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Stabilities of Disulfide Bond Intermediates in the Folding of Apamin†

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ABSTRACT: Apamin is an 18-residue bee venom peptide with the sequence CNCKAPETALCARRCQQH-amide and contains 2 disulfide bonds connecting C-1 to C-11 and C-3 to C-15. In the folding of reduced, unfolded apamin to native apamin with two disulfide bonds, the one-disulfide folding intermediate states are not populated to significant levels. To study the properties of the one-disulfide intermediates, we have synthesized two peptide models to mimic the one-disulfide intermediates, Apa-1 and Apa-2, in which two cysteines in the sequence have been replaced by alanines. These peptides can form only one of the native disulfide bonds, C-1 to C-11 in the case of Apa-1 and C-3 to C-15 in the case of Apa-2. The stabilities of these disulfide bonds have been measured as a function of pH, concentration of urea, and temperature, in order to understand which contributions stabilize the disulfide-bonded structures. Using oxidized and reduced glutathione, the equilibrium constants for forming the disulfide bonds at 25 °C and pH 7.0 are 0.018 M for Apa-1 and 0.033 M for Apa-2 and show little dependence on pH or temperature. Both disulfide bonds are destabilized slightly (by approximately a factor of 2) between 0 and 8 M urea. Circular dichroism spectra indicate that although both Apa-1 and Apa-2 exhibit some structure, Apa-2 exhibits more than Apa-1. The results suggest that in the folding of apamin, the one-disulfide intermediate containing the C-3 to C-15 disulfide bond, as in Apa-2, is favored slightly. Secondary structure provides modest stabilization to this intermediate.

Understanding the folding pathway of a protein involves identifying and characterizing the intermediates found along the pathway. This goal is made difficult because of the cooperativity of protein folding: any intermediate state will only be populated transiently, with only fully folded and denatured states significantly populated at equilibrium. Despite these difficulties, the existence of folding intermediates has long been known: kinetics measured by a single probe often exhibit multiple phases, and monitoring different probes often yields different kinetic curves (Kim & Baldwin, 1980).

Several techniques have been developed to selectively populate transient intermediates. Incorrect proline isomers can trap a nativelike intermediate along the slow folding pathway of pancreatic ribonuclease A (RNase A)[1] (Cook et al., 1979), and the stability of such an intermediate to GdmCl denaturation can be measured by double-jump experiments starting from unfolded protein (Grafl et al., 1986). A folding intermediate along the fast folding pathway of RNase A can also be transiently populated and examined by double-jump experiments starting with native protein (Hagerman et al., 1979). The nature of the structure formed along the folding pathway can be inferred by trapping exchangeable amide protons along the peptide backbone and examining by NMR when each amide proton is protected from exchange. Pioneering studies have been carried out for RNase A (Udgaonkar & Baldwin, 1988) and cytochrome *c* (Roder et al., 1988). Excellent reviews of protein folding have recently been published (King, 1989; Kim & Baldwin, 1990).

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[1] Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; Ellman's reagent or DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TFA, trifluoroacetic acid; TEA, triethylamine; GdmCl, guanidinium chloride; HPLC, high-performance liquid chromatography; CD, circular dichroism; [θ], mean residue ellipticity; NMR, nuclear magnetic resonance; RNase A, pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor.

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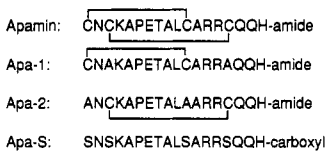

FIGURE 1: Peptide sequences and disulfide bonding arrangement of apamin and the two one-disulfide derivatives Apa-1 and Apa-2. The derivatives each have two cysteines replaced by alanines. In the peptide Apa-S, all four cysteines are replaced by serine, with a carboxy terminus. Apa-S serves as a model of the unstructured reduced peptide.

Perhaps the most successful technique for characterizing folding intermediates is the method of trapping disulfide bond formation along the folding pathway, as originally developed by Creighton on the folding of BPTI (Creighton, 1986). In this strategy, an unfolded protein with reduced cysteines is allowed to refold under oxidizing conditions. Disulfide bonds will then begin to form as the protein folds. These disulfide bonds are trapped by irreversibly reacting all remaining reduced cysteines with iodoacetic acid, thus locking in any disulfide bonds which have formed up to that point. The different disulfide-bonded intermediates are then separated and identified, and their kinetic progress is followed by trapping the folding reaction at different times (Creighton, 1978). Since these studies appeared, similar experiments have been done on ribonuclease T1 (Pace & Creighton, 1986) and RNase A (Creighton & Wearne, 1988).

Characterizing the properties of the trapped disulfide intermediates is still difficult because of the low concentrations of the intermediates formed during folding. In the case of BPTI, several of the intermediates were isolated after trapping along the folding pathway, and structural analysis was performed by NMR (States et al., 1987). The difficulties of isolating significant quantities of the low-populated intermediates can be overcome by chemically synthesizing peptides to act as models of the folding intermediates. A recent example of this was the synthesis of two peptides from BPTI which, when joined by a disulfide bond, form a structure similar to that found in native BPTI (Oas & Kim, 1988). This model was designed to mimic the properties of the corresponding one-disulfide intermediate of BPTI. The success of this technique requires that the models possess structural properties similar to the actual folding intermediate.

We have been studying the folding of apamin, a bee venom peptide consisting of 18 amino acids. The structure of apamin is stabilized by two disulfide bonds connecting C-1 to C-11 and C-3 to C-15. The structure of apamin has been solved by NMR techniques (Wemmer & Kallenbach, 1983; Pease & Wemmer, 1988) and consists of a C-terminal α-helix with disulfide bonds between two successive turns of the helix to two reverse-turn structures on the N-terminal end. The sequence of apamin is shown in Figure 1. In equilibrium mixtures of reduced and native apamin, the one-disulfide intermediates are populated only to a few percent (unpublished results). In order to investigate the stabilities of these one-disulfide intermediates, we synthesized two peptides which contain only one of the two native disulfide bonds, by replacing the other two cysteines with alanines. The peptides, labeled Apa-1 and Apa-2, are shown in Figure 1. In this paper, we report results on the stability of these disulfide bonds, as well as the influence of environment, in order to determine the importance secondary structure plays in the stability of these peptides and thus their importance as intermediates in the folding of apamin. A brief description of the techniques and preliminary results has been published (Huyghues-Despointes & Nelson, 1990).

Materials and Methods

GSH and GSSG were obtained from Sigma Chemical Co. and Amresco Research Products Co. All were dried on a freeze drier and stored desiccated in a refrigerator. Tris base and Tris-HCl were from Sigma. Urea was electrophoresis purity reagent from Bio-Rad. EDTA, disodium salt dihydrate, was from Aldrich. DTNB, TFA (HPLC spectra grade), and TEA (sequanal grade) were from Pierce. Acetonitrile was HPLC grade from various suppliers. All other chemicals were of reagent grade or better.

Apa-1 and Apa-2 were synthesized by Rheba Rutkowski in Dr. Peter S. Kim's lab (MIT) on an Applied Biosystems Model 430A peptide synthesizer using standard reaction cycles. Apa-S was synthesized using similar protocols by manual methods. The peptides were purified by reducing them with 0.1 M DTT in 0.2 M Tris buffer at pH 8.0 prior to separation using a Vydac 218TP510 semi-prep C18 column, 1.0 × 25 cm, running on a Rainin Dynamax HPLC system. The solvents were water and acetonitrile, both containing 0.1% (v/v) TFA, using a flow rate of 2 mL·min^{-1} and a 30-min gradient from 12 to 16% acetonitrile for Apa-1 and Apa-2 and from 10 to 14% acetonitrile for Apa-S. The peptides were monitored at 220 nm using an ISCO V4 variable-wavelength detector. The fractions were collected and freeze-dried. The purity of the reduced and oxidized peptides was checked by analytical HPLC. Apa-1 was used without any further purification whereas Apa-2 had to be repurified after air oxidation to separate an impurity that coeluted with the reduced peptide. Peptide identities were confirmed by amino acid analysis after a 24-h hydrolysis in 6 M HCl containing 0.1% phenol at 110 °C, using a cation-exchange column (Pickering Laboratories) operated at 65 °C, and by fluorescence detection after post-column derivatization with *o*-phthalaldehyde. Further confirmation was obtained by plasma desorption time-of-flight mass spectroscopy on a Bio-Ion 20 mass spectrometer. The dry peptides were stored frozen and desiccated.

Concentrations of GSSG were determined by measuring the absorbance at 248 nm using an extinction coefficient of 382 M^{-1}·cm^{-1} (Huyghues-Despointes & Nelson, 1990; Chau & Nelson, 1991). Concentrations of GSH solutions were measured spectroscopically by using Ellman's reagent (DTNB) and an extinction coefficient of 14 150 M^{-1}·cm^{-1} for the 2-nitro-5-thiobenzoate dianion at 412 nm (Riddles et al., 1979). Oxygen was excluded from all of the solutions to prevent air oxidation of the glutathione.

Since oxygen readily oxidizes cysteine side chains to form disulfide bonds, air was excluded from all solutions used in equilibrium experiments. In addition, 1 mM EDTA was added to the buffers to chelate trace heavy metals which catalyze the oxidation. All solutions were deoxygenated by three to five cycles of evacuating the vials for 15 min and repressurizing to atmospheric pressure with argon. All vials were kept under 4 psi of positive argon pressure throughout the experiments.

Sample Preparations and Acid Quenching. Three buffer systems were used depending on the pH range of the experiments. All buffers contained 0.2 M KCl and 1 mM EDTA. For peak identification studies, 0.1 M Tris buffer at pH 8.7 was used. For pH studies, the buffer contained both 0.1 M Tris and 50 mM potassium phosphate. The temperature and urea studies were performed at pH 7.0 using a 50 mM potassium phosphate buffer. Glutathione stock solutions were generally made fresh daily at a concentration of 10 mM GSSG or 50 mM GSH, with the actual concentrations measured as described above. For peak identification studies, mixtures were prepared which consisted of various volumes of oxidized and

reduced glutathione stock solutions to achieve the desired ratios, a volume of the peptide, and buffer to a final volume of 100 μL. The peptide was added last. For the pH, urea, and temperature studies, stock solutions containing both oxidized and reduced glutathione were prepared in buffers at 5 times the desired final concentration of all ingredients, and the pH was adjusted to 7.0. Premixing the GSH and GSSG solutions ensured that all samples contained the same glutathione concentrations. In the pH studies, 1.0-mL portions of this mixture were added to several milliliters of deoxygenated water in separate vials. After pH adjustment, the solution was brought to 5.0 mL with water. In the urea experiments, 2.0–8.0 M urea solutions were made by mixing 1.0–4.0 mL of 10 M urea with 1.0 mL of the concentrated glutathione and buffer solution, the pH was readjusted to 7.0, and the mixture was brought to 5.0 mL with water. The glutathione-containing buffer and a small volume of concentrated peptide stock solution were mixed to make a final reaction volume of 100 μL. The final peptide concentrations were in the range of 25–50 μM.

In all the experiments, the samples were prepared in 500-μL crimp-top vials sealed with Teflon-lined silicone rubber septa. As soon as all ingredients were added, each vial was vortexed and placed in a larger capped vial that was kept under argon pressure. This minimized evaporation of the sample due to slow leakage from the sample vial. After equilibrium was reached, the mixture was quenched by lowering the pH to about 2 by injecting 6 μL of 6 N HCl and quickly vortexing. Equilibrium was checked by analyzing samples at increasing times until the composition no longer changed. Equilibrium was typically achieved within 30 min at pH 8.7, 45 min at pH 7, and 90 min at pH 6. After acid quenching, the samples were analyzed by HPLC. The results are averages of at least two runs performed on different days, with duplicate HPLC runs made of most samples.

HPLC Methods. A Vydac 218TP54 reverse-phase C18 column, 0.46 × 25 cm, was used for separating the different forms of the peptides. The absorbance of the peptides was monitored at 220 nm by using Isco V4 variable-wavelength detector and a 1-mm HPLC flow cell. Water was deionized, quartz-distilled, and filtered through a Millipore Norganic removal cartridge and a 0.45-μm Millipore filter. HPLC-grade acetonitrile was filtered through a 0.45-μm Millipore filter. All solvents contained 0.1% (v/v) TFA. Solutions containing TEA were refiltered, while TFA was added without further filtration. All solutions were deaerated under vacuum before use to eliminate air bubbles in the pump system. Apa-1 elutes within 30 min isocratically in 11% acetonitrile containing 0.05% TEA (v/v) in addition to the TFA. The best separation was attained by heating the column to 40 °C by a circulating water bath and a water jacket around the column. The Apa-2 species were separated at room temperature with a gradient from 8 to 17% acetonitrile over 60 min. In the case of Apa-1, all five species separate with near-base-line resolution, whereas in the case of Apa-2, the two single mixed disulfides coelute. In both cases, oxidized and reduced glutathiones as well as buffer components eluted near the solvent front.

Identification and Quantitation of Peaks. The peaks can be identified by measuring the effect of changing the ratios of GSH to GSSG on the relative peak intensities. At equilibrium, the peptides exist in several forms, as shown in Figure 2: R, fully reduced; O, oxidized to form an intrapeptide disulfide bond; and SD_1, SD_2, and DD, the single and double mixed disulfides with glutathione. These species are shown schematically in Figure 2. Interpeptide disulfide bonds involving two peptide strands were not observed. The species are linked by several equilibrium expressions:

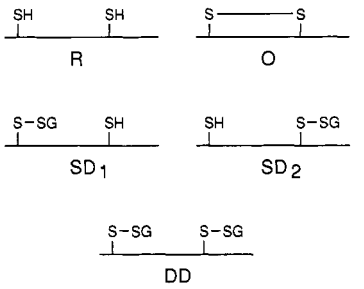

FIGURE 2: Schematic diagram of the different disulfide-bonded states of the peptides containing two cysteines. The cysteines involved in mixed disulfides in SD_1 and SD_2 were not identified for Apa-1 and are numbered arbitrarily. In Apa-2, the mixed disulfides coeluted and are combined simply as SD.

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

$$K_O = \frac{[GSH]^2}{[GSSG]}\frac{[O]}{[R]} \quad (2)$$

$$R + GSSG \rightleftharpoons SD_1 + GSH \quad (3)$$

$$K_{SD_1} = \frac{[GSH]}{[GSSG]}\frac{[SD_1]}{[R]} \quad (4)$$

$$R + 2GSSG \rightleftharpoons DD + 2GSH \quad (5)$$

$$K_{DD} = \left(\frac{[GSH]}{[GSSG]}\right)^2\frac{[DD]}{[R]} \quad (6)$$

$$SD_1 \rightleftharpoons O + GSH \quad (7)$$

$$K_O/K_{SD_1} = [GSH]\frac{[O]}{[SD_1]} \quad (8)$$

where there are expressions similar to eq 3, 4, 7, and 8 for SD_2.

The equilibrium between reduced and oxidized peptide, eq 2, follows an equilibrium expression that depends on the ratio of $[GSH]^2/[GSSG]$, while the equilibrium between reduced peptide and a single mixed disulfide, eq 4, depends on $[GSH]/[GSSG]$. The double mixed disulfide follows the square of this ratio. Since these ratios can be varied independently, a series of mixtures can be made at a constant $[GSH]/[GSSG]$ while varying $[GSH]^2/[GSSG]$, and vice versa (see Figure 3).

It is also important to determine the relative extinction coefficients of the different species, in order to accurately measure the relative concentrations of the peptide species. The relative extinction coefficients of the oxidized and reduced peaks can be determined by injecting exactly the same quantity of each form and comparing peak areas. This is conveniently done by taking equal volumes of air-oxidized peptide, injecting one sample directly while reducing the other with 0.1 M DTT in 0.2 M Tris buffer at pH 7.0 prior to injection. The peak areas of the two HPLC runs can be compared to determine the relative extinction coefficient. The relative extinction coefficients for the oxidized forms relative to the reduced forms were 0.98 for Apa-1 and 1.21 for Apa-2. The extinction coefficients for all mixed disulfide forms of Apa-1 and Apa-2 were assumed to be equal to that of the reduced peptide.

CD Measurements. Circular dichroism spectra were recorded on an Aviv 62DS circular dichroism spectrophotometer. The instrument was calibrated using a freshly made aqueous solution of (+)-10-camphorsulfonic acid at a concentration

Table I: Observed Equilibrium Constants at 25 °C and pH 7.0[a]

peptide	K_O (M)	K_{SD_1}	K_{SD_2}	K_{DD}	K_O/K_{SD_1} (M)	K_O/K_{SD_2} (M)
Apa-1	0.018 ± 0.002	2.4 ± 0.3	1.4 ± 0.2	3.9 ± 0.7	0.0075	0.013
Apa-2	0.033 ± 0.003	7.2 ± 0.5[b]		13.2 ± 4.0	0.0092[c]	

[a] For the equilibria described in eq 1–8. The values are averages and standard deviations combined from studies on the effects of pH, temperature, and urea concentration. [b] The sum of the equilibrium constants for both single mixed disulfides. [c] Assuming both K_{SD_1} and K_{SD_2} to be 3.6.

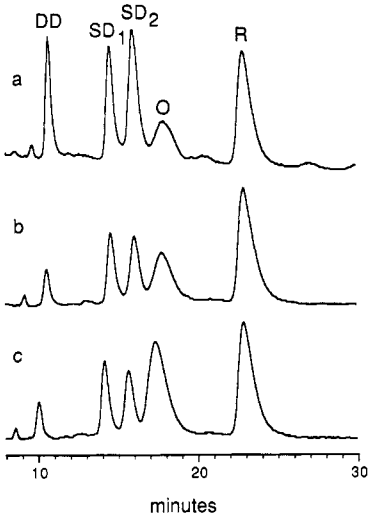

FIGURE 3: HPLC chromatograms of equilibrium mixtures of Apa-1 at various ratios of GSH/GSSG. The ratios of [GSH]/[GSSG] and $[GSH]^2/[GSSG]$ for each panel were, respectively, (a) 2.0 and 0.020, (b) 4.1 and 0.020, and (c) 4.1 and 0.010. The peak labels are described in Figure 2.

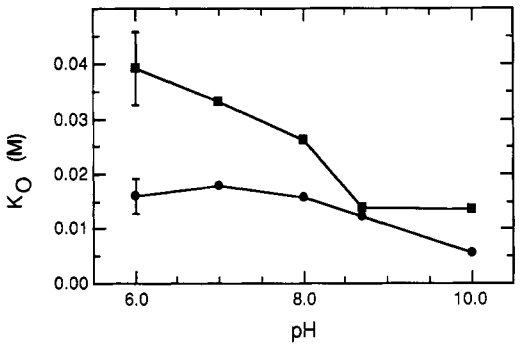

FIGURE 4: Effect of pH on the K_O in eq 2 for (●) Apa-1 and (■) Apa-2 at 25 °C. The error bars are representative of the standard deviations from multiple experiments.

of 0.92 mg·mL^{-1} as measured by the absorbance at 285 nm (Johnson, 1990). Peptide concentrations in stock solutions were determined by amino acid analysis by comparison with norleucine added as an internal standard. The buffer consisted of 0.2 M KF and 10 mM potassium phosphate buffer at pH 7.0. Three scans were averaged in the ultraviolet region between 270 and 184 nm, collecting data points every 0.5 nm with a 1.5-nm bandwidth and a 1-s time constant. Cells were 1-mm optical path length (Hellma), at peptide concentrations of 0.50–0.75 mM with respect to amino acids (28–42 μM peptide concentration). The data were linearly smoothed ±2 points and base-line-corrected. Under these conditions, the absorbance of the sample was slightly above 1.0 at 184 nm. Temperature was regulated at 25 °C by a water bath controlled by the CD computer which circulated through the metal block of the sample holder (Aviv Associates), with the temperature being measured by a thermometer imbedded in the cell block surrounding the cuvette.

For urea denaturation curves, a 10-mm path length cuvette (Hellma) was used with 2.0 mL of a solution with a peptide concentration of 0.1 mM in amino acids (5.6 μM peptide concentration). The CD was measured by averaging readings over 60 s at 220 nm as well as at 260 nm to compensate for instrumental drift. The urea concentration was increased by 1 M increments by adding solid urea to the cuvette. The appropriate quantity of urea was calculated using the equation relating solution density to urea concentration as reported by Kawahara and Tanford (1966), and the peptide concentration was corrected for the resulting volume change. The resulting urea additions (per 2 mL of starting solution) were 126, 138, 152, 169, 188, and 212 mg.

Results

Peak Identities. Figure 3 shows HPLC chromatograms of Apa-1 under a range of ratios of GSH and GSSG. All chromatograms are plotted such that the fully reduced peptide, R, is the same intensity to allow direct comparisons. Any peak involving a mixed disulfide with glutathione will vary inversely with the ratio [GSH]/[GSSG]. The oxidized peptide concentration will vary inversely with the ratio $[GSH]^2/[GSSG]$. Using panel b as a reference, panel a has the same ratio of $[GSH]^2/[GSSG]$, whereas the ratio [GSH]/[GSSG] is reduced in panel a by a factor of 2. Peak O is unchanged, whereas peaks SD_1 and SD_2 double in size, and peak DD increases about 4-fold. We cannot tell which cysteine is involved in the mixed disulfide in SD_1 and SD_2, and hence the numbering is arbitrary. Comparing panel c to panel b, the ratio of [GSH]/[GSSG] remains unchanged, whereas the ratio of $[GSH]^2/[GSSG]$ in panel c is reduced by a factor of 2. Peak O doubles in size, whereas the other peaks remain unchanged. Similar sets of experiments were used to identify the peaks in the Apa-2 chromatogram (data not shown). The assignments for Apa-2 were also confirmed by amino acid analysis of each of the peaks. The resulting values of the equilibrium constants at 25 °C and pH 7.0 for each species of Apa-1 and Apa-2 are shown in Table I.

Effects of pH. The equilibrium constant for disulfide bond formation as a function of pH for formation of oxidized peptide, K_O in eq 2, is shown in Figure 4 for both Apa-1 and Apa-2. As can be seen, there is a gradual decrease in K_O as the pH increases from 6.0 to 10.0. The change is about a factor of 3 for both Apa-1 and Apa-2. A comparable change in equilibrium constant vs pH is also seen in the single and double mixed disulfides, eq 4 and 6 (data not shown), suggesting the effect is mostly involved in the chemistry of the disulfide bond formation, as opposed to specific properties of Apa-1 and Apa-2.

Effects of Temperature. The temperature dependence of the stability of the oxidized form of Apa-1 and Apa-2 is shown in Figure 5. In the case of Apa-2, there is a very slight dependence on temperature. There is no measurable dependence on temperature for Apa-1. The mixed disulfides exhibit comparable effects of temperature (data not shown).

Effects of Urea Concentration. Figure 6 shows the effect of urea concentration on the stability of the oxidized peptides of Apa-1 and Apa-2. The equilibrium constants for Apa-1 and Apa-2 both decrease by a factor of about 2, indicating that each disulfide bond is stabilized slightly by secondary

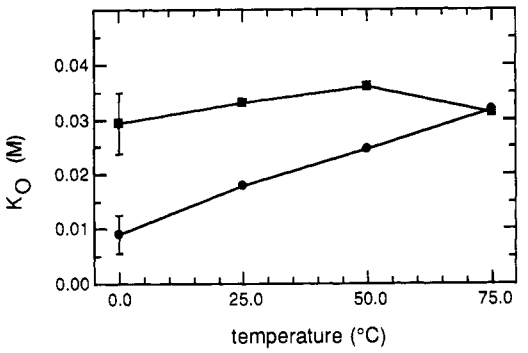

FIGURE 5: Effect of temperature on the K_O in eq 2 for (●) Apa-1 and (■) Apa-2 at pH 7.0. The error bars are representative of the standard deviations from multiple experiments.

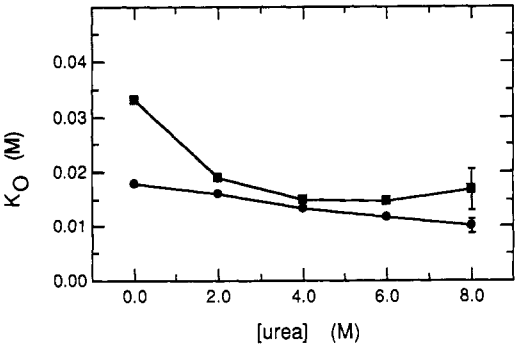

FIGURE 6: Effect of urea concentration on the K_O in eq 2 for (●) Apa-1 and (■) Apa-2 at pH 7.0 and 25 °C. The error bars are representative of the standard deviations from multiple experiments.

structure that can be denatured by urea. The curvature of the Apa-2 plot, along with its higher stability at low urea concentrations relative to Apa-1, might suggest a modest stabilization of the disulfide bond by secondary structure such as the C-terminal α-helix, which is stable in the native structure of apamin. The mixed disulfides show no measurable effect of urea (data not shown).

CD Measurements. Figure 7 shows the CD spectra of oxidized Apa-1 and Apa-2, along with those of native apamin and Apa-S. The CD spectrum of Apa-S is consistent with a lack of structure (Greenfield & Fasman, 1969), as would be expected with this peptide (see below). The CD spectrum of apamin shows significant structure, consistent with the extent of structure determined by NMR (Pease & Wemmer, 1988). The CD spectra of Apa-1 and Apa-2 both show partial structure when compared with Apa-S, with Apa-2 exhibiting somewhat more structure. The lack of an isodichroic point suggests that Apa-1 and Apa-2 exhibit different structural characteristics. The inset to Figure 7 shows the effect of urea concentration on $[\theta]_{220}$ for Apa-1, Apa-2, and Apa-S. As expected, Apa-S exhibits a minimal effect. In contrast, Apa-1 and Apa-2 both become less structured as the urea concentration increases. Apa-2 still exhibits some structure even in 6 M urea.

Discussion

Reduced and unfolded apamin spontaneously folds to native apamin under conditions where disulfide bond formation is favored. In fact, merely exposing the solution to air allows aerial oxidation to native apamin with formation of the correct disulfide bonds. This stable native structure has been determined by two-dimensional NMR studies, and indicates an α-helix from residues A-9 to Q-17 on the C-terminal end of the peptide and two reverse turns (Wemmer & Kallenbach, 1983; Pease & Wemmer, 1988). Further confirmation of

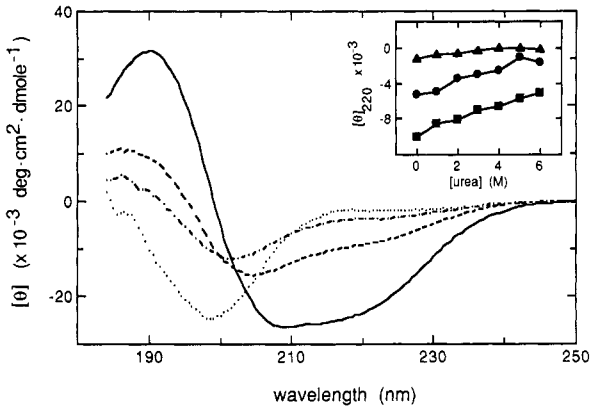

FIGURE 7: CD spectra from 250 to 184 nm of Apa-1 (-·-), Apa-2 (---), apamin (—), and Apa-S (···), plotted as $[\theta]$, the mean residue ellipticity. The inset shows $[\theta]_{220}$ plotted as a function of urea concentration for Apa-1 (●), Apa-2 (■), and Apa-S (▲).

stable structure is seen by a 20-fold reduction in the amide exchange rates of the residues in the C-terminal α-helix compared to the more exposed amide hydrogens (Dempsey, 1986). We are probing what interactions give rise to this stabilization, which of these interactions also occur in the one-disulfide intermediates, and the consequences on the folding mechanism of apamin. Toward this end, we have studied in detail the stability of the disulfide bond in two peptides containing only two cysteines, Apa-1 and Apa-2. These models of one-disulfide intermediates allow us to estimate how much stabilization arises from the nativelike structure found in native apamin. The most obvious candidate for such structure is the C-terminal α-helix; however, reverse turns and other structures might also make contributions.

Disulfide bond formation has been studied for a number of peptides containing two cysteines. In a study on the effect of distance between two cysteine residues in short synthetic peptides, the equilibrium constant was measured as a ratio of rate constants in 3 M GdmCl at pH 6.9 and 23 °C. The resulting equilibrium constants were 0.027 M with adjacent cysteines, 0.0031 M with one intervening alanine, 0.071 M with two, 0.018 M with three, 0.055 M with four, and 0.030 M when there are five intervening alanine residues (Zhang & Snyder, 1989). These equilibrium constants are between the mixed disulfide and the oxidized peptide, namely, K_O/K_{SD_1}, but they can be related to the present study by multiplying by K_{SD_1}, which is approximately 2 (Table I). In the case of two cysteines separated by six glycines, K_O is 0.060 M at pH 8.7 and 23 °C, and is independent of urea concentration (Lin & Kim, 1989). These results provide representative values for unstructured peptides.

Effective Concentrations. The goal of our studies on Apa-1 and Apa-2 was to determine if there is stabilization of the disulfide bond by the formation of secondary structure. There is a linkage between disulfide bond stability and secondary structure stabilization as a consequence of being a thermodynamic cycle (Creighton, 1983a, 1986). If disulfide bond formation is accompanied by the formation of secondary structure, this structure stabilizes the disulfide bond, and vice versa. This linkage can be described in terms of an "effective concentration", C_{eff} (Creighton, 1983a, 1986), which can be thought of as how close together the two cysteines are constrained to be. The concept of effective concentrations has been developed in several ways: as a ratio of rate constants for an intramolecular reaction and the corresponding intermolecular reaction (Kirby, 1980; Illuminati & Mandolini, 1981), as an analogous ratio of equilibrium constants

(Creighton, 1983b), or as a ratio of the reduction potentials for glutathione and the peptide thiols (Lin & Kim, 1989). The reference state for the intramolecular reaction is the intermolecular reaction at 1 M concentration of reactants. This concept assumes that the inherent chemistries for forming the disulfide bond in GSSG and in the peptide disulfide are identical and hence C_{eff} provides information about how close the two peptide cysteine thiols are constrained to be. Empirically, glutathione appears to be an excellent reference thiol, since formation of its disulfide bond does not depend on urea concentration (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).

The value of C_{eff} for a peptide disulfide bond indicates whether the two cysteines are constrained to be closer together than would be the case with an unstructured peptide. The source of this stabilization can be probed by determining under what conditions C_{eff} is significantly changed. The linkage between protein stability and the effective concentration of two cysteines is demonstrated dramatically in thioredoxin, where C_{eff} changes from 10 M in the absence of urea to 0.026 M in 7.7–9 M urea (Lin & Kim, 1989). If there is secondary structure in oxidized Apa-1 and Apa-2, we would expect to be able to detect this structure by measuring the stability of the disulfide bond under conditions which are known to affect the stability of the secondary structure. Thus, a change in C_{eff} as the secondary structure is destabilized by chemical or physical means would be a strong indication that the intermediates are stabilized by some type of secondary structure.

For the present discussion, we can consider two equilibria as candidates to define our effective concentration. The first possibility would be the equilibrium between R and O, as given in eq 1 and 2, with the result that $C_{eff} = K_O$. In this case, C_{eff} corresponds physically to the concentration of both GSH and GSSG at which equal populations of R and O exist (see eq 2). A second definition would involve the equilibrium between the mixed disulfide, SD_1, and O (eq 7 and 8). In this case, the effective concentration would be K_O/K_{SD_1} and correspond physically to the concentration of GSH required to produce equal amounts of O and SD_1. This definition results in two effective concentrations for a peptide, one for each of the two mixed disulfides SD_1 and SD_2.

The selection of a definition for effective concentration will depend on the goals of a study as well as the available data. The second definition requires measuring K_O, K_{SD_1}, and K_{SD_2} (or, equivalently, K_O/K_{SD_1} and K_O/K_{SD_2}). This definition is useful to describe the actual events in forming the disulfide bond, and differences between SD_1 and SD_2 would reflect differences in the reactivity of the two mixed disulfides. Studies of this nature have been carried out on the formation of disulfide bonds in unstructured peptides (Zhang & Snyder, 1988, 1989). The first definition is useful to investigate factors influencing the stability of a native protein or intermediates relative to the fully reduced and unfolded states. It requires no data on K_{SD_1} and K_{SD_2}. Such a definition has been used in the folding of BPTI (Creighton, 1883a,b) and thioredoxin (Lin & Kim, 1989).

In the present study, we have the luxury to select either definition (although we do not know K_{SD_1} and K_{SD_2} separately for Apa-2). Values for both definitions of effective concentration, K_O and K_O/K_{SD_1}, are listed in Table I. As can be seen, the differences in stability between Apa-1 and Apa-2 involve the formation of the mixed disulfide (eq 3), with the next step (eq 7) being similar in the two cases. The effects of temperature and pH are nearly the same on K_O and the K_{SD}'s indicating that temperature and pH affect mostly the mixed disulfides. On the other hand, urea exerts its effects exclusively on K_O, with the K_{SD}'s remaining essentially unchanged, indicating that urea is affecting mostly the formation of the intrapeptide disulfide bond (see below).

The goal of the present study is to provide information on the properties of the one-disulfide intermediates in order to understand the folding and stability of apamin. We therefore favor the first definition, $C_{eff} = K_O$. This choice is also influenced by the fact that in studying apamin folding, data on the mixed disulfides of reduced apamin and the one-disulfide intermediates cannot be obtained due to unresolved peaks in the first case and low populations in the second (J. W. Nelson and M.-H. Chau, unpublished results). This definition will also make comparisons with other work on protein folding more relevant (Creighton, 1983b; Lin & Kim, 1989).

Effects of pH. Although disulfide exchange reactions are generally written in terms of thiols, the reactions actually occur only with thiolate anions. The pK_a of thiol groups in cysteine residues in peptides is generally in the range of 8.7–8.9 (Szajewski & Whitesides, 1980; Zhang & Snyder, 1988). The rate of disulfide exchange will depend on pH when the pH is comparable to or less than the pK_a. The observed equilibrium constant can also depend on pH, but only if the pK_a's of the different thiol groups are different. If these pK_a's differ, the observed equilibrium constant will vary over the range of pH near the two pK_a's. In general, the pK_a of a nonterminal cysteine in an unstructured peptide is similar to that of glutathione (Snyder, 1987). In the case of an N-terminal cysteine residue, the ionization of the thiol is influenced by the ionization state of the amino terminus (Benesch & Benesch, 1955). This results in an effective thiol pK_a which is higher than glutathione. The effects of pH on the stability of the oxidized peptide for both Apa-1 and Apa-2 are shown in Figure 4. We found that as the pH increases from 6 to 10, the stability of the oxidized forms of both peptides decreases gradually by a factor of 2–3. The single and double mixed disulfides behave similarly to the oxidized peptide, indicating that the effect is on the properties of the mixed disulfide. We should note that the curves of stability vs pH exhibit a gradual change over 4 pH units, suggesting that the effect is due to general solvent effects rather than ionization of particular groups. If the latter were the case, the curves would exhibit most of the change over 1 or 2 pH units.

Effects of Temperature. Over the range of 0–75 °C, there is little effect of temperature on the stability of the disulfide bonds in Apa-1 and Apa-2 (Figure 5). Apa-1 exhibits a slight effect of increasing stability with increasing temperature, whereas Apa-2 shows no measurable effect. The effect of temperature is also mirrored in the mixed disulfides in both cases, and hence the slight effect of temperature might be on the inherent chemical differences between disulfide bond formation in glutathione and the peptides. In any case, the effect is small, and the van't Hoff enthalpy calculated for Apa-2 is 3.2 kcal·mol^{-1}.

Effects of Urea Concentration. Most proteins and many types of secondary structure are denatured by urea. One excellent test for the presence of secondary structure is to observe changes in structure or stability as urea is added to the solution. These tests have been used in many cases when studying the stability of α-helices (Kim & Baldwin, 1984; Marqusee et al., 1989). In the case of Apa-1 and Apa-2, there is a modest effect of urea concentration on the disulfide bond stability (Figure 6), namely, a factor of about 2 between 0 and

8 M urea. There is no measurable effect of urea concentration on the stabilities of the mixed disulfides, indicating that urea is affecting the formation of the intrapeptide bond rather than the mixed disulfide. The effect is larger in the case of Apa-2. Thus, at low urea concentrations, Apa-2 is more stable than Apa-1, whereas at higher urea concentrations, the stabilities become nearly equal. These results suggest that Apa-2 exhibits more stabilization by secondary structure than Apa-1.

Structures of Apa-1 and Apa-2. The CD spectra of Apa-1 and Apa-2 indicate these intermediates possess some secondary structure, with Apa-2 exhibiting a greater amount (Figure 7). Apamin exhibits much more structure than either intermediate, as seen by its much more intense CD spectrum (J. W. Nelson, unpublished results). Apa-S was designed to mimic the properties of unstructured reduced apamin, with the four cysteines replaced by serine and a carboxylate at the C-terminus instead of the amide as in apamin. Both of these substitutions should destabilize any α-helical structure in this peptide, since serine is considered unfavorable to helix formation (Chou & Fasman, 1974; Levitt, 1978) and a negatively charged C-terminus destabilizes α-helices (Fairman et al., 1989). The CD spectrum of Apa-S is as expected for unstructured peptides (Greenfield & Fasman, 1985; J. W. Nelson, unpublished results). As seen in Figure 7, there is no isodichroic point among the spectra, indicating that Apa-1 and Apa-2 must have structural aspects different from each other and from apamin.

The structures of both Apa-1 and Apa-2 are denatured by urea, although Apa-2 retains significant structure even in 6 M urea. By comparison of Figure 6 and the inset to Figure 7, the secondary structure stabilizes Apa-2 to a greater extent than Apa-1. However, the stabilities of Apa-1 and Apa-2 do not vary significantly with temperature. In short α-helical peptides, it generally is observed that α-helix stability decreases significantly with increasing temperature (Marqusee et al., 1989; Shoemaker et al., 1987). Thus, the stabilization of Apa-1 and Apa-2 appears to involve little enthalpic contribution. The stability might be due to the disulfide bond reducing the entropy of the peptide, thus favoring the initiation of secondary structure. The remaining secondary structure might then form with marginal stability without the need for significant enthalpic contributions.

The above experiments indicate that there is modest secondary structure stabilization of the one-disulfide intermediates. Evidence for this conclusion comes from the dependence of disulfide bond stability to denaturing effects of pH, temperature, and urea concentration. The effective concentrations of the two cysteines in Apa-1 and Apa-2 are small, and of a magnitude in the range expected for unstructured peptides. The effect of urea concentration on disulfide bond stability and structure as measured by CD indicates a modest stabilization of the disulfide bond by secondary structure. This stabilization is greater in Apa-2 and results in a 2-fold increase in the equilibrium constant of Apa-2 vs Apa-1 in the absence of urea.

What are the implications of these results on the folding of apamin? In the case of BPTI, the one-disulfide intermediates form randomly and quickly rearrange to the thermodynamically most stable forms (Creighton, 1977). These intermediates contain significant secondary structure (States et al., 1987; Oas & Kim, 1989). Formation of stable secondary structure will favor such an intermediate over unstructured intermediates, since disulfide bond formation and secondary structure are linked thermodynamically (Creighton, 1986). In apamin, we cannot answer the question of whether the secondary structure forms before the disulfide bond or whether a disulfide bond forms randomly, and subsequently rearranges to the most stable secondary structure stabilized form. Formation of the correct second disulfide bond requires one of the native C-1 to C-11 or C-3 to C-15 disulfide bonds to be present. The current studies suggest that the intermediate containing the C-3 to C-15 disulfide bond, as in Apa-2, is favored slightly, perhaps due to increased secondary structure relative to Apa-1. Selectively populating this intermediate state could accelerate the rate of folding by forming a structure conducive to forming the second disulfide bond by increasing the effective concentration of the remaining two thiols. Although the secondary structure stabilization of this intermediate is modest, it might be enough to direct and accelerate the folding process. Thus, stabilization of folding intermediates by secondary structure might to be important even in peptides as small as apamin.

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pH-Induced Folding/Unfolding of Staphylococcal Nuclease: Determination of Kinetic Parameters by the Sequential-Jump Method†

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ABSTRACT: On the basis of previous stopped-flow pH-jump experiments, we have proposed that the acid- and alkaline-induced folding/unfolding transition of staphylococcal nuclease, in the time range 2 ms to 300 s, follows the pathway $N_0 \rightleftharpoons D_1 \rightleftharpoons D_2 \rightleftharpoons D_3$, in which D_1, D_2, and D_3 are three substates of the unfolded state and N_0 is the native state. The stopped-flow "double-jump" technique has been employed to test this mechanism and to determine the rate constants which would not be accessible by the direct pH jump because of the lack of fluorescence signal, i.e., the rates for the conversion of D_1 to D_2 and of D_2 to D_3. In the forward jump, a protein solution kept at pH 7.0 was mixed with an acidic or alkaline solution to the final pH of 3.0 or 12.2, respectively. The mixed solution was kept for varying periods of time, called the delay time, t_D. A second mixing (the back jump) was launched to bring the protein solution back to pH 7.0. The time course of the Trp-140 fluorescence signals recovered in the back jump was analyzed as a function of t_D. Kinetics of the unfolding were found to be triphasic by the double-jump method, contrary to the monophasic kinetics observed by the direct pH jump. Complex kinetics of unfolding are expected with the proposed kinetic scheme. Analysis of data obtained by both the direct-jump and the double-jump experiments yielded complete sets of rate constants and activation energies for the unfolding to pH 3.0 and to pH 12.2 and for the folding from acid and alkali to pH 7.0. Fractions of protein in N_0, D_1, D_2, and D_3 states were determined to be 1.0, 0, 0, and 0, respectively, at pH 7.0; 0, 0.61, 0.28, and 0.11, respectively, at pH 3.0; and 0, 0.43, 0.30, and 0.27, respectively, at pH 12.2. ΔG of the transition between any two neighbors of the three D's was less than 0.55 kcal mol^{-1}. Thus, any obligatory pathway in an early stage of the chain folding must be determined by the kinetic barriers rather than by the stability of the intermediate states.

Staphylococcal nuclease (SNase) is a classical protein for the study of protein folding because of several convenient features. First, it consists of one peptide chain and is a single-domain protein of relatively small molecular weight, 16 800 (149 residues). Thus, its stability and pathway for folding should be more amenable to biophysical study than proteins of more complex structure. Second, there are no disulfide bridges in the structure. The unfolded peptide should behave closely like an ideal random polymer chain (Tanford, 1968). Any peptide/solvent interaction would be easier to analyze. Third, many single, double, or multiple amino acid substituted mutants are available. By comparative study of these mutants, the contribution of individual amino acids to the stability of the protein or the pathway of folding may be assessed (Shortle, 1989).

Kinetics of the folding/unfolding of SNase have been investigated in several laboratories. Schechter et al. (1970) and Epstein et al. (1971) have studied folding of the protein from

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