Disulfide Bonds: Determination, Location, and Influence on Molecular Properties of Proteins

J. S. Wall

Determination and location of cystine residues are essential to protein characterization since their disulfide bonds stabilize chainfolding and multichain structure responsible for physical and biological properties. Reduction with mercaptans, such as dithiothreitol, in dissociating solvents is the choice procedure for attaining disulfide cleavage, since other methods may cause undesirable side reactions. Under selected conditions the resulting sulfhydryls can be specifically alkylated with α,β-unsaturated compounds to prevent their reoxidation. Determination of alkylated cysteine residues supplements the cysteic acid method for cystine–cysteine analysis. Reoxidation studies on reduced proteins indicate that positions of formation of both intra- and interchain disulfide bonds are governed by amino acid sequences as well as by concentration, solvent, and pH. Location of disulfide bonds in peptides obtained by chromatography of proteolytic digests of proteins has been accelerated by automatic analysis with specific reagents. Disulfide interchange may change physical properties of proteins and must be avoided in characterization work.

The disulfide bonds present in cystine residues are common features of a majority of proteins, and serve to stabilize a variety of molecular structures. As shown in Figure 1, several different types of proteins present in cereal grains typify some of the characteristic ways in which cystine residues crosslink polypeptide chains. For example, the low-molecular-weight gliadin proteins are maintained in a folded conformation by only two intramolecular disulfide bonds (Beckwith *et al.*, 1965). Globular proteins may contain more extensive disulfide linkages. The glutenin proteins which are responsible for most of the cohesive-elastic character of wheat flour doughs consist of a linear association of polypeptide chains linked by intermolecular disulfide bonds (Nielsen *et al.*, 1962). These molecules may also contain some intramolecular disulfide bonds. Finally, there are structural proteins that are extensively crosslinked by disulfide bonds. These substances are not soluble in the most potent deaggregating solvents, such as 8 *M* urea. Only by disrupting disulfide bonds can they be dispersed. The matrix glutelin protein in corn endosperm tissues is an example we encountered (Nielsen *et al.*, 1970), but other classic examples include the keratins. Thus we find that nature, by means of disulfide bonds, is able to modify linear polypeptide chains to diverse structures with different functionalities.

DISULFIDE BOND CLEAVAGE

The disulfide bond is the most labile covalent linkage common in proteins. It is readily reduced or oxidized to yield scission products. It may also undergo sulfhydryl–disulfide interchange (Jensen, 1959), alkaline hydrolysis (Donovan, 1967), destruction by radiation (Risi *et al.*, 1967) and possibly mechanical scission (Axford *et al.*, 1962). These reactions produce difficulties in directly determining and locating disulfide linkages. But its lability also facilitates chemical modification of cystine to permit its accurate analysis, and allows one to separate and study the constituent polypeptide chains in proteins.

Three tools—oxidation, reduction, and interchange—used for modifying cystine and cleaving disulfide bonds are shown in Figure 2. Sanger (1949) oxidized cystine to cysteic acid with performic acid to separate the disulfide-linked polypeptide chains of insulin, but such treatment of other proteins may result in oxidative destruction of some amino acids. Crestfield *et al.* (1963) used sodium borohydride as a reducing agent, but it caused some chain cleavage. For proteins that can be dissolved in aprotic solvents, such as dimethyl sulfoxide, Krull and Friedman (1967) found sodium hydride to be a highly selective reducing agent. Most protein chemists prefer reducing disulfides by interchange reactions. The protein (1% solution) is equilibrated with a large excess, usually 100-fold over disulfide groups, of mercaptan, such as mercaptoethanol. Complete reduction of accessible disulfides is attained in 24 hr at pH 8. Cleland (1964) has shown that reduction is more rapid and requires less reagent when dithiothreitol is used. Also, sulfite will react with cystine disulfides to give 1 mol of cysteine and 1 mol of *S*-sulfocysteine.

But where the protein is aggregated or its molecular conformation hinders access of reagent, dissociating agents are also essential to attain complete reduction of disulfides (Cecil and Wake, 1962). In Figure 3 is shown the effect of urea concentration on the reaction of sulfite with wheat proteins (Beckwith and Wall, 1966). The formed sulfhydryl groups were determined by amperometric titration with

Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill. 61604

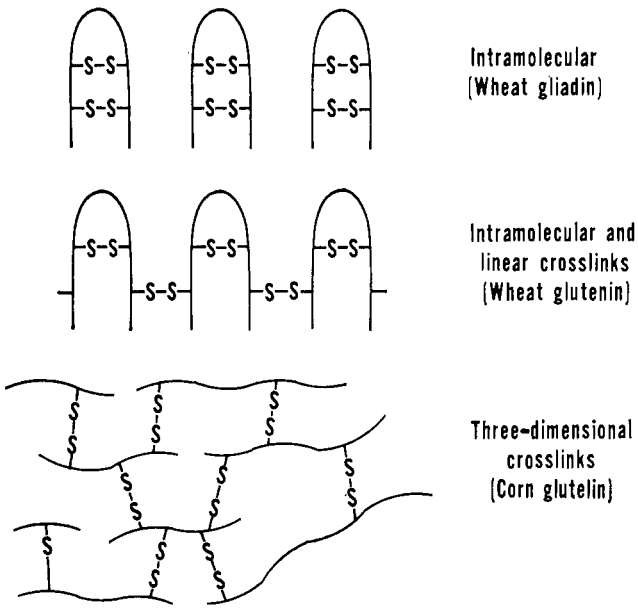

Figure 1. Disulfide bonds in cereal proteins

silver ions. Half of the gliadin disulfides, which are all intramolecular, are readily reduced in 2 *M* urea, but 5 *M* urea is required to completely cleave all the disulfide bonds. Cecil and Wake (1962) showed that intermolecular disulfides are more readily cleaved than intramolecular. This observation appears true for glutenin that has intermolecular disulfide bonds, but its high degree of aggregation requires increasing levels of urea to expose disulfides to the reducing agent. For optimal reduction the reaction is conducted for most proteins in 8 *M* urea.

ALKYLATION AND DETERMINATION

Since sulfhydryl groups are readily reoxidized by air to form disulfides, they must be blocked if one is to isolate the individual polypeptide chains. Reaction with sulfite in the presence of air and copper catalyst will quantitatively convert all cystine disulfides to *S*-sulfocysteine derivative (Swan, 1957). But this derivative is not stable. Preferably, sulfhydryl groups should be alkylated. Halogen compounds, such as iodoacetamide, are widely used for this purpose. *N*-Ethyl maleimide, an unsaturated compound, has long been used to block sulfhydryl groups. Weil and Seibles (1961) introduced another α,β-unsaturated compound, acrylonitrile, for this purpose. The reaction of this reagent with sulfhydryl compounds is diagrammed in Figure 4.

Alkylating agents are not specific for sulfhydryl groups but will attack other nucleophiles, such as the ϵ-amino group

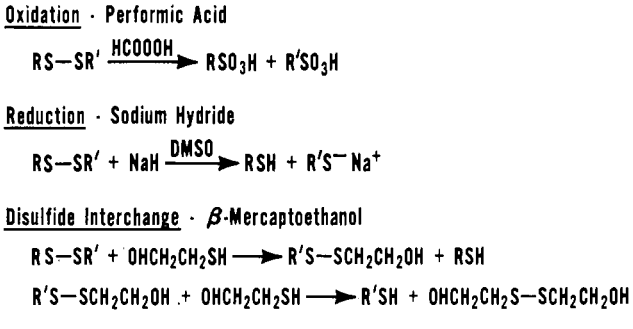

Figure 2. Methods to achieve protein disulfide cleavage

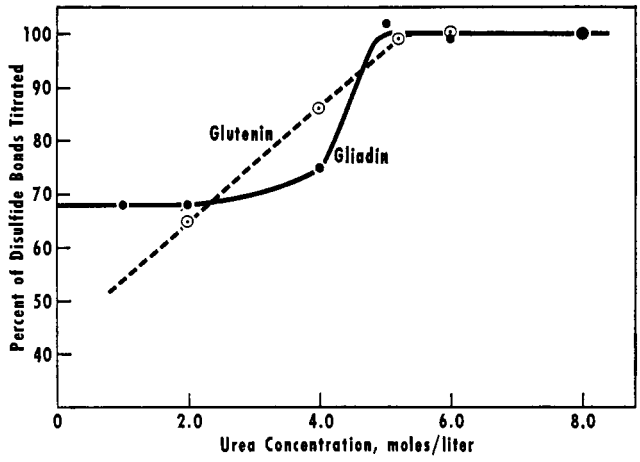

Figure 3. Effect of urea on disulfide reduction

of lysine. As seen in Figure 5, the relative rates at which acrylonitrile reacts with the amino or sulfhydryl groups in different compounds depend upon the polar and steric environments of those groups (Friedman *et al.*, 1965). In a series of amines and in a series of mercaptides of similar steric environments, the reaction rates with acrylonitrile are a function of the pK of the nucleophilic group. However, where substituents have the same pK, the mercaptide group is about 300 times more reactive than the amino. One can therefore select conditions of time, pH, and concentration of alkylating agent so that the reaction is quantitatively specific for the sulfhydryl group in proteins (Cavins and Friedman, 1968).

Disulfide groups in the presence of sulfhydryls can be determined differentially by amperometric titration before and after reaction with sulfite. The original apparatus of Benesch *et al.* (1955) has been modified to permit microanalysis, and the stability of the salt bridge has been improved by substituting polyacrylamide for agar (Rothfus, 1966).

Owing to its lability, an accurate estimation of cystine requires that it be converted to a stable derivative to permit protein hydrolysis and quantitative chromatographic isolation. Oxidation of cystine and cysteine in proteins to cysteic acid by means of performic acid is widely used to determine those amino acids. To improve recovery of cysteic acid, Moore (1963) introduced the use of hydrogen bromide to eliminate excess performic acid before concentration and acid hydrolysis. However, in some protein samples we have observed incomplete cystine oxidation by this procedure.

Quantitative determination of alkylated derivatives of cysteine has been explored as a more precise procedure for cysteine determination in reduced proteins (Kalan *et al.*, 1965). Friedman *et al.* (1970) established that an amount of vinyl pyridine equivalent to the quantity of β-mercaptoethanol used to reduce cystine residues will specifically alkylate all the sulfhydryl groups in proteins dissolved in 8 *M* urea, pH 7.5, in 90 min. The resulting *S*-β-(4-pyridylethyl)-L-cysteine residues are stable to acid hydrolysis and elute just before arginine on the short column of an amino acid analyzer

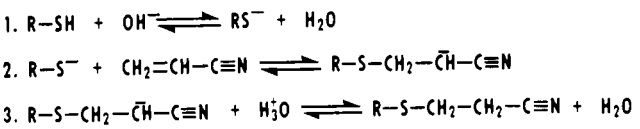

Figure 4. Reaction of sulfhydryl groups with α,β-unsaturated compounds

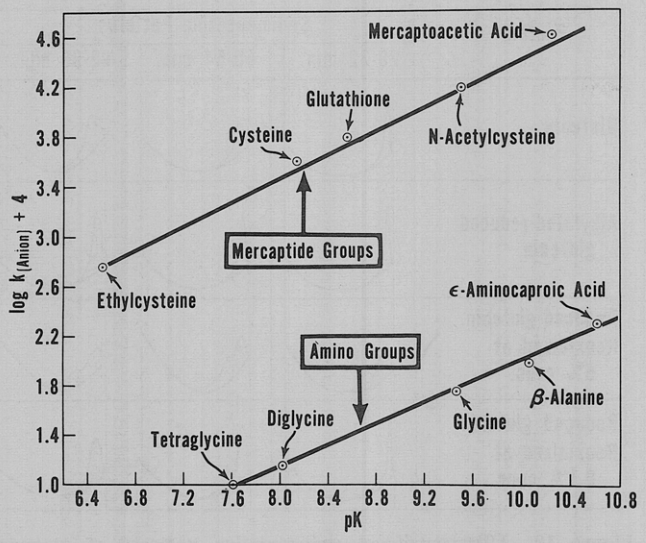

Figure 5. Log second-order anion reaction rate constants *vs.* pK for reactions of mercaptide groups and amino groups with acrylonitrile

(Figure 6). The analysis of this derivative in protein hydrolyzates permits a precise determination of total cysteine plus cysteine residues in the protein.

EFFECTS OF DISULFIDES ON PROTEIN PROPERTIES

Depending upon the location of the disulfide bond and the solvent system in which the protein is studied, dramatic changes can be demonstrated in proteins after disulfide bond cleavage. Table I summarizes properties of gliadin and glutenin before and after their disulfide bonds were reduced and the sulfhydryls alkylated. The molecular weight of the gliadin, which contains only intramolecular disulfides, was hardly

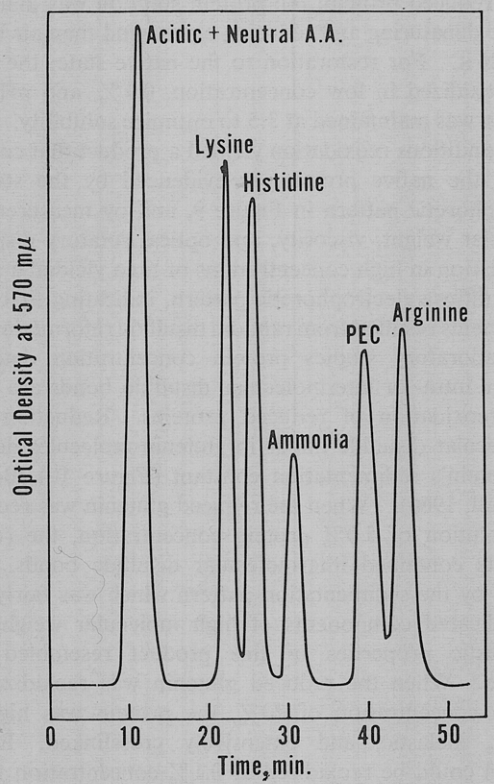

Figure 6. Chromatographic separation of β-4-pyridylethylcysteine (PEC) on a short column of an automatic amino acid (AA) analyzer

Table I. Effect of Disulfide Bond Cleavage on Molecular Properties of Gliadin and Low-Molecular-Weight Glutenin[a]

Fraction	Type of disulfide bond	Before disulfide cleavage: Molecular weight	Before disulfide cleavage: f/f_0[b]	After disulfide cleavage: Molecular weight	After disulfide cleavage: f/f_0[b]
Gliadin	Intramolecular	26,900	2.33	22,300	3.26
Low-molecular-weight glutenin	Inter- and intramolecular	125,600	3.29	36,800	3.64

[a] Determined in 8 *M* urea–0.1 *N* formic acid. [b] Frictional ratio.

altered, but in 8 *M* urea solution the frictional ratio, a measure of molecular asymmetry, was increased from 2.33 to 3.26 (Nielsen *et al.*, 1968). The disulfide bonds maintained the protein in a folded conformation even in concentrated urea. In contrast, a fraction of wheat glutenin protein that initially had a weight-average molecular weight of 125,000 was reduced to fragments averaging 36,800 upon disulfide cleavage. The frictional ratio was altered from 3.29 to 3.64, owing to cleavage of fairly linear chains. Apparently, reducing disulfide bonds converts protein molecules to the fundamental polypeptide chains which, in disassociating solvents, revert to random coil conformations. As measured by optical rotatory dispersion in acid solutions, the observed loss of α-helical contents in gliadin after reduction is consistent with this premise (Beckwith and Heiner, 1966).

But many significant changes in the protein properties following reduction are detected in nondenaturing and neutral solvents. Reduction of proteins having intramolecular disulfide bonds is usually associated with loss of biological specificity. Well-known examples of loss of enzyme activity include ribonuclease (White, 1961) and α-amylase (Isemura *et al.*, 1963). Steiner (1964) demonstrated that reduced trypsin inhibitor is unable to combine with the enzyme. As shown in Figure 7, reduced-alkylated gliadin does not exhibit antigenic activity against gliadin antibodies in immunodiffusion studies (Beckwith and Heiner, 1966).

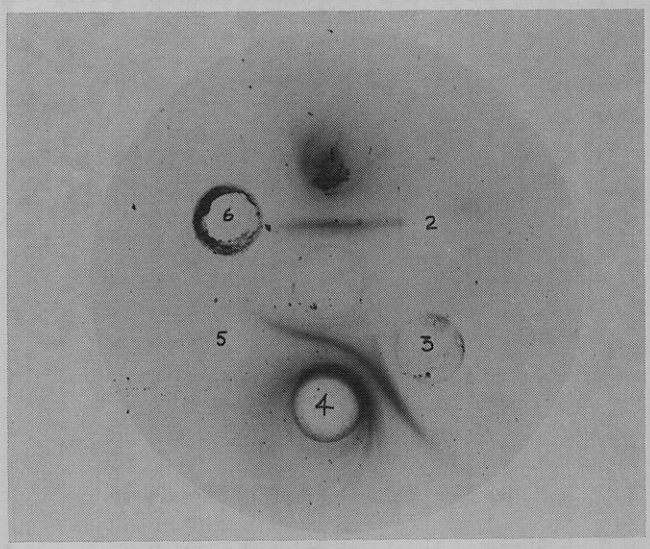

Figure 7. Loss of immunological properties of crude α-gliadin after reduction-alkylation. Ochterlony analysis of native γ-gliadin (reservoir 3) and reduced-alkylated sample (reservoir 6) against gliadin antiserum (reservoirs 1 and 4); standard gliadin solution in center well

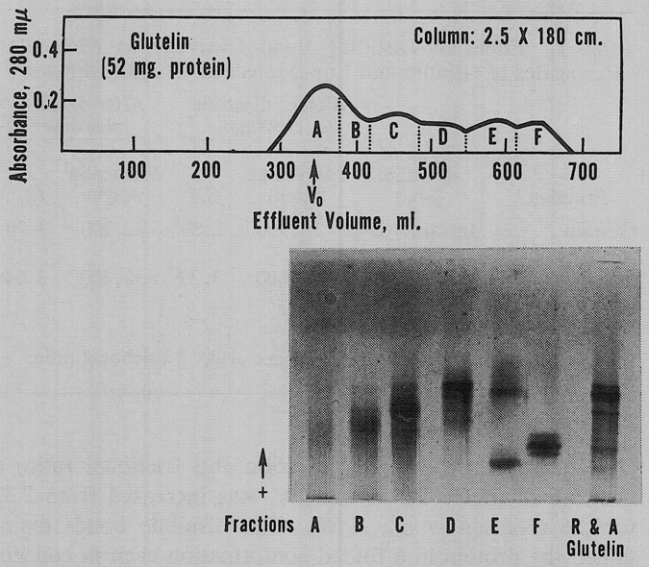

Figure 8. Upper: Chromatographic separation of reduced-alkylated corn glutelin on Sephadex G-200. Lower: Starch-gel electrophoretic patterns of fractions

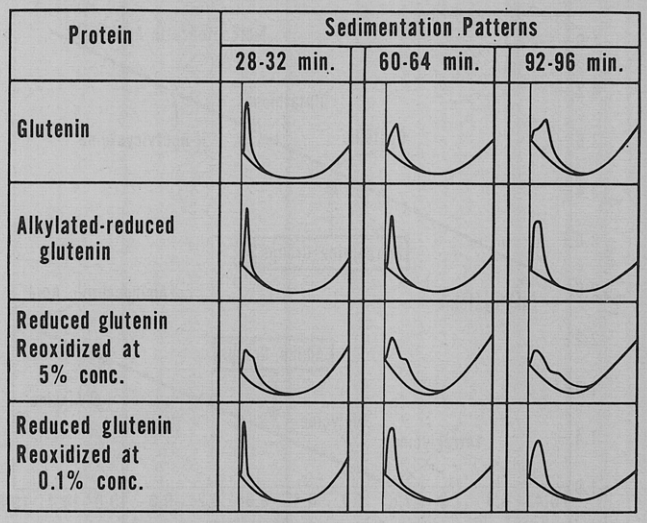

Figure 10. Ultracentrifugal sedimentation patterns of glutenin, reduced-alkylated glutenin, and reduced glutenin reoxidized at 0.1 and 5% protein concentrations

Reservoirs 1 and 4 contain antigliadin serum while reservoir 3 contains native gliadin and reservoir 6 reduced-alkylated gliadin. The loss of biological activity is a sensitive measure of changes in structure near the active site of protein interactions.

The cleavage of intermolecular disulfide bonds in proteins produces major changes in functional properties of proteins. The cohesive elastic character of hydrated wheat glutenin is lost upon breaking its disulfide bonds. The glutelin matrix protein in corn endosperm is soluble only in highly alkaline solutions. After the disulfide bonds are reduced, this protein can be dissolved in 6 *M* guanidine hydrochloride or 0.1 *M* acetic acid (Paulis *et al.*, 1969). Chromatography on Sephadex G-200 in 6 *M* guanidine hydrochloride separates the polypeptide chains of this protein into several fractions differing in molecular weight and electrophoretic migration on starch gel (Figure 8). Corn glutelin is a highly complex protein maintained by extensive three-dimensional crosslinks. It is this protein that causes difficulty during wet milling of corn; a preliminary steeping in SO_2 reducing media is required to facilitate starch and protein separation from corn grain.

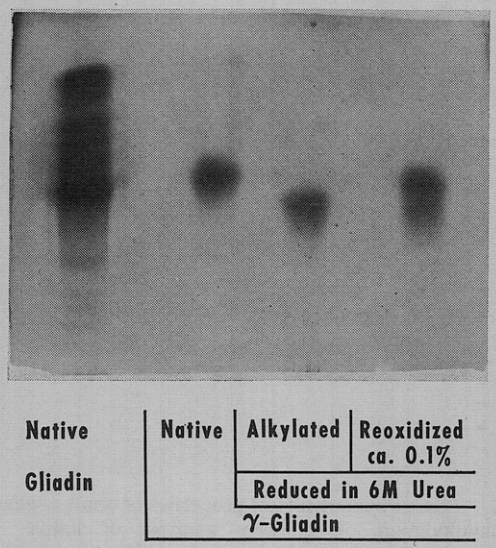

Figure 9. Starch-gel electrophoretic patterns of native, reduced-alkylated, and reduced-reoxidized γ-gliadin

REDUCTION AND REOXIDATION

The conclusion of Anfinsen (1962) that the thermodynamically most stable structure permitting intramolecular disulfide reoxidation in aqueous buffers is determined by the amino acid sequence has now been verified for many proteins. The starch-gel electrophoretic mobility of the γ-gliadin of wheat proteins (Figure 9) changes after reduction and alkylation with acrylonitrile (Beckwith *et al.*, 1965). To achieve reoxidation of the reduced protein, the protein solution was dialyzed to remove denaturing and reducing agents and then air bubbled through it. For restoration to the native state, the protein was reoxidized in low concentration, 0.1%, and pH of the solution was maintained at 3.5 to optimize solubility. Under these conditions reoxidation yielded a product that contained mainly the native protein, as evidenced by the starch-gel electrophoretic pattern in Figure 9, and by measurement of molecular weight, viscosity, and optical rotatory dispersion. Reoxidation in high concentrations of urea yielded a product with a diffuse electrophoretic pattern, indicating that several components resulted from random disulfide reformation.

In laboratory studies protein concentration determines whether intra- or intermolecular disulfide bonds are formed upon reoxidation of reduced proteins. Reduction of intermolecular disulfide bonds in glutenin molecules decreases the protein's sedimentation constant (Figure 10) (Beckwith and Wall, 1966). When the reduced glutenin was reoxidized in a solution of 5.0% protein concentration, the resulting products contained intermolecular disulfide bonds, as evidenced by the sedimentation pattern which was fairly broad but indicated components of high molecular weight. The viscoelastic properties of this product resembled native glutenin. When the reduced glutenin was reoxidized at a protein concentration of 20%, the protein was highly insoluble, inelastic, and extensively crosslinked. Reduced glutenin could be reoxidized at 0.1% concentration to yield protein of low molecular weight (Figure 10). But its rate of reoxidation was slow (11 days) compared to that of gliadin

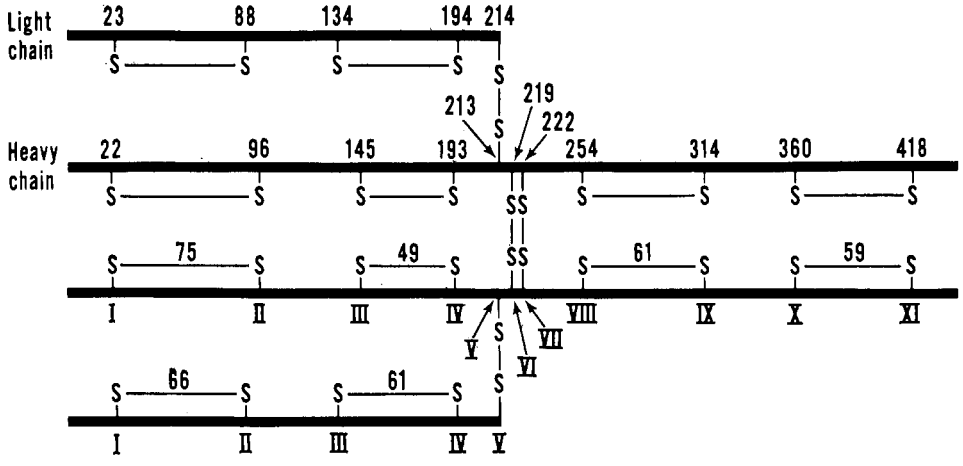

Figure 11. Disulfide bonds in human γ G-1 myeloma protein. From Edelman, G. M., Gall, W. E., *Annu. Rev. Biochem.* 38, 415 (1969). Reprinted with permission of authors and publisher

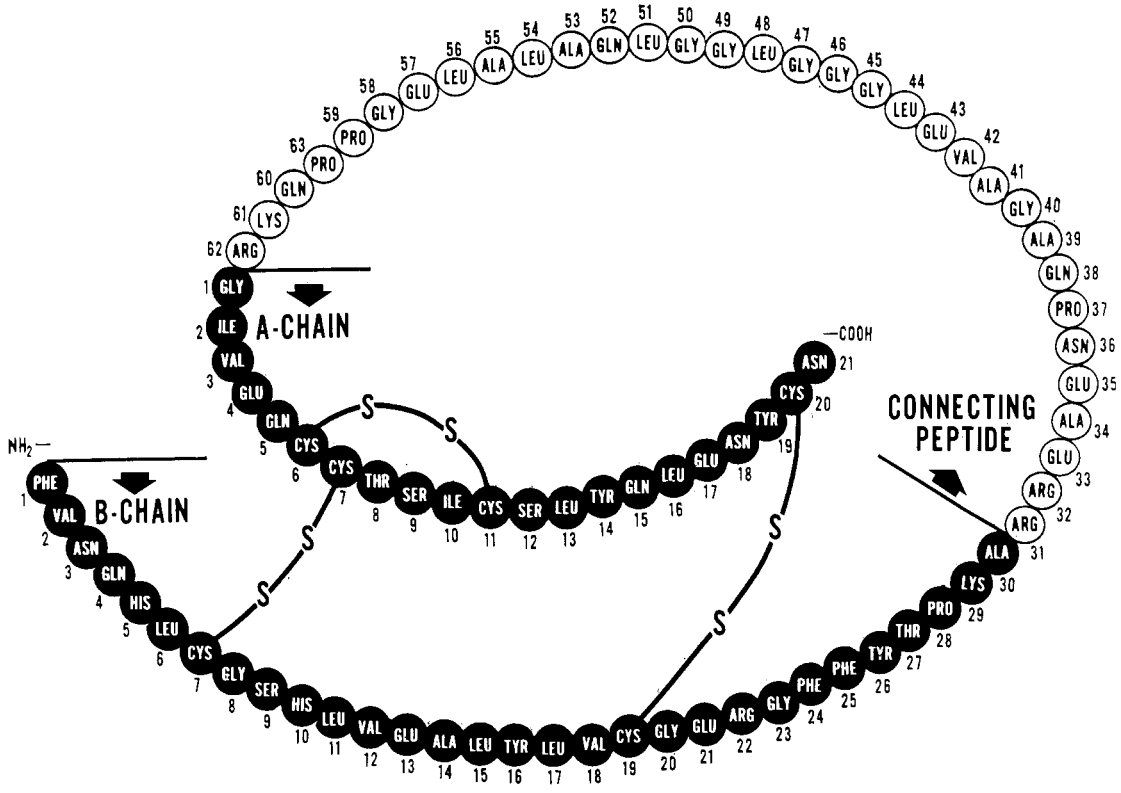

Figure 12. Proposed covalent structure of porcine proinsulin. From Chance, R. E., Ellis, R. M., Bromer, W. W., *Science* 161, 165 (1968). Reprinted with permission of authors and publisher. Copyright 1968 by American Association for Advancement of Science

(3 days). Differences between the amino acid sequences of the basic polypeptide chains of gliadin and glutenin may be responsible for the preferential development of intermolecular disulfide bonds in glutenin.

One of the most striking examples of specificity of reformation of inter- and intramolecular disulfide bonds is the reoxidation of reduced immunoglobulins. As shown in Figure 11, the human γ_1 globulins consist of two heavy chains linked by two disulfide bonds and two light chains, each of which is linked to separate heavy chains by a single disulfide bond (Edelman and Gall, 1969). Olins and Edelman (1964) separated the chains after reduction of all disulfide bonds. Reoxidation at appropriate protein concentrations gave restoration of molecular weight and return of antigen binding capability. Separated light and heavy chains readily associate to form active antibody aggregates, even when sulfhydryl oxidation is prevented. Thus the noncovalent interactions of the amino acid sequences presumably must direct disulfide bond formation between polypeptide chains.

Until recently an apparent exception to the rule that disulfide bond locations were determined by primary structure was the hormone insulin. Reduction and reoxidation of the protein, which consists of two chains, gave only a small degree of restoration of activity. Since the concept that disulfide formation was directed by primary structure was convincing, an insulin precursor was postulated. Steiner and Dyer (1967) first successfully isolated the proinsulin. The structure of proinsulin (Figure 12) was elucidated by Chance *et al.*

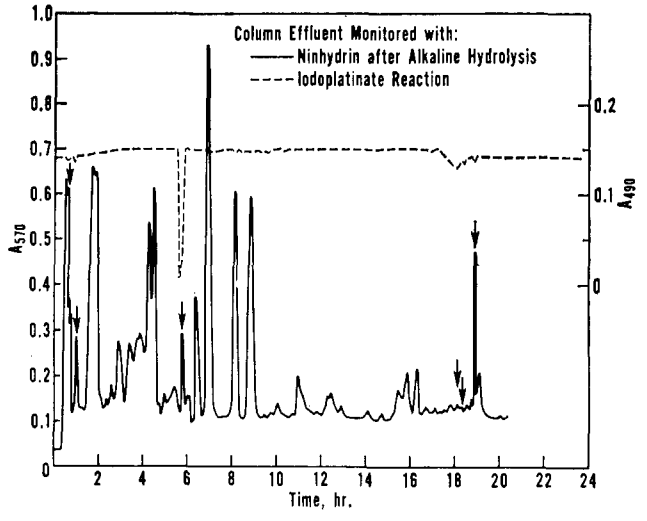

Figure 13. Cation-exchange chromatogram of native glutenin digested with pronase. Disulfide and sulfur groups detected with iodoplatinate reagent

(1968). The proinsulin is converted to insulin *in vitro* by elimination of the connecting peptide. Reduction and reoxidation of proinsulin favors reformation of the disulfide bonds in the proper position (Steiner and Clark, 1968). Primary structure appears to determine the location of disulfide crosslinks in all proteins which have been investigated.

LOCATING DISULFIDE BONDS

Considerable information about location of disulfide bonds in proteins may be obtained by separating the products of peptic or other proteolytic digests before and after disulfide cleavage. Conditions minimizing disulfide interchange (absence of reducing agents and low pH) are essential to obtain valid disulfide links (Sanger, 1949). Peptide maps obtained by combining paper electrophoresis and chromatography are frequently used to compare proteolytic digests of native and reduced proteins. A unique system has been devised by Brown and Hartley (1966) for isolation of disulfide-linked peptides in proteolytic digests. The peptides are first separated by paper electrophoresis and oxidized on the paper by performic acid vapor. Electrophoresis at right angles to the first direction produces parallel groups of cysteic acid peptides lying off a diagonal and reveals how the cysteic acid peptides were originally joined by disulfide bonds. This procedure has been applied to many proteins. With gliadin and glutenin it revealed some differences in sequences of amino acids neighboring disulfide bonds.

To survey many peptides and to isolate cystine-containing peptides readily, rapid automated procedures are used in conjunction with ion-exchange chromatography (Barber, 1967). In Figure 13 is a cation exchange chromatogram of native glutenin digested with pronase. The peptides were monitored in the effluent by reaction with ninhydrin in a Technicon Autoanalyzer. Iodoplatinate reagent was used to automatically locate sulfur-containing residues in the peptides. This reagent, of course, detects methionine and alkylated cysteine residues as well as cystine. A specific procedure for cystine disulfide bonds has been developed by Zahler and Cleland (1968) and adapted to automatic peptide analysis by Walsh *et al.* (1970). This method is based on reducing disulfides with dithioerythritol, complexing excess reagent with arsenite, and determining reduced cysteine with 5′5′-dithiobis(2-nitrobenzoic acid). Subsequent cleavage of the disulfide bonds and sequence analysis of the isolated peptides yield data that can be correlated with sequence studies on isolated polypeptide chains to locate disulfide bonds.

Even more detailed information on disulfide bond location, conformation, and influence on tertiary structure can be derived from X-ray diffraction analysis. X-ray diffraction at the 2 Å resolution level has been successful in elucidating the structure of several crystalline proteins containing disulfide bonds, including lysozyme (Blake *et al.*, 1967), ribonuclease (Kartha *et al.*, 1967), and α-chymotrypsin (Sigler *et al.*, 1968). The exact conformation of disulfide bonds in α-chymotrypsin (Figure 14) was established by X-ray diffraction analysis by Sigler and coworkers (1968). The disulfide bond is readily located in the X-ray diffraction patterns because of its density and also because it can be complexed with heavy metal ions. The spacial arrangements of chains and residues not only confirm chemical evidence for disulfide locations, but also provide information as to noncovalent bonds stabilizing the native structure.

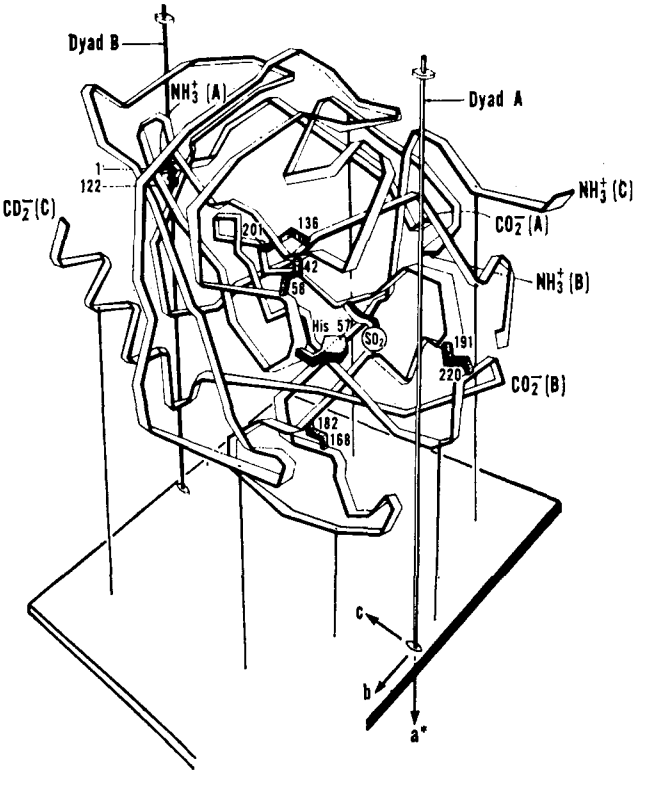

Figure 14. Conformation of polypeptide chains in α-chymotrypsin. From Sigler, P. B., Blow, D. M., Matthews, B. W., Henderson, R., *J. Mol. Biol.* 35, 143 (1968). Reprinted with permission of authors and publisher

DISULFIDE INTERCHANGE

Many changes in protein properties that accompany denaturation by solvent or heat treatment or other processes involve relocation of disulfide bonds, as outlined by Jensen (1959). Interchange of disulfide bonds is usually catalyzed by the presence of small amounts of sulfhydryl groups, as shown in Figure 15. Since the RS^- group is the active participant in interchange, such reactions are more rapid in alkaline media. Either heating the protein solution or adding denaturing solute, such as urea, enhances interchange as the resulting chain unfolding permits greater accessibility of sulfhydryls to disulfides. For example, the aggregation of

Schematic Representation of Relief of Strain in Dough by Sulfhydryl-disulfide Interchange

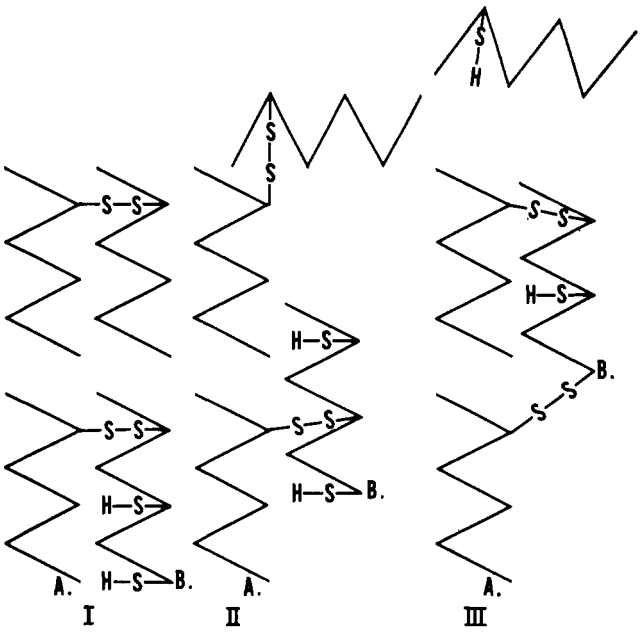

Figure 15. Disulfide interchange in dough proteins. From Sokol, H. A., Mecham, D. K., *Bakers Dig.* 34(6), 24 (1960). Reprinted with permission of authors and publisher

ovalbumin or bovine plasma albumin by heat or solvent denaturation is accompanied by a shift from intra- to intermolecular disulfide bonds (Jensen, 1959).

But more subtle phenomena, such as the improvement of wheat dough rheological behavior during mixing, may be attributed to disulfide interchange (Sokol and Mecham, 1960), as in Figure 15. Presumably, the strains from mechanical stresses of mixing are relieved by interchange transformation of highly crosslinked chains to less branched systems. Reducing agents, such as cysteine, actually decrease the dough mixing time for continuous mix dough processes (Henika and Rodgers, 1962). Oxidizing agents, including bromate, are believed to improve dough strength by eliminating some of the sulfhydryl groups involved in interchange. Consequently we find that, in an art as old as baking, skillful disulfide bond transformation is essential to attain optimal products.

CONCLUSION

We have taken a brief, hurried excursion through some areas of research on protein disulfide bonds. In a realm so extensive and varied, the route was highly biased in favor of areas with which I am familiar. I hope that I revealed some of the diversity of molecular structures that results from disulfide bonds. Also, I trust I pointed out some of the vehicles that many investigators use to explore this subject. And finally, I would like to feel that some food and agricultural chemists who have shared this bumpy path with protein chemists can visualize some profitable side trips in areas of their interest in the realm of disulfides.

ACKNOWLEDGMENT

I wish to thank my many associates, who conducted many of the experiments discussed, for their assistance in preparing this paper. Also, I wish to thank other scientists who permitted me to reproduce their important findings. Their names are indicated in the literature cited.

LITERATURE CITED

Anfinsen, C., *Brookhaven Symp. Biol.* **15,** 184 (1962).
Axford, D. W. E., Campbell, J. D., Elton, G. A. H., *Chem. Ind. (London)* **1962,** 407 (1962).
Barber, G. W., in "Technicon Symposia 1966, Automation in Analytical Chem. I," p. 401, Mediad Incorporated, White Plains, N.Y., 1967.
Beckwith, A. C., Heiner, D. C., *Arch. Biochem. Biophys.* **117,** 239 (1966).
Beckwith, A. C., Wall, J. S., *Biochim. Biophys. Acta* **130,** 155 (1966).
Beckwith, A. C., Wall, J. S., Jordan, R. W., *Arch. Biochem. Biophys.* **112,** 16 (1965).
Benesch, R. E., Lardy, H. A., Benesch, R., *J. Biol. Chem.* **216,** 663 (1955).
Blake, C. C. F., Mair, G. H., North, A. C. T. H., Phillips, D. C., Sarma, V. R., *Proc. Roy. Soc. London, Sect. B* **167,** 365 (1967).
Brown, J. R., Hartley, B. S., *Biochem. J.* **101,** 214 (1966).
Cavins, J. F., Friedman, M., *J. Biol. Chem.* **243,** 3357 (1968).
Cecil, R., Wake, R. G., *Biochem. J.* **76,** 146 (1962).
Chance, R. E., Ellis, R. M., Bromer, W. W., *Science* **161,** 165 (1968).
Cleland, W. W., *Biochemistry* **3,** 480 (1964).
Crestfield, A. M., Moore, S., Stein, W. M., *J. Biol. Chem.* **238,** 623 (1963).
Donovan, J. W., *Biochem. Biophys. Res. Commun.* **29,** 734 (1967).
Edelman, G. M., Gall, W. E., *Annu. Rev. Biochem.* **38,** 415 (1969).
Friedman, M., Cavins, J. F., Wall, J. S., *J. Amer. Chem. Soc.* **87,** 3672 (1965).
Friedman, M., Krull, L. H., Cavins, J. F., *J. Biol. Chem.* **245,** 3868 (1970).
Henika, R. G., Rodgers, N. E., U.S. Patent 3,053,666 (1962).
Isemura, T., Tagaki, T., Maeda, Y., Yutani, K., *J. Biochem.* **53,** 155 (1963).
Jensen, E. V., *Science* **130,** 1319 (1959).
Kalan, E. B., Neistadt, A., Weil, L., Gordon, W. G., *Anal. Biochem.* **12,** 488 (1965).
Kartha, G., Bello, J., Harker, D., *Nature (London)* **213,** 862 (1967).
Krull, L. H., Friedman, M., *Biochem. Biophys. Res. Commun.* **29,** 373 (1967).
Moore, Stanford, *J. Biol. Chem.* **238,** 235 (1963).
Nielsen, H. C., Babcock, G. E., Senti, F. R., *Arch. Biochem. Biophys.* **96,** 252 (1962).
Nielsen, H. C., Beckwith, A. C., Wall, J. S., *Cereal Chem.* **45,** 37 (1968).
Nielsen, H. C., Paulis, J. W., James, C., Wall, J. S., *Cereal Chem.* **47,** 501 (1970).
Olins, D. E., Edelman, G. M., *J. Exp. Med.* **119,** 789 (1964).
Paulis, J. W., Nielsen, H. C., Wall, J. S., 158th National Meeting ACS, Div. Biol. Chem., Abstr. 182, Sept. 7–12, 1969.
Risi, S., Dose, K., Rathihasamy, T. K., Augenstein, L., *Photochem. Photobiol.* **6,** 423 (1967).
Rothfus, J. A., *Anal. Biochem.* **16,** 167 (1966).
Sanger, F., *Biochem. J.* **44,** 126 (1949).
Sigler, P. B., Blow, D. M., Matthews, B. W., Henderson, R., *J. Mol. Biol.* **35,** 143 (1968).
Sokol, H. A., Mecham, D. K., *Baker's Dig.* **34**(6), 24 (1960).
Steiner, D. F., Clark, J. L., *Proc. Nat. Acad. Sci. USA* **60,** 622 (1968).
Steiner, D. F., Dyer, P. E., *Proc. Nat. Acad. Sci. USA* **57,** 473 (1967).
Steiner, R. F., *Nature (London)* **304,** 579 (1964).
Swan, J. M., *Nature (London)* **180,** 643 (1957).
Walsh, K. A. McDonald, R. M., Bradshaw, R. A., *Anal. Biochem.* **35,** 193 (1970).
Weil, L., Seibles, T. S., *Arch. Biochem. Biophys.* **95,** 470 (1961).
White, F. H., Jr., *J. Biol. Chem.* **236,** 1353 (1961).
Zahler, W. L., Cleland, W. W., *J. Biol. Chem.* **243,** 716 (1968).

Received for review September 29, 1970. Accepted January 4, 1971. Presented at Division of Agricultural and Food Chemistry Symposium on Characterization of Proteins, 160th Meeting, ACS, Chicago, Ill., September 1970. Work done at a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Reference to commercial products is not intended to be a recommendation by the Department of Agriculture over similar ones not mentioned.