Disulfide Bond Formation in Peptides by Dimethyl Sulfoxide. Scope and Applications

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Abstract: A selective and efficient method for disulfide bond formation in peptides by dimethyl sulfoxide (DMSO) is described. Facile disulfide bond formation by DMSO in aqueous buffered solutions was found to proceed in a wide range of pH. More importantly, it overcame the limitation of the conventional oxidation method with air or mixed disulfide that was applicable only at a narrow basic pH range. The sulfur–sulfur bond reaction by DMSO was selective, and no side reactions were observed with nucleophilic amino acids such as Met, Trp, or Tyr. Because of its widely applicable pH range, the DMSO oxidation method was particularly suitable for basic and hydrophobic peptides. Monocyclic disulfide formation by 20% DMSO was observed to be completed in 0.5–4 h in a series of basic and hydrophobic peptides with ring sizes varying from 6 to 11 amino acids, while similar experiments by air oxidation at basic pH required longer duration and produced incomplete reactions. A detailed kinetic study on ten peptides showed that the DMSO oxidation method was pH-independent between pH 3 and 8 with pseudo-first-order rates ranging from 0.012 to 0.14 min^{-1}. However, with peptides containing a cysteine at the amino terminus, the rates became pH-dependent with an optimal pH near neutrality. Equally facile oxidations by DMSO were observed with the basic and hydrophobic, tricyclic 29-residue human defensin. In contrast, air oxidation at basic pH of human defensin led to extensive precipitation and low yield. Our results show that DMSO is a versatile and useful oxidizing agent for peptides at a wide range of pH and may be particularly suitable for renaturation and oxidation of proteins at controlled pH.

Introduction

Disulfides in proteins play an important role in the maintenance of biological activity and conformational stability. Because of its importance, many studies have focused on the roles of disulfides as constraints to increase biological activity[1,2] and as transient intermediates in protein folding.[3,4] For these studies, it is important that these peptides or proteins with disulfides are readily accessible by the chemical synthesis. The chemical synthesis of a peptide or a protein containing one or more disulfide bonds, requires as the final step, the formation of these disulfide bonds of cysteine residues. A general scheme common to both the solution and solid-phase syntheses is the simultaneous folding and disulfide formation of the fully deblocked molecule in an aqueous solution by a mild oxidant to form the desired product with the correct disulfide bonds.[5,6] However, the problems associated with the formation of disulfide bonds have largely been overlooked.

Among the conventional methods for the formation of disulfide bonds, air oxidation in aqueous medium is the most commonly used.[5-7] Air oxidation usually requires a long duration in basic or neutral pH for completion and a high dilution of peptide or protein concentration to be effective. Nevertheless, it enjoys an advantage that it produces a harmless byproduct as H_2O in the reaction. A variation of the air oxidation method is the thiol–disulfide interchange reaction with a mixture of reduced and oxidized glutathiones.[7] The mixed disulfide interchange method is usually effective at the basic range of pH. Because the air oxidation and the mixed disulfide interchange method are slow processes, they allow equilibrations of different conformers to produce thermodynamic-controlled products. In contrast, stronger oxidizing agents such as I_2 and $K_3Fe(CN)_6$ that produce kinetic-controlled products are often used for simple peptides containing only a single disulfide bond.[8,9] These sulfur–sulfur-forming agents are such powerful oxidants that the oxidations are usually performed cautiously to prevent overoxidation. However, they have the advantage of being applicable in the acidic range but suffer from the limitation that byproducts generated usually require purification. Several nucleophilic amino acids such as Met, Tyr, Trp, and His are particularly susceptible to these strong oxidants.[10] Because of these limitations, strong oxidizing agents are seldomly used for the simultaneous refolding and disulfide formation of multidisulfide bonded peptides or proteins.

In general, the oxidation methods with air or mixed disulfides are satisfactory for most syntheses, particularly for those acidic peptides or proteins.[11] However, for basic and hydrophobic peptides that tend to aggregate and precipitate out of the solution at or near their basic or neutral isoelectric points during the folding process, the air or mixed disulfide method for oxidation is not satisfactory. We have found that this is the case in the synthesis of several basic and hydrophobic disulfide-rich peptides. In the synthesis of a series of viral growth factors,[12,13] the disulfide formation by air oxidation or mixed disulfide method produced precipitation even in the presence of a strong denaturant such as 6 M urea that resulted in unacceptably low yields of the desired product.

It would be highly desirable to devise a new method for the disulfide formation that is similar in mildness to air oxidation but can be conducted under acidic conditions with no harmful byproducts at an efficient rate. An oxidation method that appears to satisfy all these requirements is the use of dimethyl sulfoxide (DMSO). DMSO has been known to be a mild oxidizing agent

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Table I. Comparison of pH-Dependent Rates of Disulfide Formation by DMSO and Air Oxidation

pH	10^2k_1[a] (min^{-1})					
	CY-11(7)	CY-11(8)	CY-11(9)	CY-12(9)	CY-12(10)	CY-12(11)[b]
8	3.1 (1.3)[c]	3.8 (0.5)	1.4 (0.2)	2.4 (0.3)	5.4 (1.0)	4.4 (2.0)
7	11.2 (1.6)	4.6 (0.8)	2.4 (0.2)	17.0 (4.5)	11.8 (1.4)	8.1 (1.2)
6	6.6 (0.7)	6.1 (1.0)	3.7 (0.2)	1.0 (0.2)	10.3 (0.8)	5.0 (0.8)
5	3.5 (0.3)	4.0 (<0.01)	1.9 (0.1)	1.3 (0.2)	3.9 (<0.01)	1.9 (0.1)
4		2.6 (<0.01)			2.8 (<0.01)	
3		3.1 (<0.01)			2.6 (<0.01)	

[a] Pseudo-first-order rates. [b] See Figure 1 for compound designation. [c] The rates of air oxidation are in parentheses.

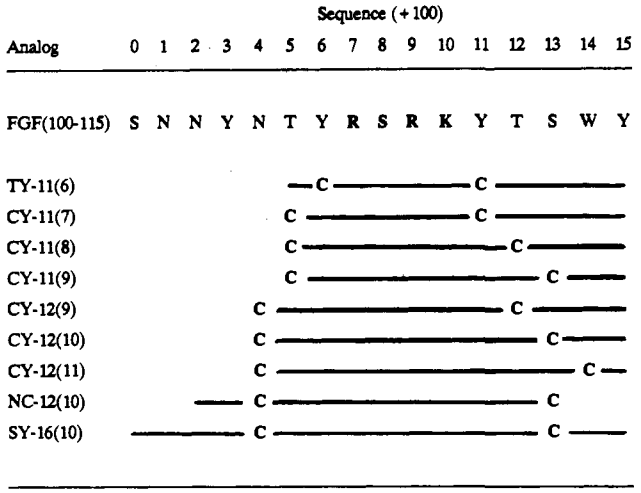

Figure 1. Model peptides based on basic fibroblast growth factor sequence 100–115. The amino acid is denoted by the one-letter code. The nomenclature of the analogue (e.g., TY-11(6)) is denoted by the amino acids at each end of its sequence (TY), the number of amino acids in the peptide chain (11) and in the disulfide loop (6, in parentheses).

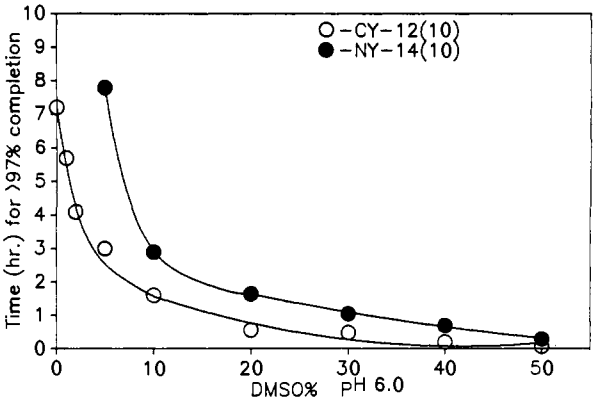

Figure 2. Concentration of DMSO vs time required for completion of disulfide formation. The sequence of the 12-residue peptide CY-12(10) is CTYRSRKYTCWY and the 14-residue peptide NY-14(10) is NYC-TYRSRKYTCWY.

for simple organic thiols producing H_2O and dimethyl sulfide as harmless byproducts.[14-18] It is miscible with H_2O at all concentrations, and thus a high concentration of DMSO could be envisioned to effect the desirable rate of reactions. Furthermore, oxidation by DMSO could also be envisioned to be performed at acidic to neutral pH range to overcome the limitation of the conventional methods of oxidation. Despite all these apparent advantages, DMSO has not been used for the folding and oxidation of synthetic peptides and proteins. In this paper, we describe the scope of disulfide formation of cysteinyl-containing peptides by DMSO and provide a general scheme for the simultaneous folding and formation of disulfide bonds in a wide range of pH for peptides.

Results and Discussion

Model Monocyclic Peptides—Synthesis and Stoichiometry of DMSO. A series of basic peptides derived from residue 93–120 of human basic fibroblast growth factor[19] was used as models to test the effectiveness of oxidation by DMSO (Figure 1). This peptide is derived from an antiparallel β-strand of FGF and is rich in aromatic as well as β-branch amino acids including a tryptophane, two threonines, and three tyrosines. This highly basic and hydrophobic sequence contained no cysteine but was converted to cysteinyl-containing sequences to suit our purpose. The cationic nature of these peptides was retained as the basic tetrapeptide, Arg107-Ser108-Arg109-Lys110, which was used as the core unit for all models. However, both the length of the peptides from 11 to 16 residues and the size of the disulfide rings from 6 to 11 amino acid residues were varied to determine the generality of the DMSO oxidation method.

The peptides were synthesized with the Boc-benzyl protecting group strategy by the solid-phase method on the *p*-methylbenzhydrylamine resin.[20] After the low–high HF cleavage,[21,22] the deblocked peptide α-carboxamide was dissolved into an aqueous acetic acid solution at a concentration of about 0.5 mg/mL, adjusted to pH 6, and diluted to the appropriate volume in DMSO to initiate the disulfide formation. For the initial study, we would like to define an optimal range of concentrations of DMSO in the aqueous reaction for the completion of the oxidation reaction. Two model peptides were used to determine the stoichiometric requirements of DMSO. Various concentrations of DMSO ranging from 1 to 50% were added to the aqueous reaction mixtures of two model peptides (Figure 2). With a 12-residue peptide, CY-12(10), disulfide formation was observed to be completed in about 1 h when the volume ratios of DMSO were between 10 and 30%. The rates increased as the concentration of DMSO increased. At 40–50% DMSO, the reaction was completed within 0.5 h. On the other hand, at concentrations below 5%, the reaction was prolonged to 2–6 h. In the absence of DMSO and in the presence of air, the reaction required more than 7 h for completion. Similarly, with a 14-residue peptide, NY-14(10), complete reaction was observed in 2–3 h in 10–30% of DMSO, and within 1 h in 40–50% of DMSO, but more than 10 h in 1–2% of DMSO and 24 h in the absence of DMSO (air oxidation). Since DMSO was intended both as a solvent and as an oxidant, a rather arbitrary midpoint concentration, 20% of DMSO by

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Table II. Rates of Disulfide Formation by DMSO (pH-Independent) and Air Oxidation

pH	10^2k_1[a] (min^{-1})			
	TY-11(6)	NC-12(10)	SY-16(10)	Ac-CY-11(8)[b,c]
8	2.7 (<0.01)[d]	1.8 (0.02)	2.2 (<0.01)	2.3 (<0.01)
7	2.6 (<0.01)	2.9 (0.002)	2.7 (<0.01)	2.0 (<0.01)
6	3.2 (<0.01)	2.7 (0.005)	2.5 (<0.01)	2.1 (<0.01)
5	2.7 (<0.01)	2.7 (0.004)	2.7 (<0.01)	2.5 (0.02)
4	2.7 (<0.01)	3.2 (0.001)	2.4 (<0.01)	2.0 (0.01)
3	2.2 (<0.01)	2.8 (0.003)	2.5 (<0.01)	2.6 (0.02)

[a] Pseudo-first-order rates. [b] See Figure 1 for compound designations. [c] N-acetylated compound of CY-11(8). [d] The rates of air oxidation are in parentheses.

volume, was used for all subsequent experiments. The choice of this working concentration of DMSO was also determined by the condition of workup since dilutions were required to lower the concentration of DMSO for purification by the reverse-phase HPLC. It should be pointed out that at such a concentration the molar excess of DMSO was about 6000-fold when compared to the peptide concentration at 0.5 mg/mL.

With 20% DMSO in aqueous solution as the folding and oxidation condition, the disulfide formation by the DMSO oxidation was rapid in all the model peptides studied. A 50% conversion to the disulfide was found to be within 5–30 min, and the complete reaction in 0.5–4 h was observed by analytical C_{18} reverse-phase HPLC (Tables I and II). At the completion of the disulfide formation, the solution was diluted 2-fold and loaded directly to a preparative reverse-phase HPLC for purification to give 32–46% overall yield. The integrity of each purified peptide was determined by Cf-252 fission ion mass spectrometry, and the observed molecular mass was found to agree with the calculated values. In contrast, parallel experiments with air oxidation at pH 8.0 in the absence of DMSO were found to require 4–72 h for completion. In some cases, air oxidation did not result in the formation of any significant amount of products (Table II).

Susceptibility of Nucleophilic Amino Acids to DMSO. To determine whether nucleophilic amino acids were susceptible to oxidative side reactions in DMSO, Met, Trp, Tyr, and His were treated in an aqueous buffered solution at pH 3–8 containing 20% DMSO for a 72-h period. The most susceptible amino acid is Met which can be converted to methionine sulfoxide [Met(O)]. However, the sulfide–sulfoxide interchange reaction is known to occur only in strongly acidic media due to the weak basicity[23] of DMSO (pK_a −1.80). Indeed, no Met(O) formation was observed in a solution of 20% DMSO at the pH range between 3 and 8 under our experimental condition (data not shown). Similarly, no oxidative reaction was observed in other nucleophilic amino acids such as Trp, Tyr, and His. Our results are consistent with the present knowledge of DMSO which is a common solvent in the solution phase of peptide synthesis and which does not cause oxidative side reactions of nucleophilic amino acid under the conventional use.

Rates of Disulfide Formation. The purified and reduced peptides were used for detailed kinetic studies in 20% DMSO at pH 3–8. For comparison, parallel experiments were performed by air oxidation at various pH without DMSO. The optimal pH range for disulfide formation in peptides and proteins by DMSO is likely to be between 3 and 8. There are two concerns for folding conditions performed at pH lower than 3. First, protein denaturation may occur. Secondly, there will be increasing danger of oxidation of methionine to methionine sulfoxide at low pHs. The pK_a of the sulfide in methionine is about −1.8, and the oxidation of methionine to methionine sulfoxide by DMSO would be favored by acidic conditions below pH 3. At pH higher than 8, disulfide interchange is usually quite rapid. This could pose a serious problem. For example, the rate constant for the disulfide interchange in a protein at pH 8 is 10 s^{-1} M^{-1}.[24] Thus, the rates of disulfide formation of a series of model peptides in DMSO were studied at pH 3–8. Purified and reduced model peptides with free

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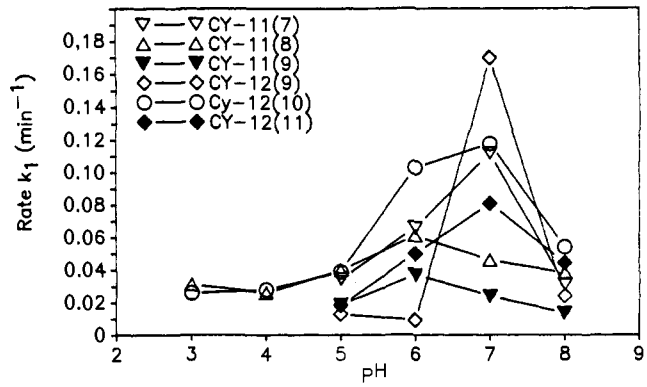


Figure 3. The pH-dependent pseudo-first-order rates of disulfide bond formation vs pH in the presence of 20% DMSO.

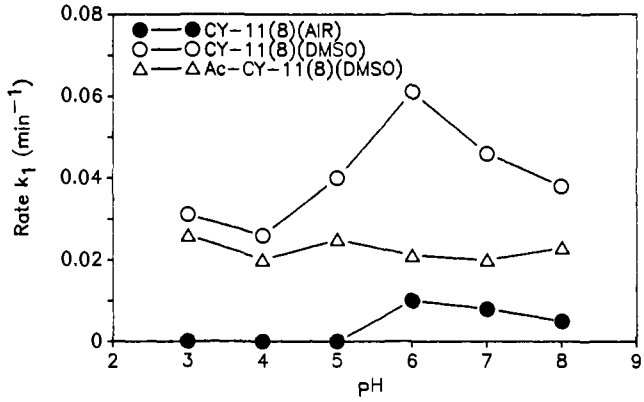


Figure 4. Differences of rate profiles of CY-11(8) in DMSO, after acetylation of the N$^{\alpha}$-amino group, and by air. Note the removal of the free amino group by acetylation of CY-11(8) rendered the rate profiles to pH-independent.

sulfhydryls were treated at various buffers in 20% DMSO. Since DMSO was used in large excess, the pseudo-first-order rate k_1 of disulfide formation was measured. The reaction products were conveniently monitored by C_{18} reverse-phase HPLC since the reduced and oxidized products were usually separated by more than 1-min apart in their elution profiles. Ten model peptides were studied in details (Figure 1). Within the range of pH 3–8, the reaction rates obeyed the first-order kinetics and varied about 17-fold, between 0.01 and 0.17 min^{-1} with half-lives between 4 and 69 min. Thus, these rate studies validated that the oxidation by DMSO at the acidic range would be useful. The size of the disulfide ring from 6 to 11 amino acid residues did not appear to significantly influence the rates of disulfide in DMSO. However, the position of the cysteine had strong effects, and two types of rate profiles were observed that were dependent on the particular location of cysteine in the amino acid sequence.

pH-Dependent Rate Constants. All six peptides CY-11(7), CY-11(8), CY-11(9), CY-12(9), CY-12(10), and CY-12(11) with a cysteine at the amino terminus showed pH dependence in their rate profiles (Table I). However, the N$^{\alpha}$-amino group of the amino terminal cysteine must be free. The optimal pH was found to be near neutrality of pH 6 and 7 (Figure 3). However, the pH dependence could be abolished when the amino terminus was acetylated as in Ac-CY-11(8) (Figure 4). In contrast, parallel experiments by air oxidation showed that rates were 2–12-fold slower at pH 7 and 8, and 10–40-fold slower at pH 5 and 6 than DMSO. Below pH 4, rates of disulfide formation by air oxidation were generally too slow to be useful (Tables I and II).

pH-Independent Rate Constants. All three peptides TY-11(6), NC-12(10), and SY-16(10) with no amino terminal cysteine exhibited pH-independent rate profiles in DMSO oxidation (Table II). While the peptide CY-12(10) containing an amino terminal cysteine exhibited pH-dependent rate profile, conversion of the amino group to an amide by acetylation as in N-acetylated peptide Ac-CY-11(8), by addition of a dipeptide as NC-12(10) or a

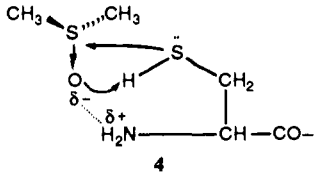
pentapeptide as in SY-16(10), led to rate profiles that were pH-independent. The rates of all four peptides at pH 3–8 varied in a very narrow range between 1.8 and 3.2 10^{-2} min^{-1} ($t_{1/2}$ = 22–39 min). In contrast, very slow rates (<0.0001 min^{-1}) were obtained by air oxidation. In two peptides, TY-11(6) and SY-16(10), no products were observed in 72 h.

Mechanistic Interpretation. Two aspects of these rate profiles need to be addressed. The first is the very large rate difference of disulfide formation between DMSO and air oxidation reactions in several peptides. This difference may in part be attributed to the solvent effect of DMSO. In the absence of DMSO, β-sheet formation is likely to be favored in several peptides that lead to aggregation and is minimized by DMSO. Another plausible explanation may be due to the solvent effect of DMSO that favors reverse turn conformation which allows the disulfide formation to occur. The second regards the pH-dependent rates when the cysteine is located at the amino position, and, more importantly, when the N^{α}-amino group is free. This aspect could be explained by the known mechanism of sulfoxide-catalyzed disulfide formation of organic thiols.[14-19]

The general mechanism of disulfide formation by DMSO has been determined by Wallach and Mahon,[14] and a simplified overall reaction can be represented by the following equation. The

$$\underset{\mathbf{1}}{RSH} + (CH_3)_2SO \rightarrow [\underset{\mathbf{2}}{(CH_3)_2S(OH)SR}] \rightarrow \underset{\mathbf{3}}{RSSR} + CH_3SCH_3 + H_2O \quad (1)$$

stoichiometry for the reaction requires 2 mol of a thiol and 1 mol of DMSO, and the reaction shows a second-order kinetics. Furthermore, the reaction is catalyzed strongly by primary and secondary amines and somewhat weakly by acids. The rate-determining step is the formation of an unstable adduct **2** that is rapidly captured by another thiol to give the disulfide. Thus, the pH-independent rates between pH 3 and 8 can be rationalized by the overall kinetics. The forward rate requires both the protonation of DMSO and formation of the thiolate anion in the second step. The protonation favored by the acidic pH is counterbalanced by the decrease in thiolate formation. This provides an explanation for our observation that the rate constants of disulfide formation is not dependent on pH between 3 and 8. The pH-dependent kinetics could be rationalized by the rate acceleration of disulfide formation at neutral pH by DMSO when the cysteine is at the amino terminus with an unprotonated α-amino group. It is plausible that the rate of acceleration observed with peptides containing a free amino terminal cysteine is due to the assistance of the free amino group as a general base and its ability in the regiosteering of the DMSO to the thiol of the cysteine. We propose that such regiospecificity is due to the weak interaction between the partial negatively charged sulfoxide and the partially positively charged unprotonated α-amine that facilitates the formation of the unstable adduct **4**. Such a regiospecific assistance and hence the acceleration rate will not be possible when the free amino group is protonated at the acidic pH, one or more amino acid residues away, or converted to an amide.



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Tricyclic Peptide—Human Defensin. Defensins are cysteinyl rich, cationic, and antimicrobial peptides produced by mammalian neutrophils as a nonadaptive mechanism in host defenses against bacteria, fungi, and viruses.[25] Defensins are stored in cytoplasmic granules and released as one of the cytotoxic effectors by the polymorphonuclear leukocytes when in contact with the pathogens. The primary sequences of 13 different defensins have been determined.[26] They all range from 29 to 32 residues with 50–80% in sequence relatedness. The structural characteristics of defensins include the invariant six cysteines and two glycines as well as two arginines that impart the cationic characteristic of defensins. The three disulfide pairs of defensins constitute a distinctive disulfide motif of this family with a pairing pattern of 1-6, 2-5, and 3-4, starting from the amino terminus (Figure 5). The solution structure of defensins have been determined and is found to consist exclusively of β-sheets and reverse turns.[27]

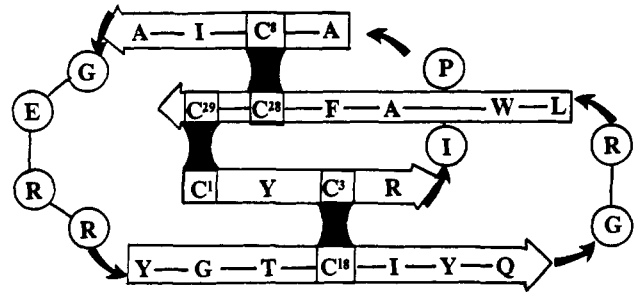

Figure 5. Sequence and disulfide pairings of human defensin. The direction of the β-strand is indicated by the open arrows and the reverse turns by solid arrows.

Table III. Comparison of Different Methods of Disulfide Formation in Defensin

run	condition[a]	pH	oxidant	yield (%)	precipitation[c]
1	urea	8.2	air	<1.0	+ + + +
2	urea	8.2	air	<1.0	+ + + +
3	guanidine·HCl	8.2	mix. disulfide	<1.0	+ + + +
4	guanidine·HCl	8.2	mix. disulfide	<1.0	+ + + +
5	guanidine·HCl high dilution[b]	8.2	DMSO	14	+
6	urea	6	DMSO	10	–
7	guanidine·HCl high dilution	6	DMSO	14	–

[a] In 2 M solution in 0.5 mg/mL of concentration. [b] Slow addition of a 6 M solution to a solution of 1 M solution containing 20% DMSO. [c] + + + +, copious amount of precipitation, nearly 80% or more of the products precipitated from the solution; +, slight amount of precipitation, less than 10% of the products precipitated from the solution; –, no precipitation observed.

The challenge for the chemical synthesis of defensins lies in the difficulties of refolding and the formation of the sulfur–sulfur bonds contributed by two factors: (1) the presence of β-sheet which tend to aggregate leading to polymeric formation and (2) the strong basic character of defensins that tend to precipitate in the basic condition during disulfide formation. In view of these difficulties, the folding and oxidation in the chemical synthesis of defensins appear to be a stern and suitable test for the sulfur–sulfur bond formation conditions by the DMSO method under acidic conditions that would minimize aggregation and precipitation.

The protection scheme for the synthesis of human defensin utilized the maximal and conventional protecting group approach of the Boc-benzyl strategy. The deprotection scheme utilized the gradative deprotection approach[28] that removed most of the side-chain protecting groups successively, while the peptide chain was anchored onto the resin support. The peptide chain, the thiol protecting groups, and the tosyl arginine were then removed by the high HF.

The folding and disulfide formation of the crude synthetic peptide were determined under a series of conditions (Table III). Seven experiments were performed, and each used 100 mg of

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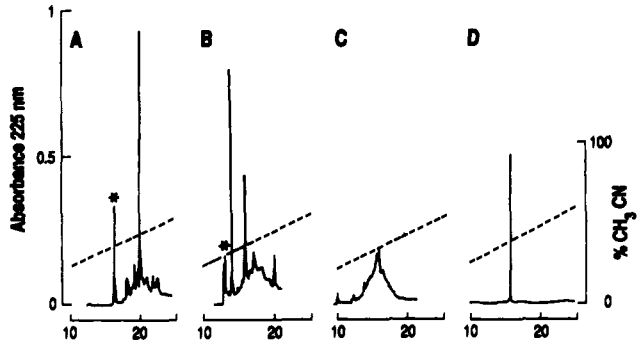

Figure 6. C_{18} reverse-phase HPLC of synthetic human defensin. (A) Crude defensin after HF cleavage. The reduced defensin eluted at about 19 min. (B) Folding and disulfide formation of crude defensin in 20% DMSO at pH 6 (run 7). The folded defensin eluted at about 14 min, 5 min ahead of the reduced and unfolded defensin. (C) Folding and disulfide formation with air oxidation after 42 h (run 1). Similar profile was obtained from run 2. (D) Purified defensin of run 7. The peak with an asterik was cresol which served as an elution standard.

peptide resin. In runs 1–4, the normal conditions of refolding were used. The crude peptide after the low–high HF treatment was solubilized in 8 M urea or 6 M guanidine hydrochloride in 0.1 M Tris buffer at pH 8.2 and was dialyzed successively in a lower concentration of the denaturants at pH 8.2 Tris buffer in the absence of air to exclude the reducing agent, dithiothreitol. Copious precipitation occurred during all stages of the dialysis. In run 1, the crude peptide contained in both solution and precipitate were folded at the 2 M urea stage by air oxidation (Figure 6). After 18 h, more precipitation was observed. The soluble portion was subjected to purification by reverse-phase HPLC and yielded 0.4 mg (<1% overall yield) of defensin. Similar procedures in run 2 with a 1:1 mixture of reduced and oxidized glutathione did not diminish the amount of precipitation during the folding or increase the yield of the product. Similar procedures with repeated using guanidine HCl as the denaturant in runs 3 and 4. However, precipitate occurred during the 4 M guanidine HCl step. Folding by the mixed disulfide procedure yielded a very small amount of defensin (<1%).

The results of runs 1 to 4 show that defensin favors aggregation at the basic pH that is near the isoelectric point of defensin. Attempts to lower the concentrations of the denaturants lead to aggregation and copious precipitation of the peptide from the solutions. In contrast, three different methods with DMSO as oxidant (runs 5–7) produced significantly higher yields and greatly minimized the precipitation problem. To minimize aggregation, a high dilution method was being used in run 5. In the high dilution method, the peptide in 6 M guanidine solution was slowly added to a solution of 1 M guanidine at pH 8.2 containing 20% of DMSO in 20 h with gentle stirring. Under such a condition, the aggregation leading to precipitation was greatly minimized, and the sulfur–sulfur bond formation was greatly accelerated. Indeed, the precipitation was minimal, and after purification by reverse-phase HPLC, 8.5 mg of defensin (14% overall yield) was obtained.

To avoid the precipitation problem completely, runs 6 and 7 were conducted at the acidic buffer range (Figure 6). In run 6, the crude peptide was dissolved in 8 M urea and run 7 in 6 M guanidine HCl, both in Tris buffered solution at pH 6. Dilution of this solution to 1 M solution at pH 6 did not result in precipitation and S–S bond formation was conducted at pH 6 in the presence of 20% DMSO. Oxidation was completed in 8 h to yield 6 (run 6) and 8.7 mg (run 7) of defensin after purification from reverse-phase HPLC. The integrity of defensin was determined by Cf-232 fission ion mass spectrometry and found to agree with the expected value. The biological activity of defensin was also found to be as active as the natural defensin.

Conclusion

Two major requirements in the disulfide formation of peptides or proteins are selectivity and efficiency. The use of DMSO for such a purpose meets both of these requirements. DMSO is a mild oxidant specific for the oxidation of sulfhydryls to sulfur–sulfur bonds. Side reactions that result in the oxidation of nucleophilic side chains of amino acids such as those of Met, Trp, Tyr, or His has not been observed within the suggested pH range of 3–8. Furthermore, the oxidation by DMSO is a very convenient and efficient process that differs in several important aspects from the conventional methods of oxidation by air or disulfide interchange.

First, DMSO is miscible at all concentrations with H_2O, and a high concentration of DMSO can be used. For example, a 20% DMSO solution in H_2O is about 3 M which will be many fold higher than the concentration of O_2 attainable in H_2O.[29] As a result, the disulfide formation by DMSO is rapid, and most disulfide formation of the monocyclic peptides is observed to be completed within 1–4 h. Furthermore, only a single step is required after the cleavage step from the resin support to the purification by HPLC. The crude peptide can be subjected to sulfur–sulfur bond formation immediately after the cleavage process in a 10–20% DMSO buffered solution and further subjected to direct purification with the reverse-phase HPLC. Second, the most important and crucial difference is perhaps the wide range of pH that DMSO can be used when compared with conventional methods. With oxidation by air or mixed disulfide interchange, the condition is usually limited to near or at basic conditions when the thiols are ionized. Thus, the cysteinyl thiols, with their pK_a between 8 and 9 are most reactive at the alkaline range. Their reactivity usually decreases by about 10-fold for each decrease of pH unit. However, a wide range of pH from pH 3 to 8 can be used with DMSO. Moreover, for most peptides, we have found that the rates of disulfide formation by DMSO are pH independent. Such a flexibility of operable pH range allows the solubilization of the peptides and proteins providing a wide range of options for the oxidation under controlled pH condition. Finally, DMSO may alleviate, in aqueous solution, the insolubility of hydrophobic peptides, such as protected peptide fragments or transmembrane peptides. This can be accomplished by using a very high concentration (e.g., >80%) of DMSO similar to those conditions often used in the solution synthesis for solubilizing protected peptide fragments. Overall, these advantages of DMSO will make it more accessible to prepare peptides with multiple disulfide bonds efficiently and in high yields.

Experimental Section

Solid-Phase Peptide Synthesis of Peptides. The monocyclic peptides were synthesized by the solid-phase method[6,21] with 4-methylbenzhydrylamine resin at a substitution level of 1.1 mmol/g, while the defensin were prepared with 4-(Boc-aminoacyloxymethyl)phenylacetamidomethyl-resin at 0.8 mmol/g substitution level.[30] Typically, 0.3–0.6 g of resin was used for each synthesis. All amino acids were protected with N^{α}-(tertbutyloxy)carbonyl (Boc). Side chain protecting groups were Arg(Tos), Asp(OcHex), Cys(Acm), Cys(4-MeBzl), Glu(OBzl), His(Dnp), Lys(2-ClZ), Ser(Bzl), Thr(Bzl), Tyr(BrZ), and Trp(For). Each synthetic cycle consisted of (i) a 20-min deprotection with 50% trifluoroacetic acid/CH_2Cl_2, (ii) neutralization with 5% diisopropylethylamine/CH_2Cl_2, and (iii) double coupling with preformed symmetrical anhydrides (6 equiv of the Boc-amino acid) for 1 h each in CH_2Cl_2 and then in dimethylformamide (DMF). Couplings of Boc-Asn-OH, Boc-Gln-OH, and Boc-Arg(Tos) were mediated by the preformed hydroxybenzotriazole active ester in DMF. Boc-Gly-OH was coupled with dicyclohexylcarbodiimide alone. All couplings were monitored by the quantitative ninhydrin test.[31]

HF Cleavage. Protected peptide–resin (0.2–0.4 g) was first treated 3–5 times with 1 M thiophenol in DMF for 8–12 h to remove the N^{im}-dinitrophenyl-protecting group of His (32) and then with 50% tri-

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fluoroacetic acid/CH_2Cl_2 (10 mL) for 5 min to remove the N^{α}-(tertbutyloxy)carbonyl group. The dried peptide–resin was treated with the low–high HF method of cleavage. For the low HF treatment, the peptide–resin was premixed with *p*-thiocresol, *p*-cresol, and dimethyl sulfide (mixture). Liquid HF at −78 °C was then added to this mixture to give a final volume of 10 mL (65:2.5:7.5:25, v/v). The mixture was equilibrated to 0 °C by stirring it in an ice bath. After 2 h, the HF and dimethyl sulfide were removed in vacuo. The high HF treatment was initiated by recharging the reaction vessel at −78 °C with 14 mL of fresh liquid HF to give a total volume of 15 mL of HF–*p*-cresol–*p*-thiocresol. The reaction was carried out at 0 °C for 1 h. HF was removed by evaporation at 0 °C. After washing with cold ether–mercaptoethanol (98:2, v/v, 30 mL) to remove *p*-thiocresol and *p*-cresol, the crude reaction mixture was extracted with different buffers at the completion of the synthesis.

Purification and Oxidative Folding of Disulfide Peptides. All peptide analogues were purified by C_{18} reverse-phase HPLC with a gradient of 5% CH_3CN containing 0.0445% CF_3CO_2H and 60% CH_3CN containing 0.039% CF_3CO_2H. Air oxidation in pH 8.0 buffer (0.1 M ammonium bicarboante/carbonate) was used for the oxidation of the monocyclic analogues. For the tricyclic defensin, the 8 M urea solution containing the crude mixture of peptide was sequentially dialyzed (Spectra Por 6, MW cutoff 1000) at 0 °C for 8–16 h against 4 L each of deaerated and N_2-purged 8, 6, 4, and 2 M urea, all in 0.1 M Tris buffer, pH 8.2. In case of guanidine HCl, the dialysis was against 4 L each of deaerated 6, 3, and 1 M guanidine HCl in 0.1 M Tris buffer, pH 8.2. Since the basic character of defensin favored aggregration leading to precipitation at the low concentrations of denaturant, a high dilution method was performed. The peptide solution at high concentrations of a denaturant (70 mL of 6 M guanidine HCl) was added slowly by peristaltic pump into a 0.1 M Tris·HCl solution (330 mL) containing 24.24% DMSO at pH 8.2. Oxidation and disulfide formation of defensin by the mixed disulfide method was performed in 2 M urea, pH 8.0 Tris HCl buffer (200 mL), in 1.5 nM oxidized and 0.75 nM reduced glutathione for 16–48 h. The clear solution was dialyzed against 8 L each of 0.1 M Tris, pH 8.0 and 1 M HOAc. The peptides were purified by C-18 reverse-phase liquid chromatography (2.5 × 30 cm) eluted with 0.05% TFA–CH_3CN. Amino acid analysis was carried out in 5.7 N HCl at 110 °C for 24 h, and the experimental results agreed well with the values. Cf-252 fission mass spectrometry wa used to measure all monocyclic peptides. The observed values agreed with the calculated values with a deviation less than 0.5 mass unit. The $(M + 4\ H)^{4+}$ of defensin was found to 843.55, and $(M + 3H)^{3+}$ was found to be 1124.4. Both gave a measured molecular weight of 3370.2 which agreed well with the calculated value of 3370.8.

Oxidation by DMSO. After the HF cleavage, the peptides derived from 200 to 400 mg of peptide resin with an initial substitution at 1.1 mmol/g was first extracted three times by a mixture of ether–mercaptoethanol (98:2, v/v) to remove the organic scavengers. The basic peptide was extracted first with 25 mL of 25% acetic acid (higher concentration when the peptide was not soluble) and a second extraction with 50 mL of 5% acetic acid. The combined acetic acid was diluted to 250 mL to a final concentration of 5% acetic acid, and its pH was adjusted to pH 6 by $(NH_4)_2CO_3$. To this solution containing the deprotected peptide at a concentration in the range of 0.5 to 1 mg/mL, 20% by volume of DMSO was added. The progress of the oxidation reaction was monitored by analytical C_{18} reverse-phase HPLC. At the completion of the reaction, usually 1–4 h, the solution was diluted 2-fold by the initial buffer (buffer A of HPLC) and loaded directly into a preparative C_{18} reverse-phase HPLC column (Vydac, 10 × 25 cm, 5 μ particle size). The desired peptide was then eluted with a linear gradient from 0 to 40–50% buffer B in 45 min. Buffer A contained 5% CH_3CN and 0.0445% CF_3CO_2H, and buffer B contained 60% CH_3CN and 0.039% CF_3CO_2H.

Oxidation by Air. The solution containing the deprotected peptide at a concentration of 0.2 mg/mL after HF was adjusted to pH 8 by Tris·HCl to a 0.1 M concentration. The reaction was slowly stirred, and the progress of the oxidation reaction was monitored by analytical C_{18} reverse-phase HPLC. The workup was similar to those samples prepared by oxidation by DMSO.

Kinetic Study. The disulfide in each of the ten purified synthetic peptides (Figure 1) at a concentration of 85 nmol in 25 μL was reduced at pH 8 with dithiothreitol (0.2 μmol/5 μL) under nitrogen for 5 min. The reduced peptide was added to a buffered solution (0.5 mL) between pH 3 and 8 containing 20% DMSO. Aliquots (20 μL each) were then withdrawn at various points in time between 2.5 and 5 min intervals and quenched by CF_3CO_2H (5 μL in 9% solution) to stop the reaction. The progress of the oxidation was analyzed immediately by C_{18} reverse-phase HPLC. The pseudo-first-order constants (k) were calculated by $kt = \ln(X_0/X_t)$, where X_0 is the initial concentration of the reactant and X_t is the concentration of the reactant remaining at t (min). Parallel experiments were performed with air oxidation to obtain the rate constants.

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Registry No. CY-11(7) reduced, 134815-95-5; CY-11(7) cyclic S–S, 134816-04-9; CY-11(8) reduced, 134815-96-6; CY-11(8) cyclic S–S, 134816-05-0; CY-11(9) reduced, 134815-97-7; CY-11(9) cyclic S–S, 134816-06-1; CY-12(9) reduced, 134815-98-8; CY-12(9) cyclic S–S, 134847-13-5; CY-12(10) reduced, 134815-99-9; CY-12(10) cyclic S–S, 134847-14-6; CY-12(11) reduced, 134816-00-5; CY-12(11) cyclic S–S, 134816-07-2; TY-11(6) reduced, 134816-01-6; TY-11(6) cyclic S–S, 134847-15-7; NC-12(10) reduced, 134816-02-7; NC-12(10) cyclic S–S, 134816-08-3; SY-16(10) reduced, 134816-03-8; SY-16(10) cyclic S–S, 134816-09-4; Ac-CY-11(8) reduced, 134847-12-4; Ac-CY-11(8) cyclic S–S, 134816-10-7; NY-14(10), 134816-11-8; DMSO, 67-68-5; human defensin, 120721-97-3.