Proton Nuclear Magnetic Resonance Investigation of the Active Site Fragment of Splenin, an Immunoregulatory Polypeptide

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Abstract: The peptide fragment Arg-Lys-Glu-Val-Tyr (SP5) is the active site fragment of splenin (formerly thymopoietin III), an immunoregulatory polypeptide isolated from bovine spleen. Like its parent polypeptide, SP5 also induces the differentiation of T and B lymphocytes. We report here an NMR investigation of the conformational properties of this active fragment in aqueous solution. All the observed NH and CH resonances of SP5 have been assigned by one-dimensional and two-dimensional NMR techniques. The variation of chemical shifts with pH, the individual amide hydrogen exchange rates, and the vicinal NH-C$^{\alpha}$H coupling constants have been measured. The data are compatible with the assumption of a highly motile dynamic equilibrium among different conformations, some of which are stabilized by internal hydrogen bonding involving the participation of Glu_3-NH, Val_4-NH, and Tyr_5-NH in the backbone and of the guanidino N$^{\epsilon}$H proton of the Arg_1 side chain. These observations provide an insight into the conformational tendencies of SP5 in aqueous solutions.

Thymopoietins (TP) I and II are polypeptide hormones isolated originally from bovine thymus.[2] Even though the bioassay used to isolate these molecules was based on their secondary effects on neuromuscular transmission,[2,3] an important biological function of both these hormones was shown to be the induction of early T lymphocyte differentiation.[4] This action was quite selective in that the thymopoietins inhibited early B cell differentiation.[5] Further, they also were shown to modulate more mature lymphocytes. The complete amino acid sequences were determined[6] and the biologically active site, common in both, was identified.[7] The fragment Arg-Lys-Asp-Val-Tyr (TP5) is the active site, corresponding to positions 32–36 of the parent hormone and is the smallest fragment that could retain the activity of the parent hormone, viz., induction of selective T-cell differentiation.[8] More recently, an immunologically cross-reactive material termed splenin (previously thymopoietin III) was isolated from bovine spleen.[6b] The complete amino acid sequence for this polypeptide was determined and was found to be closely homologous with that of thymopoietin I and II, differing only at three positions with respect to the former and at two positions with respect to the latter. In contrast to thymopoietin I and II, splenin was found to induce the differentiation of both T and B cells.[8]

The close sequence homology among the three, thymopoietins I and II, isolated from the thymus, and splenin, isolated from the spleen, suggests a common ancestral gene. However, it is interesting that Glu is present at position 34 of splenin, the putative active site region of TP-I and -II, both of which have Asp at this location.[6b] It is even more intriguing that the fragment Arg-Lys-Glu-Val-Tyr (SP5) differs from TP5 in its biological activity in that SP5 induces nonspecific differentiation of both T and B cells while TP5 induces selective T-cell differentiation.[9] A structural investigation of the parent hormones themselves is not feasible due to difficulties associated in isolating sufficient quantities needed for physicochemical investigations. On the other hand, the pentapeptide fragments can be synthesized in large enough quantities for spectroscopic studies. The retention of the respective biological activity of the parent hormones by TP5 and SP5 suggests that these fragments may be capable of assuming conformations similar to the active sites in the parent polypeptides. Therefore, a comparative study of the conformational tendencies of SP5 and of TP5 is of interest in relation to the conformation–activity relationships among these immunoregulatory peptides. Toward this end we have employed ^{1}H NMR spectroscopy to characterize the free solution conformation of SP5. We have earlier reported a characterization of TP5.[10,11]

Experimental Section

The fragment SP5 was prepared by solid-phase synthesis. Sample concentrations of 5 mM in D_2O and of 20 mM in H_2O were used for the NMR studies. The H_2O samples also contained 10% D_2O for field-frequency lock. The sample pH was adjusted by using appropriate amounts of HCl (DCl) and NaOH (NaOD) solutions of known concentration. The reported pH values are the direct pH meter readings and have not been corrected for the isotope effects for the D_2O samples. All the reported chemical shifts were referenced with respect to TSP [sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate] as the internal reference. The chemical shifts were corrected for the titration shift of this peak[12] and expressed with respect to the peak position of TSP in its fully protonated form.

The proton NMR spectra were recorded at ambient temperature (25 °C) on a Bruker WH-400 spectrometer equipped with an Aspect-2000 computer. Measurements on the low-field NH resonances in H_2O samples were made using the 2-1-4 pulse sequence.[13,14] Measurements in D_2O samples were made in the conventional FTNMR mode. Many double resonance techniques[15-18] are available for measuring labile hydrogen exchange rates of biological systems in H_2O. The chemical

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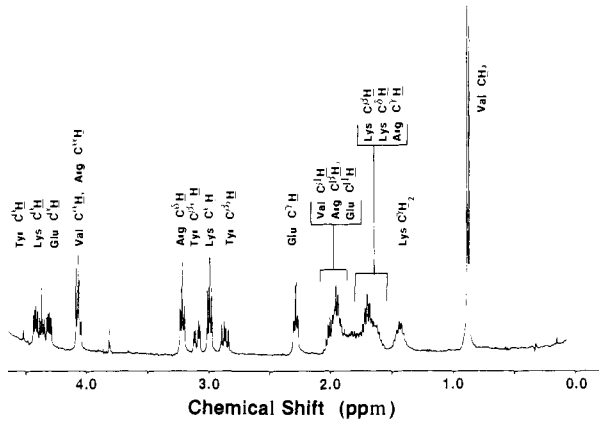

Figure 1. The 400-MHz proton Fourier transform NMR spectrum (0–4.5 ppm) of SP5 (Arg-Lys-Glu-Val-Tyr) in D_2O (pH 5.0).

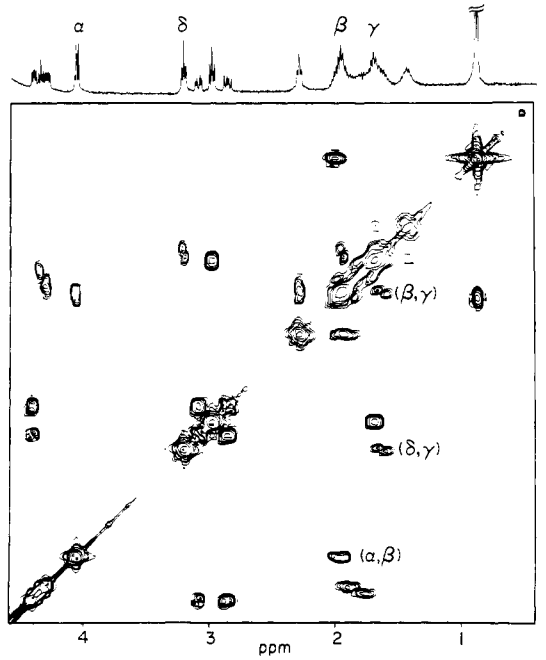

Figure 2. Contour plot of the 2D-COSY NMR spectrum of SP5 in D_2O (pH 5.0). The various cross peaks on either side of the diagonal arise due to the presence of J couplings between various spin multiplets shown in the corresponding 1D-NMR spectrum at the top. As an example, the cross peaks arising from J couplings between the α and β, β and γ, and δ and γ protons of the Arg_1 residue have been identified on the contour map.

exchange rates of individual amide hydrogens of SP5 were measured at ambient temperature by the transfer of solvent saturation/amide hydrogen saturation recovery NMR experiments.[15,16,19,20] In order to trace J connectivities between various resonances, a two-dimensional (D) ^1H shift correlated (2D-COSY) NMR experiment[21] has been employed. This technique employs a $90°-t_1-90°-$(acquisition, t_2) pulse sequence.[21] The data size used used in obtaining the 2D spectrum was 512 × 512.

Results

Assignments. A typical 400-MHz proton NMR spectrum of SP5 in D_2O showing various CH resonances in the range 0–4.5 ppm is shown in Figure 1. These resonances have been assigned on the basis of 1D- and 2D-NMR methods. The 1D-NMR methods included assignments based on multiplet structures, spin-decoupling experiments, and the characteristic pK_a values exhibited by protons adjacent to sites of ionization in the peptide. The 2D-COSY NMR method used helped us to trace the various J connected transitions in the complex 1D spectrum, and typical results are presented in Figure 2. It shows a contour plot and consists of several overlapping peaks along the diagonal with cross peaks appearing symmetrically on either side representing J connected transitions. Figure 2 also gives the corresponding 1D-NMR spectrum at the top. When the J connectivities are traced, the various ^1H resonances can be assigned to the individual amino acids in SP5 as indicated in Figure 1. The low-field region of the NMR spectrum of SP5 in H_2O is shown in Figure 3. It shows the aromatic CH resonances and the exchangeable NH resonances. The latter have been assigned on the basis of underwater decoupling experiments[10] in the conventional FT NMR mode as well as on the basis of characteristic pH dependence of chemical shift titrations.

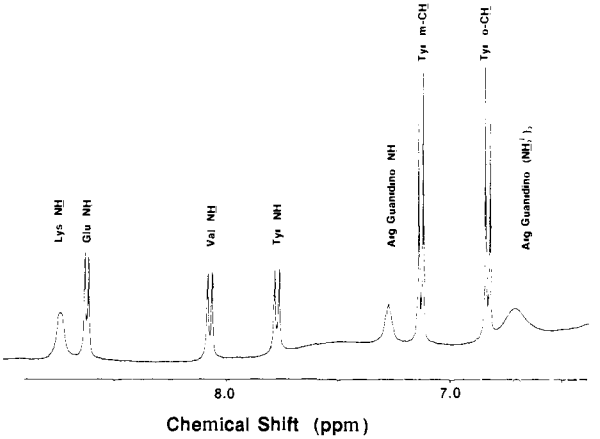

Figure 3. The 400-MHz NMR spectrum of SP5 in H_2O (pH 5.0) showing the low-field proton resonances.

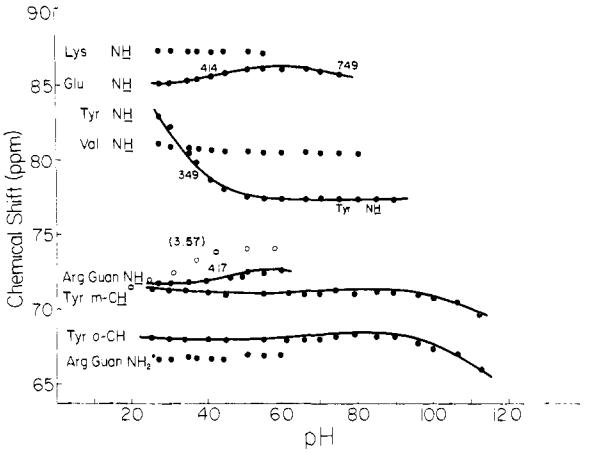

Figure 4. pH dependence of chemical shifts (closed circles) of the amide NH and the aromatic CH resonances of SP5. The pK_a values for some of the shifts are also indicated. The open circles are the titration shifts experienced by the guanidino $N^\epsilon H$ of a related peptide, TP5 (Arg-Lys-Asp-Val-Tyr).

pH Dependence of Chemical Shifts. The pK_a values of 3.5 for the titration of C-terminal carboxylate group and of 7.5 for the N-terminal α-amino group deduced from the titration shifts in D_2O (not shown) are in the general range expected for polypeptides.[22] The Glu_3 $C^\gamma H_2$ protons exhibit a titration shift with a pK_a of 4.3, due to the ionization of the side chain carboxylate group. This value is similar to that reported for Glu in other peptides.[22,23] Since the chemical shift titrations were all confined

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Table I. Vicinal NH-C$^{\alpha}$H Coupling Constants[a] for SP5

residue	J_{cor},[b] Hz
Lys	7.6 ± 0.5
Glu	8.7 ± 0.5
Val	9.8 ± 0.5
Tyr	9.8 ± 0.5

[a] Measured at pH 2.3. [b] $J_{cor} = 1.09 J_{obsd}$ values are the experimentally measured doublet separations and 1.09 refers to the corrections due to substituent effects (ref 49).

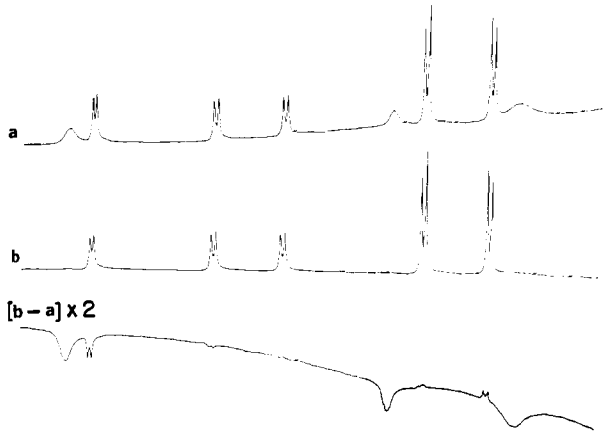

Figure 5. A typical transfer of solvent saturation NMR experiment on SP5/H_2O (pH 5.5) performed in the 2–1–4 pulse mode. The top trace (a) is the control spectrum and shows the low-field ^1H resonances. The middle trace (b) shows the spectrum obtained after steady-state saturation of the solvent resonance by multiple point irradiation with a radio-frequency field. The bottom trace shows the difference ($b - a$) amplified 2 times. The negative peaks indicate the amide hydrogens undergoing relatively significant (i.e., compared to their spin–lattice relaxation rates) chemical exchange with the solvent hydrogens. The positive peaks from the Tyr aromatic CH protons arise effect[53,54] due an intermolecular nuclear Overhauser effect[53,54] due to dipole–dipole interaction between the solute and solvent hydrogens.

to pH values below 11.2, the pK_a values for the ϵ-amino group of Lys_2 and the phenolic group of Tyr_5 could not be precisely determined. Figure 4 shows the pH dependence of chemical shifts of the various NH protons in SP5. An unusual feature readily seen in Figure 4 is the downfield shifts exhibited by Glu_3NH and Arg_1 guanidino N$^{\epsilon}$H protons, whereas intrinsic titration shifts are normally expected to be upfield.[24] The conformational implications of these downfield shifts will be discussed later.

Vicinal NH–C$^{\alpha}$H Coupling Constants. The individual NH–C$^{\alpha}$H vicinal coupling constants have been measured for SP5, and these are all listed in Table I. The magnitudes of the coupling constants suggest significant conformational averaging. Nevertheless, the observed coupling constants impose certain constraints on the various possible dominant conformations within this conformational equilibrium.

Amide Hydrogen Exchange Rates. Our laboratory has extended the double resonance technique consisting of transfer of solvent saturation/amide hydrogen saturation recovery NMR experiments[15,16] to the study of conformational properties of a number of peptides.[10,11,19,20,25–27] Most peptides appear to obey the high motility limit of conformational transitions;[28,29] i.e., the rate of

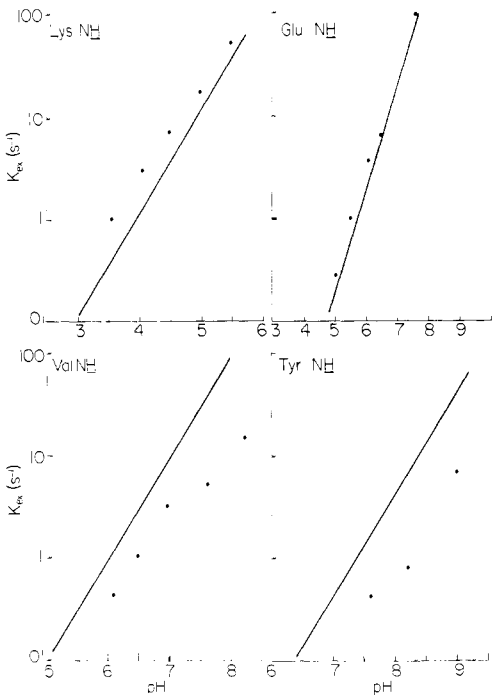

Figure 6. Comparison of experimentally determined amide hydrogen exchange rates (solid circles) of SP5 with the predicted rates (solid curves) corresponding to solvated peptides. In the panel for Tyr NH, the exchange rates (open circles) for the related peptide TP5 (measured at 25 °C) are also shown for comparison.

conformational transitions is much faster than the solvent exchange rates. In this limit the recovery of the amide hydrogen magnetization to its thermal equilibrium value in a saturation recovery NMR experiment is described by a single exponential.[20,29] A typical transfer of solvent saturation NMR experiment on SP5 is presented in Figure 5. Amide hydrogens experiencing significant chemical exchange exhibit a decrease in their intensities when the solvent is completely saturated. The chemical exchange rates of the individual amide hydrogens can be determined at any given pH by simply calculating the product of the fractional intensity change in the solvent saturation experiment and the apparent relaxation rate in the saturation recovery NMR experiment.[15,16,19,20,29] Under the high motility limit, the observed exchange rate is a weighted average of exchange rates from the various possible conformational states of the peptide.[20,29] Figure 6 presents the experimentally determined exchange rates for each of the amide hydrogens Lys NH, Glu NH, Val NH, and Tyr NH. Also shown in the figure are predicted rates (solid curves) calculated from the compilation of Molday et al.[30] for completely solvated model peptides.

Discussion

Measurement of amide hydrogen exchange rates has long been recognized as a powerful technique for gaining structural and dynamical information of proteins.[31–35] Peptide groups that are internally hydrogen bonded or remain inaccessible to the solvent within the hydrophobic interior of the protein are expected to show diminished exchange rates compared to those that are hydrogen bonded to the solvent (H_2O) molecules. Thus, a key requirement

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for a quantitative evaluation of the exchange characteristics of individual peptide groups in a protein is the availability of suitable model compound data to predict the exchange rates for the corresponding solvated amide. Such model compound data has been provided by Molday et al.[30] in 1972. The exchange rates of solvated peptides have been found to vary over an order of magnitude,[36] and this variation could be accounted for by taking into account the inductive effects due to amino acid side chains and due to ionizable groups.[30,37] The amide exchange rates have been found to be principally determined by the nearest neighbors and these effects are largely additive.[30] The model compound data provided by Molday et al. thus enable one to predict the solvated exchange rate for any given peptide group with a known primary structure within a protein. The predicted rates (solid lines) shown in Figure 6 for the peptide NH protons of SP5 take into account these primary structure effects. Even though Molday et al. have been able to convincingly predict the solvated exchange rates for two model systems, poly(DL-alanine) and *N*-acetyl-Ala-Gly-*N'*-methylamide, to an accuracy better than a factor of 2,[30] we have been considering as significant only those deviations between the predicted and observed rates that are greater than a factor of 2–3, mainly to be on the conservative side and to account for any additional influences of unknown origin on the exchange rates (e.g., variations in the inductive effects due to differences in the side chain conformations of peptides and model compounds).

Examination of Figure 6 shows that the exchange of all the secondary amides is essentially base catalyzed in the pH range employed in the studies and that there does not appear to be a significant acid catalysis. This observation is in agreement with the known behavior of secondary amides, which exhibit an exchange minimum in the pH range 2–3 and hence only base catalysis is observed above pH 3.[30,31] This is in contrast to primary amides (e.g., Asn and Gln side chain primary amides), which have an exchange minimum in the pH range $\simeq$4–5 and hence exhibit significant acid catalysis above pH 2.[38-40] At any given pH, Lys NH shows highest exchange rate, as expected due to the inductive effects of the positively charged α-ammonium group at the N terminal.[30,37] Conversely, the Tyr NH shows smallest exchange rates since the negatively charged C-terminal carboxylate (above its pK_a) decreases the base catalysis.[30] Among the backbone amide hydrogens both Val NH and Tyr NH exhibit a substantial decrease (in excess of a factor of 3) in their exchange rates compared to predicted rates. However, since the exchange lifetimes for these two amide hydrogens are relatively short ($\sim$0.1 to $\sim$1 s), rather than several hours, it is clear that these amide hydrogens experience rapid conformational transitions between solvated structures and folded structures in which the exchange is retarded. The retardation of exchange in the folded structures could be ascribed to be due to (i) internal hydrogen bonding or (ii) solvent inaccessibility. While it is perhaps reasonable to think that solvent inaccessibility could be a contributing factor for retarded exchange in large proteins with hydrophobic interior[35] or in cyclic peptides with limited conformational mobility,[41-43] it is somewhat inconceivable that in a small linear peptide such as SP5 with considerable segmental mobility a peptide NH would remain inaccessible to the solvent for significant periods of time. For example, the exchange of Tyr NH of SP5 is retarded by a factor of 6. A simple calculation using a two-state model shows that Tyr NH should remain inaccessible to the water molecules of hydration or to the charged catalytic species OH^- for 84% of the time—a requirement which is rather stringent for a highly conformationally mobile linear peptide such as SP5. It is also reasonable to assume that linear peptides cannot tolerate unsatisfied hydrogen bond donors since the expense in the free energy of breaking an amide hydrogen bond to water and not forming a compensating bond within the polypeptide is expected to be significant.[32,44] Therefore, we favor the former explanation; namely, the slow exchange of Val NH and Tyr NH reflect some conformational states in which these are internally hydrogen bonded (though not necessarily at the same time). Similar conclusions were arrived at by Oberholtzer et al. in explaining the retarded exchange in the N-linked glycopeptide of fetuin.[45] Because the saturation recovery curves were all described by single exponentials within experimental error, the transition between folded and solvated structures is fast not only on the chemical shift scale but also on the relaxation and exchange lifetime scales. This observation and the observed pH dependence of the apparent relaxation rates (not shown) suggest that the exchange behavior in this peptide is compatible with the assumption of the high motility limit. Of the remaining two peptide NH hydrogens, Glu NH shows essentially good agreement with the predicted rates, which is suggestive of a predominantly solvated environment for this amide hydrogen. However, as we will show later based on chemical shift data, minor populations ($\sim$26%) of conformations are also present in which Glu NH is also hydrogen bonded. Such minor populations cannot retard the Glu NH exchange to smaller than the factor of 2–3 uncertainty limit that we have adopted in our hydrogen exchange studies. The Lys NH exchange rates are slightly larger than predicted rates, and the reason for this is not clear. Through space effects due to the presence of a proximal positive charge could presumably account for such a behavior.[30] Nevertheless, it is clear that Lys NH also is definitely not internally hydrogen bonded. These observations of SP5 amide hydrogen exchange behavior are in general agreement with that of the pentapeptide TP5;[10,11] viz., both Lys NH and Asp NH are solvated while Val NH is internally hydrogen bonded. More recently we have also found that the Tyr NH of TP5 also exhibits diminished solvent exchange (open circles in Figure 6), an observation compatible with the assumption of internal hydrogen bonding for this amide proton. These results, together with vicinal coupling constant data, suggest some conformational similarities between TP5 and SP5.

However, SP5 differs from TP5 in certain respects in its conformational properties. Examination of Figure 4 shows that, as the pH is increased, both Glu_3 NH and Arg guanidino $N^\epsilon H$ show downfield shifts with a pK_a corresponding to the glutamic acid carboxylate group. This is a definite indication of hydrogen bonding of these two NH hydrogens with the carboxylate group of Glu_3. An internal hydrogen bonding of the Glu side chain carboxylate group with its own secondary amide hydrogen has been observed earlier in some model tetrapeptides[24] as well as in the globular protein basic pancreatic trypsin inhibitor (BPTI).[24] On the basis of NMR data on BPTI, Bundi and Wuthrich suggested a criterion for estimating the population of molecular species in which the Glu NH is hydrogen bonded to its own side chain carboxylate group; viz., a downfield shift of about 0.41 ppm (as observed for the Glu-49 NH of BPTI) may be taken to correspond to 100% population of the hydrogen-bonded species.[24] Using this criterion and noting that the downfield shift observed for Glu_3 NH in SP5 is only about 0.11 ppm, we estimate that the population of SP5 species in which the Glu_3 NH is hydrogen bonded to its own carboxylate group, thus forming a seven-membered ring system, is only about 26%. This estimate, of course, must be taken with caution due to the various assumptions involved.[24] Nevertheless, this species could not have been significantly (i.e., more than 50%) populated since the exchange data failed to detect this conformation. One possible source of uncertainty in the exchange data exists for Glu_3; viz., our theoretical calculation of solvated exchange rates for Glu_3 were based on values *suggested* by Molday et al.[30] for the inductive effects of the Glu side chain in its de-

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protonated form. A rigorous evaluation of the exchange behavior for Glu_3 NH in SP5 must await data on model compounds containing this residue. Nevertheless, the chemical shift data clearly suggest the presence of a conformation in solution in which Glu_3 NH is hydrogen bonded to its own side chain carboxylate group.

The downfield shift (pK_a = 4.17) experienced by the Arg guanidino $N^{\epsilon}H$ is suggestive of a hydrogen bond between this proton and the Glu_3 side chain carboxylate group. Figure 4 also shows, for comparison, the titration behavior (pK_a = 3.6) of this proton in the pentapeptide fragment TP5 (open circles) corresponding to the existence of a hydrogen bond between the guanidino $N^{\epsilon}H$ and the Asp_3 carboxylate group.[10] The much smaller shift observed for SP5 than for TP5 suggests that either the population of this conformation (stabilized by hydrogen bonds between the side chains of residues 1 and 3) is smaller for SP5 than for TP5 or the intrinsic strength of the hydrogen bond is weaker for SP5. We favor the former explanation since it is compatible with the presence of a minor population (~26%) of another species in which the Glu_3 carboxylate group is hydrogen bonded to its own secondary amide hydrogen.

The magnitudes of the vicinal NH–$C^{\alpha}H$ coupling constants observed for the backbone preclude the possibility of β turns[46] of type I ($\phi_{i+1} \simeq -60°$, $\psi_{i+1} \simeq -30°$, $\phi_{i+2} \simeq -90°$, and $\psi_{i+2} \simeq 0°$), type II ($\phi_{i+1} \simeq -60°$, $\psi_{i+1} \simeq 120°$, $\phi_{i+2} \simeq 80°$, and $\psi_{i+2} \simeq 0°$), and type III ($\phi_{i+1} \simeq -60°$, $\psi_{i+1} \simeq -30°$, $\phi_{i+2} \simeq -60°$, and $\psi_{i+2} \simeq -30°$) making dominant contributions to the conformational equilibrium of SP5 in solution since all these three turns predict vicinal coupling constants of the order of 3–4 Hz for the $i + 1$ residue whereas the observed coupling constants (Table I) are all much larger. The possiblity of distorted β turns with torsional angles differing from those given by Lewis et al.[46] by more than ±20° (e.g., $\phi_{i+1} \simeq -80°$) could not be excluded, but presumably they may not be favored energetically.

The diminished exchange rates for Val_4 NH and Tyr_5 NH and the observed vicinal coupling constants can be explained by the assumption of the existence of 1 → 3 bends (γ turns[47,48]) at the third and fourth residues. These turns are characterized by the torsional angles $\phi_{i+1} \simeq \mp 90°$ and $\psi_{i+1} \simeq \pm 60°$ (where the upper and lower signs correspond respectively to the equatorial and axial conformations).[48] The γ turn at position 3 permits a hydrogen bond between Val_4 NH and Lys_2 CO and a γ turn at Val_4 permits a hydrogen bond between Tyr_5 NH and Glu_3 CO. These conformations are also compatible with the vicinal NH–$C^{\alpha}H$ coupling constant data.[49] We have previously suggested, based on TP5 studies (where no data were available in our earlier studies for the exchange of Tyr_5 NH) that, in addition to a γ turn at position 3, a C_5 turn with torsional angles $\phi_{i+1} \simeq -150°$ and $\psi_{i+1} \simeq +150°$ at position 4 (Val) was also compatible with the observed NMR data on TP5.[10,11] However, the present studies on SP5 and on TP5 clearly indicate that Tyr_5 NH is also internally hydrogen bonded in both the peptides. A C_5 turn at position 4 is not favorable for the formation of this hydrogen bond. On the other hand, the assumption of a γ turn at Val_4 permits the formation of such a hydrogen bond.

In conclusion, the pentapeptide Arg-Lys-Glu-Val-Tyr exists in solution in a conformational equilibrium between folded and solvated structures. Among the folded structures we have identified conformations that are stabilized by four different hydrogen bonds. It is not necessary for any two or more of these hydrogen bonds to be present simultaneously in any given conformation though such a possibility cannot be overruled at this stage. The four hydrogen bonds that have been identified in this NMR investigation are (i) between Arg_1 guanidino $N^{\epsilon}H$ and Glu_3 carboxylate group, (ii) between Glu^3 NH and its side chain carboxylate group, (iii) between Val_4 NH and Lys_2 CO, forming a γ turn at position 3, and (iv) between Tyr_5 NH and Glu_3 CO, forming a γ turn at position 4. Construction of CPK models of SP5 suggests that it is feasible to accommodate the four hydrogen bonds in two different conformations: one stabilized by hydrogen bonds (i), (iii), and (iv) and the other stabilized by the bonds (ii), (iii), and (iv). Of course, all these observed properties should be viewed as time-averaged properties of several different conformations and provide an insight into the nature of conformational tendencies of this interesting T- and B-cell differentiating peptide fragment. It is intriguing that TP5, which has Asp at position 3, induces selective differentiation of T lymphocytes while SP5 with a Glu instead of Asp induces both T- and B-cell differentiation. The main differences between the conformational tendencies of SP5 compared to TP5 are (i) reduced population of the species with a hydrogen bond between the side chains of residues 1 and 3 and (ii) the presence of a new conformational species having a hydrogen bond between Glu_3 NH and its own carboxylate group. These observations suggest provocative and intriguing questions about the conformation–activity relationships existing among these homologous peptides with widely differing activities. Indeed, such conformation–activity studies provide rational guidelines in designing proper structure–activity experiments.[50-51] Together with studies of conformationally restricted analogues,[51,52] such studies pave the way toward the design of synthetic analogues aimed at exploiting the clinical potential of these peptides in treating immunodeficiency diseases in man.

Acknowledgment. The experiments described in this work have been performed at the NMR Core Facility of the UAB Comprehensive Cancer Center, which is supported by U.S. Public Health Service Grant CA-13148.

Registry No. SP5, 75957-60-7; splenin, 1416-60-0.

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