Effect of pH on the Conformation and Backbone Dynamics of a 27-Residue Peptide in Trifluoroethanol

AN NMR AND CD STUDY

(Received for publication, April 14, 1995, and in revised form, August 7, 1995)

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The C-terminal fragment, residues 385-411, from human fibrinogen γ-chain, i.e. K11PFRNLT1GEQOHHLG-GAKQAGD, shows multiple turn conformations in aqueous solution (Mayo, K. H., Burke, C., Lindon, J. N., and Kloczewiak, M. (1990) Biochemistry 29, 3277-3286). The present study investigates the effect of pH and trifluoroethanol on the conformation and backbone dynamics of this 27-residue peptide. Both circular dichroism (CD) and 1H-NMR data indicate the normally observed increased helical conformations as a function of increasing trifluoroethanol. 1H-NMR structural studies done in the presence of 40% trifluoroethanol, pH 5.3, yield a network of nuclear Overhauser effects consistent with significant populations of helix-like conformations. Distance geometry calculations based on nuclear Overhauser effect-derived distance constraints yield a family of structures with relatively well defined N- and C-terminal conformations and an ill defined mid-peptide region from Gly389 to Gly403. Similar conformational populations are observed at pH 2.5. CD studies, however, indicate an increase in average α-helix content on decreasing the pH from 6 to 2. This apparent conflict between CD and NMR results may be explained by a transition from multiple β-turn character at pH 5.3 to increased α-helix structure at pH 2.5. 13C-NMR relaxation data analyzed with the Lipari-Szabo model-free approach provide order parameters that demonstrate little if any influence of pH on backbone motional restrictions within the more flexible mid-peptide domain. At low pH, however, motions become less restricted within N-terminal residues Lys388-Phe400 and more restricted within C-terminal residues Ala405-Val411.

Protein folding is primarily dictated by noncovalent, relatively weak intramolecular forces, i.e. hydrogen bonding, electrostatic interactions, and hydrophobic effects (J. Jancik, 1991). The effect of pH and alcohols on protein structure and folding has been discussed widely in the literature. Depending on the protein, acids can generate either fully or partially denatured states (Kuwajima, 1992). For β-lactamase, apomyoglobin, and ferricytochrome c (Goto et al., 1990), lowering the pH to about 2 by adding HCl yields unfolded proteins whose conformations can be partially stabilized into more compact states containing substantial secondary structure by the addition of more HCl. More complex pH-induced folding transitions have been observed at pH 2.7 with barnase (Sanz et al., 1994). Alcohols have been known for some time both to denature/destabilize globular protein tertiary (Conio et al., 1970; Parodi et al., 1973) and quaternary (Y’ang et al., 1993) structure and to effect conformational stabilization of various peptides in aqueous solution (Conio et al., 1970; Parodi et al., 1973). While some of these pH- and alcohol-induced states may be true protein folding intermediates, the presence of alternatively folded structures cannot be excluded. Nevertheless, their study can shed light on general principles of protein folding and dynamics.

Recently, more and more short linear peptides are being used as models for protein folding and local structure formation. Although this approach has been exemplified with synthetic peptides derived from the α-helix protein myoglobin (Waltho et al., 1993; Shin et al., 1993a, 1993b) and the mostly β-sheet protein platelet factor-4 (Ilyina et al., 1994), studies on the ribonuclease S peptide (20 residues) (Brown and Klee, 1971) and pentapeptides like YPGDV (Dyson et al., 1988a, 1988b) first fueled the fire of interest in others. NMR and CD, in particular, have been used to show that short linear peptides can have considerable conformational populations in aqueous solution in the presence and absence of various stabilizing agents.

Trifluoroethanol is perhaps the most commonly used agent for stabilizing α-helix conformation in peptides (Moroder et al., 1975; Lu et al., 1984; Leist and Thomas, 1984; Dyson et al., 1988a, 1988b; Pena et al., 1989; Lehrman et al., 1990; Segawa, 1984). Recently trifluoroethanol has been more thoroughly studied in this function (Sönnydsen et al., 1992; Jasanoff and Fersht, 1994). Sönnydsen et al. (1992) concluded that trifluoroethanol is not a helix-inducing solvent, i.e. it does not create new structures, but rather that it is a helix-enhancing cosolvent that stabilizes helices in regions with existing α-helical propensity. The dominant effect of trifluoroethanol is caused by its significantly weaker basicity with respect to that of water (Llinas and Klein, 1975), which decreases amide proton hydrogen bonding to the solvent and strengthens intramolecular hydrogen bonds, thereby stabilizing secondary structure (Nelson and Kallenbach, 1986).

For some time, this laboratory has been interested in a peptide derived from the C-terminal region of the fibrinogen γ-chain, residues 385-411 (Mayo et al., 1990 and references therein). During NMR and CD conformational studies of this 27-residue peptide (called γ27), it was noticed that in the

* This work benefited from research grants from the W. W. Smith Charitable Trust and from the Pharmaceutical Research Institute, R. W. Johnson Co., Inc., Springhouse, PA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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†‡This paper is dedicated to the memory of Simon Pilkis, chair of the Biochemistry Department, who died suddenly August 2, 1995.

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The abbreviations used are: γ27, the 27-residue peptide derived from the C terminus of γ-chain fibrinogen, residues 385-411; NOE, nuclear Overhauser effect; NOESY, two-dimensional NMR nuclear Overhauser effect spectroscopy; HPLC, high pressure liquid chromatography; deg, degrees.
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presence or absence of trifluoroethanol, the average helix content determined by CD was increased or lowering the pH from 6 to 2. On the other hand, NOE magnitudes were decreased, suggesting the presence of either less structure or increased internal mobility at lower pH. The complications involved with interpreting NOEs from highly flexible, linear peptides arise from the fact that NOEs are sensitive to both the internuclear distance and internal motions. The present study was initiated to correlate pH-induced CD and NOE effects in γ27 with motional characteristics derived from 13C\textsubscript{H} relaxation experiments.

MATERIALS AND METHODS

Peptide Synthesis—A peptide representing amino acid residues 385–411 from human fibrinogen γ-chain (called γ27) was synthesized on a Milligen Biosearch 9600 automated peptide synthesizer. The procedures used were based on Merrifield solid phase synthesis utilizing Fmoc-BOP chemistry (Stewart and Young, 1984). After the sequence had been obtained, the peptide support and side chain protection groups were acid (trifluoroacetic acid and scavenger mixture)-deashed. Crude peptides were analyzed for purity on a Hewlett-Packard 1090M analytical HPLC using a reverse phase C18 Vydac column. Peptides generally were about 90% pure. Further purification was done on a preparative reverse phase HPLC C-18 column using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. Peptides were then analyzed for amino acid composition on a Beckman 6300 amino acid analyzer by total hydrolysis (6 N HCl at 110 °C for 18–20 h) and by mass spectrometry. Final peptide purity was greater than 95%.

Circular Dichroism—Circular dichroism (CD) spectra were measured on a Jasco J-710 air-cooled automatic recording spectropolarimeter coupled with a data processor. Curves were recorded digitally and fed through the data processor for signal averaging and base line subtraction. Spectra were recorded from 5 to 30 °C in 10 mM potassium phosphate, pH 2–6, over a 190–250-nm range using a 0.5-mm path length, thermally jacketed quartz cuvette. Temperature was controlled by using a Haake water bath. Trifluoroethanol titrations were done up to 80% trifluoroethanol. Peptide concentration was about 1.1 mg/ml. The scan speed was 5 nm/min. Spectra were signal-averaged four times, and an equally signal-averaged solvent base line was subtracted. CD spectra were analyzed by the methods of Sreerama and Woody (1993) and Chen et al. (1974) for estimation of helix content.

NMR Measurements—Freeze-dried samples for NMR measurements were dissolved in either D\textsubscript{2}O or H\textsubscript{2}O/D\textsubscript{2}O (9:1) containing 10 mM potassium phosphate. Protein concentration was in the range of 5 mM. pH was adjusted by adding microliter quantities of NaOD or DCl to the protein sample. For most experiments, the temperature was controlled at 5 °C. All NMR spectra were acquired on a Bruker AMX-600 NMR spectrometer.

For sequential assignments, two-dimensional NMR-correlated spectroscopy (Aue et al., 1976; Wider et al., 1984), double quantum-filtered two-dimensional NMR-correlated spectroscopy (Piantini et al., 1982; Shaka and Freeman, 1983), and NOESY (Jønner et al., 1979; Wider et al., 1984) experiments were performed. Two-dimensional homonuclear magnetization transfer spectra, used to identify many spin systems completely, were obtained by spin locking with an MLEV-17 sequence (Bax and Davis, 1985) with a mixing time of 64 ms. All spectra were acquired in the phase-sensitive mode (States et al., 1982). The water resonance was suppressed by direct irradiation (1 s) at the water frequency during the relaxation delay between scans as well as during the mixing time in NOESY experiments.

The majority of the two-dimensional NMR spectra were collected as 512 or 1024 t\textsubscript{1} experiments, each with 1024 or 2048 complex data points over a spectral width of 5 kHz in both dimensions with the carrier placed on the water resonance. 64 or 96 scans were generally time-averaged per t\textsubscript{1} experiment. The data were processed directly on the Bruker AMX-600 X-32 or offline on a Bruker Aspect-1 workstation using the Bruker XWIN-NMR program. Data sets were kept in both dimensions by 0–60°-shifted sine-bell or lorentzian to gaussian transformation function and generally zero-filled to 1,024 in the t\textsubscript{1} dimension prior to Fourier transformation. To obtain a quantitative description of peptide backbone dynamics, 1H-detected 13C\textsubscript{H} heteronuclear chemical shift correlation spectra (van Mierlo et al., 1993) and references therein) were accumulated to derive (1H–13C\textsubscript{H}) NOE relaxation rates on the unenriched peptides in each case. Cross-peak intensities depend on the relaxation parameter of interest. All spectra were acquired in the phase-sensitive mode by using time-proportional phase incrementation for quadrature detection in the t\textsubscript{2} dimension; 2048 data points were recorded in each quadrature channel during t\textsubscript{2} and 200 real points were recorded in t\textsubscript{1}. Spectra were acquired with a spectral width of 5000 Hz in t\textsubscript{2} and 6000 Hz in t\textsubscript{1}. The 1H carrier was placed on the HDO resonance, and the 13C carrier was set at 45.4 ppm. For T\textsubscript{1} measurements, 128 scans were acquired per t\textsubscript{1} increment; for the NOE measurement, 256 scans were acquired per increment. For measurements of T\textsubscript{1} and NOE, a relaxation delay of 5.0 s was used between scans to ensure sufficient recovery of 1H magneti- cation. For T\textsubscript{2} relaxation measurements, nine separate spectra were recorded for T = 0.01, 0.04, 0.08, 0.15, 0.2, 0.3, 0.6, 0.8, and 1.2 s. Relaxation rate constants and NOE enhancements were calculated from peak heights of the heteronuclear resonances as described by Palmer et al. (1991). Data analysis was performed on Bruker Aspect-1 or Silicon Graphics workstations using UXNMR, Aurelia (Bruker, Inc.), or FELIX (Biosym, Inc.) programs.

13C NMR relaxation data were analyzed by using the model-free formalism of Lipari and Szabo (1982a, 1982b) in which motions are described in terms of two correlation times (an overall tumbling time and an internal motion correlation time) and an order parameter (S\textsuperscript{2}), which can be related to bond angular restrictions (Lipari and Szabo, 1982b). Since S\textsuperscript{2} is least sensitive to T\textsubscript{1}, an average value of 5 × 10\textsuperscript{−11} s was used initially in the optimization routine and later varied up to 10 × 10\textsuperscript{−11} s. Then a resulting average value was fixed, and T\textsubscript{1} and S\textsuperscript{2} were allowed to vary. In either case, S\textsuperscript{2} varied by no more than 5%. Small values of the order parameter led to poor fits of the T\textsubscript{1} data whereas larger values indicated increased motional restrictions.

Distance Geometry Calculations—The structures were calculated by using the constant valence force field included in the Biosym Software (INSIGHT II 2.3, DISCOVER 2.9) (San Diego, CA) on a Silicon Graphics Indigo 2. Distance constraints were derived from NOE data as discussed under “Results.” Additional torsional restraints were applied to maintain trans-geometry and planarity for the peptide bond throughout the calculations. The starting extending polypeptide coordinate was minimized using 50 steps of steepest descent minimization and then 600 fs of molecular dynamics calculation at 1200 K with a step size of 1 fs followed by 500 steps of conjugate gradient minimization. After the triaxial bounds smoothing was done, 30 coordinate files were created by metrization and embedding. Refinement of these coordinates was done using a simulated annealing protocol with a simplified error function to further optimize the fit between the coordinates and the distance matrix. The annealing protocol was followed by 1000 steps of conjugate gradient minimization to obtain the converged distance geometry-based structures.

RESULTS

Circular Dichroism—CD spectra of γ27 obtained at pH 6, 5 °C, are displayed in Fig. 1 at different trifluoroethanol concentrations. At 0% trifluoroethanol, the γ27 CD trace is characteristic of mostly random coil conformation. As the trifluoroethanol concentration is increased, the shape of the curves becomes increasingly characteristic of helical secondary structure. The monotonic trends in ellipticities and an apparent isodichroic point at about 204 nm suggest an equilibrium between two main conformational states, helix and random coil.

Percentage of α-helicity was estimated by using the mean residue ellipticity at 220 nm (θ\textsubscript{220}) and the equation of Chen et al. (1974),

\[ \Theta = (\theta_0 - \theta_N)N/\theta_N \]  

where θ is the observed mean residue ellipticity at 220-nm wavelength, θ\textsubscript{0} is the maximum mean residue ellipticity of a helix of infinite length (Chang et al., 1978); f\textsubscript{h} is the fraction of helix in the molecule; i is the number of helical segments; N is the total number of residues, and k is a wavelength-dependent constant (2.6 at 220 nm). The number of helical segments, i, was set to 2 in order to be consistent with modeled structures generated from NOE-derived distance constraints discussed later. The expected value of the mean residue ellipticity for 100% helicity of peptides of chain length 27 residues was determined to be −32,720 deg cm\textsuperscript{2}/dmol. Using this method, calculated helicities as a function of trifluoroethanol concentration are shown in the inset to

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CD spectra of γ27. CD spectra of γ27 obtained at pH 6 and 5°C are shown at different trifluoroethanol concentrations as indicated in the figure. Trifluoroethanol concentrations are given as percentage (v/v) of composition. The peptide concentration was kept approximately constant at 40 μM. Based on CD data according to Equation 1, the calculated α-helix content in γ27 is plotted versus trifluoroethanol concentration in the inset. In the inset, data are shown for pH 6, 5°C, and 25°C. Lines drawn through points are for visual aid only.

Fig. 1. CD spectra of γ27. CD spectra of γ27 are plotted versus the solution pH from pH 2 to 6. In the inset, molar ellipticities are plotted for two wavelengths, 195 and 220 nm, as a function of the solution pH. At the top of the inset, wavelength in the 204–206-nm range is plotted versus the solution pH. Trifluoroethanol concentration was constant at 40% (v/v). The temperature was 5°C. Data points in the inset indicate the simple average of three pH titration series. Standard deviations are ±0.5 × 10^3 deg cm^2 dmol^-1 at 220 nm; ±2 × 10^2 deg cm^2 dmol^-1 at 195 nm; and ±0.2 nm for the wavelength versus pH plot. Lines connecting data points are for visual aid only.

Fig. 2. pH effect on CD spectra. CD spectra for γ27 are plotted versus the solution pH from pH 2 to 6. In the inset, molar ellipticities are plotted for two wavelengths, 195 and 220 nm, as a function of the solution pH. At the top of the inset, wavelength in the 204–206-nm range is plotted versus the solution pH. Trifluoroethanol concentration was constant at 40% (v/v). The temperature was 5°C. Data points in the inset indicate the simple average of three pH titration series. Standard deviations are ±0.5 × 10^3 deg cm^2 dmol^-1 at 220 nm; ±2 × 10^2 deg cm^2 dmol^-1 at 195 nm; and ±0.2 nm for the wavelength versus pH plot. Lines connecting data points are for visual aid only.

Fig. 3. pH 5.3 NOESY contour plots of peptide γ27. The αH-NH/aromatic and NH-NH/aromatic and NH-NH/aromatic resonance regions from a NOESY contour plot are shown. Data were collected in 60% H2O/40% perdeuterated trifluoroethanol (0.6-ml total sample volume) with 10 μM peptide γ27 at pH 5.3 and 5°C. 512 hypercomplex free induction decays containing 1024 words were collected and processed as discussed under “Materials and Methods.” The mixing time was 0.1 s. The data were zero-filled to 1024 in t2. The raw data were then multiplied by a 40°-shifted sine-squared function in t2 and t1 prior to Fourier transformation. Some sequential resonance assignments are traced out, and some longer range NOEs are indicated. Labeling of resonances is as discussed in the text.

CD data (inset) are consistent with increases in the average helix content of peptide γ27 as the pH is lowered from 6 to 2. Even though data shown in this figure have been accumulated in 40% trifluoroethanol, the same general trend is observed at lower trifluoroethanol concentrations. The isodichroic point at 200 nm supports the idea of an equilibrium between two main conformational populations, one having more helix character than the other.

NOEs and Distance Geometry Calculations—NOESY spectra of the αH-NH/aromatic and NH-NH/aromatic and NH-NH/aromatic resonance regions of γ27 (5°C and 40% trifluoroethanol) accumulated at pH 5.3 and at 2.5 are compared in Figs. 3 and 4, respectively. Since sequential resonance assignments for γ27 have been done at pH 3.5, 30°C, 0% trifluoroethanol (Mayo et al., 1990), they were easily made under the present solution conditions by using the standard approach outlined in Wu¨thrich (1986). Figs. 3 and 4 trace out some sequential assignments, which are tabulated more completely in Table I for pH 5.3 data. These data (Figs. 3 and 4) aim at identifying dNN(i, i + 1), dHH(i, i + 2), and other relatively long range NOEs that help define conformational populations for γ27. These and other NOEs are summarized in Fig. 5 for pH values of 2.5 and 5.3. A complete listing of NOEs observed at pH 5.3 is given in Table II. Numerous dNN(i, i + n) and dHH(i, i + n) NOEs are observed. At pH 5.3, most “longer range” NOEs are found within the sequence Phe389-Glu396. This network of NOEs suggests the presence of multiple-turn or helix-like structure. NOEs present at pH 5.3 normally are observed with similar or only slightly reduced magnitudes at pH 2.5. Cursory inspection of the NH-NH NOESY region may suggest large decreases in NOE magnitudes for data accumulated at pH 2.5. However, comparison with the αN region and normalization with Pro388...
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The only titratable groups in γ27 belong to the carboxylate groups of Glu396, Asp310, and Val411, and the side-chain imidazole groups of His400 and His401. pK_a values (derived from plots of chemical shift versus pH) for Glu396, Asp410, and Val411 (data not shown) range from 3.7 to 4.1. For His400 and His401, pK_a values of 6.5 and 6.6, respectively, can be estimated from the data shown in Fig. 7. Evidence for an electrostatic interaction between/among His400, His401, and Glu396 comes from the apparent Glu396 pK_a inflection observed in the titration curves of His400 and His401. NOEs observed between His400, His401, and Glu396/Gly397 side-chain proton resonances (see Fig. 3 and Table II). Marqusee and Baldwin (1987) have observed that positive-negative side-chain electrostatic interactions are most stabilizing in a helical conformation when oppositely charged residues are at the i, i + 4 positions, respectively.

Significant pH-dependent chemical shift changes (greater than 0.1 ppm) for side chains of Lys385, Ile408, Arg391, and Thr393 (data not shown) also argue for direct (although probably transient) interactions with Glu396. In a helix-like conformation (Fig. 6), Thr393 is located at the i, i + 3 position with respect to Glu396. Proximity to N-terminal residues Lys385 and Ile408 is considered plausible based on results from calculated structures. In particular, the side-chain of Lys385 can fold in toward the side-chain of Glu396 to mediate a “loose” electrostatic interaction. Within the Lys385-His401 segment, 50% of these backbone NHs are shifted more than 0.1 ppm on varying the pH between 2.5 and 5.3. In particular, two of the more shifted NHs belong to Ile408 and Phe389, supporting the idea of a possible long range structurally stabilizing effect of Glu396. Additionally, Ile408 NH is one of the most long-lived NHs at lower pH (Mayo et al., 1990). This electrostatic interaction in combination with hydrophobic side-chain clustering (Dynon et al., 1992), could explain this NH solvent protection.

Protonation/deprotonation of Asp310 and Val411 (C-terminal carboxylate) probably plays no role in the electrostatic effects of the N-terminal and mid-peptide segments. The only side chain within C-terminal residues Leu402-Val411 that shows significant chemical shifts on varying pH belongs to Ala405. Other residues have their backbone resonances more highly shifted than their side-chain resonances, suggestive of indirect, conformationally induced chemical shift changes. In particular, the noncharged amino acid residue NHs of Gln407, Ala408, and Gly409 are more degenerate at lower pH. Interestingly, side-chain proton resonances of Lys406 demonstrate a carboxylate pK_a inflection. This suggests an electrostatic interaction between Lys406 and most probably Asp310 or Val411.

γ27 Backbone Dynamics—1H-detected two-dimensional heteronuclear 13C NMR experiments (Nirmala and Wagner, 1988; Kay et al., 1989; Clore et al., 1990; Palmer et al., 1991) have been used to characterize 13C relaxation in γ27, thereby providing information on local peptide backbone motional restrictions. 13C resonance assignments were made by correlating 1H chemical shifts at pH 5.3 are listed in Table I. Typical (1H)-13C NOE data are shown in Fig. 8, and 13C of T3 and (1H)-13C, NOE data accumulated at pH 2.5 and pH 5.3 are compared in Fig. 9. Relaxation data for glycines were not derived due to resonance...
Overlap of the five glycines. Furthermore, due to spectral overlap of some other $^1$H-$^{13}$C cross-peaks, i.e., Leu$^{392}$ with Leu$^{402}$ and Gln$^{407}$, and Glu$^{396}$ with Gln$^{398}$, relaxation parameters for these cross-peaks could not be as accurately determined as those for others. $T_1$ relaxation curves, however, did appear linear; therefore, individual respective relaxation rates are similar for these partially overlapped cross-peaks.

Within the N-terminal segment Lys$^{385}$–Phe$^{389}$, both $T_1$ and NOE values are smaller for g$^{27}$ at pH 5.3, indicating decreased backbone mobility of that sequence at higher pH. This is reflected in order parameters, $S^2$, derived from these relaxation data (Fig. 9). For residues Ala$^{405}$–Val$^{411}$, $S^2$ values are larger at pH 2.5, indicating increased motional restriction of the C-terminal segment at lower pH. For residues Asn$^{390}$–His$^{401}$, $S^2$ values vary less with pH change. At either pH, the mid-peptide region residues Thr$^{393}$–His$^{400}$ generally show the smallest order parameters, indicating the presence of a relatively flexible mid-peptide segment, consistent with NOE-based distance geometry calculations, which indicate an ill-defined mid-peptide segment from about Gly$^{395}$ to His$^{401}$. The overall correlation time, $\tau_C$, was 1.9 ns at either pH value.

**DISCUSSION**

Short, linear peptides, like g$^{27}$, generally exist in solution in an ensemble of highly fluctuating structures whose NMR spectral parameters average. This is true for g$^{27}$ in aqueous solution at 30°C in the absence of trifluoroethanol (Mayo et al., 1990) where multiple turn or nascent helix (Dyson et al., 1988a, 1988b) conformation was apparent within residues 385–402. Under those solution conditions, C-terminal residues 402–411 showed no NOE structural constraints greater than $i, i+1$; however, conformational preference within that segment was

**TABLE I**

$^1$H- and $^{13}$C NMR sequence-specific resonance assignments (ppm)

| Residue | NH  | $\alpha$H | $\beta$H | Others | $^{13}$C |
|---------|-----|----------|---------|--------|--------|
| Lys$^{385}$ | 3.95 | 1.82 | $\gamma$H$_2$ 1.36 | 51.36 |
| Ile$^{386}$ | 8.45 | 8.14 | 1.72 | $\gamma$H$_2$ 1.09 | 56.9 |
| Ile$^{387}$ | 8.06 | 4.43 | 1.76 | $\gamma$H$_2$ 1.07 | 59.06 |
| Pro$^{388}$ | 4.33 | 2.06, 2.06 | $\gamma$H$_2$ 1.86 | 53.85 |
| Phe$^{389}$ | 7.60 | 4.39 | 3.03, 2.94 | 54.3 |
| Asn$^{390}$ | 8.16 | 4.54 | 2.81, 2.81 | 49.25 |
| Arg$^{391}$ | 8.18 | 4.06 | 1.84, 1.77 | 53.05 |
| Leu$^{392}$ | 7.99 | 4.24 | 1.72, 1.56 | 51.05 |
| Thr$^{393}$ | 7.84 | 4.23 | 4.17 | $\gamma$H$_2$ 1.13 | 58.62 |
| Ile$^{394}$ | 7.82 | 3.96 | 2.04 | $\gamma$H$_2$ 1.08 | 58.54 |
| Gly$^{395}$ | 8.19 | 3.87, 3.87 | $\gamma$H$_2$ 2.32, 2.28 | 53.08 |
| Gly$^{396}$ | 8.26 | 4.16 | 2.02 | $\gamma$H$_2$ 2.35, 2.29 | 52.91 |
| Gly$^{397}$ | 8.44 | 3.83, 3.88 | $\gamma$H$_2$ 2.30, 2.34 | 52.66 |
| Gly$^{398}$ | 8.20 | 4.16 | 2.06, 2.02 | $\gamma$H$_2$ 2.36 | 51.53 |
| Gly$^{399}$ | 8.21 | 4.08 | 1.96, 1.91 | $\gamma$H$_2$ 2.31 | 51.53 |
| Gly$^{400}$ | 8.18 | 4.55 | 3.21, 3.07 | $\gamma$H$_2$ 2.27 | 51.53 |
| Gly$^{401}$ | 8.32 | 4.61 | 3.25, 3.15 | $\gamma$H$_2$ 2.23 | 51.53 |
| Leu$^{402}$ | 8.32 | 4.29 | 1.63, 1.53 | $\gamma$H$_2$ 1.53 | 51.14 |
| Gly$^{403}$ | 8.37 | 3.36, 3.93 | $\gamma$H$_2$ 1.53 | 48.37 |
| Gly$^{404}$ | 8.16 | 3.89 | $\gamma$H$_2$ 1.53 | 49.14 |
| Ala$^{405}$ | 8.10 | 4.24 | 1.32 | $\gamma$H$_2$ 1.41, 1.37 | 49.14 |
| Lys$^{406}$ | 8.23 | 4.25 | 1.79, 1.71 | $\gamma$H$_2$ 1.41, 1.37 | 49.14 |
| Glu$^{407}$ | 8.51 | 4.31 | 2.09, 1.92 | $\gamma$H$_2$ 2.31 | 52.14 |
| Ala$^{408}$ | 8.35 | 4.16 | 1.34 | $\gamma$H$_2$ 1.41 | 49.14 |
| Gly$^{409}$ | 8.34 | 3.80, 3.99 | $\gamma$H$_2$ 2.31 | 52.14 |
| Asp$^{410}$ | 8.06 | 4.66 | 2.74, 2.65 | $\gamma$H$_2$ 0.84 | 50.03 |
| Val$^{411}$ | 7.68 | 4.00 | 2.04 | $\gamma$H$_2$ 0.84 | 59.31 |

**Fig. 5. Summary of NOE data for peptide g27.** The peptide sequence of g$^{27}$ is shown with a summary of identifiable NOEs given above for data accumulated at pH 5.3 and below for data accumulated at pH 2.5. NOEs are tabulated in the format discussed by Wu-¸thrich (1986). A question mark indicates ambiguity in identifying a possible NOE.
### Table II

| 1H NMR-derived distance constraints used in computer modeling |
|---------------------------------------------------------------|
| From | To | Intensity* |
| Lys385 | αH | Ile386 | NH | s |
| Ile386 | αH | Lys385 | NH | s |
| NH | Ile387 | αH | NH | w |
| Ile387 | αH | Gly385 | βH | vw |
| NH | Lys385 | γH | m |
| NH | Pro388 | βH | vw |
| NH | Pro388 | αH | s |
| Pro388 | αH | Phe389 | NH | s |
| αH | Phe389 | 2,6H | w |
| NH | Asn390 | NH | s |
| NH | Asn390 | NH | w |
| NH | Ile387 | αH | s |
| Ile387 | βH | NH2 | w |
| NH | Pro388 | αH | w |
| NH | Asn390 | αH | w |
| NH | Ile387 | γH | s |
| Ile387 | γH | Asn390 | NH | s |
| NH | Asn390 | NH | s |
| NH | Pro388 | αH | w |
| NH | Asn390 | αH | w |
| NH | Asn390 | NH | w |
| NH | Ile387 | αH | s |
| 2,6H | Leu392 | αH | w |
| 2,6H | Asn390 | αH | w |
| 2,6H | Asn390 | αH | w |
| 2,6H | Thr393 | αH | w |
| 2,6H | Ile387 | 2,6H | w |
| 2,6H | Ile387 | αH | w |
| NH | Pro388 | αH | w |
| NH | Pro388 | αH | w |
| NH | Ile387 | αH | m |
| Ile387 | γH | Asn390 | NH | s |
| αH | Asn390 | NH | w |
| NH | Ile387 | 2,6H | w |
| NH | Phe389 | 2,6H | w |
| NH | Asn390 | 2,6H | m |
| NH2 | Phe389 | γH | m |
| γNH2 | Thr393 | γH | w |
| αH | Arg391 | γH | w |
| αH | Arg391 | γH | w |
| NH | Arg391 | γH | w |
| NH | Arg391 | γH | w |
| NH | Arg391 | γH | w |
| γNH2 | Asn390 | 2,6H | w |
| γNH2 | Ile395 | 2,6H | w |
| γNH2 | Ile395 | γH | w |
| γNH2 | Ile395 | γH | w |
| γNH2 | Arg391 | NH | w |
| αH | Leu392 | NH | ms |
| αH | Thr393 | NH | w |
| NH | Ile392 | NH | s |
| NH | Ile392 | NH | s |
| NH | Asn390 | γH | w |
| NH | Asn390 | γH | w |
| NH | Ile392 | γH | w |
| NH | Thr393 | NH | s |
| NH | Arg391 | γH | w |
| NH | Arg391 | γH | w |
| NH | Asn390 | γH | w |
| NH | Thr393 | γH | w |
| NH | Arg391 | γH | w |
| NH | Arg391 | γH | w |
| NH | Arg391 | γH | w |
| NH | Asn390 | γH | w |
| NH | Leu392 | NH | s |
| NH | Leu392 | NH | s |
| NH | Glu396 | γH | w |
| NH | Leu392 | αH | w |
| NH | Leu392 | γH | w |
apparent based on chemical shift differences with fibrinogen \( g \)-chain peptide 400–411 and a 5-Hz \( J \) coupling constant for Ala\(^{408} \) (Mayo et al., 1990). This observation is supported with NMR studies on fibrinogen \( g \)-chain peptide 392–411 done by Blumenstein et al. (1992), who reported that at 5°C (also in the absence of trifluoroethanol), a significant \( \beta \)-turn population exists for the sequence Gln\(^{407} \)-Asp\(^{410} \).

| From | NH | Lys\(^{408} \) NH s |
| From | NH | Asp\(^{410} \) \( \gamma \)H w |
| From | NH | Lys\(^{406} \) \( \gamma \)H m |
| From | NH | Lys\(^{406} \) \( \delta \)H vv |
| From | NH | Ala\(^{408} \) \( \gamma \)H vv |
| From | NH | Ala\(^{408} \) \( \gamma \)H vv |
| From | aH | Gly\(^{400} \) NH s |
| From | aH | Asp\(^{410} \) NH w |
| From | NH | Asp\(^{410} \) \( \gamma \)H vv |
| From | NH | Glu\(^{407} \) \( \gamma \)H s |
| From | aH | Val\(^{411} \) NH w |
| From | aH | Asp\(^{410} \) NH s |
| From | NH | Glu\(^{407} \) \( \gamma \)H m |
| From | NH | Glu\(^{407} \) \( \gamma \)H m |
| From | aH | Val\(^{411} \) NH s |
| From | aH | Gly\(^{400} \) NH vv |
| From | NH | Gly\(^{400} \) NH s |
| From | NH | Glu\(^{407} \) NH vv |
| From | NH | Glu\(^{407} \) \( \gamma \)H vv |
| From | NH | Ala\(^{408} \) \( \gamma \)H w |
| From | aH | Val\(^{411} \) \( \gamma \)H vv |
| From | aH | Val\(^{410} \) NH s |
| From | NH | Gly\(^{400} \) NH vv |
| From | NH | Glu\(^{407} \) NH vv |
| From | NH | Ala\(^{408} \) \( \gamma \)H m |
| From | aH | Asp\(^{410} \) \( \gamma \)H vv |
| From | aH | Gly\(^{406} \) \( \varepsilon \)H vv |
| From | aH | Gly\(^{406} \) \( \varepsilon \)H vv |
| From | aH | Gly\(^{406} \) \( \varepsilon \)H vv |
| From | aH | Asp\(^{410} \) \( \gamma \)H vv |
| From | aH | Lys\(^{406} \) \( \varepsilon \)H vv |
| From | aH | Ala\(^{408} \) \( \varepsilon \)H vv |

\[ ^a \] Relative NOE intensities have been derived by volume integration and/or contour level counting and are classified as strong (s), medium (m), weak (w), and very weak (vv) with the following assigned distance bounds: strong NOEs, 1.8–2.8 Å; medium NOEs, 1.8–3.3 Å; weak NOEs, 1.8–4.0 Å, and very weak NOEs, 1.8–5.0 Å. For NOEs to side-chain groups, 0.5 Å was added to the upper distance.

These present \( \gamma \)27 NOE data accumulated in the presence of trifluoroethanol are consistent with both reports (Mayo et al., 1990; Blumenstein et al., 1992). More transient multiple turn or helix-like conformations noted at 30 or 5°C in the absence of trifluoroethanol are stabilized by the presence of trifluoroethanol, which acts as a structure-enhancing cosolvent (Sönnichsen et al., 1992; Jasanoff and Fersht, 1994), rather than as a conformation-inducing, i.e. new structure-inducing, agent. Trifluoroethanol stabilizes helix conformation in peptide sequences that have some helix propensity. The Chou-Fasman (1978) predictive secondary structure algorithm yields good probabilities for helix formation from residues Leu\(^{392} \)-Leu\(^{402} \) as well as from residues Ala\(^{405} \)-Val\(^{411} \) (Mayo et al., 1990). At pH 5.3, NOE-based distance geometry-generated structures of \( \gamma \)27 indicate that helix-like or multiple turn conformations are present within the N- and C-terminal segments, residues 391–397 and 404–408, respectively. N-terminal residues 385–387 have an extended conformation, and Pro\(^{386} \) causes a kink in the

FIG. 6. Computer-modeled structures of \( \gamma \)27. Based primarily on NOE-derived distance constraints, distance geometry, restrained minimization, and dynamics, simulated annealing calculations were performed using the XPLOR program on an SGI 480 computer. The superposition of backbone atoms of 10 structures are shown as discussed in the text. On the left side of the figure, the best overlay for N-terminal residues is shown, while on the right side, the best overlay for C-terminal residues is shown. In this figure, residues are labeled from 1 to 27 instead of from 385 to 411.

FIG. 7. Chemical shift versus pH for His\(^{400} \) and His\(^{401} \) C\(_2\). Chemical shifts for His\(^{400} \) and His\(^{401} \) C\(_2\) proton resonances are plotted versus the solution pH. The insert expands the chemical shift ordinate axis to better display the lower pH inflection in the histidine titrations curves. Data for His\(^{400} \) alone are shown connected by the solid line. Solid and open squares indicate calculated titration curves for Glu\(^{396} \) with a \( pK_a \) of 4.1 and for His\(^{400} \) with a \( pK_a \) of 6.6. These two theoretical curves sum up to yield the solid line drawn through the His\(^{400} \) data points.
structure that leads into a turn centered at 390–391. The trifluoroethanol-stabilized, N-terminal conformation, residues 385–397, is essentially the same as that observed for γ27 in aqueous solution at 30 °C (Mayo et al., 1990), once again supporting the idea that trifluoroethanol does not induce new structure formation but rather acts to enhance existing conformational populations (Sönnichsen et al., 1992; Jasani and Fersht, 1994). Within the Gly397–Gly403 segment, few “long range” NOEs are observed, which results in distance geometry calculations of a conformationally ill defined mid-peptide region. The paucity of NOEs could be the result of a more extended, solvent-exposed conformation and/or of a more flexible domain. Since average motional order parameters are reduced within this region relative to other sequences, one can conclude that the mid-peptide segment is relatively more flexible than any other segment. The lack of conformationally constraining NOEs within this region, therefore, is mostly due to the presence of an ensemble of highly fluctuating conformations. In this respect, N- and C-terminal helix-like regions are connected by a “hinge” segment. In support of this, it should be noted that glycine, which highly populates this mid-peptide segment (Gly395, Gly397, Gly403, Gly404) normally promotes increased $\psi, \phi$ angular freedom and flexibility, disrupts periodic structure, and frequently occupies the helix C-cap position (Richardson and Richardson, 1988).

In terms of the effect of pH on specific sequences within γ27, NMR data indicate that N- and C-terminal domains behave differently. Generally, the same NOEs are observed at either pH 2.5 or pH 5.3, indicating the presence of similar conformational populations. NOE magnitudes at pH 2.5, however, are reduced on average by about 10–20% relative to those observed at pH 5.3. Most NOE magnitudes observed within the mid-peptide region are unaffected by pH changes. Within the N-terminal segment, which becomes more helical at lower pH, however, NOE magnitudes are generally reduced, suggesting a more “open” or less structured γ27 N-terminal conformation at pH 2.5. This is consistent with results from protein folding studies where decreasing the pH to 2–3 denatures or unfolds protein structures. Consistent with distance geometry structural calculations, Lys385 and the N-terminal amine may interact electrostatically with Glu396, and by neutralizing Glu396 by lowering pH these charge-charge interactions are minimized or negated, causing the N-terminal segment to become less conformationally and dynamically restricted, resulting in reduced NOE magnitudes.

Unlike the N-terminal domain, the C-terminal segment, residues Ala405–Val411, becomes more motionally restricted at lower pH. In apparent contradiction to this, NOE magnitudes, particularly those of NH-NH, are reduced for these C-terminal residues, while the change in CD molar ellipticity translates into an approximately 15% increase in average helix content. For short linear peptides that exist in a highly dynamic conformational ensemble that displays some average “structure,” NOEs are difficult to interpret since they are affected both by changes in internuclear distances and by motional properties of the peptide. Increased negative CD ellipticities at 224 nm could be the result of increased β-turn character at the higher pH value, which would show a more positive absorption at 224 nm and would reduce the apparent negative ellipticity at 220 nm (Dyson et al., 1988a and 1988b). In this respect, these results suggest that the γ27 conformational ensemble is shifted to a more helical character at lower pH. Reduced NH-NH NOE magnitudes, for example, would be explained by increased average NH-NH internuclear distances in a helical conformation relative to a tight turn.

In conclusion, this study has shown that for the more hydrophobic N-terminal segment that may be partially stabilized by electrostatic interactions, lowering the pH induces a more open, more dynamic conformational ensemble, while for the C-terminal segment lowering the pH shifts this ensemble to a more helical, less flexible conformational distribution. For γ27, pH has the effect of acting at the local, rather than global, conformational level.

Acknowledgments—NMR experiments were performed at the University of Minnesota High Field-NMR Laboratory. Peptides were synthesized at the Microchemical Facility, Institute of Human Genetics, University of Minnesota. We are very grateful to Dinesha S. Walek and Marek Kloczewiak for helpful discussions on 13C relaxation studies.

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