Kinetics and Equilibria of the Thiol/Disulfide Exchange Reactions of Somatostatin with Glutathione

Dallas L. Rabenstein* and Kim H. Weaver

Department of Chemistry, University of California, Riverside, California 92521

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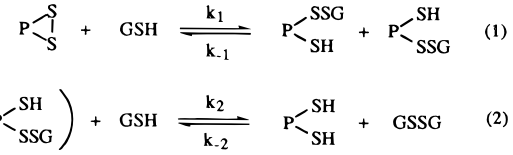
Rate and equilibrium constants are reported for the thiol/disulfide exchange reactions of the peptide hormone somatostatin with glutathione (GSH). GSH reacts with the disulfide bond of somatostatin to form somatostatin-glutathione mixed disulfides (Cys^3-SH, Cys^{14}-SSG and Cys^3-SSG, Cys^{14}-SH), each of which can react with another molecule of GSH to give the reduced dithiol form of somatostatin and GSSG. The mixed disulfides also can undergo intramolecular thiol/disulfide exchange reactions to re-form the disulfide bond of somatostatin or to interconvert to the other mixed disulfide. Analysis of the forward and reverse rate constants indicates that, at physiological concentrations of GSH, the intramolecular thiol/disulfide exchange reactions that re-form the disulfide bond of somatostatin are much faster than reaction of the mixed disulfides with another molecule of GSH, even though the intramolecular reaction involves closure of a 38-membered ring. Thus, even though the disulfide bond of somatostatin is readily cleaved by thiol/disulfide exchange, it is rapidly reformed by intramolecular thiol/disulfide exchange reactions of the somatostatin−glutathione mixed disulfides. By comparison with rate constants reported for analogous reactions of model peptides measured under random coil conditions, it is concluded that disulfide bond formation by intramolecular thiol/disulfide exchange in the somatostatin−glutathione mixed disulfides is not completely random, but rather it is directed to some extent by conformational properties of the mixed disulfides that place the thiol and mixed disulfide groups in close proximity. A reduction potential of −0.221 V was calculated for the disulfide bond of somatostatin from the thiol/disulfide exchange equilibrium constant.

Introduction

Disulfide bonds are structural elements in many biologically active peptides, including some peptide hormones and peptide toxins. For example, the nonapeptide hormones oxytocin (OT) and arginine vasopressin (AVP) both have a disulfide bond between cysteine residues at positions one and six, and the tetradecapeptide hormone somatostatin (SS) has a disulfide bond between cysteine residues at positions 3 and 14. In early reports, the biological activity of OT and AVP was accounted for in terms of a mechanism in which they interacted with their receptors by covalent bond formation via thiol/disulfide exchange reactions.[1] However, structure−activity studies have shown that the disulfide bond is not involved in the mechanism of action, but rather it is the cyclic arrangement of amino acids 1−6 that is essential for high biological activity.[2] The disulfide bonds in OT, AVP, and somatostatin serve to form and keep molecular conformations suitable for noncovalent interaction with their receptors.

Disulfide bonds are kinetically unstable structural elements, being readily cleaved by thiols via thiol/disulfide exchange.[3−7] In a previous study, we characterized the kinetics and equilibria for reduction and formation of the disulfide bonds of OT and AVP by thiol/disulfide exchange with glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) and oxidized glutathione (GSSG).[7,8] The reactions take place in two steps:



(1)

(2)

Rate constant k_1 was found to be 1−2 orders of magnitude larger than rate constants for thiol/disulfide exchange reactions of disulfide bonds between acyclic peptides. However, it was also found that, at physiological concentrations of GSH,[9] the intermediate mixed disulfides formed in this first step of the two-step reaction sequence undergo intramolecular thiol/disulfide exchange to re-form the native disulfide bonds much faster than they react with another molecule of GSH via the second reaction, even though the intramolecular thiol/disulfide exchange reactions involve closure of 20-membered rings. Furthermore, the rate constants for intramolecular thiol/disulfide exchange are significantly larger than reported for analogous reactions of model peptides under random coil conditions, which suggests that the AVP−GSH and OT−GSH mixed disulfides exist, at least part of the time, in precyclic conformations that place the thiol and mixed disulfide groups in close proximity.[7]

* To whom correspondence should be addressed. Phone: (909) 787-3585. Fax: (909) 787-4713. E-mail: dlrab@mail.ucr.edu.

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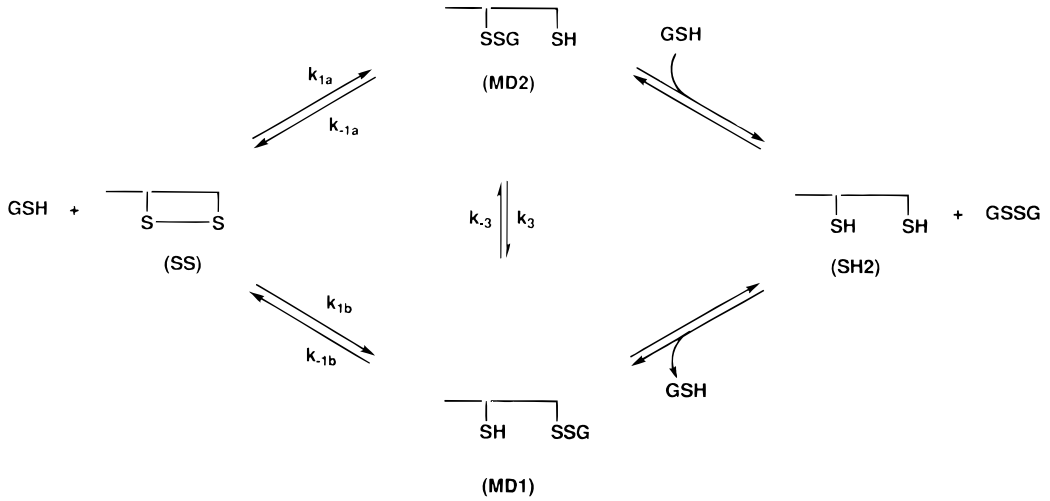

Figure 1. Thiol/disulfide exchange reactions of somatostatin (SS) with glutathione (GSH) and glutathione disulfide (GSSG). MD1 and MD2 are single somatostatin−glutathione mixed disulfides, with the disulfide bonds at Cys^{14} and Cys^{3} of somatostatin, respectively, and SH2 is the reduced, dithiol form of somatostatin.

In view of this finding, it is of interest to determine if the structurally important disulfide bonds in other peptide hormones are also rapidly reformed from their mixed disulfides by intramolecular thiol/disulfide exchange. In this paper, we report the results of a study of the kinetics and equilibria of the formation and reduction of the disulfide bond in somatostatin, which has an even larger (38 membered) disulfide-containing ring.

Ala-Gly-
Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Equilibrium constants and forward and reverse rate constants for the formation and reduction of the disulfide bond of somatostatin by thiol/disulfide exchange with the GSH/GSSG system were determined, including specific rate constants for the intramolecular thiol/disulfide exchange reactions of each of the two possible mixed disulfides. Also, the redox potential of the disulfide bond of somatostatin was determined from the equilibrium constant for its thiol/disulfide exchange reaction with GSH.

Results

The thiol/disulfide exchange reactions of somatostatin (SS) with GSH and GSSG are summarized in Figure 1. GSH reacts with the disulfide bond of SS to form a mixed disulfide (MD1 or MD2), which in turn reacts with another molecule of GSH to form reduced somatostatin (SH2) and GSSG. The reactions are reversible, and GSSG reacts with the thiol groups of SH2 to form MD1 or MD2, which in turn undergo intramolecular thiol/disulfide exchange to form the disulfide bond of SS. In addition, the mixed disulfides can interconvert by intramolecular thiol/disulfide exchange and they can react with another molecule of GSSG to form the double mixed disulfide (DMD) (not shown in Figure 1). Equilibrium constants were determined for each of the steps shown in Figure 1 and for formation of the double mixed disulfide. The kinetics of the reaction of SS with GSH to form MD1 and MD2 and of the intramolecular thiol/disulfide exchange reactions of MD1 and MD2 were characterized in terms of the rate constants shown in Figure 1, while the kinetics of the reaction of the mixed disulfides with another molecule of GSH and of SH2 with GSSG to form the mixed disulfides were characterized in terms of the rate constants defined by eq 2.

Thiol/Disulfide Exchange Equilibrium Constants. The equilibrium constants defined by eqs 3−11 were determined at 25 °C in pH 7.0 sodium phosphate buffer (0.075 M).

$$K_{1a} = \frac{[\mathrm{MD2}]}{[\mathrm{SS}][\mathrm{GSH}]} \tag{3}$$

$$K_{1b} = \frac{[\mathrm{MD1}]}{[\mathrm{SS}][\mathrm{GSH}]} \tag{4}$$

$$K_{2a} = \frac{[\mathrm{SH2}][\mathrm{GSSG}]}{[\mathrm{MD2}][\mathrm{GSH}]} \tag{5}$$

$$K_{2b} = \frac{[\mathrm{SH2}][\mathrm{GSSG}]}{[\mathrm{MD1}][\mathrm{GSH}]} \tag{6}$$

$$K_{1} = \frac{[\mathrm{MD1}] + [\mathrm{MD2}]}{[\mathrm{SS}][\mathrm{GSH}]} \tag{7}$$

$$K_{2} = \frac{[\mathrm{SH2}][\mathrm{GSSG}]}{([\mathrm{MD1}]+[\mathrm{MD2}])[\mathrm{GSH}]} \tag{8}$$

$$K_{\mathrm{ov}} = \frac{[\mathrm{SH2}][\mathrm{GSSG}]}{[\mathrm{SS}][\mathrm{GSH}]^2} \tag{9}$$

$$K_{3} = \frac{[\mathrm{MD1}]}{[\mathrm{MD2}]} \tag{10}$$

$$K_{4} = \frac{[\mathrm{DMD}][\mathrm{GSH}]^2}{[\mathrm{SH2}][\mathrm{GSSG}]^2} \tag{11}$$

The concentrations of SS and SH2 and of the two somatostatin−glutathione mixed disulfides were determined in equilibrium mixtures by HPLC. A typical chromatogram is shown in Figure 2. The peaks for SS and SH2 were assigned by comparison with chromatograms of each compound. The peaks labeled MD1 and MD2 were assigned previously by ^{1}H NMR to the specific

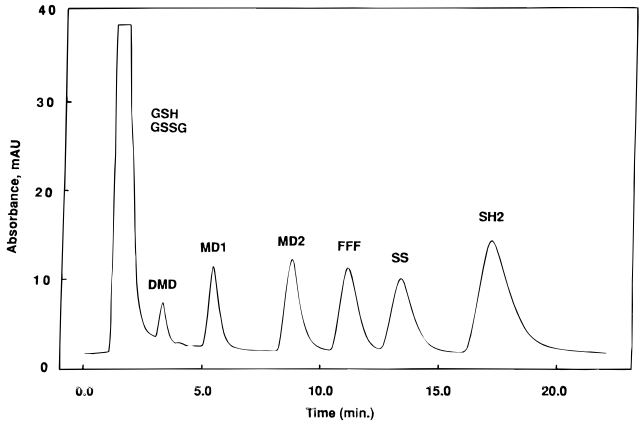

Figure 2. Chromatogram of a somatostatin/GSH reaction mixture at equilibrium (25 °C, pH 7.0 phosphate buffer). The peak labels are defined in the text. The concentrations determined from the peak areas are 5.73 μM SS, 14.9 μM SH2, 3.18 μM MD1, and 5.73 μM MD2. The concentration of the internal standard FFF was 15.0 μM. The mobile phase contained pH 3.0 phosphate buffer (0.1 M) and 26% acetonitrile.

somatostatin−glutathione mixed disulfides[10]

Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
|
γ–Glu–Cys–Gly

(MD1)

Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
|
γ–Glu–Cys–Gly

(MD2)

and the peak labeled DMD is for the double mixed disulfide

Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
| |
γ–Glu–Cys–Gly γ–Glu–Cys–Gly

(DMD)

The peak labeled FFF is for (phenylalanyl)(phenylalanyl)-phenylalanine, which was added as an internal intensity standard.

To establish that the reactions are reversible, equilibrium was approached from both directions.[11] After a solution prepared by combining stock solutions of GSH, GSSG, and somatostatin had reached equilibrium, as indicated by HPLC analysis, the equilibrium was shifted twice, first by addition of additional GSH and then by addition of additional GSSG. After each addition, aliquots of the resulting solutions were analyzed as a function of time until there was no further change in concentration. Equilibrium constants calculated using concentrations from the three separate equilibrium conditions were equal within experimental error. For example, the three values determined for K_1 from such an experiment were 121, 128, and 129 M^{-1} and the values determined for K_2 were 20.6, 20.8, and 22.4 M^{-1}. These results verify that the thiol/disulfide exchange reactions are reversible under the conditions used and that equilibrium was reached. The average values obtained for the equilibrium constants from 20 separate experiments over a range of reactant concentrations are reported in Table 1. Also listed for comparison in Table 1 are literature values for the analogous equilibrium constants for the OT/GSH and AVP/GSH systems.[7]

Table 1. Equilibrium Constants for Thiol/Disulfide Exchange Reactions of Somatostatin, Arginine Vasopressin, and Oxytocin with Glutathione

	somatostatin[a−c]	arginine vasopressin[d]	oxytocin[d]
K_{1a}, M^{-1}	71 ± 9		
K_{1b}, M^{-1}	50 ± 6		
K_1, M^{-1}	121 ± 13	60	140
K_{2a}	0.35 ± 0.03		
K_{2b}	0.49 ± 0.06		
K_2	0.20 ± 0.02	0.24	0.26
K_{ov}, M^{-1}	24.7 ± 3.5	14.5	36
K_3	0.71 ± 0.06		
K_4	7 ± 2		
$E^{o\prime}$, V	−0.221	−0.228	−0.216

[a] 25 °C and 0.075 M pH 7.00 phosphate buffer. [b] Reactant concentrations covered the following ranges: 8−30 μM for the total somatostatin concentration; 4−18 mM for the GSH concentration; and 0.4−2 mM for the GSSG concentration. [c] The average relative standard deviation of the equilibrium constants is 13%. [d] 25 °C and pH = 7.00; 0.15 M KCl.[7]

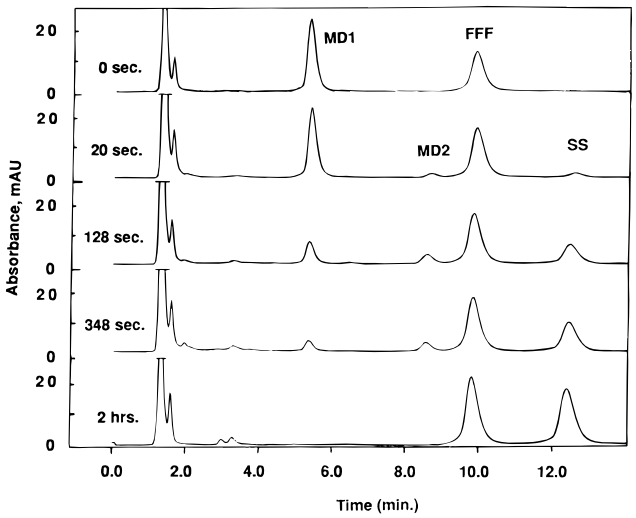

Figure 3. Chromatograms of samples taken as a function of time after raising the pH of an ~4 μM solution of MD1 and internal standard FFF to 7.0.

Kinetics of Thiol/Disulfide Exchange Reactions. Rate constants k_{-1a} and k_3 and rate constants k_{-1b} and k_{-3} (Figure 1) were determined directly by monitoring the conversion of MD2 and MD1, respectively, to SS and the other mixed disulfide. The procedure involved increasing the pH of solutions of MD1 or MD2 (at concentrations in the 1−10 μM range) from pH ~2.5, where thiol/disulfide exchange is very slow, to pH 7.0. Aliquots were then removed as a function of time, quenched and analyzed by HPLC. Representative chromatograms from a study of the conversion of MD1 to MD2 and SS are presented in Figure 3. At time 0, the chromatogram only shows peaks for MD1 and the internal intensity standard FFF. In the chromatogram for the aliquot taken at 20 s, peaks are also observed for both MD2 and SS, indicating that the cyclization reaction to form SS and the isomerization reaction to form MD2 are taking place at similar rates. As the reaction proceeds, the SS peak increases in intensity, and eventually both MD1 and the MD2 formed from MD1 are converted to SS. Since the solution only contains MD1 at $t = 0$, the cyclization and

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Table 2. Rate Constants for Thiol/Disulfide Exchange Reactions of Somatostatin, Arginine Vasopressin, and Oxytocin with Glutathione[a]

	somatostatin[a]	arginine vasopressin[b]	oxytocin[b]
k_{1a}, M^{-1} s^{-1}	0.39 ± 0.09		
k_{-1a}, s^{-1}	0.0055 ± 0.0011		
k_{1b}, M^{-1} s^{-1}	0.49 ± 0.18		
k_{-1b}, s^{-1}	0.0097 ± 0.0034		
k_1, s^{-1}	0.88 ± 0.20	38	110
k_{-1}, s^{-1}	0.0072 ± 0.0020	0.63	0.76
k_3, s^{-1}	0.0030 ± 0.0006		
k_{-3}, s^{-1}	0.0039 ± 0.0009		
k_2, M^{-1} s^{-1}	1.83 ± 0.22	0.74	0.84
k_{-2}, M^{-1} s^{-1}	9.5 ± 0.6	3.1	3.2

[a] 25 °C, pH 7.00, 0.15 M phosphate buffer. [b] 25 °C, pH 7.00, 0.15 M KCl.[7]

Table 3. Intrinsic Equilibrium and Rate Constants for Thiol/Disulfide Exchange Reactions of Somatostatin with Glutathione

equilibrium constants		rate constants	
K^i_{1a}, M^{-1}	7.3	k^i_{1a}, M^{-1} s^{-1}	33.6
K^i_{1b}, M^{-1}	290	k^i_{-1a}, s^{-1}	4.58
K^i_1, M^{-1}	297	k^i_{1b}, M^{-1} s^{-1}	42.2
K^i_{2a}	0.036	k^i_{-1b}, s^{-1}	0.147
K^i_{2b}	2.8	k^i_3, s^{-1}	2.50
K^i_2	0.0355	k^i_{-3}, s^{-1}	0.059
K^i_{ov}, M^{-1}	10.5		
K^i_3	42.4		

interconversion reactions are the only reactions that are taking place initially, and thus, k_{-1b} and k_{-3} were obtained directly from the initial rates of formation of SS and MD2, respectively. Values determined for k_{-1a}, k_3 and k_{-1b}, k_{-3} from multiple experiments with MD2 and MD1, respectively, are presented in Table 2. Also listed in Table 2 are values for k_{1a} and k_{1b}, which were calculated from k_{-1a} and K_{1a} and k_{-1b} and K_{1b} using the relations $k_{1a} = K_{1a}k_{-1a}$ and $k_{1b} = K_{1b}k_{-1b}$.

The value listed in Table 2 for rate constant k_1 (eq 1) was calculated from k_{1a} and k_{1b} using the relation $k_1 = k_{1a} + k_{1b}$. Rate constant k_1 was also estimated from the initial rate of disappearance of SS by reaction with GSH. The procedure involved addition of GSH to a solution of SS and FFF, and then aliquots were removed as a function of time, quenched, and analyzed by HPLC. SS concentrations in the range 16−34 μM were used. To approximate pseudo-first-order reaction conditions, GSH concentrations in the 2−10 mM range were used. Because the rate of the reaction was found to be too fast at pH 7.0 to follow accurately by removing and quenching aliquots as a function of time, the reaction was run at lower pH where a smaller fraction of the GSH is in the reactive thiolate form. Rate constant k_1 was determined from the initial rate measured in 12 experiments in the pH range 5.3−6.2. Each of these rate constants was then used to estimate a value for k_1 at pH 7.00 by accounting for the increased concentration of the reactive thiolate form at pH 7.00; a pK of 8.93 was used for the thiol group of GSH.[12] An average value of 2.00 ± 0.55 M^{-1} s^{-1} was estimated for k_1 at pH 7.00.

Rate constant k_{-2} (eq 2) was determined by reaction of reduced somatostatin with GSSG at pH 7.0. The reduced somatostatin was prepared by electrochemical reduction of a SS/FFF solution.[13] The procedure for determination of k_{-2} involved addition of GSSG to the resulting solution of SH2/FFF at pH 7.0, and then aliquots were removed as a function of time, quenched, and analyzed by HPLC. Rate constant k_{-2} was determined from the initial rate of the reaction. The value reported in Table 2 is the average from six experiments in which the concentration of SH2 and GSSG were in the range of 20−50 μM and 0.3−0.5 mM, respectively. The value listed for rate constant k_2 was calculated from k_{-2} and K_2.

Discussion

Thiol/disulfide exchange occurs by an S_N2 displacement mechanism in which a thiolate anion, the reactive form of the thiol in thiol/disulfide exchange reactions, approaches the disulfide bond along its S−S axis.[3,5,14,15] The equilibrium and rate constants reported in Tables 1 and 2 are for pH 7.00, where only a fraction of the thiol groups of GSH, MD1, MD2, and the dithiol form of somatostatin are in the thiolate form, and thus they are conditional constants for pH 7.00. pH-independent, intrinsic equilibrium, and rate constants for the reactions in terms of thiolate species are reported in Table 3. The intrinsic equilibrium constants, K^i, were calculated using the values reported in Table 1 by accounting for the fractional thiolate concentrations at pH 7.00 with equations of the type $K^i_{1a} = (\alpha_{MD2}/\alpha_{GSH})K_{1a}$ where α is the fraction of the thiol group of the compound indicated in the thiolate form at pH 7.00. α was calculated to be 0.0116 for GSH, 0.0661 for the Cys^3 thiol group of MD1, 0.001 20 for the Cys^{14} thiol group of MD2 using pK_a values of 8.93 for GSH, 8.15 for the Cys^3 thiol group of MD1, and 9.92 for the Cys^{14} thiol group of MD2.[12,16,17] The fraction of the dithiol form of somatostatin with both thiol groups ionized was calculated to be 7.9×10^{-5} using pK_a values of 8.15 and 9.92 for the Cys^3 and Cys^{14} thiol groups. The intrinsic rate constants were calculated from the conditional rate constants in Table 2 using relationships of the type $k^i_{1a} = k_{1a}/\alpha_{MD2}$.

Kinetic Stability of the Disulfide Bond of Somatostatin. A main objective of this research was to characterize the kinetic stability of the disulfide bond of somatostatin with respect to cleavage by thiol/disulfide exchange. The results in Table 2 indicate that the disulfide bond of somatostatin is readily cleaved by a two-step reaction with GSH and that the rate constants for its two possible reactions with GSH in the first step (k_{1a} and k_{1b} in Figure 1) are essentially equal. Also, they are of a magnitude that is typical of rate constants for reaction of thiol-containing amino acids and peptides with intermolecular peptide disulfide bonds, which suggests that, even though it is part of a ring system, the disulfide bond of somatostatin is not particularly unstable and it reacts as a normal disulfide bond. For comparison, rate constants for the second-order reaction of GSH and cysteine with the disulfide bond of GSSG at pH 7.0 are 0.41 and 0.33 M^{-1} s^{-1}, respectively.[6] This is in contrast

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(16) The pK_a's of the mixed disulfides of somatostatin cannot be measured due to intramolecular thiol/disulfide exchange. The pK_a of Cys^3 was estimated to be 8.15 using the pK_a value reported for Cys^6 of arginine vasopressin in D_2O.[17] The pK_a of Cys^{14} was estimated to be 9.92 using the pK_a's reported for Cys^6 of tocinoic acid in D_2O.[17] The pK_a values for D_2O solution were converted to H_2O solution using the relation $pK_a(H_2O) = pK_a(D_2O) - 0.50$.[6]
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to the rate constants measured for reaction of GSH with the disulfide bonds of arginine vasopressin and oxytocin (k_1, eq 1), which are significantly larger than rate constants for typical thiol/disulfide exchange reactions,[7] and they are larger than rate constant k_1 for somatostatin.[18]

With respect to the kinetic stability of somatostatin, it is important to consider the fate of the mixed disulfides formed in the first step. The mixed disulfides can react with another molecule of GSH to give fully reduced somatostatin in the second step or they can undergo intramolecular thiol/disulfide exchange to re-form the disulfide bond of somatostatin. Rate constants k_{-1} and k_2 (eqs 1 and 2) can be used to determine the relative tendencies for reaction by these two pathways if k_2 is converted to the pseudo-first-order rate constant k_2' (= k_2[GSH]). Using the results in Table 2 for somatostatin, it can be shown that $k_{-1} > k_2'$ when [GSH] < 0.004 M; i.e., intramolecular thiol/disulfide exchange to re-form the disulfide bond of somatostatin is faster than reaction with another molecule of GSH when [GSH] < 0.004 M. This suggests that in human blood plasma, where the concentration of GSH and other nonprotein thiols is much less than 0.004 M,[9,19] mixed disulfides of somatostatin will tend to undergo intramolecular thiol/disulfide exchange to re-form the native disulfide bond rather than react with another molecule of thiol to give the reduced, dithiol form of somatostatin. That is, even though the disulfide bond of somatostatin is susceptible to cleavage by thiol/disulfide exchange, biologically active somatostatin is readily re-formed by intramolecular thiol/disulfide exchange. This is an important concept to consider when investigating the behavior of other disulfide-containing peptide hormones in biological systems and the use of disulfide bonds as structural elements in peptide and peptidomimetic drugs.

A similar analysis using the rate constants in Table 2 for arginine vasopressin and oxytocin indicates that the tendency for formation of their disulfide bonds by intramolecular thiol/disulfide exchange is even greater.[7] Specifically, $k_{-1} > k_2'$ when [GSH] < 0.85 and 0.90 M for arginine vasopressin and oxytocin, respectively. This even greater tendency for intramolecular thiol/disulfide exchange is most likely because smaller rings are formed by the disulfide bond in arginine vasopressin and oxytocin (20-membered as compared to the 38-membered ring of somatostatin). Nevertheless, the rate constant k_{-1} for somatostatin is surprisingly large. This can be seen, for example, by comparison of k_{-1} to rate constants reported for the analogous reactions of a homologous series of peptides of the type Cys-(Ala)$_n$-Cys, where n varies from 1 to 5, which corresponds to ring sizes of 11, 14, 17, 20, and 23 atoms for the disulfide form of the peptides.[20] The rate constants for the homologous series were measured under conditions where the mixed disulfides are random coil, and they were reported as intrinsic rate constants, k^i_{-1}, i.e., rate constants for conditions where the reacting thiol groups are completely in the deprotonated thiolate form. For purposes of comparison with the values reported in Table 2, we have converted them to conditional rate constants at pH 7.0 by accounting for the protonation state of the thiol group at pH 7.0 using the relation $k_{-1} = \alpha k^i_{-1}$, where α is the fraction in the thiolate form.[21] The values calculated for k_{-1} are 0.06, 0.12, 0.011, 0.011, and 0.0068 s^{-1} for $n = 1$, 2, 3, 4, and 5, respectively. Comparison with the values listed in Table 2 indicates that k_{-1} for arginine vasopressin and oxytocin is some 60−70 times larger than k_{-1} for the $n = 4$ random coil homolog, which also forms a 20-membered ring. No rate constants are available for homologs that form a 38-membered ring for comparison with k_{-1} for somatostatin. However, k_{-1} for somatostatin is essentially the same as k_{-1} for the $n = 5$ homolog, which forms a 23-membered ring; considering the steady decrease in k_{-1} for the series of random coil homologs as the size of the ring increases, it seems likely that k_{-1} for reaction of a random coil mixed disulfide to form a 38-membered ring would be significantly less than is found for somatostatin. That is, this analysis suggests that disulfide bond formation by intramolecular thiol/disulfide exchange in the somatostatin−glutathione mixed disulfides is not completely random, but rather it is directed to some extent by conformational properties of the mixed disulfides that place the thiol and the mixed disulfide groups in close proximity.

That some fraction of the somatostatin−glutathione mixed disulfides exist in precyclic conformations is supported by evidence from ^{1}H NMR studies of the two somatostatin−glutathione mixed disulfides.[10] ^{1}H NMR results, as well as results from semiempirical energy calculations and time-resolved fluorescence spectroscopy experiments, indicate that the cyclic disulfide form of somatostatin exists as an equilibrium of several rapidly interconverting, low-energy conformations, including conformations that have β_{II} turns over Trp^8-Lys^9 and Thr^{10}-Phe^{11}.[10,22−27] Nuclear Overhauser enhancement (NOE) data and the temperature coefficients of selected NH chemical shifts, together with chemical shift data for C_αH and NH protons, indicate that the mixed disulfides are interconverting between multiple conformations, some of which have secondary structure, and that some elements of the secondary structure are similar to those of the cyclic disulfide form of somatostatin.[10]

Kinetics of Intramolecular Thiol/Disulfide Exchange Reactions of Somatostatin−Glutathione Mixed Disulfides. The rate constants in Table 2 are for pH 7.00, where only a small fraction of the various thiol groups are in the reactive thiolate form. The pK_a values of 8.15 for the Cys^3 thiol group of MD1 and 9.92 for the Cys^{14} thiol group of MD2[16] indicate that a much smaller fraction of MD2 will be in the thiolate form at pH 7.0, and thus, k_{-1a} is predicted to be much less than k_{-1b}. However, rate constants for thiol/disulfide exchange also depend on the nucleophilicity of the thiolate anion,

(18) The comparison is made in terms of k_1 because values have not been reported for k_{1a} and k_{1b} for arginine vasopressin and oxytocin.

(19) The concentrations of cysteine, cysteinglglycine, and homocysteine in human plasma are in the region of 9, 3, and 0.25 μM, respectively.[9b]

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as indicated by its Bronsted basicity, and the Bronsted basicities of the central and leaving group sulfur atoms.[3]

The dependence on the Bronsted basicity of the thiolate nucleophile and the central and leaving group sulfurs is given by eq 12

$$\log k^{i} = C + \beta_{nuc} pK_a^{nuc} + \beta_c pK_a^{c} + \beta_{lg} pK_a^{lg} \quad (12)$$

where k^i is the intrinsic rate constant.[3] The constant C and the Bronsted coefficients β_{nuc}, β_c, and β_{lg} have been estimated to be 7.0, 0.50, −0.27, and −0.73, respectively, using rate data for intermolecular thiol/disulfide exchange reactions.[3] While these values for C and the Bronsted coeficients are not directly applicable to rate constants for intramolecular thiol/disulfide exchange, they can be used to account qualitatively for some features of the rate constants in Tables 2 and 3. For example, k^i_{-1a} is predicted by eq 12 to be significantly larger than k^i_{-1b}, because the Bronsted basicity of the thiolate anion of MD2 is larger and that of the central sulfur is smaller in the reaction described by k^i_{-1a}. Likewise, k^i_3 is predicted to be much larger than k^i_{-3}, as observed, because the Bronsted basicity of the thiolate nucleophile is larger and that of the leaving group is smaller in the reaction described by k^i_3. Thus, the smaller fraction of Cys^{14} in the thiolate form is compensated for by the greater nucleophilicity of the Cys^{14} thiolate anion and the dependence of thiol/disulfide exchange rate constants on the Bronsted basicity of the central and leaving group sulfur atoms, with the result that k_{-1a} and k_{-1b}, and k_3 and k_{-3}, are similar in magnitude.

Thiol/Disulfide Exchange Equilibria and the Redox Potential of the Somatostatin Disulfide Bond. Even though the disulfide bond in somatostatin is part of a much larger ring system, and the rate constants for its thiol/disulfide exchange reactions with GSH are significantly different from those of arginine vasopressin and oxytocin, the stepwise and overall equilibrium constants for its thiol/disulfide exchange reactions with GSH are essentially the same as those for arginine vasopressin and oxytocin at pH 7.00. The equilibrium constants are ratios of rate constants, i.e., $K_1 = k_1/k_{-1}$, $K_2 = k_2/k_{-2}$, and $K_{ov} = (k_1k_2)/(k_{-1}k_{-2})$. Rate constant k_1 for reaction of GSH with somatostatin is significantly less than for reaction with AVP and oxytocin, but k_{-1} is also much less with the apparently fortuitous result that their ratio, and thus K_1, is essentially the same for somatostatin, AVP, and oxytocin. The rate constant for the second step in the overall reduction reaction, k_2, is the same within a factor of 2−3 for the three compounds, as is rate constant k_{-2}, as might be expected since both k_2 and k_{-2} are for thiol/disulfide exchange reactions of acyclic compounds.[6] Thus, K_2 and K_{ov} are also similar for somatostatin, AVP, and oxytocin. It also is of interest to note that the equilibrium constants are similar in magnitude to those for the reaction of two one-disulfide analogs of apamin, in each of which two of the four cysteines of apamin are replaced by serine.[28] The two cysteines in the two analogs are separated by either 9 or 11 amino acids, as compared to 10 for somatostatin.

The equilibrium constant for the overall reaction of somatostatin with GSH is related to the half-cell potential for its disulfide bond:

$$E^{\circ\prime}_{SS/SH2} = E^{\circ\prime}_{GSSG/GSH} + (RT/nF) \ln K_{ov} \quad (13)$$

A value of −0.221 V was calculated for $E^{\circ\prime}_{SS/SH2}$ using the value reported in Table 1 for K_{ov} and $E^{\circ\prime}_{GSSG/GSH} = -0.262$ V.[29]

$E^{\circ\prime}_{SS/SH2}$ is similar to $E^{\circ\prime}$ for the disulfide bonds of AVP and oxytocin (−0.228 and −0.216 V, respectively), as expected since K_{ov} is similar for somatostatin, AVP, and oxytocin. It is also of interest to note that $E^{\circ\prime}_{SS/SH2}$ is also similar to $E^{\circ\prime}$ values reported for the disulfide bonds of octapeptide models for the active sites of the thiol−protein oxidoreductases thioredoxin, thioredoxin reductase, glutaredoxin, and protein disulfide isomerase, even though the model peptides form smaller (14-membered) disulfide-containing rings. The redox potentials of the model peptides, all of which are of the general sequence Ac-X-Cys-X-X-Cys-X-X-X-NH_2, are −0.212, −0.232, −0.237, and −0.227 V, respectively.[30] It will be of interest to determine the effect of the kinetics of the intramolecular thiol/disulfide exchange reactions on the equilibrium constants and redox potentials for the model peptides, as was done above for somatostatin, AVP, and oxytocin.

Finally, $E^{\circ\prime}$ values for a series of nonpeptide molecules that form 5−11 membered disulfide-containing rings range from −0.354 V to −0.240 V.[31] For example, the $E^{\circ\prime}$ value for dithiothreitol (DTT), lipoic acid, and 6,6′-sucrosedithiol, which form 6-, 5-, and 11-membered rings, are −0.327, −0.288, and −0.245 V, respectively. $E^{\circ\prime}$ for somatostatin is less reducing; however, it is larger than might be expected, considering that formation of the disulfide bond of somatostatin involves closure of a 38-membered ring.

Experimental Section

Chemicals. Somatostatin was obtained from Bachem, Inc., Torrance, CA. Glutathione, glutathione disulfide, dithiothreitol (DTT), and FFF were supplied by Sigma Chemical Co. HPLC-grade acetonitrile and reagent grade phosphoric acid were purchased from Fisher Scientific Co. The peptide content of the GSH, GSSG, and somatostatin was determined by 500 MHz ^{1}H NMR using an internal intensity standard. The GSH was found to be 99.7% GSH and 0.3% GSSG. The GSSG was found to be 95% GSSG; the remainder is assumed to be water because no extra resonances were observed in the ^{1}H NMR spectrum. The somatostatin was determined to be 68.7% somatostatin, with the balance being trifluoroacetate and water.

Reduced somatostatin was prepared either by reduction of somatostatin with DTT or by electrochemical reduction under nitrogen in a glove bag.[13] Somatostatin was dissolved in 0.15 M KCl to give concentrations of 20−50 μM, FFF stock solution was added, and the solution was then reduced at −1.0 V using a three-electrode system (a Hg pool working electrode, an Ag/AgCl reference electrode in saturated KCl and a Pt auxiliary electrode in 0.15 M KCl). After 25−30 min, 90−95% of the somatostatin was reduced as determined by HPLC.

Synthesis and Isolation of Mixed Disulfides. The two somatostatin−glutathione mixed disulfides were synthesized by reacting somatostatin with a mixture of GSH and GSSG. Typical concentrations were 0.5−2 mM GSSG, 1−8 mM GSH, and 100−400 μM somatostatin. Mixtures were allowed to react for ∼5 min at pH 7.0 and then were quenched by lowering the pH to 2.5. The best yields of mixed disulfide were obtained with GSH:GSSG ratios of 5:1 to 10:1.

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The mixed disulfides were isolated by reversed-phase HPLC on a 10 mm × 250 mm C18 reversed-phase column. Because the mixed disulfides readily undergo intramolecular thiol/disulfide exchange reactions at neutral pH, they were isolated at pH 2.5 with a mobile phase containing 0.1% trifluoroacetic acid in acetonitrile (29%) and water. Because less than 20% of the somatostatin was converted to mixed disulfide in the reaction, the native disulfide and reduced dithiol forms of somatostatin were also collected and reacted again with a mixture of GSH and GSSG to form more mixed disulfide. The isolated mixed disulfides were lyophilized, rechromatographed to increase purity, and then stored in dry ice until used.

Kinetic and Equilibrium Studies. Reaction mixtures were analyzed by HPLC on a 3.2 mm × 100 mm C18 reversed-phase column (particle size 3 μm) and a 15 mm × 3.2 mm guard column. The detector was set at 215 nm. Chromatographic conditions were optimized by varying the pH, the percent acetonitrile, and the concentration of phosphate in the mobile phase. A pH 3.0 mobile phase containing 26% acetonitrile and 0.1 M phosphate buffer was found to give good separation of the reduced, dithiol, and mixed disulfide forms of somatostatin and the internal standard FFF. The mobile phase was prepared by adding phosphoric acid and acetonitrile to water that had been purified with a Millipore water purification system and adjusting the pH with sodium hydroxide. The mobile phase was filtered through a 0.5 μm filter to remove any particulates. The mobile phase was sparged daily with helium to remove dissolved gas to prevent postcolumn degassing, which caused a noisy base line.

The response of the detector was calibrated by measuring the ratio of analyte to internal standard peak areas vs the concentration of analyte for somatostatin and reduced somatostatin.[11] Somatostatin and reduced somatostatin concentrations ranged from 0.1 to 100 μM and 0.1 to 50 μM, respectively. The concentration of FFF was 19−21 μM. The reduced somatostatin solutions used to calibrate detector response were prepared by reducing somatostatin with DTT. Detector response was found to be linear over the above concentration ranges for somatostatin and reduced somatostatin. The limit of detection for somatostatin was determined to be ~18 nM at a signal-to-noise ratio of 3, which corresponds to ~0.36 pmol. Detector calibration factors were determined for each mixed disulfide by measuring a chromatogram for an aliquot of a solution of the mixed disulfide. The pH of the remaining solution was then increased to allow the mixed disulfide to convert to the native disulfide form by intramolecular thiol/disulfide exchange, and a chromatogram was measured for an aliquot of the solution. The calibration factor for the mixed disulfide was determined from the relative peak areas for the cyclic and mixed disulfide forms and the calibration factor for the cyclic disulfide.

All solutions used in the kinetic and equilibrium studies were deoxygenated by bubbling with oxygen-scrubbed argon through a glass dispersion tube. To exclude oxygen, experiments were conducted in a nitrogen-filled glovebag. Samples were then removed from the glovebag and analyzed by HPLC. The general procedure used in the kinetic experiments has been described previously.[7,11] To summarize the procedure for measurement of k_1, aliquots of GSH, somatostatin, and FFF standard solutions in pH 5.6−5.7 buffer (0.0375 M phosphate, 0.0375 M acetate) were combined. Samples were then removed as a function of time, quenched by lowering the pH to 2.5, and analyzed by HPLC. The procedure for determining k_{-2} was similar and involved mixing aliquots of standard solutions of GSSG, reduced somatostatin, and FFF in 0.15 M pH 7.0 buffer.

Equilibrium constants for the thiol/disulfide exchange reactions were determined at pH 7.0 in 0.075 M phosphate buffer by measuring the concentrations of the native disulfide, reduced dithiol, and mixed disulfide forms of somatostatin in solutions containing known, excess concentrations of GSH and GSSG, i.e., in solutions containing a GSH/GSSG redox buffer.[11] The procedure involved combining appropriate volumes of standard solutions; the reaction mixture was then allowed to react for 1 h, after which time an aliquot was removed, quenched, by lowering the pH with HCl to minimize air oxidation after removal from the glove bag, and analyzed by HPLC. After an additional 20 min, another aliquot was removed, quenched, and analyzed by HPLC. This procedure was repeated two more times to ensure that equilibrium was achieved, as indicated by no further change in concentration with time. The equilibrium concentrations of somatostatin, reduced somatostatin, and the two somatostatin−glutathione mixed disulfides were determined from the HPLC analysis, while the equilibrium concentrations of GSH and GSSG were calculated from their initial concentrations and the equilibrium concentrations of the various forms of somatostatin.

Kinetics experiments were conducted by combining stock solutions of the reactants, which were equilibrated at 25 °C before combining and were maintained at 25 °C throughout the experiment. Aliquots were removed as a function of time and quenched by lowering the pH to 2.5. The quenched solutions were then analyed by HPLC.

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