Self-Assembly and Gelation of Oxidized Glutathione in Organic Solvents

Robert P. Lyon and William M. Atkins*

Contribution from the Department of Medicinal Chemistry, Box 357610, University of Washington, Seattle, Washington 98195-7610

Received November 21, 2000

Abstract: The oxidized disulfide form of the ubiquitous tripeptide glutathione (γ-glu-cys-gly) (GSSG) is shown to produce transparent, thermoreversible gels in aqueous solutions of dimethyl sulfoxide, dimethylformamide, and methanol, at GSSG concentrations as low as 1.5 mM. The gels bind Congo Red and exhibit dramatic green birefringence when observed between crossed polarizers, characteristic of amyloid structures. By transmission electron microscopy, the gels appear to consist of a network of fibrous structures about 75 nm in diameter. Several structurally related peptides, including the glutathione isomer glu-cys-gly and the aspartyl analogue of glutathione (β-asp-cys-gly), failed to produce gels under similar conditions. These results suggest that the interactions which produce gelation are highly specific and that the unusual peptide geometry introduced by γ-glu-cys linkage is critical to the gelation behavior. ^{1}H NMR indicates solvent-dependent perturbation of the γ-glutamyl α- and β-protons and circular dichroism reveals a shift in the geometry of the disulfide bond under conditions producing gelation. We propose that in appropriate organic solvents, GSSG self-assembles into an extended network of β-sheetlike structures capable of immobilizing bulk solvent. While obviously speculative, it is interesting to consider possible physiological consequences of glutathione self-recognition in such processes as abnormal protein aggregation and the thiol−disulfide exchange which is believed to participate in protein folding.

Considerable attention has been given to polymers and small molecules capable of self-assembly into extended noncovalent structures such as nanotubes and tapes.[1−3] Under appropriate conditions, these assembled arrays are often observed to trap bulk solvent and result in the formation of transparent gels.[4−6] Such gels constructed of biocompatible materials have been sought by researchers in fields such as biomaterials, biosensors, tissue engineering, and drug delivery.[7,8] For these applications, peptides and proteins have emerged as ideal gelling agents. Numerous proteins and peptides, both natural and synthetic, have been shown to produce aggregates and gels in various solvent systems.[3,4,8,9] While large proteins in denaturing conditions can cause gelation by nonspecific tangling of the protein backbones, the formation of gels by small proteins and peptides is often the result of highly specific interactions to form long, noncovalent supramolecular assemblies. Considerable work has focused on engineering peptides to manipulate these interactions and control their gelation properties.[3,10,11] A common structural motif of the peptides which have resulted from these efforts is the antiparallel β-sheet. Several peptides have been designed to form extended intermolecular β-sheets in appropriate solvents, causing gelation at sufficiently high concentrations.[3,4] A set of design principles has emerged from these studies for the engineering of peptides which form β-sheet arrays. These principles include the following: a minimum of six residues per strand; strong attractive forces between the side chains of adjacent strands (hydrophobic, hydrogen bonding, and electrostatic); an element of lateral recognition to configure adjacent strands in an antiparallel fashion; and solvent interactions to maintain solubility in the desired solvent.[3,4]

The self-assembled β-sheets of these engineered peptides resemble the abnormal protein aggregates characteristic of numerous diseases, including the prion rods of spongiform encephalopothies, polyglutamine fibrils of Huntington's disease, and amyloid plaques of Alzheimer's disease (AD).[4] Indeed, a fragment consisting of residues 1−28 of the AD amyloid β-protein has been reported to form gels in aqueous solutions.[9] The interactions which produce aggregates and gels of peptides in various solvent systems may thus represent models of pathological in vivo protein aggregation and abnormal protein folding. Recent observations that many unrelated proteins may form amyloid fibrils under appropriate conditions has suggested that the ability to adopt an amyloidogenic conformation is independent of sequence, being rather a characteristic of the conserved polypeptide backbone.[12−14]

* To whom correspondence should be addressed.

(1) Voyer, R. *Bioorg. Chem.* **1997**, *184*, 1−37.
(2) Hartgerink, J. D.; Granja, J. R.; Milligan, R. A.; Ghadiri, R. *J. Am. Chem. Soc.* **1996**, *118*, 43−50.
(3) Aggeli, A.; Bell, M.; Boden, N.; Keen, J. N.; McLeish, T. C. B.; Nyrkova, I.; Radford, S. E.; Semenov, A. *J. Mater. Chem.* **1997**, *7*, 1135−1145.
(4) Aggelli, A.; Bell, M.; Boden, N.; Keen, J. N.; Knowles, P. F.; McLeish, T. C. B.; Pitkeathly, M.; Radford, S. E. *Nature* **1997**, *386*, 259−262.
(5) Hanabusa, K.; Miki, T.; Taguchi, Y.; Koyama, T.; Shirai, H. *J. Chem. Soc., Chem. Commun.* **1993**, 1382−1384.
(6) Wang, C.; Stewart, R. J.; Kopecek, J. *Nature* **1999**, *397*, 417−420.
(7) Holmes, T. C.; de Lacalle, S.; Su, X.; Liu, G.; Rich, A.; Zhang, S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6728−6733.
(8) Tan, M. M.; Corley, C. A.; Stevenson, C. L. *Pharm. Res.* **1998**, *15*, 1442−1448.
(9) Kirschner, D. A.; Inouye, H.; Duffy, L. K.; Sinclair, A.; Lind, M.; Selkoe, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6953−6957.
(10) Osterman, D. G.; Kaiser, E. T. *J. Cell. Biochem.* **1985**, *29*, 57−72.
(11) Krejchi, M. T.; Atkins, E. D. T.; Waddon, A. J.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *Science.***1994**, *265*, 1427−1432.
(12) Chiti, F.; Webster, P.; Taddei, N.; Clark, A.; Stefani, M.; Ramponi, G.; Dobson, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3590−3594.

10.1021/ja0040417 CCC: $20.00
Published on Web 04/13/2001

The ubiquitous tripeptide glutathione (γ-glu-cys-gly, GSH) is present in nearly all known aerobic organisms. Glutathione concentrations in human tissues are typically in the 1−10 mM range.[15,16] The multiple, well-characterized functions of GSH include detoxification of exogenous electrophiles and reactive oxygen species, maintenance of cellular thiol status, and serving as a cofactor in the biosynthesis of endogenous compounds.[16−18] Additionally, there is indirect evidence that glutathione may have roles in protein folding, signaling, and regulation via thiol−disulfide exchange.[19,20] Its role as an antioxidant is mediated by recycling of glutathione between its reduced form as a free thiol (GSH) and its oxidized disulfide (GSSG) form. Glutathione is an unconventional peptide in that the glu-cys linkage is via the side chain carboxylate of glutamate (see Figure 1).

We have observed that GSSG produces transparent, thermoreversible gels in dimethyl sulfoxide (DMSO) and in aqueous solutions of dimethylformamide (DMF) and methanol. By electron microscopy, these gels appear as a fibrous network of filaments approximately 75 nm wide. The filaments stain with Congo Red and exhibit strong green birefringence, suggestive of a crossed β-sheet structure similar to β-amyloid. Analogue studies indicate that the γ-glu-cys peptide linkage of glutathione is a structural motif critical to the gelation behavior. GSSG is considerably smaller than other natural peptides which have been shown to adopt an amyloidogenic configuration, and its peptide backbone is unique due to the γ-glu-cys linkage. Previous spectroscopic studies suggest that in aqueous solution, GSSG adopts an intramolecular, antiparallel β-sheet type conformation stabilized by the restricted rotation about the disulfide bond.[21,22] We propose that in appropriate organic solvents, these stable β-sheet units self-assemble into an extended network of intermolecular, antiparallel sheets. This network immobilizes bulk solvent and results in the formation of transparent gels. It is interesting to speculate that this unexpected self-recognition behavior of such an abundant and multifunctional peptide may have physiological consequences for protein regulation, aggregation, and folding.

Materials and Methods

Glutathione (reduced and oxidized), glutathione ethyl ester, and *S*-methyl glutathione were purchased from Sigma. The tripeptides glu-cys-gly and β-asp-cys-gly were purchased from Synpep. Other reagents and solvents were purchased from Aldrich.

Preparation of Gels. Gels in DMSO were prepared by dissolution of glutathione with gentle heating to about 60 °C. Gels in aqueous DMF and methanol were prepared by addition of the organic solvent to concentrated aqueous solutions of glutathione. For glutathione derivative and analogue studies, solutions of 10 mg/mL were prepared and observed for 1 month for gelation behavior.

Visible and Polarization Microscopy. Firm gels of GSSG in DMSO (65 mM) were sliced with a razor into thin sections and placed on microscope slides. To 1 mL of a stock solution of 80% ethanol/20% DMSO saturated with Congo Red and sodium chloride were added 10 μL of 1% NaOH. Alkaline stain solution was applied to a slide for 2 min then poured off, and the gel sections were rinsed with several portions of 80% ethanol/20% DMSO. Stained sections were air-dried overnight to remove excess DMSO. Drying was necessary to make the sections suitably thin for polarization microscopy. Dried gel samples were visualized at 40× or 100× magnification, and birefringence was determined between crossed polarizers. Two control slides were also prepared. These consisted of 65 mM glu-cys-gly ("isoglutathione") plus Congo Red in DMSO and 65 mM GSSG plus Congo Red in 50/50 water/DMF (neither of these solutions produce gels). In each case, the solution was applied to a slide and allowed to dry prior to microscopy observations.

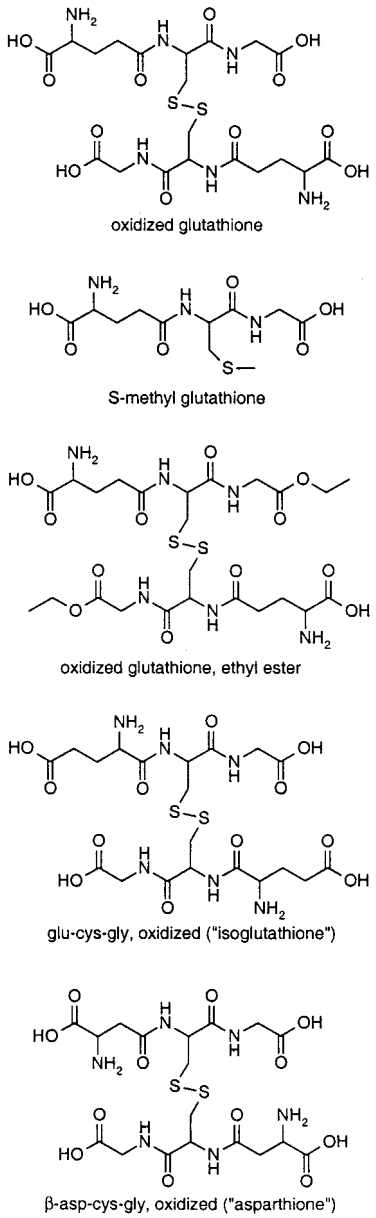

Figure 1. Structures of oxidized glutathione (GSSG) and the glutathione analogues and derivatives discussed in this paper.

Electron Microscopy. For transmission electron microscopy, Formvar coated copper grids (200 mesh, Fullam, Inc., Latham, NY) were touched to the surface of the gel, then washed with ethanol and negatively stained with uranyl acetate in 95% ethanol. TEM was performed on a JEOL electron microscope with an acceleration potential of 80 keV.

Spectroscopy. (a) Proton NMR: Glutathione (GSH or GSSG) was dissolved at 3 mg/mL in either d_6-DMSO or 90% H_2O/10% D_2O.

(13) Guijarro, J. I.; Sunde, M.; Jones, J. A.; Campbell, I. D.; Dobson, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4224−4228.
(14) Gross, M.; Wilkins, D. K.; Pitkeathly, M. C.; Chung, E. W.; Higham, C.; Clark, A.; Dobson, C. M. *Protein Sci.* **1999**, *8*, 1350−1357.
(15) Slivka, A.; Spina, M. B.; Cohen, G. *Neurosci. Lett.* **1987**, 74, 112−118.
(16) Meister, A. *Pharmacol. Ther.* **1991**, *51*, 155−194.
(17) Hayes, J. D.; McLellan, L. I. *Free Radical Res.* **1999**, *31*, 273−300.
(18) Sies, H. *Free Radical Biol. Med.* **1999**, *27*, 916−921.
(19) Cotgreave, I. A.; Gerdes, R. G. *Biochem. Biophys. Res. Commun.* **1998**, *242*, 1−9.
(20) Darby, N. J.; Freedman, R. B.; Creighton, T. E. *Biochemistry* **1994**, *33*, 7937−7947.
(21) Howarth, O. W.; Lian, L. Y. *J. Chem. Soc., Perkin. Trans. 2* **1982**, 263−267.
(22) Schwartz, A. L.; Cutnell, J. D. *J. Magn. Reson.* **1983**, *53*, 398−411.

Table 1. ^{1}H NMR Chemical Shift Data

sample	Glu β_1	Glu β_2[a]	Glu γ	Cys β_1	Cys β_2	Glu α	Gly α	Cys α	Cys N−H	Gly N−H
GSH/H_2O	2.15		2.53	2.94	2.94	3.80	3.95	4.55	8.46	8.50
GSSG/H_2O	2.14		2.52	2.96	3.26	3.80	3.95	4.65	8.58	8.53
$\Delta\delta$	−0.01		−0.01	+0.02	+0.32	0	0	+0.10	+0.12	+0.03
GSH/DMSO	1.85	1.93	2.32	2.69	2.82	3.33	3.70	4.36	8.37	8.68
GSSG/DMSO	1.94		2.35	2.89	3.15	3.47	3.71	4.52	8.56	8.62
$\Delta\delta$	+0.09	+0.01	+0.03	+0.20	+0.33	+0.14	+0.01	+0.16	+0.19	−0.06

[a] The two β-protons of glu are nonequivalent, and in some cases are resolvable.

Spectra were obtained on a Varian INOVA 500 MHz NMR.

(b) Circular dichroism: Spectra of GSSG in 90% methanol were obtained in 5 mm path length cells on a Jasco J-720 spectropolarimeter.

(c) Mass spectrometery: Gel samples were dissolved in water and analyzed by direct injection ESI-MS on a Fisons VG Quattro II mass spectrometer.

Results

General Observations. Glutathione solutions up to 80 mM have been prepared by heating DMSO to 60 °C. Upon cooling, the rate of gelation is dependent upon glutathione concentration and whether the starting material was reduced (GSH) or oxidized (GSSG) glutathione. In the case of GSSG, increased viscosity is immediately observed at concentrations above 16 mM. At 8 mM, formation of rigid transparent gels is observed within 1 week. No gelation is observed at concentrations below 8 mM (4.9 mg/mL). However, if GSH is dissolved in DMSO, gelation is noticeably slower, requiring several days at 32 mM and up to 2 weeks at 16 mM (note that upon oxidation, GSSG molar concentration is half that of the original GSH concentration). However, the gels which eventually result from GSH solutions are qualitatively stiffer than those from GSSG solutions, perhaps due to aggregates assembling from fewer nucleation sites and thus forming fewer, but more extensive intermolecular arrays. The gelation of GSH/DMSO solutions can be accelerated by bubbling compressed air through the solution, while gelation time is increased approximately 3-fold by the addition of 1 molar equiv of dithiothreitol (DTT). These gels are thermostable to about 50 °C, becoming transparent fluids above this temperature, and re-gelling upon cooling. Upon standing at room temperature, GSSG/DMSO gels are stable for months, while addition of 1 molar equiv of DTT causes dissolution within 24 h.

Solutions prepared by adding DMF to aqueous GSSG solutions produce gels within minutes at concentrations as low as 5 mM (3 mg/mL) in 90% DMF. These gels are thermostable to about 65 °C. Above this temperature, they produce a milky white suspension which, upon cooling, clarifies nearly completely and re-gels. Solutions prepared by adding methanol to aqueous GSSG solutions require cooling to about 0 °C to produce transparent gels at concentrations as low as 1.5 mM (0.9 mg/mL) in 90% methanol. In some cases, the solution remains gelled after warming to room temperature. In all solvent systems, gels form with up to 20% water, although the minimum GSSG concentration necessary to form gels increases with increasing aqueous fraction. It is notable that GSSG forms gels in both polar aprotic (DMSO and DMF) and protic (methanol) organic solvents.

Behavior of Glutathione Analogues and Derivatives. The glutathione analogues and derivatives illustrated in Figure 1 were assayed for their ability to produce gels in 90% methanol, 90% DMF, and DMSO. Because of its inability to form a disulfide, *S*-methyl glutathione was selected to verify that the disulfide is required for gelation. "Isoglutathione" and "asparthione" were chosen to test the relevance of the unusual geometry of the γ-glutamyl function of glutathione to the gelation behavior. Glutathione ethyl ester was selected to assess the importance of the hydrogen bond-donating and ionizable terminal carboxylic acid function. Of these analogues and derivatives, only the oxidized form of glutathione ethyl ester was found to exhibit any gelation activity, forming transparent gels in 90% methanol and 90% DMF. In DMSO, the reduced thiol form of the ethyl ester is readily soluble, but the disulfide is practically insoluble, and does not form gels. Neither "isoglutathione" nor "asparthione" displayed any gelation activity, suggesting that the γ-glutamyl structural element is critical for gelation.

Spectroscopy. Proton NMR was employed to probe for conformational differences between GSH and GSSG in organic solvents which do not exist in aqueous systems. Therefore, spectra were obtained for the reduced and oxidized forms of glutathione in DMSO and in 90% H_2O/10% D_2O. Table 1 summarizes the observed chemical shifts, along with changes in chemical shift which occur upon oxidation of GSH to GSSG within each solvent system. Upon oxidation, significant changes in the chemical shift of the cysteinyl protons occurred within both solvent systems, while the γ-glutamyl α- and β-protons exhibited significant changes only in DMSO.

Circular dichroism spectra of GSSG in 90% methanol were obtained at variable temperature and concentration (Figure 2) to probe conformational changes occurring upon gelation. These spectra are similar to those of GSSG in aqueous solutions which have been published previously.[23,24] These authors have assigned the negative bands (212 and 270 nm) to disulfide transitions and the positive band at 230 nm to the cys-gly peptide bond. A fourth band near 240 nm is clearly present in our spectra, but is nearly absent in aqueous solutions.[23] Figure 2A illustrates the concentration dependence of the CD spectrum at −10 °C, a temperature that results in gelation at concentrations of 1 mM and above. The intensity of the disulfide band at 212 nm decreases dramatically with increasing concentration, and is completely absent in the gelled samples at 1 and 2 mM. By contrast, the disulfide band at 270 nm is relatively unaffected by variations in concentration below the threshold for gelation, but in the gelled samples the intensity of this band increases significantly and it appears to blue-shift. The maxima of this peak is difficult to discern, however, because the sign of the band at 240 nm appears to invert to become negative in the gelled samples, coalescing with the disulfide band. These long-wavelength changes are also observed in Figure 2B, which illustrates the temperature dependence of the spectrum at 1 mM. The fluid → gel transition between 5 and −10 °C again produces a marked increase in the intensity of the long-wavelength disulfide band and an inversion of the sign of the 240 nm band. At 0.25 mM (Figure 2C), below the gelation threshold, temperature has little influence on these bands, with the greatest

(23) Coleman, D. L.; Blout, E. R. *J. Am. Chem. Soc.* **1968**, *90*, 2405−2415.

(24) Ottnad, M.; Ottnad, C.; Hartter, P.; Jung, G. In *Glutathione: Proceedings of the 16th Conference of the German Society of Biological Chemistry*; Flohe, L., Benohr, H. Ch., Sies, H., Waller, H. D., Wendel, A., Eds.; Georg Thieme Verlag: Stuttgart, 1973; pp 20−28.

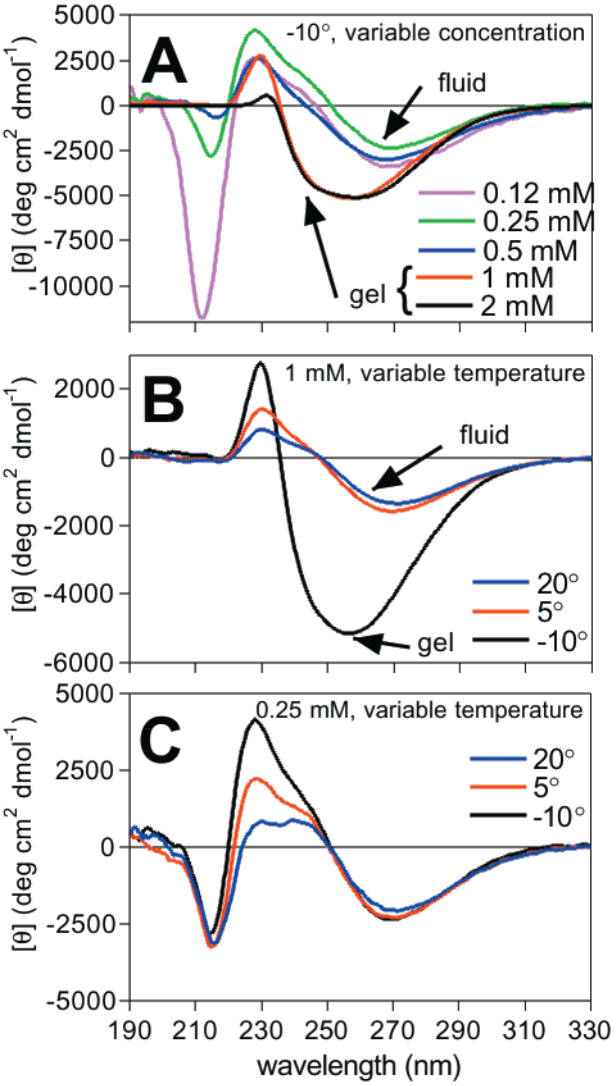

Figure 2. Circular dichroism spectra of GSSG in 90% methanol as a function of temperature and concentration: (A) variable concentration at a constant temperature of −10 °C (1 and 2 mM samples are in the gel phase, others are fluid); (B) variable temperature at a constant concentration of 1 mM (again, −10 °C sample in the gel phase); and (C) variable temperature at a constant concentration of 0.25 mM. All samples are fluid.

impact being seen on the cys-gly peptide bond transition at 230 nm. Collectively, these results suggest that gelation is coupled to an alteration of the local structure of the disulfide.

Visible Microscopy. To determine if the GSSG aggregates which produce gels can be characterized as amyloid, we stained gel slices with an alkaline Congo Red solution and observed them between crossed polarizers. The staining and subsequent appearance of green birefringence is considered the most specific method for the determination of amyloid.[25] GSSG/DMSO gel slices stained readily with Congo Red, and when partially dried appear pale red in color with darker red fibers visible. Under crossed polarizers, the thin sections exhibit strong green birefringence, as shown in Figure 3. These results are consistent with Congo Red binding to a cross-β sheet fibril structure. None of the control slides exhibited any birefringence (not shown).

Electron Microscopy. TEM images revealed tangles of fibrous structures approximately 75 nm in diameter. The structures appeared to consist of straight fibers with multiple

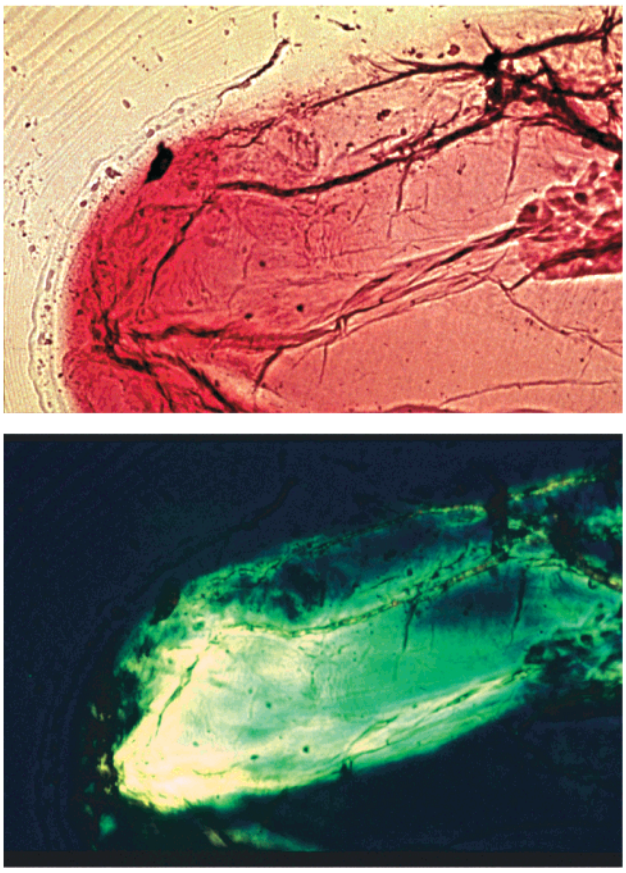

Figure 3. Congo Red staining and birefringence of GSSG/DMSO gel slice. Top: Gel slice stained with Congo Red and observed at 40× magnification. Bottom: Same gel slice observed between crossed polarizers, giving the green birefringence characteristic of amyloid structures.

branching "nodes", which intersect and tangle to form a 3-dimensional weblike fiber network. Examples illustrating these structures are shown in Figure 4. The morphology of these structures appears distinct from typical unbranched amyloid fibrils.

Discussion

Previous NMR studies of GSSG in aqueous media have indicated that its two tripeptide strands adopt an antiparallel, extended conformation.[21,22] This conformation is stabilized by hydrogen bonding between the glycyl amide nitrogens and the γ-glutamyl amide carbonyls, as well as the restricted rotation of the disulfide bond. Although the hydrogen bonding pattern is typical of an antiparallel β-sheet, the disulfide between the strands distinguishes this structure from a "true" β-sheet. Because of this, one would not necessarily expect GSSG to exhibit the spectroscopic signatures of a classic β-sheet. However, similar antiparallel β-like conformations have been reported for other small acyclic peptides with a central cystine residue.[26,27] Furthermore, the CD spectrum of GSSG is similar to that of a cystine peptide which has been shown by NMR and X-ray crystallography to adopt an antiparallel intramolecular β-type conformation.[26] This β-type intramolecular conformation thus appears to be a common structural motif among small acyclic cystine peptides. However, GSSG is the first such

(25) Westermark, G. T.; Johnson, K. H.; Westermark, P. *Methods Enzymol.* **1999**, *309*, 3−25.

(26) Raj, P. A.; Soni. S. D.; Ramasubbu, N.; Bhandary, K. K.; Levine, M. J. *Biopolymers* **1990**, *30*, 73−85.

(27) Ueyama, N.; Araki, T. *J. Am. Chem. Soc.* **1978**, *100*, 4603−4605.

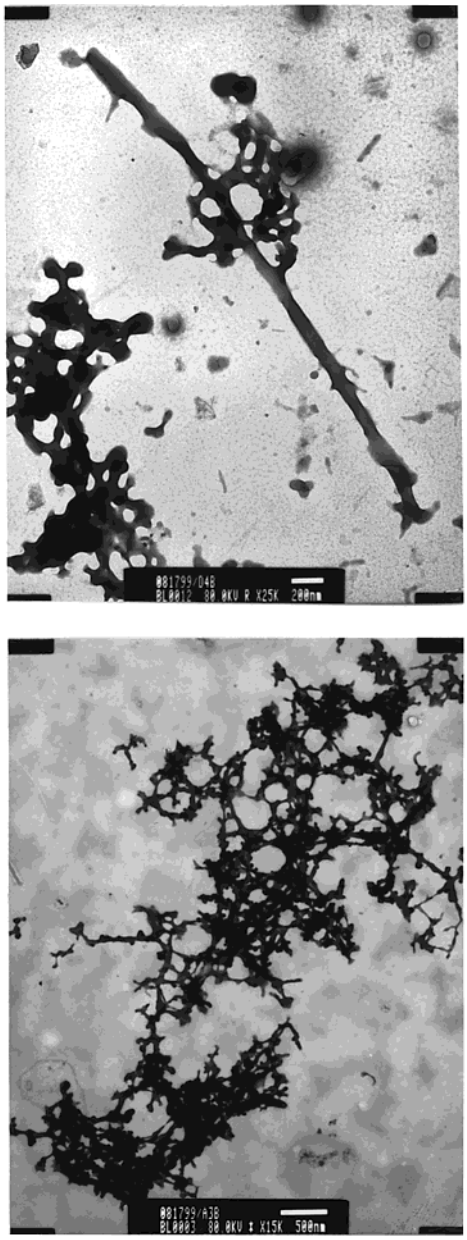

Figure 4. Electron micrographs of GSSG/DMSO gel. Top: Fiber about 2 μm in length with minimal branching nodes (bar = 200 nm). Bottom: Tangle of fibers of varying lengths, with multiple nodes producing a weblike network (bar = 500 nm).

peptide to be reported to self-assemble into extended intermolecular aggregates in organic solvents, and our studies suggest that the γ-glu-cys bond is required for this unique behavior.

Many peptides and proteins have been observed to form gels in denaturing organic solvents. However, glutathione is an unusual and interesting case for several reasons. First, it is significantly smaller than most peptide gelling agents. It has been suggested that the minimum required peptide length to produce stable extended β-sheet structures is six residues.[3,10,11] While GSSG satisfies this stipulation, its nonlinear geometry dictates that each strand is only three residues long. Because of this small size, the extended intermolecular array is assembled by remarkably few contacts. Second, glutathione represents an atypical peptide structure. In addition to its nonlinearity by virtue of a central cystine residue, GSSG also possesses two side-chain peptide bonds (the γ-glu-cys linkages). The results from our analogue studies indicate that the gelation behavior is highly specific to this structure. The oxidized disulfide forms of the glutathione isomer glu-cys-gly as well as the analogue β-asp-cys-gly failed to exhibit gelation in any of the solvents tested. These stringent structural requirements for gelation suggest that GSSG gels are stabilized by highly ordered and specific intra- and intermolecular interactions. Finally, the ubiquitous nature of glutathione in biological systems and its high concentrations (up to 10 mM) in our own cells make it a particularly relevant peptide for study.

Our initial observation was that reduced glutathione, GSH, produces gels in DMSO over a period of several days. However, three lines of evidence demonstrate that it is GSSG which is responsible for gelation. First, the *S*-methyl conjugate of glutathione, which cannot oxidize to GSSG, does not exhibit gelation. Second, mass spectra (not shown) of gels prepared with GSH indicate the presence of substantial quantities of GSSG, confirming that the oxidation occurs over the time scale of gelation. Acceleration of this oxidation by bubbling air through GSH/DMSO solutions also accelerates gelation, while addition of DTT slows gelation considerably and causes the dissolution of preexisting gels. Finally, solutions prepared with GSSG produce gels much more quickly than solutions prepared with GSH. Thus, oxidation represents a "molecular switch" that converts soluble GSH into a species capable of self-assembly into noncovalent arrays. The redox environment therefore presents an opportunity for control or regulation of the gelation process.

In aqueous media, NMR studies suggest that the terminal groups of GSSG have minimal interactions with each other,[21] and it appears probable that these groups would be highly solvated. In organic solvents, a cross-strand interaction between these terminal groups may become more favorable than solvation. Indeed, our NMR data of the glutamyl α- and β-protons (Table 1) suggest that in DMSO, there is a change in the environment of the N-terminus upon oxidation of GSH to GSSG. These protons are shifted downfield by 0.14 and 0.09 ppm, respectively. In aqueous media, these protons do not exhibit any significant shift upon oxidation of GSH to GSSG. Only the cysteinyl protons exhibit large changes upon oxidation, as they would be expected to. The N-termini of GSSG differ from typical cystine peptides due to the γ-glu-cys linkage, which creates an unusually long distance between this bond and the N-terminus. How this affects the conformation of the N-termini is unclear, but its presence is critical for the gelation behavior, as demonstrated by the analogue studies.

Previous studies in aqueous solutions suggest that GSSG has three CD bands: two associated with the disulfide and one arising from the cys-gly peptide bond.[23,24] Due to the long distance from the chiral glutamyl α-carbon to the γ-glu-cys peptide bond, it is not believed that this chromophore is a significant source of asymmetry in the CD spectrum. The work of Coleman and Blout [23] with derivatives of L-cystine revealed that GSSG gives rise to an anomalously broad long-wavelength disulfide band in aqueous media, and they suggested that this may be due to an unidentified fourth band of negative sign near 240 nm. This band is clearly present in our spectra (Figure 2), and we propose that this relatively weak band may be assigned to the γ-glu-cys peptide bond. In the transition from fluid to gel in 90% methanol, this band reverses in sign from positive to negative and increases in magnitude (Figure 2A,B), suggesting a major perturbation in the geometry of its associated chromophore. If our assignment of this band to the γ-glu-cys peptide bond is correct, the observed perturbation further implicates the γ-glu-cys moiety in the gelation process. The other significant change which occurs in the CD spectrum upon

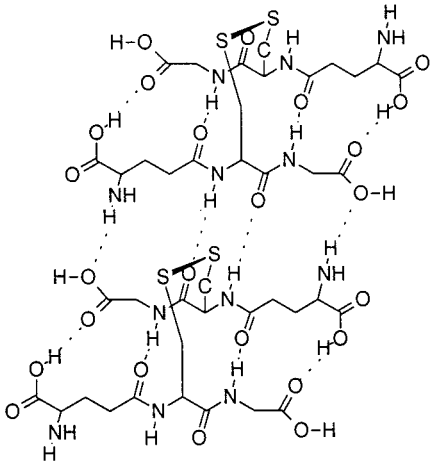

Figure 5. Model of intra- and intermolecular hydrogen bonding which could stabilize an extended GSSG β-sheetlike structure in organic solvents.

gelation is the increase in intensity and the blue shift of the long wavelength disulfide transition ($\pi \rightarrow \sigma^*$). The disulfide group possesses inherent asymmetry due to the fact that it can adopt a left- or right-handed screw sense, with a significant barrier to rotation between the two. The presence of nearby asymmetric centers breaks the degeneracy of the two senses, so in derivatives of L-cystine such as GSSG one screw sense is energetically favored over the other. However, solutions typically contain a mixture of the two rotamers, so intensities of this band are usually very low. The observation that the intensity of this band increases upon gelation suggests that the energy differential between the two rotamers becomes greater in the gel phase. This suggests that only one rotamer is responsible for self-assembly, thus introducing a further element of specificity. Unfortunately, there is disagreement in the literature as to whether the sign of the long-wavelength disulfide band can be reliably used to determine absolute screw sense, so we make no attempt to do so here. There is also disagreement as to whether the short-wavelength disulfide band arises from a $\pi \rightarrow \sigma^*$ or $\sigma \rightarrow \sigma^*$ transition. Our data indicate that in 90% methanol, this band is highly sensitive to concentration (Figure 2A), and is completely absent under gel-forming conditions. The significance of this spectral change is not clear at present. Regardless of this uncertainty, the CD data demonstrate that the local chirality of the disulfide bond and possibly also the γ-glu-cys peptide bond are coupled to intermolecular self-assembly, as manifested in gelation.

It is notable that the barrier to rotation about the disulfide bond results in minimal entropic cost for the formation of the proposed intramolecular interactions within the antiparallel strands of GSSG. Because the strands are already effectively "locked" into a β-type conformation, the entropy loss associated with assembly into an extended intermolecular β-sheet is lower than it would be for conformationally unrestricted tripeptides. We presume that this allows the formation of stable intermolecular sheets with peptide strands which are shorter than would be possible with linear peptides. The observed Congo Red birefringence (Figure 3) suggests that the extended GSSG structure is very similar to amyloid, with extended sheets running perpendicular to the direction of the strands. A model that illustrates an extended GSSG β-sheet is shown in Figure 5. This model shows all of the terminal groups in an un-ionized state, although we have not elucidated their protonation state in each of the solvent systems which produce gels. However, the observation that the glycyl ethyl ester of GSSG is capable of forming gels indicates that gelation does not require the C-terminus to act as a hydrogen bond donor or to form a salt bridge. Thus our proposed model suggests that the glycyl carboxyl group acts only as a hydrogen bond acceptor. The design principles of cross-strand attractive forces and lateral strand recognition which have been proposed by Aggeli et al. [3] for engineering β-sheet peptides are uniquely addressed by this model. The disulfide can be considered as an extremely strong (and conformationally restricted) cross-strand attraction within each GSSG unit, and the geometry of the γ-glutamyl residue allows for specific intermolecular recognition. Indeed, the γ-glu-cys linkage appears to be a requirement for intermolecular assembly. The observation that the unique structural features of GSSG allows its aggregation into supramolecular structures may provide additional design principles for the engineering of self-assembling peptides.

The ubiquitous nature and multiple functions of glutathione in biological systems raise questions about possible physiological significance of its self-assembly behavior. Concentrations required for localized microscopic effects of such behavior may be significantly lower than those required for a macroscopic event such as gel formation. Partially folded or unfolded proteins may be expected to have exposed sections of uncharged amino acid residues which resemble the low-dielectric conditions of polar organic solvents such as methanol and DMF. It is interesting to speculate that the self-recognition properties of glutathione within such an environment may have functional implications. For example, our Congo Red birefringence results suggest that GSSG may possess a face complimentary to amyloid aggregates, and thereby interact with unfolding intermediates of β-amyloid protein. There is also increasing interest in the function of transiently formed glutathione mixed disulfides in protein folding, which serve to direct the formation of appropriate protein disulfides in a process mediated by protein disulfide isomerase.[19] However, the mechanistic details of how glutathione performs this function are poorly understood. The interactions which we propose here to stabilize an extended GSSG sheet (Figure 5) could also be formed by two glutathione-protein mixed disulfides, with the two glutathionyl moieties presenting complementary hydrogen bonding and electrostatic sites. In this manner, glutathiolation of two protein cysteine residues may provide a handle for molecular recognition of each other, in a manner analogous to the self-recognition that drives the intermolecular assembly of GSSG reported here. Human lens proteins are also frequently glutathiolated,[19] and the role of glutathione in the process of cataract formation and lens opacification is an area of intense research. In light of the ubiquitous nature of glutathione and its extraordinary range of physiological functions, it is possible that the remarkably specific self-assembly properties described here serve important roles in biological systems.

Acknowledgment. The authors would like to acknowledge Michael Dabrowski for assistance with electron microscopy, Dr. Siddhartha Sarma for assistance with NMR, and Dr. Terrance Kavanagh for assistance with visible microscopy. This work was supported by NIH Training Grant T32 GM07750 (R.P.L.) and by the Department of Medicinal Chemistry at the University of Washington, Seattle.

JA0040417