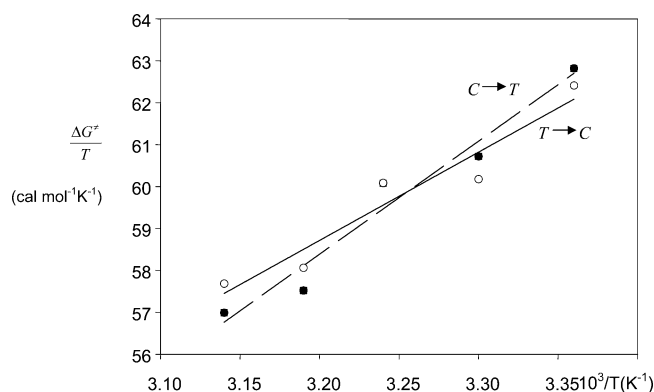


FIGURE 4: Plots of $\Delta G^\ddagger/T$, the free energy of activation divided by the absolute temperature, vs $1/T$, the reciprocal of the absolute temperature, for the ($C \rightarrow T$) conversion (black symbols), and the ($T \rightarrow C$) conversion (open symbols), where *C* indicates the state of $P_5\text{-Pro}^{14}\text{Asp}$ OMTKY3 with cis $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond and *T* indicates the state with trans $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond. The lines represent least-squares fits each set of data points.

Prolyl peptide bond isomerizations are characterized by large positive activation enthalpies. The activation enthalpies for the $C \rightarrow T$ and $T \rightarrow C$ transitions of $P_5\text{-Pro}^{14}\text{Asp}$ OMTKY3 were determined from measurements of the temperature dependence of the interconversion rates. The measured exchange rate constants varied from 0.25 s^{-1} at $25\text{ }^\circ\text{C}$ to 2.1 s^{-1} at $45\text{ }^\circ\text{C}$. Figure 4 shows the plot of $\Delta G^\ddagger/T$ versus $1/T$. From these data one obtains $\Delta H^\ddagger = 27.9 (\pm 3.8)$ kcal mol^{-1} and $\Delta S^\ddagger = 30.9 (\pm 12.4)$ cal $\text{mol}^{-1}\text{ K}^{-1}$ for forward ($C \rightarrow T$) reaction and $\Delta H^\ddagger = 21.7 (\pm 4.0)$ kcal mol^{-1} and $\Delta S^\ddagger = 10.8 (\pm 12.7)$ for reverse ($T \rightarrow C$) reaction, where the errors (in parentheses) were determined from least-squares fitting. The activation enthalpy ΔH^\ddagger determined for the reaction is comparable to others reported for prolyl peptide bond isomerizations in model peptides and other proteins (24, 25).

For a cis prolyl peptide bond, the distance between the H^α of the preceding residue and the H^α of the proline is shorter than the distance between the H^α of the preceding residue and the H^δ of the proline; for a trans peptide bond,

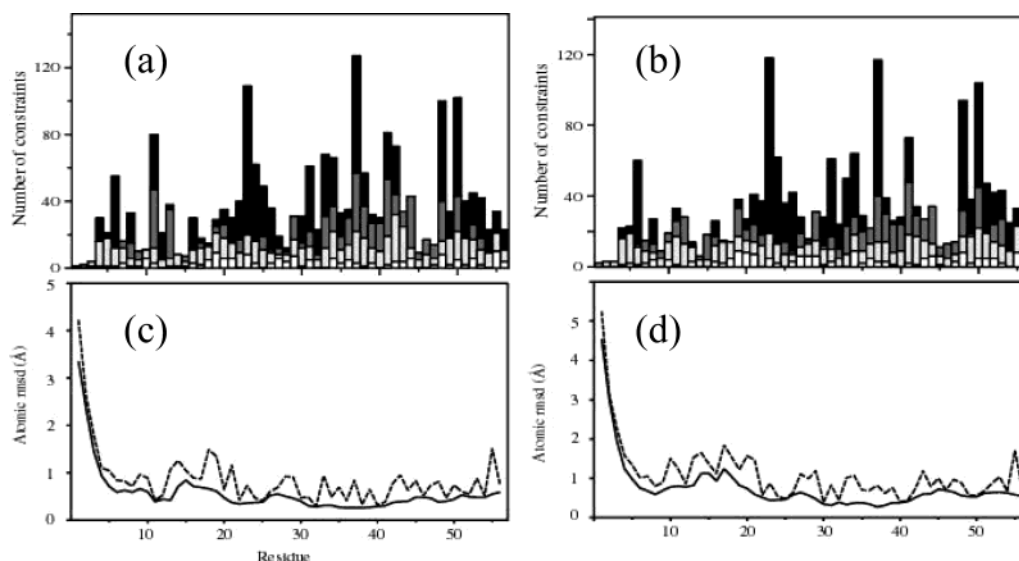


FIGURE 5: Summary of the numbers of structural constraints determined for the *C* (cis $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond) and *T* (trans $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond) states of $P_5\text{-Pro}^{14}\text{Asp OMTKY3}$ and the rmsd values of the family of conformers representing the structures. Number of NOE upper distance limits per residue plotted as a function of residue number for (a) the *C* state and (b) the *T* state. The numbers of intrasidue, sequential, medium, and long-range constraints are distinguished, respectively, as white, light gray, dark gray, and black bars. Average backbone (solid line) and heavy atom (dashed line) rmsd values are given for (c) the family of 20 *C*-state conformers and (d) the family of 20 *T*-state conformers.

these relative distances are reversed (26). Thus species with cis or trans prolyl peptide bonds can be distinguished on the basis of NOEs that report on these relative distances. $[\text{H}-\text{H}]\text{-NOESY}$ data for $P_5\text{-Pro}^{14}\text{Asp OMTKY3}$ at pH 2.8 show an NOE between $P_7\text{-Pro}^{12}\text{H}^\alpha$ and $P_8\text{-Tyr}^{11}\text{H}^\alpha$ (both from the *C* state), which identifies the peptide bond as cis, and NOEs between $P_7\text{-Pro}^{12}\text{H}^\alpha$ and $P_7\text{-Pro}^{14}\text{H}^{\delta 2}$ and $\text{H}^{\delta 3}$ (all from the *T* state), which identify the peptide bond as trans (see Figure S3 of the Supporting Information).

Finally, cis and trans prolyl peptide bonds also can be distinguished on the basis of chemical shift comparisons. A prolyl $\delta^{13}\text{C}^\gamma$ generally is 1.5–2.0 ppm to higher frequency in the cis configuration than in the trans (27, 28). Moreover, pairwise graphical comparisons of $\delta^{13}\text{C}^\beta$ and $\delta^{13}\text{C}^\gamma$ chemical shifts provide an additional statistic for distinguishing cis and trans Xaa–Pro peptide bond configurations (29). In the present case, the $P_7\text{-Pro}^{12}$ chemical shifts for $^{13}\text{C}^\gamma$ in the *C* (24.4 ppm) and *T* states (27.3 ppm) are consistent with the $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond being cis in *C* and trans in *T*.

Structure Calculations. Prior to calculation of the final structures, the configuration of the $P_8\text{-Tyr}^{11}\text{-P}_7\text{-Pro}^{12}$ peptide bond was constrained to be cis in the conformers representing the *C* state and trans in those representing *T*. The inverse configuration was tried with each set of input data (trans for *C* and cis for *T*), but these led to large numbers of constraint violations.

Starting with the NOE cross-peak lists, hydrogen bond constraints and disulfide bonds constraints for $P_5\text{-Pro}^{14}\text{Asp OMTKY3}$ described in Materials and Methods, the DYANA software package yielded 1,143 unique distance constraints for the *C* state and 1,063 for the *T*. Thus the average constraints per residue were 20.4 for *C* and 19.0 for *T*. The distributions of distance constraints for *C* and *T*, plotted as a function of residue number, are shown in Figure 5a,b. For both conformers, more constraints were distributed in the segments $P_4'\text{-Pro}^{22}\text{-P}_{10}'\text{-Asn}^{28}$, $P_{12}'\text{-Thr}^{30}\text{-P}_{24}'\text{-Val}^{42}$, and

$P_{30}'\text{-Leu}^{48}\text{-P}_{38}'\text{-Cys}^{56}$ than in other regions. No medium- or long-range NOEs were observed for the first three N-terminal residues. Many more constraints were found for $P_8\text{-Tyr}^{11}$ in the *C* state (80) than in the *T* state (33).

The 20 conformers representing the *C* state are shown in Figure 6a, and those representing the *T* state are shown in Figure 6b. Structure statistics are summarized in Table 1. None of the 20 structures had NOE violations greater than 0.30 Å or any violations of van der Waals contacts.

Superposition of residues 6–56 for the final ensemble of 20 torsion angle dynamics conformers yielded root-mean-square deviations from the mean backbone structures of 0.58 Å (for the family of *C* conformers) and 0.75 Å (for the family of *T* conformers). The average backbone and heavy atom rmsd values relative to the mean structures of each conformer are shown as a function of residue number in Figure 5c,d. The atomic rms deviations for the N-terminal residues are relatively high in both conformers. Rmsd values for residues in the segment $P_9\text{-Glu}^{10}\text{-P}_1\text{-Leu}^{18}$ were higher in the *T* state than in the *C* by 0.26–0.58 Å but were nearly equivalent elsewhere. The *C* state more closely resembles structures of wild-type OMTKY3 determined at higher pH values (30–33).

Dependence of the Peptide Bond Configuration on pH. $[\text{H}-\text{H}]\text{-TOCSY}$ spectra were collected from solutions of $P_5\text{-Pro}^{14}\text{Gly OMTKY3}(6\text{--}56)$, $P_5\text{-Pro}^{14}\text{Ala OMTKY3}(6\text{--}56)$, $P_5\text{-Pro}^{14}\text{Asp OMTKY3}(6\text{--}56)$, and wild-type OMTKY3(1–56) at pH values ranging from neutral to acidic. Similar reversible pH-dependent conformational transitions were observed for all three variants. However, the number of residues with chemical shifts perturbed by the conformational transition was found to depend on the nature of the side chain of $P_5\text{-X}^{14}$. In $P_5\text{-Pro}^{14}\text{Ala OMTKY3}(6\text{--}56)$, very few residues exhibited doubled peaks. However, in $P_5\text{-Pro}^{14}\text{Gly OMTKY3}(6\text{--}56)$ and $P_5\text{-Pro}^{14}\text{Asp OMTKY3}(6\text{--}56)$, more than two-thirds of the residues were perturbed by the conformational transition.

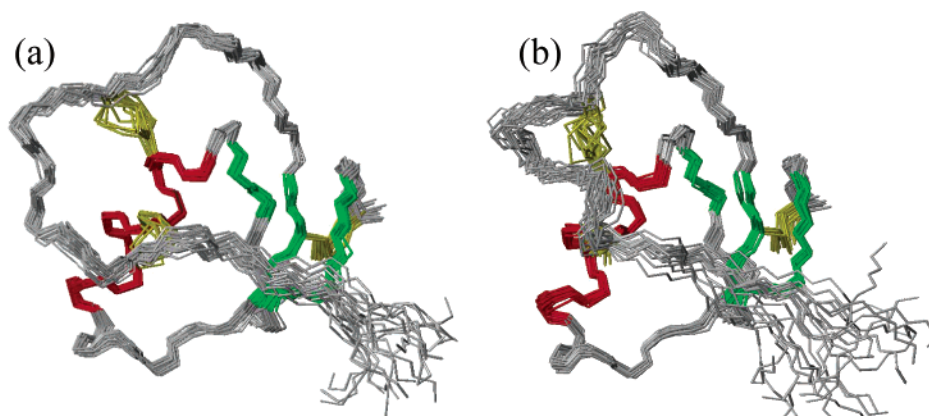


FIGURE 6: Representation of the structures of the two forms of P₅-Pro¹⁴Asp OMTKY3 present in solution at pH 2.5: (a) the *C* state with cis P₈-Tyr¹¹-P₇-Pro¹² peptide bond and (b) the *T* state with trans P₈-Tyr¹¹-P₇-Pro¹² peptide bond. Each structure is represented by a superposition of the 20 conformers with lowest target functions from 50 calculated in the last round of refinement. Disulfide bridges are shown in yellow, β -strands in green, and α -helices in red.

Table 1: Statistics for the Three-Dimensional Structures of the *C* and *T* States of P₅-Pro¹⁴Asp OMTKY3

parameters	<i>C</i> state (cis P ₈ -Tyr ¹¹ -P ₇ -Pro ¹² peptide bond)	<i>T</i> state (trans P ₈ -Tyr ¹¹ -P ₇ -Pro ¹² peptide bond)
NOE Constraints		
long	474	431
medium	236	218
short	272	252
intraresidue	163	163
Ramachandran Statistics		
most favored	73.3	70.7
additional allowed	23.5	24.1
generously allowed	2.6	4.0
disallowed	0.5	1.2
target function		
family	0.38 ± 0.07	0.20 ± 0.05
minimized average	0.19	0.31
upper limit violations		
number > 0.1 Å	6 ± 2	3 ± 2
sum of violations (Å)	2.1 ± 0.4	1.7 ± 0.3
maximum violation (Å)	0.19 ± 0.02	0.15 ± 0.04
Van der Waals violations		
number > 0.1 Å	0 ± 0	0 ± 0
sum of violations (Å)	1.5 ± 0.2	1.0 ± 0.2
maximum violation (Å)	0.16 ± 0.04	0.10 ± 0.03
Rmsd (6–56) to the Mean Coordinates, Å		
backbone	0.58 ± 0.12	0.75 ± 0.18
heavy atom	1.15 ± 0.16	1.39 ± 0.20

The intensities of pairs of NMR peaks in the [¹H-¹H]-TOCSY data from a given variant at different pH values were integrated to obtain the relative occupancies of each conformer. Figure 7 shows the pH dependence of the relative populations of the *T* states of four P₅ variants of OMTKY3(6–56): Pro (wild-type), Ala, Asp, and Gly. All four variants showed similar peak doublings for residues P₄-Ala¹⁵, P₆-Cys²⁴ (except for wild-type which showed no doubling), P₂₂-Ala⁴⁰, and P₂₃-Val⁴¹, even though some of these residues (e.g., P₄-Ala¹⁵ and P₂₃-Val⁴¹) are spatially distant from one another (33). The four variants showed different mole fractions of the *T* state at the high and low pH extremes studied: ~0.2 to ~0.6 for P₅-Pro¹⁴Asp, ~0.2 to ~0.7 for P₅-Pro¹⁴Gly, ~0.05 to ~0.3 for P₅-Pro¹⁴Ala, and ~0 to ~0.2 for wild type. All three showed transition midpoints near pH 3. The nonsigmoidal nature of the transition made it difficult to determine accurate pH_{mid} values.

For comparison purposes, [¹H-¹H]-TOCSY spectra were collected from two additional variants (data not shown): P₅-Pro¹⁴Glu OMTKY3(6–56) and P₅-Pro¹⁴His OMTKY3(6–56). The relative occupancies of *T* states in these two variants were both higher than that in wild-type OMTKY3 (6–56), but not as high as that in P₅-Pro¹⁴Gly OMTKY3(6–56) or P₅-Pro¹⁴Asp OMTKY3(6–56).

Mutants at P₁-Leu¹⁸ or P₆-Lys¹³. The relative population of *T* state was found to be elevated when P₁-Leu¹⁸ was replaced by Gly¹⁸ (but not Ala¹⁸) (see Figure S4 of the Supporting Information) or when P₆-Lys¹³ is replaced by Asp¹³ or Glu¹³. The ¹H^N of P₆-Glu¹³ in the *C* state of P₆-Lys¹³Glu OMTKY3 showed biphasic pH dependence (Figure 8a), as did the relative population of the *T* state with similar pH_{mid} values (Figure 8b). By contrast, the ¹H^N of P₆-Asp¹³ in the *C* state of P₆-Lys¹³Asp OMTKY3 showed monophasic pH dependence (Figure 8c), as did the relative population of the *T* state with a similar pH_{mid} value (Figure 8d). These results suggest that the state of the P₆ residue is somehow coupled to the *C/T* transition.

Effect of the Conformational Transition on pK_a Values. The inherent high sensitivity of the [¹H-¹⁵N]-HSQC experiment enabled us to follow peaks over the whole pH titration range and to determine the pK_a values for all six carboxyl groups in P₅-Pro¹⁴Asp OMTKY3(6–56) in both the *C* and *T* conformational states. The pH titration results are presented in Table 2.

¹⁵N Transverse Relaxation Measurements. The backbone ¹⁵N *R*₂ relaxation rates for residues in the *C* and *T* states of P₅-Pro¹⁴Asp OMTKY3 determined at 750.13 MHz (¹H frequency) at pH 2.5 and 25 °C are shown in Figure 9. As discussed above, the exchange rate between the two conformers under these conditions is 0.25 s⁻¹, which is much slower than the *R*₂ rates expected for a protein of this size (34). Thus, chemical exchange effects were expected to result in only minor errors in the experimental *R*₂ values for the two states.

DISCUSSION

Protein folds and stabilities, and hence functions, can be greatly altered by cis/trans isomerizations of prolyl peptide bonds or by changes in the conformations of disulfide bridges (25, 35–39). Efforts have been made to explore how internal

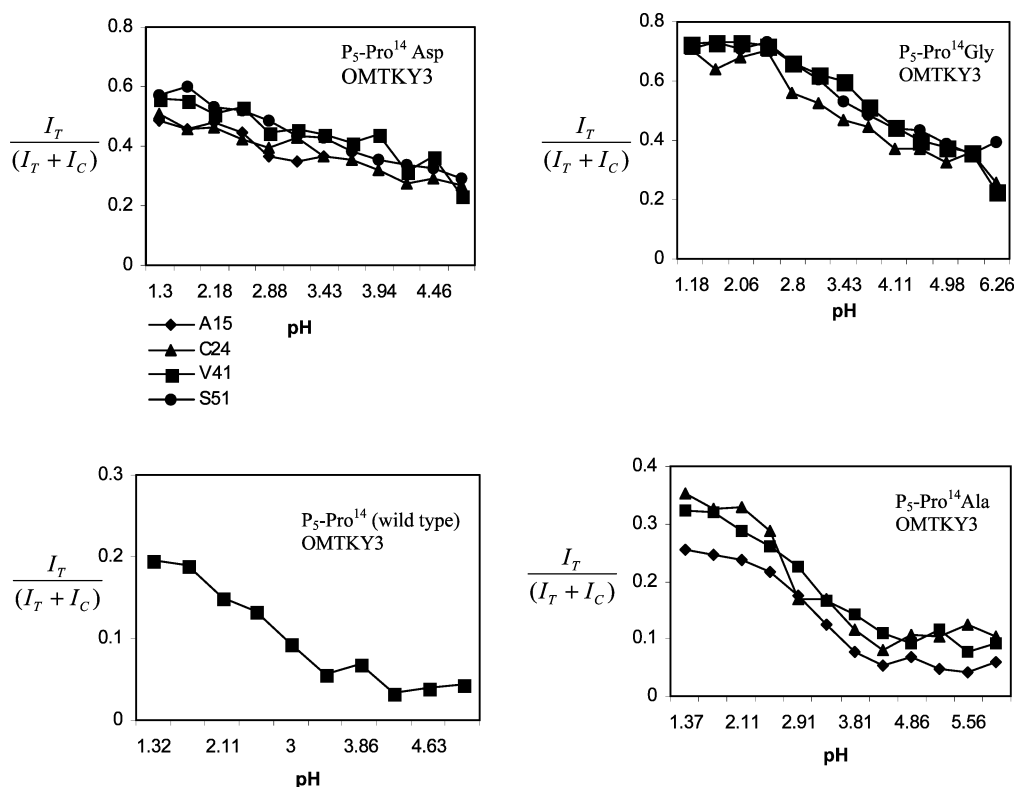


FIGURE 7: The pH dependences of the mole fraction of the low-pH conformer in OMTKY3 variants: P₅-Pro¹⁴ OMTKY3 (wild type), P₅-Pro¹⁴Ala OMTKY3, P₅-Pro¹⁴Asp OMTKY3, and P₅-Pro¹⁴Gly OMTKY3. Populations were determined from the relative volumes of [¹H-¹H]-TOCSY peaks corresponding to each state: $I_T/(I_T + I_C)$, where I_T and I_C refer to the intensities of peaks in the *T* state and *C* state, respectively.

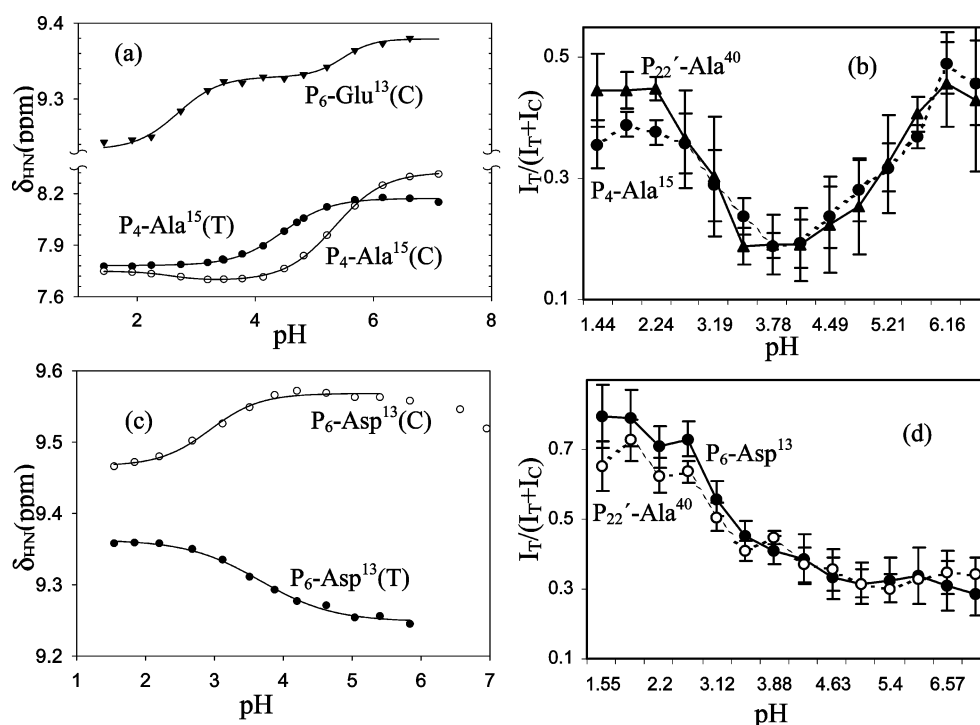


FIGURE 8: Effects of pH on P₆-Lys¹³Asp/Glu OMTKY3: (a) Titration curves for ¹H^N signals from P₆-Glu¹³ and P₄-Ala¹⁵ in the *C* state of P₆-Lys¹³Glu OMTKY3. (b) Relative populations of the *T* state as a function of pH for P₆-Lys¹³Glu OMTKY3. (c) Titration curves for ¹H^N signals from P₆-Asp¹³ in the *C* and *T* states of P₆-Lys¹³Asp OMTKY3. (d) Relative populations of the *T* state as a function of pH for P₆-Lys¹³Asp OMTKY3. Curves in panels a and c represent best fits to theoretical titration equations. Error estimates in panels b and d were derived from the noise of the spectra.

dynamics, hydrophobic, and electrostatic effects in proteins influence the relative populations of isoforms (20, 26, 40–45). In this regard, advanced NMR techniques provide an effective approach for identifying conformational equilibria

(14, 20, 46), as well as for determining the structural and dynamic features of each conformer (29, 47).

Comparison of the Structures of the C and T States with that of Wild-Type OMTKY3. Several NMR and X-ray

Table 2: Titration Parameters^a

	P ₁₂ -Asp ⁷		P ₉ -Glu ¹⁰		P ₅ -Asp ¹⁴		P ₁ '-Glu ¹⁹		P ₉ '-Asp ²⁷		P ₂₅ '-Glu ⁴³		Cys56 (P ₃₈ ')	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T
pK _a	2.83 (±0.11)	3.34 (±0.07)	4.27 (±0.05)	4.05 (±0.08)	3.76 (±0.03)	3.66 (±0.06)	3.40 (±0.06)	3.71 (±0.07)	2.22 (±0.04)	2.22 (±0.04)	4.90 (±0.08)	4.58 (±0.10)	2.27 (±0.05)	2.27 (±0.07)
Hill coefficient	0.69 (±0.08)	0.75 (±0.09)	0.88 (±0.08)	0.57 (±0.07)	1.04 (±0.07)	0.91 (0.11)	1.16 (±0.17)	1.07 (±0.18)	0.92 (±0.05)	0.92 (±0.05)	0.77 (±0.08)	0.63 (±0.08)	0.86 (±0.04)	0.81 (±0.05)

^a Derived from the pH dependence of ¹⁵N NMR signals from six residues of P₅-Pro¹⁴Asp OMTKY3 in the *C* and *T* states.

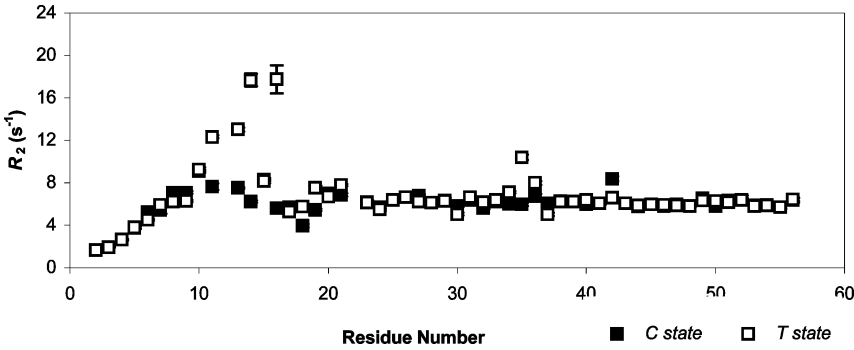


FIGURE 9: Comparison of ¹⁵N *R*₂ relaxation rates plotted as a function of the amino acid sequence for the two states of P₅-Pro¹⁴Asp OMTKY3 at pH 2.5. The error bars represent standard deviations returned upon nonlinear analysis of the relaxation data.

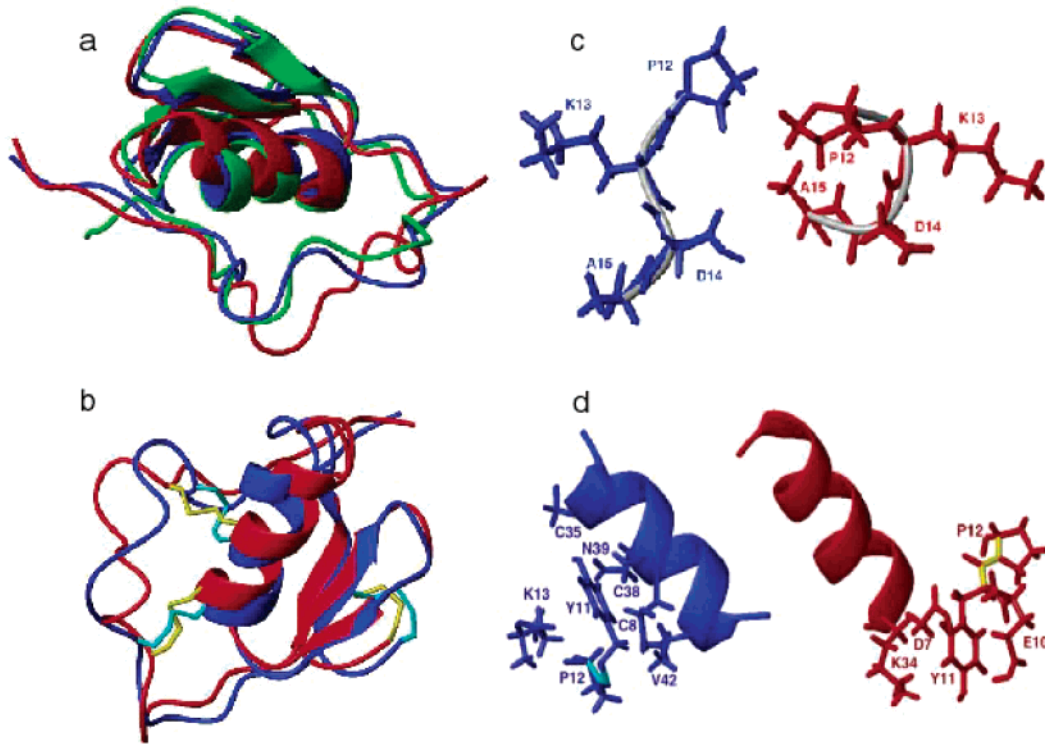


FIGURE 10: Comparisons of the structures of the *C* and *T* forms of P₅-Pro¹⁴Asp OMTKY3 at pH 2.5. (a) *C* state structure (in blue) and *T* state structure (in red) compared with the structure of wild-type OMTKY3 (in green) determined at pH 4.1 (32). (b) Superposition of the mean structures of the *T* state (in red) and the *C* state (in blue). The disulfide bridges in the *C* state are shown in cyan; those in the *T* state are shown in yellow. (c) Detailed view of main chain conformations and side chain packing of residues from P₇-Pro¹² to P₄-Ala¹⁵ in the *C* state (left, in blue) and *T* states (right, in red). (d) Comparison of the region of the P₈-Tyr¹¹-P₇-Pro¹² peptide bond in the *C* state (left, in blue) and *T* state (right, in red). P₈-Tyr¹¹-P₇-Pro¹² peptide bond is shown in cyan in the *C* state and yellow in *T* state. Residues making contacts with the P₈-Tyr¹¹ ring in each state are shown in the same color as the backbone ribbon.

structures are available for wild-type OMTKY3 (30–33, 48). Comparison is made here (Figure 10a) between the mean structures of the *C* and *T* states of P₅-Pro¹⁴Asp OMTKY3 and that of wild-type OMTKY3 determined by NMR at pH 4.1 (33). The three-stranded β -sheet is the most conserved core element in the three structures, with no greater than 0.6 Å backbone rmsd between OMTKY3 and *C* or *T*. The

region exhibiting the largest differences is the reactive-site loop of the *T* state. The backbone rmsd values between the reactive-site loop of wild-type OMTKY3 (P₁₃-Val⁶-P₃'-Arg²¹) and the corresponding loops of the *T* state and *C* state are 2.7 and 1.1 Å, respectively. The backbone of the reactive site loop of *T* diverges significantly from the highly conserved backbone of OMTKY3 and other members of the

Table 3: Angles Specifying the Conformation of the Backbone of the Reactive Site Region

	P ₆	P ₅	P ₄	P ₃	P ₂	P ₁	P ₁ '	P ₂ '	P ₃ '
	ϕ, ψ	ϕ, ψ	ϕ, ψ	ϕ, ψ	ϕ, ψ	ϕ, ψ	ϕ, ψ	ϕ, ψ	ϕ, ψ
wild-type OMTKY3 <i>C</i> state	-118, 144	-89, 14	-165, 121	-101, 134	-55, -163	-117, -12	-46, 153	-97, 93	-122, 84
P ₅ -Asp ¹⁴ OMTKY3 <i>C</i> state	-97, 147	-86, 19	-153, 169	-144, 173	-101, 156	-92, -23	-60, 151	-106, 117	-141, 99
P ₅ -Asp ¹⁴ OMTKY3, <i>T</i> state	-64, -18	-85, -11	-116, 85	176, 83	-96, -149	-65, -11	-67, 111	-109, 121	-138, 109

family of canonical proteinase inhibitors (3, 31, 49–53). Although similar in the P₂'–P₃' region, large local conformational differences are observed in P₆–P₁ region. In particular, the backbone torsion angles of P₆ (Lys13) and P₅ (Asp14) in *T* are more characteristic of a ₃₁₀-helix, whereas they are more extended in wild-type OMTKY3. In addition, the α -helix in *T* deviates from its alignment in *C* and wild-type OMTKY3, owing to reasons discussed below.

The local structure of the *C* state of P₅-Asp OMTKY3 at pH 2.5 is similar to that of wild-type OMTKY3 at pH 4.0 (32) (Table 3), with the exception of a small deviation in the region P₄–P₂, which presumably results either from the pH difference or the P₅ residue substitution. This structural comparison is further supported by the chemical shift comparison of wild-type OMTKY3 with the two states of P₅-Asp OMTKY3 (see Figure S5 of the Supporting Information).

Structural Differences between the *C* and *T* States. The minimized mean structures for the two states are compared in Figure 10b. The *C* and *T* states share similar structural elements, such as the three-stranded antiparallel β -sheet and the β -turn connecting strands β_1 and β_2 . Although the overall backbone rms deviation between the mean structures of *C* and *T* is 3.03 Å, the backbone rms deviation of the β -sheet in the two is only ~0.26 Å. The α -helix is also preserved in the two states (backbone rmsd 0.34 Å) with the largest difference being in the orientation of the two ends of the helix. Most of the hydrogen bonds stabilizing the β -sheet and the α -helix are also present in both states (see Table S1 of the Supporting Information). The side chain of P₁-Leu¹⁸ has a similar orientation in the *C* and *T* states, highly exposed to the solvent in both.

The side chains of P₇-Pro¹² and P₄-Ala¹⁵ are farther apart in *C* than in *T* (Figure 10c). In the *C* state, the side chain of P₂₄'-Val⁴² and the P₇-Pro¹² ring make many hydrophobic contacts. As a result in the *C* state, the P₇-Pro¹²–P₄-Ala¹⁵ backbone is rather extended. In the *T* state, as a consequence of disruption of the hydrophobic interactions between P₇-Pro¹² and P₂₄'-Val⁴², the side chain of P₇-Pro¹² projects away from the helix and becomes closely packed with the side chain of P₄-Ala¹⁵ (Figure 10c). Concurrently, the side chain of P₆-Lys¹³, which contacts the side chain of P₂₁'-Asn³⁹ in *C* state, moves closer to the side chains of P₅-Asp¹⁴ and other residues within the reactive-site loop.

Relocation of the Tyr-11 Ring. The aromatic ring of P₈-Tyr¹¹ moves from the center of the α -helix in *C* to the N-terminus of the helix in *T*. This relocation of the phenyl ring disrupts the hydrophobic cluster of the *C* state and loosens the contacts between the reactive-site loop and the α -helix. As shown in Figure 10d, the relocated P₈-Tyr¹¹ ring in *T* contacts several other residues, including P₁₂-Asp⁷, P₄-Ala¹⁵, and P₁₆'-Lys³⁴. In addition, relocation of the P₈-Tyr¹¹ ring results in large structural perturbations of residues in the loop containing the P₈-Tyr¹¹–P₇-Pro¹² peptide bond as

well as in the N- and C-terminal residues of the α -helix. Our structural analysis suggests that the aromatic ring of P₈-Tyr¹¹ plays a vital role in stabilizing the *cis* conformation of the peptide bond. In the *C* state, the aromatic ring of P₈-Tyr¹¹ is attached to the middle of the α -helix, with its plane parallel to the axis of the helix (Figure 10c). The hydrophobic ring of P₈-Tyr¹¹ makes many contacts with the side chains of surrounding residues (P₁₁-Cys⁸, P₆-Lys¹³, P₁₇'-Cys³⁵, P₂₀'-Cys³⁸, P₂₁'-Asn³⁹, and P₂₄'-Val⁴²). The relative spatial arrangement between the reactive-site loop and the helix is stabilized further by packing between the ring of P₇-Pro¹² and the side chain of P₂₄'-Val⁴².

Differences in Hydrogen Bonding. In the *C* states of wild-type OMTKY3 (5) and P₅-Asp OMTKY3, H^N of P₂-Thr¹⁷ exhibits a negative titration shift (to lower frequency with increasing pH), which has been attributed to the hydrogen bond between the side chains of P₂-Thr¹⁷ and P₁'-Glu¹⁹ (5). In the *T* state, however, the H^N of P₂-Thr¹⁷ exhibits a positive titration shift. This result is indicative of a difference in hydrogen bonding between P₂-Thr¹⁷ and P₁'-Glu¹⁹ in *C* and *T*. Coupling between the change in peptide bond configuration at P₈-Tyr¹¹–P₇-Pro¹² and the change in hydrogen bonding at P₂-Thr¹⁷ and P₁'-Glu¹⁹ probably is mediated by changes in the contacts among P₂-Thr¹⁷, P₁₅'-Asn³³, and P₁₆'-Lys³⁴, which are present in *C* but disrupted in *T*. Structural comparison of the two states also reveals that a hydrogen bond between the amide group of P₄-Ala¹⁵ and the backbone oxygen of P₇-Pro¹² exists in *T* but not in *C*.

Differences in the Conformations of Two Disulfide Bridges. Of the three disulfides the ¹³C α and ¹³C β resonances of two (P₁₁-Cys⁸–P₂₀'-Cys³⁸ and P₃-Cys¹⁶–P₁₇'-Cys³⁵) showed large chemical shift perturbations (>|0.5 ppm|). The side chain resonances of the third disulfide bridge (P₆'-Cys²⁴–P₃₈'-Cys⁵⁶) exhibited no chemical shift perturbations. Disulfide bridge P₆'-Cys²⁴–P₃₈'-Cys⁵⁶ is well defined, with a right-handed conformation in both the *C* and *T* states. Although the conformations of the other two disulfide bridges are less clearly defined, comparison of the structures for *C* and *T* (Figure 6) shows that these two disulfides adopt very different conformations in the two forms. The C α –C α distances for each of these is longer by >1 Å in *T* than in *C*. Since these two disulfides covalently bond the reactive-site loop to the α -helix, the changes in their geometries probably arise from strains imposed by the structural rearrangement of this region of the protein.

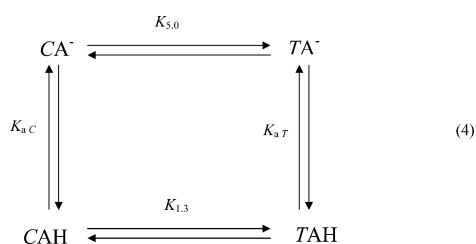
Factors Contributing to Differential Stabilization of the *C* and *T* States. It has been recognized that an aromatic residue preceding a proline can have a large effect on the relative stabilities of the *cis* and *trans* peptide bond configurations (42, 54, 55). Surveys of the Protein Data Bank by MacArthur et al. (56) indicated that a *cis* peptide bond is more prevalent in Xxx–Pro peptide bonds when Xxx is an aromatic residue. From NMR investigations of *cis*/*trans* isomerization of the model peptide acetyl-Gly-Xxx-Pro-Gly

carboxamide, where Xxx = Tyr, Phe, Trp, etc., Wu and Raleigh suggested that interactions between the aromatic ring and the prolyl ring stabilize the *cis* state of the Xxx–Pro peptide bond (57). The present results show how the packing of the aromatic ring of P₈-Tyr¹¹ with residues in the α -helix play a vital role in stabilizing the *cis* conformation of the P₈-Tyr¹¹–P₇-Pro¹² peptide bond.

How can the structural results rationalize the experimental order of stabilization of the *trans* state in P₅-Pro¹⁴Xxx OMTKY3 mutants: Xxx = Gly > Asp > Glu > Ala > His > Pro? Analysis suggests that the presence of C ^{β} and C ^{δ} substituents on residue 14 interfere with the packing between the side chains of P₇-Pro¹² and P₄-Ala¹⁵ in the *T* state (Figure 10c). This may explain why replacement of P₅-Pro¹⁴ by Gly greatly elevates the population of the *T* state and why the *C* state predominates in wild-type OMTKY3, P₅-Pro¹⁴Ala OMTKY3, and P₅-Pro¹⁴His OMTKY3. This kind of steric argument, however, fails to explain why the *T* state is favored when residue 14 is Asp or Glu. Thus a second mechanism must be invoked. A plausible mechanism is stabilization of *T* by electrostatic interaction between the side chain of P₆-Lys¹³ and the acidic side chain at residue P₅-Asp/Glu¹⁴.

A precedent for such a dual mechanism comes from work of Hodel et al. (58) on the configuration of the Lys¹¹⁶-Pro¹¹⁷ peptide bond of staphylococcal nuclease (SNase). In wild type SNase, the *cis* form is much more highly populated than the *trans* form (26, 58). Replacement of Lys¹¹⁶ by Gly, Asp, or Asn stabilizes the *trans* form relative to the *cis* form, whereas replacement by Ala, Met, or Gln has no effect on the equilibrium. These results were explained in terms of two distinct mechanisms: reduction of the steric barrier (in the case of Gly) and side-chain tertiary interactions (in the case of the Asp mutant).

Dependence of the $C \rightleftharpoons T$ Equilibrium on pH. Between pH 5.0 and 1.3, $I_T/(I_T + I_C)$ changes from 0.22 to 0.55 (Figure 7); thus, $K = [T]/[C]$ changes from 0.28 to 1.2 over this pH range. This result suggests that the *T* state has a higher affinity for one or more protons than the *C* state. This effect can be modeled by a coupled equilibrium of the form shown in eq 4, where the acidic site(s) affected is represented in the *C* state as CA[−] and in the *T* state as TA[−].



Because of the equilibrium condition, $\text{p}K_{aC} + \text{p}K_{5.0} = \text{p}K_{aT} + \text{p}K_{1.3}$. In addition, from the change in the *cis/trans* equilibrium noted above, $\text{p}K_{5.0} - \text{p}K_{1.3} = 0.63$. Thus, over this pH range, $\Delta \text{p}K_a = \text{p}K_{aT} - \text{p}K_{aC} = 0.63$.

The side chains of P₁₂-Asp⁷ and P₁'-Glu¹⁹ showed higher $\text{p}K_a$ values in the *T* than the *C* form (Table 2). The side chain of P₅-Asp¹⁴ and the C-terminal carboxyl P₃₈'-Cys⁵⁶ had similar $\text{p}K_a$ values in the two states. And the side chains of P₉-Glu¹⁰ and P₂₅'-Glu⁴³ exhibited lower $\text{p}K_a$ values in the *T* state than the *C* state. Not surprisingly, residues exhibiting large $\text{p}K_a$ differences in *C* and *T* lie in regions with major

chemical shift differences, whereas those with minimal $\text{p}K_a$ difference lie in regions with minor shift differences (Figure 3). The data of Table 2 yield 21.62 as the sum of the six $\text{p}K_a$ values for the *T* form and 20.98 as the sum of the six $\text{p}K_a$ values for the *C* form. Thus, the aggregate $C \rightarrow T$ $\text{p}K_a$ difference of 0.64 is in excellent agreement with the value of 0.63 predicted from the pH dependence of the *cis/trans* equilibrium.

To test this model, another double mutant, (P₁₂-Ala⁷, P₅-Asp¹⁴) OMTKY3, was constructed, and [¹H–¹H]-TOCSY data for this mutant were collected at pH 6.0 and 2.2 (Supporting Information, Figure S6). The spectra were assigned by reference to spectra of P₅-Asp¹⁴ OMTKY3. The $\text{p}K_a$ of P₁₂-Asp⁷ was determined to be 0.5 pH unit higher in the *T* state than in *C* state (Table 2). The expectation was that, in the absence of other effects of the mutation, removal of a titrating group with a positive $C \rightarrow T$ $\text{p}K_a$ difference should stabilize the *trans* configuration of the P₈-Tyr¹¹–P₇-Pro¹² peptide bond. Indeed, the double mutant exhibited the expected increased stabilization of *T*. The fractional occupancy of *T* in this mutant was 0.5–0.6 at pH 6.0 (vs 0.22 for the single mutant) and ~ 0.7 at pH 2.2 (vs 0.5 for the single mutant).

In wild-type OMTKY3, the low $\text{p}K_a$ of P₁₂-Asp⁷ has been attributed to a hydrogen bond between the side chains of P₁₂-Asp⁷ and P₁₀-Ser⁹ (5, 59, 60). This hydrogen bond is weakened in the *T* state compared the *C* as shown by the pH titration shift of the P₁₀-Ser⁹ H^N (0.5 ppm in *C*, 0.3 ppm in *T*). A second factor contributing to the elevated $\text{p}K_a$ of Asp⁷ in *T* compared to *C* may be the increased hydrophobic environment of the P₁₂-Asp⁷ side chain in *T* (9, 61–65). Structural comparison (Figure 10d) reveals relatively close contacts between the side chains of P₁₂-Asp⁷ and P₈-Tyr¹¹ in *T* state but not in *C*.

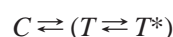
Results from pH titration studies of various OMTKY3 mutants indicate that the hydrogen bond between the side chains of P₁'-Glu¹⁹ and P₂-Thr¹⁷ lowers the $\text{p}K_a$ of P₁'-Glu¹⁹ by 0.4–0.5 pH unit (11). Our structural and pH titration results suggest that the higher $\text{p}K_a$ value observed for P₁'-Glu¹⁹ in *T* probably arises from the effect of breaking or weakening this hydrogen bond.

Comparison of the Two Low-pH Conformational Transitions: In 1967, Donovan reported a structural transition at low pH in whole chicken ovomucoid. A blue shift of the absorption spectrum was found to be accompanied by a 300% increase in fluorescence when chicken ovomucoid solutions were made acidic (6). These changes are reversible and have the pH dependence of a single acidic group of $\text{p}K_a = 2.8$. In later studies, March (66) assigned P₁₃'-Tyr³¹ as the residue in all three domains responsible for the change in the optical spectrum, and Croll (67) observed a transition with a pH_{mid} of about 2.5 that perturbed the chemical shifts of ¹³C NMR signals of chicken ovomucoid third domain assigned to ¹³C ^{γ} and ¹³C ^{ϵ} of P₁₃'-Tyr³¹. Both authors explained the spectral results in terms of protonation of the side chain of P₉'-Asp²⁷ at low pH, which disrupts the hydrogen bond between the aromatic ring of P₁₃'-Tyr³¹ and the side chain of P₉'-Asp²⁷. The present studies show that the actual $\text{p}K_a$ of P₉'-Asp²⁷ is about 2.7 in both conformational states, a value that is close to the pH_{mid} for both the Donovan transition and for the $C \rightleftharpoons T$ transition. Although the two transitions occur with similar pH_{mid} values, it appears

that they are independent. The P₈-Tyr¹¹-P₇-Pro¹² cis/trans isomerization is slow on the NMR time scale and is characterized by a low Hill coefficient, whereas the residues affected by the Donovan transition show single peaks and a Hill coefficient close to unity (67).

Internal Dynamics of the Two States. The *C* and *T* states of P₅-Asp¹⁴ OMTKY are equally populated at pH 2.5, indicating that their free energies are equivalent. However, our structural analysis revealed that the *C* → *T* transition results in the loss of many hydrophobic contacts between the P₈-Tyr¹¹ ring and other groups. How does the *T* state compensate for this energy loss? To address this question, we explored the dynamic features of the two states by measuring ¹⁵N transverse relaxation.

As shown in Figure 9, the average ¹⁵N *R*₂ relaxation rate for residues in *C* state is 6 s⁻¹, with the rates for the N-terminal residues much slower than average and those for the P₁₁-Cys⁸-P₄-Ala¹⁵ loop residues slightly faster than average. Although the ¹⁵N *R*₂ rates were similar in the two states for most residues, several residues exhibited much faster relaxation in *T* than in *C*: these included the stretches P₈-Tyr¹¹-P₃-Cys¹⁶ (except P₄-Ala¹⁵ and P₁₆'-Lys³⁴-P₁₈'-Asn³⁶). We attribute the increased ¹⁵N *R*₂ relaxation rates for these residues to conformational exchange within the trans state on the μs to ms time scale within that state. If we assume that the exchange process within *T* is two-state (between states *T* and *T**), the overall exchange process is described as:



Although the origin of this slow exchange event remains unclear, the increased loop mobility undoubtedly contributes entropic stabilization to the *T* state.

Multiple exchange processes on different time scales reflect the complexity of the free energy landscape for the protein. A similar example was provided by an SH3 domain studied by Kay and co-workers (14, 47), which exhibited both a conformational exchange between the folded and unfolded states, with a *k*_{ex} of 2.2 s⁻¹, and a more rapid exchange process on the microsecond–millisecond time scale within the unfolded state.

After this manuscript was submitted, a paper appeared by Hemmi et al. (68) describing a silver pheasant ovomucoid third domain variant containing an engineered disulfide bridge between cysteines mutated in at positions P₅ and P₂₁'. The inserted disulfide bridge is homologous to one present in the ascidian trypsin inhibitor (69). Interestingly, in the context of the present study, the configuration of the P₈-Tyr-P₇-Pro peptide bond in the disulfide variant was determined to be trans (68), rather than cis as in wild-type silver pheasant ovomucoid third domain. The disulfide variant retained activity as a potent inhibitor of α-chymotrypsin and *Streptomyces griseus* proteases A and B but lost most of its activity as an inhibitor of porcine pancreatic elastase (68).

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SUPPORTING INFORMATION AVAILABLE

Six figures and three tables as referenced in the text are available free of charge via the Internet at <http://pubs.acs.org>.

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