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Helical Formation in Isolated Fragments of Bovine Growth Hormone

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ABSTRACT: The peptide 109–133 was isolated from bovine growth hormone (bGH) and studied for helix formation in aqueous solutions. This fragment was shown to contain helical structure by far-ultraviolet circular dichroism in aqueous solutions. The amount of helix was dependent on pH and peptide concentration. The peptide has maximum helicity between pH 4 and 5 and at high peptide concentration. Under these conditions for maximal helix population, this fragment is approximately 100% helical. Secondary structure predictions suggest that residues 110–127 have a strong propensity to form an amphipathic helix. We have also studied a related peptide, 96–133, and show by gel filtration that it undergoes an increase in molecular weight that directly correlates with a coil to helix transition. A comparison of the helical content of 96–133 to 109–133 and circular dichroism studies of peptide 96–112 suggest that the helix of 96–133 is limited to the 109–133 region. Current models for α-helix formation predict that peptides the size of 109–133 should not contain measurable helicity in aqueous solutions. Our studies show that the unusual stability of helix 109–133 is due to electrostatic interactions and probable intermolecular packing between hydrophobic faces of the amphipathic surfaces of the helices. The implications of helix formation in these fragments to a framework model of protein folding for bGH are discussed.

The framework model of protein folding states that formation of secondary structure precedes tertiary structure (Kim & Baldwin, 1982). Previous folding studies of bovine growth hormone (bGH) are consistent with a framework-type model of folding (Brems et al., 1987). bGH is a member of the class of large pituitary polypeptide hormones and has 191 amino acids. Circular dichroism studies indicate that it is approximately 50% helical (Holladay et al., 1974). Equilibrium denaturation of bGH is not a two-state process, and at least four species have been identified: unfolded, native, intermediate (that contains helical structure but lacks tertiary structure), and an associated intermediate (that contains helical structure but lacks tertiary structure). Kinetic folding studies of bGH showed that formation of helical structure precedes the tertiary structure (Brems et al., 1987).

It is commonly accepted that the secondary structure of globular proteins is unstable in the absence of long-range tertiary interactions. This notion came from studies of protein fragments that corresponded to helical regions of the intact protein (Epand & Scheraga, 1968; Taniuchi & Anfinsen, 1969) and the two-state equilibrium denaturation of most proteins (Creighton, 1984). The general lack of stability of isolated secondary structure is sometimes considered as evidence against the feasibility of the framework model. Because of the unusual stability of bGH helical structure, we reasoned that it may be possible to find appropriate fragments of bGH that are helical in aqueous solution. If so, these highly stable helices would likely represent the helical regions that make up the framework of an early folding intermediate. Previous studies of fragments derived from bGH have revealed that 96–133 is biologically active and contains measurable helical structure in aqueous solutions (Chen & Sonenberg, 1977). We have reinvestigated the helical formation of this fragment and have obtained a truncated form, 109–133, that retains all of the helix that is observed in 96–133. The sequence of peptide 96–133 is as follows: VFTNS^{100}LVFGTSDRVY^{110}EKLKDLEEGI^{120}LALMRELEDG^{130}TPR.

Experimental Procedures

Materials

Fragment 96–133 was derived from bGH that was obtained by expression of bGH in *Escherichia coli* using recombinant

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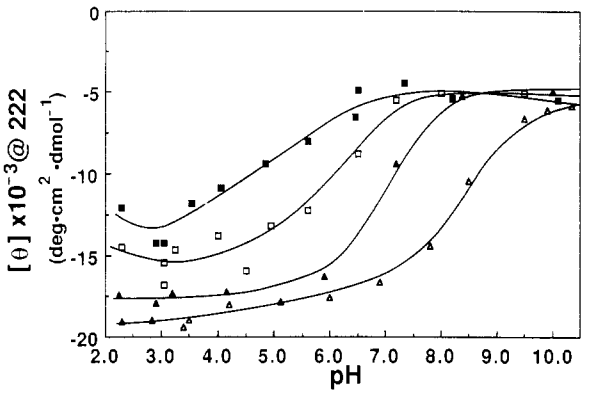

FIGURE 1: Effect of pH and peptide concentration on the helicity of fragment 96–133. The measurements were obtained at (△) 0.3, (▲) 0.04, (□) 0.01, and (■) 0.006 mg/mL. All solutions contained 1 mM each of sodium citrate, sodium phosphate, and sodium borate and were adjusted to the different pHs by addition of concentrated hydrogen chloride or sodium hydroxide.

DNA technology (Souza et al., 1984; Olsen, 1985). Fragment 96–133 was prepared by limited trypsin digestion and isolated as described by Gráf and Li (1974). The high-performance liquid chromatography (HPLC) methods used were described previously (Brems et al., 1985). A MicroPak TSK G-2000H exclusion column (7.5 mm × 30 cm) from Toya Soda Manufacturing Co. was used for the size exclusion chromatography. A Vydak C-4 column (4.6 mm × 25 cm) was used for the reverse-phase chromatography. Clostripain was obtained from Sigma and activated according to their instructions.

Methods

Preparation of Fragment 109–133. This fragment was derived from a limited clostripain digestion of fragment 96–133. Twenty milligrams of 96–133 was reacted with 0.2 mg of clostripain in 1 mL of 0.1 M sodium phosphate, pH 7.6, and 2.5 mM dithiothreitol for 12 h. The reaction products were separated by reversed-phase HPLC utilizing an acetonitrile/water gradient containing 0.1% trifluoroacetic acid. The purified fragment 109–133 was identified by complete N-terminal sequence analysis using a gas-phase sequencer (Applied Biosystems, Inc., Model 470 A protein sequencer). The purified fragment 109–133, when rerun on reverse-phase HPLC, was greater than 95% a single peak as detected by the UV absorbance at 214 nm.

Preparation of Peptide 96–112. This peptide (0.5 mmol) was synthesized on an Applied Biosystems 430A peptide synthesizer using standard methodology. The peptide was purified by reverse-phase HPLC utilizing a Vydak C-4 column (20-30 μm, 300 Å). The identity of the peptide was determined by fast atom bombardment mass spectrometry (MH^+, m/z 1961), and the amino acid composition was confirmed by analysis on a Beckman 7300 analyzer after acid hydrolysis.

Peptide concentrations were measured by ninhydrin determination following alkaline hydrolysis (Rosen, 1957). Circular dichroism data were obtained on a Jasco J-500C spectropolarimeter. Estimates of protein secondary structure based on the circular dichroism spectra were made by using the CONTIN program that analyzes the circular dichroism spectrum directly as a linear combination of the circular dichroism spectra of 16 proteins whose secondary structures are known from X-ray crystallography (Provencher et al., 1978).

RESULTS

Helical Content of Peptide 96–133 Is Dependent on Peptide Concentration. Chen and Sonenberg (1977) reported that peptide 96–133 is helical in aqueous solutions. They showed

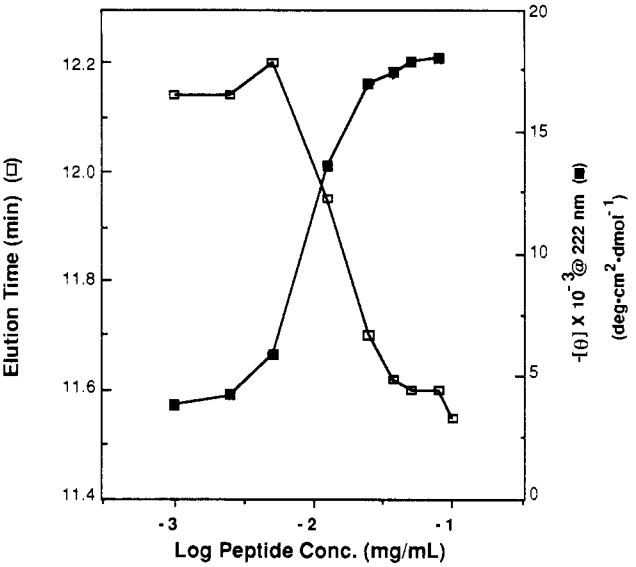

FIGURE 2: Effect of peptide 96–133 concentration on its helicity and elution from size-exclusion chromatography. The circular dichroism measurements were obtained in 1 mM sodium citrate, sodium phosphate, and sodium borate, at pH 4.5, and the size-exclusion results were obtained in 2 mM sodium citrate and 0.1 M NaCl at pH 4.5. The peptide concentrations for the size-exclusion results represent the concentrations of the samples used for injection and have been corrected for a 20-fold dilution that occurs upon chromatography.

that there was a helix to coil transition when the pH changed from 5 to 9. Figure 1 shows that the helical content of 96–133 is also dependent on peptide concentration. The pK_a of the helix to coil transition increased with the peptide concentration. Under all conditions studied, increased peptide concentration resulted in greater helicity. The concentration effect on the pH-induced helix to coil transition indicates that electrostatic interactions are involved in the association.

In order to more directly monitor the association reaction, the gel filtration of peptide 96–133 was studied as a function of concentration. As shown in Figure 2, as the peptide concentration was increased, the elution time from gel filtration decreased, indicating the formation of a species with a larger Stokes radius. After correction for the dilution effect that occurred upon gel filtration, the formation of a larger species directly correlated with an increased helix content. At high peptide concentration, the associated peptide has a limited size. At the most dilute peptide concentrations, the elution from gel filtration does not demonstrate a concentration dependence and retains approximately 15% helix as measured by circular dichroism (assuming 100% helix has a mean residue ellipticity of −30 000 deg·cm²·dmol⁻¹).

Preparation of Peptide 109–133. Limited proteolysis of 96–133 with clostripain resulted in cleavage at Arg–108. The elution profile from reverse-phase HPLC of the reaction products is illustrated in Figure 3. Since 96–133 contains a single Tyr at 110, the detection at 280 nm of the chromatogram is indicative of peptides containing the single Tyr. The peak that eluted at approximately 24 min (Figure 3) was peptide 109–133 as determined by amino acid sequence analysis by sequential Edman degradation. This peptide was then studied for helix formation.

Fragment 109–133 Is Helical in Aqueous Solution and Retains All of the Helix of the Larger Peptide 96–133. Under solution conditions for maximum helicity, the fragment 96–133 is approximately 60% helical. This could reflect that the helix is localized to 60% of the molecule or that 60% of the molecules are completely helical. The latter case represents a marginally stable equilibrium condition that might be easily perturbed

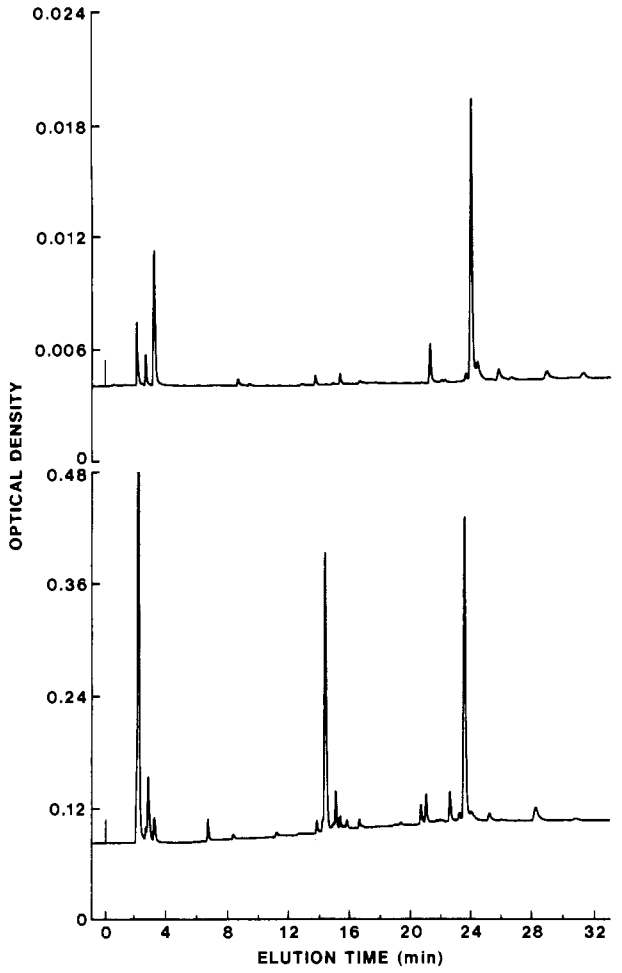

FIGURE 3: Reverse-phase HPLC of limited clostripain digestion of fragment 96–133. For both chromatograms, equal amounts of reaction products were injected, and the top chromatogram was detected by the absorbance at 280 nm and the bottom at 214 nm.

by small amounts of denaturant. The former case represents a stable helix that may show some stability toward small amounts of denaturant. Urea denaturation of the 96–133 helix demonstrated a pretransition plateau (unpublished data), suggesting that the helix is stable and probably localized to 60% of the molecule. Secondary structure predictions indicated that the region from 110–127 should be helical. It thus seemed possible to further localize the helix by removing the N-terminal segment (96–108). For this purpose, the truncated peptide 109–133 was prepared. Figure 4 shows the far-ultraviolet circular dichroism spectra of fragments 109–133 and 96–112 in aqueous solution. The spectrum of 109–133 is consistent with α-helix and demonstrates minima at 223 and 208 nm and a maximum at 193 nm. The spectrum of 96–112 indicates that it is not helical. Also illustrated in Figure 4 is a computer-simulated fit for a peptide containing 88% helicity that is in good agreement with the spectrum of 109–133. Similar analysis of 96–133 under conditions of greatest helicity estimated 55% helicity. Truncation of 96–133 to 109–133 removes 34% of the molecule, and the total mean residue ellipticity increased 30%, thus indicating that the helix in 96–133 is localized to the 109–133 segment. These results show that the isolated N-terminal portion (96–112) of fragment 96–113 is nonhelical but the isolated C-terminal portion (109–133) is helical.

Helical Content of 109–133 Is Dependent on Peptide Concentration and pH. The effect of pH and concentration on the helicity of fragment 109–133 is demonstrated in Figure

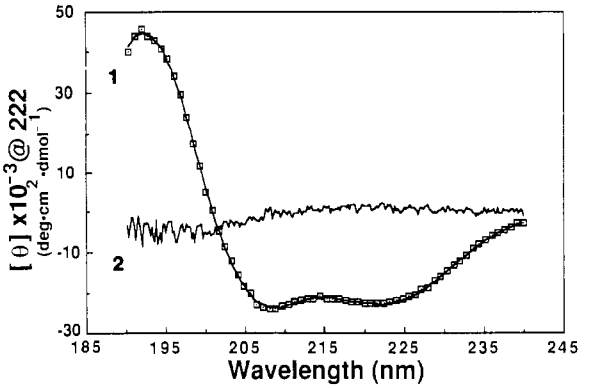

FIGURE 4: Far-ultraviolet circular dichroism spectra of fragments 109–133 and 96–112. The spectrum of 109–133 (□) was obtained in water, pH 4.5, at 0.0243 mg/mL. Line 1 represents a theoretical spectrum derived from the CONTIN program for a protein of 88% α-helix. Line 2 represents the spectrum of 96–112 in water, pH 4.5, at 0.0165 mg/mL.

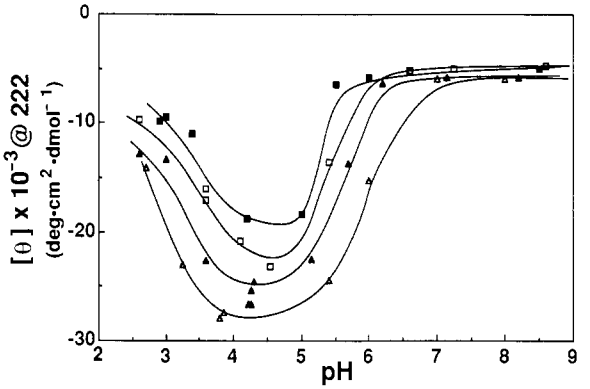

FIGURE 5: Effect of peptide concentration and pH on the helicity of fragment 109–133. The measurements were obtained at (△) 0.13, (▲) 0.05, (□) 0.02, and (■) 0.01 mg/mL. The solution conditions were identical with those of Figure 1.

5. At all peptide concentrations, the helix was maximally populated between pH 4 and 5. Variation of pH showed that protonation of one or more groups with a pK_a between pH 5 and 6 resulted in greater helix content, while protonation of one or more groups with a pK_a between pH 3 and 4 decreased the helix. At all pHs below alkaline, the helix content was directly dependent on peptide concentration. At pHs greater than 7, the helix content demonstrated approximately 15% helix (assuming 100% helix has a mean residue ellipticity of −30 000 deg·cm^2·dmol^{-1}) and appears independent of peptide concentration. Addition of 6 M guanidine hydrochloride to the peptide at pH 8 diminishes the mean residue ellipticity to less than −500 deg·cm^2·dmol^{-1}, indicating that the mean residue ellipticity of −5000 deg·cm^2·dmol^{-1} observed in alkaline pH at all peptide concentrations is significant. Similar to 96–133, the pK_a of the helix to coil transition varied with concentration, indicating that electrostatic interactions are involved in the association of fragment 109–133.

Discussion

About 20 years ago, several laboratories studied the problem of whether peptides could form α-helix in aqueous solution, and the answers to these investigations were usually negative (Epand & Scheraga, 1968; Taniuchi & Anfinsen, 1969). Short peptides are predicted not to show measurable helix formation in water according to the Zimm–Bragg equation (Zimm & Bragg, 1959) and the "host–guest" studies of α-helix formation using random copolymers of amino acids (Sueki et al., 1984). Two exceptions of small peptides (less than 40 residues) derived from globular proteins that are known to have

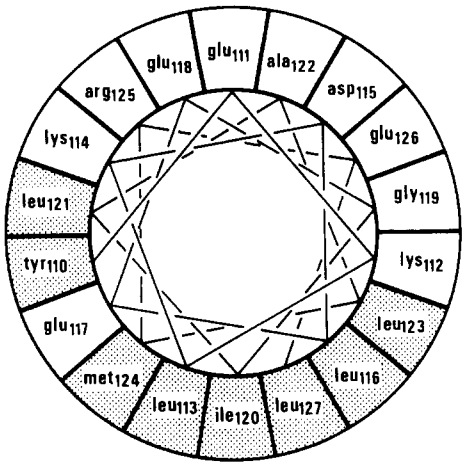

FIGURE 6: Axial projection of the potential α-helix structure of residues 110–127. The top side of the figure illustrates the hydrophilic surface, and the bottom side of the figure illustrates the hydrophobic surface of the putative amphiphilic helix.

helical structure in aqueous solution are the S-peptide (and its derivatives) from ribonuclease S (Brown & Klee, 1971; Bierzynski et al., 1982; Kim et al., 1982; Gallego et al., 1983; Shoemaker et al., 1985, 1987; Rico et al., 1986) and the active fragment 96–133 from bGH (Chen & Sonenberg, 1977). Studies of S-peptide and its derivatives have indicated that its unusual helix stability can be attributed to a favorable interaction between charged residues of the peptide and the helix dipole. Our studies of helix 109–133 show that it is concentration and pH dependent. Figure 2 demonstrates that as the concentration was increased, the peptide increased in both molecular weight and helical content. The concentration dependence of the helix indicates that intermolecular interactions are important to helix stability. At dilute peptide concentrations (Figure 2) and at alkaline pH (Figure 5), the helix does not show a concentration dependence but does have measurable helicity. The data suggest that the monomer is partially helical and that increased intermolecular interactions enhance the helix stability. Secondary structure predictions using the Chou–Fasman (1974) and Robson (1971) algorithms predict that residues 110–127 of bGH have a high propensity for α-helix formation. Modeling of this helix indicates that it is likely amphipathic (Figure 6). The increase in helicity that occurred upon association could result from intermolecular packing between hydrophobic surfaces of the amphipathic helices. The pH dependence indicates that electrostatic interactions are also important for helix stability. The pH dependence showed maximum helix stability between pH 4 and 5. Figure 5 shows that protonation of one or more charged groups between pH 5 and 6 resulted in an increase in helicity and that protonation of one or more different charged groups with a pK_a between 3 and 4 decreased helicity. The only charged residues of the peptide with pK_a's in this pH range are the following: Glu-111, -117, -118, -126, and -128; Asp-115 and -129; and the C-terminal carboxyl. The ionized Glu-126 and -128, Asp-129, or the C-terminal carboxyl would be expected to have unfavorable interactions with the helix dipole (Blagdon & Goodman, 1975). Protonation of one or more of these groups would thus have a helix-stabilizing effect. At the N-terminus of the helix, ionized Glu-111 would be expected to have favorable interactions with the helix dipole. Protonation of this group would thus have a helix-destabilizing effect. The model of the predicted amphipathic helix (Figure 6) indicates that Glu-117 invades the hydrophobic face and could potentially interfere with an ideal hydrophobic surface. Protonation of Glu-117 may relieve this interference and could stabilize helicity by allowing preferred intermolecular hydrophobic packing. Although highly speculative, these proposed charge effects could explain the observed results.

It is of interest to compare the results of fragment 96–133 to 109–133. Previous workers have identified that fragment 96–133 forms a stable helix in aqueous solution (Chen & Sonenberg, 1977). They showed that 96–133 undergoes a helix to random coil transition between pH 5 and 10. Our studies show that the pH dependence of this helix to coil transition is also affected by the peptide concentration (Figures 1 and 5). Increased concentrations of the peptide enhanced helix stability and increased the pK_a of the pH-dependent transition. These data demonstrate that association can have large effects on the pK_a of this helix to coil transition. Comparing the helical content of 96–133 to 109–133 under conditions for maximal helix population (55% to 88%, respectively) would suggest that the helix of fragment 96–133 is localized within segment 109–133. Fragment 96–112 was void of measurable helix which further suggests that the region 96–108 does not directly affect the helix. The pH dependence of the helix in fragments 96–133 and 109–133 is different (Figures 1 and 5). It is possible that the position of the N-terminal amine relative to the helix may be important to helix stability.

An inevitable question is the following: What relationship is there between structure formed by a fragment and structure in the intact protein? For S-peptide from ribonuclease S, studies have shown that the helix is limited to certain residues, including ones that are helical in the intact protein, and that a functional helix termination signal exists in the isolated peptide (Kim & Baldwin, 1984). Synthetic analogues of S-peptide of different helix stabilities were reconstituted with S-protein, and the resulting thermostabilities of the semisynthetic ribonuclease S were studied (Mitchinson & Baldwin, 1986). In all cases, the thermostability of the semisynthetic ribonuclease S was related to the helical stability of the isolated peptide. These observations suggest that information gained from fragment studies will be relevant to intact protein.

How proteins fold up has long been a mystery. Folding studies of bGH demonstrate that folding is a sequential process with populated equilibrium and kinetic intermediates. These intermediates are characterized by the lack of tertiary structure but having substantial secondary structure (α-helix). These folding intermediates indicate that the helical structure of bGH is unusually stable, even in the absence of tertiary structure. The folding results are consistent with a simple framework model for protein folding in which the amino acid sequence directly determines the position of the α-helix and β-sheets, and these secondary structures then pair to form the tertiary fold. It may be envisaged that the pairing of secondary structures and the formation of the tertiary fold increase the overall stability of the secondary structure. Our observations that fragment 109–133 has stable helical structure in aqueous solution are consistent with that predicted from the folding studies. Since helix 109–133 can form in the absence of the rest of bGH, it quite likely represents a region of the intact protein that serves as part of the helical framework for an early folding intermediate. In dilute peptide concentrations, or alkaline pH, the helix 109–133 is partially stable and does not show a dependence on peptide concentration. At nonalkaline pH, the helix is further stabilized by helix pairing as a result of increased peptide concentration. We suggest that *folding* of intact bGH occurs by initial formation of helical structure (of which helix 109–133 is part) and becomes increasingly more stable as helices pair. The self-association observed for

helix 109–133 may reflect indirectly the helix pairing that occurs in bGH.

Equilibrium folding studies of bGH have shown that, under conditions for partial denaturation, association occurs (Havel et al., 1986; Brems et al., 1986). The residual helical structure present in the partially denatured species was implicated in the mechanism of association. It was found that the fragment 96–133 was able to decrease the population of the associated intermediate. To explain these results, we suggested that in the native state the hydrophobic face of the amphipathic helix 110–127 is packed in a lipophilic environment. Upon partial denaturation, the hydrophobic face of helix 110–127 becomes exposed. This metastable form is the species from which association occurs. The association is stabilized by the hydrophobic interactions resulting from intermolecular packing of the lipophilic faces of the helices. The results reported here in which both fragment 96–133 and fragment 109–133 self-associate are consistent with the results reported on the associated intermediate. If the associated equilibrium intermediate is shown to be important to the folding pathway, then the self-association of helix 109–133 would be directly relevant.

The implications of these observations for studying the mechanism of protein folding are considerable. Progress in the field has been held up by the difficulty of finding folding intermediates whose structures can be characterized (Baldwin, 1986). Characterizing structures of a peptide like 109–133 is not a serious problem provided that there is a single dominant structure. Thus, the way may be open for analyzing the structure of folding intermediates of bGH and finding out what interactions stabilize these structures at the beginning of the folding pathway. To this end, high-field NMR is currently being used to study the structure of fragments 96–133 and 109–133.

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Registry No. bGH 109–133, 110904-77-3; bGH 96–133, 37239-89-7; bGH 96–112, 110875-12-2.

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