

An Experimental Approach to Evaluating the Role of Backbone Interactions in Proteins Using Unnatural Amino Acid Mutagenesis[†]

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Received April 2, 1997; Revised Manuscript Received July 2, 1997[®]

ABSTRACT: The contribution of backbone hydrogen bonds in α -helices to the overall stability of a protein has been examined experimentally by replacing several backbone amide linkages in α -helix 39–50 of T4 lysozyme with ester linkages. T4 lysozyme variants wherein the backbone amide bonds between residues Ser³⁸ and Leu³⁹, Lys⁴³ and Leu⁴⁴, or Ala⁴⁹ and Ile⁵⁰ are replaced with ester bonds were generated by incorporating α -hydroxy acids at positions 39, 44, or 50, respectively, using unnatural amino acid mutagenesis. The stabilities of the proteins were determined from their thermal denaturation curves as monitored by circular dichroism. Comparison of the thermal stabilities of the amide- and ester-containing proteins shows that the ester substitution has a similar thermodynamic effect at all three positions. At the N- and C-terminal positions, where only one hydrogen-bonding interaction is perturbed, the ester substitution is destabilizing by 0.9 and 0.7 kcal/mol, respectively. Introduction of the ester linkage in the middle of the helix, which alters two hydrogen-bonding interactions, destabilizes the protein by 1.7 kcal/mol. The values obtained from these ester to amide mutations are compared to the values from similar mutations that have been made in other secondary structures and bimolecular complexes.

The α -helix was first proposed as a secondary structural element in proteins in 1951 on the basis of hydrogen-bonding interactions between i and $i + 4$ backbone amide groups (Pauling et al., 1951). Since that time, the contribution of these hydrogen bonds to the overall stability of the α -helix has been a matter of some debate (Dill, 1990; Doig & Williams, 1992; Honig & Yang, 1995; Khechinashvili, 1990; Klotz & Franzen, 1962; Murphy & Gill, 1991). One view holds that protein stability is determined primarily by hydrophobic and packing interactions (Kauzmann, 1959; Klotz & Franzen, 1962; Privalov & Gill, 1988). Hydrogen bonds in the folded state of the protein are said to be isoenergetic with respect to those to water in the unfolded state and so contribute primarily to the specificity of side chain and backbone interactions. Alternatively, hydrogen bonds have been proposed to make a significant energetic contribution to the thermodynamic stability of the folded protein (Creighton, 1991; Doig & Williams, 1992; Honig & Yang, 1995; Murphy & Gill, 1991; Pace et al., 1996). The contributions of backbone hydrogen bonds, as opposed to side chain hydrogen bonds, are of particular interest since they are thought to be critical in the formation of secondary structures and are an essential part of a number of models of protein folding (Dill, 1990).

Several experimental approaches have been taken to determine the thermodynamic consequences of hydrogen bond formation. Both protein and peptide folding have been characterized thermodynamically using calorimetry (Makhatazde & Privalov, 1995; Scholtz et al., 1991). These thermodynamic data have been compared to those obtained from the study of small molecules, such as the dissolution of cyclic amide crystals in water and the aqueous association of small molecules (Doig & Williams, 1992; Klotz & Franzen, 1962; Murphy & Gill, 1991; Susi et al., 1964). Site-directed mutagenesis has been used to assess the energetic consequences of deleting or perturbing one or both members of a hydrogen bond between two side chains in a protein or between a side chain and a ligand (Alber et al., 1987; Chen et al., 1993; Matthews, 1995; Murray et al., 1994; Schreiber & Fersht, 1995; Shirley et al., 1992; Thorson et al., 1995a,b). Similarly, the free energy of the binding interactions between proteins and small molecules has been determined by chemically perturbing atoms in the ligand that form hydrogen bonds to the protein (Morgan et al., 1991; Murray et al., 1994). While all of these experimental studies provide insights into the energetics of amide–amide interactions in solution, they do not provide a measure of the strength of backbone amide–amide interactions in proteins, where the geometric constraints and electronic environment are distinct. To date, direct examination of these backbone hydrogen bonds has been limited almost exclusively to computational methods (Honig & Yang, 1995; Tirado-Rives et al., 1993; Yang & Honig, 1995a,b).

Here, we report on an experimental approach for assessing the contribution of backbone hydrogen bonds to protein stability. Specifically, several backbone amide linkages in an α -helix in T4 lysozyme (T4L)¹ have been replaced with ester linkages using amber suppressor tRNAs that have been acylated chemically with α -hydroxy acids. This approach parallels that of Kent in which chemically synthesized

[†] The authors are grateful for financial support for this work from the National Institutes of Health (Grant R01GM49220). P.G.S. is a Howard Hughes Medical Institute Investigator and a W. M. Keck Foundation Investigator. J.T.K. was supported by a fellowship from the California division of the American Cancer Society. V.W.C. was supported by predoctoral fellowships from the NSF, the American Chemical Society, and the Howard Hughes Medical Institute.

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[®] Abstract published in *Advance ACS Abstracts*, September 1, 1997.

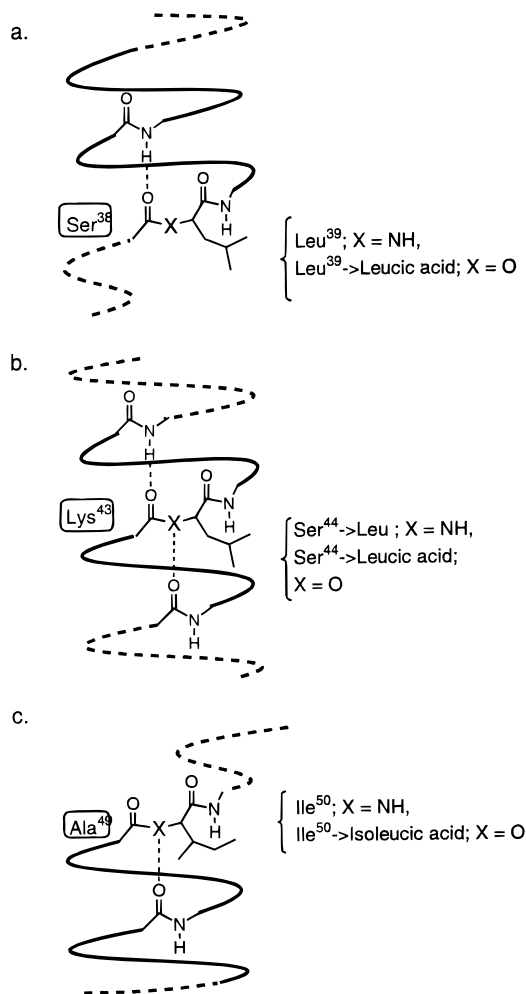


FIGURE 1: Schematic representation of sites of α -hydroxy acid incorporation: (a) N-terminal mutation of Leu^{39} to leucic acid, (b) intrahelix mutations of Ser^{44} to Leu and Ser^{44} to leucic acid, and (c) C-terminal mutation of Ile^{50} to isoleucic acid.

peptides containing ester linkages were used to study the binding energetics of protein/peptide complexes (Lu et al., 1997). The biosynthetic machinery of *Escherichia coli* has long been known to recognize α -hydroxy acids as well as α -amino acids (Chladek & Sprinzl, 1985; Fahrestock et al., 1970). Consequently, by using unnatural amino acid mutagenesis, as opposed to traditional site-directed mutagenesis, it is possible to site-specifically alter the protein backbone (Ellman et al., 1992). The ester group should be a fairly conservative replacement for an amide group since the two have similar conformational preferences (Blom & Gunthard, 1981; Ingwall & Goodman, 1974; Wiberg & Laiding, 1987). The primary effects of the ester substitution are 2-fold: a decrease in the basicity (Arnett et al., 1974) and, as a consequence, the hydrogen bond-accepting ability of the carbonyl oxygen and the replacement of the main chain NH hydrogen bond donor with the electronegative O atom of the ester. The α -hydroxy acids were incorporated at the N terminus, middle, and C terminus of α -helix 39–50 in T4 lysozyme (Figure 1), and the thermal stabilities of the wild-type (wt) and main chain amide ester mutant proteins were determined. The energetic consequences of these mutants

are discussed in the context of previous work on the contributions of hydrogen bonds to protein stability and molecular complexation.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were purchased from New England Biolabs; reagents and enzymes used for sequencing were purchased from U.S. Biochemicals. Oligonucleotides were synthesized on an Applied Biosystems PCR Mate DNA synthesizer. All chemicals were purchased from Aldrich. The dinucleotide pdCpA aminoacylated with leucine was provided by J. Thorson (University of California, Berkeley). All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX300 spectrometer and were referenced to tetramethylsilane. Fast atom bombardment mass spectra (FAB MS) were recorded by the University of California Mass Spectra laboratory with *m*-nitrobenzyl alcohol as the matrix. High-pressure liquid chromatography (HPLC) was performed on a Waters Model 600E apparatus equipped with a Rainin Dynamax UV-1 detector. A Waters Porasil column (M20 10/50 OSD-3) was used for preparative chromatography, and a Rainin Microsorb C-18 column was used for analytical chromatography. Thin-layer chromatography (TLC) was performed on silica gel (Merck Fertigplatten, 60F-254, Art. 5765). All column chromatography and flash chromatography was carried out on silica gel (Merck Kieselgel 60, Art. 9385). Autoradiography was conducted using a Molecular Dynamics Model 445 phosphorimager.

(S)-Methyl 2-(Benzyloxy)-4-methylpentanoate. To a solution of methyl 2-hydroxy-4-methylpentanoate (1.8 g, 12 mmol) in dry CH_2Cl_2 (2 mL) was added benzyltrichloroacetimidate (4.1 g, 16.4 mmol). The solution was chilled in an ice bath, and then trifluoromethanesulfonic acid (30 μL , 0.3 mmol) was added dropwise. After addition of the acid, the ice bath was removed, and the solution was stirred for 3 h at room temperature. The reaction mixture then was poured into hexane (50 mL) and neutralized with 1 N NaHCO_3 (50 mL). The aqueous layer was extracted with ether (3×100 mL). The combined organic extracts were washed with saturated aqueous NaCl and dried over MgSO_4 , and the solvent was removed *in vacuo*. The resulting residue was chromatographed on silica with 10% ethyl acetate/hexane to afford the benzyl ether as a colorless oil (1.51 g, 47%): $R_f = 0.35$; $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 4.71 (d, $J = 9$ Hz, 1), 4.41 (d, $J = 9$ Hz, 1), 4.37 (m, 5), 4.02 (dd, $J = 8, 2$ Hz, 1), 3.77 (s, 3), 1.87 (m, 1), 1.75 (m, 1), 1.51 (m, 1), 0.92 (d, $J = 3$ Hz, 3), 0.83 (d, $J = 3$ Hz, 3).

(S)-Methyl 2-(2-Nitrobenzyloxy)-4-methylpentanoate. To a solution of (S)-methyl 2-(benzyloxy)-4-methylpentanoate (0.72 g, 3.2 mmol) and trifluoroacetic anhydride (6 mL) in a 0 $^\circ\text{C}$ bath was added finely ground $\text{Cu}(\text{NO}_3)_2$ (1.4 g, 6.4 mmol). The reaction mixture was stirred at 0 $^\circ\text{C}$ for 1 h, giving a clear blue solution. The solution was concentrated *in vacuo*, and the residue was dissolved in CH_2Cl_2 (100 mL) and washed with saturated aqueous NaHCO_3 (50 mL) and saturated aqueous NaCl (50 mL). The organic fraction was dried over MgSO_4 and the solvent removed *in vacuo* to afford a crude mixture of the *ortho* and *meta* isomers (1:4). The isomers were separated by flash chromatography using 20% ethyl acetate/hexane to afford the desired *ortho*-substituted product (100 mg, 11%): $R_f = 0.35$; $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ 8.06 (d, $J = 8$ Hz, 1), 7.87 (d, $J = 8$ Hz, 1), 7.63

¹ Abbreviations: T4L, T4 lysozyme; OMTK3, turkey ovomucoid third domain; CHYM, α -chymotrypsin; BPTI, basic pancreatic trypsin inhibitor (bovine).

(dd, $J = 8$ Hz, 1), 7.42 (dd, $J = 8$ Hz, 1), 4.99 (d, $J = 14$ Hz, 1), 4.83 (d, $J = 14$ Hz, 1), 4.10 (dd, $J = 5$ Hz, 1), 3.74 (s, 3), 1.88 (m, 1), 1.80 (m, 1), 1.58 (m, 1), 0.95 (d, $J = 7$ Hz, 3), 0.92 (d, $J = 7$ Hz, 3).

Cyanomethyl 2-(2-Nitrobenzyloxy)-4-methylpentanoate. To a solution of methyl 2-(2-nitrobenzyloxy)-4-methylpentanoate (100 mg, 0.36 mmol) in ethanol (5 mL) was added solid KOH (40 mg, 1.8 mmol). The resulting solution was stirred for 1 h at room temperature. The reaction mixture was then poured into a mixture of ethyl acetate (25 mL) and H₂O (10 mL) and acidified by dropwise addition of 1 N HCl. The aqueous layer was then extracted with ethyl acetate (2 × 20 mL); the combined organic extracts were dried over MgSO₄, and the solvent was evaporated under reduced pressure. The residue was dissolved in chloroacetonitrile (1 mL, 15.8 mmol). Freshly distilled triethylamine (0.1 mL, 0.72 mmol) was added dropwise, and the resulting solution was stirred for 1 h at room temperature. The solution was concentrated *in vacuo*, and the residue was applied directly to a column of silica and eluted with 20% ethyl acetate/hexane to afford the active ester as a yellow oil (49 mg, 44%): $R_f = 0.2$ – 0.3 ; ¹H NMR (CDCl₃, 300 MHz) δ 8.07 (d, $J = 8$ Hz, 1), 7.84 (d, $J = 8$ Hz, 1), 7.67 (t, $J = 8$ Hz, 1), 7.45 (t, $J = 8$ Hz, 1), 4.98 (d, $J = 13$ Hz, 1), 4.87 (d, $J = 13$ Hz, 1), 4.79 (d, $J = 8$ Hz, 1), 4.20 (dd, $J = 8$ Hz, 1), 1.87 (m, 1), 1.79 (m, 1), 1.63 (m, 1), 0.97 (d, $J = 6$ Hz, 3), 0.95 (d, $J = 6$ Hz, 3).

5'-Phospho-2'-deoxycytidyl(3'-5')-2'(3')-O-[(S)-2-(o-nitrobenzyloxy)-4-methylpentanoyl]adenosine. The hydroxyacyl-pdCpA was prepared and isolated as reported for aminoacyl-pdCpAs in 40–70% yield (Cornish et al., 1994b; Robertson et al., 1991): MS m/e 989 (MH⁺).

(2S,3S)-Cyanomethyl O-(Nitroveratryloxycarbonyl)-2-hydroxy-3-methylpentanoate. (2S,3S)-2-Hydroxy-3-methylpentanoic acid (isoleucic acid) was prepared by diazotization and subsequent hydrolysis of the parent amino acid as reported (Winitz et al., 1956). To a solution of (2S,3S)-2-hydroxy-3-methylpentanoic acid (0.5 g, 3.8 mmol) and nitroveratryl chloroformate (NVOC-Cl) (1.0 g, 3.8 mmol) in dry THF (20 mL) was added dry triethylamine (1.0 mL, 7.6 mmol). The reaction mixture was stirred for 3 h at room temperature. The reaction mixture was then poured into an ice-cold mixture of water (50 mL) and ether (50 mL). The aqueous layer was made basic by dropwise addition of saturated aqueous NaHCO₃. The organic layer was extracted with H₂O (2 × 100 mL). The combined aqueous fractions were acidified dropwise with 1 N HCl and then extracted with ethyl acetate (2 × 50 mL). The combined organic fractions were dried over MgSO₄ and concentrated *in vacuo* to afford the crude carbonate (400 mg). Chloroacetonitrile (1 mL, 15.8 mmol) and dry triethylamine (1 mL, 7.6 mmol) were added to the crude carbonate. After 1 h, the reaction mixture was poured into H₂O (60 mL). The solution was made basic by dropwise addition of saturated aqueous NaHCO₃, and the aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed with saturated NaCl (50 mL), dried over MgSO₄, and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel using 30% ethyl acetate/hexane to afford the desired cyanomethyl ester (87 mg, 7%): $R_f = 0.2$ – 0.3 ; ¹H NMR (CDCl₃, 300 MHz) δ 7.75 (s, 1), 7.06 (s, 1), 4.93 (d, $J = 4.5$ Hz, 1), 5.72 (d, $J = 15$ Hz, 1), 5.55 (d, $J = 15$ Hz, 1), 4.04 (s, 3), 3.97 (s, 3), 2.08 (m, 1), 1.50 (m,

1), 1.34 (m, 1), 1.06 (d, $J = 6.9$ Hz, 3), 0.96 (t, $J = 7.5$ Hz, 3); MS m/e 417 (MLi⁺).

5'-Phospho-2'-deoxycytidyl(3'-5')-2'(3')-O-[(2S,3S)-O-(NVOC)-2-hydroxy-3-methylpentanoyl]adenosine. The hydroxyacyl-pdCpA was prepared and isolated as reported for aminoacyl-pdCpAs in 40–70% yield (Cornish et al., 1994b; Robertson et al., 1991): MS m/e 990 (MH⁺).

Preparation of α -Hydroxyacyl-tRNAs. The truncated amber suppressor tRNA_{CUA}(-CA) was prepared by run-off transcription of *FokI*-digested *pYPhe2* as reported (Ellman et al., 1991). The α -hydroxy- or α -aminoacyl-pdCpAs then were coupled to this tRNA_{CUA}(-CA) using T4 RNA ligase, and the nitroveratryl or nitrobenzyl protecting groups were removed by photolysis as reported (Ellman et al., 1991; Mendel et al., 1995).

Construction of T4L Mutant Genes. Mutant proteins were constructed by the method of Kunkel et al. (1987) using an M13mp18 derivative containing a 650 base pair (bp) *Bam*HI/*Hind*III fragment that encodes the cysteine free form of T4L (Perry & Wetzel, 1987). The oligonucleotides 5'-AGT-ATT-ACG-CCC-CTA-AGC-TTT-ATC-TAA and 5'-GA-TTT-AGC-AGC-ATT-CTA-TGA-TGG-ACT-TTT-TG were used to generate the pT4L50am and pT4L39am mutants, respectively. The mutated *Bam*HI/*Eco*RI fragment then was subcloned into the expression vector *pHSe54,97.TA*. The mutants pT4L44am and pT4L40am were reported previously (Cornish et al., 1994a). Mutations in the M13mp18 derivatives were verified by dideoxy sequencing (Sanger et al., 1977), and the entire coding region in *pHSe54,97.TA* was sequenced for each mutant.

In Vitro Protein Synthesis and Protein Purification. *In vitro* protein synthesis was carried out as described (Cornish et al., 1994b; Ellman et al., 1991). The *in vitro* reactions were carried out under conditions where the amount of protein produced when the aminoacyl-tRNA was replaced by an unacylated tRNA (the level of read-through) was judged to be less than 1% on the basis of SDS-PAGE and autoradiography of the soluble fractions from *in vitro* reactions charged with [³⁵S]Met. Proteins were isolated from 10 mL *in vitro* reaction mixtures. The proteins were purified by ion-exchange chromatography as reported (Cornish et al., 1994b), except that the protein solutions were desalted by passage through a PD-10 (Pharmacia) size-exclusion column. Each of the purified proteins was determined to be greater than 95% homogeneous on the basis of SDS-PAGE with silver staining.

Confirmation of Ester Incorporation by Alkaline Hydrolysis. The extent of ester incorporation was assessed by selective hydrolysis of the ester backbone linkage under alkaline conditions. A crude *in vitro* reaction mixture (10 μ L) which had been charged with [³⁵S]Met was treated with a solution of 1:1 concentrated ammonium hydroxide/10% SDS (2 μ L) for 0.5–1.5 h at 90 °C. The solutions then were neutralized by treatment with 30% acetic acid (0.5 μ L). Separation of full-length and truncated protein by SDS-PAGE, followed by quantitation with a phosphorimager, indicated that less than 5% of the full-length protein remained after hydrolysis. Typically, less than 2% of the full-length protein is observed after hydrolysis. However, ester linkages generated from hydroxy acids containing bulky sidechains (isoleucic acid versus leucic acid or lactic acid) tend to be more resistant to alkaline hydrolysis. Wild-type protein undergoes no appreciable hydrolysis under these conditions.

Thermal Stability Measurements. The thermal stability of the proteins was measured by monitoring the ellipticity at 223 nm at 1 °C temperature increments from 20 to 65 °C in 20 mM K₃HPO₄ (pH 2.5) and 25 mM KCl as described previously (Becktel & Baase, 1987; Cornish et al., 1994b). After each melt, the samples were returned to 20 °C and the θ_{223} was observed to be at least 80% of its initial value. In addition, the mutant proteins containing backbone ester linkages were shown by SDS-PAGE with silver staining not to have undergone hydrolysis during the thermal denaturation. The CD wavelength scans and melting curves were measured on an AVIV model 62DS circular dichroism spectrometer equipped with an AVIV thermoelectric temperature controller and a thermostatically controlled cuvette holder. The $\Delta\Delta G^\circ (\Delta G^\circ_{\text{amide}} - \Delta G^\circ_{\text{ester}})$ was calculated from $\Delta T_m (T_{m,\text{amide}} - T_{m,\text{ester}})$ using the equation $\Delta\Delta G^\circ = \Delta T_m \Delta S^\circ$, where $\Delta S^\circ = 274 \pm 12 \text{ cal deg}^{-1} \text{ mol}^{-1}$ as described (Zhang et al., 1991). A positive $\Delta\Delta G^\circ$ indicates that the wt protein is more stable than the mutant protein.

Catalytic Activity Measurements. The catalytic activity of T4 lysozyme was estimated from the rate of hydrolysis of NAP IV cell walls (Ellman et al., 1992; Nelson & Gold, 1982). All mutant proteins were shown to have activities similar to that of the wt protein on the basis of quantification of radiolabeled protein in crude *in vitro* reactions using a phosphorimager.

Calculation of Solvent Accessible Surface Areas. Solvent accessible surface areas (SASs) were determined on the basis of the crystal structure of wt T4L using Macromodel 4.5 with a 1.4 Å probe atom (Mohamadi et al., 1990). These values were compared to those found for the corresponding atoms in local minima (OPLS/water dielectric continuum) of the extended chain conformations of the peptides Ac-Pro-Leu-Ser-Asp-NH₂ and Ac-Leu-Ala-Ile-Gly-NH₂. The areas calculated for the amide NH or ester O between residues Ser³⁸ and Leu³⁹ are as follows: amide proton, SAS_{extend} = 3.9 Å², SAS_{fold} = 8.5 Å²; ester oxygen, SAS_{extend} = 2.1 Å², SAS_{fold} = 3.6 Å². The area calculated for the amide or ester carbonyl between residues Ala⁴⁹ and Ile⁵⁰ is as follows: carbonyl oxygen, SAS_{extend} = 22.2 Å², SAS_{fold} = 21.5 Å².

RESULTS

α -Hydroxy Acid Incorporation into the Protein Backbone. Using an amber suppressor tRNA chemically acylated with an α -hydroxy acid, three main chain amide linkages in helix 39–50 in T4 lysozyme (T4L) were replaced with ester linkages. The protein T4L provides a good model for this work. The thermodynamic stabilities and structures of the wt protein and several mutant proteins have been well characterized (Matthews, 1995). In addition, a variety of synthetic amino acids have been incorporated into the protein previously using unnatural amino acid mutagenesis (Cornish et al., 1995; Ellman et al., 1991; Mendel et al., 1995).

The ester substitutions were engineered at the linkages between residues Ser³⁸ and Leu³⁹, Lys⁴³ and Ser⁴⁴, and Ala⁴⁹ and Ile⁵⁰ by incorporating α -hydroxy acids at positions 39, 44, and 50, respectively (Figure 1). At sites 39 and 50, the α -hydroxy acid that has the wild-type side chain was employed: leucic acid at site 39 and isoleucic acid at site 50. For synthetic ease, leucic acid was incorporated at site 44 and compared to the corresponding T4L Ser⁴⁴ → Leu mutant. Previous work by Matthews and co-workers has

established that this mutation does not alter the structure or activity of the protein significantly (Blaber et al., 1994). The backbone substitutions were made at both the N and C termini as well as in the middle of the helix since the structural and therefore energetic consequences of the mutations will differ. There is no evidence for direct helix-capping interactions with either the Ser³⁸–Leu³⁹ or Ala⁴⁹–Ile⁵⁰ amide bonds (Matthews, 1995; Nicholson et al., 1991). Thus, in the absence of significant structural perturbations, the energetic cost of the amide to ester substitution in the middle of the helix should equal the sum of the effects of the N-terminal and C-terminal mutations.

The α -hydroxy acids were incorporated in response to *amber* codons using a synthetic hydroxyacyl-tRNA_{CUA}. Notably, the efficiencies with which α -hydroxy acids are inserted using an *amber* suppressor tRNA are lower than those for the corresponding α -amino acids. At all three sites, the α -hydroxy acids were incorporated with 20% efficiency, in comparison to 60% efficiency for the α -amino acids. Suppression experiments with nonacylated tRNAs yielded less than 3% of the full-length protein. The homogeneity of the α -hydroxy acid mutants was confirmed by hydrolysis of the ester bonds in the mutant proteins under basic conditions. Selective and essentially complete cleavage of the backbone ester bond was confirmed by denaturing polyacrylamide gel analysis of [³⁵S]methionine-labeled proteins.

Relative Thermodynamic Stabilities of the Proteins. Wild-type T4L and the Leu³⁹ → leucic acid, Ser⁴⁴ → Leu, Ser⁴⁴ → leucic acid, and Ile⁵⁰ → isoleucic acid mutant proteins were purified to homogeneity from 10 mL *in vitro* reaction mixtures by ion-exchange chromatography. The proteins were all judged to be greater than 95% homogeneous on the basis of SDS-PAGE and silver staining. The structural integrity of the mutant proteins was verified by several experiments. First, the circular dichroism (CD) spectra between 205 and 300 nm for the mutant proteins are indistinguishable from those of the wt protein. Second, the wt and mutant have similar specific activities as determined using a cell wall lysis assay. Previous work by Matthews and co-workers has shown that limited structural perturbations that result from substitution of Ser⁴⁴ for Pro in the 39–50 helix of T4L significantly decrease the catalytic activity of the enzyme (Blaber et al., 1994). Third, the combined energetic effect of the N- and C-terminal main chain amide to ester mutations is nearly identical to that resulting from introduction of the ester in the middle of the helix. This additivity suggests that the amide to ester mutation does not cause significant structural perturbations.

The relative thermodynamic stabilities of the amide- and ester-containing proteins were determined from their thermal denaturation curves as monitored by CD spectroscopy (Table 1). The mutant proteins could be reversibly denatured with no evidence of ester hydrolysis detectable by SDS-PAGE

² Heat of inactivation assays (Cornish et al., 1994b; Wells & Powers, 1986) of wt T4L, Leu³⁹ → leucic acid, Ser⁴⁴ → Leu, Ser⁴⁴ → leucic acid, Asp⁶¹ → Leu, Asp⁶¹ → leucic acid, Asn⁶⁸ → Leu, and Asn⁶⁸ → leucic acid suggest that the energetic effects of the amide → ester substitutions are relatively insensitive to local context (J. T. Koh, V. W. Cornish, and P. G. Schultz, unpublished results). The ester mutations decrease the thermal stability of T4L by a similar magnitude at both N-terminal positions, Leu³⁹ and Asp⁶¹, and at both internal positions, Ser⁴⁴ and Asn⁶⁸.

Table 1: Relative Thermodynamic Stabilities of the T4 Lysozyme Mutants Containing Amide and Ester Backbone Linkages^a

protein	T_M (°C)	ΔT_M (°C)	$\Delta\Delta G^\circ$ (kcal/mol)
wt (Leu ³⁹)	45.19 ± 0.04	—	—
Leu ³⁹ → leucic acid	41.95 ± 0.06	-3.24 ± 0.10	0.89 ± 0.03
Ser ⁴⁴ → Leu	47.19 ± 0.19	—	—
Ser ⁴⁴ → leucic acid	40.87 ± 0.10	-6.31 ± 0.30	1.73 ± 0.08
wt (Ile ⁵⁰)	45.19 ± 0.04	—	—
Ile ⁵⁰ → isoleucic acid	42.55 ± 0.10	-2.64 ± 0.10	0.72 ± 0.03

^aThe ΔT_M and $\Delta\Delta G^\circ$ reported refer to the difference in stability between the ester- and the amide-containing proteins. A positive $\Delta\Delta G^\circ$ indicates that the wt protein is more stable than the corresponding ester-containing protein.

with silver staining. The Leu³⁹ → leucic acid and Ile⁵⁰ → isoleucic acid mutations destabilize the protein by 0.9 and 0.7 kcal/mol, respectively. The Leu⁴⁴ → leucic acid mutation results in a 1.7 kcal/mol loss in stability. In each case, the ester mutation is destabilizing, consistent with the fact that an ester is a weaker hydrogen bond acceptor than an amide and is not a hydrogen bond donor. The N- and C-terminal mutations have similar energetic effects despite the fact that the N-terminal mutation only weakens a hydrogen bond while the C-terminal mutation eliminates the hydrogen bond altogether and potentially introduces a repulsive lone pair–lone pair interaction.

DISCUSSION

Thermodynamic Analysis of the N-Terminal Mutation Ser³⁸-Leu³⁹. The experimental $\Delta\Delta G^\circ$ measured upon deleting or perturbing one member of a hydrogen bond pair does not provide a measure of the strength of a hydrogen bond (Fersht, 1988). Rather, in the case of the amide to ester substitution, the $\Delta\Delta G^\circ$ reflects the difference between the amide interactions in the folded and unfolded states and the ester interactions in the folded and unfolded states. In the folded α -helix, the main chain amide carbonyl of residue i forms a hydrogen bond with the amide NH group of residue $i + 4$. In addition to the actual strength of the ($i, i + 4$) hydrogen bond, the free energy of this interaction depends on local dipole effects, the interaction of the amide group with local water molecules, and other electrostatic interactions unique to the helix environment (Yang & Honig, 1995a). The free energy of the amide group in the α -helix will also be governed by local van der Waals interactions and the conformational entropy associated with the amide group itself.

The main chain amide interactions are expected to change considerably in the unfolded, extended conformation of the protein. The amide groups now form hydrogen bonds to water, resulting in altered water structure and entropy. Dipole–dipole interactions should decrease since the backbone amide groups are more distant from one another and no longer aligned. Electrostatic interactions in general will be affected by the transfer of groups from the lower dielectric environment of the protein to water upon unfolding. Whereas van der Waals interactions of the backbone amides with the rest of the protein will change upon unfolding, the conformational entropy associated with the amide group should remain relatively constant since the amide has a strong conformational preference for the *trans* conformation (Blom & Gunthard, 1981; Wiberg & Laiding, 1987). Thus, in the case of a main chain amide group, the $\Delta G^\circ(F \rightarrow U)$ represents

not only the free energy of the hydrogen bond but also that of other electrostatic interactions, solvation, van der Waals interactions, and entropy. Determining the magnitude of any one of these interactions using a mutagenesis approach would require that the amide group be substituted with a group that perturbed only that particular interaction in the folded state. Because such a substitution is not chemically feasible, we chose to replace the amide group with the isoelectronic analogue, the ester group. The experimental $\Delta\Delta G^\circ$ then represents

$$\begin{aligned} \Delta\Delta G^\circ(\text{obs}) &= \Delta G^\circ_{\text{amide}}(F \rightarrow U) - \Delta G^\circ_{\text{ester}}(F \rightarrow U) \\ &= [G^\circ_{\text{amide}}(U) - G^\circ_{\text{amide}}(F)] - \\ &\quad [G^\circ_{\text{ester}}(U) - G^\circ_{\text{ester}}(F)] \end{aligned}$$

The thermodynamic effects of the amide to ester substitution are most easily assessed at the N terminus of the helix where only one $i, i + 4$ hydrogen bond interaction is affected (Figure 1a). In addition, the amide NH and ester O groups of the backbone amide at position i are both exposed to solvent in the folded and unfolded forms of the protein and should not contribute to the energy of folding.³ The change in free energy ($\Delta\Delta G^\circ$) upon replacing the amide backbone linkage at Ser³⁸-Leu³⁹ with an ester linkage can be therefore described as

$$\begin{aligned} \Delta G^\circ_{\text{amide}}(F \rightarrow U) &= G^\circ_{\text{amide}}(U) - G^\circ_{\text{amide}}(F) \\ &= G^\circ_{\text{a}}(\text{C}=\text{O}^{\text{a}}_i \cdots \text{H}_2\text{O}) + \\ &\quad G^\circ_{\text{a}}(\text{N}-\text{H}^{\text{a}}_{i+4} \cdots \text{H}_2\text{O}) - \\ &\quad [G^\circ_{\text{a}}(\text{C}=\text{O}^{\text{a}}_i \cdots \text{H}-\text{N}^{\text{a}}_{i+4}) + \\ &\quad G^\circ_{\text{a}}(\text{H}_2\text{O} \cdots \text{H}_2\text{O})] + \\ &\quad \Delta G^\circ_{\text{a}}(F \rightarrow U)(\text{van der Waals}_a) + \\ &\quad \Delta G^\circ_{\text{a}}(F \rightarrow U)(\text{conformational entropy}_a) + \\ &\quad \Delta G^\circ_{\text{a}}(F \rightarrow U)(\text{electrostatics}_a) \end{aligned}$$

and

$$\begin{aligned} \Delta G^\circ_{\text{ester}}(F \rightarrow U) &= G^\circ_{\text{ester}}(U) - G^\circ_{\text{ester}}(F) \\ &= G^\circ_{\text{e}}(\text{C}=\text{O}^{\text{e}}_i \cdots \text{H}_2\text{O}) + \\ &\quad G^\circ_{\text{e}}(\text{N}-\text{H}^{\text{a}}_{i+4} \cdots \text{H}_2\text{O}) - \\ &\quad [G^\circ_{\text{e}}(\text{C}=\text{O}^{\text{e}}_i \cdots \text{H}-\text{N}^{\text{a}}_{i+4}) + \\ &\quad G^\circ_{\text{e}}(\text{H}_2\text{O} \cdots \text{H}_2\text{O})] + \\ &\quad \Delta G^\circ_{\text{e}}(F \rightarrow U)(\text{van der Waals}_e) + \\ &\quad \Delta G^\circ_{\text{e}}(F \rightarrow U)(\text{conformational entropy}_e) + \\ &\quad \Delta G^\circ_{\text{e}}(F \rightarrow U)(\text{electrostatics}_e) \end{aligned}$$

where $G^\circ_{\text{x}}(\text{C}=\text{O}^{\text{x}} \cdots \text{H}-\text{N}^{\text{x}})$ is the free energy of the backbone hydrogen bond in the α -helix; $G^\circ_{\text{x}}(\text{C}=\text{O}^{\text{x}} \cdots \text{H}_2\text{O})$ and G°_{x} -

³ The assumptions about the solvent exposure of the amide hydrogen and the ester oxygen at the helix termini are supported by calculations of the solvent accessible surface areas based on the wt crystal structure and on the extended conformation of representative model peptides (see Experimental Procedures).

($X^{\dots}H_2O$) are the free energies of the backbone hydrogen bonds to water in the unfolded conformation, $\Delta G^{\circ}_x(F \rightarrow U)$ -(electrostatic_x) is the difference in free energy of the remaining electrostatic interactions in the folded relative to the unfolded conformation, $\Delta G^{\circ}_x(F \rightarrow U)$ (van der Waals_x) represents the changes in van der Waals interactions, and $\Delta G^{\circ}_x(F \rightarrow U)$ (conformational entropy_x) represents the changes in conformational entropy upon folding. Contributions by the side chains as well as the rest of the protein are assumed to be identical for the amide-containing and ester-containing proteins.

A number of terms contained in ΔG°_{amide} and ΔG°_{ester} are likely to be close in energy. $G^{\circ}_x(H_2O \dots H_2O)$ and $G^{\circ}_x(N-H_{i+4} \dots H_2O)$ are likely to be equivalent between the amide- and ester-containing proteins. Because the ester and amide carbonyl groups are isosteric, the values of $\Delta G^{\circ}_x(F \rightarrow U)$ -(van der Waals_x) should be similar. The values of $\Delta G^{\circ}_x(F \rightarrow U)$ (conformational entropy_x) should also be roughly the same for both the ester and amide bond since esters also display a strong conformational preference for the *trans* conformation (Blom & Gunthard, 1981; Ingwall & Goodman, 1974; Wiberg & Laiding, 1987). Thus,

$$\begin{aligned} \Delta \Delta G^{\circ}(\text{obs}) &= \Delta G^{\circ}_{amide}(F \rightarrow U) - \Delta G^{\circ}_{ester}(F \rightarrow U) \\ &= G^{\circ}_a(C=O_i^a \dots H_2O) - \\ &\quad G^{\circ}_e(C=O_i^e \dots H_2O) - \\ &\quad [G^{\circ}_a(C=O_i^a \dots H-N_{i+4}^a) - \\ &\quad G^{\circ}_e(C=O_i^e \dots H-N_{i+4}^a)] + \\ &\quad [\Delta G^{\circ}_a(F \rightarrow U)(\text{electrostatics}) - \\ &\quad \Delta G^{\circ}_e(F \rightarrow U)(\text{electrostatics})] \end{aligned}$$

which can also be written as

$$\begin{aligned} \Delta \Delta G^{\circ}(\text{obs}) &= \Delta G^{\circ}(U)(\text{carbonyl-water interaction}) - \\ &\quad \Delta G^{\circ}(F)(\text{"backbone hydrogen bond"}) + \\ &\quad \Delta \Delta G^{\circ}(F \rightarrow U)(\text{electrostatics}) \end{aligned}$$

The $\Delta \Delta G^{\circ}$ for the water-octanol partitioning coefficients of amides and esters provides one measure for $\Delta G^{\circ}(U)$ -(carbonyl-water interaction) since water-saturated octanol might be expected to approximate the environment of the folded protein where the functional groups remain partially solvent-exposed. For *N*-methylacetamide and methylacetate, the $\Delta \Delta G^{\circ}$ (octanol \rightarrow water partitioning) is -1.7 kcal/mol (Leo et al., 1971). This value can be used as an upper limit to the difference in solvation energies of the ester and amide carbonyl groups, since both the carbonyl group and the amide NH or ester oxygen contribute to the overall value of $\Delta \Delta G^{\circ}$ -(octanol \rightarrow water partitioning). However, on the basis of the comparison of the vapor-water equilibria values for acetamide, *N*-methylacetamide, and *N,N*-dimethylacetamide with those for esters, aldehydes, and ketones, it has been suggested that solvation of the carbonyl group dominates the observed difference in the equilibria (Hine, 1975; Wolfenden, 1978). Consequently,

$$\begin{aligned} \Delta G^{\circ}(F)(\text{"backbone hydrogen bond"}) - \\ \Delta \Delta G^{\circ}(F \rightarrow U)(\text{electrostatics}) = \\ \Delta \Delta G^{\circ}(\text{octanol} \rightarrow \text{water partitioning}) - \Delta \Delta G^{\circ}_{obs} \geq \\ -1.7 \text{ kcal/mol} - 0.89 \text{ kcal/mol} = -2.59 \text{ kcal/mol} \end{aligned}$$

This analysis suggests that the difference in the strengths of the backbone amide and ester hydrogen bonds is less than or equal to 2.6 kcal/mol (in favor of the amide). However, other electrostatic interactions also contribute to this difference. For example, there are notable differences in the distribution of charge within amide and ester groups that will interact differently with the helix dipole. Another possibility is a change in protein structure, although the catalytic activities of the WT and mutant proteins are similar.

Impact of the Ester Substitution at Lys⁴³-Leu⁴⁴ and Ala⁴⁹-Ile⁵⁰. The energetic consequences of the Ala⁴⁹-Ile⁵⁰ mutation should be associated largely with the substitution of the backbone NH group with the ester oxygen group since the carbonyl group of Ile⁵⁰ is solvent-exposed in both the native and unfolded states. The substitution of Ile⁵⁰ to isoleucic acid results in a 0.7 kcal/mol decrease in the stability of the mutant protein relative to that of the native protein. This decrease in stability again reflects the relative strengths of the intrahelix hydrogen bond, differences in solvation of the amide NH and ester O groups in the unfolded state, and the additional electrostatic effects described above. Moreover, this value will be affected by any potential repulsive interactions present between the ester oxygen and the carbonyl of oxygen Leu⁴⁶ in the native protein. Given the similarity of the values of $\Delta \Delta G^{\circ}$ for the ester substitutions at the N- and C-terminal sites, the latter effect appears to be relatively small. The local helical conformation could be altered to accommodate such repulsive interactions and at the same time allow hydrogen bonding of the ester groups to solvent. However, the side chain of Ile⁵⁰ is anchored by several contacts to the body of the protein, and the ester oxygen is not as accessible to solvent as it is at the Lys⁴³-Leu⁴⁴ linkage.

The ester substitution at the middle of the helix resulting from the Leu⁴⁴ to leucic acid mutation leads to a 1.73 kcal/mol decrease in protein stability. At this site $\Delta \Delta G^{\circ}$ reflects differences in the hydrogen bond strengths and electrostatic, entropic, and van der Waals interactions associated with both the amide and ester carbonyl groups (N-terminal effects) as well as the amide NH and ester oxygen groups (C-terminal effects). The energetic cost of the internal mutation is close to the sum of the effects of the N-terminal and C-terminal mutations. In this case, the difference in solvation can be approximated directly from the water-octanol partition coefficients as -1.7 kcal/mol (Leo, 1971). This places a value of 3.43 kcal/mol on the intrinsic difference in interactions of the ester and the amide group within the helix (again in favor of the amide).

Comparison to Other Hydrogen Bonding Interactions. The contribution of side chain hydrogen bonds to protein stability typically has been examined by deleting one member of the hydrogen bond pair and, more recently, by modulating the strength of the hydrogen bond (Thorson et al., 1995b). For example, hydrogen bonds between a glutamate carboxylate and a tyrosine hydroxyl group have been evaluated by measuring the change in protein stability when various fluorotyrosine analogues with altered pK_a s are substituted for tyrosine. A clear correlation was demonstrated between hydrogen bond strength and protein stability. When a tyrosine hydroxyl group that is hydrogen-bonded to an uncharged residue is substituted with hydrogen or a weakly hydrogen bonding isostere, the decreases in protein stability have been on the order of 0.5–1.8 kcal/mol (Dill, 1990;

Jackson, 1994; Serrano et al., 1992; Thorson et al., 1995a). The observed $\Delta\Delta G^\circ$ of each hydrogen bond altered or removed from the amide to ester mutations at all three positions in the T4L α -helix is about 0.8 kcal/mol. This difference in stability is on the low end of that observed for side chain mutations and may reflect the fact that the ester group is almost isosteric with respect to the amide group and retains some of the electrostatic character of the amide group. In contrast, side chain mutations typically result in more significant changes in solvation and van der Waals interactions in addition to the loss of a hydrogen bond. Alternatively, the energetic difference between these sets of mutations may indicate that hydrogen bonding interactions are less stabilizing in the backbone of this solvent-exposed α -helix than in isolated, buried side chain hydrogen bonds.

One can also compare the energetic consequences of an amide to ester mutation of backbone-backbone hydrogen bonds in helices with similar structural mutations in other complexes or secondary structures. In an examination of the molecular interactions between thermolysin and a phosphonate inhibitor, Bartlett and co-workers synthesized a series of structurally related phosphonates and determined their relative binding energies (Morgan et al., 1991). In particular, the inhibition constants (K_i s) were compared for two inhibitors which differed only in the substitution of a backbone amide linkage by an ester linkage. In the enzyme/inhibitor complex, the amide carbonyl group of the inhibitor hydrogen bonds to the guanidinium group of Arg²⁰³, while the amide NH group appears to hydrogen bond to the side-chain amide carbonyl of Asn¹¹² (Figure 3a). Replacing the amide bond of the inhibitor with an ester bond increased the K_i by a factor of 92, or 2.7 ± 0.1 kcal/mol. This example is analogous to the amide to ester mutation of the Lys⁴³-Leu⁴⁴ linkage in the middle of the helix in T4L (Figure 1b). Notably, the amide to ester mutation in the α -helix only decreases the stability of the protein by 1.7 ± 0.1 kcal/mol. The difference in the observed $\Delta\Delta G^\circ$ s of these two mutations likely reflects the fact that the inhibitor forms a hydrogen bond to a charged group which would be expected to show a greater sensitivity to the nature of the acceptor (Hine, 1975). However, it should also be noted that the amide and ester groups of these thermolysin inhibitors are far less solvent-exposed than those of the Lys⁴³-Leu⁴⁴ linkage which lie on the solvent-exposed face of the helix.

Chemical methods have been used to introduce esters into the backbone of small proteins as well. For example, Groeger *et al.* (1994) were able to synthesize ester-containing mutants of the bovine pancreatic trypsin inhibitor (BPTI) using an enzymatic-chemical semisynthesis. The mutated amide linkage forms an intermolecular hydrogen bond between the amide NH of Gly¹⁷ of a BPTI mutant and the amide carbonyl of Phe⁴¹ chymotrypsin (Figure 3e). In this case, the carbonyl of the mutated amide linkage does not participate in hydrogen bonding. Only the NH of the amide participates in an intermolecular hydrogen bond which is removed by ester substitution. This situation is similar to the ester substitutions made at the C-terminal Ala⁴⁹-Ile⁵⁰ amide linkage of T4L which destabilized the protein by 0.7 kcal/mol. However, the analogous ester substitution to the intermolecular backbone-backbone interaction between BPTI and chymotrypsin destabilizes the complex by 1.9 kcal/mol, more than twice as much as at the C terminus of the α -helix in T4L. There are a number of environmental

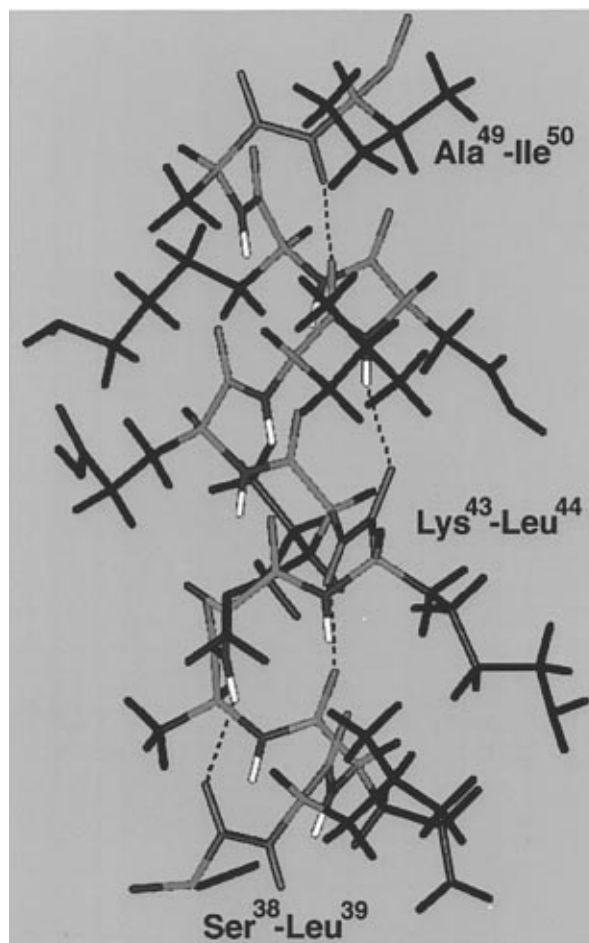


FIGURE 2: Structure of the 39–50 α -helix of T4 lysozyme Ser⁴⁴ \rightarrow Leu based on the reported crystal structure (Matthews, 1995). Hydroxy acids were substituted by Ile⁵⁰, Leu⁴⁴, and Leu³⁹. The three amide linkages modified by ester substitution (Ser³⁸-Leu³⁹, Lys⁴³-Leu⁴⁴, or Ala⁴⁹-Ile⁵⁰) are highlighted (pink). Hydrogen bonds affected by substitution are shown (---).

differences between these two linkages that may account for the different energetic consequences of ester substitution. Most notable is the observation that the Ala¹⁶-Gly¹⁷ linkage of BPTI is virtually inaccessible to solvent in the protein-protein interface of the complex and the carbonyl oxygen of this linkage does not appear to participate in hydrogen bonding.

Recently, the synthesis of the proteinase inhibitor turkey ovomucoid third domain (OMTK3) containing backbone ester mutations has also been achieved using native chemical ligation (Lu et al., 1997). In the complex of OVMK3 with α -chymotrypsin (CHYM), a backbone amide linkage has been substituted with an ester linkage. The amide NH group of OVMK3 in the native structure donates a hydrogen bond to the amide carbonyl of Ser²¹⁴ of CHYM and the side chain hydroxyl of Ser¹¹⁹. The amide carbonyl group of OVMK3 is intramolecularly hydrogen bonded to the neutral side chain of Asn³³ (Figure 3b). Substitution of this backbone amide group with an ester causes a 1.6 kcal/mol drop in the stability of the complex of OVMK3 with CHYM and a drop of 0.8–2.0 kcal/mol in stability with a variety of other proteinases. Of the six proteinase complexes examined, five show values of $\Delta\Delta G$ that are substantially higher than those found for the analogous C-terminal helical ester substitutions in T4L. However, the precise effect of this mutation in the OVMK3/CHYM complex is difficult to ascertain because the serine

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BI9707685