Characterization of Tertiary Interactions in a Folded Protein by NMR Methods: Studies of pH-Induced Structural Changes in Human Growth Hormone†

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ABSTRACT: The pH-induced conformational changes in human growth hormone (hGH) have been studied, using a new quantitative NMR approach that combines ^{13}C labeling of specific backbone carbonyl carbons with a complete spectral analysis of the corresponding ^{13}C resonances. Thus, a complete analysis of the carbonyl resonances of the 26 Leu residues of hGH and their variation with pH provided detailed information about the equilibrium folding processes of the protein, including information about the kinetics of the folding. By combining this information with the pH dependence of readily identifiable ^{1}H resonances, the pH-induced changes observed in the carbonyl carbon spectra can be associated with specific regions in the protein and can be ascribed to a series of localized adjustments in the tertiary structure, brought about by changes in the hydrogen bond interactions or electrostatic interactions between different residues in the globular folded protein. The preexchange lifetimes of these adjustments range from a fraction of a millisecond to a few milliseconds.

Understanding the principles of protein folding has been a challenge of long standing in the area of molecular biology. According to the framework model (Kim & Baldwin, 1982) the protein folding process involves the early formation of hydrogen-bonded secondary structures. These events are then followed by reorganization to yield tightly packed domains constituting the tertiary structure. The tertiary structure is stabilized by interactions between different elements of the secondary structure. These interactions are defined by only a few residues, and whereas amino acid substitution at other residue positions are allowed, even minor changes at sites directly involved in the tertiary interactions may affect the folding of the protein drastically (Hughson & Baldwin, 1989). Changing the charge distribution in the protein by changing pH generally affects the stability of the protein and may lead to an unfolding of the native state (Nall et al., 1988).

Like the other members of the family of growth hormones, the globular structure of human growth hormone (hGH)[1] has been shown to be sensitive to changes in pH. The pH-induced changes of hGH have been found to be reversible (Aloj & Edelhoch, 1972; Turner et al., 1983), but unlike bovine growth hormone (Holzman et al., 1990) there does not seem to be any indications of a general unfolding of the secondary structure of hGH (Bewley & Li, 1967; Aloj & Edelhoch, 1972). Thus, the conformational changes can be considered a reorganization of the globular structure imposed by a change in favorable tertiary interactions. With the presence of different stable tertiary structures within virtually the same framework of secondary structures, hGH offers the opportunity to study in detail the stabilizing interactions responsible for the second stage in the folding within the framework model.

For the investigation of the conformational changes in hGH we here present a new quantitative NMR approach that combines specific isotopic labeling with a complete spectral analysis. Thus, it is shown that specific isotope labeling of the backbone carbonyl carbons of the leucine residues of hGH allows the structural changes to be monitored at 26 different sites distributed throughout the protein, and it is demonstrated that a complete analysis of the pH dependence of the ^{13}C NMR spectrum can provide specific information about the equilibrium folding processes in hGH, including information about the kinetics. Even though the individual signals from the carbonyl carbons of the 26 leucine residues have not yet been assigned, it is possible, in combination with titration data obtained from ^{1}H NMR spectra of hGH, to correlate the structural changes with specific regions of the protein.

MATERIALS AND METHODS

Unlabeled, authentic hGH was prepared by recombinant DNA technology according to Dalbøge et al. (1987). Biosynthetic hGH, labeled with ^{13}C in the carbonyl carbons of the 26 leucine residues, was purified from *Escherichia coli* strain MC1061 auxotrophic for the amino acid leucine. Fermentation with [1-^{13}C]leucine (99% Stohler/Kor) as the only amino acid added to the medium, and purification and removal of the amino terminal extension were carried out as described previously (Christensen et al., 1992, 1986; Dalbøge et al., 1987). As judged by IE-HPLC, the product was more than 99% pure.

Samples for proton NMR measurements were prepared by dissolving 20 mg of recombinant hGH in 1.0 mL of D_2O, giving a final concentration of 0.9 mM hGH. Samples for ^{13}C NMR were prepared by dissolving 15 mg of 99%

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[1] Abbreviations: hGH, human growth hormone; pGH, porcine growth hormone; NMR, nuclear magnetic resonance; FID, free induction decay; TMS, tetramethylsilane; WALTZ, wideband alternating-phase low-power technique for zero residue splitting; NOESY, two-dimensional NOE spectroscopy; NOE, nuclear Overhauser enhancement; TOCSY, total correlation spectroscopy; COSY, two-dimensional correlation spectroscopy; CD, circular dichroism; IE-HPLC, ion-exchange high-performance liquid chromatography; ppm, parts per million; SD, standard deviation.

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[1-^{13}C]Leu-hGH in 1.5 mL of D_2O (99.9%, Norsk Hydro). Lower concentrations were used near the isoelectric pH of 4.9 (Bewley & Li, 1975). The pH was determined at 24 °C by a Radiometer PHM80 meter equipped with a 3-mm glass electrode. Reported values refer to direct meter readings (Kalinichenko, 1976). The solution pH was adjusted with dilute DCl or NaOD, and pH measurements were performed before and after data acquisition. Measurements of pH agreed to within 0.05 unit before and after spectral acquisition. The ionic strength of the samples varied from 0.010 to 0.100 M during the titration process.

NMR experiments were performed on a Bruker AM 500 spectrometer, operating at 500.13 MHz for ^{1}H nuclei and at 125.76 MHz for ^{13}C nuclei. One-dimensional proton spectra were recorded at 293 K and 305 K. Residual HOD was suppressed by saturation prior to data acquisition. Proton chemical shifts are expressed relative to TMS and were measured with respect to internal 1,4-dioxane at 3.74 ppm. The carbon-13 spectra were recorded at 293 K, with a spectral width of 10 000 Hz in 8192 data points. Broadband proton decoupling was applied using a WALTZ-decoupling sequence (Shaka et al., 1983), and a decoupler power of ca. 0.5 W. The number of averaged transients for each spectrum varied from 12 900 to 47 200. Carbon-13 chemical shifts are referenced relative to external TMS.

The pH dependence of the ^{13}C NMR spectra was analyzed by the method of concerted nonlinear least-squares fitting (Abildgaard et al., 1991), i.e., a simultaneous fit to all spectra in the titration data set, using the analytical expression for discrete Fourier-transformed NMR spectra (Abildgaard et al., 1988). The spectra were scaled to the same overall integrated intensity prior to the least-squares analysis and weighted according to their relative noise level. Including a possible pH dependence of the chemical shift and the line width, the applied expression takes the form

$$\hat{S}_n = \hat{S}(\nu_n, \mathrm{pH}) = \sum_{l=1}^{L} A_l T e^{i\phi_l} \frac{1 - e^{[i2\pi[\nu_{0,l}(\mathrm{pH}) - n/N_1T] - R_{2,l}(\mathrm{pH})]NT}}{1 - e^{[i2\pi[\nu_{0,l}(\mathrm{pH}) - n/N_1T] - R_{2,l}(\mathrm{pH})]T}} \quad (1)$$

Here A is the signal intensity, N is the number of data points in each FID sampled at intervals T, and N_1 is the number of data points describing the Fourier-transformed spectrum. Each signal is characterized by its resonance frequency, ν_0, the effective transverse relaxation rate, R_2, and the phase parameter, ϕ. In the description of the carbonyl spectra we assume that each carbonyl carbon can exist in one of two interconverting conformations, A and B, characterized by resonance frequencies ν_{0A} and ν_{0B}, respectively. If exchange between the two sites is fast on the NMR time scale, only an average signal will be observed with the resonance frequency given by

$$\nu_0(\mathrm{pH}) = \nu_{0A} x_A + \nu_{0B}(1 - x_A) \quad (2)$$

$$x_A = \frac{1}{1 + 10^{\mathrm{pH} - \mathrm{p}K_a}} \quad (3)$$

where K_a is the apparent ionization constant for the titration. In the region of fast exchange the line width of the observed signal is given by (Kaplan & Fraenkel, 1980)

$$\Delta\nu_{1/2} = x_A \Delta\nu^A_{1/2} + x_B \Delta\nu^B_{1/2} + 4\pi x^2{}_A x^2{}_B (\Delta\nu_{AB})^2 \tau \quad (4)$$

where $\Delta\nu^A_{1/2}$ and $\Delta\nu^B_{1/2}$ are the line widths in the two forms A and B, respectively, while $\Delta\nu_{AB}$ is the chemical shift difference in Hz between the two sites A and B. Here $\tau^{-1} = x_A/\tau_A$, where $\tau_A^{-1} = k_A$ is the pH independent, first-order rate constant for the interconversion of A to B. Therefore eq 4 can be rewritten as

$$\Delta\nu_{1/2} = x_A \Delta\nu^A_{1/2} + x_B \Delta\nu^B_{1/2} + 4\pi x_A x^2{}_B (\Delta\nu_{AB})^2 k_A^{-1} \quad (5)$$

Thus, the measured exchange rate is the rate of exchange between the conformational states at low and high pH, respectively, of the individual carbons within hGH. Consequently, each resonance is fully described by eight parameters: the chemical shift of the two sites, δ_A and δ_B; the apparent pK_a; the mean preexchange lifetime, τ_A; the intensity of the signal, A; the line width in the two sites, $\Delta\nu^A_{1/2}$ and $\Delta\nu^B_{1/2}$; and the phase, ϕ.

The pH dependence of the ^{1}H NMR chemical shifts was analyzed by a nonlinear least-squares fit of the equation describing a three-step titration

$$\delta_{obs} = \delta_1 x_1 + \delta_2 x_2 + \delta_3 x_3 + \delta_4 x_4 \quad (6)$$

where δ_{obs} is the observed chemical shift at any pH, while δ_1–δ_4 are the chemical shifts of the four species present with molar fractions x_1–x_4, respectively. For p$K_{a1} \ll$ p$K_{a2} \ll$ pK_{a3}, the molar fractions are given by

$$\begin{aligned} x_1 &= 1 - \frac{1}{1 + 10^{\mathrm{p}K_{a_1} - \mathrm{pH}}} \\ x_2 &= \frac{1}{1 + 10^{\mathrm{p}K_{a_1} - \mathrm{pH}}} - \frac{1}{1 + 10^{\mathrm{p}K_{a_2} - \mathrm{pH}}} \\ x_3 &= \frac{1}{1 + 10^{\mathrm{p}K_{a_2} - \mathrm{pH}}} - \frac{1}{1 + 10^{\mathrm{p}K_{a_3} - \mathrm{pH}}} \\ x_4 &= \frac{1}{1 + 10^{\mathrm{p}K_{a_3} - \mathrm{pH}}} \end{aligned} \quad (7)$$

where K_{ai} is the apparent ionization constant of the group represented by the subscript i. A possible cooperativity of the dissociation can be analyzed by a modified form of the Hill equation, which for a two-state transition takes the form (Markley, 1973)

$$\delta_{obs} = \delta_1 x_1 + \delta_2 (1 - x_1) \quad (8)$$

where

$$x_1 = \frac{1}{1 + 10^{n(\mathrm{pH} - \mathrm{p}K_a)}} \quad (9)$$

and the Hill coefficient n is a convenient indication of the cooperativity.

For partial assignment of proton resonances, ^{1}H–^{1}H NOESY, TOCSY, and COSY NMR spectra were acquired at 293 K. The NOESY and TOCSY spectra were recorded with hGH dissolved in H_2O at pH 3.6. The COSY spectrum was recorded with hGH dissolved in D_2O at pD 3.3. A mixing time of 200 ms was used for the NOESY spectrum. The TOCSY spectrum was recorded using a spin-lock period of 22 ms.

RESULTS

Titration of Leucine ^{13}C-Labeled Carbonyl Resonances. The carbonyl region of the ^{13}C NMR spectrum of [1-^{13}C]-Leu-hGH is shown in Figure 1. Significant changes occur in the spectrum when the pH is lowered from neutral to acidic, giving rise to a completely different appearance of the spectrum. The spectral changes associated with the titrations were found to be fully reversible under the conditions used in this study. At increased ionic strength (close to 0.1

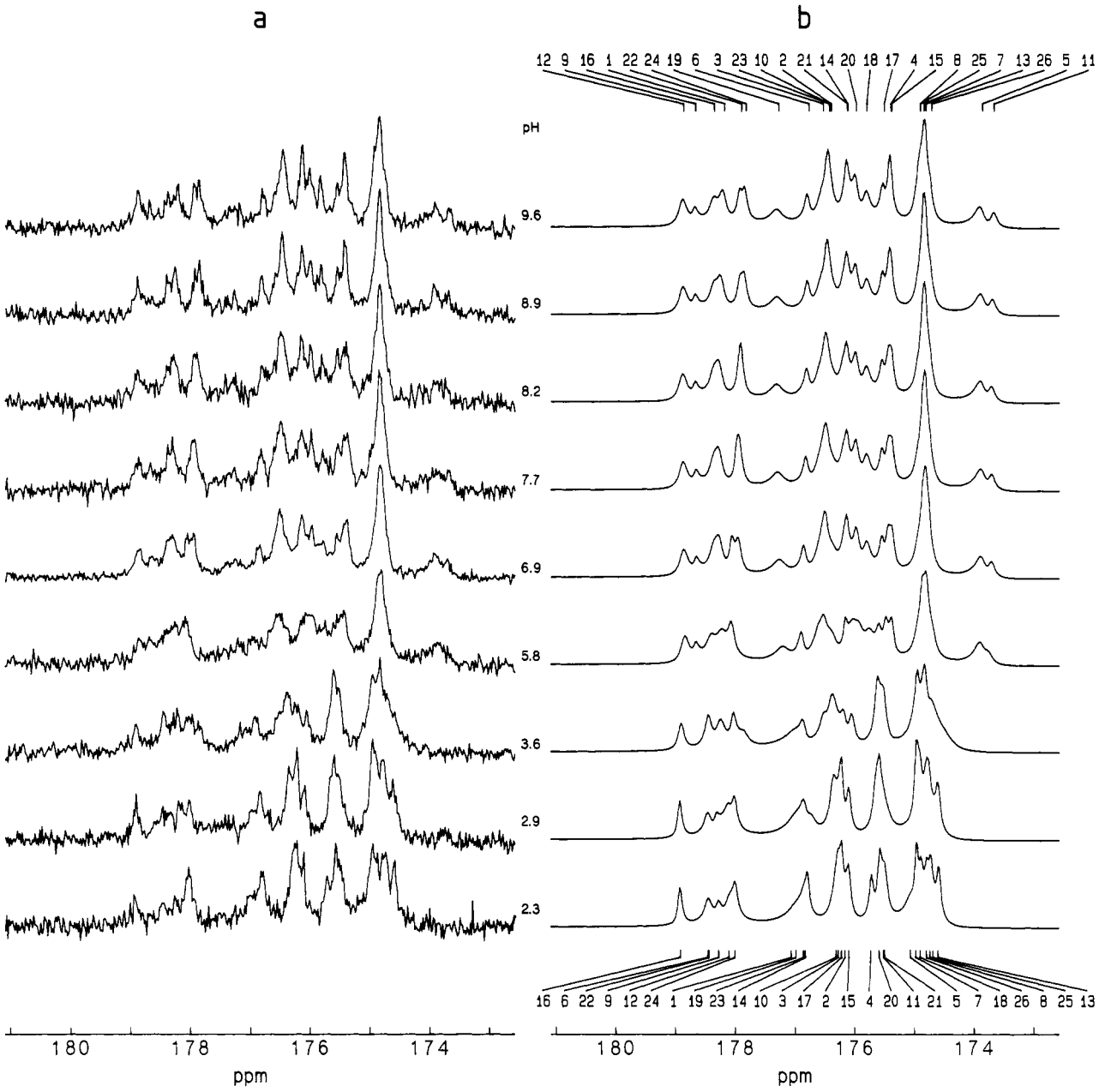

FIGURE 1: (a) Carbonyl region of the 125.76 MHz ^{13}C NMR spectrum of [1-^{13}C]Leu human growth hormone at various pH values in D_2O at 293 K. (b) Corresponding spectra calculated from the parameters derived from the experimental data in (a) by nonlinear least-squares fit and presented in Table I. All spectra are phase corrected. The position at the two extreme pH values of each of the 26 Leu carbonyl signals are indicated in the calculated spectra.

M), however, the titration curves did not show complete reversibility in the acidic range. This could be due to a shift in pK_a of the ionizable groups, resulting from the increased ionic strength (Russell & Fersht, 1987). However, at an even higher ionic strength (approximately 0.2 M) the acidic form of hGH was found to associate, as evidenced by an insoluble precipitate. Therefore, it is possible that the irreversible titrations observed for hGH at high ionic strength are due to a partly aggregation. Similar observations have been made for the acid-transformed bovine growth hormone (Burger et al., 1966). Also this growth hormone shows a reversible molecular transition at low salt concentrations (<0.1 M), while aggregation and precipitation occur at higher salt concentrations (>0.1 M) and low pH.

To test whether the observed spectral changes of hGH at low ionic strength and acidic pH also can be ascribed to an aggregation, a series of ^1H NMR spectra were obtained over a concentration range of 0.01–1.0 mM at pH 3.1. However, no measurable change in either the line width or the chemical shift of the signals was observed over this concentration range, implying that hGH does not aggregate significantly under the conditions of the NMR experiments. Also the reversibility of the spectral changes during forward and backward titrations shows that variations in the salt concentration below 0.1 M do not lead to any observable shifts in the pK_a values of the ionizable groups. Consequently, the observed spectral changes are neither due to aggregation or to any other salt-dependent effect on hGH.

Examination of the pH dependence of the leucine carbonyl ^{13}C NMR spectra shows that the majority of the resonance positions shift considerably with pH and that the line width of several of the signals vary significantly. On the other hand, a precise monitoring of these pH-induced changes by simple inspection is not possible due to the substantial overlap in the spectra. However, by a direct and concerted nonlinear least-squares analysis (Abildgaard et al., 1991) of all the spectra

in Figure 1a, using the theoretical model (eqs 1–3 and eq 5) that describes the spectra and their pH dependence, the available spectral and kinetic information as well as the apparent pK_a values can be extracted from the data with the maximum obtainable precision. As demonstrated previously (Abildgaard et al. 1991), this approach improves the ability to retrieve the parameters for overlapping resonances considerably and minimizes the number of independent parameters that describe the spectra. Thus, the 26 Leu carbonyl signals in Figure 1a, and their variations as a function of pH, are described by only 208 parameters, while the spectra are defined by 5900 spectral data points. It should be noted that the analysis also includes the imaginary part (not shown in Figure 1) of the spectra; that is, all the available spectral information is included in the analysis (Abildgaard et al., 1988). It should also be emphasized that, in the concerted analysis applied here, the signal-to-noise ratio is no longer related to the individual spectra. Rather, it is the signal-to-noise ratio of the total data set that matters.

The result of the analysis is presented in Figure 1b and in Table I. Despite the low signal-to-noise ratio in the individual spectra, estimates of all the 208 parameters that describe the 26 individual Leu carbonyl carbons and their variation with pH could be derived. This includes estimates of the titration parameters, despite the relatively large pH increments in these data. This is particularly important here, where the low solubility near the isoelectric point prohibits the recording of useful ^{13}C spectra in the region around pH 4.9. It should be noted, however, that the use of relatively few points on the pH axis necessitates a higher precision of the pH measurements, especially for the determination of pK_a. Thus, it is likely that the reported standard errors for the pK_a's in Table I, derived by assuming that the errors in the pH measurements are negligible, are somewhat optimistic. More reliable standard errors may be obtained by the method of total least-squares analysis (Golub & van Loan, 1980). On the other hand, given a certain accuracy in the pH measurements, the lack of observations close to the transition zone does not necessarily prohibit a good estimation of the pK_a values, provided that the precision of the chemical shifts is sufficiently high. In fact, close to the transition pH, a high precision in the chemical shift values may be poorly utilized unless the accuracy of the pH measurements are unusually high.

The change in chemical shift, $\Delta\delta$, caused by the deprotonation can be ascribed to a combination of structural changes and proximal electrostatic interactions (Saitô, 1986). Furthermore, studies of proteins with virtually pH-invariant structures (Tüchsen & Hansen, 1988) show that electrostatic interactions with ionizable groups produce only minor changes in the carbonyl carbon chemical shifts (<0.1 ppm). In contrast, the shifts presented in Table I fall, with only a few exceptions, in the range from 0.2 to 1.8 ppm. In addition, the rate constants that are obtained for the exchange processes associated with the interconversions are substantially slower than direct ionization events ($\tau < 500\ \mu s$; Eigen et al., 1960; Eigen, 1963). Therefore, the observed changes of the chemical shifts can be attributed to changes in the microscopic environment of the carbonyl carbons, caused by pH-induced changes in the protein structure.

The parameters presented in Table I, correspond to a single apparent titration. It is possible, however, that the leucine carbonyl ^{13}C chemical shifts are influenced by the titration of more than one acidic group. In principle, the applied model can be extended to cover this situation, but in the present case the small number of observations along the pH direction does not allow such a detailed description. Thus if multiple, apparent titrations occur, the derived parameters in Table I are average values of the parameters, characterizing each one of the titrations. As a consequence, parameters for such multiple structure transitions will be poorly defined, showing apparently large standard deviations for the calculated parameters. However, the ^{13}C shifts of carbonyl carbons are dominated by short-range interactions. Therefore, major changes in chemical shift of the signal correspond to local structural changes, implying that multiple, apparent titrations are less likely to occur.

Table I: Spectral Parameters (at 293 K) Derived for Carbonyl Carbon Resonances of Leucine Residues[a] in hGH by Nonlinear Least-Squares Analysis of Titration Data Shown in Figure 1a[b]

signal	δ_A (ppm)	δ_B (ppm)	$\Delta\delta$ (ppm)	pK_a	τ_A[c] (s)
1	176.79	178.266	−1.47	2.93	0.0013
	(0.14)	(0.004)	(0.14)	(0.18)	(0.0005)
2	176.13	176.378	−0.25	3.1	–
	(0.02)	(0.014)	(0.04)	(0.2)	
3	176.23	176.583	−0.36	3.14	–
	(0.02)	(0.013)	(0.03)	(0.08)	
4	175.763	175.376	0.386	3.26	0.0014
	(0.006)	(0.003)	(0.009)	(0.04)	(0.0005)
5	175.12	173.879	1.25	3.58	0.0007
	(0.02)	(0.007)	(0.02)	(0.04)	(0.0002)
6	178.50	176.870	1.63	3.77	–
	(0.03)	(0.004)	(0.04)	(0.03)	
7	174.978	174.867	0.111	3.78	0.042[d]
	(0.004)	(0.004)	(0.008)	(0.16)	(0.011)
8	174.732	174.938	−0.206	3.78	0.003
	(0.007)	(0.011)	(0.018)	(0.09)	(0.004)
9	178.26	178.644	−0.38	3.82	–
	(0.01)	(0.008)	(0.02)	(0.08)	
10	176.30	176.500	−0.20	3.97	–
	(0.01)	(0.009)	(0.02)	(0.14)	
11	175.54	173.718	1.82	4.16	0.0002
	(0.02)	(0.006)	(0.03)	(0.04)	(0.0008)
12	178.098	178.852	−0.76	4.28	0.0016
	(0.009)	(0.007)	(0.02)	(0.05)	(0.0005)
13	174.601	174.815	−0.21	4.3	0.071[d]
	(0.005)	(0.007)	(0.01)	(0.2)	(0.015)
14	176.829	176.15	0.68	4.48	0.0109[d]
	(0.007)	(0.02)	(0.03)	(0.11)	(0.0017)
15	176.098	175.433	0.665	4.55	0.0008
	(0.005)	(0.004)	(0.008)	(0.04)	(0.0003)
16	178.916	178.366	0.550	4.72	0.0027
	(0.004)	(0.005)	(0.008)	(0.05)	(0.0009)
17	176.217	175.540	0.677	4.84	0.0028
	(0.005)	(0.004)	(0.009)	(0.07)	(0.0007)
18	174.900	175.814	−0.914	4.87	0.0026
	(0.006)	(0.011)	(0.017)	(0.09)	(0.0006)
19	176.983	177.20	−0.22	4.90	–
	(0.014)	(0.03)	(0.04)	(0.35)	
20	175.586	175.982	−0.396	5.24	0.0044
	(0.003)	(0.004)	(0.007)	(0.09)	(0.0008)
21	175.496	176.179	−0.683	5.27	0.0006
	(0.008)	(0.006)	(0.014)	(0.04)	(0.0002)
22	178.437	177.918	0.519	5.52	–
	(0.007)	(0.011)	(0.018)	(0.04)	
23	176.858	176.458	0.401	5.60	0.0027
	(0.009)	(0.008)	(0.017)	(0.08)	(0.0007)
24	178.006	178.092	−0.086	5.97	0.009
	(0.004)	(0.006)	(0.010)	(0.14)	(0.006)
25	174.691	174.843	−0.152	6.08	0.004
	(0.014)	(0.006)	(0.021)	(0.17)	(0.004)
26	174.798	174.72	0.078	6.53	0.020
	(0.005)	(0.10)	(0.014)	(0.17)	(0.011)

[a] The signals are given in order of increasing apparent pK_a value of their pH-induced shift. [b] Figures in parentheses are the calculated standard errors. [c] Preexchange lifetimes too short to be detected within experimental error has been indicated by –. [d] The exchange rate does not fulfill the condition for fast exchange, indicating that two separate resonances should be observable. Thus, the lifetime, τ_a, and the pK_a value given for this signal is less reliable than might be deduced from the reported standard deviation.

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        10         20         30         40         50
FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ KYSFLQNPQT
        60         70         80         90        100
SLCFSESIPT PSNREETQQK SNLELLRISL LLIQSWLEPV QFLRSVFANS
       110        120        130        140        150
LVYGASDSNV YDLLKDLEEG IQTLMGRLED GSPRTGQIFK QTYSKFDTNS
       160        170        180        190
HNDDALLKNY GLLYCFRKDM DKVETFLRIV QCRSVEGSCG F
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FIGURE 2: Amino acid sequence of human growth hormone. The 26 leucine residues are underlined.

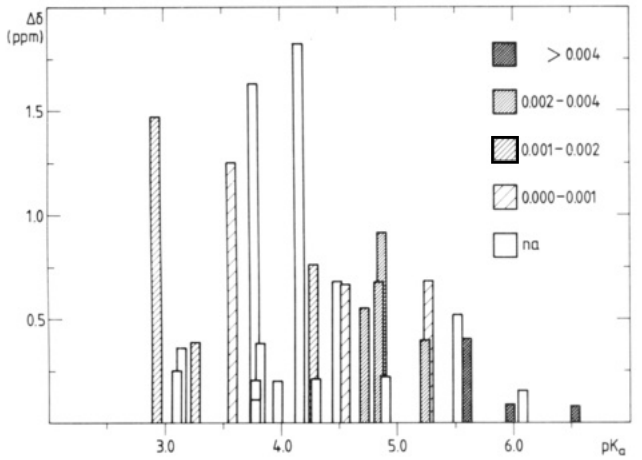

FIGURE 3: Summary of the apparent titration parameters for the carbonyl resonances given in Table I. The height of the bars corresponds to the magnitude of the pH-induced chemical shift. The pattern code shows the τ_A in seconds, as indicated (na = not available).

Table II: Leucine Signals from Table I Arranged in Groups with Identical pK_a Values[a]

group no.	signals no.	pK_a	τ_A (s)
I	1, 2, 3, 4	3.18	0.0013
II	5	3.58	0.0007
III	6, 7, 8, 9, 10	3.80	
IV	11	4.16	0.0002
V	12, 13	4.28	
VI	14, 15	4.53	
VII	16, 17, 18, 19	4.80	0.0027
VIII	20, 21	5.26	0.001
IX	22, 23	5.54	0.0027
X	24, 25	6.02	0.006
XI	26	6.53	0.02

[a] Signal numbers refer to the numbers in Table I.

The distribution of the 26 leucine residues in hGH is indicated in Figure 2. Although the leucine ^{13}C resonances have not yet been assigned to specific residues in the protein, it is clear that the structural changes involve most parts of the globular structure, considering the distribution of the leucine residues throughout the molecule and the general and extensive variation of the carbonyl spectrum with pH.

The data in Table I are summarized in Figure 3. It is clear from this figure that the structural transitions occur over a wide pH range. Since the carbonyl carbons, participating in the same structural transition, will have similar pK_a values, clusters are expected to be seen in the diagram. Taking the uncertainty of individual pK_a values into account, several groups of signals are found. For a signal to be a member of a certain group, the criterion has been used that the observed pK_a value must fall within 1 SD from the weighted average of the pK_a value of the group. The resulting groupings of the signals are shown in Table II.

Four resonances form a group (group I) that participates in a structural change with an apparent pK_a value of 3.18. As found for both resonances 1 and 4, the preexchange lifetime, τ_A, for the carbonyl carbons of the protonated form that are involved in this transition is on the order of 1.3 ms corresponding to a rate constant of $k_A = 770\ s^{-1}$. Five resonances form group III with a pK_a value of 3.80. The rate constant associated with this transition is too fast to be detected. The preexchange lifetime determined for resonance 7 is considered to be an artifact, due to the very small value of $\Delta\nu_{AB}$ (14 Hz) and the considerable crowding in this region of the spectrum. Four resonances form a distinct group, VII, with an average pK_a value of 4.80 and a τ_A close to 2.7 ms ($k_A = 370\ s^{-1}$). As for resonance 19, it is not clear whether this resonance is actually a member of this group. The large uncertainties of the spectral parameters describing resonance 19 indicate that it is subjected to more than one structural transition. Among the remaining signals no clear grouping can be found, suggesting that the observed spectral changes are either due to highly localized perturbations or are the effective, average values of two or more structural transitions. For the signals 24–26, only insignificant pH-induced shifts were observed.

Titration of Specific Proton Resonances. In order to correlate the pH-induced changes observed in the carbonyl carbon spectra with specific regions in the protein, the pH dependence of readily identifiable ^1H NMR signals in hGH were investigated. Two sets of titrations were performed (293 and 305 K, respectively). The ^1H NMR spectrum of hGH in D_2O at 305 K is shown in Figure 4, and the pH dependence of the chemical shifts at 293 K of the well-resolved resonances marked A–L are presented in Figure 5. Up to three significant, apparent titrations were fitted by using eqs 6 and 7. In cases where it would obviously lead to improvements in the fit, Hill coefficients were fitted as well. The parameters derived from the fitting of the data at 293 and 305 K are given in Tables III and IV.

The resonances A–C in Figure 4 have previously been assigned to the C2H imidazole-ring protons of the three histidines His-18, His-151, and His-21, respectively (Turner et al., 1983). The C2H signal of His-151 mainly follows a simple one-proton titration, with values of pK_a and chemical shifts virtually identical to the values found in small peptides and random-coil proteins (Markley, 1973; Bundi & Wüthrich, 1979). At 305 K a small, secondary shift with an apparent pK_a value of 8.1 is observed.

The C2H resonances of His-18 and His-21 show a more complex pH dependence. Apart from the major shift, caused by direct ionization of the imidazole ring, both signals are influenced by one or two more titrations (Tables III and IV). The titration of the imidazole ring of His-18, giving the major change in the chemical shift of the C2H proton signal, has an abnormally high pK_a value (7.686 at 293 K, and 8.16 at 305 K), in agreement with the previous suggestion (Turner et al., 1983) that this residue is involved in an ion-pair interaction with a *de*protonated carboxyl group. The value of the additional apparent titration, observed at acidic pH, shows that the interacting acidic group is likely to be an Asp or Glu side-chain carboxylate group with a correspondingly low pK_a value (3.39 at 293 K). For the primary titration of His-18, a significant improvement in the overall RMS deviation at 293 K was obtained using a variable Hill coefficient for the titration characterized by pK_{a3}. The fitted Hill coefficient of 2.55 (0.16) indicates that the titration of His-18 is not a simple one-proton event. However, at 305 K the Hill coefficient did not differ significantly from unity. A minor shift with an apparent pK_a value of 6.59 is observed for the His-18 C2H signal at 293 K. This value is close to the pK_a value for the

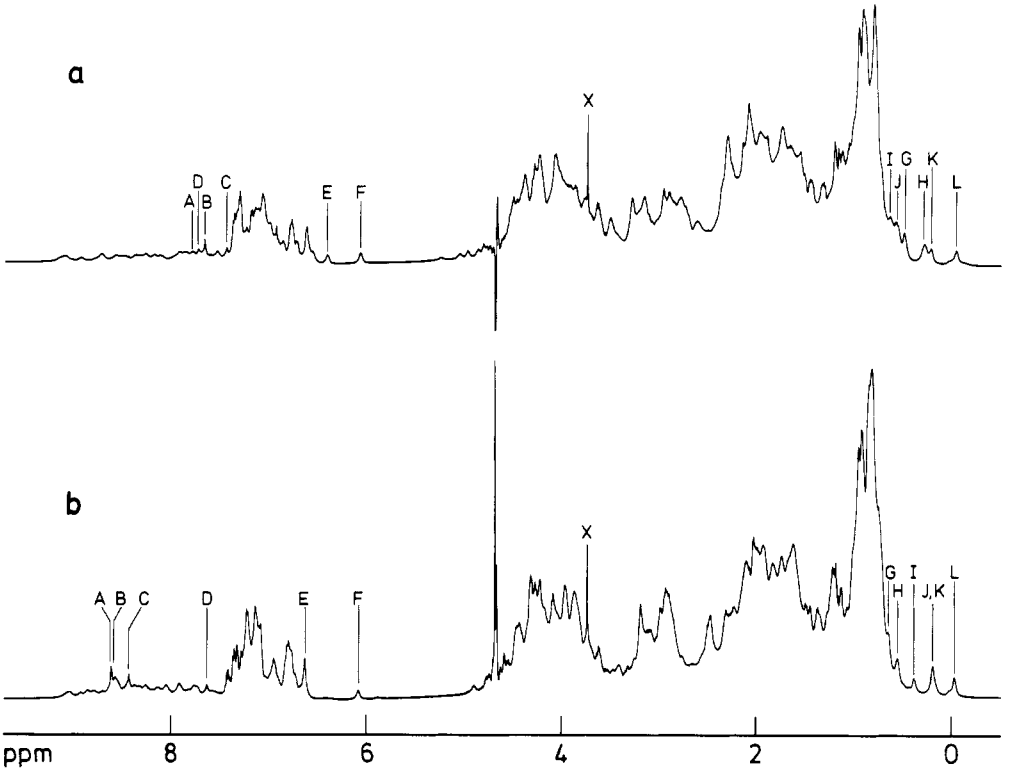

FIGURE 4: 500 MHz ^{1}H NMR spectra at 305 K of human growth hormone dissolved in D_2O at (a) pD 8.5 and (b) pD 2.1. The signal marked × is from dioxane.

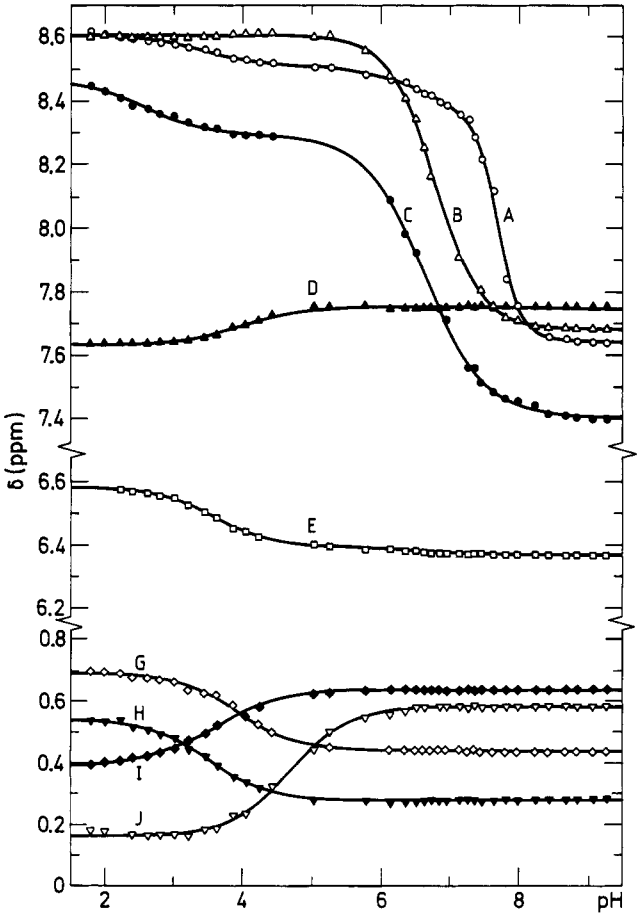

FIGURE 5: Dependence on pH at 293 K of the ^{1}H signals indicated in Figure 4. The solid lines correspond to the parameters given in Table III.

primary titration of His-21 (6.62 at 293 K), indicating an interaction between these two histidine residues. Similarly, at 305 K His-21 shows a titration with an apparent pK_a value (2.8) identical to the apparent, acidic pK_a value of His-18 and with a titration shift (approximately 0.2 ppm) that is even larger than for His-18; that is, the titration of the acidic group that interacts with His-18 has an even larger effect on the chemical shift of His-21. Again, this indicates that an interaction is taking place between the two histidine residues.

The low-field aromatic resonance D (7.63 ppm at pD 2.1, Figure 5) has an intensity of only one proton and shows a doublet fine structure at resolution enhancement. In the TOCSY spectrum recorded at pH 3.6 at 293 K (not shown), a connectivity with a proton at 6.92 ppm is observed. Thus, a possible assignment for resonance D is the C4H or the C7H ring proton of the only Trp residue in position 86. Other possibilities are the C2H or C6H of a rigid (not flipping) phenylalanine or one of the ring protons of a rigid tyrosine ring. However, no other J connectivities are observed for the signal scalar coupled to D, which might imply that D is belonging to a rigid tyrosine ring. At pH titration the signal follows a titration curve with a pK_a of 4.00 and 4.27 at 293 and 305 K, respectively, leading to an upfield shift of 0.1 ppm at low pH.

Resonance E (6.47 ppm at pH 3.6, 293 K) with an intensity corresponding to two protons was assigned to the C3,5H ring protons of a Tyr residue, with the C2,6H proton at 6.62 ppm. The chemical shift shows two pH-dependent perturbations; a primary upfield shift on deprotonation with a pK_a of 3.58 and a secondary transition with a pK_a of 6.42 (293 K). In the NOESY spectrum of hGH in H_2O, an NOE connection is seen between signals D and E. Signal E is also showing an NOE to the C2,6H protons of an unnamed phenylalanine residue with the chemical shift of C2,6H, C3,5H, and C4H at 7.14, 6.80, and 7.40 ppm, respectively. The proton in E that is coupled to D also has an NOE to the C4H proton of the unnamed phenylalanine residue, indicating a close contact between the side chains of the two tyrosines, D and E, and the

Table III: Ionization Constants and Chemical Shifts[a] for the ^1H Resonances of hGH at 293 K

	signal	δ_1 (ppm)	pK_{a1}	δ_2 (ppm)	pK_{a2}	δ_3 (ppm)	pK_{a3}	δ_4 (ppm)
A[b]	His-18, C2H	8.607 (0.004)	3.39 (0.08)	8.508 (0.003)	6.59 (0.11)	8.346 (0.019)	7.686 (0.018)	7.643 (0.002)
B[c]	His-151, C2H	8.6039 (0.0016)	6.779 (0.008)	7.681 (0.002)				
C	His-21, C2H	8.457 (0.016)	2.69 (0.14)	8.286 (0.007)	6.623 (0.019)	7.407 (0.005)		
D	Tyr, C2,6H	7.6348 (0.0013)	4.00 (0.03)	7.7518 (0.0007)				
E	Tyr, C3,5H	6.5832 (0.0015)	3.580 (0.018)	6.3921 (0.0017)	6.42 (0.11)	6.3690 (0.0007)		
F	Tyr, C3,5H	6.076 (0.010)						
G	Leu, CH_3	0.691 (0.002)	3.91 (0.02)	0.4402 (0.0010)				
H	Val/Leu, CH_3	0.5413 (0.0005)	3.487 (0.015)	0.2780 (0.0010)				
I	Ile, CH_3	0.388 (0.003)	2.65 (0.05)	0.427 (0.008)	3.72 (0.04)	0.6356 (0.0007)		
J	Val, CH_3	0.1625 (0.0019)	4.68 (0.03)	0.5825 (0.0012)				
K	Leu, $C^{\gamma 1}H_3$	0.198 (0.008)						
L	Leu, $C^{\gamma 2}H_3$	− 0.065 (0.011)						

[a] Parameters were obtained by nonlinear least-squares fit of eqs 6 and 7. Figures in parentheses are the calculated standard error derived from the diagonal elements of the variance–covariance matrix. [b] Parameters obtained with a fitted Hill coefficient of 2.55 (0.16) for pK_{a3}. [c] Parameters obtained with a fitted Hill coefficient of 1.29 (0.02) for pK_{a1}.

Table IV: Ionization Constants and Chemical Shifts[a] for the ^1H Resonances of hGH at 305 K

	signal	δ_1 (ppm)	pK_{a1}	δ_2 (ppm)	pK_{a2}	δ_3 (ppm)	pK_{a3}	δ_4 (ppm)
A	His-18, C2H	8.625 (0.015)	2.8 (0.4)	8.54 (0.04)	4.8 (0.5)	8.412 (0.009)	8.16 (0.03)	7.551 (0.011)
B	His-151, C2H	8.593 (0.004)	6.83 (0.04)	7.72 (0.04)	8.1 (0.3)	7.627 (0.006)		
C	His-21, C2H	8.472 (0.008)	2.78 (0.07)	8.260 (0.006)	6.65 (0.03)	7.477 (0.019)	8.3 (0.3)	7.405 (0.007)
D	Tyr, C2,6H	7.6268 (0.0012)	4.50 (0.05)	7.7270 (0.0008)				
E	Tyr, C3,5H	6.600 (0.006)	3.98 (0.07)	6.430 (0.007)	6.4 (0.4)	6.4033 (0.0016)		
F	Tyr, C3,5H	6.071 (0.012)						
G	Leu, CH_3	0.651 (0.002)	3.3 (0.3)	0.71 (0.04)	4.16 (0.12)	0.508 (0.005)	6.3 (0.3)	0.483 (0.001)
H	Val/Leu, CH_3	0.568 (0.002)	3.703 (0.019)	0.2811 (0.0010)				
I	Ile, CH_3	0.380 (0.007)	2.7 (0.5)	0.42 (0.02)	4.01 (0.07)	0.6358 (0.0012)		
J	Val, CH_3	0.1985 (0.0008)	5.20 (0.04)	0.531 (0.012)	7.0 (0.5)	0.549 (0.001)		
K	Leu, $C^{\gamma 1}H_3$	0.201 (0.016)						
L	Leu, $C^{\gamma 2}H_3$	− 0.02 (0.02)						

[a] Parameters were obtained by nonlinear least-squares fit of eqs 6 and 7. Figures in parentheses are the calculated standard error derived from the diagonal elements of the variance–covariance matrix.

unnamed phenylalanine.

The chemical shift of resonance F (6.09 ppm at pH 3.6), with an intensity of two protons, is almost unaffected by the changes in pH. On the basis of the doublet fine structure and a single connectivity to a resonance at 6.73 ppm in the TOCSY spectrum, it can be assigned to the upfield-shifted signal from the C3,5H ring protons of a Tyr residue.

The six resonances G-L (Figure 4), all with intensities corresponding to three or six protons (vide infra), can be assigned to methyl groups with chemical shifts moved upfield relative to the corresponding signals in small peptides. The upfield-shifted position can be ascribed to the proximity of the methyl groups to aromatic side chains, and as such, the shift of these resonances is very sensitive to conformational changes. A doublet fine structure of the three-proton resonance G makes it assignable to the CH_3 group of a Val or Leu residue. Further, in the COSY spectrum at pD 3.3 a connectivity to a signal at 2.04 ppm, which again correlates to a signal at 2.43 ppm, suggests that it is a Leu. A major pH-induced change in chemical shift is observed with a pK_a value of 3.91 (293 K). In addition, two minor apparent titrations are observed at 305 K. The corresponding pK_a values of 3.3 and 6.3 are relatively close to the values found for His-18 and His-21, indicating a relation to these residues. Resonance H has an intensity corresponding to six protons and correlates to two resonances at 1.78 and 1.82 ppm, respectively; i.e., the two methyl groups

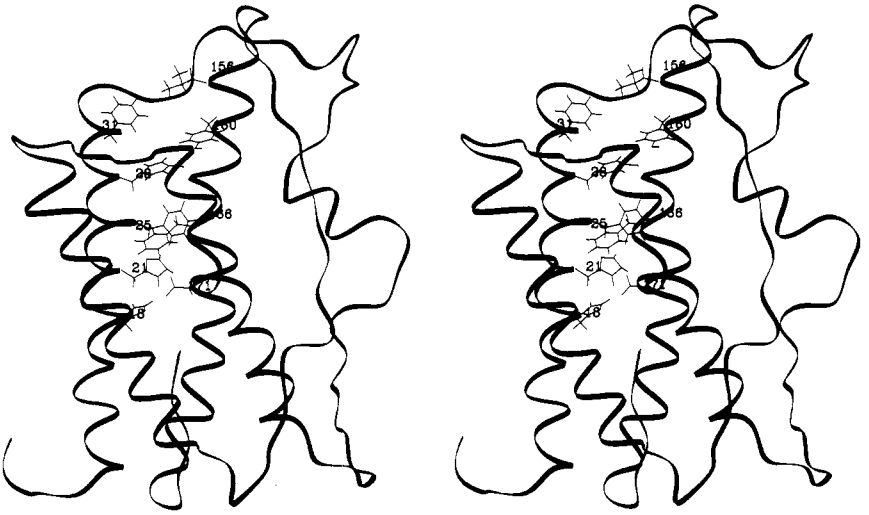

FIGURE 6: Stereoview of the hGH molecule illustrating the helix–helix contact area between helix 1 and helix 4 (see text). The interactions between the aromatic side chains of the two helices are indicated, as well as the close proximity of His-18 and Asp-171 that allows the formation of a salt bridge between these two residues, the position of His-21, and the close proximity of Phe-31 and Leu-156 that gives rise to the extreme chemical shift of the CH_3 protons of the leucine. For further details see text.

in the H resonance correlate to different protons and must, therefore, belong to different residues. While the resonance at 1.78 is correlated to a signal in the $C^{\alpha}H$ region (3.88 ppm) and, therefore, can be assigned to a CH_3 group of a Val, the correlation of the resonance at 1.82 is unclear. Even so, H shows only one single and well-defined titration shift with a pK_a value of 3.71, indicating that the two CH_3 groups of the resonance are in adjacent locations. Resonance I also correlates to two resonances (0.58 and 1.07 ppm, respectively, at pD 3.3) but has the intensity of only three protons. It was, therefore, assigned to an Ile. On protonation it moves upfield, with the major titration shift corresponding to a pK_a value of 3.72 (293 K). An additional pH-induced shift of this resonance with a pK_a value of 2.65 (293 K) is also observed. The three-proton resonance J and a resonance at 0.78 ppm (pD 3.3) are both correlated to a signal at 1.66 ppm, which, in turn, is correlated to a signal in the α-region at 4.18 ppm. Resonance J is therefore assigned to a Val. At protonation, resonance J is upfield shifted following a simple ionization curve with a pK_a value of 4.68 (293 K). Its line width is markedly greater at the midpoint of the titration. The broadening is attributed to exchange effects associated with the structural interconversion caused by the ionization process (vide infra). From the exchange broadening the exchange rate was estimated to $k_A \approx 300\ s^{-1}$. The methyl groups K and L both correlate to the same β proton. They are, therefore, assigned to the $C^{\gamma 1}H_3$ and $C^{\gamma 2}H_3$ groups of the same Val residue. They show no significant pH dependence.

DISCUSSION

In globular proteins the dispersion of the backbone carbonyl carbon resonances, corresponding to a given type of amino acid residue, is due mainly to the differences in local conformation, as defined by the backbone dihedral angles and the manner of hydrogen bonding (Saitô, 1986; Ando et al., 1988). In random coil proteins the backbone carbonyl carbons of the same type of residue are effectively independent of the neighboring residues (Howarth & Lilley, 1978), and for leucine residues in proteins in aqueous solution the random coil chemical shift value of the ^{13}C carbonyl carbon is 175.6 ppm (Howarth & Lilley, 1978). Consequently, the large dispersion of 5 ppm of the resonances from the 26 hGH leucine residues, observed in the entire pH region in Figure 1, shows that although numerous pH-induced conformational changes occur, these changes do not lead to any substantial unfolding of the protein. These findings are in accordance with the results from optical rotatory dispersion measurements (Bewley & Li, 1967) and CD spectra (Aloj & Edelhoch, 1972), which show that the secondary structure of hGH is virtually unaffected by changes in pH.

The three-dimensional structure of hGH is presently not known in detail, and our knowledge of the folding is based on secondary structure predictions (Chou & Fasman, 1974a,b; Kawauchi & Li, 1974) and the results derived from various physicochemical methods. Recently, the crystal structure at 2.8-Å resolution of the highly homologous porcine growth hormone (pGH) has been reported in some detail (Abdel-Meguid et al., 1987). Due to the extensive degree of amino acid sequence homology with human growth hormone, particularly within the four helical regions known for pGH, it is reasonable to assume that the three-dimensional structure of hGH is similar to that of pGH.

Given the validity of this assumption, the structure of hGH is, basically, composed of a tightly packed bundle of four antiparallel α-helices made up of residues 6–33 (helix 1), 75–96 (helix 2), 106–129 (helix 3), and 154–183 (helix 4). Although the atomic coordinates for pGH are presently not available, some of the general features of the helix–helix packing can be established. Thus, helix 1 and helix 4, both 45 Å in length, form one-third of a turn of an antiparallel coiled-coil α-helix (Crick, 1953; Schulz & Schirmer, 1979). This helix–helix packing is especially favored by side-chain meshing of hydrophobic residues at the central positions of the contact area (i, $i + 3$, and i', $i' - 4$, where $i = j + 7k$ and $i' = j' - 7k$ while $k = 1, 2, \ldots$), by polar residues at the outer surface positions ($i + 1$, $i + 2$, $i + 5$, and $i' - 2$, $i' - 5$, $i' - 6$), and by charged residues in favorable positions for the formation of salt bridges or hydrogen bonds with their counter parts ($i + 4$, $i + 6$, and $i' - 3$, $i' - 8$, respectively). Letting $j = 7$ and $j' = 181$, the resulting helix–helix packing enables the formation of a salt bridge between His-18 and Asp-171, as suggested by the abnormal pK_a value of His-18 and its significant apparent titration at acidic pH. A model of hGH showing this interaction is sketched in Figure 6. The structure shown in Figure 6 was derived from the coordinates of the calculated structure of bovine growth hormone (Carlacci et

al., 1991) using the program HOMOLOGY (Biosym Technologies, Inc.). HOMOLOGY facilitates creating a three-dimensional structure for a protein from a known amino acid sequence and a known structure for a related protein. This initial structure was subjected to alternating restrained energy minimization and restrained molecular dynamics (Kaptein et al., 1985) using the program DISCOVER (Biosym Technologies, Inc.). With the interhelical packing scheme shown in Figure 6, the His-21 resides at the helix–helix contact area inaccessible to the solvent. This position explains the low exchange rate constant of the His-21 imidazole C2H proton previously observed (Turner et al., 1983). Moreover, the spatial arrangement of the two helices will allow for favorable interactions between the aromatic side chains (Serrano et al., 1991) of the residues His-18, His-21, Phe-166, Phe-25, Tyr-28, Tyr-160, and Phe-31, as indicated in Figure 6. Thus, the close contact between His-21 and Phe-166 and Phe-25 is probably responsible for the upfield-shifted position of the His-21 C2H proton resonance (resonance C, Figure 5). The three interacting aromatic side chains (D, E, and the unnamed phenylalanine) may be part of this set of aromatic side chains. The fact that resonance E is affected by the titrations of the side chain of His-21 supports this suggestion. A tentative assignment would then be: E and D are Tyr-28 and Tyr-160, in some order, and the unnamed phenylalanine is either Phe-31 or Phe-25. Finally, the significant change of the shielding of the His-21 C2H proton by titration of the acidic group that interacts with His-18 suggests that the change in ion-pair interaction causes a change in the relative position of helix 1 and helix 4. This change alters the relative position of the stacked aromatic side chains, leading to the observed changes in chemical shift for these resonances.

Unlike His-151 and His-21, the pK_a value of His-18 shows a profound temperature dependence (an increase of 0.5 going from 293 to 305 K). Therefore, this pK_a value provides information about the ion-pair interaction. Qualitatively, the temperature variation shows that the deprotonation of the imidazole cation decreases with increasing temperature, indicating that the stability of the salt bridge increases with the temperature. Quantitatively, the difference between the two pK_a values corresponds to a $\Delta H \approx -68$ kJ mol^{-1} for the deprotonation of the imidazole group of His-18. This contrasts with the ΔH value for the deprotonation of a noninteracting imidazole group in a protein which is *positive* and on the order of 30 kJ mol^{-1} (Roberts et al., 1969; Fersht, 1985). Therefore, the ΔH value obtained here indicates that the salt bridge formation is opposed by an increase in enthalpy of about 95 kJ mol^{-1}. This clearly shows that the salt bridge formation is entropy driven (Cantor & Schimmel, 1980b). Further, the change of the dissociation constant, caused by the ionic interaction in the salt bridge, is given by the free energy change, ΔG_c, which is related to the pK_a values by the equation (Cantor & Schimmel, 1980a,b)

$$\mathrm{p}K_a' = \mathrm{p}K_{a,0} + \frac{\Delta G_c}{2.303RT} \qquad (10)$$

Here pK_a' is the observed pK_a value and p$K_{a,0}$ is the pK_a value in the absence of the electrostatic interaction. With a p$K_{a,0}$ of a noninteracting histidine of 6.81 at 305 K (Markley, 1973), the pK_a' value of 8.16 found here corresponds to a free energy of stabilization of the salt bridge of approximately −7.2 kJ mol^{-1} at 305 K, according to eq 10. Assuming that the free energy change responsible for the lowering of the pK_a (2.8 at 305 K) of the carboxylic acid of the salt bridge is the same, we find that the p$K_{a,0}$ in the absence of the interaction is approximately 4.1. This is consistent with a side-chain carboxyl group of an Asp residue (Nozaki & Tanford, 1967; Cantor & Schimmel, 1980a) and, thus, with the proposition that the interacting group is Asp-171 (vide supra).

The close resemblance between the weighted average of pK_{a1} for His-18 and His-21 at 293 K and the weighted average pK_a of the leucine carbonyls in group I (Table II), suggests that the structural changes associated with the leucine carbonyl carbons in group I are linked to the titration of the Asp-171 side-chain group. The fact that only four leucine carbonyl resonances are affected significantly by the change in the His-18 ion-pair interaction suggests that the conformational transition, associated with the formation and breaking of the salt bridge between His-18 and Asp-171, is confined to the region around His-18 and His-21. A deformation of the two helices in this region (helix 1 and helix 4), accompanied by a change in the hydrogen bonding of the secondary structure and the main-chain dihedral angles, might lead to the observed changes of the chemical shifts of the carbonyl carbons (Ando et al., 1984; Saitô, 1986). As indicated by the resonances 1 and 4 (Table I), the interconversion between the two forms is relatively fast (k_A = 770 s^{-1}, τ_A = 1.3 ms).

The increase in the strength of the electrostatic interactions with temperature is probably responsible for the greater number of pH-induced shifts observed for the proton resonances at 305 K. With the increased strength of these interactions, their disruption is expected to have a more far-reaching effect on the structure and, thus, on the chemical shift of the proton signals.

Fluorescence emission spectroscopy of hGH (Aloj & Edelhoch, 1972) has shown that the pH dependence of the quenching of the tryptophanyl fluorescence from the single tryptophan residue in position 86 fits a simple ionization process with a pK_a of 5.0 at 298 K. The only proton signal with an apparent titration in this range is resonance J, assigned to an upfield-shifted CH_3 group of a Val residue. This residue shows an apparent titration shift with a pK_a of 4.68 at 293 K and 5.20 at 305 K, which, by interpolation, gives a predicted pK_a of 4.9 at 298 K. The pGH crystal structure shows that Trp-86 resides in helix 2. This helix, in turn, is packed in the interior of the molecule between the coiled-coil α-helix (helix 1 and helix 4), helix 3, and the irregular peptide fragment connecting helix 3 with helix 4. Assuming the same α-helical structure around Trp-86 in hGH, the Val-90 residue is close to Trp-86. This position may result in a considerably upfield shift of the Val-90 CH_3 group due to a ring current shift from Trp-86. Therefore, both the pK_a of the apparent titration of the Val CH_3 resonance, *J*, and its extreme chemical shift suggest that this resonance can be assigned to one of the CH_3 groups of Val-90. The apparent pK_a of 4.8 for the Leu carbonyl resonances forming group VII implies that this group is affected by the protonation event responsible for the change in the tryptophanyl fluoresence emission and the concomitant apparent ^{1}H titration shift of Val-90.

The observations just mentioned seem to indicate that localized conformational changes accompany the titration of a single acidic group around pH 5. A local deformation of helix 2, leading to a change in the main-chain dihedral angles, can explain the change in the position of the Val-90 CH_3 group with respect to Trp-86, as well as the change in chemical shift of the four leucine carbonyl carbons forming group VII. The three leucines close to Trp-86 (Leu-80, Leu-81, and Leu-82) may be members of group VII. An ionization process with a pK_a value of this size can be ascribed to the deprotonation of an Asp or a Glu side-chain group that either

interacts with a nearby negatively charged group or is buried inside a hydrophobic environment of the protein. This makes Glu-174 a possible candidate for the acidic group. This residue is probably buried in the interior of the helix 1, helix 4, helix 2 contact area where it may be partly stabilized by one or more of the nearby polar groups Met-14, Met-170, and Arg-178. Thus, the exchange rate constant, k_A = 370 s^{-1} (τ_A = 2.7 ms), observed for the leucine carbonyl carbons in group VII, as well as for the Val-90 CH_3 resonance (≈300 s^{-1}, vide supra) may reflect the rate of rupture of the local structure necessary to make the Glu-174 accessible to the solvent.

The upfield-shifted methyl resonances G–I all show apparent titrations around pH 4. Several Leu carbonyl resonances also titrate in this region (Figure 3). A possible assignment to signal G is the leucine residue in position 156. As shown in Figure 6, Leu-156 located near Phe-31 could be shifted upfield due to the interaction with the aromatic side chain. A minor pH-induced shift with an apparent pK_a of 6.3, observed for signal G, seems to indicate that the local structure around Leu-156 is slightly dependent on the titration of His-21. At least two additional structural transitions are seen. One at an pK_a of 3.8 might involve the resonances G and I since both have a major apparent titration at the same pK_a. However, it is difficult to draw any definite conclusions about the assignment of the signals based upon the information obtained so far.

CONCLUSIONS

The present study demonstrates that the backbone carbonyl ^{13}C NMR resonances are sensitive probes of the local structure of folded proteins, and the investigation here of hGH shows that the combination of specific isotope labeling and a complete least-squares analysis allows the detection of even small changes in the globular structure of a relatively large protein.

In combination with titration shifts observed in the proton spectrum, the study here also shows that the pH-induced conformational changes in hGH can be described as a series of localized adjustments of the tertiary structure imposed by the change in favorable interactions between different residues in the globular folded protein. No indications of a profound unfolding of the globular structure were observed. The conformational changes associated with the deprotonation of acidic side-chain groups involved in ion-pair interactions are relatively fast, with preexchange lifetimes on the order of 1 ms or less. The structural changes associated with the deprotonation of the buried Glu-174 side chain is somewhat slower, presumably because a more drastic perturbation of the folded structure is required to permit water to penetrate into the interior of the protein.

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