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Supplementary Material Available: Tables of crystal data, structure solution and refinement (S1), atom coordinates (S2), bond lengths and angles (S3), and anisotropic thermal parameters (S4) for both structures 1,3-[$(E_2N_2C)C_6H_4(CN_2E_2)$] (E = S, Se) (10 pages). Ordering information is given on any current masthead page.

Local Effect of Glycine Substitution in a Model Helical Peptide

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Abstract: The amino acid glycine strongly destabilizes α-helical structure in proteins as well as in model helical peptides. We have investigated the role of a single glycine substitution in a helical host peptide system. Quantitatively, a single glycine → leucine substitution has about one-third of the effect on the stability of helix as does triple substitution of these residues in the middle of the helix. The single glycine perturbs the distribution of helix in the peptide. NMR experiments detect a strong local drop in helix structure at the residues flanking the site of substitution, in addition to overall loss in helicity of the peptide seen at all positions in the chain.

Introduction

The α-helix[1] is the single most abundant secondary structure in globular proteins.[2] The factors that stabilize α-helical structure are of fundamental importance in understanding how structure is acquired,[3] both in isolated fragments of proteins and in intact protein chains. In some, but not all,[4] cases α-helices serve as early intermediates in the folding of globular proteins. Polypeptides of different composition[3] and peptides of different sequence[5-10] exhibit varying degrees of helicity, so that each side chain in a helix appears to influence the helical structure present. Alanine and leucine are well-known to stabilize α-helical structure, for example, whereas proline and glycine destabilize helices.[9,10] But how or why a particular side chain influences the stability of α-helix is not clear; it seems likely that no single mechanism will account for the role of all side chains. The intrinsic strength of hydrogen bonds in the helix must depend on the nature of the donor and acceptor side chains. Inspection of helix wheels[11] shows that longer range interactions among side chains along the helix are possible, particularly if the spacing of groups is appropriate. Thus charges or nonpolar side chains spaced at intervals of i, $i + 4$ lie on the same face of the helix and can presumably influence each other. On the other hand differences among chemically similar side chains such as leucine, isoleucine, and valine[6c,7c,9,10] in identical environments imply that there are short-range effects of single substitution as well. The conformational restriction imposed by α-helix formation has been shown to be a significant factor in the helix propensities of chemically similar side chains.[12]

We have investigated[10] a series of peptides that we refer to by the standard one-letter abbreviations for the three "guest" amino acids which are introduced into the central positions in "host" chains with the sequence

succinyl-Tyr-Ser-Glu_4-Lys_4-XXX-Glu_4-Lys_4-NH_2

CD spectroscopy of ten substituted chains allows us to assign an order of relative helix stabilizing effect to the different guest amino acids in the series:[10] Ala > Leu > Met > Gln > Ile > Val > Ser > Thr > Asn > Gly. This order is consistent with that determined in a series of host-guest coiled-coil peptides by O'Neil and DeGrado,[9] but not with host-guest experiments on polyamino acids with alkylated glutamic acid as host side chains.[5] NMR analysis of members of the series indicates that each is partially helical, with the helix favoring the N terminus of the chain.[13] This analysis is based on distance criteria and coupling constants and shows that the chemical shift of the Cα protons in the host blocks of these peptides affords a useful measure of the helix probability.

The question that concerns us here is how a single substitution stabilizes α-helix relative to the triple substitutions used previously

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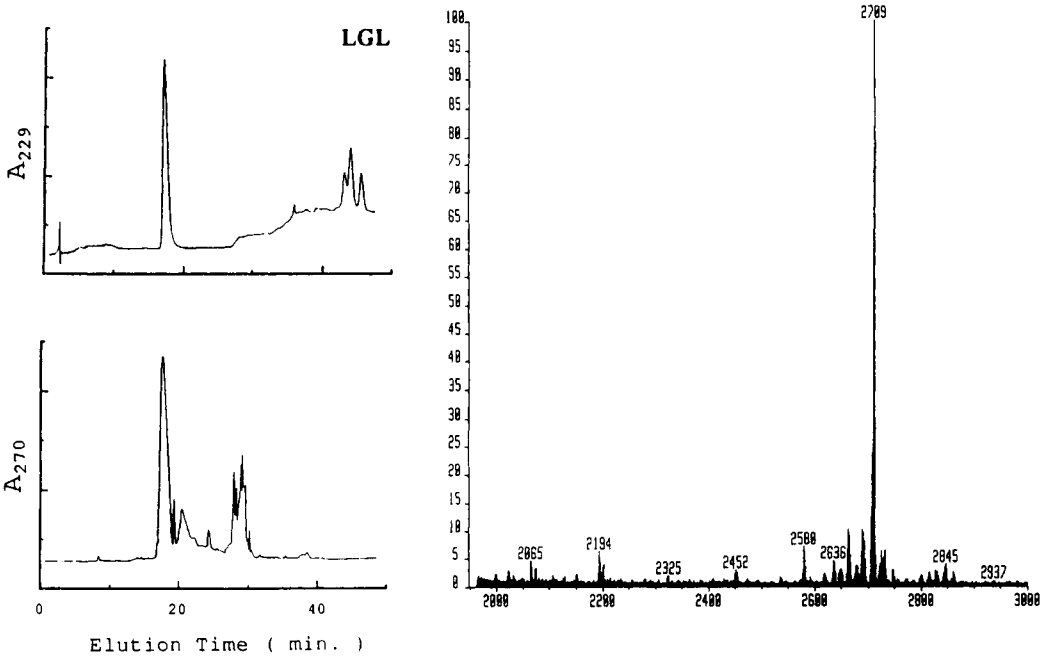

Figure 1. (a, left) HPLC chromatographic elution profiles for crude LGL (lower panel) and after reverse phase purification (upper panel). The ordinate is scaled so that the major peak in each profile has the same amplitude at 270 and 229 nm. (b, right) FABMS of the pure peptide LGL. An ion corresponding to the calculated average M + H is observed at m/z 2709.

in this system. We compare here the extent and distribution of helical residues in LGL, in which a single glycine residue is flanked by two leucine residues, with a chain containing three adjacent guest Leu residues, LLL, and one with three guest Gly residues, GGG. The single substitution of Gly in LGL is found to reduce the helix content by an amount roughly one-third the difference between LLL and GGG. Detailed analysis of the effect of the substitution by ^{1}H NMR shows that the helical structure is reduced sharply in the local vicinity of the Gly in LGL; the effect extends in both the N and C directions from the site of substitution, diminishing with distance from the Gly.

Experimental Section

Peptide Synthesis. The syntheses of peptides LLL and GGG are as previously described.[10] Peptide LGL was prepared by using solid-phase peptide synthesis on a Milligen/biosearch 9600 synthesizer. 9-Fluorenylmethyloxycarbonyl (Fmoc) protection was employed for the α-amino function of amino acids. 4-Methylbenzhydrylamine (MBHA) resin was used as the solid support, and 5-(4′-(Fmoc-aminomethyl)-3′,5′-dimethoxyphenoxy)valeric acid was used as the linker to give the peptide amide after cleavage at the end of synthesis. The coupling was performed by using the BOP/HOBT method.[14] The NH_2 terminal of peptide was succinylated by succinic anhydride in dimethylformamide with an equivalent of triethylamine. Cleavage and deprotection were effected by treatment with TFA reagent (90% trifluoroacetic acid, 5% thioanisole, 3% ethanedithiol, and 2% anisole) at room temperature for 2 h and precipitated with ether. Crude peptides were purified by reverse-phase preparative HPLC on an Econosil C18-10μ column (250 mm × 22.5 mm) monitored at 270 nm with a gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid. The purified peptides were desalted on a Sephadex G-10 column and checked for purity on an analytic Delta Pak C18-15μ HPLC column (300 mm × 3.9 mm) monitored at 229 nm (see Figure 1a).

All the protected amino acid residues, MBHA resin, and PAL linke were purchased from MilliGen/Biosearch. 1-Hydroxybenzothiazole (HOBT) was obtained from Keystone Biotec. BOP reagent was supplied by Peptides International. All other reagents were obtained from Aldrich.

Mass Spectrometry. The correct primary ion molecular weight of the purified peptides were confirmed by fast atom bombardment mass spectrometry (M-Scan, Inc., West Chester, PA) as shown in Figure 1b. Fast atom bombardment analysis was carried out on M-Scan's VG Analytical ZAB-2SE high-field mass spectrometer operating at V_{acc} = 8 kV. A cesium ion gun was used to generate ions for the mass spectra which were recorded with a PDP 11-250J data system. Mass calibration was performed with cesium iodide or cesium iodide/glycerol.

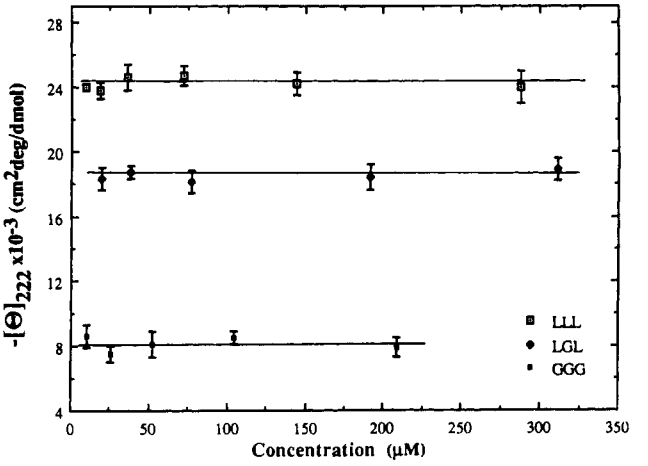

Figure 2. Concentration dependence of the ellipticity at 222 nm for peptides LLL, LGL, and GGG. The uncertainty is based on three measurements in each case.

Circular Dichroism (CD) Measurements. Circular dichroism spectra were run on a modified Cary 60 spectropolarimeter (Aviv DS60) equipped with an HP Model 89100A temperature controller. The wavelength was calibrated with (+)-10-camphorsulfonic acid.[15] The concentration of stock peptide solution was determined by using the absorption of Tyr in 6 M guanidine hydrochloride (ultrapure reagent from Schwarz) at 275 nm as a reference.[16] All measurements were carried out in 10 mM with 1 mm path length cells. The pH was adjusted with HCl and NaOH. The concentration in CD experiments were 25–40 μM, except when concentration dependence was studied.

^{1}H NMR Spectroscopy. NMR spectra were taken on a Bruker AM-500 spectrometer. Standard 2D experiments (HOHAHA, NOESY) were run on samples of ca. 5 mM peptide in 10% D_2O and room temperature, as described previously.[13]

Results and Discussion

It is essential to establish that the structure in LGL is intramolecular.[10] Figure 2 shows that the mean residue ellipticity,

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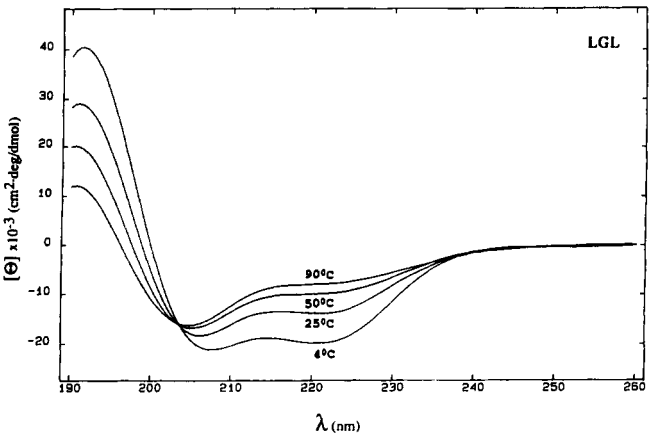

Figure 3. CD spectra of peptide LGL in 10 mM KF, neutral pH, at 4, 25, 50, and 90 °C. This experiment tests the hypothesis that the helix structure in LGL results from a tight association between chains.

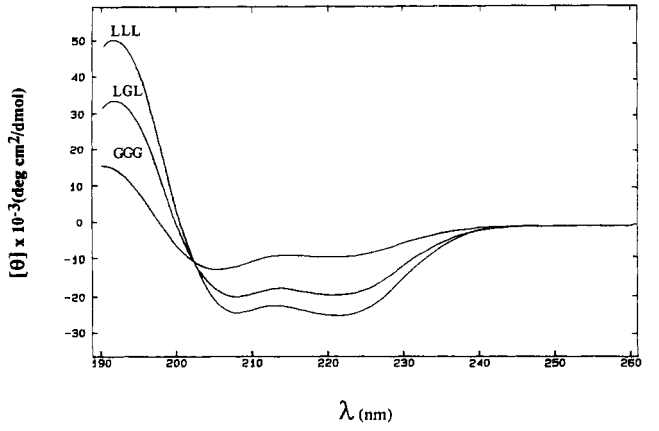

Figure 4. CD spectra of the three peptides LLL, LGL, and GGG in 10 mM KF, neutral pH, at 4 °C.

$[\theta]_{222}$, is independent of the peptide concentration for the three peptides of this study, over a range from about 10–300 μM in peptide. Figure 3 shows the CD spectra of LGL at four different temperatures. The gradual loss of helicity with temperature from 4 to 90 °C reveals a weak cooperativity in thermal transition. Figure 4 compares the CD spectra of LGL with those of LLL and GGG. Peptide absorption bands near 200 nm become optically active in α-helices, producing a characteristic double minimum in the CD at 208 and 222 nm and a maximum at 195 nm.[17] The mean residue CD values at these wavelengths are widely used as a measure of helicity.[17] Figure 5 shows the calibration curve for LLL in the presence of increasing concentration of the helix-forming solvent, TFE (insert), from which we derive the value $[\theta]_{222} = -32000 \pm 700$ deg·cm^2/dmol for the maximally helical peptide. Titration of the peptides of this study with the denaturing solvent guanidine–HCl is shown also. In each case, we obtain $[\theta]_{222} = 0 \pm 500$ deg·cm^2/dmol for an apparently fully coiled peptide. On the basis of these values, we can estimate from Figure 4 that at 4 °C, LLL contains about 75% helix, GGG about 25%, while LGL has 58%. The results are summarized in Table I. We have previously estimated the equilibrium constants s for adding a residue of any of 10 amino acids to a nucleated helix[10] using a multistate statistical model for the process of helix formation.[18] The model allows one to fit s values to the CD spectrum of a given peptide by a simple iterative procedure. From this model we calculate that a single glycine substitution destabilizes the helix in LLL by 0.60 ● 0.05 kcal/mol, a value in reasonable agreement

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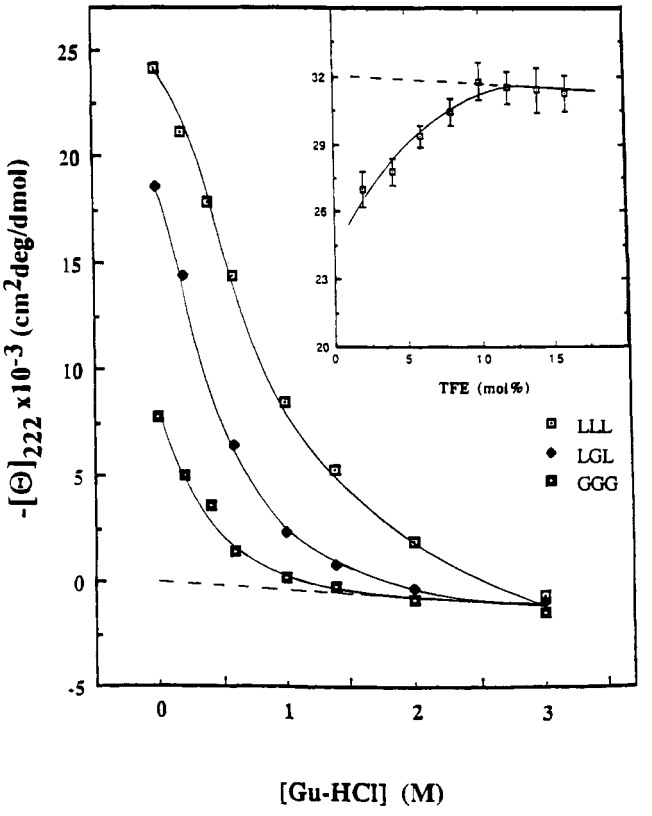

Figure 5. Guanidine–HCl concentration dependence of $[\theta]_{222}$ for peptides LLL, LGL, and GGG. The insertion shows TFE dependence of $[\theta]_{222}$ for LLL.

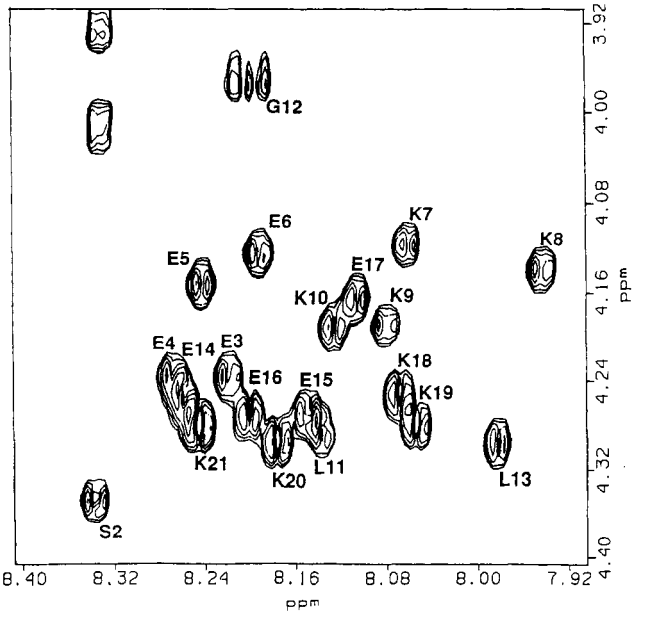

Figure 6. The α-amide region of the TOCSY spectrum (19) of LGL peptide (~5 mM) in 10% D_2O at 25 °C.

Table I. Helix Content and Free Energy of Substitution of Three Synthetic Peptides

peptide	$-[\theta]_{222}$[a]	f,[b] %	$\Delta\Delta G$[c]
LLL	24100 ± 700	75 ± 3	−1.84 ± 0.15
LGL	18500 ± 550[d]	58 ± 3	−1.20 ± 0.10
GGG	18100 ± 450	25 ± 2	0.00

[a] Mean residue ellipticity (cm^{2}deg/dmol) of the peptides at 222 nm. [b] $f = [\theta]_{obs}/[\theta]_{100}$, the fraction of helix. $[\theta]_{100} = -32{,}000 \pm 700$ cm^{2}deg/dmol as shown in Fig. 5. [c] Free energies per chain relative to GGG calculated from a multistate model of the helix-coil transition (18). [d] The helicity estimated from the multistate model is −18,900.

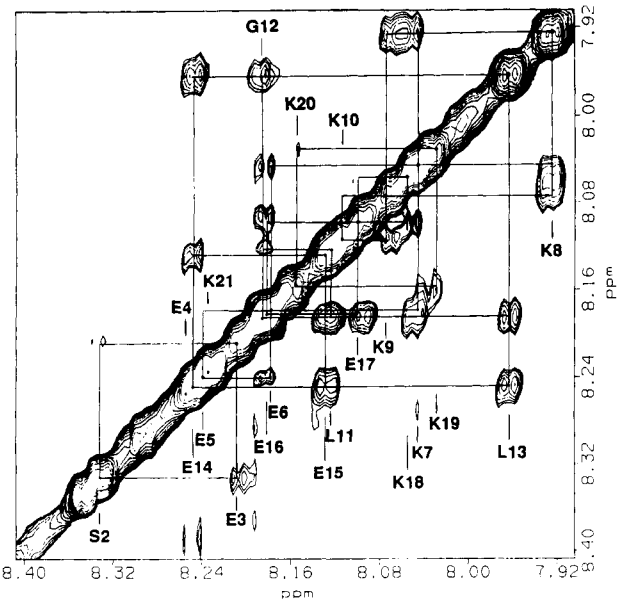

Figure 7. The amide–amide region of the NOESY spectrum of LGL peptide in 10% D_2O at 25 °C.

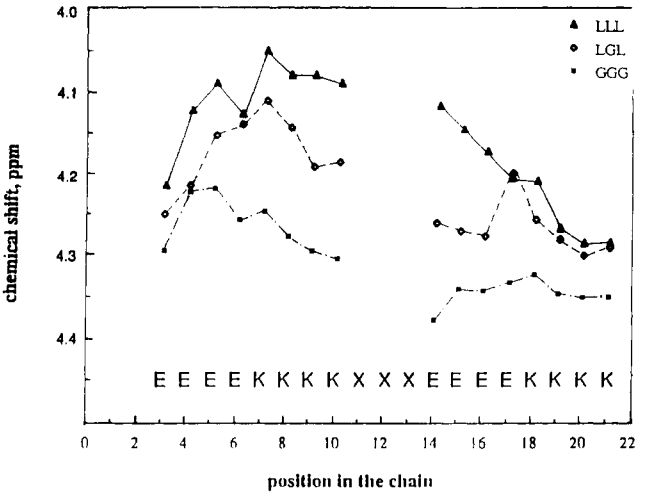

Figure 8. Diagram of α-proton chemical shifts for peptides LLL, LGL, and GGG vs position in the chain. Chemical shifts were measured from TOCSY experiment.

with estimates from peptide models.[5,9,10]

Use of ^1H NMR criteria for helical residues defines the extent and position of the helix in a given peptide more precisely.[13] Sequential assignments[19] of the ^1H spectra of LLL and GGG have been reported.[13] We have performed 2D TOCSY and NOESY spectroscopy on LGL yielding complete assignment of its ^1H spectrum. Figure 6 shows the assigned fingerprint region of the ^1H NMR spectrum of LGL derived from a standard sequential assignment.[20] As in the case of the AAA and LLL peptides, the low values of $^3J_{\alpha N}$ coupling constants and the presence of short-range (i, i + 1 NH–NH) and medium-range (i, i + 3 NH–CαH) NOE's identify helical residues unequivocally. Figure 7 shows the short-range i, i + 1 amide–amide connectivities in the NOESY spectrum of LGL. The frequency of the Cα proton resonances of the invariant residues in these peptides has been found to provide a useful measure of local helicity in these peptides:[13,21] the further upfield a given Cα shift, the greater the helix content at that position in the chain. Figure 8 contrasts the CαH chemical shifts in these molecules. In agreement with the CD spectral analysis, the LGL peptide has a helix content intermediate between those of LLL and GGG.

It is apparent from Figure 8 however that the local distribution of helix in the two chains which contain appreciable helix structure differs drastically. The helix in LLL can be seen to extend nearly to the N terminus, reaching a maximum near K7, and declining gradually from residue E14 toward the C terminus. The helix in LGL behaves similarly, except for a sharp decrease in the vicinity of the Gly residue (Figure 8). Gly is thought to destabilize α-helix primarily because of its increased conformational freedom in the coil.[12] The distribution of helical residues in LGL suggests that each residue in the chain can access the coil as well as helix state, so that the system is in fast exchange on the NMR time scale. An independent indication for fast exchange is that, in all these peptides, the two β-protons of the side chains are equivalent in chemical shift. The picture which results then is that despite the stabilizing influence of the flanking Leu residues on the overall helicity in LGL, the single glycine locally diminishes the helical structure in a manner that attenuates with distance from the site of substitution. In the isolated α helix, there is rapid interconversion between helix and coil at any site, with consequent loss in local as well as overall helical structure.

The triple substitution (GGG) diminishes the stability of the helix by approximately three times as much as the single substitution (LGL) relative to LLL, indicating no strong local interactions among the glycines. This result suggests that the data obtained from triple substitutions, which were used to amplify the potentially small free energy differences, are appropriate for application to single substitutions, hence that the observed differences reflect intrinsic propensities of the test residues. Both additive and nonadditive double mutations in proteins have been described.[22,23] For example, the $\Delta\Delta G$ (1.10 kcal/mol) of double Gly → Ala substitutions at positions 46 and 48 in the N terminal helix of λ repressor is found to be less than the sum of single changes at each position (Gly46 → Ala/0.66 kcal/mol, Gly → Ala/0.87 kcal/mol). The difference in this case might be due to specific effects on helix nucleation rather than propagation.[22] In phage T4 lysozyme, the stabilization arising from two double mutants (E128A/V131A/0.42 kcal/mol) within a single α-helix is equal to the sum of two single mutants (E128A/0.16 kcal/mol, V131A/0.26 kcal/mol).[23]

Quantitative differences in helix stability at the site of substitution in LGL relative to LLL can also be compared with estimates from globular proteins. A series of single site substitutions in a hydrophic helical site Ile3 or T4 lysozyme gives a $-\Delta\Delta G$ value of 2.5–2.7 kcal/mol for GLy → Leu,[24] much larger than the results obtained here. Most of this effect can be attributed to hydrophobic interactions, since if we correct for the contribution of hydrophobic free energy ($-\Delta G_{tr}$ for Leu relative to Gly was estimated as about 2.15–2.56 kcal/mol)[25] the result is close to that obtained here. The difference between Gly → Ala is only 0.7–1.4 kcal/mol,[24] but the hydrophobicity of Ala is much smaller than that of Leu ($-\Delta G_{tr}$ relative to Gly is 0.20–0.80 kcal/mol).[25] It is also close to our result (Gly → Ala/0.79 ± 0.07 kcal/mol)[10] after correction with hydrophobic contribution. On the other hand, the $\Delta\Delta G$ value of substitutions at a solvent exposed helical position in λ repressor for Gly → Ala (0.66–0.87 kcal/mol)[22] can be compared directly with our result since no hydrophobic interaction is involved.[10] Thus the quantitative value determined from LGL agrees reasonably with measurements at solvent exposed sites in helices of globular proteins. Whether the substitution of single

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glycine has a similar local structural effect in these molecules remains to be seen.

Conclusions

The conclusions of this study are that the effect of a single glycine substitution in an isolated α-helix reduces the mean helix content of the peptide as one would predict given the s value for this side chain.[9,10] However, an individual glycine exerts a strong local destabilizing effect on the helix at and close to the substitution site. The helical structure in these peptides is likely to reflect an equilibrium between helix and coil that is rapid on the NMR time scale at 25 °C.

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Registry No. LLL, 131131-67-4; GGG, 132791-42-5; LGL, 131131-69-6; G, 56-40-6.

Determination of pK_as of Ionizable Groups in Proteins: The pK_a of Glu 7 and 35 in Hen Egg White Lysozyme and Glu 106 in Human Carbonic Anhydrase II

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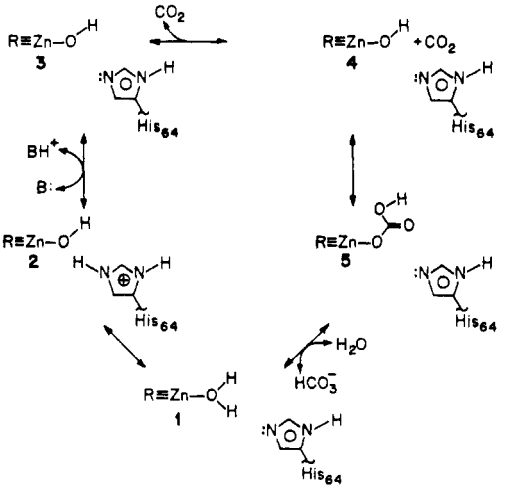
Abstract: We report a method by which molecular dynamics-free energy perturbation simulations can be used to estimate the pK_a of ionizable groups in proteins. The method has been tested to demonstrate its effectiveness in determining the pK_a of Glu 7 and 35 in hen egg white lysozyme, where the former has a pK_a of 2.6 and the latter a perturbed pK_a of 6.0-6.5 (in the presence of un-ionized Asp 52). We predict that the pK_a of Glu 7 is 3.1 ± 3.1, while we predict that Glu 35 has a pK_a of 12.0 ± 1.3. These test simulations indicate that (1) our approach is capable of predicting the perturbation of the pK_a of glutamic acid to higher values, (2) the precision of the approach depends on the conformational flexibility of the glutamic acid side chain, and (3) the accuracy of the approach, on average, is ±3 pK_a units. This approach was then applied to the problem of the pK_a of Glu 106 in human carbonic anhydrase II (HCAII). The activity of HCAII is dependent on a group whose pK_a is around 7.0. Glu 106 has been implicated as this group, but this requires the pK_a of this residue to be around 7. We predict that this group has a pK_a of 2.2 ± 2.8, which, even given the accuracy of our method, suggests that this group is not the activity-linked group. The present work demonstrates that our approach can be fruitfully applied to chemically important questions and that free energy methods can be applied to the determination of pK_a's in proteins with an accuracy to about ±3 pK_a units.

Introduction

The catalytic mechanism of human carbonic anhydrase (HCA) has been studied in detail.[1-4] The catalysis is dependent on a group whose pK_a is around 7.[1] After much debate it was decided that a zinc-bound water best satisfied this criterion, which led to the formulation of the zinc-hydroxide mechanism (see Scheme I).[1] However, one mechanism that is significantly different than the zinc-hydroxide mechanism, but cannot be ruled out is the proton shuttle mechanism of Kannan et al. (see Scheme II).[2,3] The zinc ion sill has a similar role, but the proton is not shuttled out of the active site via His 64 (**1** → **2**). Instead, this mechanism involves the active site residues Thr 199 and Glu 106 in a proton relay (forward A → B).[2-4] The one drawback of this mechanism, though, is that it requires that the activity-linked group whose pK_a is around 7 be Glu 106 (C → D). The pK_a of a glutamic acid is normally around 4.0, but highly perturbed pK_a's for glutamic acids have been observed in proteins.[5] Experimentally it

Scheme I



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