Contribution to Global Protein Stabilization of the N-Capping Box in Human Growth Hormone†

Eugene A. Zhukovsky,*,‡ Michael G. Mulkerrin,§ and Leonard G. Presta‡

Departments of Protein Engineering and Medicinal and Analytical Chemistry, Genentech, Inc., South San Francisco, California 94080

Received April 26, 1994; Revised Manuscript Received June 8, 1994®

ABSTRACT: In this work we have investigated the contribution to protein stability of residues forming the boundaries of α-helices. At the N-terminus of helix 2 of human growth hormone there are two residues, Ser71 and Glu74, which form two reciprocal hydrogen bonds between the side chains and the backbone nitrogens of either residue (the N-capping box). In order to evaluate the stabilizing effect of each hydrogen bond, site-directed mutagenesis was employed. In addition, the effect of side-chain negative charge on helix stabilization, via charge dipole interaction, was assessed. Ultraviolet spectroscopy and near- and far-UV CD spectroscopy as well as guanidine hydrochloride protein denaturation were used as assays to monitor the conformational and free energy of stabilization changes induced by the point mutations. The results of these experiments can be summarized as follows: (a) receptor binding studies showed that the tertiary conformation of each mutant was similar to that of the native hormone, (b) far-UV CD spectroscopic analyses showed that the overall α-helical content was unchanged in the mutants, (c) UV absorption and CD spectroscopic analyses indicated small alterations in helical packing in those mutants in which the hydrogen bond between the side chain of Ser71 and backbone NH of Glu74 was disrupted, (d) the hydrogen bond involving the side chain of Ser71 contributes at least 1.0 kcal/mol to protein stabilization and has a 2-fold larger stabilizing effect than that of the hydrogen bond involving the Glu74 side chain, and (e) the putative charge–dipole interaction of Glu74 with the α-helix dipole does not contribute to the stabilization of the tertiary conformation of human growth hormone.

In globular proteins, about 30% of all residues are part of α-helices (Creighton, 1983), and in some proteins α-helices may be the first secondary structural elements to form during folding (Roder et al., 1988). Two hypotheses have been proposed which suggest how the primary amino acid sequence could function in formation and stabilization of α-helices. According to the first hypothesis, termed helix capping (Presta & Rose, 1988), the first four amide groups at the N-terminus and the last four carbonyl groups at the C-terminus of a helix differ from the internal helical residues in that they do not form intrahelical backbone hydrogen bonds. It was proposed that side chains of polar or charged amino acids that flank the helix termini can form hydrogen bonds to these amide and carbonyl groups, providing start/stop signals and stabilizing the α-helix. An analysis of amino acid positional preference in α-helices also noted that polar and charged residues were more prevalent at helix termini whereas the middle of an α-helix contained predominantly hydrophobic amino acids (Richardson & Richardson, 1988). More recently, a specific hydrogen-bonding pattern, termed the N-capping box, was found to be particularly utilized at helix N-termini (Harper & Rose, 1993). This motif consists of a reciprocal main-chain–side-chain hydrogen-bonding network between the first residue, whose α-carbon is located on the cylinder of the helix but has nonhelical backbone conformation (cap residue), and the residue which is located three positions downstream (N + 3 residue). Recent studies of the proteins barnase (Serrano & Fersht, 1989; Serrano et al., 1992), T4 lysozyme (Bell et al., 1992), and cytochrome b_5 (Lecomte & Moore, 1991) and peptide systems (Lyu et al. 1992, 1993; Forood et al., 1993; Heinz et al., 1993; Fairman et al., 1989; Bruch et al., 1991; Chakrabartty et al., 1993; Zhou et al., 1994) have presented evidence consistent with and contradictory to this hypothesis. The second hypothesis, termed charge–dipole interaction, suggests that charged side chains at helix termini can form a stabilizing electrostatic interaction with the helix dipole (Blagdon & Goodman, 1975; Hol et al., 1978; Nicholson et al., 1988, 1991). Note that these two hypotheses are not mutually exclusive since charged side chains can also function as hydrogen bond donors or acceptors to the helix backbone groups.

This study has evaluated the helix capping and charge–dipole hypotheses using site-directed mutagenesis of human growth hormone (hGH).[1] hGH is a single-chain protein consisting of 191 residues with two disulfide bonds. The crystal structure of hGH shows that it is an antiparallel, four α-helix bundle with a helical content of about 60% (DeVos et al., 1992). The single Trp86 residue, which is conserved in growth hormones (Nicoll et al., 1986), occupies a hydrophobic pocket formed between helices 2 and 4. The presence of this unique tryptophan offers a convenient spectroscopic probe for monitoring the conformation of this protein (Mulkerrin & Cunningham, 1993). hGH is a very stable molecule with a ΔG_{H_2O} (free energy of unfolding in aqueous solution) of ca. 14 kcal/mol (Brems et al., 1990; DeFelippis et al., 1993). Thus, hGH provides an excellent system for introducing

† E.A.Z. is a postdoctoral fellow of Genentech, Inc.
‡ Department of Protein Engineering.
§ Department of Medicinal and Analytical Chemistry.
® Abstract published in *Advance ACS Abstracts,* August 1, 1994.

[1] Abbreviations: hGH, human growth hormone; hGHbp, human growth hormone binding protein; BIAcore, Pharmacia Biosensor; UV CD, ultraviolet circular dichroism; GuHCl, guanidine hydrochloride. Residues in hGH are presented in three-letter code. Mutant proteins are designated by single-letter code for the wild type, followed by the residue number, followed by the code for the new residue; in cases of double mutants point mutations are separated with a slash.

0006-2960/94/0433-9856$04.50/0

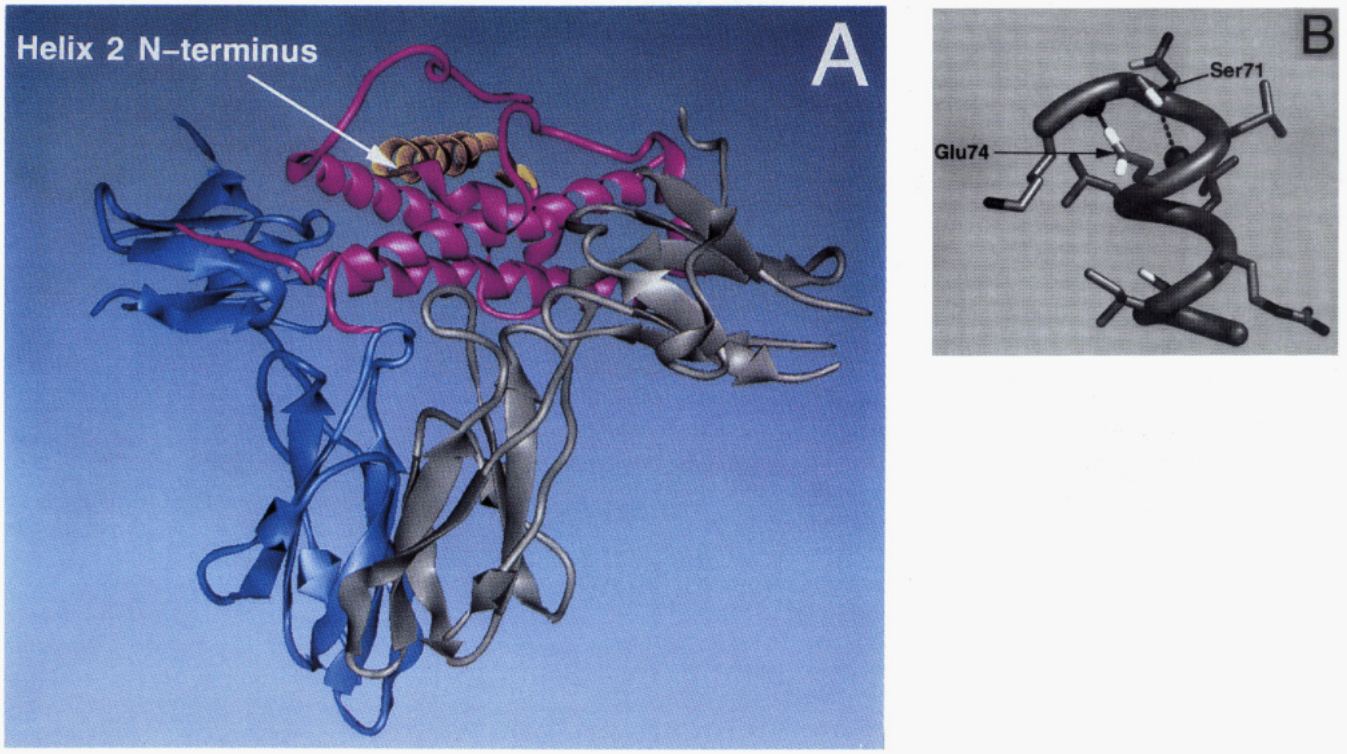

FIGURE 1: (A) Crystal structure of the complex between hGH and hGHbp (DeVos et al., 1992). The two hGHbp molecules forming the complex are in blue and gray. hGH is in magenta with helix 2 in yellow. (B) The N-capping box of helix 2 in hGH formed by the capping residue, Ser71, and the $N + 3$ residue, Glu74. The network of two reciprocal hydrogen bonds between the side chains and backbone amides of the N-capping box is shown as black dashed lines. Oxygen atoms are shown in white and nitrogen atoms are shown in black; carbon atoms of side chains and the backbone are shown in gray.

potentially destabilizing mutations while leaving the molecule stable enough for purification and characterization.

hGH has an N-capping box formed by Ser71 and Glu 74 at the N-terminus of helix 2 (Figure 1). The Glu74 NH–Oγ Ser71 hydrogen bond will be denoted as the cap hydrogen bond and the Ser71 NH–Oϵ Glu74 hydrogen bond as the $N + 3$ hydrogen bond. By replacement of one or both residues at these positions the energy of stabilization provided by the two N-capping box hydrogen bonds and the charged Glu74 side chain has been evaluated using equilibrium denaturation. The helix 2 N-capping box in hGH contributes about 1.5 kcal/mol to the protein stabilization energy. Almost all of this stabilization comes from the two hydrogen bonds and not from the charge–dipole interaction. This result is consistent with the helix capping hypothesis and suggests that capping interactions are an important component of the forces providing protein conformational stabilization (Lattman & Rose, 1993).

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 polynucleotide kinase, and T4 ligase were purchased from New England Biolabs and T4 DNA polymerase and Sequenase V.2 from U.S. Biochemical Corp. [α-^{32}P]dATP was purchased from Amersham Corp. and guanidine hydrochloride (GuHCl) from ICN. Human growth hormone binding protein (hGHbp) was kindly donated by B. Cunningham (Genentech, Inc.), and the pb0720 plasmid was obtained from H. Lowman (Genentech, Inc.).

Modeling. The crystal structure coordinates of hGH (DeVos et al., 1992) were obtained from A. DeVos (Genentech, Inc.). InsightII version 2.2 software (Biosym Technologies Inc.) was used to model amino acid substitutions. The steric environment of each substitution in the mutant hGH models was assessed visually in order to determine allowed conformations of the side chain and to locate possible interactions with the residues surrounding the N-capping box of helix 2. Panels A and B of Figure 1 were produced using the Midas program (UCSF).

Site-Directed Mutagenesis. All mutants were prepared by site-directed mutagenesis employing the method of Kunkel et al. (1987) on the pb0720 vector plasmid in which the gene for hGH is under the control of the *phoA* promoter. This vector was derived from pb0475 (Cunningham et al., 1989) by deleting the *Bam*HI restriction site at the *f1 ori*. All mutations were confirmed by the dideoxy sequencing method (Sanger et al., 1977).

Mutant Expression and Purification. Plasmid vectors containing the wild-type or mutant hGH were transformed into the protease-deficient 34B8 strain of *Escherichia coli* and grown in low-phosphate media to induce the alkaline phosphatase promoter (Chang et al., 1987). The proteins were purified from cell pellets obtained from 500-mL shake flasks or cell pastes derived from 10-L fermentation runs (Cunningham & Wells, 1989). Purification was achieved by processing the crude extract by ion-exchange chromatography on a mono Q column (Pharmacia LKB Biotechnology Inc.). The eluted active fraction was then buffer-exchanged into 10 mM Tris-HCl, pH 8.0–150 mM NaCl, concentrated to approximately 1 mg/mL, and its concentration was determined from the absorption spectra using $\epsilon_{277nm}^{0.1\%} = 0.82$ cm^{-1} for the wild type and mutants (Mulkerrin & Cunningham, 1993). The purity of the proteins was in the range of 95–99% as determined by Coomassie-stained sodium dodecyl sulfate–polyacrylamide gels. N-Terminal amino acid sequence analysis and electrospray mass spectrometry of hGH and all of the mutants permitted unambiguous confirmation of protein identity.

Affinity Constant Measurements. Characterization of the affinity binding parameters of the complex of wild-type or

mutant hGH with its receptor was performed using the extracellular domain of the receptor (hGHbp). Association, dissociation, and affinity constants were determined by employing the Pharmacia Biosensor (BIAcore) as previously described (Cunningham & Wells, 1993). A mutant (S201C)-hGHbp was used, which assured the formation of a 1:1 complex of hGH:hGHbp due to blocking of receptor dimerization (Cunningham & Wells, 1993). Five 2-fold serial dilutions of ligands, starting from 300 nM, were used to determine the association rate constant, k_{on}. The dissociation rate constant, k_{off}, was measured under ligand-saturating concentrations of 5 μM. The diagram in Figure 2 represents the average of triplicate determinations.

Spectroscopy and Processing of Spectra. All spectra were recorded at protein concentrations of approximately 45 μM at 20 °C in 10 mM Tris-HCl, pH 8.0–150 mM NaCl buffer and were smoothed employing AVIV (Lakewood, NJ) software. Absorption spectra were measured on an AVIV 14DS spectrophotometer in the wavelength range of 350–250 nm at intervals of 0.1 nm in a thermostated rectangular cuvette with a 1-cm path length. Light scattering was corrected as described previously (Schauenstein & Bayzer, 1955; Mulkerrin & Cunningham, 1993). The final spectra represent the average of 10 scans with a time constant of 5 s. These spectra permitted calculation of the precise protein concentration, indispensable for rigorous comparison of the CD spectra, and the second derivative of the near-UV spectra employing AVIV software. Far-UV CD spectra were recorded on an AVIV 60DS spectropolarimeter in the wavelength range of 250–200 nm in 0.2-nm intervals in a thermostated circular cuvette with a 0.01-cm path length. The final far-UV CD spectra represent the average of five scans with an integration of 2 s. Results are reported as mean residue weight ellipticity (Θ_{MRW}, in deg cm^2 dmol^{-1}) and were calculated by employing the appropriate mean residue weights for each protein.

Equilibrium Denaturation Assays. In order to quantify the contributions to global protein stability of both the hydrogen-bonding network and charge–dipole interactions of the N-capping box, equilibrium denaturation of the hGH mutants was performed to determine the change in the free energy of unfolding between the wild type and the mutants, $\Delta\Delta G$. Due to the particularly high stability of hGH [ΔG_{H_2O} = 14 kcal/mol (Brems et al., 1990; DeFelippis et al., 1993)] and its tendency to aggregate at high temperatures, neither urea nor thermal denaturation could be used to induce full unfolding of the molecules, and GuHCl was selected as denaturant. Since the GuHCl absorption below 230 nm interferes with the accurate determination of protein ellipticity, unfolding was monitored by far-UV CD spectroscopy at 230 nm. Hence, the systematic uncertainty associated with data acquisition at high concentrations of denaturant due to interference by GuHCl was minimized.

There are three methods available to analyze the denaturation profiles of proteins: the linear extrapolation method (Greene & Pace, 1974), the denaturant binding model (Tanford, 1970), and the residue transfer method (Pace, 1975). We have chosen to use the linear extrapolation method. This is the simplest method, and it is used in the majority of protein denaturation studies (Brems et al., 1990; Serrano & Fersht, 1989; Sancho et al., 1992; Shortle & Meeker, 1986; Shirley et al., 1992). ΔG_{H_2O} (free energy of unfolding in the absence of the denaturant), $D_{50\%}$ (the denaturant concentration at which the apparent free energy of unfolding, ΔG_u, is zero), and m (the slope of ΔG_u vs D) were determined as described in Santoro and Bolen (1988). To eliminate the errors in estimation of ΔG_{H_2O}, associated with large extrapolation from about 4 to 0 M GuHCl, the difference in the free energy of unfolding between the wild type and the mutant was calculated by employing the equation (Horovitz et al., 1992):

$$\Delta\Delta G = \langle m \rangle (D_{50\%\ wt} - D_{50\%\ mut}) \quad (1)$$

where $\langle m \rangle$ is the average value of m for the wild type and all mutants (except S71V/E74L). GuHCl-induced equilibrium denaturations were monitored by employing far-UV CD spectroscopy. The reactions were performed at 20 °C in 10 mM Tris-HCl, pH 8.0–150 mM NaCl buffer at protein concentrations of approximately 0.04 mg/mL. It has previously been determined that hGH follows a two-state model for denaturation in the concentration range of 0.01–2 mg/mL (Brems et al., 1990; DeFelippis et al., 1993); the reversibility of unfolding was determined by the full recovery of original ellipticity upon dilution of the denatured samples to facilitate refolding. All determinations of the denaturation parameters were performed in duplicate or triplicate. In a typical experiment, 20–25 samples containing protein solutions with increasing concentrations of the denaturant, spanning the range of 0–8 M, were prepared. Ellipticity, Θ_{obs}, was measured in circular cuvettes, with a path length of 0.5 cm, at 230 nm. A total of 200 data points were taken in a 5-min trace and averaged. In order to eliminate errors in the determination of GuHCl concentrations introduced by pipetting, the refractive index of each sample was measured. This allowed accurate determination of GuHCl concentration (Pace et al., 1990). The calculation of ΔG_u, taking into account the slopes of the pre- and posttransitional baselines, was performed as previously described (Horovitz et al., 1992). The data in panels A and B of Figure 5 were fit to eq 4 and 3, respectively, from Santoro and Bolen (1988) by employing the general curve and linear fit options of the KaleidaGraph program, version 2.1.4 (Synergy Software, PCS Inc.).

RESULTS

Modeling Prior to Mutant Construction. Modeling techniques were used to identify those substitutions at residues Ser71 and Glu74 which would eliminate one or all of the interactions provided by the N-capping box (i.e., hydrogen bonds and charge–dipole interactions) and which would not significantly perturb the tertiary structure of the mutant relative to that of the wild type. Since positions 71 and 74 are located on the surface of the protein, they should easily accommodate residues with side chains of differing size. Hence, any observed trends and changes in the structure, binding affinity, and thermodynamic stability of the mutants, relative to the wild type, should be attributable to changes in the hydrogen-bonding and charge–dipole interactions.

Six groups of mutants were constructed to test the contribution of the N-capping box to the global stability of hGH. First, a positive control, S71T, in which the hydrogen bond network is essentially that of the wild type, was constructed. Second, the side chains of the residues comprising the N-capping box were truncated in two single mutants, S71A and E74A, and a double mutant, S71A/E74A. Third, hydrophobic residues at positions 71 and 74 were introduced to challenge the hydrophilic end of the helix (mutants S71V, E74L, and S71V/E74L). Fourth, the effect of hydrophilic substitutions was evaluated: mutants E74T, E74S, S71Q, and S71Q/E74S. In these four mutants, modeling showed that the substituted amino acid side chains might be able to

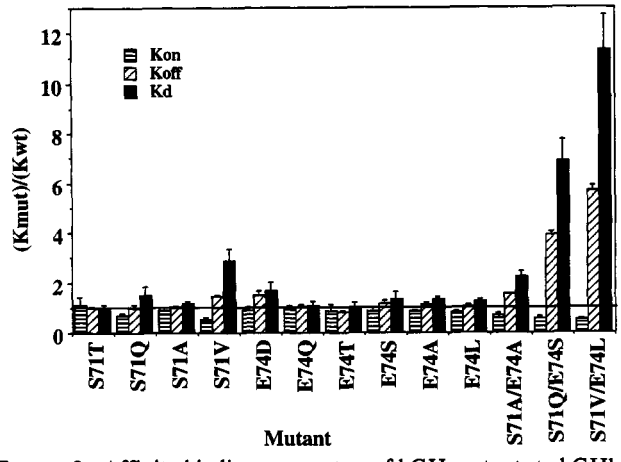

FIGURE 2: Affinity binding parameters of hGH mutants to hGHbp as measured on the BIAcore. Association (k_{on}), dissociation (k_{off}), and affinity constants (K_d) are presented as ratios of K_{mut}/K_{wt}. All of the measurements represent the average of at least three determinations. The average standard deviation for k_{on} is 17%, for k_{off} is 10%, and for K_d is 20%.

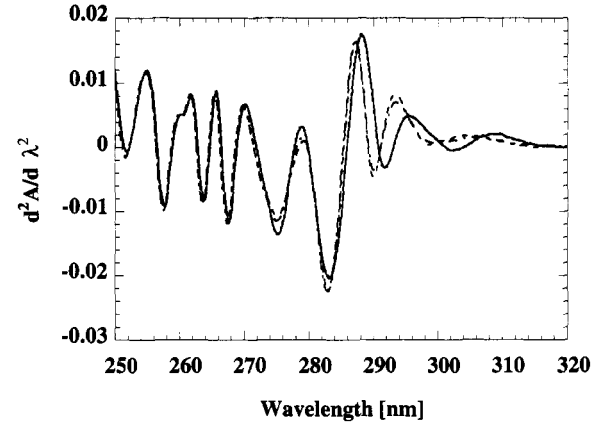

FIGURE 3: Second derivative of the near-UV absorption spectra of native hGH (—) and selected single mutants exhibiting spectra identical to and different from that of the wild type [S71T (- - -) and S71V (– • –), respectively] and a double mutant S71Q/E74S (– –). Changes in the spectra of the mutants relative to that of the wild type are localized to the 290–310-nm region.

form hydrogen bonds with the appropriate amide protons. However, they could only do so by severely restricting the side-chain conformation, which would make the formation of such a bond highly unlikely. Fifth, to test the possible stabilizing effect of the charged residue, Glu74, the E74Q mutant was constructed which lacked the negative charge of the Glu side chain but retained the hydrogen-bonding capacity of the wild-type Glu residue. Sixth, the E74D mutant was constructed to test the effect of the charge at position 74 in the absence of the hydrogen bond formed between the side chain of this residue and the proton of the amide nitrogen of Ser71. The Asp side chain can only make a hydrogen bond with the Ser71 NH if the Asp side-chain conformation is severely restricted.

Binding Assays. As seen in Figure 1A, the helix 2 N-terminus is not part of the binding surface of hGH. Thus, mutations introduced at this site are unlikely to disrupt the binding of the hormone to the receptor, and binding assays were used to screen for changes, relative to the wild type, in the tertiary structures of the mutant proteins.

Binding characteristics of the mutants to hGHbp are presented in Figure 2. All binding parameters—k_{on}, k_{off}, and K_d—are shown as ratios of K_{mut}/K_{wt}. The majority of the mutants displayed binding properties indistinguishable from those of the wild type. None of the mutants exhibited the 20–60-fold reduction of binding to hGHbp observed in previous alanine scanning experiments (Cunningham & Wells, 1989, 1993). This suggests that no changes of the tertiary structure which affect the binding surface of the proteins have taken place. The S71V mutant exhibited a marginal change in binding properties—its K_d was approximately 3-fold greater than that of the wild type. The double mutants S71V/E74L and S71Q/E74S showed K_ds increased by 11- and 7-fold, respectively, relative to the wild type. The extent of the decrease in binding of these double mutants was about 3-fold larger than would be expected from the measured K_ds of the corresponding single mutants if changes in binding were simply additive. This suggests that there may be an interaction between the two residues of the N-capping box in these mutants. Without crystal structures of the single and double mutants it is difficult to ascribe a specific structural change which would correlate with the nonadditivity of these mutants.

Spectroscopic Analysis of the Mutants. Spectroscopic analyses of the mutant proteins were used to further assess the impact of each mutation upon the hGH secondary and tertiary structures. Near-UV spectroscopy (250–350 nm) is used to monitor changes in the local environment of aromatic chromophores and disulfide bonds and provides a method for tracking the tertiary structural variations which often may accompany site-directed mutagenesis (Mulkerrin & Cunningham, 1993). The most sensitive chromophore to changes in environment is the tryptophan residue. Trp86, located on helix 2 in the hydrophobic interior of the protein, is the sole tryptophan residue in hGH; it has been shown to be a sensitive probe of structural changes in hGH mutants (Mulkerrin & Cunningham, 1993). In solution tryptophan possesses two vibronic bands, ^1L_a centered at 292 nm and ^1L_b at 291 nm (Bewley & Li, 1984; Mulkerrin & Cunningham, 1993). However, in hGH the tryptophan absorption bands are shifted to around 303 and 292 nm, respectively. It has been shown that a large part of this shift is attributable to hydrogen bond formation between the indole NH of Trp86 and the side chain of the Asp169 residue located on helix 4 (Bewley & Li, 1984; DeVos et al., 1992).

It is difficult to determine the precise locations of the absorption maxima of individual chromophores under the relatively broad spectral envelope of all the contributing chromophore bands in a zero-order spectrum. The second derivative (second order) of the absorption spectrum provides a more accurate determination of the absorption maxima of the contributing protein chromophores (Butler & Hopkins, 1970). The minima in the second-order spectra correspond to the maxima of the chromophore absorption bands in the zero-order spectrum.

The second derivatives of the near-UV absorption spectra of hGH and the S71T, S71V, and S71Q/E74S mutant proteins are presented in Figure 3. In the mutants which exhibited spectral changes, perturbations typical of tryptophan absorption are observed in the 290–310-nm region. The most dramatic shift toward shorter wavelengths (blue shift) relative to that of the wild type is exhibited by the wavelength maximum of the ^1L_a absorption band; thus, only the ^1L_a bands are presented in Table 1. As seen in Table 1 (and Figure 3) the "positive control" mutant, S71T, did not exhibit spectral perturbations, suggesting no disruption of the hGH structure. This table also shows that all of the substitutions at position 74, except for the E74L mutant, displayed ^1L_a band maxima within one standard deviation of that of the wild type. In contrast, substitutions at position 71, in which the hydrogen

Table 1: Wavelength Maxima of the ^1L_a Absorption Band of Trp86 and the Shift of These Maxima in the Mutants Relative to That of the Wild Type

protein	^1L_a (nm)[a]	$\Delta^1L_{a\ wt-mut}$ (nm)
wild type	302.8	
S71T	302.5	0.3
S71A	301.9	1.0
S71Q	301.4	1.4
S71V	302.2	2.6
E74Q	302.5	0.3
E74D	302.5	0.3
E74T	302.8	0.0
E74S	302.5	0.3
E74A	302.4	0.4
E74L	301.9	0.9
S71A/E74A	300.3	2.5
S71Q/E74S	299.3	3.5
S71V/E74L	299.0	3.8

[a] The average standard deviation of determination of ^1L_a maxima is 0.3 nm.

bond between the side chain at position 71 and the backbone NH of Glu74 is removed, exhibited a blue shift of the ^1L_a absorption band. Similar deviations were seen for the E74L mutant and the double mutants. These perturbations ranged from 0.9 to 3.8 nm. Since the absorption maxima in such mutants are blue shifted, this suggests that the Trp86–Asp169 hydrogen bond is weaker than in the wild type (Bewley & Li, 1984; Mulkerrin & Cunningham, 1993). Also note that the observed shift of the ^1L_a band in all double mutants is larger than the sum of the shifts of the single mutants. This nonadditivity may be indicative of an interaction of the two residues occupying positions 71 and 74.

Far-UV CD spectroscopy (200–250 nm) is a convenient probe of the secondary structure of proteins in general and α-helical content (Greenfield & Fasman, 1969) and packing in particular (Manning et al., 1988; Manning & Woody, 1991; Cooper & Woody, 1990). The structure of hGH is highly helical, and substantial changes in helicity resulting from the amino acid substitutions were not anticipated. However, the prospect of subtle variations in helical packing remained a possibility (Mulkerrin & Cunningham, 1993).

The far-UV CD spectrum of hGH is dominated by the presence of α-helices and contains two characteristic minima at 208 and 222 nm (Holladay et al., 1974). Upon replacement of the residues forming the hydrogen bond network of the N-capping box, the overall shape of the spectrum remained similar to that of the wild type (Figure 4) though the intensities of the CD signals of some single (Figure 4B) and double (Figure 4C) mutants became slightly diminished. Upon comparison of Figure 4 with Table 1, it is apparent that the mutants exhibiting lower intensity in the far-UV CD spectra are the same mutants in which the ^1L_a absorption bands are blue shifted relative to that of the wild type. The far-UV CD difference spectra (data not shown) revealed that these variations in intensity increase from long to short wavelengths. In summary, the data from the far-UV CD spectra lead us to conclude that the mutations introduced into the sequence of hGH do not perturb (i.e., unwind) the α-helices in the mutant proteins.

Equilibrium Denaturation Assays. Data from selected GuHCl denaturation experiments are presented in Figure 5A and were analyzed using the nonlinear least-squares technique of Santoro and Bolen (1988). Plots of ΔG_u vs [GuHCl] for the transition region of the equilibrium denaturation experiments for native protein and selected mutants are presented in Figure 5B. Note that the final estimates of ΔG_{H_2O} derived from the method of Santoro and Bolen (Figure 5A) and that

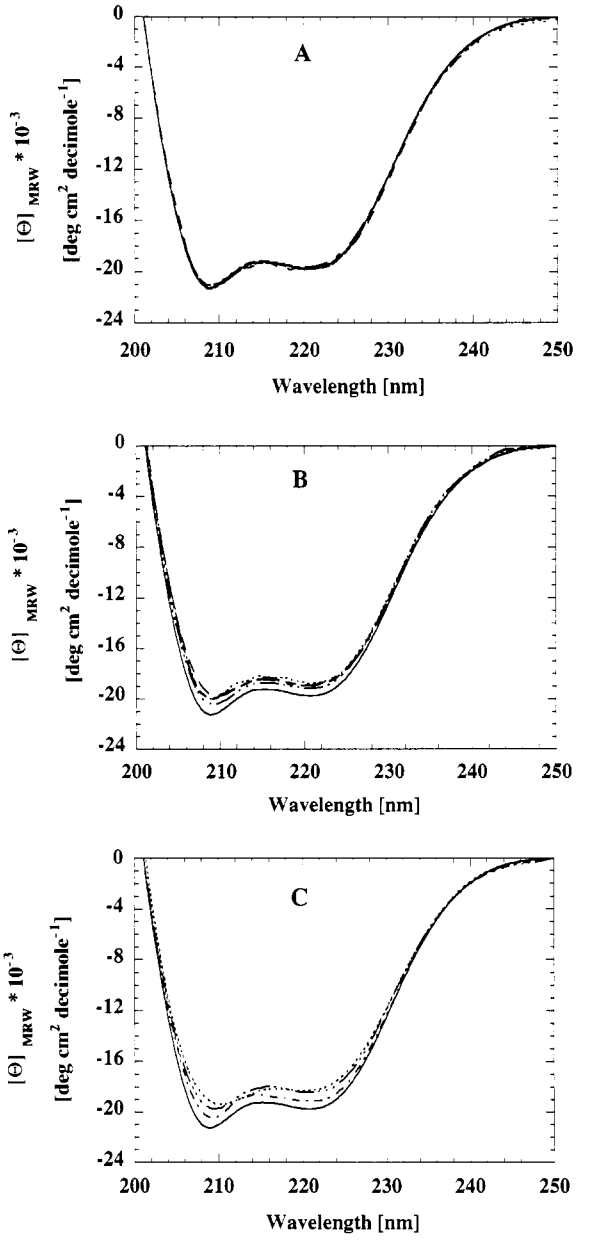

FIGURE 4: Far-UV CD spectra of hGH (—) and the hGH mutants: (A) mutants exhibiting spectra identical to that of the wild type, S71T (– · –), E74A (– –), E74T (- - -), E74S (– – –), E74Q (- - -), and E74D (•••); (B) single mutants exhibiting spectra different from that of the wild type, S71Q (–···–), S71A (– –), S71V (- - -), and E74L (– – –); (C) spectra of the double mutants, S71A/E74A (– ··· –), S71Q/E74S (– · –), and S71V/E74L (- - -). Note that in (B) and (C) the shape of the spectra is identical to that of the wild type, but the intensity of the CD signals in the mutants is decreased relative to native hGH.

of the conventional presentation of the linear extrapolation technique (Figure 5B) are internally consistent and that it is purely a matter of convention to present the data in the form discussed below.

Table 2 presents the parameters of the equilibrium denaturation process. A one-way analysis of variance (ANOVA) of m did not produce any evidence that these values are mutant dependent ($p = 0.93$, 13 proteins) except for the S71V/E74L mutant. These observations allowed calculation of the average m for these 13 proteins, and the variation in estimates of m between the mutants was considered to be due to experimental error. In the case of the S71V/E74L mutant its own value of m was used to estimate ΔG_{H_2O} since it was 36% smaller than $\langle m \rangle$.

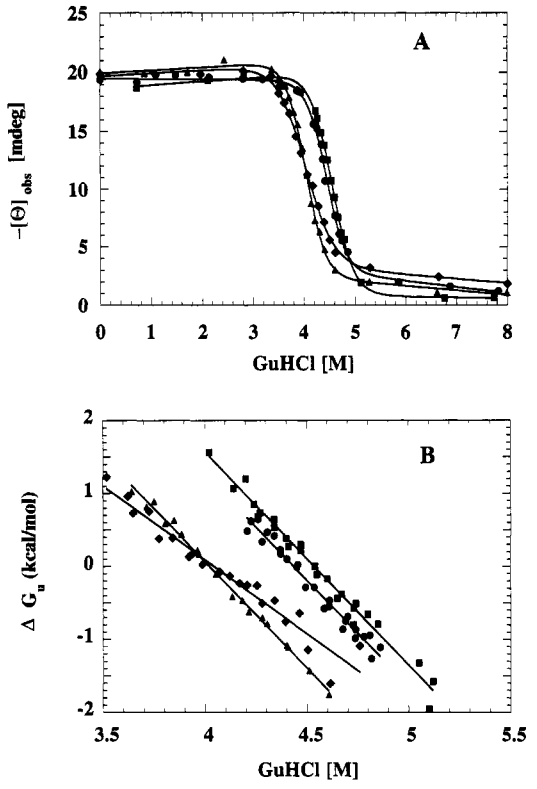

FIGURE 5: Equilibrium denaturation profiles of native hGH (●) and three selected mutants, S71T (■), S71A/E74A (▲), and S71V/E74L (◆). (A) Equilibrium denaturation data fit to a nonlinear least-squares algorithm (Santoro & Bolen, 1988); (B) ΔG_u versus [GuHCl] plots of the data from the transition regions of the same mutants as in (A). Note the difference of the slope of S71V/E74L relative to those of native hGH and the other mutants.

Table 2: Parameters of the Equilibrium Denaturation Process for hGH and the hGH Mutants

mutant	ΔG_{H_2O}[a] (kcal/mol)	m[b] (kcal mol^{-1} M^{-1})	$D_{50\%}$[c] (M)	$\Delta\Delta G$[d] (kcal/mol)
wt	14.15	−3.18	4.45	0.00
S71T	13.43	−2.96	4.54	−0.33
S71A	12.89	−3.12	4.13	0.97
S71V	12.83	−3.16	4.06	1.19
S71Q	12.69	−3.15	4.03	1.28
E74D	12.17	−2.85	4.27	0.55
E74Q	12.91	−2.94	4.39	0.18
E74A	12.74	−2.97	4.29	0.49
E74S	13.04	−3.14	4.15	0.91
E74T	12.43	−2.91	4.27	0.55
E74L	12.91	−3.08	4.19	0.79
S71Q/E74S	11.89	−3.04	3.91	1.64
S71A/E74A	11.93	−2.96	4.03	1.28
S71V/E74L	8.24	−2.03	4.06	4.88

[a] The average standard deviation of ΔG_{H_2O} was 0.88 kcal/mol. [b] The within-mutant average standard deviation of m values was 0.22 kcal mol^{-1} M^{-1}; the between-mutant average value of $\langle m \rangle$ = 3.04 kcal mol^{-1} M^{-1}, derived by averaging of m for 13 proteins (S71V/E74L was excluded), had a standard deviation of 0.11 kcal mol^{-1} M^{-1}. [c] The within-mutant average standard deviation of $D_{50\%}$ was 0.03 M. [d] The value of $\Delta\Delta G$ was calculated not from the values of ΔG_{H_2O} but by employing eq 1; the average standard deviation of $\Delta\Delta G$ was 0.14 kcal/mol.

The parameter m has been interpreted as a measure of the difference between the solvent-exposed nonpolar surface area of the denatured and native states of proteins and/or the cooperativity of protein unfolding (Greene & Pace, 1974; Shortle & Meeker, 1986; Shortle et al., 1988, 1990, 1992). Thus, the marked change in the value of m for the S71V/E74L mutant could be attributed, according to Shortle and Meeker (1986), either to the presence of equilibrium intermediates during protein unfolding or alternatively to changes in the solvation properties of the surface area of the denatured state. Note that the values of the equilibrium denaturation parameters for native protein in Table 2 are consistent with those reported previously (Brems et al., 1990).

Four observations are apparent from the results presented in Table 2. First, the positive control mutant, S71T, has a free energy of unfolding similar to that of the wild type. Second, comparing the free energy of unfolding of S71V, S71A, and S71Q (with an average value of 1.15 ± 0.16 kcal/mol) with that of E74A, E74T, E74D, E74L, and E74S (with an average value of 0.66 ± 0.18 kcal/mol) suggests that the hydrogen bonding provided by the cap residue, Ser71, is approximately 2-fold more stabilizing than that provided by the $N+3$ residue, Glu74. Also, the $\Delta\Delta G$ values of the Ser71 mutants were not particularly sensitive to the size or polarity of the side chain as long as the hydrogen bond to the NH of Glu74 was unlikely to be formed. The destabilizing effect of the E74L substitution (0.79 kcal/mol) is slightly higher than the average effect of the Ala, Thr, and Asp substitutions (0.53 ± 0.03 kcal/mol), possibly due to the unfavorable contribution from the increased solvent-accessible nonpolar surface area in the folded protein. The only result which was not easily interpretable was the relatively increased destabilization of the E74S mutant (0.91 kcal/mol) compared to that of E74T (0.55 kcal/mol). Neither the spectroscopic data, binding assays, nor the modeling analysis provides insight as to the possible origin of this effect.

The third observation is that among the double mutants only the S71A/E74A mutant has a $\Delta\Delta G$ which is approximately the sum of its constituent single mutants. The S71Q/E74S mutant is 0.55 kcal/mol more stable than would have been estimated from the sum of the $\Delta\Delta G$ values for the S71Q and E74S single mutants, suggesting that the side chains in this double mutant may be interacting with each other. The S71V/E74L double mutant has to be considered separately from all other mutants (discussed above). It appeared to be approximately 3 kcal/mol more destabilized than would have been calculated from the sum of the $\Delta\Delta G$ of the S71V and E74L single mutants. This result, in conjunction with the spectroscopic and binding assays, suggests that the cumulative hydrophobic effects of Val and Leu substitutions in the double mutant may be significantly more destabilizing than in the corresponding single mutants.

Finally, the magnitude of the charge–dipole stabilization provided by the charge of Glu74 can be evaluated by comparing the data for the mutants at position 74. Only a marginally destabilizing effect is observed upon the elimination of the charge in the E74Q mutant (0.18 kcal/mol). However, the protein is destabilized approximately 3-fold more by the E74D substitution (0.55 kcal/mol) in which the charge of the side chain is retained but the formation of the hydrogen bond to the NH of the cap is unlikely. Further, no stabilizing effect of the charge at position 74 is apparent when $\Delta\Delta G$ for the E74T (0.55 kcal/mol) and E74A (0.49 kcal/mol) mutants are compared with that of the E74D mutant (0.55 kcal/mol). Therefore, in the absence of hydrogen-bonding capacity of the side chain at position 74, its charge does not contribute to the free energy of stabilization of the native state of hGH.

DISCUSSION

There are two approaches to investigating the stabilizing interactions in α-helices: studying an α-helix in the context of a full-length protein or as an isolated α-helical peptide. The appealing feature of peptides is the absence of tertiary structural interactions, characteristic of proteins, which

complicate an analysis of intrahelical stabilization. However, studies on peptides can suffer from low inherent helicity of the peptide, fraying of the helical ends (Chakrabartty et al., 1991; Waltho et al., 1993; Strehlow et al., 1991), possibility of formation of 3_{10} helices instead of α-helices (Miick et al., 1992), and peptide oligomerization (Fersht & Serrano, 1993). On the other hand, in the full-length protein, despite the difficulty of accounting for all of the tertiary interactions, the ends of the helices are better defined in terms of structure and location.

This study has assessed the contribution to α-helical stability of hydrogen bond and charge–dipole interactions at the N-terminus of an α-helix in hGH. The amino acid side chains involved in these interactions comprise a structure referred to as an N-capping box (Harper & Rose, 1993; Presta & Rose, 1988). In particular, residues Ser71 and Glu74 in helix 2 of hGH form a hydrogen bond network in which each side chain forms a hydrogen bond to the backbone NH of the other residue (Figure 1b). To evaluate the importance of these two hydrogen bonds, as well as the putative charge–dipole interaction between the Glu74 side chain and helix 2 N-terminus, the two side chains were substituted with several different amino acids. Note that the capping hypothesis suggests a mechanism by which the terminal residues can form hydrogen bonds capable of simultaneously stabilizing and serving as start/stop signals for the folding of α-helices. Thus, the content of this hypothesis is twofold—thermodynamic and kinetic. Thermodynamically, it suggests a mode for stabilization of α-helices, and kinetically, its intrinsic start/stop signals for the formation of a particular kind of secondary structure (in this case α-helical) ought to affect the process of folding itself. This investigation has only tested the thermodynamic consequences of the capping hypothesis.

Spectroscopic, binding, and denaturation assays were employed to monitor any changes in hGH upon disruption of the hydrogen-bonding network of the N-capping box. Quantitative comparative analysis of the mutants is possible only for those cases in which there is no dramatic perturbation of the tertiary structure; only in such cases can changes in the structural and thermodynamic stability characteristics of the protein be unambiguously attributed to interactions of the N-capping box constituents. The spectroscopic and binding assays permitted characterization of the structural fidelity of the hGH mutants. In the near-UV absorption spectra of the mutants at position 71, the observed blue shift of the ^1L_a band may be a result of weakening of the hydrogen bond between Trp86 and Asp169, attributable to either increased hydrogen bond length or altered hydrogen bond geometry. Since Trp86 is located on the same helix approximately 15 Å from the substitutions in the N-capping box, these mutations may exert their effect on the Trp86–Asp169 hydrogen bond through a rotation or translation of helix 2 with respect to the other helices of the four-helix bundle. Each of the second derivative minima outside the region characteristic of tryptophan absorption (290–310 nm) was identical to that of the wild type; i.e., the local environment of the other chromophores was not significantly perturbed. However, minor changes in the overall tertiary conformation of these mutants cannot be ruled out since tyrosine and phenylalanine are less sensitive to changes in their environment and, therefore, may not exhibit environmentally induced changes in their absorption characteristics.

The far-UV CD spectra showed no alteration in the helical content. Thus, no unambiguous confirmation of the unwinding of the N-terminus of helix 2, attributable to the elimination of the cap hydrogen bond, was found. Some of the mutants, however, displayed small changes in the intensity of the signal in the far-UV CD spectra. This phenomenon has been previously described (Cooper & Woody, 1990; Manning et al., 1988; Manning & Woody, 1991), and it has been suggested that such systematic spectral variations are indicative not of changes in the overall helicity of protein, but rather of alterations in the helical packing (Mulkerrin & Cunningham, 1993). The same subset of mutants which exhibited small changes in far-UV CD spectra displayed a blue shift of the ^1L_a absorption band of the Trp86 residue. Hence, in the mutants in which residue 71 cannot form the hydrogen bond network of the N-capping box, a rigid body shift of helix 2, indicated by the near-UV absorption spectra, would also explain the small changes in intensity of the far-UV CD spectra. An alternative interpretation of such changes is that alterations in the far-UV component of the tryptophan spectrum may contribute to the observed ellipticity. Further, the relative intensities of the CD signals depend on the protein sample concentrations. Therefore, perturbations of the tryptophan chromophore may also cause small changes in absorptivity of the mutants and consequently errors in concentration determination.

At position 74 the only single substitution which produced small alterations in the far-UV CD and near-UV absorption spectra was E74L. Modeling of this mutant revealed no steric hindrance against placing a leucine at this position. However, since the residue occupying position 74 is solvent exposed, leucine may increase the solvent-accessible nonpolar surface area in the folded protein. Alternatively, Leu74 could interact favorably with the side chains of Phe139 and Lys70 to form a hydrophobic pocket. If indeed this occurred, then it might cause a small distortion at the N-terminus of helix 2 which may explain its slightly larger $\Delta\Delta G$ compared to the Ala, Thr, and Asp substitutions.

The perturbations in the tertiary structure of the mutant proteins observed spectroscopically were not significant enough to substantially alter their binding properties. Binding assays showed that only the S71V, S71Q/E74S, and S71V/E74L mutants exhibited a relatively small reduction in binding relative to native hGH (Figure 2).

Equilibrium denaturation of the mutants, using GuHCl, was employed to quantify changes in the free energy of unfolding. Previous studies have shown that changing the stability of one secondary structural element, e.g., an α-helix, could alter the global stability of a protein (Mitchinson & Baldwin, 1986; Zhang et al., 1991). Hence the difference in the free energy of unfolding, $\Delta\Delta G$, between the mutants and native hGH should reflect the contribution of the N-capping box of helix 2 to the global stability. However, in order for $\Delta\Delta G$ to be a reliable measure of the difference in stability of a mutant and native hGH, there should be no significant difference in the folded, tertiary structure between the two proteins. The spectroscopic and binding assays demonstrated that this was achieved for all the mutants.

All of the mutants presented in Table 2 are destabilized to various degrees except the positive control mutant S71T, which is marginally more stable than native hGH. Those mutants in which Ser71 was substituted with a residue unable to form the cap hydrogen bond were on average destabilized by 1.15 kcal/mol relative to the wild type. Further, the stabilizing character of the cap hydrogen bond was explicitly demonstrated by the comparison of the two isosteric mutants, S71T and S71V. The difference between the side chains of Val and Thr is that the latter possesses an hydroxyl group which can form the cap hydrogen bond whereas the methyl group of the

Table 3: Comparison of the Change in Free Energy, $\Delta\Delta G$, upon Substitution of the Cap Residue in Three Proteins: hGH, T4 Lysozyme (Bell et al., 1992), and Barnase (Serrano & Fersht, 1989; Serrano et al., 1992)

	$\Delta\Delta G^a$ (kcal/mol)			
			barnase	
substitution	hGH	T4 lysozyme	helix 1 (residue 6)	helix 2 (residue 26)
S → T	−0.33	−0.20	−0.27	−0.54
S → A	0.97	1.30	1.88	1.40
S → V	1.19	1.30		1.55
S → Q	1.28		1.39	1.04

[a] The published $\Delta\Delta G$ values have been recalculated using Ser, instead of the native Thr, in the cap position of T4 lysozyme and barnase by subtracting the value of $\Delta\Delta G$ for the S → T transition from the value of $\Delta\Delta G$ for the Ala, Val, and Gln mutants.

former cannot. From Table 2 the stabilization attributable to the formation of this bond, in S71T relative to S71V, is calculated to be 1.52 kcal/mol. This clearly demonstrates that the formation of the cap hydrogen bond stabilizes the α-helix and consequently the overall tertiary structure of hGH.

Of the two hydrogen bonds in the N-capping box, the cap hydrogen bond is more important since removal of this hydrogen bond destabilizes hGH approximately 2-fold more than does removal of the $N + 3$ hydrogen bond, even though the latter has a charged acceptor (compare S71A and E74A). The relatively greater importance of the cap hydrogen bond may arise from two factors. First, its backbone nitrogen donor is part of the helix, whereas the backbone nitrogen donor of the $N + 3$ hydrogen bond is outside the helical cylinder (Figure 1B). Second, the Ser71 side chain also forms a hydrogen bond with the side chain of Ser132. Therefore, when Ser71 is substituted with an amino acid that cannot form these two hydrogen bonds, the mutant protein loses the energy of stabilization corresponding not to one but two intramolecular hydrogen bonds. Finally, our study of the N-capping box in hGH implies that, in the mutants which lacked the intramolecular hydrogen bonds of the wild type, the hydrogen-bonding capacity of the amides would be satisfied by water molecules since the mutagenized residues are solvent exposed. Thus, the changes in stability that we have observed can be attributed to the stabilization afforded by *intraprotein* hydrogen bonds relative to those made with the solvent water molecules.

Comparison of mutants at position 74 also permits evaluation of the importance of the charge–dipole interaction at the helix 2 N-terminus. Substitution of Glu74 with Gln74 removed the charge but retained hydrogen-bonding capacity and was only marginally destabilizing (0.18 kcal/mol). In contrast, removing the $N + 3$ hydrogen bond while retaining the negative charge (E74D mutant) was 3-fold more destabilizing than the E74Q substitution. A rigorous analysis of the charge–dipole interaction would entail a study of the pH dependence of $\Delta\Delta G$ for native hGH and each mutant. However, since the experiments were performed at pH 8.0, the Glu and Asp side chains should be fully ionized, and our results establish the absence of any significant stabilization even in the presence of a fully ionized side chain. Furthermore, comparison of E74D with the E74A, E74T, and E74L mutants demonstrates that once the $N + 3$ hydrogen bond is removed, the charge on the side chain is inconsequential to the global stability.

The results for hGH are in agreement with those of previous studies on T4 lysozyme (Bell et al., 1992) and barnase (Serrano & Fersht, 1989; Serrano et al., 1992) (Table 3). Both T4 lysozyme and barnase have Thr instead of Ser at the cap position. To facilitate comparison of hGH with the T4 lysozyme and barnase, their $\Delta\Delta G$ values have been recalculated using Ser as the standard. Table 3 shows that Thr is slightly more stabilizing than Ser for all three proteins and that removal of the cap hydrogen bond destabilizes the proteins by at least 1 kcal/mol. This value is consistent with estimates of the "average" intramolecular hydrogen bond in proteins (Shirley et al., 1992; Fersht, 1987). It is also consistent with the results of calculations of the $\Delta\Delta G$ based on the modified Lifson–Roig theory, which suggests that the contribution of the N-capping residues differs over a range of 2 kcal/mol (Doig et al., 1994). In hGH, the charge–dipole interaction was only marginally destabilizing (0.18 kcal/mol); other groups have shown that charge–dipole interactions increase the stability of α-helices in some proteins by 0.6–2.0 kcal/mol (Nicholson et al., 1988, 1991; Serrano & Fersht, 1989; Sancho et al., 1992). For the two helices evaluated in barnase, there is a considerable variation of the stabilization afforded by the cap hydrogen bond (Table 3). This suggests that the importance of hydrogen bonds and charge–dipole interactions at helix termini may vary between particular helices, even among those within a protein. Given that the proteins in Table 3 were analyzed using three different techniques (GuHCl, thermal, and urea-induced denaturation), the observed trends attributable to the cap hydrogen bond are remarkably similar, lending unambiguous support for the cap hypothesis (Presta & Rose, 1988). Additional support for this hypothesis can be found in studies of peptides (Bruch et al., 1991; Chakrabartty et al., 1993; Forood et al., 1993; Lyu et al., 1992, 1993; Zhou et al., 1994).

ACKNOWLEDGMENT

The authors thank Bart de Vos for crystallographic coordinates of hGH; Henry Lowman for providing pb0720 plasmid; Brian Cunningham for donating human growth hormone binding protein; Peter Ng, Parkash Jhurani, and Mark Vasser for synthetic oligonucleotides; Brad Snedecor and Han Chen for fermentation pastes; Jim Bourell and Victor Ling for mass spectrometry of the proteins; Chris Grimley for the N-terminal end analysis; Richard Morse for assistance in preparation of the manuscript; and Tom Bewely for helpful discussions.

REFERENCES

Bell, J. A., Becktel, W. J., Sauer, U., Baase, W. A., & Matthews, B. W. (1992) *Biochemistry 31*, 3590.
Bewley, T. A., & Li, C. H. (1984) *Arch. Biochem. Biophys. 233*, 219.
Blagdon, D. E., & Goodman, M. (1975) *Biopolymers 14*, 241.
Brems, D. N., Brown, P. L., & Becker, G. W. (1990) *J. Biol. Chem. 265*, 5504.
Bruch, M. D., Dhingra, M. M., & Gierasch, L. M. (1991) *Proteins: Struct., Funct., Genet. 10*, 130.
Butler, W. L., & Hopkins, D. W. (1970) *Photochem. Photobiol. 12*, 439.
Chakrabartty, A., Schellman, J. A., & Baldwin, R. L. (1991) *Nature (London) 351*, 586.
Chakrabartty, A., Doig, A. J., & Baldwin, R. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11332.
Chang, C. N., Rey, M., Bochner, B., Heyneker, H., & Gray, G. (1987) *Gene 55*, 189.
Cooper, T. M., & Woody, R. W. (1990) *Biopolymers 30*, 657.
Creighton, T. E. (1983) in *Proteins: Structure and Molecular Properties*, Freeman, New York.
Cunningham, B. C., & Wells, J. A. (1989) *Science 244*, 1081.
Cunningham, B. C., & Wells, J. A. (1993) *J. Mol. Biol. 234*, 554.

Cunningham, B. C., Jhurani, P., Ng, P., & Wells, J. A. (1989) *Science 243*, 1330.
DeFelippis, M. R., Alter, L. A., Pekar, A. H., Havel, H. A., & Brems, D. N. (1993) *Biochemistry 32*, 1555.
DeVos, A. M., Ultsch, M. H., & Kossiakoff, A. A. (1992) *Science 255*, 306.
Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795.
Doig, A. J., Chakrabartty, A., Klingler, T. M., & Baldwin, R. L. (1994) *Biochemistry 33*, 3396.
Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M., & Baldwin, R. L. (1989) *Proteins: Struct., Funct., Genet. 5*, 1.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301.
Fersht, A. R., & Serrano, L. (1993) *Curr. Opin. Struct. Biol. 3*, 75.
Forood, B., Feliciano, E. J., & Nambiar, K. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 838.
Greene, R. F., & Pace, C. N. (1974) *J. Biol. Chem. 249*, 5388.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108.
Harper, E. T., & Rose, G. D. (1993) *Biochemistry 32*, 7606.
Heinz, D. W., Baase, W. A., Dahlquist, F. W., & Matthews, B. W. (1993) *Nature (London) 361*, 561.
Hol, W. G. H., Van Duijnen, P. T., & Berendsen, H. J. C. (1978) *Nature (London) 273*, 443.
Holladay, L. A., Hammonds, R. G., & Puett, D. (1974) *Biochemistry 13*, 1653.
Horovitz, A., Matthews, J. M., & Fersht, A. R. (1992) *J. Mol. Biol. 227*, 560.
Kunkel, T. A., Roberts, J. D., & Zahour, R. A. (1987) *Methods Enzymol. 154*, 367.
Lattman, E. E., & Rose, G. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 439.
Lecomte, J. T. J., & Moore, C. D. (1991) *J. Am. Chem. Soc. 113*, 9663.
Lyu, P. C., Zhou, H. X., Jelveh, N., Wemmer, D. E., & Kallenbach, N. R. (1992) *J. Am. Chem. Soc. 114*, 6560.
Lyu, P. C., Wemmer, D. E., Zhou, H. X., Pinker, R. J., & Kallenbach, N. R. (1993) *Biochemistry 32*, 421.
Manning, M. C., & Woody, R. W. (1991) *Biopolymers 31*, 569.
Manning, M. C., Illangasekare, M., & Woody, R. W. (1988) *Biophys. Chem. 31*, 77.
Miick, S. M., Martinez, G. V., Fiori, W. R., Todd, A. P., & Millhauser, G. L. (1992) *Nature (London) 359*, 653.
Mitchinson, C., & Baldwin, R. L. (1986) *Proteins: Struct., Funct., Genet. 1*, 23.
Mulkerrin, M. G., & Cunningham, B. C. (1993) in *Protein Folding: In Vivo and In Vitro* (Cleland, J. L., Ed.) pp 240–253, American Chemical Society, Washington, DC.
Nicholson, H., Becktel, W. J., & Matthews, B. W. (1988) *Nature (London) 336*, 651.
Nicholson, H., Anderson, D. E., Dao-Pin, S., & Matthews, B. W. (1991) *Biochemistry 30*, 9816.
Nicoll, C. S., Mayer, G. L., & Russell, S. M. (1986) *Endocr. Rev. 7*, 169.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1.
Pace, C. N., Shirley, A. B., & Thomson, J. A. (1990) in *Protein Structure: a Practical Approach*, IRL Press, Oxford.
Presta, L. G., & Rose, G. D. (1988) *Science 240*, 1632.
Richardson, J. S., & Richardson, D. C. (1988) *Science 240*, 1648.
Roder, H., Elöve, G. U., & Englander, S. W. (1988) *Nature (London) 335*, 700.
Sancho, J., Serrano, L., & Fersht, A. R. (1992) *Biochemistry 31*, 2253.
Sanger, F., Niklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063.
Schauenstein, E., & Bayzer, H. (1955) *J. Polym. Sci. 16*, 45.
Serrano, L., & Fersht, A. R. (1989) *Nature (London) 342*, 296.
Serrano, L., Sancho, J., Hirshberg, M., & Fersht, A. R. (1992) *J. Mol. Biol. 227*, 544.
Shirley, B. A., Stanssens, P., Hahn, U., & Pace, C. N. (1992) *Biochemistry 31*, 725.
Shortle, D., & Meeker, A. K. (1986) *Proteins: Struct., Funct., Genet. 1*, 81.
Shortle, D., Meeker, A. K., & Freire, E. (1988) *Biochemistry 27*, 4761.
Shortle, D., Stites, W. E., & Meeker, A. K. (1990) *Biochemistry 29*, 8033.
Shortle, D., Chan, H. S., & Dill, K. A. (1992) *Protein Sci. 1*, 201.
Strehlow, K. G., Robertson, A. D., & Baldwin, R. L. (1991) *Biochemistry 30*, 5810.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1.
Waltho, J. P., Feher, W. A., Merutka, G., Dyson, H. J., & Wright, P. E. (1993) *Biochemistry 32*, 6337.
Zhang, X.-J., Baase, W. A., & Matthews, B. W. (1991) *Biochemistry 30*, 2012.
Zhou, H. X., Lyu, P., Wemmer, D. E., & Kallenbach, N. R. (1994) *Proteins: Struct., Funct., Genet. 18*, 1.