pH Dependence of Hydrogen Exchange from Backbone Peptide Amides in Apamin

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ABSTRACT: The kinetics of hydrogen exchange of the 11 most protected backbone amides of bee venom apamin have been measured between pH 1 and pH 8.5 by using time-resolved and saturation-transfer NMR spectroscopy. The five amides most protected from base-catalyzed exchange, those of residues 5 and 12–15, show highly correlated exchange behavior in the base-catalyzed regime. It is proposed that the intramolecular hydrogen bonds stabilizing these amides define a stable cooperative unit of secondary structure in apamin (a C-terminal helix and an N-terminal β-turn). This conformational unit is further stabilized (by 5–6 kJ mol^{-1}) on titration of the Glu-7 side-chain carboxyl group. The relative contributions of specific intramolecular interactions to this conformational stabilization are estimated. The pH_{minima} in the pH-dependent single amide exchange curves are compared with values predicted by correcting for sequence-dependent contributions to amide exchange rates [Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150–158]. The lack of correlation suggests that the "open" conformers from which amide exchange occurs are nonrandom. This conclusion is dependent on the assumption that acid-catalyzed exchange occurs via N-protonation so that residual conformational effects on exchange rates in the open conformers will affect acid- and base-catalyzed rates in approximately equal and opposite ways. A strong correlation between the measured pH_{minima} and the amide proton chemical shifts is observed, however, and this may be most easily accommodated if acid-catalyzed exchange occurs by the imidic acid mechanism (via amide O-protonation).

Apamin (Habermann & Reiz, 1965; Shipolini et al., 1967) is a small basic peptide of 18 amino acids from bee venom that blocks a Ca^{2+}-activated increase in permeability to potassium in cultured neurons (Hugues et al., 1982a), hepatocyte (Burgess et al., 1981; Cook et al., 1983), and certain smooth muscle preparations (Vladimirova & Shuba, 1978; Banks et al., 1979), possibly by binding to a Ca^{2+}-dependent potassium channel (Hugues et al., 1982b; Seager et al., 1984; Lazdunski et al., 1985). Two disulfide bonds cross-link the primary sequence of apamin, and studies using a variety of spectroscopic methods have shown that the peptide is conformationally stable in aqueous solution (Miroshnikov et al., 1978; Bystrov et al., 1980; Walde et al., 1981). A revised model for the conformation of apamin has recently been proposed on the basis of the complete assignment of the proton nuclear magnetic resonance (NMR)[1] spectrum, together with NOE data and the identification of exchange-protected backbone amides (Wemmer & Kallenbach, 1983). A major feature of this model is the identification of a helix that comprises the C-terminal 9–10 amino acid residues of the peptide. Contained within this helix are two arginine residues whose side-chain guanidino functions are important for biological activity (Vincent et al., 1975; Cosand & Merrifield, 1977; Granier et al., 1978) and may be involved in the putative binding of the peptide to a potassium channel.

The model proposed by Wemmer and Kallenbach (1983) for the conformation of apamin in water is probably a very good indication of the major elements of secondary structure stabilized by the disulfide bonds. It is generally recognized, however, that the interpretation of spectroscopic data from peptides in terms of a single fixed conformation is a necessary simplification of the true conformational properties of the peptide backbone in solution, whatever the extent to which secondary structure is stabilized. An analysis of the conformational fluctuations undergone by the peptide backbone, and of temperature and pH-dependent shifts in conformation or conformational stability, is of interest, particularly in understanding how conformational constraints on side-chain groups (topological structure and stability) are imposed by the conformational properties of the peptide backbone. Although the study of minor conformational states in conformationally stable polypeptides is difficult because of the low contribution of these states to the "conformational average" detected spectroscopically, conformational fluctuations can be studied if they are important for allowing exchange of peptide amides with solvent. This arises from the idea that intramolecularly hydrogen-bonded amides exchange only when conformational fluctuations result in the opening of these hydrogen bonds, exposing the amide to attack by solvent catalyst (water protons or hydroxyl anions). An analysis of single amide exchange rates can give information about the stability of hydrogen-bonded secondary structure in different regions of the peptide backbone, the dependence of conformational stability on environmental factors (pH, temperature, ligand binding, and presence of denaturants), the degree to which conformational fluctuations in units of secondary structure are correlated (a measure of conformational cooperativity), and, in favorable cases, some properties of the "open" conformations from which amide exchange occurs (Woodward et al., 1982; Wagner, 1983; Englander & Kallenbach, 1984).

In this paper, the pH dependence of exchange of the 11 most protected backbone amides in apamin measured by NMR is presented. The excellent resolution of the amide region of the proton NMR spectrum allows accurate rate determinations to be made over a wide range of conditions of temperature and pH and in the presence of denaturants. Moreover, the relative simplicity of the structure of apamin makes possible the detailed interpretation of pH-dependent exchange data without

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; pH_{min}, the pH of the minimum in the pH-dependent amide exchange curves.

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the ambiguities that can confuse interpretations of pH-dependent amide exchange from proteins (Englander & Kallenbach, 1984). From the pH-dependent exchange data, it is possible to identify a stable unit of secondary structure that appears to undergo a cooperative "structure-opening" reaction allowing concerted exchange of amide protons with solvent and to follow pH-dependent shifts in the conformational properties of the peptide backbone.

Materials and Methods

The purification (Banks et al., 1981) and characterization (Dempsey, 1982) of the samples of apamin used in this study have been described.

NMR spectra were obtained on the Bruker 300 MHz and Oxford Instruments 470 MHz spectrometers of the Oxford Enzyme Group. Nuclear Overhauser experiments were done as described by Dubs et al. (1979) using short preirradiation times (<0.5 s) to minimize the effects of spin diffusion. NOE difference spectra were generated by intersubtracting the summed free induction decays from 256 on- and off-resonance preirradiated accumulations.

First-order hydrogen–deuterium exchange rates with rate constants less than about 0.1 min^{-1} were determined from the decay of intensity of individual amide resonances with time after dissolving the peptide in D_2O. The measurement of peak heights rather than integrated intensities yielded more accurate first-order rate constants. The lag time before spectral accumulations were begun (the time for mixing, introducing the sample into the probe, and temperature equilibration) was about 2 min, and each time point required accumulation for at least 1 min (32 scans) to generate adequate signal to noise. Exchange rates with half-lives of about 4 min or greater could be measured with good accuracy by using this method.

First-order amide exchange rates in the range 12–3000 min^{-1} were measured by saturation transfer from water under steady-state conditions. These experiments were done in 90% H_2O/D_2O and involved two measurements. At a pH where amide chemical exchange is slow, the spin–lattice decay rates (T_1) were measured by the inversion–recovery method with saturation of solvent (gated off during the inversion and observe pulses and spectral acquisition). First-order exchange rates (k_a) were determined over the pH range at which transfer of saturation on preirradiation of the solvent could be observed by using the relationship (Gadian, 1982):

$$k_a = (M_0 - M_a)/(M_a T_{1a})$$

A value of M_0 was determined by measuring the amide intensity at pH 3 (where chemical exchange is very slow compared with T_1) with gated preirradiation of solvent. This corrects for the solvent to amide NOE, leaving saturation transfer as the sole contribution to intensity changes on preirradiation of solvent. The use of this method rests on the assumption that the intrinsic T_1 of the observed amide does not change significantly with pH. This was found to be the case at least over the pH range (pH 2–5 for the highly protected amides) where chemical exchange makes no contribution to the measured relaxation rates.

Exchange experiments were done at 20 mM apamin concentration and were 20 mM in buffer ions. Buffer ions were replaced with 20 mM KCl for the saturation transfer experiments where pH variations were necessary. pH values are direct meter readings using the hydrogen electrode. Temperatures were controlled with a thermostated air flow and were calibrated with methanol or ethylene glycol (Van Geet, 1968). Exchange rates determined at temperatures within 5 deg of 20 °C were normalized to 20 °C by using the activation energies for the acid- and base-catalyzed exchange reactions given in Englander et al. (1979) to generate the curves for exchange at 20 °C in Figure 3. Saturation transfer rates were measured over a range of temperatures to extend the accessible pH range, and the activation energies for the rate-limiting step of the base-catalyzed exchange reactions were determined from an Arrhenius plot. Apart from small linear changes in chemical shift of hydrogen-bonded amides expected on increasing temperature, the NMR spectrum of apamin is unaffected by temperature at least up to 80 °C.

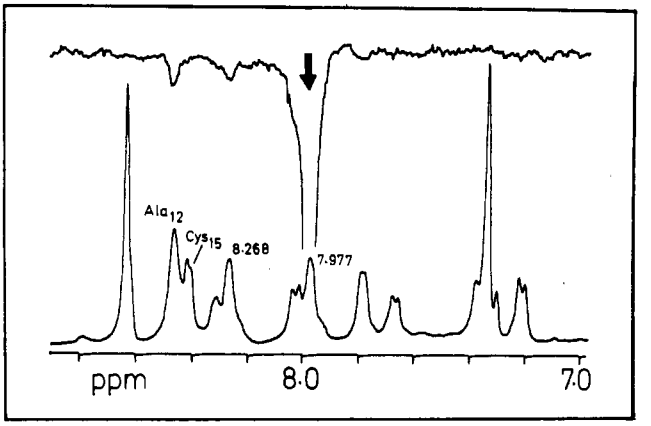

FIGURE 1: Low-field region of the 300-MHz NMR spectrum of apamin (15 mM, pD 2.5, 15 °C), 2 h after dissolving in D_2O (bottom), and the difference NOE spectrum obtained after a presaturating pulse (0.4 s) on the amide at 7.977 ppm (arrowed).

No correction for isotope effects was made in the comparison of time-resolved amide exchange measurements (H → D exchange) and saturation-transfer exchange measurements (H → H exchange). Amide exchange isotope effects have been measured by Englander et al. (1979) and were shown to be very small.

Results

Assignment of Arginine Amides. Using two-dimensional NMR methods, Wemmer and Kallenbach (1983) produced a complete assignment of the proton NMR spectrum of apamin, correcting and supplementing the assignments of Bystrov et al. (1980) which were obtained from one-dimensional NMR experiments. Our assignments have been consistent with the assignments of Wemmer and Kallenbach except for inconsistencies in the NOE data for the amide protons of arginine residues 13 and 14. This is illustrated in Figure 1 which shows the amide region of the proton NMR spectrum in D_2O (after partial exchange-out of quickly exchanging amides to improve spectral resolution) and the difference NOE obtained with a presaturating pulse (0.4 s) on the amide resonance at 7.977 ppm (Arg-14 NH in the assignments of Wemmer and Kallenbach). Small NOEs are seen to the upfield Arg NH signal (8.269 ppm; Arg-13 NH according to Wemmer and Kallenbach) and more strongly to the amide of Ala-12. An NOE to the Ala-12 β-methyl is also observed (not shown). Intra-amide NOEs observed on irradiation of the downfield Arg amide (8.269 ppm) cannot be unambiguously interpreted due to limited spectral resolution in this region of the spectrum. However, no NOE to the β-methyl of Ala-12 is seen. These NOEs are consistent with the assignment of the amide resonance at 7.977 ppm to Arg-13 NH and that at 8.268 ppm to Arg-14 NH (a reversal of the assignments of these resonances given by Wemmer and Kallenbach). The full amide resonance assignments are shown in the low-field region of the proton NMR spectrum of apamin in Figure 2.

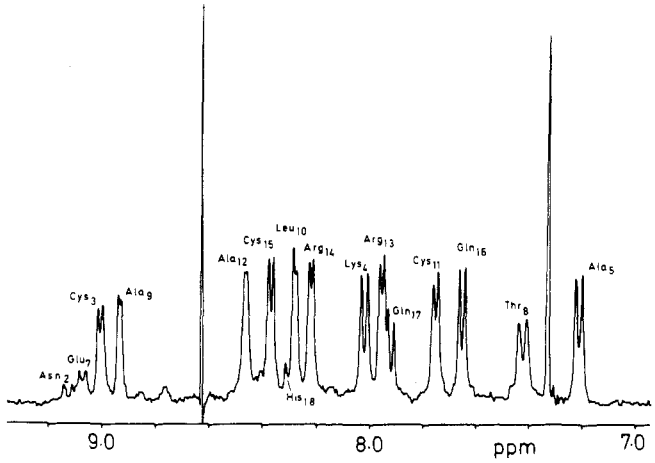

FIGURE 2: Low-field region of the 300-MHz NMR spectrum of apamin (15 mM, pD 2.2), 20 min after dissolving in D_2O, with the assignments of peptide amides.

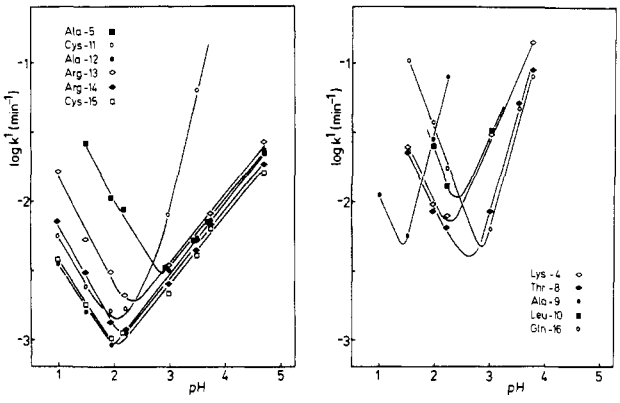

FIGURE 3: pH dependence of pseudo-first-order exchange rates for H–D exchange of peptide amides in apamin obtained by following the decay in intensity of amide resonances after dissolving in D_2O (20 °C, 15 mM apamin, 20 mM buffer ions).

pH Dependence of Amide Hydrogen Exchange. Figure 3 shows the pH dependence of hydrogen–deuterium exchange of the 11 most protected peptide amides of apamin over the pH range accessible to measurement by following the decay of resonance intensities in the amide region of the proton NMR spectrum. The amides of residues 11–15 are hydrogen-bonded within a C-terminal helix (Wemmer & Kallenbach, 1983). The amide of Ala-5 probably forms a hydrogen bond with the peptide carbonyl of Asn-2 (Okhanov et al., 1980; Wemmer & Kallenbach, 1983). The latter interpretation is supported by NOE data (an NOE between the amides of Ala-5 NH and Lys-4 NH is the largest intra-amide NOE in apamin and is indicative of a type 1 β-turn involving residues 2–5) and is consistent with other NMR data for β-turn amides (Smith & Pease, 1980) in which the transannular hydrogen bond is long (with the amide resonance correspondingly shielded as observed). Other intramolecular hydrogen bonds include (Bystrov et al., 1980; Wemmer & Kallenbach, 1983; unpublished results) Thr-8 NH to Ala-5 carbonyl, Lys-4 NH to Asn-2 side-chain amide, Ala-9 NH to Thr-8 side-chain hydroxyl, and, with decreasing stability, the amides of Gln-16, Gln-17, and possibly His-18 in a helical structure.

The pH dependence for exchange of the amides of Asn-2 and Glu-7 is not shown because of their anomalous behavior due to hydrogen bond formation to the side-chain carboxyl of Glu-7 when the latter function titrates (Okhanov et al., 1980). Although there is good evidence that conformations involving these charge-stabilized hydrogen bonds are highly

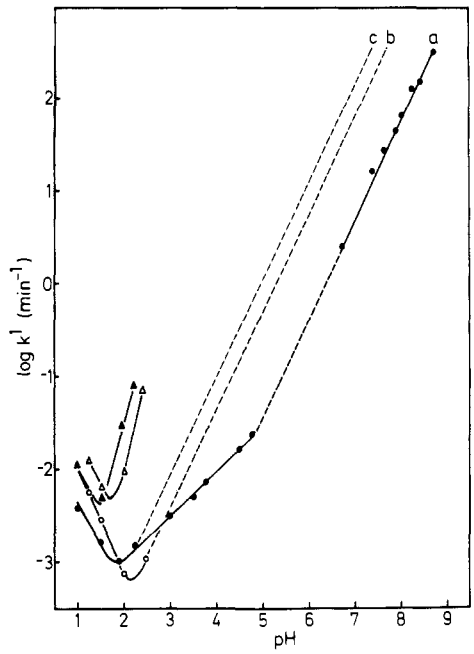

FIGURE 4: pH dependence of hydrogen exchange of Cys-15 NH at low (●) and high (○) ionic strength (0.6 M KCl). High pH points were obtained from saturation transfer experiments. Exchange rates for Ala-9 NH at low (▲) and high (△) ionic strength are also shown. Curve b is the pH dependence for Cys-15 NH expected at high ionic strength and curve c at low ionic strength, if first-order dependence on OH^- concentration was followed over the complete base-catalyzed limb of the exchange curve.

populated (see Discussion), the amides of Asn-2 and Glu-7 exchange rapidly at pH values around the pK_a of the Glu-7 carboxyl. This may be attributed to intramolecular catalysis of base-catalyzed exchange of these amides promoted by the rapid carboxyl–carboxylate fluctuation. A similar effect is observed in the bovine pancreatic trypsin inhibitor where the amide NH of Glu-49, hydrogen-bonded to the side-chain carboxyl of the same residue, exchanges much more rapidly than expected (Wagner, 1983). Although extensive pH-dependent exchange data for the peptide amide of Ala-9 could not be obtained due to resonance overlap, similar intramolecular catalysis of base-catalyzed exchange by the side-chain hydroxyl of Thr-8, the likely hydrogen bond acceptor for Ala-9 NH (unpublished results), may be responsible for the greater than first-order dependence of exchange rate on OH^- concentration reproducibly observed (Figures 3 and 4). Intramolecular catalysis of amide exchange by side-chain functions in polypeptides and proteins is assumed to be negligible (Molday et al., 1972; Englander & Kallenbach, 1984). It is possible, however, that such catalysis may occur if the amide is hydrogen-bonded to the side-chain catalytic function (carboxylate or hydroxyl). Greater than first-order dependence on OH^- concentration is also displayed by the amides of Thr-8, Cys-11, and Gln-16 (Figure 3). This is due to a destabilization of hydrogen bonds involving these amides as the Glu-7 carboxyl titrates (see Discussion). The amides of Cys-3, Gln-17, and His-18 exchange too quickly for reliable pH-dependent data to be obtained, and exchange rates for Cys-11 NH above pH about 4 could not be measured because of resonance overlap.

In Figure 4 the exchange curve of Cys-15 NH has been extended in the base-catalyzed limb by using rates measured in saturation transfer experiments. The curves for the amides of residues 5 and 12–14 have been omitted for clarity but closely follow that for Cys-15 over the whole of the base-catalyzed limb accessible to measurement. This close corre-

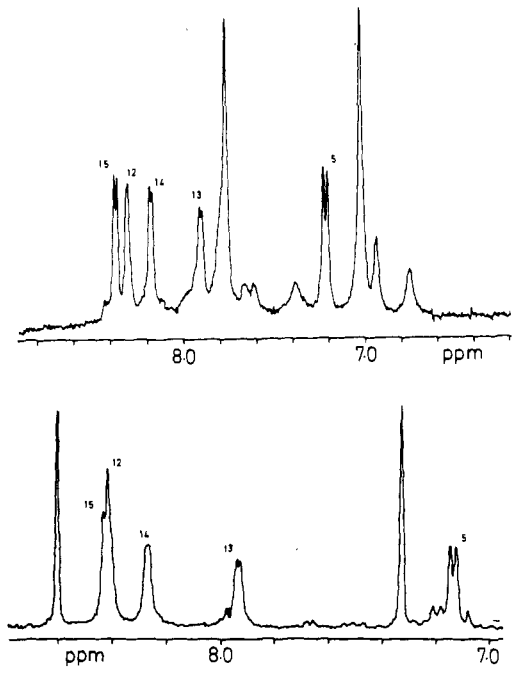

FIGURE 5: Amide region of the NMR spectrum of apamin in 20 mM acetate, pH 4.7, 20 °C, 200 min after dissolving in D_2O (lower). The upper spectrum is the amide region at pH 7.5, 40 °C, in 90% H_2O–10% D_2O obtained after a presaturating pulse (4 s) on water.

spondence in exchange rates is illustrated in the two spectra of Figure 5 which show the amide region of the NMR spectrum after exchange-out at pH 4.7 for 200 min at 20 °C (lower spectrum) and with presaturation of the solvent resonance at pH 7.5 (40 °C) where all but the strongly protected amides have disappeared by magnetization transfer from the saturated solvent resonance (upper spectrum). The accessible pH range of the saturation transfer experiments was extended by measuring exchange rates at three temperatures (23, 40, and 60 °C). The three sets of data fitted onto the curve shown in Figure 4 by using a value for the activation energy for the rate-limiting step of the exchange process of 4.3 kJ mol^{-1}.

Also shown in Figure 4 are the exchange curves in the acid-catalyzed regime for Ala-9 and Cys-15 NHs measured in 0.6 M KCl. These rates were measured to determine the contribution of the high fixed charge density on apamin (net positive charge below pH 2.5 of 5) to the observed exchange kinetics. The effect of high ionic strength is to increase the pH_{min} (the pH in the exchange curves at which exchange is slowest) by about 0.3 pH unit. A similar shift in the pH-dependent exchange curves by about 0.3 pH unit to higher pH in 0.6 M NaCl was observed for all the amides measured (not shown), indicating that, at least in its effect on amide exchange in apamin, the global electrostatic effect arising from fixed charges of amino acid side chains approximates to a smeared charge having uniform distribution. At the low ionic strength of the exchange experiments illustrated in Figures 3 and 4 (20 mM in buffer ions and 20 mM in peptide) the net electrostatic effect (Kim & Baldwin, 1982) arising from the high fixed charge density of apamin therefore decreases acid-catalyzed exchange rates by about 2-fold and increases base-catalyzed rates by a similar factor. This effect is approximately one-third of the fixed charge effect on amide exchange in polylysine measured by Kim and Baldwin (1982) in the same ionic strength rate; the positive charge density on apamin is similarly about one-third to one-quarter that of polylysine.

Base-catalyzed rates in high ionic strength solutions above pH 3 were not determined because of possible complications in interpretation arising from the effects of high salt concentrations on the salt bridge between the α-amino group and the Glu-7 δ-carboxylate [Bystrov et al. (1980) and Figure 6; see Discussion]. For those amides unaffected by titration of the Glu-7 carboxyl (such as Ala-9 NH; Figure 4) base-catalyzed rates were indeed found to increase 2–3-fold in 0.6 M NaCl. The dotted curves in Figure 4 (curves b and c) illustrate the base-catalyzed pH dependence for exchange of Cys-15 expected at low (curve b) and high ionic strength (curve c) if first-order dependence on hydroxyl concentration was obeyed over the whole of the base-catalyzed limb. The vertical displacement of the experimental curve (curve a) from the extrapolated curve having the same pH_{min} (curve b) gives a measure of the extent to which the non-first-order dependence on hydroxyl concentration between pH 2.5 and pH 5 results in the further suppression of the base-catalyzed exchange rates of the most slowly exchanging amides in apamin (the amides of residues 5 and 12–15).

Discussion

Correlated Amide Exchange. The most notable feature of the amide exchange data in Figures 3 and 5 is the close correspondence in exchange rates of five backbone peptide amides over the whole of the base-catalyzed limb accessible to measurement by the methods used in this study. Four of these amides (the amides of residues 12–15) are hydrogen-bonded within a C-terminal helix (Wemmer & Kallenbach, 1983), and the fifth (Ala-5 NH) probably forms the transannular hydrogen bond of a β-turn (Bystrov et al., 1980). This correlation extends over at least 6 pH units, and the set of five highly protected amides has suppressed exchange rates (less than first-order dependence on hydroxyl concentration) between pH 2 and pH 5.

The set of five amides highly protected to base-catalyzed exchange unambiguously defines the stable unit of secondary structure of apamin. Above pH 5 these amides exchange at least 20 times more slowly than other backbone amides in apamin. Although it is difficult to prove so from amide exchange data, the marked similarity in base-catalyzed exchange rates among these amides and their common responsiveness to conformational effects between pH 2.5 and pH 5 (Figure 4; see below) strongly suggest that a cooperative conformational fluctuation(s) in the backbone encompassing the C-terminal helix and an N-terminal β-turn limits the exchange of these amides. This supports the conclusion of Englander and Kallenbach (1984), on the basis of single pH exchange measurements in the acid- (pH 2.0) and base-catalyzed (pH 3.7) regime, that the helical amides of apamin exchange via cooperative "structure-opening" fluctuations involving the whole helix.

The base-catalyzed rates of the slowly exchanging set follow first-order dependence on hydroxyl concentration at high pH at least up to pH 8.5 (Figure 4), indicating that their exchange obeys the EX_2 mechanism in which the structure-opening conformational fluctuations limiting exchange are faster than the intrinsic chemical exchange rates (Englander & Kallenbach, 1984). The intrinsic chemical exchange rates are readily calculated (Englander et al., 1979); the first-order rate constant for chemical exchange of a "free" amide at pH 8.5 (20 °C) is about 200 s^{-1} (when a correction is made for an electrostatic effect of positive amino acid side chains at low ionic strength). Conformational fluctuations limiting exchange of the stable amides must be faster than 200 s^{-1}. Because the amides in the stable set exchange much more slowly (at high pH) than other apamin amides, it is likely that the concerted fluctuation limiting exchange is the slowest "breathing mode" in the set of conformational fluctuations that characterize the dynamic

behavior of apamin in water. We note that there are small differences in the measured exchange rates among the slowly exchange amides which remain when the rates are corrected for sequence-dependent inductive contributions to exchange (Molday et al., 1972). However, these differences are generally no greater than a factor of 2–3. It is well established that both global (Kim & Baldwin, 1982; see below) and local (Matthew & Richards, 1983; Shire et al., 1975) electrostatic contributions can have large effects on the chemical properties (like hydrogen exchange) of reactive groups in proteins. It is therefore not possible to assign small differences in exchange rates to real variations in fluctuational modes limiting exchange of these amides unless it can be established that exchange occurs from "random-coil" open conformers in which residual conformation-dependent contributions to exchange are absent.

Peptide amides that are not part of the stable cooperative unit defined by the amides of residues 5 and 12–15 must exchange through more rapid, local fluctuations that do not perturb the stability of the cooperative unit. These include (Bystrov et al. 1980; Wemmer & Kallenbach, 1983; unpublished results) the amides of residues 16–18 which display helix-fraying behavior as the helix becomes progressively deconstrained toward the C-terminus (Wemmer & Kallenbach, 1983) and amides forming hydrogen bonds to side-chain groups (Asn-2 and Glu-7 NHs to the side-chain carboxylate, Lys-4 NH to the side-chain amide of Asn-2, and Ala-9 NH to the side-chain hydroxyl of Thr-8). At low pH the stable unit of secondary structure may include the amide of Cys-11. It will be shown below that titration of the Glu-7 carboxyl results in a shift in the conformational properties of the turn region of the peptide backbone that results in the progressive destabilization of Cys-11 NH above pH 3 (Figure 3). The β-turn proposed to contain a Thr-8 NH to Ala-5 carbonyl hydrogen bond might have been expected to form part of the stable unit of secondary structure in apamin because it lies within the main backbone of the peptide. However, the Thr-8 amide undergoes rather rapid base-catalyzed exchange over the titration range of the Glu-7 δ-carboxyl (figure 3) whereas the inductive effect of the carboxylate would be expected to cause a small *suppression* of the exchange of Thr-8 NH over the titration range (Molday et al., 1972). It must be concluded that the hydrogen bond involving the Thr-8 NH is destablized by titration of the Glu-7 carboxylate and that the amide can exchange through independent local fluctuations of the backbone.

The use of the hydrogen-exchange method to identify the stable conformational backbone structure of polypeptides is probably the most effective way of evaluating the success of theoretical or empirical conformer predictions. Wemmer and Kallenbach have already shown that the NMR-derived conformation of apamin is generally consistent with the theoretical structure prediction of Hider and Ragnarrson (1981). The latter authors used structural parameters based on secondary-structural preferences of amino acid residues in proteins (Levitt, 1978) to predict a common structural unit for apamin and other homologous bee venom peptides comprising an N-terminal β-turn covalently linked to an α-helical segment by disulfide bonds. The present work illustrates that the conformation of apamin is based on such a structural unit. The problems of applying conformer predictions based on conformational preferences of amino acids in proteins to small disulfide-bonded polypeptides has been commented on (Walde et al., 1981), and the success in the case of apamin may result because the disulfide bonds stabilize an inherently favorable conformer (which is thus predictable in comparison with structural preferences in proteins) and do not markedly influence the folding pattern. This idea is supported by the finding that the reduced peptide spontaneously reoxidizes to the native conformer even under conditions highly unfavorable for disulfide bond formation (20 mM apamin, pH 2.0, 4 °C; unpublished results). This would be unlikely to occur if rather unfavorable backbone conformers were required in order to bring the correct cysteine thiol pairs into juxtaposition.

pH-Dependent Changes in Conformation and Stability in Apamin. What is the origin of the suppressed dependence on hydroxyl concentration of base-catalyzed exchange for the slowly exchanging amides between pH 2 and pH 5? At the relatively low ionic strength of the exchange experiments in this study it is likely that the suppression of base-catalyzed rates contains some contribution from the decrease in electrostatic screening that results from decreasing the net positive charge on the peptide from +5 (at pH 2) to +4 (pH 5) as the side-chain carboxyl of Glu-7 titrates (pK_a = 3.6; Bystrov et al., 1980; Figure 6). However, this effect is insufficient to account fully for the decreased pH dependence over this range. This is illustrated in Figure 4 where the effect of increasing ionic strength on the *acid*-catalyzed exchange of Cys-15 NH and on the exchange of Ala-9 NH is shown. Addition of 0.6 M KCl, an ionic strength that almost completely suppresses the effect of bound ions and electrostatic screening on amide exchange rates from charged polymers (Kim & Baldwin, 1982), results in a small increase in acid-catalyzed exchange rates and an increase in pH_{min} by about 0.3 pH unit. Base-catalyzed exchange rates for Ala-9 NH can be measured at pH values below those which the Glu-7 carboxyl titrates (because of the considerably suppressed pH_{min} value for this amide), and these rates are similarly decreased by about 3-fold in 0.6 M KCl. The *net* global electrostatic effect on amide exchange in apamin resulting from the high fixed charge density on the peptide results in a decrease in the pH_{min} by about 0.3 pH unit at low ionic strength as the acid- and base-catalyzed rates are altered by 2–3-fold. The decrease in the global electrostatic effect on titration of a *single* charged group (the Glu-7 carboxyl) will therefore be expected to have an insignificant effect on the measured exchange rates (approximately equivalent to 0.3/5 pH units which is probably within the experimental error of the rate and pH measurements.[2]

We believe that the suppressed pH dependence of the five protected amides between about pH 2.5 and pH 5 results from the stabilization of the conformation of apamin on titration of the Glu-7 carboxyl. There are two potential contributions to conformational stabilization, and these are both evident in the pH titration data of Figure 6. On titration of the carboxyl the amides of Glu-7 and Asn-2 undergo large titration shifts to low field characteristic of hydrogen-bond formation, these hydrogen bonds being formed with the Glu-7 carboxylate as the hydrogen-bond acceptor (Okhanov et al., 1980). It has also been shown that the glutamic acid carboxylate and the α-amino group participate in a salt bridge interaction (Okhanov et al., 1980). This interaction results in the suppression of the pK_a of the carboxyl by about 0.6–0.8 pH unit which is reflected in the effect of high ionic strength on the titration

[2] It can be predicted that, in the general case, the *global* electrostatic effect arising from side-chain fixed charges, while having a significant effect on the pH_{min} in single amide exchange titration curves, will be unlikely to have a large effect on the pH dependence of exchange rates unless a large number of side-chain groups titrate with similar pK_a's. Even in these cases, however, *local* electrostatic effects or pH-dependent changes in conformational properties are more likely to dominate pH-dependent exchange behavior in complex polypeptides and proteins.

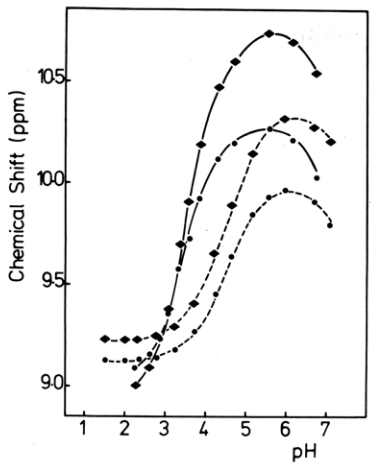

FIGURE 6: pH titration of the proton NMR chemical shifts of Glu-7 NH (♦) and Asn-2 NH (●) in 20 mM KCl (—) and 2 M NaCl (---).

of the amide resonances of Glu-7 and Asn-2 (Figure 6).

It is possible to show from the pH-dependent exchange data in Figure 4 that the salt bridge interaction does not contribute significantly to conformational stability of the peptide backbone of apamin. Over the pH range at which the α-amino group titrates (pK_a = 6.5; Okhanov et al., 1980) there is apparently no destabilization of conformation of the stable unit (resulting in a greater than first-order dependence on hydroxyl concentration) that should result if suppression of the salt bridge interaction destabilized the backbone conformation. It can be concluded, therefore, that the stabilization of the conformation of apamin over the titration range of the Glu-7 carboxyl that results in the suppressed pH dependence of the stable set of peptide amides arises from the formation of the two further intramolecular hydrogen bonds. It is possible to estimate the free energy of stabilization of the backbone conformation from the formation of these hydrogen bonds based on the vertical shift of the base-catalyzed exchange for the highly protected set of amides relative to the extrapolated curves expected if no suppression of the base-catalyzed rates occurred (Figure 4). The exchange curves are shifted vertically by 10–15-fold corresponding to a free energy of stabilization of 5–6 kJ mol^{-1} at 20 °C.[3]

In addition to stabilizing a low-energy conformer of apamin, titration of the Glu-7 carboxyl appears to cause significant changes in the local conformational properties of regions of the peptide backbone. Below pH 2.5 the amide of Cys-11 is highly protected from exchange (Figure 3) and probably forms the last stable hydrogen bond (to the amide carbonyl of Glu-7 for an α-helix) of the helix at low pH (see Figure 7). The peptide backbone turns at the N-terminal end of the helix to

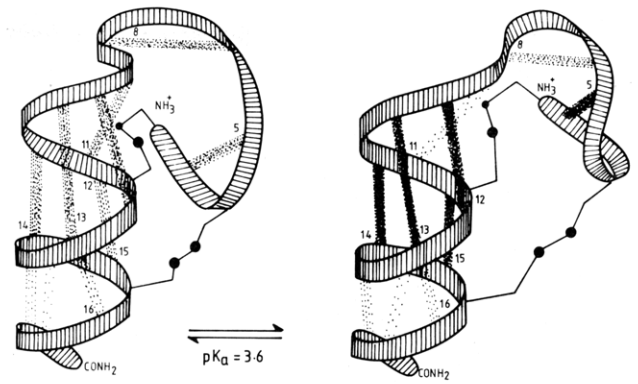

FIGURE 7: Schematic illustration of the conformational properties of apamin in water determined from analyzing pH-dependent amide exchange kinetics. The degree of protection of hydrogen-bonded amides to exchange is indicated by the density of strippling. Intramolecular hydrogen bonds involving side-chain functions, including those to the Glu-7 carboxylate responsible for the pH-dependent changes in conformational properties illustrated (see text), have been omitted for clarity. The hydrogen bond lengths are arbitrary.

join the N-terminal peptide segment which is additionally connected to the helix by two disulfide bonds (Figure 7). As the Glu-7 carboxyl titrates, the Cys-11 amide becomes rapidly destablized to exchange (showing greater than first-order dependence on OH^- concentration between pH 2.5 and pH 3.5) until above pH 3.7 it exchanges more than 10 times faster than the helical amides of residues 12–15. This indicates that the structural reorientations associated with the interactions involving the Glu-7 carboxylate are incompatible with the continuation of the C-terminal end of the helix to include the Cys-11 amide in a helix hydrogen bond.

Wemmer and Kallenbach (1983) have pointed out that the C-terminal end of the helix in apamin undergoes helix fraying as the amides of residues 16, 17, and 18 become progressively deconstrained. The data in Figure 3 show that the helix stops more abruptly at the N-terminal end, with the amide of Cys-11 at very low pH and with the amide of Ala-12 at high pH. At pH 2, for example, the amide of Cys-11 is stabilized about 30-fold relative to Leu-10 NH, and at pH 4, the highest pH for which an exchange rate for Cys-11 NH can be measured, the amide of Ala-12 is already stabilized about 20-fold relative to the amide of Cys-11. It is particularly interesting that the Cys-11 amide hydrogen bond can be "lost" from the end of the helix without measurable effect on the stability of the next hydrogen bond (that involving the amide of Ala-12). This is probably a reflection of the dominant role of the disulfide bond in stabilizing the apamin helix and the contribution of the amide to Glu-7 carboxylate hydrogen bonds to conformational stability that is itself transmitted to the helix through the disulfides. Indeed, it does not require any significant changes in conformation of the N-terminal end of the apamin helix to release the Cys-11 amide from hydrogen bonding to the Glu-7 carbonyl (the expected hydrogen bond acceptor) but only an unfavorable reorientation of the latter function as the Glu-7 side chain reorients to form the observed pH-dependent hydrogen bonds.

pH_{minima} for Hydrogen Exchange in Apamin. Sequence-dependent inductive effects that can influence the rates of hydrogen exchange of peptide amides have been characterized by Molday et al. (1972) using small conformationally unstructured model peptides. These effects tend to alter the base- and acid-catalyzed rates in approximately equal and opposite ways (Molday et al., 1972) and thus have a marked effect on pH_{min}, the pH at which amide exchange is slowest. It is reasonable to expect that amide exchange from a "random

[3] It now becomes possible to account more completely for the titration behavior of the carboxylate hydrogen-bonded amides Asn-2 and Glu-7. The titration shift for the Glu-7 amide at low ionic strength [about 1.8 ppm (Figure 6)] is much larger than the maximum expected for the intraresidue Glu δ-carboxylate to the amide hydrogen bond in polypeptides [about 1.0 ppm (Bundi & Wuthrich, 1979)]. The enhanced titration shifts arise from titration-induced reorientations in conformation of the N-terminal region of apamin which bring the α-amino group and the amides of residues 2 and 7 closer together as the hydrogen bond and salt bridge interactions occur. The titration shifts then have contributions from the increased polarization of the amide bonds expected on hydrogen bond formation and an additional deshielding effect from the proximal α-amino group. The effect of high salt concentrations (or of titrating the α-amino group) in decreasing these titration shifts (Figure 6) is not due to the suppression of the hydrogen bond interactions by weakening the salt bridge but rather to the suppression of the deshielding inductive effect of the proximal α-amino group.

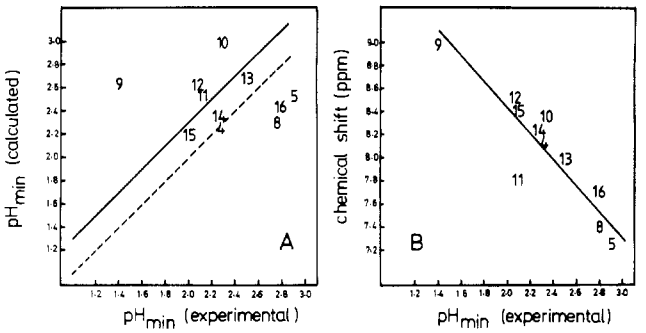

FIGURE 8: (A) Comparison of the experimental pH_{min} (Figure 3) with the expected pH_{min} values calculated from the sequence-dependent factors of Molday et al. (1972). The relationship (---) for a perfect correlation is modulated by the global charge effect (Figure 4) to give the expected experimental line (—). (B) Relationship between the experimental pH_{min} values (Figure 3) and the proton NMR chemical shifts of the amide resonances at pH 2.2, 20 °C.

coil" open conformation would have pH_{min} values similar to those predicted from the sequence-dependent inductive effects determined by Molday et al. (1972). Amides exchanging from conformers that were nonrandom might be affected by residual electrostatic and other conformation-dependent factors, and the pH_{min} of these amides should correlate less well with the values predicted from the data of Molday et al. (1972). It is apparent (Figure 8A) that even when corrected for global electrostatic effects that suppress all pH_{min} values by about 0.3 pH unit (Figure 4), the measurable pH_{min} for the amides of apamin correlate very poorly with the pH_{min} values expected purely from sequence-dependent inductive effects (the coefficient of correlation, $r = 0.150$). This indicates that the open states from which the amides exchange with solvent are nonrandom. This is perhaps not surprising for apamin considering that in all possible open conformers the two intramolecular disulfide bonds remain intact (but see below).

Although the pH_{min} for exchange of amides in apamin do not correlate with the predicted pH_{min} values, a strong correlation with the amide proton NMR chemical shifts is observed (Figure 8B; $r = 0.902$). The amide chemical shifts in apamin are widely dispersed compared with the random coil shifts of the reduced peptide (unpublished results) and the differences, at least for the slowly exchanging amides, will be dominated by conformational effects in the stable conformation(s). Thus, if the stabilization of amides to exchange by 100-fold indicates that the equilibrium constant for the closed (intramolecularly hydrogen bonded) to open conformational transition has a value of about 100, then the observed chemical shift will be weighted by this factor toward values corresponding to the closed conformers. The pH_{min} values, on the other hand, should reflect properties of the conformations from which amide exchange occurs and these are expected to be open conformers in which intramolecular hydrogen bonds involving the amides in consideration are transiently broken. The observed correlation between pH_{min} values (a property of the open state) and amide proton chemical shift (a property of the closed conformers) is therefore unexpected. The data obtained so far is insufficient to interpret completely the significance of the observed correlation, and the various possibilities will not be discussed here. However, one chemical exchange mechanism that might be influenced by the properties of the closed, hydrogen-bonded state is the imidic acid mechanism for the acid-catalyzed exchange, a mechanism for which experimental support has recently been presented (Perrin et al., 1984). In the imidic acid mechanism the amide proton is acidified by rate-limiting protonation at the amide carbonyl, and this might occur while the amide hydrogen remains intramolecularly hydrogen bonded. That the observed correlation is related to acid- (rather than base-) catalyzed exchange can be seen in Figure 3 where, for the highly exchange-protected amides, differences in pH_{min} arise almost completely from differences in acid-catalyzed exchange rates.

Conclusions

The disulfide bonds of apamin stabilize a cooperative unit of secondary structure comprising a C-terminal helix and an N-terminal β-turn so that backbone peptide amides within the cooperative unit have exchange rates suppressed about 100-fold relative to amides in random-coil peptides. Further conformational stability (corresponding to a free energy of stabilization of 5–6 kJ mol^{-1} at 20 °C) is provided by hydrogen bonds involving the Glu-7 carboxylate and the amides of residues 2 and 7. A salt bridge between the Glu-7 carboxylate and the N-terminal amino group does not contribute significantly to conformational stability.

While the salt bridge and hydrogen bond interactions involving the Glu-7 carboxylate were previously observed (Bystrov et al., 1980), the detailed nature of pH-dependent changes in local conformation and conformational stability was not. The present work illustrates the complementary nature of the hydrogen exchange method which allows the determination of changes in conformational properties of hydrogen-bonded secondary structure that are "invisible" or difficult to assign from other NMR methods (such as pH titration effects on chemical shift, ring-current shifts, or NOE intramolecular distance constraints) which, because these parameters are weighted averages over a "conformational ensemble", are interpreted in terms of static conformers. A further advantage is the ability to identify units of cooperative secondary structure; this provides a relatively unambiguous test of the success of low-energy conformer predictions and should be useful in analyzing the normal modes of structural fluctuations in polypeptides (Sheridan et al., 1983). Any molecular dynamical calculation of the conformational fluctuations of apamin, for example, should aim to generate the concerted backbone fluctuations that appear to be required to allow concerted exchange of the stable set of amides.

A paradox arises in the interpretation of the variation of pH_{min} values in the pH-dependent exchange curves of amides in polypeptides. Within the assumption that acid- and base-catalyzed exchanges occur with rate-limiting protonation or deprotonation, respectively, at amide nitrogen, a comparison with calculated pH_{min} values (as is done here) is a valid approach to determining whether residual conformation-dependent effects modulate exchange from open conformers in polypeptides. This arises because residual conformation-dependent electrostatic effects [like sequence-dependent inductive effects (Molday et al., 1972)] will be expected to act on acid- and base-catalyzed exchange in approximately equal and opposite ways. With the possibility that acid-catalyzed exchange may occur via protonation at amide oxygen, this symmetry in the acid- and base-catalyzed exchange mechanism is lost (Perrin & Lollo, 1984), and it is no longer possible to compare experimental and calculated values of pH_{min} in this way. A comparison of experimental base-catalyzed exchange rates (the mechanism of which process is unambiguous) with predicted rates cannot be used to give information about the open state because residual conformation-dependent effects on base-catalyzed exchange in the open state and the direct effect arising from restricted access to the exchanging amide by limited opening of hydrogen-bonded structure cannot readily be separated. The mechanism of acid-catalyzed exchange in

polypeptides therefore remains of interest, and a more detailed study of acid-catalyzed amide exchange in apamin is in progress.

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