Chain Reversals in Model Peptides: Studies of Cystine-Containing Cyclic Peptides. 1. Conformational Free Energies of Cyclization of Hexapeptides of Sequence Ac-Cys-X-Pro-Gly-Y-Cys-NHMe

P. J. Milburn, Y. Konishi, Y. C. Meinwald, and H. A. Scheraga*

Contribution from the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301. *Received November 21, 1986*

Abstract: The chemical equilibrium between the oxidized (cyclic) and reduced (acyclic) forms of hexapeptides of sequence Ac-Cys-X-Pro-Gly-Y-Cys-NHMe was examined in aqueous solution at 25 °C, pH 8.0, to obtain quantitative information about their propensity to form chain reversals. The method of disulfide exchange was employed, giving highly reproducible observed apparent equilibrium constants. Appropriate macroscopic acid dissociation constants were determined and used to correct for the effects of ionization, yielding standardized equilibrium constants. The standard Gibbs free energy changes for these processes were derived and used, in comparison with an arbitrary standard process, to determine the relative facility with which a given peptide adopts chain-reversal conformations. An automated HPLC method for the quantitative analysis of the thiol/disulfide equilibrium mixtures is described. The results indicate a distinct dependence of chain-reversal formation upon both composition and sequence. Comparison is made with studies of other model peptide systems, with generally good agreement. A correlation with statistical analyses of protein structure for β-bend-forming potentials of amino acid residues was observed. Limitations and potential uses of this approach to chain-reversal formation in peptides are discussed.

Considerable effort has been made to comprehend the factors that determine the expression of chain reversals in a given peptide sequence, consequent to their recognition as a basic element of backbone structure in proteins.[1-3] Early theoretical work originated the nomenclature of chain-reversal study.[3-8] Contemporary investigations of the data available from crystal structure analyses revealed that a significant proportion of the amino acid residues in proteins were involved in chain reversals comprised of four residues, termed β-turns.[4,7-10] A chain reversal comprised of three residues, the γ-turn, was also proposed,[5] and a lone example was observed in thermolysin.[11] Venkatachalam[3] described six types of β-bend, defining a set of dihedral angles typifying each. Initially, only well-defined β-turns were considered,[9] and much emphasis was placed upon the presence of a hydrogen bond between residues $i + 3$ and i.[12] Lewis et al.,[4] in their survey of protein crystal coordinates, recognized the frequent occurrence of distorted bends and identified a bend if the distance from C_i^α to C_{i+3}^α was less than 0.7 nm and residues $i + 1$ and $i + 2$ were not in an α-helix. Kuntz[10] and Crawford et al.,[7] in similar analyses of protein structure, identified such features as "corners" and "open-turns", which also emphasized the existence of chain reversals quite dissimilar to those illustrated by Venkatachalam.[3] Lewis et al.[8] provided a comprehensive nomenclature for β-turns defining an additional four types. Out of 135 bends, which they found in 8 proteins, 129 belonged to the set of 10 types of β-turns; the remaining chain reversals were comprised of 5 residues.

The β-turn is by far the most frequent chain reversal in proteins.[13,14] The analysis of Lewis et al.[4] led them to postulate that short-range interactions dominated the adoption of a β-turn conformation by a given amino acid residue and to ponder whether β-turns occur in smaller structures. In retrospect, examples of β-turns in peptides were already apparent.[15-17] Consequently, many empirical studies were devoted to the identification and characterization of chain reversals in both naturally occurring and model peptides.[13] Contemporary theoretical studies were devoted to considering the energetics of bend formation of isolated peptides corresponding to known β-bends in proteins,[18,19] and in general models,[6,20] and to examining the conformational properties of oligopeptides of physiological significance.[13] Such studies established that short-range interactions are sufficient to allow the adoption of a conformation corresponding to a chain reversal.[21]

Subsequently, various authors turned their attention to the prediction of bend formation in a given sequence on the basis of the properties of the constituent amino acids. Lewis et al.[4] analyzed the crystal data of three proteins to derive bend probabilities as a function of amino acid sequence. This theme was also adopted

* To whom requests for reprints should be addressed.

(1) Blake, C. C. F.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sarma, V. R. *Proc. R. Soc. London, B* **1967**, *B167*, 365-377.
(2) Geddes, A. J.; Parker, K. D.; Atkins, E. D. T.; Beighton, E. *J. Mol. Biol.* **1968**, *32*, 343-358.
(3) Venkatachalam, C. M. *Biopolymers* **1968**, *6*, 1425-1436.
(4) Lewis, P. N.; Momany, F. A.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 2293-2297.
(5) Némethy, G.; Printz, M. P. *Macromolecules* **1972**, *5*, 755-758.
(6) Chandrasekaran, R.; Lakshminarayanan, A. V.; Pandya, U. V.; Ramachandran, G. N. *Biochim. Biophys. Acta* **1973**, *303*, 14-27.
(7) Crawford, J. L.; Lipscomb, W. N.; Schellman, C. G. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 538-542.
(8) Lewis, P. N.; Momany, F. A.; Scheraga, H. A. *Biochim. Biophys. Acta* **1973**, *303*, 211-229.
(9) Dickerson, R. E.; Takano, T.; Eisenberg, D.; Kallai, O. B.; Samson, L.; Cooper, A.; Margoliash, E. *J. Biol. Chem.* **1971**, *246*, 1511-1535.
(10) Kuntz, I. D. *J. Am. Chem. Soc.* **1972**, *94*, 4009-4012.
(11) Matthews, B. W. *Macromolecules* **1972**, *5*, 818-819.
(12) IUPAC-IUB commission on biochemical nomenclature: *J. Biol. Chem.* **1972**, *247*, 977-983.
(13) Smith, J. A.; Pease, L. G. *CRC Crit. Rev. Biochem.* **1980**, *8*, 315-339.
(14) (a) Richardson, J. S. *Adv. Protein Chem.* **1981**, *34*, 167-399. (b) Hruby, V. J. *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1974; Vol. 3, pp 1-188. (c) Ovchinnikov, Y. A.; Ivanov, V. T. *The Proteins*, 3rd ed.; Neurath, H., Hill, R. L., Eds.; Academic: New York, 1982; Vol. 5, pp 307-642.
(15) Shields, J. E.; McDowell, S. T. *J. Am. Chem. Soc.* **1967**, *89*, 2499-2500.
(16) (a) Ueki, T.; Ashida, T.; Kakudo, M.; Sasada, Y.; Katsube, Y. *Nature (London)* **1967**, *216*, 1205-1207. (b) Ueki, T.; Ashida, T.; Kakudo, M.; Sasada, Y.; Katsube, Y. *Acta Crystallogr., Sect. B: Struct. Sci.* **1969**, *25*, 1840-1849.
(17) (a) Urry, D. W.; Ohnishi, M.; Walter, R. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *66*, 111-116. (b) Urry, D. W.; Walter, R. *Proc. Natl. Acad. Sci., U.S.A.* **1971**, *68*, 956-958.
(18) Simon, I.; Némethy, G.; Scheraga, H. A. *Macromolecules* **1978**, *11*, 797-804.
(19) (a) Hiltner, W. A.; Walton, A. G. *J. Mol. Biol.* **1975**, *92*, 567-572. (b) Hurwitz, F. I.; Hopfinger, A. J. *Int. J. Pept. Protein Res.* **1976**, *8*, 543-550.
(20) (a) Maigret, B.; Pullman, B. *Theor. Chim. Acta* **1974**, *35*, 113-128. (b) Nishikawa, K.; Momany, F. A.; Scheraga, H. A. *Macromolecules* **1974**, *7*, 797-806.
(21) Anfinsen, C. B.; Scheraga, H. A. *Adv. Protein Chem.* **1975**, *29*, 205-300.

0002-7863/87/1509-4486$01.50/0

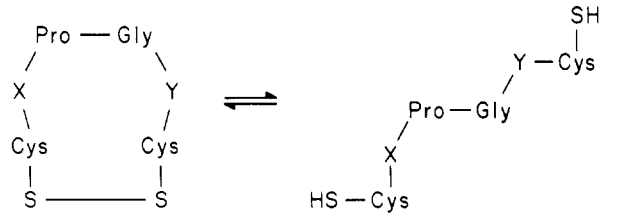

Figure 1. Representation of the equilibrium between the cyclic and acyclic forms of a hexapeptide. X is Gly, Lys, Phe, or Val; Y is Gly. In one additional peptides, X is Gly and Y is Lys. The terminal blocking groups (Ac and NHMe for the N- and C-terminal Cys residues, respectively) are not shown. Ac and NHMe are abbreviations for CH_3CO and $NHCH_3$, respectively.

by Crawford et al.,[7] Burgess et al.,[22] and Chou and Fasman[23,24] in order to predict the occurrence of β-bends in proteins. Zimmerman and Scheraga[25] carried out a statistical mechanical analysis of the conformational space of a large set of dipeptides in order to calculate the probability of their adoption of a bend conformation. Some inconsistencies were apparent between these approaches, and it was not clear how appropriate either approach might be in a given circumstance. Consequently, the need to develop experimental methods for estimating tendencies toward the formation of chain reversals was apparent.

Until the elegant work of Sato et al.,[26] experimental studies of chain reversals had primarily been devoted to elucidating the various spectral properties in microscopic detail.[13,27,28] However, the method of Sato et al.,[26] based upon exciton transfer between terminal chromophores of a tetrapeptide, allowed the bend-forming tendency to be estimated, under favorable conditions, in a variety of nonaqueous solvents. Recently, Boussard and Marraud[29] have reported a method for estimating the proportion of β-folded conformations of terminally blocked dipeptides in apolar solution, which was based upon a knowledge of the fine details of the infrared absorption spectra.

In this paper, we introduce a method for obtaining a numerical estimate of the propensity of a given tetrapeptide sequence for the formation of a chain reversal in *aqueous* solution. The method is based upon the measurement of the chemical equilibrium between cyclic and acyclic forms of [Cys[1]], [Cys[6]]-hexapeptides.

Methods

1. Disulfide-Exchange Equilibrium. In order to determine the conformational equilibrium between chain reversals and other conformations, we have examined the chemical equilibrium between the oxidized and reduced peptides of the type shown in Figure 1 in water at 25 °C, pH 8.0. All of these peptides contain the Pro-Gly sequence, which has a high propensity for forming a β-bend.[4]

The relative stabilities of chain reversals were studied with the disulfide-exchange method[30] by measuring the apparent observed equilibrium constant defined as follows:

$$\{\text{peptide-X}\}_r + \{\text{peptide-Y}\}_{ox} \overset{K'_{8.0}}{\rightleftharpoons} \{\text{peptide-X}\}_{ox} + \{\text{peptide-Y}\}_r \quad (1)$$

The subscripts ox and r denote oxidized and reduced species, respectively, where

$$K'_{8.0} = [X_{ox}][Y_r]_o/[X_r]_o[Y_{ox}] \quad (2)$$

for the equilibrium observed at pH 8.0 and $[X_r]_o$ and $[Y_r]_o$ are the *analytical* concentrations of the respective reduced peptides. It should be noted that, in solution at pH 8.0, the terminally protected bis(thiol) peptides are distributed between three macroscopic ionization states, namely, the dianion, monoanion, and uncharged species. Hence

$$[X_r]_o = [X_r^{2-}] + [X_r^-] + [X_r] \quad (3)$$

with a similar expression for $[Y_r]_o$. The equilibrium concentrations of the peptides were obtained by means of a reversed-phase high-performance liquid chromatography (RP-HPLC) method (see below). Following specific resolution, the molar quantities were deduced from a digital integration of the output from an automated, post-column-derivitization analyzer,[31] specific for the disulfide and thiol functions. The sulfite/2-nitro-5-thiosulfobenzoate (NTSB) assay described by Thannhauser et al.[32] was employed in the quantitative analysis of the sulfur-containing groups.

The half reactions for the equilibrium may be written as

$$\{\text{peptide-X}\}_r \rightleftharpoons \{\text{peptide-X}\}_{ox} + 2e^- + 2H^+ \quad (4a)$$

and

$$\{\text{peptide-Y}\}_{ox} + 2e^- + 2H^+ \rightleftharpoons \{\text{peptide-Y}\}_r \quad (4b)$$

Hence, we may define a formal (pH independent; see part 3 of this section) equilibrium constant K' as

$$K' = \{[X_{ox}]/[X_r]\}\{[Y_r]/[Y_{ox}]\} \quad (5)$$

Now

$$[X_r]_o = [X_r]\{1 + K_{X1}/(H^+) + K_{X1}K_{X2}/(H^+)^2\} \quad (6)$$

where $[X_r]_o$ is the sum of the concentrations of the reduced {peptide-X} in all of its states of ionization (eq 3) and

$$K_{X1} = (H^+)[X_r^-]/[X_r]$$
$$K_{X2} = (H^+)[X_r^{2-}]/[X_r^-] \quad (7)$$

where K_{X1} and K_{X2} are the macroscopic acid dissociation constants of $\{\text{peptide-X}\}_r$. Similarly

$$[Y_r]_o = [Y_r]\{1 + K_{Y1}/(H^+) + K_{X1}K_{X2}/(H^+)^2\} \quad (8)$$

Consequently, the formal equilibrium constant, K', is related to the observed equilibrium constant, $K'_{8.0}$, as follows:

$$K' = K'_{8.0}\left[\frac{\{1 + K_{X1}/(H^+) + K_{X1}K_{X2}/(H^+)^2\}}{\{1 + K_{Y1}/(H^+) + K_{Y1}K_{Y2}/(H^+)^2\}}\right] \quad (9)$$

Hence, the pH-independent equilibrium constant K' is generated from the pH-dependent equilibrium constant $K'_{8.0}$ by using a correction factor which is a function of pH and the several macroscopic acid dissociation constants of the solutes. Thus, K' describes the equilibrium in a standard state where the reduced peptides are chemically homogeneous (in the bis(thiol) form), and hence, in the low pH limit the observed equilibrium constant would

(22) Burgess, A. W.; Ponnuswamy, P. K.; Scheraga, H. A. *Isr. J. Chem.* **1974**, *12*, 239–286.
(23) Chou, P. Y.; Fasman, G. D. *Biochemistry* **1974**, *13*, 222–245.
(24) Chou, P. Y.; Fasman, G. D. *J. Mol. Biol.* **1977**, *115*, 135–175.
(25) (a) Zimmerman, S. S.; Shipman, L. L.; Scheraga, H. A. *J. Phys. Chem.* **1977**, *81*, 614–622. (b) Zimmerman, S. S.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4126–4129. (c) Zimmerman, S. S.; Scheraga, H. A. *Biopolymers* **1977**, *16*, 811–843; **1978**, *17*, 1849–1869, 1871–1884, 1885–1890.
(26) Sato, K.; Kawai, M.; Nagai, U. *Biopolymers* **1981**, *20*, 1921–1927.
(27) (a) Deslauriers, R.; Leach, S. J.; Maxfield, F. R.; Minasian, E.; McQuie, J. R.; Meinwald, Y. C.; Némethy, G.; Pottle, M. S.; Rae, I. D.; Scheraga, H. A.; Stimson, E. R.; Van Nispen, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2512–2514. (b) Deslauriers, R.; Evans, D. J.; Leach, S. J.; Meinwald, Y. C.; Minasian, E.; Némethy, G.; Rae, I. D.; Scheraga, H. A.; Somorjai, R. L.; Stimson, E. R.; Van Nispen, J. W.; Woody, R. W. *Macromolecules* **1981**, *14*, 985–996. (c) Maxfield, F. R.; Bandekar, J.; Krimm, S.; Evans, D. J.; Leach, S. J.; Némethy, G.; Scheraga, H. A. *Macromolecules* **1981**, *14*, 997–1003. (d) Bandekar, J.; Evans, D. J.; Krimm, S.; Leach, S. J.; Lee, S.; McQuie, J. R.; Minasian, E.; Némethy, G.; Pottle, M. S.; Scheraga, H. A.; Stimson, E. R.; Woody, R. W. *Int. J. Pept. Protein Res.* **1982**, *19*, 187–205.
(28) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1–109.
(29) Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1985**, *107*, 1825–1828.
(30) (a) Eldjarn, L.; Pihl, A. *J. Am. Chem. Soc.* **1957**, *79*, 4589–4593. (b) Jocelyn, P. C. *Eur. J. Biochem.* **1967**, *2*, 327–331.
(31) Thannhauser, T. W.; McWherter, C. A.; Scheraga, H. A. *Anal. Biochem.* **1985**, *149*, 322–330.
(32) Thannhauser, T. W.; Konishi, Y.; Scheraga, H. A. *Anal. Biochem.* **1984**, *138*, 181–188.

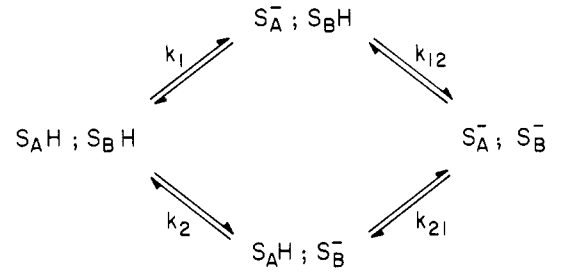

Figure 2. Representation of ionization equilibria of the reduced form of a peptide containing two ionizable groups, S_AH and S_BH.

be sensibly equal to the formal (pH-independent) equilibrium constant. In practice, chemical stability and reactivity dictate that the experiments be performed at pH 8.0, corresponding to conditions appropriate for the base-catalyzed oxidative refolding of reduced and denatured proteins.[33]

In the case of the lysine-containing molecules, which are tribasic acids, one obtains the following relationship:

$$K' = K'_{8.0} \times \left[\frac{\{1 + K_{X1}/(H^+) + K_{X1}K_{X2}/(H^+)^2 + K_{X1}K_{X2}K_{X3}/(H^+)^3\}}{\{[1 + K_{X_{ox}}][1 + K_{Y1}/(H^+) + K_{Y1}K_{Y2}/(H^+)^2]\}} \right] \quad (10)$$

where $K_{X_{ox}}$ is the acid dissociation constant of the lysine-containing $\{\text{peptide-X}\}_{ox}$ and K_{X1}, K_{X2}, and K_{X3} are the three macroscopic acid dissociation constants of $\{\text{peptide-X}\}_r$.

Given the pertinent macroscopic acid dissociation constants, we may compute a formal equilibrium constant, K', for the oxidation–reduction process and, hence, a relative standard free energy of cyclization, $(\Delta G°)'$.

It should be emphasized that the formal equilibrium constant, K', as defined above is an apparent equilibrium constant in the sense that we have omitted the activity coefficients from its definition. The true equilibrium constant, K, is related to the apparent equilibrium constant, K', as follows:

$$K' = K\left[\frac{f_{X_{ox}} f_{Y_r}}{f_{X_r} f_{Y_{ox}}}\right] \quad (11)$$

where f is the activity coefficient of the peptide species in eq 5, denoted by the subscript. While it has been shown recently that the activity coefficients of terminally blocked dipeptides in water are sensitive to composition, sequence, and stereochemistry,[34] we are assuming that the activity coefficient *ratio* of oxidized and reduced species will not differ significantly from one system to the next; i.e., $(f_{ox}/f_r)_X \simeq (f_{ox}/f_r)_Y \simeq (f_{ox}/f_r)_Z$, etc., where f is the activity coefficient of the species with a given subscript. Consequently, it is desirable that the equilibrations be performed at minimal concentrations in order that the higher order terms in the activity expansion are negligible. One must recognize, however, that the values of K' obtained pertain only to the stated equilibrium conditions.

2. Determination of Dissociation Constants. In this study, we have opted to use titration monitored by ^{13}C NMR spectroscopy to determine microscopic acid dissociation constants, following the method detailed by Rabenstein,[35] and, subsequently, to compute the macroscopic constants.

The acid dissociation of a terminally blocked bis(thiol) hexapeptide (a dibasic acid) may be described in terms of its microscopic states of ionization, as shown in Figure 2. At any pH, the proportion of thiol A (S_AH) ionized is given by eq 12a.

$$P_A = \frac{k_1/k_2 + k_{21}/(H^+)}{(H^+)/k_2 + k_1/k_2 + 1 + k_{21}/(H^+)} \quad (12a)$$

Similarly, the expression for the degree of ionization of thiol B is eq 12b, where k_1, k_2, and k_{21} are the microscopic acid dissociation constants defined in Figure 2.

$$P_B = \frac{1 + k_{21}/(H^+)}{(H^+)/k_2 + k_1/k_2 + 1 + k_{21}/(H^+)} \quad (12b)$$

The degree of ionization of each thiol, P_A and P_B, was determined experimentally as a function of pH by observing the ^{13}C chemical shifts of the pertinent α- and β-carbon atoms in the cysteine side chains. For *either* the α- *or* β-carbon atom in cysteine A we may write

$$P_A = \frac{\delta_{S_A} - \delta_{S_AH}}{\delta_{S_A^-} - \delta_{S_AH}} \quad (13a)$$

where δ_{S_AH}, $\delta_{S_A^-}$, and δ_{S_A} are the ^{13}C chemical shifts for thiol A of the conjugate acid, conjugate base, and the weighted average in the partially ionized state, respectively. Similarly, for cysteine B

$$P_B = \frac{\delta_{S_B} - \delta_{S_BH}}{\delta_{S_B^-} - \delta_{S_BH}} \quad (13b)$$

Values for the microscopic dissociation constants, k_1, k_2, k_{12}, and k_{21}, are obtained numerically by fitting to the experimental data, viz., chemical shift as a function of pH.

The macroscopic acid dissociation constants, pertinent to the cyclic/acyclic equilibria under investigation, are related to the microscopic constants

$$K_1 = k_1 + k_2 \qquad K_2 = 1/(k_{12}^{-1} + k_{21}^{-1}) \quad (14)$$

with the following definitions of K_1 and K_2:

$$K_1 = \frac{(H^+)[\{\text{peptide}\}^-]}{[\{\text{peptide}\}]}$$

$$K_2 = \frac{(H^+)[\{\text{peptide}\}^{2-}]}{[\{\text{peptide}\}^-]} \quad (15)$$

The lysine-containing bis(thiol) hexapeptides are tribasic acids, and the method for calculating the three macroscopic acid dissociation constants is described fully in the Appendix.

The macroscopic dissociation constant, K_a, of lysine in the lysine-containing cyclic disulfides was obtained directly by fitting the data for chemical shifts as a function of pH, where K_a is related to the degree of ionization as follows:

$$P = \frac{K_a/(H^+)}{1 + K_a/(H^+)} \quad (16)$$

The δ and ϵ ^{13}C resonances of the lysine residue were used to monitor its titration.

3. Conformational Free Energy of Cyclization. Having obtained a formal apparent (pH-independent) equilibrium constant, K', one is able to compute the corresponding apparent standard Gibbs free energy change $(\Delta G°)'$. In the disulfide-exchange equilibrium, this parameter expresses the difference between the free energies of cyclization of the peptides sampled. Formally, one may envisage two thermodynamic components which contribute to differences in the Gibbs free energy of oxidation of the peptides, namely, chemical and conformational. The former component arises from the processes of proton loss and electronic rearrangement which are concomitant with the oxidation. Sequence-dependent differences in thiol deprotonation are annulled in the process of standardization (i.e., transforming $K'_{8.0}$ to K'). Free energy differences arising from electron rearrangement, however, are *assumed* to be negligible, viz., the nature of the α-substituent of the $(n \pm 1)$ residue does not affect the formation of the S–S bond. While this does not seem unreasonable *a priori*, it should be noted

(33) Thannhauser, T. W.; Scheraga, H. A. *Biochemistry* **1985**, *24*, 7681–7688.

(34) (a) Blackburn, G. M.; Lilley, T. H.; Milburn, P. J. *J. Chem. Soc., Chem. Commun.* **1985**, 229–300. (b) Kent, H. E. Ph.D. Thesis, University of Sheffield, 1983. (c) Milburn, P. J. Ph.D. Thesis, University of Sheffield, 1984.

(35) Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797–2803.

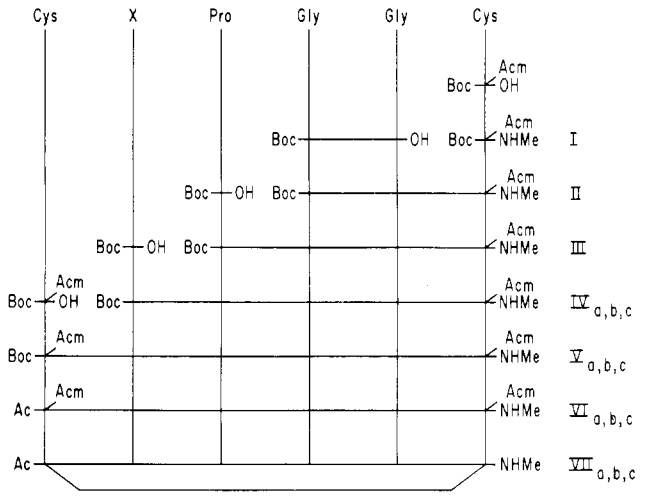

Figure 3. Schematic representation of the synthesis of the cyclic CFPGGC, CVPGGC, and CGPGGC disulfide-containing hexapeptides. For a, b, and c, X ≡ Phe, Val, and Gly, respectively.

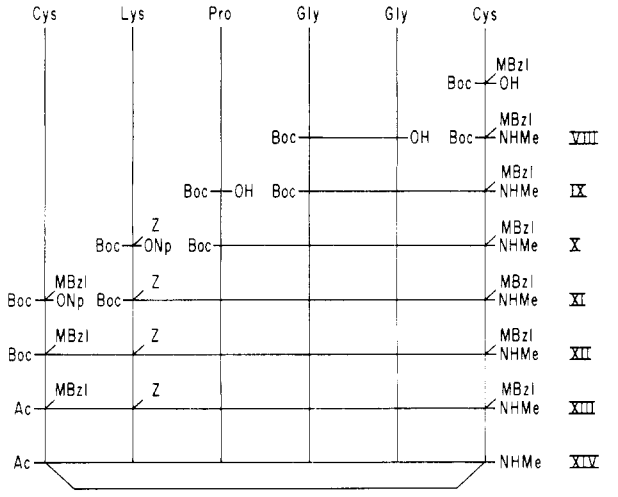

Figure 4. Schematic representation of the synthesis of the cyclic CKPGGC disulfide-containing hexapeptide.

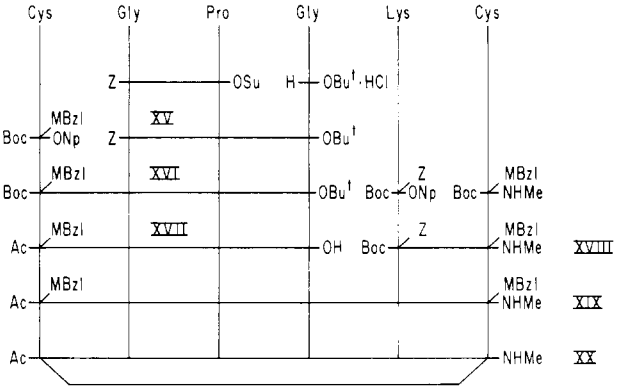

Figure 5. Schematic representation of the synthesis of the cyclic CGPGKC disulfide-containing hexapeptide.

that electronic energies are generally very much larger than conformational energies, and consequently, even slight inequalities may contribute significantly to the observed free energy differences. The conformational component arises from the formation of conformational states which are allowed in the cyclic peptide from conformations of the acyclic peptide which could not be incorporated into the 20-membered ring. By assuming that the difference in the standard free energy of cyclization $(\Delta G°)'$ is substantially due to conformational processes, we may parametrize a conformational property of the peptides. We shall term this parameter the conformational free energy of cyclization and identify it with the propensity to form chain reversals. For the purpose of comparison, one must arbitrarily assign a reference process. In this study, we have chosen to define the cyclization of the peptide Ac-Cys-Val-Pro-Gly-Gly-Cys-NHMe as our standard process. We have, thus, obtained the relative free energy of cyclization for a series of peptides. This expresses the ease of formation of a chain reversal in a Cys-X-Pro-Gly-Y-Cys sequence relative to that in a Cys-Val-Pro-Gly-Gly-Cys sequence.

Experimental Section

1. Peptide Synthesis. The three cyclic hexapeptides (CFPGGC, VII_a; CVPGGC, VII_b; and CGPGGC, VII_c) were synthesized according to the scheme in Figure 3 (it should be noted that this nomenclature refers to the terminally blocked peptide, with Ac and NHMe at the N- and C-terminus, respectively). The *tert*-butyloxycarbonyl (Boc) group was employed for α-amino protection and the acetamidomethyl (Acm) function[36] for thiol protection. At first, the C-terminal *N*-methylamide function was introduced by the mixed anhydride method,[37] with subsequent couplings executed with dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBT).[38] Prior to coupling, removal of Boc was performed by treatment with HCl-saturated ethyl acetate except for the compounds III, V_a, V_b, and V_c, which were insoluble in this solvent. In these cases, anhydrous trifluoroacetic acid (TFA) was used instead, and the resulting TFA salts were exchanged to HCl salts prior to the DCC/HOBT coupling. Acetylation of V_c to yield VI_c proceeded satisfactorily using *p*-nitrophenyl acetate (AcONp), i.e., when residue 2 was Gly, but the reactions were sluggish when residue 2 was Phe (V_a) or Val (V_b). In these cases, acetylation was achieved with acetic anhydride.

All of the *S*-acetamidomethyl peptide derivatives shown in Figure 3 were more soluble in water than in organic solvents. Consequently, they were isolated from reaction mixtures by passage of an aqueous solution through a mixed-bed ion-exchange resin and recrystallized from appropriate organic solvents in good yields. The identity of all of the intermediates was confirmed by ^{1}H NMR, and their purity was determined by TLC and melting point determinations.

The thiol groups in the linear hexapeptides VI_a, VI_b, and VI_c were deprotected and oxidized in one step by using iodine in methanol.[39] The cyclic peptides were isolated by chromatography on a Dowex I (acetate-form) column followed by preparative RP-HPLC on a C_{18} column with gradient elution in 0.09% aqueous TFA/acetonitrile. The yields of the monomeric species from oxidative cyclization varied from 30% to 54%.

An alternative cyclization procedure was investigated in the synthesis of CFPGGC (VII_a). The thiol functions were liberated by treatment with mercuric acetate in aqueous acetic acid,[36] and subsequently, oxidation was performed in dilute aqueous solution at pH 8.0 by perfusion with oxygen. In this case, the yield of the desired peptide was 20%, and consequently, the iodine oxidation (vide supra) was deemed superior.

The peptide CKPGGC (XIV) was prepared as outlined in Figure 4. As in the previous scheme, the Boc group was the α-amino protecting function. The benzyloxycarbonyl function (Z) was used for ϵ-amino protection of lysine and *p*-methoxybenzyl (MBzl) groups[40] for thiol protection. The C-terminal *N*-methylamide was generated by the mixed anhydride method. Peptide couplings proceeded without problems and were, successively, two DCC/HOBT condensations followed by two *p*-nitrophenyl (ONp) active ester[41] couplings. Treatment with HCl-saturated ethyl acetate afforded N-terminal deprotection. Acetylation was performed under mild conditions by using AcONp. The linear hexapeptide, XIII, was deprotected by solvolysis in anhydrous methanesulfonic acid (MSA) and oxidatively cyclized in water at pH 8.0 with oxygen. The cyclic hexapeptide (XIV) was purified by elution from Dowex I (acetate form) and subsequent preparative RP-HPLC fractionation. The yield of the cyclization step was 36%.

(36) Veber, D. F.; Milkowski, J. D.; Varga, S. L.; Denkewalter, R. G.; Hirschmann, R. *J. Am. Chem. Soc.* **1972**, *94*, 5456–5461.
(37) (a) Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1967**, *89*, 5012–5017. (b) Meienhofer, J. *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1979; Vol. 1, pp 263–314.
(38) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788–798.
(39) (a) Kamber, B. *Helv. Chim. Acta* **1971**, *54*, 927–930. (b) Marbach, P.; Rudinger, J. *Helv. Chim. Acta* **1974**, *57*, 403–414.
(40) (a) Akabori, S.; Sakakibara, S.; Shimonishi, Y.; Nobuhara, Y. *Bull. Chem. Soc. Jpn.* **1964**, *37*, 433–434. (b) Fujii, N.; Yajima, H. *J. Chem. Soc., Perkin Trans. 1* **1981**, 789–796.
(41) (a) Bodanszky, M.; du Vigneaud, V. *J. Am. Chem. Soc.* **1959**, *81*, 5688–5691. (b) Bodanszky, M. *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1979; Vol. 1, pp 105–196.

Table I. Analytical Data for Cystine-Containing Cyclic Hexapeptides

peptide	amino acid analysis[a]	FABMS $(M + H)^+$ $(m/z)^b$
CFPGGC (VII_a)	Gly, 2.00; Cys, 1.97; Phe, 1.06; Pro, 1.03	636 (636)[c]
CVPGGC (VII_b)	Gly, 2.00; Cys, 1.67; Val, 1.04; Pro, 1.05	589 (589)
CGPGGC (VII_c)	Gly, 3.00; Cys, 2.10; Pro, 1.10	546 (546)
CKPGGC (XIV)	Gly, 2.00; Cys, 1.91; Lys, 1.08; Pro, 1.01	617 (617)
CGPGKC (XX)	Gly, 2.00; Cys, 1.93; Phe, 1.10; Pro, 1.00	617 (617)

[a] Normalized to glycine. [b] Fast-atom bombardment mass spectrometric ratio of mass to charge for the protonated molecualr ion $(M + H)^+$. [c] The theoretical mass to charge ratio of the protonated molecular ion is given in parentheses.

The peptide CGPGKC (XX) was prepared as portrayed in Figure 5. The N-terminal tetrapeptide, XVII, and the C-terminal dipeptide, XVIII, were obtained by using either the *N*-hydroxysuccinimido[42] or *p*-nitrophenyl ester condensations. The two fragments were coupled with DCC/HOBT to yield the linear hexapeptide, XIX. This molecule proved to be insoluble in most solvents and hence was not separated from the dicyclohexylurea (DCU) byproduct. Removal of the DCU was accomplished after deprotection, oxidation, and purification as described for XIV.

The identity and purity of the cyclic disulfide-containing hexapeptides were established by analytical RP-HPLC, TLC, amino acid analysis, ^1H NMR, and FAB mass spectrometry. The analytical data are summarized in Table I. The detailed experimental procedures and the properties of the intermediates are available as supplementary material.

The bis(thiol) CGPGGC, CFPGGC, and CVPGGC acyclic hexapeptides were prepared from the bis(acetamidomethyl) derivatives by treatment with mercuric acetate.[36] The deprotection of the CFPGGC peptide was performed in aqueous acetic acid of sufficient concentration to allow dissolution of the peptide. The product mixture, after precipitation of mercury as HgS and subsequent filtration, was diluted and lyophilized. The lyophilizate was dissolved into an aqueous mixture of 0.09% TFA and 2.0% acetonitrile, and the product was fractionated by preparative RP-HPLC. A yield of 90% was obtained. A similar procedure was followed with CVPGGC; although deprotection was complete prior to lyophilization, as monitored by Ellman's titration,[43] a mixture of approximately 1:2:1 bis(thiol), mono(acetamidomethyl), and bis(acetamidomethyl) derivatives was obtained. This procedure yielded similar results for CGPGGC. The recondensation of thiol and acetamidomethanol under the conditions of lyophilization was circumvented by performing the deprotection in 0.09% TFA and, following removal of mercury, directly fractionating the reaction mixture by preparative RP-HPLC. Yields were of the order of 70% for both peptides.

The bis(thiol) forms of the two lysine-containing peptides were prepared in an essentially quantitative fashion by reduction of the cyclic disulfides in an aqueous solution of tris(hydroxymethyl)methylamine (Tris) pH 8.0, 0.1 M with DL-dithiothreitol (DTT). Purification was achieved by preparative RP-HPLC. The overall yields with respect to the bis[(*S*)-(*p*-methoxybenzyl)] peptides were better by this route than those obtained in attempts to purify the bis(thiol) peptides directly after deprotection with MSA. It appeared that the bis(thiol) ϵ-amino lysine-containing peptides were not stable under the conditions of purification.

The identity and purity of the bis(thiol) hexapeptides were established by ^1H and ^{13}C NMR and analytical RP-HPLC and by their chemical relationship to the corresponding cyclic disulfide species.

The stereochemical purity of the products was not examined explicitly since there was no method available to us which might not itself cause racemization, particularly at the cysteine residues. We have, however, observed relatively small quantities of material which have highly similar elution properties to the desired products in our RP-HPLC procedure. These materials were separated at the preparative RP-HPLC stage, and we consider it likely that at least some of these eliminated molecules are other diastereoisomeric forms of the product.

2. Reversed-Phase HPLC. Analytical RP-HPLC was performed using an IBM LC/9533 ternary gradient liquid chromatograph. Detection was achieved by an Isco Model 1840 absorbance detector or by the disulfide/thiol detection system (DTDS).[31]

Table II. Linear Composition Gradients of RP-HPLC Eluant Used in the Analysis of the Disulfide-Exchange Equilibrium Mixture

peptide mixture	initial composition, % CH_3CN (v:v)	final composition, % CH_3CN (v:v)	gradient duration, min
GVPGGC + CFPGGC	11	23	120
CVPGGC + CGPGGC	11	16	90
CFPGGC + CGPGGC	11	23	120
CVPGGC + CKPGGC	5	16	150
CVPGGC + CGPGKC	5	16	150

The product mixtures from the syntheses of the oxidized and reduced hexapeptides were analyzed by elution from a Waters Nova Pak C_{18} column with a gradient of acetonitrile in water, both as 0.09% TFA solutions. Chromatograms were generated by using both detection systems, and thus, disulfide/thiol-containing fractions were identified among those visualized at 210 nm. The flow rate was 0.5 mL/min, and the linear gradients, expressed as proportion of acetonitrile (v:v), were 2–20% for the peptides CGPGGC, CKPGGC, and CGPGKC; 15–25% for the peptide CFPGGC; and 8–25% for the peptide CVPGGC. The durations of the gradients were 40 and 60 min, respectively, for the oxidized and reduced peptides.

Preparative-scale RP-HPLC was executed with a Rainin Dynamax Macro C_{18} column with a Spectra Physics SP 8000 liquid chromatograph coupled to a Model 770 spectrophotometric detector. The gradients determined in the analytical-scale analyses were run for 50 min at a flow rate of 20 mL/min. The chromatograms were generated with photometric detection at 220 nm.

Analyses of the equilibrium mixtures were performed on a Waters RCM 100 radial compression module loaded with a Nova-PAK C_{18} cartridge. The flow rate was 0.3 mL/min, and the 0.09% TFA acetonitrile/water gradients employed are given in Table II. The DTDS, described in detail by Thannhauser et al.,[31] was employed as such with a single, but crucial, modification. The column effluent was led directly to the reaction coil by means of a minimal length (20 cm) of 0.3-mm diameter capillary tubing, rather than as done previously by splitting the flow and passing it through a peristaltic pump. This resulted in greatly enhanced resolution and order of magnitude increase in sensitivity over the system previously described.[31] In this configuration, however, the DTDS destroys the entire sample fraction. The reaction coil employed was of a length such that the total reaction time was of the order of 50 min. Light was excluded, so that the photocatalyzed destruction of 2-nitro-5-thiobenzoate (NTB) in the assay mixture[44] was prevented.

The assay mixture was of the following composition: 0.5 mM NTSB, 200 mM Na_2SO_3, 0.1 M glycine, 3 mM disodium ethylenediaminotetraacetate (EDTA), and 0.03% Brij detergent, pH 9.5. Determination of the kinetics of formation of NTB[32] by reaction with each of the cyclic peptides showed that reaction was complete after 10 min with the exception of the peptide CGPGGC, which required 20 min. The assays were found to be quantitative in all cases by comparison to independent nitrogen analysis.[32] The response of the DTDS was found to be linear over the concentration range employed in this study, and the reproducibility of the integrated output was found to match the uncertainty of the sample injection volume.

3. Disulfide-Exchange Equilibrations. All solutions were prepared with double deionized water of HPLC-grade purity which had been thoroughly purged with prepurified nitrogen. The stock buffer was 0.111 M Tris/HCl and 0.111 mM EDTA, pH 8.0, which was degassed by aspiration immediately prior to use. The oxidized-peptide solution was prepared simply by dissolution into freshly degassed water to a concentration of approximately 1.2 mg/mL (2 mM). The reduced peptide was dissolved into a degassed aqueous solution of EDTA (10 μM) in hydrochloric acid (10 μM), pH 5.0, to a final concentration of approximately 1.2 mg/mL (2 mM).

The stock buffer (900 μL) was delivered into a reaction vial equipped with a magnetic stirring bar. After the addition of an aliquot of the oxidized-peptide solution (x μL; $x \equiv 40$, 50, or 60 μL) the vial was sealed with a serum cap. A stream of argon, which was prehumidified by passage through an aqueous Tris/HCl solution (0.1 M, pH 8.0), was passed over the solution, and the vial was immersed in a water thermostat set to 25.00 ± 0.1 °C. The solution was stirred for 10 min to allow thermal equilibration and gaseous exchange, and an aliquot of the reduced-peptide solution ($100 - x$, μL) was added by means of a syringe, thus initiating the reaction. In all cases, a period of 60 min was found to be sufficient to allow for complete equilibration. During this time the mixture was stirred magnetically under a gentle stream of argon.

(42) Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839–1842.
(43) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
(44) Damodaran, S. *Anal. Biochem.* **1985**, *145*, 200–204.

Disulfide exchange was quenched by the addition of aqueous TFA (0.3 M, 200 μL) from a syringe, inducing a pH drop from 8.0 to 2.0. The choice of final pH was determined by the effective pH of the initial RP-HPLC eluant. A sample of the equilibrium mixture, in this metastable state, was injected directly onto the C_{18} RP-HPLC column with a minimum of delay.

In order to ascertain the effectiveness of the pH drop method, a solution of reduced DTT (approximately 100 μM) and *N,N*-diacetyl-*N'*,*N'*-dimethylcystinamide (≃100 μM) in sodium citrate buffer (0.1 M, pH 2.0) was incubated under the conditions described (vide supra). A sample, withdrawn after 15 min, was analyzed by the RP-HPLC/DTDS method. No reaction was detected, demonstrating that oxidation of DTT both atmospheric and by *intermolecular* disulfide exchange was prevented (kinetically) under these conditions, despite the thermodynamic favorability of this reaction.[45] Thus, the analytical concentrations obtained from the RP-HPLC assay will have been unaffected by oxidation or disulfide exchange and, hence, correspond to the respective concentrations of the equilibrium mixture.

In the investigation of the disulfide-exchange equilibrium of a given pair of peptides, a set of six experiments was performed. Both {peptide-X} and {peptide-Y} were used as the reductant in three differing mole ratios. Hence, the equilibrium is approached from "both sides", which probes for the presence of any kinetic barriers to the attainment of the equilibrium state.

The DTDS was calibrated immediately *prior* to the injection of the equilibrium mixture by injecting an aliquot of a cystine solution whose concentration had been determined by using the photometric NTSB assay.[32] For a given batch of NTSB reagent, the response of the system was found to be stable.

4. ^{13}C NMR Titrations. Spectra were recorded with a Varian XL-400 instrument equipped with a 5-mm broad-band probe operating at 100.57 MHz while the probe temperature was maintained at 25.0 ± 0.1 °C. Proton-decoupled spectra were obtained by using the Waltz 16 broad-band decoupling sequence.[46] The sensitivity of the probe was measured by using dioxane (40%) in benzene-d_6 and was found to give a signal-to-noise ratio of 160:1. Potassium formate was used as an internal reference; with this instrument, we obtained a chemical shift of 170.94 ppm with respect to an external standard, being a coaxial insert containing ^{13}C enriched (5–10%) tetramethylsilane (Me_4Si) as a 25% solution in acetone-d_6.

All solutions were prepared and titrated in a nitrogen atmosphere. The NMR samples were prepared by dissolving the bis(thiol) peptides (5–8 mg) and approximately 0.1 equiv of DTT (0.2 mg) into the solvent mixture. The latter was prepared as follows: deuterium oxide (50 μL); dipotassium EDTA (50 μL, 5.0 mM); potassium formate, pH 6.0 (100 μL, 0.25 M); and double deionized water (300 μL). All aqueous solutions were freshly prepared by using degassed HPLC grade water. The solutions were titrated by the addition of minute amounts of 3 M KOH and 3 M HCl, and pH measurements were made directly in the NMR tube with an Ingold 6030-02 combination pH electrode. Appropriate signal-to-noise ratios were obtained after 1600–4000 acquisitions. The data were smoothed by multiplying the FID by an exponential function with a frequency constant of 3.0 Hz. Spectral assignments were made by reference to the compilations of Howarth and Lilley.[47] Resonances were assigned only to a residue type; i.e., while two cysteine residues were distinguished, sequential assignment was not attempted. The two cysteine residues were classified simply as downfield (Cys_A) and upfield (Cys_B). The downfield resonances of both the α- and β-pairs pertained to the same residue, confirmed by the similarity of their ionization behavior. Measurements were made over the pH range of 6–12. Inclusion of DTT was found to be essential in order to prevent oxidation of the peptides over the time course of the experiments, and, in all cases, no problems were encountered from resonance overlap.

5. Calculation of Microscopic Acid Dissociation Constants. Initial values ("best guess") of the various microscopic constants were optimized to fit the data for pH and ^{13}C NMR chemical shifts. The calculated parameters were related to the experimental parameters by using the degree of ionization as described above. In order to avoid distortion of the fit by weighting the experimental values of δ_{S_AH} and $\delta_{S_A^-}$, etc. (observed at the end points of titration), these constants in eq 13a and 13b were taken as variable parameters. The initial values of the variable end point parameters were those observed experimentally. All parameters were optimized simultaneously without weighting unless convergence did not occur. In the latter case, the end point chemical shifts were held constant in the first round of optimization in order to obtain good starting values for the microscopic dissociation constants. Subsequently, a full optimization was performed. The nonlinear least-squares routine employed was LMDIF from the Minpack library.

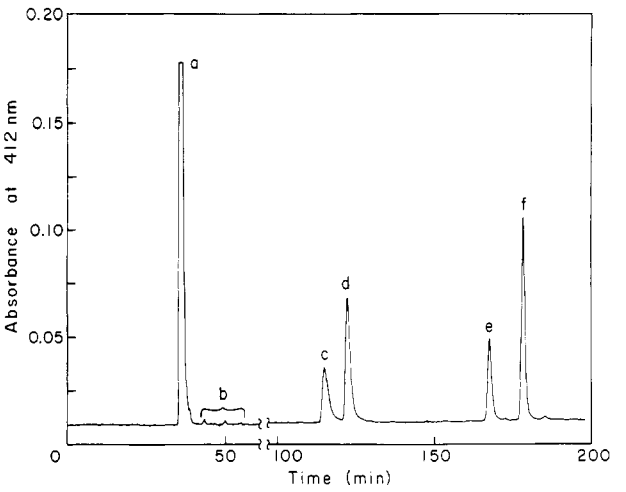

Figure 6. Disulfide/thiol analysis of an equilibrium mixture between CKPGGC and CVPGGC: (a) cystine calibration; (b) injection artifacts; (c) oxidized CKPGGC; (d) reduced CKPGGC; (e) Oxidized CVPGGC; (f) reduced CVPGGC.

Table III. Equilibrium Concentrations (μM) for Disulfide Exchange between CGPGGC and CFPGGC at 25 °C and pH 8.0[a]

$[CGPGGC_{ox}]$	$[CGPGGC_r]_o$	$[CFPGGC_{ox}]$	$[CFPGGC_r]_o$	$K'_{8.0}$
74.7	21.7	29.8	*43.5*[b]	5.01
92.1	17.2	26.2	*24.8*	5.07
71.1	17.2	37.0	*45.1*	5.03
51.3	*5.8*[b]	60.7	34.0	4.99
63.7	*37.8*	19.0	54.4	4.82
59.1	*8.2*	71.1	49.2	4.98

[a] Where $K'_{8.0} = [CGPGGC_{ox}][CFPGGC_r]_o/[CGPGGC_r]_o[CFPGGC_{ox}]$. [b] Entries in italics denote the reductant species.

Table IV. Observed Equilibrium Constants for Disulfide-Exchange Reactions at 25 °C and pH 8.0

forward reaction	$K'_{8.0}$
$CVPGGC_{ox} + CFPGGC_r \rightarrow$	1.23 (0.05)[a]
$CVPGGC_{ox} + CGPGGC_r \rightarrow$	6.40 (0.22)
$CFPGGC_{ox} + CGPGGC_r \rightarrow$	4.98 (0.16)
$CVPGGC_{ox} + CKPGGC_r \rightarrow$	1.21 (0.11)
$CVPGGC_{ox} + CGPGKC_r \rightarrow$	1.42 (0.08)

[a] The term in parentheses is the range of 95% confidence for the mean of six determinations.

Results

A typical chromatogram obtained by using the DTDS is depicted in Figure 6. The chromatogram clearly illustrates the stoichiometries of the NTSB assay, i.e., one disulfide or two thiol functions per molecule. The low flow rate (0.3 mL/min) is reflected in the protracted elution of buffer components. It should be noted that no significant dimer formation was observed under the conditions of equilibration, in contrast to those of synthesis, where the concentrations were higher.

The numerical output from digital integration of the chromatogram was substituted directly into eq 2, yielding the value of $K'_{8.0}$. The stoichiometric factor appears both in the denominator and numerator and, hence, cancels. The molar concentrations at equilibrium were computed from the integrated DTDS output by using the calibration constant obtained from the standard cystine solution. A representative set of results is presented in Table III. The remaining sets of data are available in the supplementary material. Mean values of $K'_{8.0}$ were computed for each system, and these are presented in Table IV.

The microscopic acid dissociation constants (Tables V and VI) were computed from the data for chemical shifts as a function of pH (which are presented in the supplementary material).

(45) Cleland, W. W. *Biochemistry* **1964**, *3*, 480–482.
(46) Shaka, A. J.; Keeler, J.; Freeman, R. *J. Magn. Reson.* **1983**, *53*, 313–340.
(47) Howarth, O. W.; Lilley, D. M. J. *Prog. Nucl. Magn. Reson. Spectrosc.* **1978**, *12*, 1–40.

Table V. Microscopic Acid Dissociation Constants for the Dibasic Bis(thiol) Peptides at 25 °C

	$CGPGGC_r$	$CFPGGC_r$	$CVPGGC_r$
10^9k_1[a]	1.01 (0.03)[b]	1.12 (0.03)	1.58 (0.03)
10^9k_2	1.73 (0.04)	1.63 (0.05)	2.43 (0.05)
10^9k_{12}	1.90 (0.14)	3.05 (0.25)	4.75 (0.29)
$10^{10}k_{21}$	9.22 (0.21)	5.98 (0.13)	8.41 (0.17)
$(\sum F/\{M-N\})^{1/2}$[c]	0.70	0.06	0.08

[a] Microscopic acid dissociation constants are defined in Figure 2. [b] Terms in parentheses are estimates of the systematic error. [c] The standard error of the optimization, $(\sum F/\{M-N\})^{1/2}$, is also given, where $\sum F$ is the sum of the residuals, M is the number of nonlinear equations relating experimental and variable parameters, and N is the number of variable parameters.

Table VI. Microscopic Acid Dissociation Constants for the Lysine-Containing Bis(thiol) Peptides at 25 °C

	$CKPGGC_r$	$CGPGKC_r$
10^9k_1[a]	1.64 (0.09)[b]	1.43 (0.07)
10^9k_2	2.45 (0.18)	2.06 (0.21)
$10^{11}k_3$	1.02 (0.89)	9.03 (77)
10^9k_{12}	1.75 (0.02)	1.76 (0.05)
10^9k_{21}	1.17 (0.01)	1.22 (0.09)
$10^{12}k_{13}$	9.44 (0.17)	9.53 (0.15)
$10^{10}k_{31}$	15.1 (6.4)	1.51 (9.3)
$10^{10}k_{23}$	24.2 (16)	2.51 (18)
10^9k_{132}	7.77 (0.15)	8.05 (0.15)
$10^{11}k_{213}$	4.19 (0.21)	4.36 (0.27)
10^9k_{321}	4.85 (1.0)	4.84 (5.6)
$(\sum F/\{M-N\})^{1/2}$[c]	0.07	0.08

[a] Microscopic acid dissociation constants are defined in Figure 7. [b] Terms in parentheses are estimates of the systematic error. [c] Standard error of the optimization.

It was not possible to derive analytical estimates of the errors in the microscopic acid dissociation constants, because of the nonlinear nature of the optimization. Instead, we have performed a crude numerical examination of the results. Assuming that the only significant systematic experimental error arises from the measurement of pH (±0.01 unit), we have recalculated the microscopic constants using pH readings incremented to both limits and, thus, have approximated the range of systematic error for each parameter. These values were used to estimate the uncertainties in the derived macroscopic acid dissociation constants.

The standard error in the chemical shifts obtained from optimizations was between 0.06 and 0.08 ppm for the bis(thiol) peptides. This represents less than 5% of the total change in chemical shift for all resonances observed (in some cases as little as 2%). The quality of the fits was better for the lysine-containing cyclic disulfides.

The generation of macroscopic acid dissociation constants from microscopic parameters for the peptides has been described (vide supra), the results being given in Table VII. These values have been used to calculate formal equilibrium constants, K', from the observed equilibrium constants, $K'_{8.0}$, according to eq 9 and 10.

The formal equilibrium constants displayed in Table VIII were used to calculate standard Gibbs free energy changes, $(\Delta G°)'$, which are also given in Table VIII. The error expressed in the values of K' is an approximation calculated from the 95% confidence interval of $K'_{8.0}$ and the appropriate estimated systematic errors in the macroscopic acid dissociation constants. The errors expressed in $(\Delta G°)'$ were calculated by using the error distribution function $\partial(\Delta G°)' = RT\partial(K')/K'$.

Discussion

The observed equilibrium constants for disulfide exchange at 25 °C, pH 8.0, are seen to be independent of the nature of the reductant, mole ratio, and concentration within the experimental regime. This demonstrates an absence, under these conditions, of both kinetic barriers to equilibration and significant second-order effects, such as specific solute–solute interaction.

Table III reveals that, typically, the results are only slightly scattered for a given system. Consequently, the mean values of the observed equilibrium constants are found to have rather small limits of 95% degree of confidence (Table IV). In a preliminary series of experiments, both peptides were added to the equilibration mixture in the oxidized form and, after preincubation, partially reduced in situ by 0.5 equiv of DTT. The results of these experiments were identical with those presented here (Table IV) within the range of 95% confidence, reinforcing the reproducibility of these results and providing an alternative protocol for the experiment.

The observed (pH-dependent) equilibrium constants indicate that some of the peptides are preferentially oxidized under the conditions of the experiment. In particular, the CGPGGC peptide appears to cyclize more readily than the others. The peptide CVPGGC cyclizes with least facility at pH 8.0. These observations reflect our synthetic experiences in that the oxidized peptide CGPGGC was obtained in the highest yield, while the peptide CVPGGC gave the lowest yield (but the highest yield of the cyclic dimer; data not presented).

The microscopic acid dissociation constants obtained from the ^{13}C NMR titrations are presented in Tables V and VI. Those for the dibasic peptides exhibit the minor differences in magnitude that one might expect for a set of peptides differing only slightly in sequence and with the same net charge. The microscopic constants calculated for the two lysine-containing peptides are also unremarkable in the sense that their relative values are compatible with one's chemical intuition. It is seen, for example, that the dissociation associated with one of the thiols occurs more readily in both peptides. This would be anticipated for a cysteine residue adjacent to a lysine residue. Also, we note that the constants relating to species unlikely to exist in aqueous solution have large uncertainties in the results of the nonlinear least-squares fit, e.g., the nonionized peptide and the constants k_3, k_{31}, and k_{32} (see Figure 7).

Since free energies of ionization are large compared to those of conformational processes, one might expect that relatively small differences in the acid dissociation constants of the peptides could lead to significant differences between $K'_{8.0}$ and K'. These ionization effects would modify the apparent ease with which the peptides are oxidized in a solution at a pH near their pK. The various macroscopic acid dissociation constants of the peptides studied (Table VII) do bear a close resemblance to each other. These small differences, however, are sufficient to constitute significant, but minor, corrections to the observed equilibrium constants, $K'_{8.0}$ (Table IV), as embodied in the formal equilibrium constants, K' (Table VIII). It should be noted that the corrections arising from the ionization of the lysine residue at pH 8.0 were insignificant.

Observation of the results of the equilibria of the peptide CVPGGC yields relative conformational free energies of cycli-

Table VII. Macroscopic Acid Dissociation Constants at 25 ● 0.1 °C

peptide	K_1	K_2	K_3
$CGPGGC_r$	2.74 (0.04)[a] × 10^{-9}	6.21 (0.30) × 10^{-10}	
$CFPGGC_r$	2.75 (0.08) × 10^{-9}	5.00 (0.25) × 10^{-10}	
$CVPGGC_r$	4.01 (0.08) × 10^{-9}	7.10 (0.30) × 10^{-10}	
$CKPGGC_r$	4.10 (0.18) × 10^{-9}	7.09 (0.23) × 10^{-10}	4.13 (0.38) × 10^{-11}
$CGPGKC_r$	3.58 (0.29) × 10^{-9}	7.13 (0.50) × 10^{-10}	4.30 (1.7) × 10^{-11}
$CKPGGC_{ox}$	2.67 (0.06) × 10^{-11}		
$CGPGKC_{ox}$	2.46 (0.05) × 10^{-11}		

[a] Terms in parentheses are estimates of systematic error obtained as described in text.

Table VIII. Formal Equilibrium Constants and Standard Gibbs Free Energies for Peptide Cyclizations at 25 °C and pH 8.0

forward reaction	K'	$-(\Delta G°)'$, kcal mol^{-1}
$CVPGGC_{ox} + CFPGGC_r \rightarrow$	1.11 (0.05)	0.06 (0.03)
$CVPGGC_{ox} + CGPGGC_r \rightarrow$	5.78 (0.20)	1.04 (0.02)
$CFPGGC_{ox} + CGPGGC_r \rightarrow$	4.99 (0.14)	0.95 (0.02)
$CVPGGC_{ox} + CKPGGC_r \rightarrow$	1.21 (0.11)	0.11 (0.05)
$CVPGGC_{ox} + CGPGKC_r \rightarrow$	1.37 (0.08)	0.19 (0.03)

Table IX. Standard Gibbs Free Energies for Peptide Cyclizations at 25 °C, pH 8.0, Expressed Relative to the Peptide CGPGGC

forward reaction	$(\Delta G°)'$, kcal mol^{-1}
$CGPGGC_{ox} + CFPGGC_r \rightarrow$	0.98
$CGPGGC_{ox} + CVPGGC_r \rightarrow$	1.04
$CGPGGC_{ox} + CGPGKC_r \rightarrow$	0.85
$CGPGGC_{ox} + CKPGGC_r \rightarrow$	0.93

zation, $(\Delta G°)'$. This parameter has a zero value for the reference process, by definition, which is the cyclization of CVPGGC. Hence, the peptides adopt cyclic conformations more readily in the order CGPGGC > CGPGKC > CKPGGC > CFPGGC > CVPGGC. Immediately, one observes that there is a small but significant difference in the conformational free energy of cyclization between CKPGGC and CGPGKC, indicating the dependence of this process upon sequence in addition to composition. These results indicate that the replacement of another amino acid by a glycyl residue at position 2 or 5 results in a marked facilitation of ring closure. The appropriate comparisons of the conformational free energies of cyclization are presented in Table IX.

If one subtracts the value of $(\Delta G°)'$ for the peptide CFPGGC from that for CGPGGC (both being obtained with reference to CVPGGC), one obtains the value −0.98 kcal/mol. This Gibbs free energy change corresponds to the difference between the conformational cyclization free energies of CGPGGC and CFPGGC. Reference to Table VIII shows that the direct experimental estimate of this parameter is −0.95 kcal/mol, identical with this calculated value within the experimental uncertainty. Hence, the conformational free energies of cyclization that we have obtained are additive, i.e., independent of the nature of the equilibrium mixture. This important observation was tested further by performing a series of four equilibrations with the peptides CKPGGC and CFPGGC. Defining the forward reaction as the oxidation of CKPGGC, we obtained a value of $K'_{8.0} = 0.97$ (0.04). Substitution of the appropriate macroscopic dissociation constants (Table VII) into eq 10 yielded a value of 1.09 (0.04) for K'. This converts to −0.05 (0.02) kcal/mol for the conformational free energy of cyclization of CKPGGC with respect to CFPGGC. The value of this parameter calculated from the values of $(\Delta G°)'$ with respect to CVPGGC (Table VIII) is seen to be −0.05 kcal/mol. The free energy parameters are, therefore, additive in this case also.

The cyclic hexapeptides are necessarily constrained to conformations which constitute chain reversals by their covalent structure. The distance between the C^α atoms of [Cys^1] and [Cys^6] is confined within a distribution defined by bond vibrational and torsional potentials. In a sample of five globular proteins, Wako and Scheraga[48] found an average C^α to C^α distance of 0.52 ± 0.04 nm within a cystine residue. Thus, in crude terms, the "most open" turn allowed in the cyclic cystine peptides is one in which the C^α_i to C^α_{i+5} distance is of the order of 0.52 nm. Therefore, the above preferential series corresponds to the propensity toward chain reversal of the given peptide sequence. It should be noted that, in addition to intramolecular contributions, $(\Delta G°)'$ incorporates contributions from solvation (i.e., hydration) which accompany conformational change.

It is not unreasonable to assume that, among the ensemble of conformations adopted by these peptides in solution, there will exist a significant proportion of β-turn conformations. This postulate is well supported by the known conformational properties of the neurohypophyseal peptides oxytocin[17,49-53] and vasopressin.[54,55] These hormones incorporate a disulfide-containing, 20-atom cyclic (tocin) moiety, which is flexible[50,51] and appears to adopt an ensemble of rapidly interconverting β-turn[52,53] and 1-5-turn[53] conformations. Additionally, studies of linear peptides have set precedent for β(II)-conformations being adopted by the sequences VPGG[56,57] and GPGG.[57]

Clearly, one should not expect that a unique β-turn conformation will dominate the conformational ensemble of the cystine peptides or, indeed, that other chain-reversal conformations such as γ-turns[5,11] or open-turns[7,8,10] will not contribute significantly. Studies of 1-penicillamino derivatives of oxytocin[58] set a precedent for the occurrence of conformations corresponding to two consecutive γ-turns in the 20-membered cyclic moiety. The 20-membered cycle may also accommodate the multiple β-turns described by Isogai et al.[59] In this sense, it seems that, in the absence of specific short-range interactions with the disulfide function, these peptides would constitute excellent general model systems with which to study the relative propensity of a given peptide sequence to form chain reversals.

We have assumed that the intrinsic free energy of formation of a disulfide bond should be essentially identical in all of the peptides, such that comparison of the free energies of cyclization of the peptides indicates the different chain-reversal probabilities in the set of *acyclic* peptides. It is likely, however, that some chain reversals in the acyclic conformational ensemble may not be incorporated into the corresponding cyclic ensemble. If this condition applies to conformations which dominate the set of chain reversals in an acyclic peptide, the free energy comparison would be erroneous. This caveat should be recalled when employing this method to estimate propensities toward formation of a chain reversal.

At the present time, it is not appropriate to make a detailed analysis of the results in terms of specific conformations. We are, however, currently undertaking both theoretical[60] and experimental[61] conformational analyses of these molecules and will, thus, postpone such discussions until their completion.

While exercising due caution, it is enlightening to compare our results with those of other studies directed toward examining the β-turn-forming potential of peptide sequences. In making these

(48) Wako, H.; Scheraga, H. A. *J. Protein Chem.* **1982**, *1*, 5-45.

(49) Hruby, V. J. *Perspectives in Peptide Chemistry*; Weiland, T., Geiger, R., Eberle, A., Eds.; Karger: Basel, NY, 1981; pp 207-220.

(50) (a) Brewster, A. I. R.; Hruby, V. J. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3806-3809. (b) Glasel, J. A.; Hruby, V. J.; McKelvy, J. F.; Spatola, A. F. *J. Mol. Biol.* **1973**, *79*, 555-575. (c) Hruby, V. J.; Deb, K. K.; Fox, J.; Bjarnason, J.; Tu, A. T. *J. Biol. Chem.* **1978**, *253*, 6060-6067.

(51) Wood, S. P.; Tickle, I. J.; Treharne, A. M.; Pitts, J. E.; Mascarenhas, Y.; Li, J. Y.; Husain, J.; Cooper, S.; Blundell, T. L.; Hruby, V. J.; Buku, A.; Fischman, A. J.; Wyssbrod, H. R. *Science (Washington, DC)* **1986**, *232*, 633-636.

(52) Kotelchuck, D.; Scheraga, H. A.; Walter, R. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3629-3633.

(53) Brewster, A. I. R.; Hruby, V. J.; Glasel, J. A.; Tonelli, A. E. *Biochemistry* **1973**, *12*, 5294-5304.

(54) (a) Hruby, V. J.; Upson, D. A.; Yamamoto, D. M.; Smith, C. W.; Walter, R. *J. Am. Chem. Soc.* **1979**, *101*, 2717-2721. (b) Hruby, V. J.; Deb, K. K.; Spatola, A. F.; Upson, D. A.; Yamamoto, D. *J. Am. Chem. Soc.* **1979**, *101*, 202-212. (c) Deslauriers, R.; Smith, I. C. P.; Walter, R. *J. Am. Chem. Soc.* **1974**, *96*, 2289-2291.

(55) Krishna, N. R.; Huang, D. H.; Glickson, J. D.; Rowan, R., III; Walter, R. *Biophys. J.* **1979**, *26*, 345-366.

(56) (a) Urry, D. W.; Mitchell, L. W.; Ohnishi, T. *Biochem. Biophys. Res. Commun.* **1974**, *59*, 62-69. (b) Urry, D. W.; Long, M. M.; Ohnishi, T.; Jacobs, M. *Biochem. Biophys. Res. Commun.* **1974**, *61*, 1427-1433. (c) Khaled, M. A.; Renugopalakrishnan, V.; Urry, D. W. *J. Am. Chem. Soc.* **1976**, *98*, 7547-7553. (d) Khaled, M. A.; Urry, D. W. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 485-491.

(57) (a) Kopple, K. D.; Go, A.; Pilipauskas, D. R. *J. Am. Chem. Soc.* **1975**, *97*, 6830-6838. (b) Kopple, K. D.; Go, A. *Biopolymers* **1976**, *15*, 1701-1715.

(58) Meraldi, J.-P.; Hruby, V. J.; Brewster, A. I. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1373-1377.

(59) Isogai, Y.; Némethy, G.; Rackovsky, S.; Leach, S. J.; Scheraga, H. A. *Biopolymers* **1980**, *19*, 1183-1210.

(60) Shieh, H.; Némethy, G.; Scheraga, H. A., work in progress.

(61) Milburn, P. J.; Scheraga, H. A., work in progress.

comparisons, we have assumed that, in putative β-turn conformations, residues 2 to 5 inclusively comprise the turn, while the cysteine residues adopt extended conformations in order to allow ring closure. It should be noted that cysteinyl residues have a relatively high probability of occurrence at the first and fourth position of β-turns;[24] consequently, alternative β-turns and multiple β-turns are likely to occur in addition to that described above.

Nagai and co-workers[26,62] have developed a method for determining the β-turn preferences of tetrapeptide sequences by observing the circular dichroism (CD) of their *N*-(2,4-dinitrophenyl)-*p*-nitroanilides. Their results show that [Gly[1]], [Gly[4]] tetrapeptides have a relatively high propensity toward formation of β-turns,[63] which is consistent with our findings. By varying residues 2 and 3 they observed[64] a good correlation with the β-bend potentials of Chou and Fasman.[24] A similar study[65] of residues 1 and 4 did not yield this correlation, a difference rationalized as arising from the limited chain length of the model and the effects of the terminal substituents on the conformational space of the terminal residues.

The equilibrium constants obtained for the standardized disulfide exchange (Table VIII) were compared with the β-bend-forming potentials of residue 1 (in the 1–4 bend) listed by Chou and Fasman.[24] With regard to the positional bend potentials, there is minimal agreement. Glycine is predicted[24] to favor β-turns as observed in our studies. The peptide CFPGGC should form β-turns more readily[24] than CVPGGC as we have observed, although the Chou–Fasman potentials suggest that the difference should be more pronounced. The results for CKPGGC, however, disagree with the predictions[24] since the peptide contains the central four residues least likely to form β-turns, but our results reveal that chain reversal in this peptide is more favorable than one in the valine- and phenylalanine-containing peptides. Interestingly, the central four residues of the peptide CGPGKC are predicted[24] to have the second highest bend potential of this set, in accordance with our results. Furthermore, comparison of our results with the bend potentials calculated *irrespective* of position[24] reveals a qualitative agreement. This latter observation suggests that the 20-membered peptide ring may assume β-turn conformations in varying sequential positions; e.g., in CKPGGC, the lysyl residue may occur as residue i, $i + 1$, and $i - 1$ in β-turns.

Pohl[66] considered the deduction of conformational energies from the crystal coordinates of proteins. He assumed that individual influences are averaged out for a large number of observations and that the ensemble of conformational states observed has the properties of a Boltzmann distribution. The normalized frequency of occurrence of a given conformational state should, thus, have the properties of a pseudopartition function. Consequently, parameters such as those obtained by Chou and Fasman[24] from proteins should be proportional, as a first approximation, to those obtained from studies of small peptides, whose conformational states are truly represented by a Boltzmann distribution. Our results and those of Sato et al.[64] suggest that the parameters obtained by Chou and Fasman[24] for the prediction of the tendency toward formation of β-turns may, indeed, be applicable to oligopeptides in solution. As a corollary, appropriate small-molecule model systems might therefore be useful for the parametrization of this conformational property of proteins. Clearly, a considerably enlarged data set is required before wholely reliable conclusions may be drawn.

Theoretical analysis of the conformational probability for the 160 000 possible tetrapeptides of naturally occurring amino acid residues is not feasible currently. Even farther from practical realization would be an experimental regime of such dimensions. Consequently, parametric schemes are essential to the solution of the predictive problem associated with chain reversals. Currently, statistical analysis of protein structure represents the most practical means of obtaining the large amount of data required. It is, therefore, gratifying to note that this source of information displays some correlations with the limited experimental data available. In some cases, however, the statistical data base is rather limited since certain residues occur only rarely in the crystal structures available.[67] An experimental technique, such as the one presented herein, which correlates with the β-turn potentials, for example, would thus be able to supplement the existing statistically derived parameters.

The methods of determining the β-turn-forming tendencies of peptides developed by Nagai and his collaborators[26] and by Boussard and Marraud[29] both suffer from limitations which are not apparent in the method presented in this paper. The spectral observation of chain reversals in linear peptides requires that the sequence under observation have at least a moderate tendency toward forming such conformations in order that a measurable population exists in solution. By employing cyclic hexapeptides, we have a system in which chain reversals are enforced by the covalent connectivity. It is this property of cyclic peptides which has resulted in their popularity as models of chain reversals in proteins.[68] This principle has also been employed by Balaram and co-workers[69] in a series of spectral studies of conformationally restrained peptides cyclized by disulfide bonds. For various reasons, neither the infrared[29] nor the chirooptical[26] method is applicable to aqueous solutions. In principle, however, the cyclic disulfide method could be adapted to nonaqueous or mixed-solvent systems if so desired.

Our method is not affected by the inclusion of chromophoric residues into the sequence under investigation as is the chirooptical method.[26] It is likely, however, that the incorporation of an additional cysteine into the sequence (e.g., in the X or Y positions) would render the cyclic disulfide method somewhat cumbersome. Oxidation of the tris(thiol) peptide might yield three monomeric species and numerous oligomeric species, both reducing synthetic yield and, potentially, rendering the disulfide-exchange equilibrium analysis to be impossible.

Aside from the obvious synthetic requirements, the disulfide-exchange method demands that a set of suitable chromatographic conditions be discovered in order to assay the equilibrium mixture. Additionally, one must evaluate the macroscopic acid dissociation constants for each peptide studied since relatively small differences in acidity of the thiol groups lead to significant corrections to the observed apparent equilibrium constants. The estimation of these dissociation constants represents the most onerous part of our procedure, since the equilibrium constants may be estimated simultaneously for as many peptides as allowed by the chromatographic resolution. To illustrate this, we performed a series of experiments using a ternary mixture of CGPGGC, CFPGGC, and CVPGGC and using, in turn, each peptide as the reductant. The values of $K'_{8.0}$ so obtained were 1.23 (0.01), 6.09 (0.08), and 4.91 (0.33), which are sensibly identical with the corresponding equilibrium constants given in the first three rows of Table IV.

It is our opinion that this technique will be widely applicable in the study of chain-reversal conformations. The distinction between "good" and "bad" bend-forming sequences may now be expressed in terms of a standard Gibbs free energy difference, potentially of use in the design of protein modifications. The relationship between the structure of oligopeptides, expressed by

(62) (a) Sato, K.; Taki, N.; Nagai, U.; Higashijima, T. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2476–2479. (b) Sato, K.; Higashijima, T.; Sugawara, R.; Nagai, U. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3699–3702. (c) Higashijima, T.; Sato, K.; Nagai, U. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3323–3328. (d) Sato, K.; Kawai, M.; Nagai, U. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 1527–1531. (e) Sato, K.; Nagai, U. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3329–3336.

(63) (a) Kawai, M.; Sato, K.; Sugawara, R.; Nagai, U. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3005–3008. (b) Kawai, M.; Sato, K.; Nagai, U. *Int. J. Pept. Protein Res.* **1984**, *24*, 607–612.

(64) Sato, K.; Nagai, U.; Higashijima, T. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 1657–1660.

(65) Sato, K.; Sugawara, R.; Nagai, U. *Int. J. Pept. Protein Res.* **1984**, *24*, 600–606.

(66) Pohl, F. M. *Nature (London), New Biol.* **1971**, *234*, 277–279.

(67) Kolaskar, A. S.; Ramabrahmam, V.; Soman, K. V. *Int. J. Pept. Protein Res.* **1980**, *16*, 1–11.

(68) Deber, C. M.; Madison, V.; Blout, E. R. *Acc. Chem. Res.* **1976**, *9*, 106–113.

(69) (a) Rao, B. N. N.; Kumar, A.; Balaram, H.; Ravi, A.; Balaram, P. *J. Am. Chem. Soc.* **1983**, *105*, 7423–7428. (b) Ravi, A.; Balaram, P. *Tetrahedron* **1984**, *40*, 2577–2583.

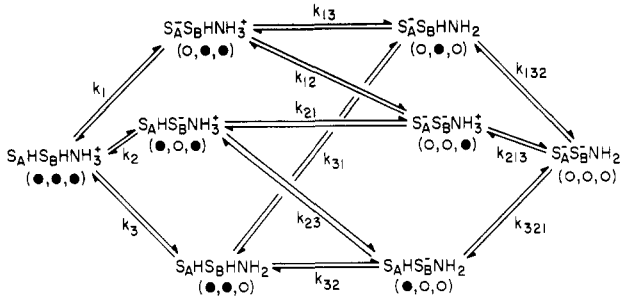

Figure 7. Ionization equilibria of the reduced form of a peptide containing three acidic groups: S_AH, S_BH, and NH_3^+.

adoption of chain-reversal conformations, and function, expressed in biological activity, may now be investigated quantitatively. The potential significance of chain reversals (not necessarily the native conformation uniquely) postulated to be important in *directing* the folding of nascent polypeptides may now be evaluated in energetic terms. Additionally, we foresee a role for this technique in the construction of predictive schemes for peptide and protein conformation (*vide supra*).

The dominance of short-range interactions in the formation of chain reversals has been clearly demonstrated by the results of statistical analyses of protein structure in the crystalline state[4,23,24,67] and by analysis of chain-reversal formation in solutions of oligopeptides.[26,29] The results presented herein further attest to the validity of this principle. In this context, short-range interactions refer to those between the side chain and backbone of a residue. Palau et al.[70] have considered the limitations of conformational predictions based purely on parameters pertaining to short-range interactions and have concluded that it is essential to consider longer range interactions in proteins. Clearly, parametrization of the conformational properties of amino acid residues which are based predominantly upon their short-range interactions may provide only an initial estimate of conformational probability which, in most instances, will be modified by higher order parameters arising from longer range effects.

Conclusion

We have developed a technique which allows one to obtain, for the first time, quantitative estimates of the propensity toward chain-reversal formation for peptides in aqueous solution. These initial results have reinforced the hypothesis[4] that short-range interactions dominate the tendency of a peptide chain to fold back on itself. Our results are qualitatively predicted by using statistical parameters derived from protein crystal structures by Chou and Fasman,[24] following the initial success of Lewis et al.[4,8] in their calculations of bend probabilities. We consider this method to be of great potential in the study of chain-reversal formation and, in consequence, are studying farther cystine-containing peptides.

Acknowledgment. This work was supported by research grants from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (AM-08465) and from the National Science Foundation (DMB84-01811). We are indebted to the NSF Regional Instrumentation Facility, The Johns Hopkins University School of Medicine, for obtaining the FAB mass spectra and to T. W. Thannhauser for performing the amino acid analyses. We thank V. G. Davenport for the provision of facilities for integration of chromatograms. It has been brought to our attention that Creighton[71] had suggested the study of sulfhydryl/disulfide equilibria to measure "the tendency for small peptides to form bends".

Appendix

An ionization scheme for the lysine-containing bis(thiol) peptides is depicted in Figure 7.

The macroscopic acid dissociation constants are defined as follows:

$$k_1 = \frac{[(0,\bullet,\bullet)]\,(H^+)}{[(\bullet,\bullet,\bullet)]} \quad \text{(A1)}$$

$$k_2 = \frac{[(\bullet,0,\bullet)]\,(H^+)}{[(\bullet,\bullet,\bullet)]} \quad \text{(A2)}$$

$$k_3 = \frac{[(\bullet,\bullet,0)]\,(H^+)}{[(\bullet,\bullet,\bullet)]} \quad \text{(A3)}$$

$$k_{13} = \frac{[(0,\bullet,0)]\,(H^+)}{[(0,\bullet,\bullet)]} \quad \text{(A4)}$$

$$k_{12} = \frac{[(0,0,\bullet)]\,(H^+)}{[(0,\bullet,\bullet)]} \quad \text{(A5)}$$

$$k_{21} = \frac{[(0,0,\bullet)]\,(H^+)}{[(\bullet,0,\bullet)]} \quad \text{(A6)}$$

$$k_{23} = \frac{[(\bullet,0,0)]\,(H^+)}{[(\bullet,0,\bullet)]} \quad \text{(A7)}$$

$$k_{32} = \frac{[(\bullet,0,0)]\,(H^+)}{[(\bullet,\bullet,0)]} \quad \text{(A8)}$$

$$k_{31} = \frac{[(0,\bullet,0)]\,(H^+)}{[(\bullet,\bullet,0)]} \quad \text{(A9)}$$

$$k_{132} = \frac{[(0,0,0)]\,(H^+)}{[(0,\bullet,0)]} \quad \text{(A10)}$$

$$k_{213} = \frac{[(0,0,0)]\,(H^+)}{[(0,0,\bullet)]} \quad \text{(A11)}$$

$$k_{321} = \frac{[(0,0,0)]\,(H^+)}{[(\bullet,0,0)]} \quad \text{(A12)}$$

P_A, P_B, and P_C are the fractional degrees of dissociation of the acidic functions S_AH, S_BH, and NH_3^+, respectively, where

$$P_A = \frac{\text{total concentration of microspecies deprotonated at site A}}{\text{total concentration of all microspecies}}$$

Thus

$$P_A = \frac{[(0,\bullet,\bullet)]+[(0,\bullet,0)]+[(0,0,\bullet)]+[(0,0,0)]}{[(\bullet,\bullet,\bullet)]+[(0,\bullet,\bullet)]+[(\bullet,0,\bullet)]+[(\bullet,\bullet,0)]+[(0,\bullet,0)]+[(0,0,\bullet)]+[(\bullet,0,0)]+[(0,0,0)]} \quad \text{(A13)}$$

Substituting for the microscopic dissociation constants and obtaining concentrations in terms of the undissociated species shows that

$$P_A = \frac{\frac{k_1}{(H^+)}[(\bullet,\bullet,\bullet)]\left\{1+\frac{k_{13}}{(H^+)}+\frac{k_{12}}{(H^+)}+\frac{k_{132}k_{13}}{(H^+)^2}\right\}}{\frac{k_1}{(H^+)}[(\bullet,\bullet,\bullet)]\left\{\frac{(H^+)}{k_1}+1+\frac{k_{12}}{k_{21}}+\frac{k_{13}}{k_{31}}+\frac{k_{13}}{(H^+)}+\frac{k_{12}}{(H^+)}+\frac{k_{23}k_{12}}{k_{21}(H^+)}+\frac{k_{132}k_{13}}{(H^+)^2}\right\}} \quad \text{(A14)}$$

Hence

$$P_A = \frac{1+\frac{k_{13}}{(H^+)}+\frac{k_{12}}{(H^+)}+\frac{k_{132}k_{13}}{(H^+)^2}}{1+\frac{(H^+)}{k_1}+\frac{k_{12}}{k_{21}}+\frac{k_{13}}{k_{31}}+\frac{k_{13}}{(H^+)}+\frac{k_{12}}{(H^+)}+\frac{k_{23}k_{12}}{k_{21}(H^+)}+\frac{k_{132}k_{13}}{(H^+)^2}} \quad \text{(A15)}$$

Expressions for P_B and P_C are obtained similarly, yielding

$$P_B = \frac{\frac{k_{12}}{k_{21}}+\frac{k_{12}}{(H^+)}+\frac{k_{23}k_{12}}{k_{21}(H^+)}+\frac{k_{132}k_{13}}{(H^+)^2}}{D} \quad \text{(A16)}$$

(70) Palau, J.; Argos, P.; Puigdomenech, P. *Int. J. Peptide Protein Res.* **1982**, *19*, 394–401.
(71) Creighton, T. E. *J. Mol. Biol.* **1975**, *96*, 767–776.

$$P_C = \frac{\frac{k_{13}}{k_{31}} + \frac{k_{13}}{(H^+)} + \frac{k_{23}k_{12}}{k_{21}(H^+)} + \frac{k_{132}k_{13}}{(H^+)^2}}{D} \quad \text{(A17)}$$

where D is the denominator in the expression for P_A (eq A15).

The values of the seven microscopic constants embodied in the expressions for the degree of dissociation were obtained by fitting to the data sets of the ^{13}C NMR titrations. The values of the remaining five macroscopic constants were obtained from the following relationships:

$$k_2 = k_1k_{12}/k_{21} \quad \text{(A18)}$$

$$k_{213} = k_{13}k_{132}/k_{12} \quad \text{(A19)}$$

$$k_{321} = k_{21}k_{213}/k_{23} \quad \text{(A20)}$$

$$k_{32} = k_{31}k_{132}/k_{321} \quad \text{(A21)}$$

$$k_3 = k_{23}k_2/k_{32} \quad \text{(A22)}$$

The numerical values of the macroscopic acid dissociation constants K_1, K_2, and K_3 may be computed from the microscopic constants according to the following relationships:

$$K_1 = k_1 + k_2 + k_3 \quad \text{(A23)}$$

$$K_2 = \frac{(k_{13} + k_{12} + k_{23}k_{12}/k_{21})}{(1 + k_{12}/k_{21} + k_{13}/k_{31})} \quad \text{(A24)}$$

$$K_3 = \frac{1}{(k_{132}^{-1} + k_{213}^{-1} + k_{321}^{-1})} \quad \text{(A25)}$$

Registry No. I, 108562-76-1; II, 108562-77-2; III, 108562-78-3; IVa, 108562-79-4; IVb, 108562-80-7; IVc, 108562-81-8; Va, 108562-82-9; Vb, 108562-83-0; Vc, 108562-84-1; VIa, 108562-85-2; VIb, 108562-86-3; VIc, 108562-87-4; VIIa, 108562-88-5; VIIb, 108594-51-0; VIIc, 108562-89-6; VIII, 108562-90-9; IX, 108562-91-0; X, 108562-92-1; XI, 108562-93-2; XII, 108562-94-3; XIII, 108562-95-4; XIV, 108594-52-1; XV, 39622-01-0; XVI, 108562-96-5; XVII, 108562-97-6; XVIII, 108562-98-7; XIX, 108562-99-8; XX, 108563-00-4; BOC-Cys(Acm)-OH, 19746-37-3; BOC-Gly-OH, 31972-52-8; BOC-Pro-OH, 15761-39-4; BOC-Phe-OH, 13734-34-4; BOC-Val-OH, 13734-41-3; BOC-Gly-OH, 4530-20-5; BOC-Cys(MBzl)-OH, 18942-46-6; BOC-Lys(Z)-ONp, 2389-46-0; BOC-Cys(MBZl)-ONp, 53843-86-0; Z-Gly-Pro-OSu, 38417-02-6; H-Gly-OBu-*t*-HCl, 27532-96-3.

Supplementary Material Available: Data on peptide synthesis, Tables 1-4 of data from the disulfide-exchange equilibrations, and Tables 5-11 of data obtained from the peptide titrations (21 pages). Ordering information is given on any current masthead page.

Linear Alkane Radical Cations Prepared in Synthetic Zeolites by Irradiation at 4 K: ESR Evidence for Ion–Molecule Reaction To Form 1-Alkyl Radicals

Kazumi Toriyama,* Keichi Nunome, and Machio Iwasaki*

Contribution from the Government Industrial Research Institute, Nagoya, Hirate, Kita, Nagoya 462, Japan. *Received August 18, 1986*

Abstract: A new method is explored for preparing radical cations of alkanes in the system other than halogenated and neon matrices. The radical cations are produced from *n*-C_6H_{14} and *n*-C_8H_{18} adsorbed in synthetic zeolite (ZSM-5) by X-irradiation at 4 K. The ESR spectra show that the cations exhibit the planar extended structure without showing coexistence of the gauche conformer. With increasing the concentration of alkanes, the parent molecules are increasingly adsorbed as a dimer, from which the 1-alkyl radical is preferentially formed during irradiation at 4 K. This indicates that the *n*-alkane radical cation undergoes prompt deprotonation via the ion–molecule reaction when a neighboring alkane molecule exists as a proton acceptor in the adsorbed site. The formation of the 1-alkyl radical is consistent with the high unpaired electron density in the in-plane C–H bonds at the chain end of the radical cations. These results are closely related to our previous finding of the selective formation of the 1-alkyl radicals in the 4 K radiolysis of crystalline neat linear alkanes.

The radical cations of alkanes are one of the most fundamental and important reaction intermediates, which are of wide interest in chemistry. We have reported the first clear and unescapable spectroscopic evidence of $C_2H_6^+$ in SF_6,[1] although shortly before our observation the inconclusive spectrum of $C_2Me_6^+$ in CBr_4 and its intuitive interpretation were reported by Symons and Smith,[2] followed by the controversial argument by Wang and Williams.[3] We have further shown that radical cations of a variety of alkanes can be stabilized in SF_6 and in perfluorocarbons as well as in fluorochlorocarbons irradiated at low temperature.[4-8] On the basis of the systematic studies by ESR spectroscopy, we have characterized structures and reactions of prototype radical cations of a series of alkanes including linear,[1,4,5] branched,[1,5,6] cyclo,[7] and

(1) Iwasaki, M.; Toriyama, K.; Nunome, K. *J. Am. Chem. Soc.* **1981**, *103*, 3591.
(2) (a) Smith, T. G.; Symons, M. C. R. *J. Chem. Res. (S)* **1979**, 382. (b) Symons, M. C. R. *Chem. Phys. Lett.* **1980**, *69*, 198.
(3) Wang, J. T.; Williams, F. *J. Phys. Chem.* **1980**, *84*, 3156.
(4) Toriyama, K.; Nunome, K.; Iwasaki, M. *J. Phys. Chem.* **1981**, *85*, 2149.
(5) (a) Toriyama, K.; Nunome, K.; Iwasaki, M. *J. Chem. Phys.* **1982**, *77*, 5891. (b) Iwasaki, M.; Toriyama, K.; Nunome, K. *Radiat. Phys. Chem.* **1983**, *21*, 147.
(6) (a) Nunome, K.; Toriyama, K.; Iwasaki, M. *J. Chem. Phys.* **1983**, *79*, 2499. (b) Nunome, K.; Toriyama, K.; Iwasaki, M. *Chem. Phys. Lett.* **1984**, *105*, 414.
(7) (a) Iwasaki, M.; Toriyama, K.; Nunome, K. *Faraday Discuss. Chem. Soc.* **1984**, *78*, 19. (b) Iwasaki, M.; Toriyama, K.; Nunome, K. *J. Chem. Soc., Chem. Commun.* **1984**, 143. (c) Ushida, K.; Shida, T.; Iwasaki, M.; Toriyama, K.; Nunome, K. *J. Am. Chem. Soc.* **1983**, *105*, 5496. (d) Toriyama, K.; Iwasaki, M.; Nunome, K. *J. Chem. Soc., Chem. Commun.* **1984**, 202. (e) Toriyama, K.; Nunome, K.; Iwasaki, M.; Shida, T.; Ushida, K. *Chem. Phys. Lett.* **1985**, *122*, 118.
(8) (a) Toriyama, K.; Iwasaki, M.; Nunome, K. *J. Chem. Soc., Chem. Commun.* **1983**, 1346. (b) Nunome, K.; Toriyama, K.; Iwasaki, M. *Tetrahedron* **1986**, *22*, 6315.

0002-7863/87/1509-4496$01.50/0