DISULFIDE AND SULFHYDRYL GROUPS IN GLYCININ[1]

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ABSTRACT

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The number of disulfide (SS) bonds in glycinin and their rate of cleavage by dithiothreitol were determined at various concentrations of urea. A progressive increase in SS bond scission was observed with increasing urea concentration, reaching the maximum of 20 SS bonds at $8M$ urea. In addition, a linear relationship was obtained for the rate of SS cleavage as a function of urea concentration. These results indicate that most of the SS bridges are buried in the interior of the protein molecule. On exposure to alkaline denaturation, a maximum of 9.2 sulfhydryl (SH) groups were obtained per mole of glycinin at pH 11.9. Since glycinin in $6M$ urea contains only 1.7 SH groups per mole, the remaining SH groups are probably the result of the alkaline cleavage of some susceptible SS bonds.

Glycinin is the major storage protein of soybean seeds (*Glycine max*) located in cotyledonary subcellular particles called protein bodies (1-3). Results of extensive studies on the structure of the protein have been published (4-12). The number of sulfhydryl (SH) and disulfide (SS) groups in glycinin is not known, although these moieties have been implicated in the formation of SS polymers (4), heat denaturation (13,14), and intersubunit association of the protein (11,12,15). In view of the involvement of the SS bonds in the stabilization of the structure of glycinin and in its denaturation, we have attempted to explore quantitatively their presence and reactivity in urea solutions and alkaline environment. The SS cleaving reagent dithiothreitol (DTT) (16) was used to measure spectrophotometrically the number and reactivity of SS bonds in urea, whereas p-hydroxymercuribenzoate (PHMB) (17) was used to quantitate SH groups.

MATERIALS AND METHODS

DTT, PHMB, and EDTA were obtained from Sigma Chemical Co., St. Louis. Ultrapure urea was purchased from Schwarz/ Mann Co., Orangeburg, NY. All other chemicals were of reagent grade. Glycinin was prepared as previously described (4,18).

Spectrophotometric measurements were performed with a Beckman Model 25 double-beam recording spectrophotometer. Matched quartz cuvettes of 1-cm path length were numbered and used in the same sequence for all experiments. A London Radiometer PHM 62 pH meter was used for all pH adjustments.

SS Bonds

The assay for the measurement of SS bonds depends on the spectrophotometric quantitation of oxidized DTT (310 nm) produced from the cleavage of SS groups in the protein by the DTT reagent (16). Appropriate blanks have to be provided to correct for possible oxidation of DTT and possible

increase of 310-nm absorbance due to scattering from the denatured protein solution. Therefore, the following solutions and procedure were used:

1. Stock buffer solution—$0.05M$ potassium phosphate buffer pH 7.6 made $10^{-3}M$ in EDTA.
2. Buffer-urea solution ($8M$)—urea (4.8 g) dissolved and made to 10 ml with stock buffer solution, prepared fresh just before assay.

3. DTT solution ($0.05M$)—DTT (7.7 mg) dissolved in 0.1-ml stock buffer solution, prepared fresh.
4. Protein-buffer-urea solution—10 mg protein dissolved in 1.5-ml buffer-urea solution. Centrifuge at 2,000 g for 15 min to obtain clear solution for spectrophotometric studies. Protein concentration is determined spectrophotometrically.

All of the solutions placed in the cuvettes are flushed with nitrogen. Urea denaturation of glycinin takes place within a few seconds. In this procedure, however, approximately 30 min of preincubation time of the protein with urea was allowed to ensure maximum unfolding. For the estimation of the protein concentration, a 100-μl aliquot of the protein-buffer-urea solution was mixed with 1.5 ml of the buffer-urea solution, and the absorbance at 280 nm was measured against buffer-urea solution as reference. An E $^{1\%}_{1cm}$ value of 8.2 was used to obtain the concentration of glycinin (g).

Possible changes in light scattering at 310 nm were measured by recording the absorbance of the protein-buffer-urea solution against the buffer-urea solution. Corrections were made by subtracting this absorbance from that obtained in the presence of DTT. The test solution contained 1.2 ml of the protein-buffer solution mixed with 10 μl of the DTT solution. The reference was 1.2 ml of the buffer-urea solution mixed with 10 μl of the DTT solution. The absorbance at 310 nm was recorded as a function of time immediately after the addition of DTT. Estimation of the number of SS bonds was made as described previously (16). Five experiments were performed for each molar concentration of urea.

SH Groups

The method that Boyer (17) described was used to measure SH groups in glycinin. This involves spectrophotometric measurement of mercaptide formation employing PHMB as reagent. The following solutions were prepared:
1. Stock buffer solution—$0.05M$ potassium phosphate buffer pH 7.6.
2. Buffer-$6M$ urea solution—urea (3.6 g) dissolved and made to 10 ml with stock buffer solution.
3. PHMB solution (1.8×10^{-3} M)—PHMB (6.6 mg) solubilized in minimum $0.1N$ KOH and made to 10 ml with stock buffer, prepared fresh.
4. Protein-buffer-urea solution—3 mg glycinin dissolved in 3 ml of the buffer-urea solution and centrifuged at 2000 g for 15 min to obtain a clear supernatant. Protein concentration was determined spectrophotometrically as above.

The absorbance at 250 nm due to the protein was measured by reading 1.2 ml of the protein-buffer-urea solution against buffer-urea solution as blank. Subsequently, 10 μl of the PHMB solution were added to both the blank and the sample, and the increase in absorbance at 250 nm was recorded. A molar

extinction coefficient of 7600 for mercaptide formation was used for estimating the number of available SH groups.

In the case of alkali (1.0N KOH) titration, the difference in absorbance (ΔA) of the protein solution treated with the necessary volume of alkali to obtain a certain pH value versus the protein solution diluted with the same volume of pH 7.6 buffer is estimated at 250 nm. This is necessary to correct for the increase in the 250-nm absorbance due to the ionization of tyrosine groups at higher pH values. As an example, 1.2 ml of the protein-buffer-urea solution is treated with a volume (*e.g.*, 0.75 ml) of 1N KOH to bring the pH to 11.9. The blank contains 1.2 ml of the protein-buffer-urea solution plus 0.75 of the stock buffer solution. The ΔA at 250 nm is subsequently subtracted from the ΔA value of the test solution. The test solution contains 1.2 ml of the protein-buffer-urea solution, 0.75 ml 1N KOH and 10 μl PHMB solution and is read against a blank containing 1.2 ml of the protein-buffer-urea solution, 0.75 ml buffer solution, and 10 μl PHMB solution. Experiments as above were performed between pH 7.6 and 13.0. The final calculations on the number of SH groups take into consideration the dilution factor due to the addition of alkali.

RESULTS AND DISCUSSION

SS Bonds

The effect of varying concentrations of urea on the number and rate of cleavage of SS bonds in glycinin by DTT is shown in Fig. 1. A maximum of 20 SS
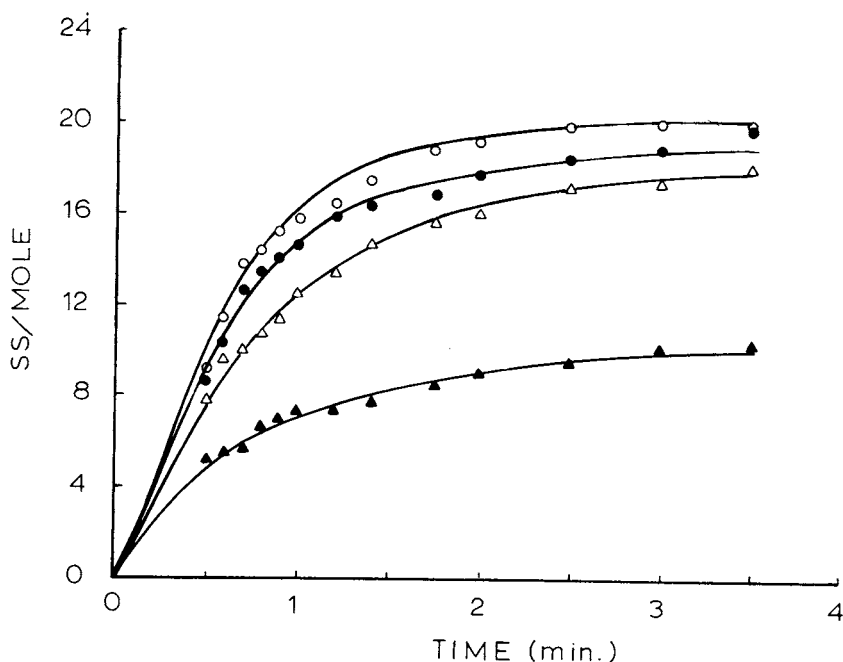

Fig. 1. Time course of glycinin SS bond cleavage by DTT in various urea concentrations (▲, 2M urea; △, 4M urea; ●, 6M urea; o, 8M urea).

bonds were found using $8M$ urea concentration. A lower number of SS bonds were reactive at $4M$ and $6M$ urea, whereas a dramatic decrease was observed at $2M$ urea concentration. The rate of the reaction was demonstrated to follow pseudo first-order kinetics as illustrated in Fig. 2. A plot of $\ln(dx/dt)$ versus time produced the reaction rate constant (k_e) for each urea concentration. When the reaction rate constants were plotted as a function of urea molarity, a linear relationship was obtained (Fig. 3).

The data presented above suggest that most of the SS bonds in glycinin are buried in the interior of the protein molecule and become available on unfolding of the protein with urea. Data in the absence of urea could not be obtained
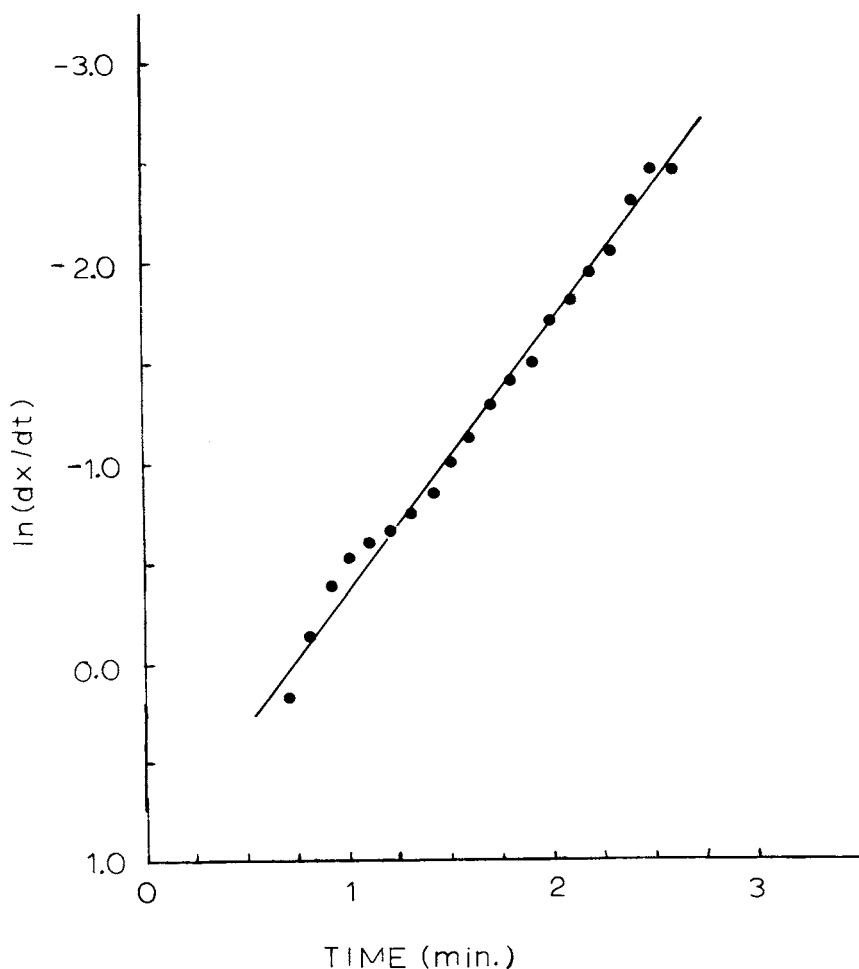

Fig. 2. Typical pseudo first-order reaction plot of SS bond cleavage by DTT (number of SS bonds per mole of glycinin) in the presence of $6M$ urea.

accurately because of light scattering interference due to the high concentrations of protein employed for the spectrophotometric measurements. These problems were alleviated by the presence of urea in the solutions. The finding that glycinin contains at least 20 SS bonds per molecule (320,000 daltons) agrees well with amino acid analysis data showing 44 ± 6 half cystine residues per mole of glycinin estimated on the basis of a 350,000 daltons mol wt (8). On this basis (*i.e.*, 350,000 mol wt), glycinin should contain approximately 21.8 SS bonds per molecule plus 1.8 SH groups (see below), thus producing 45.4 half cystine residues.

The observed increase in the reaction rate of SS cleavage in the presence of increasing concentrations of urea could be explained as follows: Glycinin may contain several areas of different structural compactness. On addition of urea, and depending on its concentration, only certain regions become unfolded. DTT may attack the SS bonds of the "loose" regions until the maximum number of SS bonds is cleaved. Increased concentration of urea may loosen up more structural
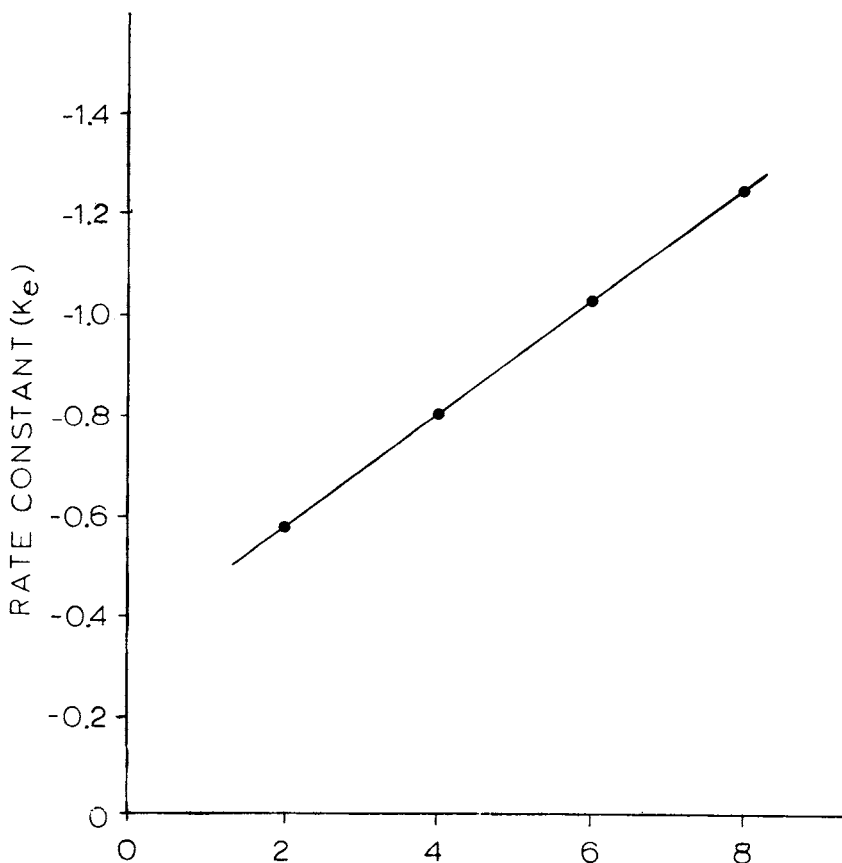

Fig 3. Pseudo first-order rate constants of SS bond cleavage at varying molar concentrations of urea.

regions, thus making available more SS bonds for interaction. The increase in the reaction rate constant with increasing urea concentration, however, may be due to alteration of the "reactivity" of SS bonds related to steric factors in the neighborhood of the cystine molecules.

SH Groups

Determination of the SH groups of glycinin with PHMB as a function of pH is depicted in Fig. 4. No detectable SH groups were found up to pH 10.5. Above pH 11.0, SH groups appear that reach a maximum of 9.2 groups per mole of glycinin (320,000 mol wt) at pH 11.9. Thus, there appears to be a narrow pH region where SS bonds are cleaved during alkaline denaturation. A matter of interest was knowing whether these groups are buried in the native protein molecule and are being exposed during alkaline denaturation, or whether they are the result of SS bond cleavage by the alkali. In a control experiment in which glycinin was exposed to $6M$ urea and PHMB, only 1.7 SH groups per mole of protein could be detected. Therefore, the conclusion was that most of the SH groups are formed by OH⁻ scission of exposed SS bonds. The pH effect on the unfolding of glycinin has been studied previously (8) by spectrophotometric titration of tyrosine phenoxy groups. The two curves (Fig. 4, present paper; Fig. 3, Reference 8) of SH group formation and tyrosine exposure as a function of pH closely resemble each other. The new information that has been obtained from these studies is that alkaline denaturation of glycinin above pH 11.0 also involves cleavage of some susceptible SS bonds.

The effect of OH⁻ on the SS bond of proteins which has been discussed previously (19), may involve the formation of either an R-SOH moiety that can
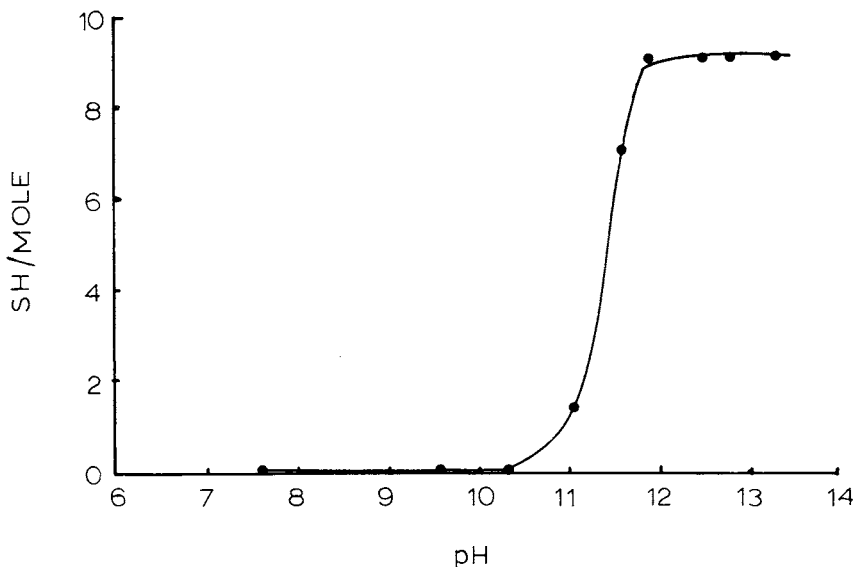

Fig. 4. Reactivity of SH groups in glycinin treated with alkali at indicated pH.

be further oxidized to cysteic acid, or the generation of a dehydroalanine residue, which by interaction with lysine can form lysinoalanine at high pH. The two schemes of cleavage can be illustrated as follows:

$$R\text{-}S\text{-}S\text{-}R + OH^- \underline{\quad\quad} RS^- + R\text{-}SOH$$
$$R\text{-}S\text{-}S\text{-}R + OH^- \underline{\quad\quad} R\text{-}S\text{-}S^- + H_2C = C$$

From a practical standpoint, some of the above interactions may take place during the first step (*i.e.*, alkaline denaturation) of soybean protein fiber formation (20). In this respect, the suggested (20) SH-SS interchange may indeed be involved in the mechanism of protein cross-linking in spun fibers. Kelley and Pressey (20), however, did not predict the possible formation of new SH groups by the alkaline treatment of the protein SS groups. The present results provide the first experimental evidence for the presence of buried SS groups in glycinin and the formation of SH groups on alkaline denaturation.

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