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# Direct observation of better hydration at the N terminus of an α-helix with glycine rather than alanine as the N-cap residue

(protein stability/protein folding)

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ABSTRACT **The structural basis for the stability of N termini of helices has been analyzed by thermodynamic and crystallographic studies of three suitably engineered mutants of the barley chymotrypsin inhibitor 2 with Ser, Gly, or Ala at the N-cap position (residue 31). Each mutant has a well-organized shell of hydration of the terminal NH groups of the helix. The three structures are virtually superimposable (rms separations for all atoms, including the common water molecules, are 0.15–0.17 Å) and show neither changes in conformation at the site of substitution nor changes in the crystal packing. The only changes on going from Ser-31 to Ala-31 to Gly-31 are in the position of a water molecule (Wat-116). This is bound to the Ser-$O_\gamma$ atom in the Ser-31 structure but is in a weak hydrogen bonding position with the NH of residue 34 (O· · ·N = 3.28 Å) in the Ala-31 mutant, partly replacing the strong Ser-31-$O_\gamma$· · ·N34 hydrogen bond (O· · ·N = 2.65 Å). The corresponding water molecule completely replaces the Ser hydroxyl hydrogen bond to N34 on mutation to Gly (2.74 Å). The only other change between the three structures is an additional water molecule in the Ala-31 structure (Wat-150) that partly compensates for the weak Wat-116· · ·N34 hydrogen bond. Perturbation of solvation by the side chain of Ala is consistent with earlier hypotheses on the importance of exposure of the termini of helices to the aqueous solvent.**

Factors contributing to the stability of the ubiquitous α-helix are being clarified from statistical survey combined with experimental data on engineered proteins and synthetic peptides (reviewed in ref. 1). The first residue at the N terminus of the helix whose α-carbon lies on the cylinder containing the helix backbone is defined as the N-cap residue (2). It is most commonly Ser, Gly, Asp, or Thr, in order of decreasing occurrence (2, 3). Ser, Asp, and Thr are particularly effective N-caps, because their side chains can solvate the exposed NH groups of the first turn of the helix (4). The order of stability of N-cap residues in two helices of the protein barnase is Thr ≈ Asp ≈ Ser ≥ Gly ≈ Asn ≥ Gln ≈ Glu > His ≥ Ala ≥ Val ≫ Pro (5, 6). It was suggested that the differences in stability between Gly, Ala, and Val as N-caps of the helices of barnase correlated with the steric bulk of the residue; i.e., Gly allows better hydration of the end of the helix than does Ala, which in turn allows better hydration than Val (5). Gly destabilizes proteins because it stabilizes the unfolded state by its additional degrees of rotational freedom (7). Analysis of the relative stabilizing effects of Gly versus Ala at all solvent-exposed positions in both helices of barnase showed that the variation in stability depends on two further factors (6, 8). First, Ala buries more solvent-accessible hydrophobic surface area on folding from an extended conformation than does Gly (9). The amount buried depends on the nature of the surrounding side chains. Second, the side chain of Ala can sterically prevent NH and CO groups from making good hydrogen bonds with solvent, which is especially important for the N and C termini of the helices (8). The data fit an empirical equation that relates $\Delta\Delta G_{Ala\rightarrow Gly}$ (the change in stability on mutating Ala to Gly) to both $\Delta A_{HP}$ (the solvent-accessible hydrophobic surface area of the helix containing Ala minus the equivalent for that containing Gly) and $\Delta A_{HB}$ (solvent-accessible surface area of NH and CO groups in the Ala-containing helix that require solvation minus the equivalent area of Gly-containing helix):

$$\Delta\Delta G_{Ala\rightarrow Gly} = 1.82\ (\pm 0.25) - (0.046 \pm 0.013)\Delta A_{HP} - (0.19 \pm 0.03)\Delta A_{HB}\ (\text{kcal·mol}^{-1}), \quad [1]$$

(modified from and recalculated as described in refs. 6 and 8, using latest unpublished data from this laboratory on barnase; 1 kcal = 4.18 kJ). The term in $\Delta A_{HP}$ is probably reliable because of commonly found relationships between free energies of transfer of hydrophobic surface groups and surface (9), but the term in $\Delta A_{HB}$ lacks precedence and requires further research before its importance can be generalized.

The stabilities and crystal structures of six mutants at the N-cap residue (Thr-59) of the long helix of T4 phage lysozyme have been determined. The relative stabilities are Thr ≈ Ser ≈ Asp > Asn > Gly ≈ Ala ≈ Val (10). In none of these structures, however, was a new water molecule seen to replace the hydrogen-bonding function of the γ-hydroxyl in the wild-type Thr variant. Bell *et al.* (10) disputed the hypothesis of Serrano *et al.* (6, 8) that Gly and Ala are better helix-capping residues than Val because a smaller residue allows better hydration at the end of the helix. However, Serrano *et al.* (6) noted that the side chain of residue Asp-61 in T4 lysozyme moves to become a surrogate N-cap on mutation of Thr-51 to Ala, Gly, and Val, so lessening the need for external solvation.

In the present study, we report the energetics and crystal structures of a series of mutants at the N-cap, position 31, of the barley chymotrypsin inhibitor 2 (CI2); Ser-31, Ser-31 → Ala, and Ser-31 → Gly. As this region of the protein is disordered in the crystals of wild-type protein (11, 12), we have constructed a pseudo-wild-type (pseudo-wt) protein (Glu-33 → Ala and Glu-34 → Ala) which is suitable for crystallographic study and have used this as the reference protein. These mutations also remove the possibility of the side chain of Glu-33 becoming a surrogate N-cap. The protein was also truncated by the deletion of the N-terminal tail of 20 residues that is disordered in both the crystal and solution structures (11–13) to give a 64-residue fragment that is very suitable for x-ray studies.

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Abbreviations: CI2, chymotrypsin inhibitor 2; pseudo-wt, pseudo-wild-type.

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## EXPERIMENTAL PROCEDURES

**Materials.** A single copy of the truncated CI2 gene (expressing residues Met-20–Gly-83) was subcloned in pTZ18U to create the high-level expression vector pCI2, and mutagenesis, expression and purification were performed as described (14). Residues Glu-33 and Glu-34 were first mutated to Ala to give the pseudo-wt. All protein samples were homogeneous as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis and isoelectric focusing.

**Equilibrium Denaturation Measurements.** Changes in the free energy of unfolding of the three proteins were determined at 25°C in 50 mM Mes·HCl buffer at pH 6.3 by equilibrium denaturation using guanidinium chloride and fluorescence spectroscopy (14, 15).

**Crystallographic Methods.** Crystals were grown by using the hanging-drop method under conditions similar to those used for wild-type CI2. This yielded crystals isomorphous with wild type in space group *P*622. In this space group, the structure of the free inhibitor is disordered because most of the hydrogen bonding network supporting the loop in the complex with subtilisin is lost. Disorder is due to forced packing contacts, which are not resolved by refinement with two molecules per asymmetric unit in a lower-order space group. The Glu-33 and Glu-34 side chains form particularly bad density/contacts. After finding bad density for some 23 side chains (out of 65), we decided to use the double mutant Glu-33 → Ala and Glu-34 → Ala as a pseudo-wt CI2. The pseudo-wt protein crystallizes readily in the orthorhombic space group $P2_12_12_1$.

The structure of the pseudo-wt CI2 was solved by rotation and translation functions using the wild type as a starting model and was refined with the Hendrickson–Konnert restrained least-squares program PROLSQ (16). Water molecules were added in chemically reasonable positions and kept after cycles of refinement when their thermal values were less than three times the mean *B* value for the protein atoms. The crystal structures of the Ser-31 → Ala (S31A) and Ser-31 → Gly (S31G) mutants were refined from the refined pseudo-wt-CI2 atom coordinates; see Table 1 for all relevant details.†

†The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (references: pseudo-wt CI2, 1YPC; pseudo-wt-CI2 S31A, 1YPA; pseudo-wt-CI2 S31G, 1YPB).

## RESULTS

**Stability of Proteins.** The free energy of unfolding of the pseudo-wt (E33A,E34A) at 25°C, pH 6.3, in 50 mM Mes·HCl is 7.6 kcal·mol$^{-1}$. On mutation of Ser-31 to Gly (S31G, E33A,E34A), the free energy increases by 0.84 ± 0.13 kcal·mol$^{-1}$ (±SE) and on mutation of Ser-31 to Ala (S31A,E33A,E34A) to 0.88 ± 0.13 kcal·mol$^{-1}$.

**Surface Area Calculations.** The mutant S31A,E33A,E34A has a solvent-accessible surface area for the NH groups of residue 31, Val-32, Ala-33, and Ala-34 of 6.8 Å$^2$ less than that for S31G,E33A,E34A, whereas the solvent-accessible hydrophobic surface area is greater by 18.9 Å$^2$, calculated as described (6). Substituting these values into Eq. 1 indicates that the mutation Gly-31 → Ala in pseudo-wt should destabilize the protein by 0.3 ± 0.4 kcal·mol$^{-1}$.

**Description of the α-Helix of CI2.** CI2 has an irregular three-turn helix, residues 31–43, in which the backbone does not form all of the possible $i + 4$ hydrogen bonds (i.e., from the CO of residue $i$ to the NH of residue $i + 4$). The expected hydrogen bonds NH37···O33, NH38···O34, NH41···O37, and NH42···O38 are absent. The carbonyl oxygen atoms of residues 33 and 34 make strong hydrogen bonds with water molecules, Wat-126 and Wat-138, respectively (Fig. 1). The N-cap side-chain OH of Ser-31 makes a hydrogen bond with the main-chain NH group of residue 34 ($i$, $i + 3$) and with water Wat-116. Ser-31 has main-chain torsion angles in the β-conformation ($\phi = -70°$ and $\psi = 165°$; see Table 2) that are typical of N-cap residues; see, for example, in barnase, where the N-cap residue Thr-6 of barnase helix 1 has $\phi = -98°$, $\psi = 156°$, and for helix 2 Thr-26 has $\phi = -76°$, $\psi = 170°$. The $\chi_1$ torsion angle for the Ser-31 side chain is +62°.

In the pseudo-wt structure, the helix is further distorted at Leu-40 with a $\psi$ value of −14°. The N terminus of the CI2 helix is bound by Wat-118 and Wat-122, to residues in the turn 72–76. This end of the helix is rich in water molecules that form a strongly hydrogen-bonded network both to the other molecule in the asymmetric unit and to symmetry-related molecules. In particular, at the N terminus of the CI2 helix the exposed hydrogen bond donor groups from the backbone NHs extend the pattern by hydrogen bonds NH31···Wat-119, NH32···Wat-118, and NH33···Wat-117 (Fig. 1).

Atoms within both residues of the turn, 72–76, and of the helix are also connected by hydrogen bonds with residues in the reactive site loop of a symmetry-related molecule at −1

Table 1. Data collection and refinement statistics

| Protein* | Pseudo-wt CI2 (E33A,E34A) | Pseudo-wt-CI2 S31A (E33A,E34A) | Pseudo-wt-CI2 S31G (E33A,E34A) |
|---|---|---|---|
| Wavelength, Å | 0.92 | 1.54 | 1.54 |
| Cell dimensions, Å | | | |
| *a*, *b*, *c* | 29.1, 33.7, 56.10 | 29.13, 33.62, 56.40 | 29.16, 33.65, 55.99 |
| Resolution, Å | 21.5–1.74 | 14.5–2.0 | 14.5–2.0 |
| Total reflections collected | 16,840 | 14,613 | 14,561 |
| Unique reflections | 5,758 | 3,752 | 3,689 |
| Completeness of data (highest-resolution bin in parentheses), % | 96.3 (95.8) | 94.9 (98.5) | 93.0 (96.4) |
| $R_{merge}$ on intensities (highest-resolution bin in parentheses), % | 2.9 (7.7) | 7.8 (8.2) | 7.7 (12.9) |
| Average multiplicity (highest-resolution bin in parentheses) | 2.8 (1.9) | 3.9 (3.7) | 3.8 (3.8) |
| Overall ⟨*F*/SD⟩ (highest-resolution bin in parentheses) | 38.4 (18.2) | 55.1 (44.6) | 50.2 (41.2) |
| rms deviation from ideal values | | | |
| Bond length, Å | 0.012 | 0.009 | 0.009 |
| Bond angle, ° | 0.032 | 0.033 | 0.033 |
| Crystallographic residual, % | 17.3 | 17.4 | 17.8 |
| Mean *B* all atoms, Å$^2$ | 10.8 | 10.3 | 11.6 |

*Crystals grown from 16–45% saturated $(NH_4)_2SO_4$ in 50 mM Tris·HCl, pH 8.0, space group $P2_12_12_1$.

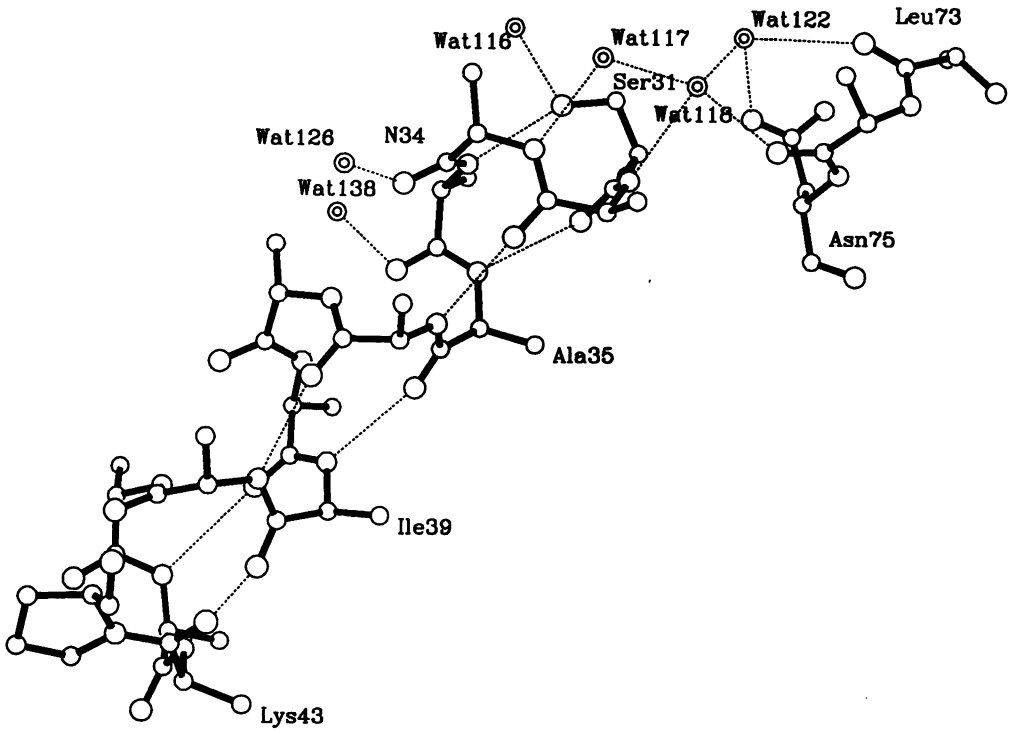


FIG. 1. View of the $\alpha$-helix, showing part of the local water structure surrounding the helix and the connection via water molecule Wat-118 to the turn composed of residues 72–76. Only the side chains of residues Ser-31, Pro-44, and Asn-75 are shown; for the remaining residues only $C_\beta$ is shown. Hydrogen bonds are drawn as broken lines.

$-x, -\frac{1}{2}+y, \frac{1}{2}-z$, in particular Gln-47, Glu-60, and Arg-62. Here, the side chain of Asp-74 makes an intramolecular salt bridge with Arg-62, which appears to compensate for the observed strained main-chain torsion angles for Asp-74 (with $\phi = 70°$ and $\psi = 27°$). In the free wild-type CI2 structure, almost all of the hydrogen bonding network supporting the loop is lost; the two Arg residues (Arg-62 and Arg-65) providing the hydrogen bonds that appear to fix the loop region in the structure of the complex between subtilisin and CI2 are much less ordered than observed in both the subtilisin complex and the pseudo-wt-CI2 structures.

In the crystal structure of the pseudo-wt CI2 molecule, the helix participates in additional interactions with parts of other, symmetry-related, molecules. The helix packs against two symmetry-related molecules. Its N terminus is connected by hydrogen bonds to water with C-terminal residues of the helix in a symmetry-related molecule at $-1-x, \frac{1}{2}+y, \frac{1}{2}-z$. In addition, the C terminus of the helix within the asymmetric unit packs against the N terminus of the helix at $-1-x, -\frac{1}{2}+y, \frac{1}{2}-z$. These interactions give for the pseudo-wt-CI2 structure a well-defined set of connections between Ser-31-$O_\gamma$···Wat-116···O40-(symmetry) and O35···Wat-138···Wat-139-(symmetry)···O44-(symmetry).

**Comparison of Pseudo-wt CI2 with Mutant Structures.** There is no significant movement of any of the protein atoms upon substitution of the Ser for either Ala or Gly at position 31 (Fig. 2). The rms discrepancy between all common atoms in the three structures is essentially just experimental error, with values for pseudo-wt CI2 to pseudo-wt-CI2 S31A of 0.17 Å, and for pseudo-wt CI2 to pseudo-wt-CI2 S31G of 0.15 Å, and for pseudo-wt-CI2 S31A to pseudo-wt-CI2 S31G of 0.16 Å.

Table 2. Geometry of helix formed by residues 31–43 (pseudo-wt CI2)

| Residue | $\phi$, ° | $\psi$, ° | N—H···O H bond | Distance, Å | Energy,* kcal/mol | Angle,† ° | N—H···$OH_2$ H bond | Distance, Å | Angle,† ° |
|---|---|---|---|---|---|---|---|---|---|
| Ser-31 | −75 | 165 | | | | | N31···Wat-119 | 2.99 | 161 |
| Val-32 | −62 | −44 | | | | | N32···Wat-118 | 3.01 | 165 |
| Ala-33 | −58 | −46 | | | | | N33···Wat-117 | 2.90 | 149 |
| Ala-34 | −67 | −43 | | | | | N34···$O_\gamma$31 | 3.08 | 162 |
| Ala-35 | −61 | −46 | N35···O31 | 2.99 | −2.4 | 163 | | | |
| Lys-36 | −59 | −40 | N36···O32 | 2.99 | −2.6 | 168 | | | |
| Lys-37 | −58 | −41 | N37···O33‡ | 3.44 | −1.4 | | | | |
| Val-38 | −75 | −46 | N38···O34§ | 3.48 | −1.2 | | | | |
| Ile-39 | −59 | −44 | N39···O35 | 2.97 | −2.5 | 169 | | | |
| Leu-40 | −69 | −14 | N40···O36 | 2.89 | −2.8 | 165 | | | |
| Gln-41 | −80 | −40 | | | | | | | |
| Asn-42 | −78 | −38 | N42···O38 | 3.37 | −1.5 | | | | |
| Lys-43 | −138 | 80 | N43···O39 | 2.73 | −3.0 | 153 | | | |
| Pro-44 | −54 | −30 | | | | | | | |

*Calculated from the program DSSP (17).
†The angles for amide to oxygen are N—H···O and for oxygen to oxygen are HO—H···O=C.
‡O33···Wat-126 is 2.70 Å, 125°.
§O34···Wat-138 is 3.03 Å, 112°.

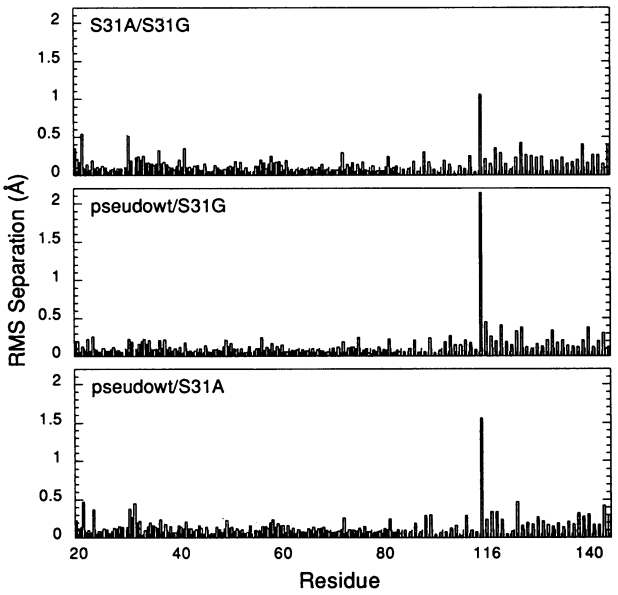


FIG. 2. Separation between equivalent atoms in the different mutant structures of pseudo-wt CI2, after superposition, as a function of residue number. Solid bars, rms separation between main-chain atoms; open bars, rms separation between side-chain atoms, followed by that between the common water molecules. (*Top*) Between pseudo-wt-CI2 S31A and pseudo-wt-CI2 S31G. (*Middle*) Between pseudo-wt CI2 and pseudo-wt-CI2 S31G. (*Bottom*) Between pseudo-wt CI2 and pseudo-wt-CI2 S31A.

In each of the three structures, the solvent structure is well defined and the temperature factors of the water molecules compare well with the average temperature factor of all atoms used in the refinement (see Table 3). There are 45 pairs of water molecules that occupy equivalent positions in each of the two structures pseudo-wt CI2 and pseudo-wt-CI2 S31A and 41 pairs between pseudo-wt CI2 and pseudo-wt-CI2 S31G.

Table 3 lists the hydrogen bond partners and geometry of all the waters associated with the helix in pseudo-wt CI2, including the symmetry-related contacts. It is clear that within the series Ser → Ala → Gly the water structure is constant with the exception of Wat-116 and that the mutation has no other effect upon either the geometry of the protein or the crystal packing. Further, although in the crystal structure of pseudo-wt CI2 the helix residues 31–43 form strong intramolecular contacts, these interface hydrogen bonds are not a complicating factor in considering the effects of the mutations studied here.

The water oxygen labeled Wat-116 in each of the three structures is the only atom seen to be in a different position in the series Ser → Ala → Gly. In the structure of the pseudo-wt-CI2 protein, the N-cap Ser hydroxyl group makes a hydrogen bond to the amide nitrogen of the $i + 3$ residue (Ala-34) of the helix. Replacing the Ser by Ala allows one water to approach the amide nitrogen of residue 34. Only a weak hydrogen bond is possible, however, with N···O of 3.28 Å, with N—H···O (water) angle of 123°, compared with an N···$O_\gamma$ distance of 3.08 Å and N—H···$O_\gamma$ angle of 162° for pseudo-wt CI2. The methyl group of the Ala in pseudo-wt-CI2 S31A prevents further approach of a water molecule towards the nitrogen of residue 34, with the Ala-31-$C_\beta$ to Wat-116 distance of 3.42 Å. The removal of the hydroxyl group from residue 31 and the movement of Wat-116 towards the N-cap hydrogen bonding position is accompanied by the insertion of a second water molecule, Wat-150, in the pseudo-wt-CI2 S31A structure. This water makes hydrogen bonds between Wat-116 and Wat-119 (see Fig. 3).

In the pseudo-wt-CI2 S31G structure, a water molecule, Wat-116, takes up the N-cap hydrogen bond position with N34 with N···O (water) of 2.74 Å and N—H···O (water) of 143°. This water is now too far removed from Wat-119 to allow for the bridging water molecule (Wat-150) observed in

Table 3. Water hydrogen bonding

| | Pseudo-wt-CI2 | | Pseudo-wt-CI2 S31G | | Pseudo-wt-CI2 S31A | |
|---|---|---|---|---|---|---|
| H bond partners | Distance, Å | Angle,* ° | Distance, Å | Angle,* ° | Distance, Å | Angle,* ° |
| Wat-115···Glu-60-$O_{\varepsilon 2}$ | 2.55 | 158 | 2.55 | 152 | 2.60 | 146 |
| Wat-115···Arg-62-$N_{\eta 1}$ | 2.88 | 146 | 2.93 | 148 | 2.98 | 150 |
| Wat-115···Wat-117† | 2.81 | | 2.94 | | 3.17 | |
| Wat-116···Leu-40-O‡ | 2.76 | | 3.06 | | | |
| Wat-116···Wat-150 | | | 2.82 | | | |
| Wat-116···Ser-31-$O_\gamma$ | 2.65 | 116 | | | | |
| Wat-116···Ala-34-N | | | 3.28 | 123 | 2.74 | 143 |
| Wat-117···Ala-33-N | 2.90 | 149 | 2.83 | 155 | 2.83 | 163 |
| Wat-117···Wat-118 | 2.65 | | 2.85 | | 2.77 | |
| Wat-117···Gln-41-$O_{\varepsilon 1}$‡ | 2.68 | | 2.71 | | 2.84 | |
| Wat-117···Gln-41-$N_{\varepsilon 2}$‡ | · | | | | 2.88 | |
| Wat-118···Val-32-N | 3.01 | 165 | 3.01 | 154 | 2.93 | 156 |
| Wat-118···Wat-122 | 3.28 | | 3.34 | | | |
| Wat-118···Asp-74-O | 2.78 | 142 | 2.74 | 137 | 2.82 | 145 |
| Wat-119···Xaa-31-N | 2.99 | 161 | 2.88 | 162 | 3.08 | 166 |
| Wat-119···Wat-150 | | | 3.02 | | | |
| Wat-122···Asn-75-$O_{\delta 1}$ | 2.80 | 125 | 2.69 | 115 | | |
| Wat-122···Leu-73-O | 3.12 | 153 | 2.81 | 154 | | |
| Wat-138···Ala-34-O | 3.03 | 112 | 3.07 | 111 | 3.03 | 112 |
| Wat-138···Wat-139‡ | 3.06 | | 2.92 | | 3.21 | |
| Wat-139···Wat-149† | 2.87 | | 3.13 | | 2.92 | |
| Wat-139···Pro-44-O | 3.17 | 113 | 3.38 | 103 | 3.15 | 115 |
| Wat-138···Wat-136† | 3.36 | | 3.31 | | 3.15 | |

*The angles for amide to oxygen are N—H···O and for oxygen to oxygen are HO—H···O=C.
†Symmetry $-x, -\frac{1}{2} + y, \frac{1}{2} - z$.
‡Symmetry $-1 - x, \frac{1}{2} + y, \frac{1}{2} - z$.

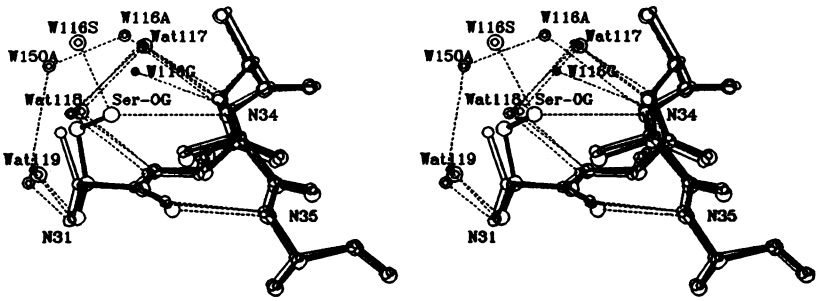


FIG. 3. Stereo diagram showing the N-terminal region of the helix. Residues 31–35 are presented, together with water molecules Wat-116, Wat-117, Wat-118, Wat-119, and, for pseudo-wt-CI2 S31A, Wat-150. W116A indicates Wat-116 bound to Ala-31, etc. Only the side chain for residue 31 in pseudo-wt CI2 and pseudo-wt-CI2 S31A is drawn. Atoms for pseudo-wt CI2 (Ser-31) are drawn with large circles connected by solid bonds. Atoms for pseudo-wt-CI2 S31A are drawn with medium-size circles with open bonds. Atoms for pseudo-wt-CI2 S31G are drawn with small circles with dashed bonds. Hydrogen bonds are drawn as broken lines.

the pseudo-wt-CI2 S31A structure and the change in position of Wat-116 is the only difference between the structures of pseudo-wt CI2 and pseudo-wt-CI2 S31G. The Wat-116 molecule in the S31G structure cannot take up a position exactly comparable to that occupied by $O_\gamma$ in the Ser-31 structure but occupies a site that is 3.80 Å from the $C_\alpha$ of Gly-31 while maintaining a strong hydrogen bond with the $i + 3$ amide nitrogen.

## DISCUSSION

The pseudo-wt CI2 is a highly suitable protein for extended theoretical and experimental studies on protein folding, consisting of just 64 amino acid residues. Its crystal structure, refined to 1.75-Å resolution in this study, does not suffer from the serious problems observed in the wild-type structure (11, 12). A well-resolved shell of water molecules is seen to solvate the NH groups at the N terminus of the helix in two mutants that have had the N-cap residue Ser-31 changed to an Ala or a Gly. The side chain of Ala-31 sterically inhibits a water molecule, Wat-116, from taking up its optimal position for hydrogen bonding that is observed in the mutant with Gly-31 at the N-cap. These data support the hypothesis of Serrano *et al.* (5, 6), following the arguments of Presta and Rose (4), that it is important for the NH groups in the first turn of an $\alpha$-helix to be optimally exposed to solvent when there is not a side chain that can form intramolecular hydrogen bonds with those NH groups. The differences between the structural results observed here and those presented by Bell *et al.* (10) for T4 lysozyme can be readily accommodated. The side chain of Asp-61 becomes located over the end of the long helix of lysozyme on mutation of the N-cap Thr-59 to Ala. The example of lysozyme is also complicated by the high degree of side-chain mobility of the residues at the N terminus of the relevant helix, with average temperature factors for the side-chain atoms greater than 50 Å$^2$. All other things being equal, the observations here and the correlations in ref. 5 and 6 imply that the greater the degree of exposure of polar groups to solvent, the greater the stability of the protein until there is unrestricted solvation.

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