and many alternative methods of partitioning the charge density to give atomic point charges, such as a dipole-conserving analysis,[40] are better for calculating intermolecular electrostatic energies. An alternative approach to deriving point charge models is to fit the charges directly to the electrostatic potential outside the molecule. This requires the evaluation of the potential, by integration over the ab initio charge density, at a large grid of points, and thus is computationally expensive for large molecules. The published errors from such potential fitting studies[2-4,27] suggest that the optimum atomic point charge model will predict the electrostatic potential with errors that are not negligible in comparison with those resulting from the use of transferable models. In addition, there is no reason to suppose that the errors involved in assuming transferability between molecules, or conformations, will be smaller for potential-derived charges than for a more complete distributed multipole representation. (Indeed, the potential-derived charges for a few amides[27] suggest that they may be less transferable.) Thus, the way forward for the accurate modeling of electrostatic interactions is first to go to a distributed multipole model and, when it is necessary to assume that an electrostatic model is transferable between molecules, use the recipes derived in this study.

Future Developments. We have established a successful transferable electrostatic model for amides and peptides with hydrocarbon side chains. In order use this approach to model a wide range of polypeptides, we need atomic multipole moments for all the naturally occurring amino acids. Since these include charged side chains and aromatic rings, which would probably be even more susceptible to intramolecular polarization than hydrocarbon side chains, the transferable PEPTIDE model will be more appropriate than a transferable ATOM model. Fortunately, the required calculations on the dipeptides of all the protein residues can now be performed with the direct SCF method,[34] and this work is in progress. This will provide a model for examining the intermolecular electrostatic interactions of polypeptides at a new level of accuracy and reliability. However, analogous critical studies of the other terms in inter- and intramolecular force fields, and improvements in simulation methods, are needed for molecular modeling to achieve its full potential for providing insight into molecular processes.

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Supplementary Material Available: Table of multipole moments of amides and dipeptides (8 pages). Ordering information is given on any current masthead page.

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Local Structure in Ribonuclease A. Effect of Amino Acid Substitutions on the Preferential Formation of the Native Disulfide Loop in Synthetic Peptides Corresponding to Residues Cys^{58}-Cys^{72} of Bovine Pancreatic Ribonuclease A

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Abstract: The possible existence of local structure in a 15-residue peptide fragment of bovine pancreatic ribonuclease A has been examined. Three peptides, corresponding to the amino acid sequence 58-72 of the native protein and two homologues thereof with amino acid substitutions at residues 66 ($Lys^{66} \rightarrow Gln$) and 67 ($Asn^{67} \rightarrow Ala$), respectively, have been synthesized by classical solution procedures. These peptides, all containing three Cys residues at positions 58, 65, and 72, were studied in disulfide-exchange equilibration experiments under strongly oxidizing conditions at 23 °C and pH 8.0. By use of an RP-HPLC method for the separation of the various species, it was demonstrated that the intramolecular equilibrium constnt K_{exp} for the two possible isomers of the native sequence, containing intramolecular eight-residue cyclic disulfides, was 3.58 ± 0.10 in favor of the disulfide bond between Cys^{65} and Cys^{72}, which is the disulfide bond present in the native protein. Amino acid substitutions at positions 66 ($Lys^{66} \rightarrow Gln$) or 67 ($Asn^{67} \rightarrow Ala$) did not result in marked changes in the standard free energy difference between the native and nonnative eight-residue disulfide loops, K_{exp} being 3.02 ± 0.17 for the Gln^{66} and 4.49 ± 0.23 for the Ala^{67} homologue. Possible reasons for this behavior are discussed.

It has been known for three decades that all the information required for a protein to attain its native three-dimensional structure (under appropriate solution conditions) is contained solely in its amino acid sequence.[1] However, the question as to *how* proteins fold, i.e., as to what are the detailed molecular mechanisms that lead from the unfolded polypeptide chain to a biologically active conformation, still awaits a final resolution. Regarding the early stages of the folding process, it has become increasingly clear that these are dominated by short-range interactions[2] resulting in the formation of locally ordered structures along the overall unfolded polypeptide chain.[3-12] These early-forming "chain-

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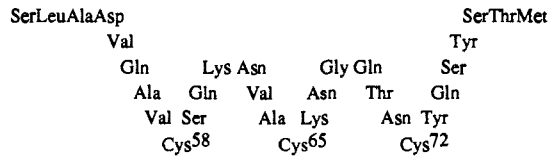

Figure 1. Amino acid sequence of RNase A M-peptide.[30,33]

folding initiation structures" (CFIS),[4,6,12-14] which are in rapid equilibrium with unfolded conformations, restrict the accessible conformational space of certain parts of the polypeptide chain, thus initiating the folding process and directing subsequent folding events.

Much of the evidence for the existence of these CFIS comes from NMR studies on medium-sized to small protein fragments that often reveal the presence of even small amounts of ordered structure in the conformational equilibrium that would not be detectable by any other spectroscopic method. This subject has recently been reviewed.[12,15]

In the case of bovine pancreatic ribonuclease A (RNase A), which is the subject of this report, attention was initially focused on the N-terminal fragments [residues 1–13 (C-peptide)[16,17] and 1–20 (S-peptide)[18-21]], which are capable of adopting nativelike helical conformations as isolated peptides under appropriate experimental conditions. However, experimental and theoretical studies in this [6,12,13,22-27] as well as other laboratories[28,29] have identified several additional sites besides the N-terminal helix that may form CFIS.

We have recently provided experimental evidence for the presence of nativelike local interactions in an isolated proteolytic fragment of RNase A encompassing residues 50–79 (RNase M-peptide)[30] (Figure 1). In this peptide, the formation of the native eight-residue disulfide loop between Cys^{65} and Cys^{72} is favored over the nonnative one of equal ring size (Cys^{58}-SS-Cys^{65}) by $\Delta G° = 1.1$ kcal/mol ($K = 5.9 \pm 0.8$),[30] although no such preference should be expected based on simple statistical considerations.[31,32] In order to evaluate the importance of specific amino acid residues for the observed standard free energy difference between the two 8-residue disulfide loops, we have now studied the thermodynamic properties of three homologous 15-residue peptides consisting of the minimum sequence for competitive loop formation, viz., CH_3CO-[RNase 58–72]-$NHCH_3$. One of the peptides corresponds to the wild-type sequence, RNase A 58–72 (denoted as [58–72]KN), while the two others contain single amino acid substitutions at positions 66 (Lys → Gln; denoted as [58–72]QN) and 67 (Asn → Ala; denoted as [58–72]KA), respectively. The choice of these particular substitutions was based on two observations: (i) Residues Lys^{66} and Asn^{67} are completely conserved in all mammalian pancreatic RNase A's that have been sequenced so far (39 species).[34,35] (ii) In the crystalline protein,[36] residues Cys^{65}-Lys^{66}-Asn^{67}-Gly^{68} form a type III β-turn, this particular sequence having one of the highest β-turn probabilities among all possible tetrapeptides, based on statistically derived conformational probability parameters for single amino acid residues.[37,38] The formation of β-turns (or chain reversals) has been proposed to play an important role in the initiation of protein folding;[39] local interactions defining a turn structure in the sequence Cys^{65}–Gly^{68} may therefore contribute significantly to the differential stability of the native and nonnative eight-residue disulfide isomers in the M-peptide.

While the replacement of Lys^{66} by Gln serves primarily to evaluate the importance of a positively charged side chain in position 66, it should only slightly disfavor β-turn formation at residues 65–68.[37,38] In contrast, substitution of Ala for Asn should result in a dramatic decrease in the β-turn potential at this site;[37,38] if a β-turn at Cys^{65}-Lys^{66}-Asn^{67}-Gly^{68} were of significant importance for the greater stability of the native loop, this drop in the β-turn potential should be reflected in a change in the thermodynamic properties of this homologous peptide, i.e., in a decrease or even inversion of the standard free energy difference between the native and nonnative loops as compared to the wild-type sequence.

Rationale of the Procedure. In order to study the effect of amino acid substitutions on the relative thermodynamic stabilities of the native and nonnative intramolecular eight-residue disulfide loops, the different peptides were each equilibrated with a mixture of 2,2′-dithiobis(ethylamine) (cystamine) and β-mercaptoethylamine (cysteamine) at pH 8.0 and 23 °C. Under these conditions, either of the loops is present as a mixture of three different species, depending on the state of the thiol group that does not participate in the intramolecular disulfide bond. Consequently, three equilibrium constants that relate the native and nonnative disulfide loops may be defined as follows, where R is $CH_2CH_2NH_2$:

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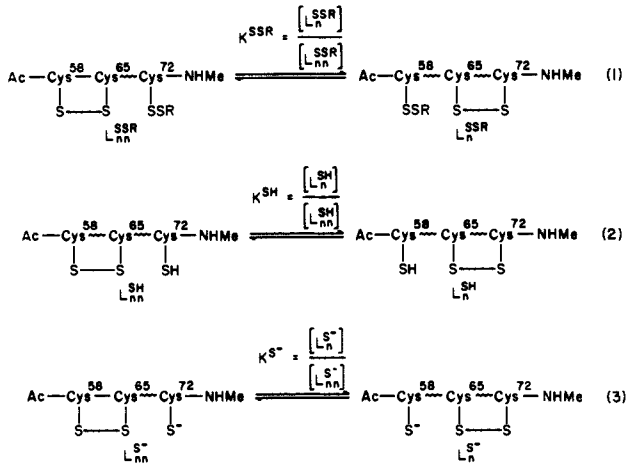

Termination of the disulfide-exchange reactions was achieved by blocking the free thiol groups with 2-aminoethyl methanethiosulfonate (AEMTS),[40] which converts all the species in eqs 2 and 3 to disulfides of the same form (i.e., same R group) as the ones represented in eq 1. This facilitates the subsequent chromatographic analysis, because a smaller number of oxidized peptides has to be separated. However, the experimentally determined apparent equilibrium constant

$$K_{exp} = [L_n^{SSR}]_b/[L_{nn}^{SSR}]_b \quad (4)$$

where $[L_n^{SSR}]_b$ and $[L_{nn}^{SSR}]_b$ are the concentrations of L_n^{SSR} and L_{nn}^{SSR} in the equilibrium mixture *after* blocking with AEMTS, does not a priori correspond to any of the equilibrium constants defined in eqs 1–3 because K_{exp} of eq 4 is actually

$$K_{exp} = \frac{[L_n^{SSR}] + [L_n^{SH}] + [L_n^{S^-}]}{[L_{nn}^{SSR}] + [L_{nn}^{SH}] + [L_{nn}^{S^-}]} \quad (5)$$

The equilibrium concentrations of $L_n^{S^-}$ and $L_{nn}^{S^-}$ are related to $[L_n^{SSR}]$ and $[L_{nn}^{SSR}]$, respectively, by the equilibrium constants for the following reactions:

$$L_n^{S^-} + RSSR \overset{K_1}{\rightleftharpoons} L_n^{SSR} + RS^- \quad (6)$$

$$L_{nn}^{S^-} + RSSR \overset{K_2}{\rightleftharpoons} L_{nn}^{SSR} + RS^- \quad (7)$$

In addition, $[L_n^{SH}]$ and $[L_{nn}^{SH}]$ are related to $[L_n^{S^-}]$ and $[L_{nn}^{S^-}]$, respectively, through the acid dissociation constants K_a of the thiol groups of Cys^{58} and Cys^{72}:

$$[L_n^{SH}] = [L_n^{S^-}][H_3O^+]/K_a[Cys^{58}] \quad (8)$$

$$[L_{nn}^{SH}] = [L_{nn}^{S^-}][H_3O^+]/K_a[Cys^{72}] \quad (9)$$

By combining eqs 6–9, eq 5 can be rewritten as

$$K_{exp} = K^{SSR}\left[\frac{1 + \frac{1}{K_1}\frac{[RS^-]}{[RSSR]}\left(1 + \frac{[H_3O^+]}{K_a[Cys^{58}]}\right)}{1 + \frac{1}{K_2}\frac{[RS^-]}{[RSSR]}\left(1 + \frac{[H_3O^+]}{K_a[Cys^{72}]}\right)}\right] \quad (10)$$

For very low values of the ratio $[RS^-]/[RSSR]$, K^{SSR} can therefore be approximated by K_{exp}. This is easily understood since, in the limiting case of high cystamine concentration, the species represented in eqs 2 and 3 will be populated only scarcely and, therefore, contribute very little to K_{exp} as expressed by eq 4 or, equivalently, eq 5.

K^{SSR}, however, does not represent solely the conformational energy difference between the native and nonnative eight-residue intramolecular disulfide loops, since the relative stabilities of L_n^{SSR} and L_{nn}^{SSR} also depend on the free energy difference between the

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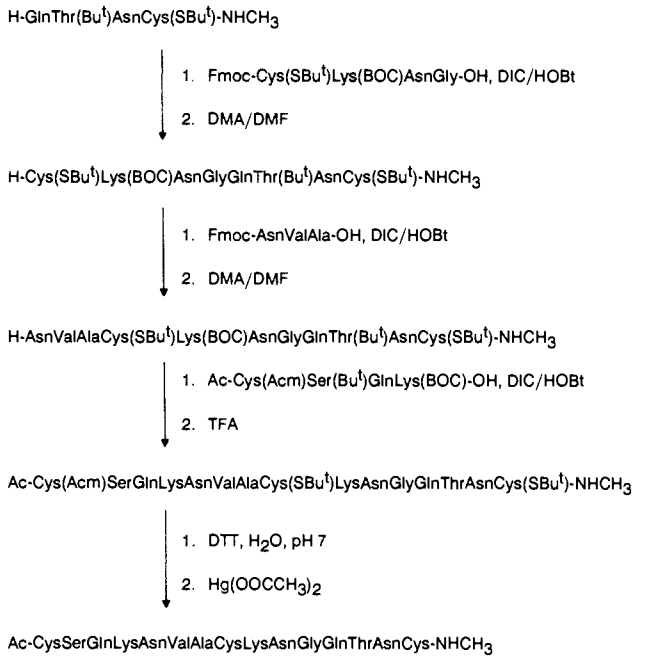

Figure 2. Schematic representation of the various fragment condensation steps in the final assembly of [58–72]KN. The syntheses of [58–72]QN and [58–72]KA followed analogous routes.

mixed disulfides of cysteamine with Cys^{58} and Cys^{72}, respectively. This is reflected in eq 11

$$K^{SSR} = K^{SH}K_1'/K_2' \quad (11)$$

where K_1' and K_2' are the equilibrium constants for the reactions

$$L_n^{SH} + RSSR \overset{K_1'}{\rightleftharpoons} L_n^{SSR} + RSH \quad (12)$$

$$L_{nn}^{SH} + RSSR \overset{K_2'}{\rightleftharpoons} L_{nn}^{SSR} + RSH \quad (13)$$

and are related to K_1 and K_2 through the K_a's of the corresponding Cys residues. However, since amino acid replacements in positions 66 and 67 are highly unlikely to exert any effect on K_1' and K_2', any *differences in* K^{SSR} for the three peptides would have to be attributed to a *change in* K^{SH} and thus to a change in the intrinsic free energy differences between the native and nonnative disulfide loops.

Experimental Section

1. Peptide Synthesis. The three peptides were synthesized by classical solution procedures employing a strategy allowing for the final removal of all side-chain protecting groups under mild conditions. Thus, the ϵ-NH_2 group of Lys was protected with the acid-labile *tert*-butyloxycarbonyl (BOC) group and the hydroxyl functions of Thr and Ser were protected as *tert*-butyl (Bu^t) ethers. The *tert*-butylthio (SBu^t)[41,42] (Cys^{65} and Cys^{72}) and the acetamidomethyl (Acm)[43] group (Cys^{58}) were used for the protection of the thiol group[44] on Cys. Intermediates bearing no acid-labile side-chain protecting groups were synthesized by using the BOC group for N^{α} protection, which was removed by treatment with HCl/ethyl acetate (AcOEt) or, alternatively, anhydrous trifluoroacetic

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Table I. Results of Amino Acid Analyses of Tryptic Digestion Products for the Various Oxidized Peptide Species Obtained in Disulfide-Exchange Equilibration Experiments with [58–72]KN[a,b]

	A I 58–61	A II 62–72[c]	B I 58–61	B II 67–72	B III 62–66	C I 62–66	C II 58–61 67–72[c]	D I 67–72	D II 58–66[c]
Asx		2.76 (3)		1.81 (2)	1.05 (1)	1.00 (1)	1.87 (2)	1.83 (2)	1.00 (1)
Glx	0.95 (1)	1.10 (1)	1.05 (1)	1.03 (1)			2.08 (2)	1.08 (1)	1.06 (1)
Ser	1.06 (1)		1.06 (1)				1.04 (1)		1.03 (1)
Gly		1.09 (1)		0.98 (1)			1.09 (1)	1.04 (1)	
Thr		1.00 (1)		1.08 (1)			0.98 (1)	1.04 (1)	
Ala		0.94 (1)			0.96 (1)	1.03 (1)			0.95 (1)
Val		1.09 (1)			1.02 (1)	1.03 (1)			0.98 (1)
Cys[d]	0.45 (1)	1.38 (2)	0.35 (1)	0.36 (1)	0.37 (1)	0.38 (1)	1.46 (2)	0.47 (1)	1.69 (2)
Lys	0.99 (1)	0.98 (1)	0.99 (1)		0.97 (1)	0.95 (1)	0.95 (1)		1.98 (2)

[a] Capital letters refer to the peak labeling in Figure 3; roman numerals indicate the order of elution of the various tryptic peptides from the RP-HPLC column. [b] Arabic numerals indicate those parts of the amino acid sequence of [58–72]KN to which the various tryptic digestion products correspond. Theoretical values are given in parentheses. [c] These fragments contain two different particles that are held together by a disulfide bond. [d] Cys values are not corrected for destruction during hydrolysis.

acid (TFA). The base-labile N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)[45] or the N^{α}-benzyloxycarbonyl (Z) group [removed with 15% dimethylamine (DMA)/DMF or by catalytic hydrogenation, respectively] was employed in all other cases, depending on the presence or absence of Cys in the respective intermediates, since the removal of the Z group by catalytic hydrogenation in the presence of Cys can be accomplished only under special conditions.[46]

The general synthetic scheme is based on the synthesis of four protected fragments encompassing residues 58–61, 62–64, 65–68, and 69–72, respectively, which were subsequently assembled in a stepwise fashion. The shorter fragments were generally synthesized by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) procedure[47] or the *N*-hydroxysuccinimide esters (OSu)[48] of the suitably protected amino acids in cases where the C-terminus of the corresponding fragment was left unprotected. The *N*-methylamide of BOC-Cys(SBu^t)-OH was prepared by the mixed anhydride (MA) method,[49] and acetylation of the N-terminal tetrapeptide methyl ester was accomplished by using *N*-acetoxysuccinimide (AcOSu). Activation with diisopropylcarbodiimide (DIC)[50]/HOBt was used for the condensation of fragments (Figure 2).

Removal of the Lys, Thr, and Ser side-chain protecting groups from the fully assembled pentadecapeptides was achieved by treatment with anhydrous TFA. Subsequent cleavage of the SBu^t groups from the corresponding Cys residues with dithiothreitol (DTT) proceeded without any problems. At this stage, the peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC). Removal of the Acm group(s) was accomplished by treatment with mercuric acetate [$Hg(OAc)_2$] in aqueous acetic acid and subsequent treatment with a large excess of DTT followed by purification by preparative RP-HPLC. The purity of the final products was established by analytical RP-HPLC, amino acid analysis, and FAB-MS. [58-72]KN: Amino acid analysis (AAA) (not corrected for losses during hydrolysis) Asx 2.95 (3), Glx 1.99 (2), Ser 0.90 (1), Gly 1.00 (1), Thr 1.00 (1), Ala 1.01 (1), Val 1.07 (1), Cys 2.19 (3), Lys 2.10 (2); FAB-MS, 1653.5 (MH^+) (MH^+ calcd for $C_{63}H_{109}N_{23}O_{23}S_3$ 1653.9). [58–72]QN: AAA, Asx 3.03 (3), Glx 3.08 (3), Ser 0.98 (1), Gly 1.08 (1), Thr 0.91 (1), Ala 1.01 (1), Val 1.05 (1), Cys 2.41 (3), Lys 0.93 (1); FAB-MS, 1652.7 (MH^+) (MH^+ calcd for $C_{62}H_{105}N_{23}O_{24}S_3$ 1653.9). [58–72]KA: AAA, Asx 1.95 (2), Glx 1.98 (2), Ser 0.91 (1), Gly 1.02 (1), Thr 1.01 (1), Ala 1.96 (2), Val 1.06 (1), Cys 1.95 (3), Lys 2.11 (2); FAB-MS, 1609.3 (MH^+) (MH^+ calcd for $C_{62}H_{108}N_{22}O_{22}S_3$ 1610.8). In every case, the material used for the equilibrium experiments was rechromatographed on a semipreparative RP-HPLC column immediately before use. The optical purity of the final products was established by enzymatic procedures.[51] In particular, the absence of D-Ala at position 64 and D-Lys at position 61 (non-Gly fragment condensation sites) could be demonstrated explicitly by treatment with Leu-aminopeptidase (at the level of the 11-residue intermediates) and trypsin, respectively.

2. Reverse-Phase High-Performance Liquid Chromatography. (RP-HPLC). Analytical RP-HPLC was generally carried out on a Spectra Physics SP 8000 liquid chromatograph coupled to a Model 770 spectrophotometric detector (except for the *quantitative* analyses of the disulfide-exchange equilibration mixtures). Various Waters Nova Pak C_{18} columns (0.5 × 10 cm or 0.8 × 10 cm) were used in combination with a Waters RCM 100 radial compression module. Preparative-scale RP-HPLC was carried out on a Rainin Dynamax Macro C_{18} column on the same instrument. Details of the chromatographic conditions for the analysis and purification of synthetic intermediates and the final products are given in the supplementary material.

Quantitative analysis of the disulfide-exchange equilibration mixtures was carried out on a Spectra Physics SP 8800 liquid chromatograph using a custom-packed 0.8 × 10 cm Nova Pak C_{18} cartridge (Batch 1040 packing material). Peaks were visualized with the disulfide detection system (DDS) described by Thannhauser et al.[52] employing the modification introduced by Milburn et al.,[53] which results in the destruction of the entire sample in the post-column derivatization process. Elution was achieved using linear gradients of 0.09% TFA/90% aqueous CH_3CN (B) in 0.09% TFA/H_2O (A). The gradients were as follows (% A + % B = 100%): 7% B isocratic for 15 min, 7% B to 19% B in 90 min (i.e., 19% B at t = 105 min) for [58–72]KN and [58–72]QN; 8% B isocratic for 15 min, 8% B to 20% B in 90 min for [58–72]KA. The flow rate was 0.3 mL/min in all cases.

Separations of disulfide-exchange equilibration mixtures *without* destruction of the sample (UV detection at 210 nm) were carried out on the same column using a linear gradient from 5% B to 20% B in 60 min at a flow rate of 0.8 mL/min.

A gradient from 0% B to 20% B in 60 min at a flow rate of 0.8 mL/min was employed in the analysis of the tryptic digestion mixtures.

3. Disulfide-Exchange Equilibrations. The concentrations of compounds containing free thiol groups were determined with Ellman's reagent;[54] concentrations of disulfide-containing compounds were established by the method of Thannhauser et al.[55] Disulfide-exchange equilibration experiments (cf. also ref 30) were carried out under Ar at pH 8.0 (100 mM Tris-HCl, 1 mM EDTA buffer) at 23 ± 0.2 °C for 3.5 h. The solution (1 mL) was 4.2–7.2 μM in peptide and the initial concentration of cystamine hydrochloride, $[cystamine]_0$, was 4–11 mM; the ratio of $[cystamine]_0/[free\ SH]_{total}$ was adjusted to between 50 and 200 by addition of cysteamine. A 120–200-fold excess of AEMTS·HBr was used to quench the exchange reaction and the entire mixture (1.3 mL) was analyzed by RP-HPLC using the DDS. Oxidized peptides that were used as alternative starting points in the quantitative disulfide-exchange equilibration experiments were recovered from separations of equilibration mixtures without sample destruction. The experimental procedures for the identification of the various oxidized peptide species by means of their tryptic digestion patterns were essentially as described in ref 30.

Results

Figure 3 shows a typical chromatogram obtained after incubation of [58–72]KN with an excess of cystamine at pH 8.0 and 23 °C for 3.5 h and subsequent quenching with AEMTS. As can

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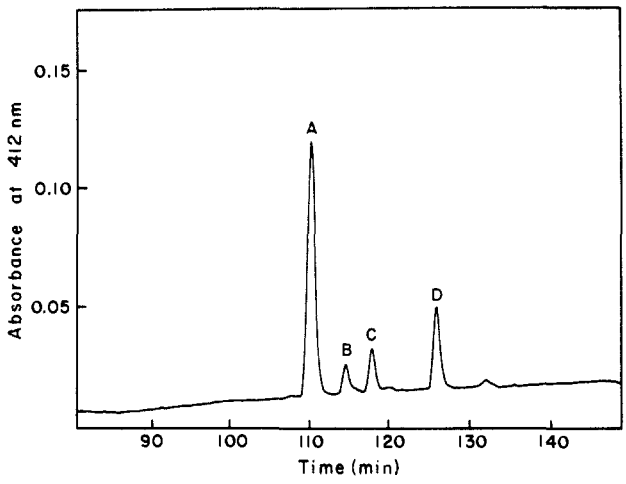

Figure 3. RP-HPLC analysis of disulfide-exchange equilibration experiments with [58–72]KN at 23 °C and pH 8.0 after quenching with AEMTS. The chromatogram was obtained with the disulfide detection system (DDS).[52,53] A, oxidized peptide species incorporating the native sulfide loop (L_n^{SSR}); B, tris-AET derivative; C, oxidized peptide species incorporating a disulfide bond between Cys^{58} and Cys^{72}; D, oxidized peptide species incorporating the nonnative eight-residue disulfide loop (L_{nn}^{SSR}).

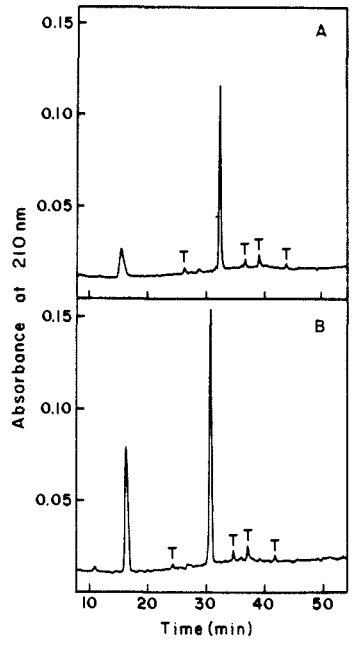

Figure 4. RP-HPLC traces of tryptic digests of A, peak A of Figure 3, and B, peak D of Figure 3. Peaks labeled with T were also present in the trypsin control.

be seen, four major (A–D) and a few minor products were obtained, one of which (B) was readily identified as tris-(2-aminoethylthio)-[58–72]KN (tris-AET[58–72]KN) on the basis of chromatographic comparison with an authentic sample (prepared by reacting fully reduced [58–72]KN with excess AEMTS). Assignment of the other major peaks was made on the basis of their tryptic digestion patterns, which are distinctly different for the three possible monomeric cyclic disulfides.

The RP-HPLC chromatograms of the tryptic digests of peaks A and D (Figure 3) are depicted in Figure 4, parts A and B, respectively; the tryptic peptides were identified by amino acid analysis, and the results are summarized in Table I together with those obtained for tryptic peptides derived from peaks B and C (Figure 3).

Since tryptic cleavage at Lys sites that are located within one of the eight-residue disulfide loops does not result in the formation of two separate peptides (because the tryptic fragments are still covalently connected by the disulfide bond), tryptic digestion of the oxidized peptide species embodying the native disulfide loop (Cys^{65}-SS-Cys^{72}) provides only two peptides comprising residues 58–61 and 62–72, respectively. Analogously, the oxidized peptide species incorporating the nonnative disulfide loop (Cys^{58}-SS-Cys^{65}) gives rise to two peptides comprising residues 58–66 and 67–72, respectively. Therefore, peak A (Figure 3) could be readily identified as the native disulfide isomer, whereas peak D corresponds to the species with a disulfide bond between Cys^{58} and Cys^{65}. Similarly B and C were identified as tris-AET[58–72]KN and the isomer containing a disulfide bond between Cys^{58} and Cys^{72}, which in the case of B confirmed the initial assignment.

Table II. Experimentally Observed Equilibrium Constants for Disulfide-Exchange Reactions at 23 °C and pH 8.0

starting peptide[a]	K_{exp}[b]
[58–72]KN; 3 SH	3.61 ± 0.07[c]
[58–72]KN; L_n^{SSR}	3.53 ± 0.13
[58–72]QN; 3 SSR	2.97 ± 0.01
[58–72]QN; L_n^{SSR}	3.05 ± 0.16
[58–72]KA; 3 SH	4.46 ± 0.13
[58–72]KA; L_n^{SSR}	4.52 ± 0.18

[a] 3 SH, fully reduced peptide; L_n^{SSR}, peptide incorporating the native eight-residue disulfide loop and the third thiol present as mixed disulfide with cysteamine; 3 SSR, tris-AET derivative. [b] Average value and standard deviation for three independent experiments. [c] Average value and standard deviation for four independent experiments.

The chromatograms of the quenched disulfide-exchange equilibration mixtures of [58–72]QN and [58–72]KA were qualitatively very similar to the one shown in Figure 3; especially, the order of elution of the various oxidized species was the same in all three cases. It should be noted that the distinction between the three intramolecular disulfide isomers was less straightforward in the case of [58–72]QN than for [58–72]KN and [58–72]KA. Replacement of Lys^{66} removes one of the tryptic cleavage sites and, although the native disulfide isomer is easily recognized because of the formation of the tetrapeptide 58–61, cleavage at Lys^{61} does not result in any fragmentation for either of the two other isomers (Cys^{58}-SS-Cys^{65} or Cys^{58}-SS-Cys^{72}). However, under more stringent conditions (higher trypsin concentration, longer reaction times) partial cleavage also occurs at Cys(AET) sites, which allowed unambiguous assignment of the two nonnative disulfide isomers.

The results of the disulfide-exchange equilibration experiments are summarized in Table II. K_{exp} refers to the ratio of peak areas (obtained with the DDS) of the native (Cys^{65}-SS-Cys^{72}) and nonnative (Cys^{58}-SS-Cys^{65}) disulfide isomers *after* quenching of the reaction with AEMTS, i.e., $K_{exp} = [L_n^{SSR}]_b/[L_{nn}^{SSR}]_b$ (eq 4).

It should be emphasized that, in all cases, the same values for K_{exp} were obtained (within the experimental error) when the equilibrium was approached from different sides. This proves that K_{exp} indeed reflects the different thermodynamic stabilities of the native and nonnative eight-residue disulfide species and is not due to a high kinetic barrier for the conversion of the native to the nonnative isomer.

The same values for K_{exp} (within the experimental error) were found when disulfide exchange was quenched by directly lowering the pH to 2, without blocking the free SH groups,[53] which is a reasonable finding in view of the large excess of cystamine applied in the equilibration experiments (cf. section "Rationale of the Procedure") (data not shown). This confirms that the data presented in Table II are not dependent on the use of AEMTS as the blocking reagent.

Discussion

The data presented in Table II show that there is a clear preference for the formation of the native (Cys^{65}-SS-Cys^{72}) over the nonnative (Cys^{58}-SS-Cys^{65}) eight-residue disulfide loop in all cases, and only small differences (in terms of standard free energy) are observed between the three homologous peptides. The value of K_{exp} obtained for the wild-type peptide is 3.58 ± 0.10 (ΔG° = −0.75 ± 0.02) (average value and standard deviation for seven independent experiments) and is thus somewhat lower than in the RNase M-peptide (5.9 ± 0.8).[30] Since K_{exp} corresponds closely

to K^{SSR} as defined in eq 1, this difference does not necessarily reflect a change in the "conformational" equilibrium constant K^{SH} (eq 2), but could be accounted for simply by a different ratio of K_1'/K_2' (eq 11) in [58–72]KN and the M-peptide, respectively. Although data obtained for the M-peptide[30] suggest that K_1'/K_2' may not be too different from unity, any interpretation of the difference in K_{exp} between [58–72]KN and the M-peptide in terms of differences in local interactions would be highly speculative, since K_1'/K_2' has not been determined for either peptide. We shall, therefore, focus on the comparison of [58–72]KN with the homologous peptides [58–72]QN and [58–72]KA, respectively, where the differences in K_{exp} clearly reflect the differences in the equilibrium constants K^{SH} among the three peptides (cf. section "Rationale of the Procedure").

Despite the universal conservation of Lys and Asn in positions 66 and 67, respectively, of the amino acid sequences of mammalian pancreatic ribonucleases,[34,35] amino acid substitutions at these positions do not result in any dramatic change in the standard free energy difference between the native and the nonnative eight-residue disulfide isomers as compared to the wild-type peptide. Although [58–72]QN exhibits a slight decrease in K_{exp} [$K_{exp} = 3.02 \pm 0.17$; $\Delta G° = -0.65 \pm 0.03$ kcal/mol (average and standard deviation of six independent experiments)], the result clearly indicates that the positively charged Lys side chain is not of primary importance for the preferential formation of the native disulfide loop, at least not under the conditions of ionic strength used in our experiments (which are those used by Konishi et al.[56] in a whole series of regeneration experiments with disulfide-reduced RNase A). It has been pointed out that Lys[66] is somewhat close to the active site of the enzyme and may therefore be conserved for catalytic reasons,[34,57] although it is not usually invoked in the catalytic mechanism of RNA cleavage.

Even more surprising, substitution of Ala for Asn[67] leads to a 30% increase in K_{exp} [$K_{exp} = 4.49 \pm 0.23$; $\Delta G° = -0.88 \pm 0.03$ kcal/mol (average and standard deviation of six independent experiments)], instead of a marked shift in favor of the nonnative eight-residue disulfide isomer, as might have been expected for reasons discussed above. There are obviously two alternative initial conclusions that may be drawn from this finding: (i) Residues 65–68 (in [58–72]KN) are not present in a β-turn conformation to any significant extent to account for the free energy difference between the two eight-residue disulfide isomers. This also raises the question whether the protein conformation in this highly solvent-exposed region in solution is actually identical with the one in the crystal structure. However, recent NMR studies of RNase A in solution[58] confirm the presence of a β-turn at Cys[65]-Lys[66]-Asn[67]-Gly[68]. (ii) The β-turn exists, but is not disturbed when Asn[67] is replaced by Ala. This possibility seems quite unlikely, even given the limited reliability of Chou–Fasman-type conformational prediction parameters.[59] The predicted decrease in the β-turn potential is so significant that a destabilizing effect on the native eight-residue disulfide loop should be observed, if the turn contributed markedly to its thermodynamic stability.

It should be noted, however, that, according to statistical analyses of protein crystal structures, the probability of occurrence of Ala in the second position (position $i + 1$) of a β-turn is approximately twice as high as for occurrence in the third position ($i + 2$);[37,38,60] this is in agreement with conformational energy calculations on X-Ala and Ala-X dipeptide units[61] that consistently show a higher β-turn probability for Ala-X than for X-Ala sequences in positions $i + 1$ and $i + 2$. In addition, the latter calculations[61] predict a high β-turn potential for the Ala-Gly dipeptide, Gly also being by far the most frequently occurring amino acid at the $i + 2$ position of type II β-turns.[38] It is, therefore, conceivable that substitution of Ala for Asn[67] could result in a shift of the location of the β-turn in [58–72]KA to residues Lys[66]-Ala[67]-Gly[68]-Gln[69], thus retaining a conformation where the closer proximity of Cys[65] and Cys[72] (as compared to Cys[58] and Cys[65]) still favors formation of the native disulfide isomer.

These ideas are compatible with conformational energy calculations using ECEPP.[62] These calculations indicate that the Cys[65]-SS-Cys[72] eight-residue disulfide loop with Ala in position 67 is significantly (~10 kcal/mol) more stable with a type II β-turn at residues 66–69 than in the native conformation[36] with a type III turn at positions 65–68. We want to emphasize that these ideas (including the computational result) are not meant to offer a rationale for the observed (small) difference in values of $\Delta G°$ between [58–72]KN and [58–72]KA, but rather to try to rationalize the surprising finding of the *absence* of any significant change in the equilibrium constant upon replacement of Asn[67] with Ala. Alternatively, formation of a β-turn at positions 65–68 could also be an event subsequent to the formation of the disulfide bond between Cys[65] and Cys[72], whose preferential formation could be determined by parameters that were not addressed in this study.

Conclusions

The results of the disulfide-exchange experiments on [58–72]KN clearly demonstrate that local interactions exist in this peptide that suffice to favor the formation of the native disulfide bond between Cys[65] and Cys[72] significantly over the nonnative eight-residue disulfide isomer (using a reference state with the third Cys residue forming a mixed disulfide with cysteamine). None of the single amino acid substitutions (Lys[66] → Gln; Asn[67] → Ala) investigated caused a major change in the standard free energy difference between the two isomers, although Lys[66] and Asn[67] are highly conserved in all ribonucleases from mammalian species. In the case of [58–72]KA, this may be attributed to a change in local interactions without significantly changing the stability of the native loop.

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Supplementary Material Available: Data on peptide synthesis, Tables 1–3 of data from the disulfide-exchange equilibrations, and Tables 4 and 5 of data from amino acid analyses and tryptic peptides from digestions of oxidized peptide species derived from [58–72]QN and [58–72]KA, respectively (26 pages). Ordering information is given on any current masthead page.

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