Cooperative Disulfide Bond Formation in Apamin†

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ABSTRACT: Apamin is being studied as a model for the folding mechanism of proteins whose structures are stabilized by disulfide bonds. Apamin consists of 18 amino acid residues and forms a stable structure consisting of a C-terminal α-helix and two reverse turns. This structure is stabilized by two disulfide bonds connecting Cys-1 to Cys-11 and Cys-3 to Cys-15. We used glutathione and dithiothreitol as reference thiols to measure the stabilities of the two disulfide bonds as a function of urea concentration and temperature in order to understand what contributes to the stability of the native structure. The results demonstrate modest contributions from secondary structure to the overall stability of the two disulfide bonds. The equilibrium constants for disulfide bond formation between the fully reduced peptide and the native structure with two disulfide bonds at 25 °C and pH 7.0 are 0.42 M^2 using glutathione and 2.7×10^{-5} using dithiothreitol. The equilibrium constant decreases by a factor of approximately 4 in 8 M urea and decreases by a factor of 3 between 0 and 60 °C. At least three one-disulfide intermediates are found at low concentrations in the equilibrium mixture. Using glutathione, the equilibrium constants for forming the one-disulfide intermediates with respect to the reduced peptide are approximately 0.025 M. The second disulfide bond forms with an equilibrium constant of approximately 17 M. Thus, apamin folding is very cooperative, but the native structure is only modestly stabilized by urea- or temperature-denaturable secondary structure.

Apamin is a neurotoxic peptide isolated from bee venom. Because of the biological activity of apamin on the central nervous system (Habermann, 1972), extensive studies have been made to investigate the relationship between structure and function (Vincent et al., 1975; Granier et al., 1978). The solution structure of this peptide has been studied extensively by means of CD[1] (Miroshnikov et al., 1978), energy minimization calculations (Freeman et al., 1986), and NMR (Hider & Ragnarsson, 1980; Bystrov et al., 1980; Okhanov et al., 1980; Wemmer & Kallenbach, 1983). The structure has been refined recently by distance geometry derived from NMR distance constraints (Pease & Wemmer, 1988). The structure consists of a C-terminal α-helix and two reverse turns. Disulfide bonds connect two successive turns of the helix to the reverse turns. Synthetic apamin exhibits full biological activity (Van Rietschoten et al., 1975). Thus, the three-dimensional conformation and the function of apamin are inherent in its amino acid sequence. Native apamin has an extremely stable structure which is maintained over a broad range of pH and solution polarity, addition of protein denaturant, and even the chemical modification of the many side-chain functional groups (Miroshnikov et al., 1978). However, reduced apamin is fully unfolded. Of the side chains investigated, only the two arginines, Arg-13 and Arg-14, were found to be critical to the neurotoxicity (Granier et al., 1978; Miroshnikov et al., 1978). The sequence of apamin is shown in Figure 1.

The conformation of apamin is obviously stabilized by the two disulfide bonds. How do the two disulfide bonds form and stabilize the final conformation? Does secondary structure play any role in forming and stabilizing the disulfide bonds? To better understand the structure and stability, it is important to investigate the folding mechanism. We have been studying the folding of apamin and attempting to identify and characterize the folding intermediates. Folding of apamin is very cooperative, and as a result, one-disulfide intermediates are populated to only a few percent at equilibrium. Nevertheless, we were able to identify at least three one-disulfide folding intermediates in the equilibrium mixture and study the stabilities of their disulfide bonds. In order to assess the importance of secondary structure on the stability of apamin, we measured the stabilities of the disulfide bonds as a function of urea concentration and temperature.

We have previously studied the stability and structure of two peptides designed to model one-disulfide intermediates of apamin, in which two cysteines, either Cys-1 and Cys-11 or Cys-3 and Cys-15, were replaced by alanines (Huyghues-Despointes & Nelson, 1991). This allows formation of only one nativelike disulfide bond. Both intermediate models exhibited partial structure by CD, although the intermediate model containing the Cys-3 to Cys-15 disulfide bond exhibited a greater extent of both structure and stability.

Materials and Methods

Apamin was purchased from Sigma or synthesized by Multiple Peptide Systems (San Diego, CA). Both samples were purified by reverse-phase HPLC and characterized by mass spectroscopy. GSH and GSSG were obtained from both Sigma Chemical Co. and Amresco Research Co. DTT$\begin{smallmatrix}\mathrm{SH}\\\mathrm{SH}\end{smallmatrix}$ and DTT$\begin{smallmatrix}\mathrm{S}\\\mathrm{S}\end{smallmatrix}$ were obtained from Aldrich Chemical Co. DTT$\begin{smallmatrix}\mathrm{S}\\\mathrm{S}\end{smallmatrix}$ was purified to eliminate strong oxidizing agents by a procedure

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[1] Abbreviations: DTT$\begin{smallmatrix}\mathrm{SH}\\\mathrm{SH}\end{smallmatrix}$, reduced dithiothreitol; DTT$\begin{smallmatrix}\mathrm{S}\\\mathrm{S}\end{smallmatrix}$, oxidized dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; CD, circular dichroism; C_{eff}, effective concentration; BPTI, bovine pancreatic trypsin inhibitor.

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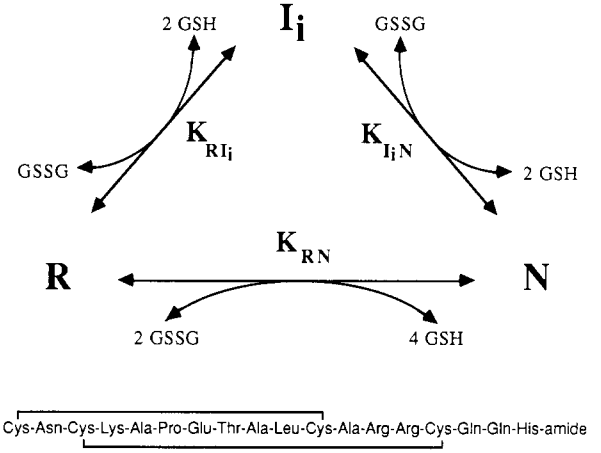

FIGURE 1: Disulfide equilibria between apamin and glutathione. R is the reduced and unfolded peptide, N is the native peptide, and I_i represents the one-disulfide folding intermediates. The sequence and disulfide bond arrangement of apamin are shown at the bottom.

described by Creighton (1977b). All were dried on a freeze dryer and stored desiccated in a refrigerator. TFA (HPLC spectra grade) was obtained from Pierce Chemical Co. Urea was an electrophoresis purity reagent from Bio-Rad. These reagents were used without further purification.

All buffer solutions contained 50 mM potassium phosphate, 0.2 M KCl, and 1 mM EDTA, adjusted to pH 7.0. All thiol solutions were made fresh daily. In order to avoid air oxidation of sulfhydryl groups, buffers used to prepare all solutions were degassed as previously described (Chau & Nelson, 1991). All reaction vials were kept under positive argon pressure during equilibration.

A schematic diagram of the equilibria using glutathione as the reference thiol is shown in Figure 1. R is the reduced and unfolded peptide, N is the native peptide, and I_i represents the six possible one-disulfide intermediates. Knowing the concentration of each species, the equilibrium constants K_{RN}, K_{RI_i}, and K_{I_iN} for the disulfide exchange reaction can be calculated from eqs 1 to 6. These equilibrium constants are related to one another by eq 7.

$$R + 2GSSG \rightleftharpoons N + 4GSH \quad (1)$$

$$K_{RN} = \frac{[GSH]^4}{[GSSG]^2}\frac{[N]}{[R]} \quad (2)$$

$$R + GSSG \rightleftharpoons I_i + 2GSH \quad (3)$$

$$K_{RI_i} = \frac{[GSH]^2}{[GSSG]}\frac{[I_i]}{[R]} \quad (4)$$

$$I_i + GSSG \rightleftharpoons N + 2GSH \quad (5)$$

$$K_{I_iN} = \frac{[GSH]^2}{[GSSG]}\frac{[N]}{[I_i]} \quad (6)$$

$$K_{RN} = K_{RI_i}K_{I_iN} \quad (7)$$

All concentrations of dithiothreitol and glutathione in equilibrium mixtures were measured directly from HPLC peaks. The correlations between peak area and concentration were determined for each species as previously described (Chau & Nelson, 1991). The relative extinction coefficients of oxidized and reduced apamin were measured by preparing solutions containing equimolar amounts of apamin (10 nmol) with equal volumes of either 0.1 M DTT$\begin{smallmatrix}\mathrm{S}\\|\\\mathrm{S}\end{smallmatrix}$ or 0.1 M DTT$\begin{smallmatrix}\mathrm{SH}\\|\\\mathrm{SH}\end{smallmatrix}$. Both samples were acid quenched with the same volume of 6 M HCl before HPLC analysis. The relative extinction coefficient of native (N) compared to reduced (R) apamin at 220 nm was found to be 1.57. The intermediates (I_i) were assumed to have extinction coefficients equal to reduced apamin. The recovery of reduced apamin was sensitive to the performance of the column, and consequently the relative extinction coefficient serves as a useful check of the performance of the column.

The effect of urea on the stability of N at 25 °C was measured at urea concentrations from 0 to 8 M. The urea solutions were prepared by dissolving solid urea in pH 7.0 buffer. Solutions of DTT$\begin{smallmatrix}\mathrm{S}\\|\\\mathrm{S}\end{smallmatrix}$ (0.1 M), DTT$\begin{smallmatrix}\mathrm{SH}\\|\\\mathrm{SH}\end{smallmatrix}$ (0.1 M), GSSG (0.02 M), and GSH (0.05 M) were made with degassed urea buffers. The pH of each solution was adjusted to pH 7.0 with degassed 1 M KOH. The thiol stock solutions were degassed for an additional 15 min. Mixtures of DTT$\begin{smallmatrix}\mathrm{S}\\|\\\mathrm{S}\end{smallmatrix}$ and DTT$\begin{smallmatrix}\mathrm{SH}\\|\\\mathrm{SH}\end{smallmatrix}$, or GSH and GSSG (0.1 mL), were added to a sample of apamin (5.0 nmol) predried by purging with argon in a sealed vial. Since the concentration of DTT$\begin{smallmatrix}\mathrm{S}\\|\\\mathrm{S}\end{smallmatrix}$ had to be at least 50 times higher than that of DTT$\begin{smallmatrix}\mathrm{SH}\\|\\\mathrm{SH}\end{smallmatrix}$ in order to achieve comparable concentrations of R and N, the final concentration of DTT$\begin{smallmatrix}\mathrm{SH}\\|\\\mathrm{SH}\end{smallmatrix}$ was less than 1 mM. The concentration of GSH was 100-fold greater than that of the peptide in order to speed up the reactions. The mixture was allowed to reach equilibrium in an oxygen-depleted environment for at least 1 h. Portions of the equilibrium mixture were withdrawn at increasing time and acidified to pH 2 with 6 M HCl until the composition no longer charged, indicating that equilibrium was achieved. Acidification prior to injection was essential to obtain reproducible integration of GSH and GSSG peaks (Chau & Nelson, 1991). The equilibria were achieved within 2 h in all cases. Results from at least two injections were averaged to contribute to each equilibrium constant.

Samples for the temperature study were prepared in the same manner as described using pH 7.0 phosphate buffer. The reaction mixture was sealed and incubated in a water bath at the desired temperature from 0 to 60 °C for 2 h. Acid quench was done at the equilibration temperature. Duplicate samples were withdrawn from the equilibrium mixture for HPLC analysis. All HPLC analyses were carried out at room temperature.

HPLC Methods. A Rainin Dynamax HPLC was used with a Vydac 218TP54 reverse-phase C18 analytical column, 0.46 × 25 cm, for separating the equilibrium mixtures. Acetonitrile and water were used as mobile phases at a flow rate of 1.0 mL·min^{-1}. Injections were made by a Hewlett-Packard 1050 Series autosampler. Absorbance of the eluent was measured by an ISCO V4 absorbance detector monitoring at 220 nm with a 1-mm HPLC flow cell. Water was deionized, quartz distilled, and filtered through a Millipore Norganic organic removal cartridge and a 0.45-μm Millipore filter before use. All solvents contained 0.1% (v/v) TFA. Different gradients were employed to facilitate good separation of each thiol species and different forms of the peptides. GSH and GSSG elute early in a low percentage of acetonitrile. As a result, a shallow gradient of 0–10% acetonitrile over 10 min was used

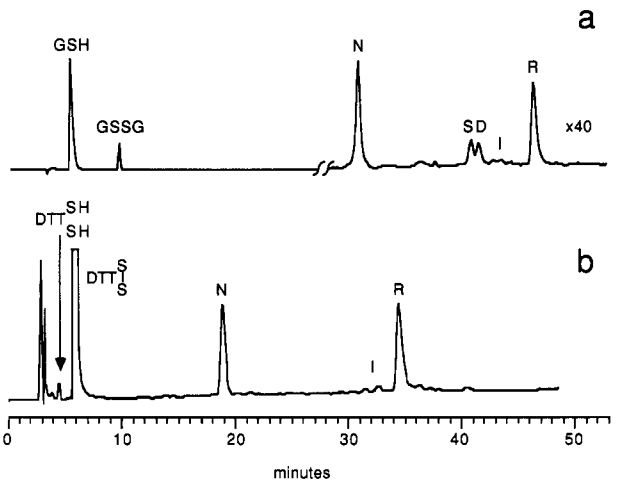

FIGURE 2: (a) HPLC chromatogram of an equilibrium mixture of apamin and glutathione. The peptide portion of the chromatogram is shown expanded 40-fold. N is the native form, R is the reduced form, I labels the one-disulfide intermediates, and SD labels the mixed disulfides with glutathione. The concentration of GSH was 40 mM and that of GSSG was 2 mM. Double- and even triple-mixed disulfides could be observed under some conditions and elute before the SD peaks. (b) HPLC chromatogram of an equilibrium mixture of apamin and dithiothreitol. The concentration of DTT (SH, SH) was 0.4 mM and that of DTT (S, S) was 77 mM. The peaks of N and R run at different retention times in the two chromatograms due to differences in the gradient used to separate the glutathione and dithiothreitol components.

for glutathione mixtures. DTT (SH, SH) and DTT (S, S) were well resolved and elute within 10 min isocratically in 10% acetonitrile. Both gradients were followed by a 10–16% acetonitrile gradient over 40 min to elute the peptide components including native and reduced apamin, all mixed disulfides, and one-disulfide intermediates. All components were well resolved, allowing accurate integration of peak areas. Representative chromatograms are shown in Figure 2. Integrations were carried out using the Rainin Dynamax HPLC reprocessing and analysis program.

Results

The equilibrium constant for forming native apamin using glutathione as the reference thiol (eq 1 and Figure 2a) is 0.42 M^2 at 25 °C. The corresponding equilibrium constant using dithiothreitol as the reference thiol is 2.7×10^{-5}. The value determined using dithiothreitol is less accurate than that determined using glutathione, since the concentration of DTT (SH, SH) had to be kept below 1 mM, resulting in less accurate integration of its peak on the HPLC chromatogram. However, dithiothreitol does not readily form mixed disulfides with peptide cysteines. As seen in Figure 2b, no new one-disulfide intermediates appear in the region otherwise obscured by mixed disulfides between apamin and glutathione. Thus, all of the intermediates are observed using glutathione as the reference thiol. All further studies were done using glutathione. On the basis of comparisons of duplicate runs and experiments performed on successive days, we estimate the errors in K_{RN} using glutathione as the reference thiol to be on the order of 10%.

The dependence of K_{RN} on urea concentration is shown in Figure 3a. K_{RN} decreases gradually by approximately a factor of 4 with increasing urea concentration up to 8 M. The

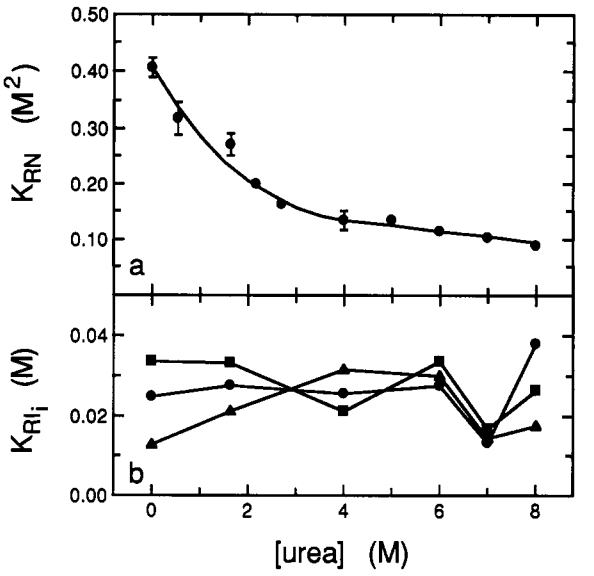

FIGURE 3: (a) Effect of urea concentration on K_{RN} in eq 2 at pH 7.0 and 25 °C. The error bars represent the standard deviations from multiple experiments. Error bars not shown are approximately the size of the circles. The line is a smooth curve through the data. (b) Equilibrium constants for first (■), second (●), and third (▲) resolved one-disulfide intermediate peaks plotted as a function of urea concentration. The intermediates are not populated enough to allow accurate integration of peak areas.

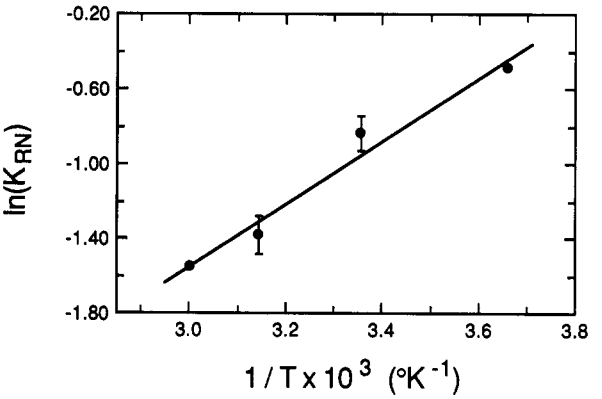

FIGURE 4: Temperature dependence of K_{RN} at pH 7.0 from 0 to 60 °C, plotted as ln K_{RN} vs $1/T$. The error bars represent the standard deviations from duplicate experiments. Error bars not shown are approximately the size of the circles. The line shows the least-squares fit and corresponds to an enthalpy of −3.4 kcal·mol^{-1}.

equilibrium constants for forming the one-disulfide intermediates are shown in Figure 3b. They are basically independent of urea concentration. The scattering of the data is due to integrations of very small peak areas that result in relatively large errors.

The temperature dependence of K_{RN} between 0 and 60 °C is shown in Figure 4. K_{RN} decreases by a factor of approximately 3 between 0 and 60 °C. The van't Hoff enthalpy is calculated to be -3.4 ± 0.4 kcal·mol^{-1}.

Discussion

Glutathione vs DTT. Glutathione is a frequently used reference thiol for the study of the stability and kinetics of disulfide bond formation (Loechler & Hollocher, 1975; Shaked et al., 1980; Creighton, 1983, 1986; Zhang & Snyder, 1989; Lin & Kim, 1989; Huyghues-Despointes & Nelson, 1991). Among its advantages is the similarity between the disulfide bond in GSSG and those found in peptides. The pK_a of the thiol group in GSH is nearly the same as that found in cysteines contained within peptides (Zhang & Snyder, 1988), simplifying the effects of pH on disulfide bond equilibria. The

equilibrium expression for formation of an intrapeptide disulfide bond depends on the ratio of $[GSH]^2/[GSSG]$ (see eq 4), thus allowing determination of equilibrium constants over a broad range of values. For example, Lin and Kim (1989) were able to measure the equilibrium constant between glutathione and the disulfide bond in thioredoxin over the range of 10–0.026 M as they denatured the protein with urea. The major disadvantage of glutathione is the stable formation of mixed disulfides between peptide cysteines and glutathione. The peaks corresponding to the mixed disulfides with apamin elute just before the one-disulfide intermediates, and hence some of the one-disulfide intermediates might be obscured by the much larger mixed disulfide peaks (see Figure 2a). Dithiothreitol solves this problem, while at the same time contributing some of its own. Disulfide formation in DTT$\begin{matrix}\text{S}\\|\\\text{S}\end{matrix}$ involves the formation of a six-membered ring, which makes DTT$\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix}$ a much more potent reducing agent (Creighton, 1986). This also means that dithiothreitol does not form stable mixed disulfides with peptide thiols. This advantage is offset by the large reducing potential, which makes it difficult to measure disulfide bond stabilities. The equilibrium constant expression depends on the ratio of reduced to oxidized dithiothreitol, and DTT$\begin{matrix}\text{S}\\|\\\text{S}\end{matrix}$ has a solubility limit of about 0.1 M. With apamin, this requires that the concentration of DTT$\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix}$ be on the order of 1 mM or less. The strong tendency of DTT$\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix}$ to oxidize also means air oxidation is more problematic, even more so because of its low concentration.

We were able to measure the equilibrium between native and reduced apamin using dithiothreitol as the reference thiol. Due to problems described above, the measurement is not as accurate as with glutathione. The HPLC chromatogram of such an equilibrium measurement is shown in Figure 2b. The most important result of this experiment is the observation that no one-disulfide intermediates appear in the chromatogram at a position of the mixed disulfides with glutathione. The value of the equilibrium constant between reduced and native apamin, using dithiothreitol as the reference thiol, was measured to be 2.7×10^{-5}. The largest uncertainty in this value arises from the small area of the DTT$\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix}$ peak (see Figure 2b). In fact, we could not reliably make the measurement when we added urea.

Comparing the values obtained using glutathione, 0.42 M^2, with that using dithiothreitol allows us to estimate the equilibrium between glutathione and dithiothreitol, shown in eq 8. The resulting value is $(0.42\ M^2/2.7 \times 10^{-5})^{1/2}$ or 125 M

$$\text{DTT}\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix} + \text{GSSG} \rightleftharpoons \text{DTT}\begin{matrix}\text{S}\\|\\\text{S}\end{matrix} + 2\text{GSH} \qquad (8)$$

at pH 7.0. We can compare this with a value of 1160 M at pH 8.7 determined by comparing rate constants of disulfide bond formation in BPTI measured using glutathione and dithiothreitol (Creighton & Goldenberg, 1984) and a value of 8800 M at pH 7 measured using a lipoamide–lipoamide dehydrogenase coupled to the formation of NADH by glutathione reductase (Szajewski & Whitesides, 1980). Because of the discrepancies in these values for this important equilibrium, we measured the equilibrium between glutathione and dithiothreitol directly by HPLC methods and obtained a value of 200 M at pH 7.0 (Chau & Nelson, 1991).

Effective Concentrations. The equilibrium constant for an intrapeptide disulfide using glutathione as the reference thiol nominally has the units of concentration. One way to consider this is to interpret the equilibrium constant in terms of an "effective concentration", C_{eff} (Creighton, 1983, 1986). For our purposes, we will define C_{eff} to be equal to the equilibrium constant for disulfide bond formation, an example of which is given by eqs 3 and 4 (Huyghues-Despointes & Nelson, 1991; Lin and Kim, 1989). This concept assumes that the inherent chemistry for forming the disulfide bond in GSSG and in the peptide disulfide is identical. C_{eff} provides information about the probability that the two peptide cysteine thiols are at the proper distance and geometry to form a bond (Flory et al., 1976). See Huyghues-Despointes and Nelson (1991) for a more detailed description of effective concentrations. Empirically, glutathione appears to be an excellent reference thiol, since formation of its disulfide bond does not depend on urea (Creighton, 1977b). Also, the peptide acetyl-Cys-$(Gly)_6$-Cys-amide, which is not expected to form any structure, has a C_{eff} of about 0.07 M, which does not depend on urea concentrations up to 8 M (Lin & Kim, 1989).

Equilibrium constants or effective concentrations have been measured for many unstructured peptides. In a series of experiments with different spacings between cysteines, the equilibrium constants varied from about 0.018 to 0.055 M when there were three to five intervening alanine residues between two cysteines in synthetic peptides (Zhang & Snyder, 1989). Thus, formation of a disulfide bond in an unstructured peptide is associated with effective concentrations in the range of tens of millimolar. Comparing the measured value of C_{eff} for a peptide disulfide bond with that expected in an unstructured peptide indicates whether the two cysteines are constrained by structure in conformations favorable for disulfide bond formation. The factors stabilizing these structures can be probed by determining under what conditions C_{eff} is significantly changed.

Stabilities of the One-Disulfide Intermediates. There are six possible one-disulfide folding intermediates for apamin. The one containing a disulfide bond between Cys-1 to Cys-3 is not likely to be observed due to the steric constraint imposed in a small ring: the equilibrium constant for forming the disulfide loop in a peptide containing two cysteines that are separated by one alanine residue is reported to be only 0.003 M (Zhang & Snyder, 1989). We were able to identify at least three one-disulfide intermediates at equilibrium in the present study (see Figure 2a). Despite the low concentrations of these intermediates, the stabilities of their disulfide bonds could be estimated and are shown in Figure 3b. Their equilibrium concentrations are low and hence not accurately measured.

To circumvent the problem of low concentrations of intermediates, we have studied two synthetic peptides based on apamin's sequence as models to mimic the properties of the one-disulfide intermediates (Huyghues-Despointes & Nelson, 1991). In these peptides, two cysteines were replaced by alanine, and hence only one of the nativelike disulfide bonds, connecting either Cys-1 to Cys-11 or Cys-3 to Cys-15, could form. These peptide disulfide bonds have effective concentrations of 0.018 and 0.033 M, respectively, both of which decrease by a factor of approximately 2 between 0 and 8 M urea (Huyghues-Despointes & Nelson, 1991). Both one-disulfide models exhibit partial structure by CD, with the model

containing the disulfide bond between Cys-3 and Cys-15 exhibiting a greater extent of structure. The effective concentrations exhibit very little temperature dependence. These models probably provide an accurate picture of the properties of the one-disulfide intermediates in apamin. The value of approximately 0.025 M for the three intermediates found in the equilibrium mixtures in the present study lies in the middle of the values obtained for the one-disulfide models. We can conclude that the one-disulfide intermediates form with a low effective concentration, but with formation of some structure and exhibiting a small stabilization by urea-denaturable structure.

Formation of the Second Disulfide Bond. The first disulfide bond forms with an equilibrium constant, K_{RI_i}, of approximately 0.025 M. The overall equilibrium constant between reduced and native apamin, K_{RN}, is about 0.42 M^2. Thus, formation of the second disulfide bond has an equilibrium constant, K_{I_iN}, of approximately 17 M (see eq 7). This 640-fold increase in the effective concentration for forming the second disulfide bond indicates a very cooperative folding process. The first disulfide bond apparently promotes structure in the peptide such that the second disulfide bond forms with great facility.

Effect of Urea and Temperature. Increasing temperature or urea concentration generally destabilizes secondary structure (Marqusee et al., 1989). Since there is no effect of urea on the chemistry of disulfide bond formation (Creighton, 1977b; Lin & Kim, 1989), the effect of urea on the equilibrium constants reveals how secondary structure stabilizes the disulfide-bonded structures. A similar effect on the equilibrium constants is also expected when the temperature is raised (Marqusee et al., 1989). The present study shows that secondary structure stabilization contributes to the stability of the disulfide bond and consequently to the structure formed in apamin. K_{RN} shows a modest dependence on both urea and temperature, suggesting a similar secondary structure destabilization in both cases. However, the effect is small, with K_{RN} decreasing by only a factor of about 4 between 0 and 8 M urea, corresponding to a free energy of only 0.8 kcal·mol^{-1} at 25 °C. The measured enthalpy is only −3.4 kcal·mol^{-1}. Native apamin has been shown to be extremely stable against denaturation by CD studies (Miroshnikov et al., 1978). Reduced apamin loses essentially all of its structure. The addition of urea, therefore, will not have much effect on the peptide, no matter whether it is oxidized or reduced. Our results rationalize this lack of urea denaturation: urea- or temperature-denaturable secondary structure does not contribute a lot to apamin's stability!

Cooperativity of Folding. The equilibrium constant for forming the first disulfide bond in apamin is approximately 0.025 M, while that for forming the second disulfide bond is 17 M. This represents a high cooperativity during the folding of apamin from the reduced to the oxidized form. The formation of the first disulfide bond stabilizes some structure that apparently brings the other two thiol groups into close proximity. This cooperativity results in a very low population of one-disulfide folding intermediate states at equilibrium as is generally observed in the folding of globular proteins (Privalov, 1979). The calculated population distribution throughout the folding transition of apamin is shown in Figure 5. The total one-disulfide intermediate concentration never exceeds about 5% of the total peptide.

The addition of urea only modestly affects the formation of the disulfide bonds, and hence cooperativity in folding is still observed even in high concentrations of urea. What, then,

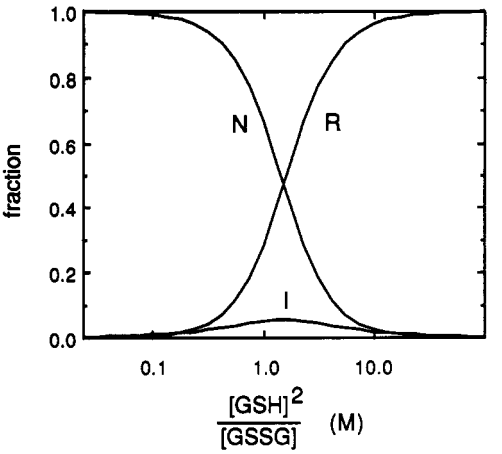

FIGURE 5: Calculated population distribution for R, N, and the combined one-disulfide intermediates, I, as a function of reducing potential expressed on a log scale of $[GSH]^2/[GSSG]$. Equilibrium parameters used were 0.42 M^2 for K_{RN} and 0.025 M for each of three one-disulfide intermediates. The total intermediate population is approximately 5% of the total peptide at the midpoint of the transition curve.

contributes to this cooperative effect? After one of the two native disulfide bonds forms, connecting either Cys-1 to Cys-11 or Cys-3 to Cys-15, the other two cysteines are separated by six residues, in addition to the two cysteine side chains and disulfide bond. These two intermediates have equilibrium constants of approximately 0.018 and 0.033 M and exhibit some structure as observed by CD (Huyghues-Despointes & Nelson, 1991). The second disulfide bond is accompanied with a significant increase in the amount of structure. However, this structure is destabilized only modestly by urea or temperature. At present, we can only speculate on what stabilization is occurring when the second disulfide bond forms. The formation of the first disulfide bond could overcome the entropy required for initiating structure, resulting in the formation of a marginally stable structure. Once the initiation of part of the structure is promoted by the first disulfide bond, the second disulfide bond might act cooperatively, inducing the rest of the structure to form without providing much additional stability.

Comparison with Other Work. Lin and Kim (1989) have studied the effects of urea on the stability of the disulfide bond in thioredoxin. Thioredoxin contains two cysteines, although the conformation of the protein is not affected significantly by forming a disulfide bond. It has been shown in their study that the effective concentration of the two cysteines is approximately 10 M in the folded protein and drops to about 0.026 M when unfolded by urea. Thus, formation of the disulfide bond in the native protein is 400 times more favorable than that in the unfolded protein. It is interesting that the effective concentrations in the unfolded and native thioredoxin are very close to those we measure for the first and second disulfide bonds in apamin. Thus, the formation of the first disulfide bond in apamin increases the effective concentration for forming the second by the same amount that thioredoxin folding increases the effective concentration of its two cysteines. However, we must be careful in interpreting this comparison, since the purpose of the two cysteines in thioredoxin is to reduce proteins such as ribonucleotide reductase and not to stabilize the structure of thioredoxin. The high effective concentration of the cysteines increases the reduction potential of thioredoxin.

Perhaps a more relevant comparison is with the folding of BPTI, whose structure is stabilized by three disulfide bonds.

BPTI spontaneously unfolds when its disulfide bonds are reduced (Creighton, 1977a). The linkage between folding and disulfide bond formation has been studied in detail by Creighton. In this case, all disulfide-bonded intermediates are stabilized significantly by secondary structure, and adding urea or guanidinium chloride destabilizes the preferred one- and two-disulfide folding intermediates, resulting in statistical distributions of disulfide bonds (Creighton, 1977b). BPTI consists of only 58 amino acids, and thus secondary structure stabilization of disulfide bond intermediates does not require a large protein.

Disulfide bond formation has also been studied in α-conotoxin GI, a snail toxin peptide consisting of 13 amino acids (Zhang & Snyder, 1991). The natural, active form of this peptide has two disulfide bonds in the same topology as apamin. However, after reduction and reoxidation, only 71% of the peptide re-forms the native disulfide bond arrangement, with little apparent cooperativity in forming the two disulfide bonds (Zhang & Snyder, 1991). Thus, disulfide bond formation in this conotoxin does not exhibit the cooperativity observed in apamin.

Considering our results on both apamin and the one-disulfide intermediate models (Huyghues-Despointes & Nelson, 1991), the folding of apamin appears to be surprisingly similar to that of BPTI in some respects. We can envision the following scenario: In apamin, the first disulfide bond forms with a low stability and rearranges to the preferred intermediate containing a disulfide bond between Cys-3 and Cys-15, with an equilibrium constant of approximately 0.033 M. This intermediate contains some secondary structure as seen by CD which provides a small amount of stability to the intermediate. This stabilization is modest, since the equilibrium constant is small in magnitude and urea decreases the equilibrium constant for these intermediates by a factor of about 2 (Huyghues-Despointes & Nelson, 1991). This structure might be important in bringing the remaining two cysteines into proximity. The second disulfide bond then forms with an equilibrium constant more than 100-fold larger than the first and is accompanied by the formation of significantly more secondary structure as seen by CD and NMR. The main difference between BPTI and apamin is that secondary structure stabilization is more modest in apamin—the equilibrium constant between reduced and native apamin decreases by only a factor of 4 in high concentrations of urea. This model would imply that both the first and second disulfide bonds induce structure by promoting the initiation of structure by perhaps overcoming the entropy required for initiation. The propagation of the secondary structure accompanying formation of the second disulfide bond might provide only a small increment to the stability.

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