

## Review

# Stability of Protein Pharmaceuticals

Mark C. Manning,<sup>1,2</sup> Kamlesh Patel,<sup>1</sup> and Ronald T. Borchardt<sup>1</sup>

---

Recombinant DNA technology has now made it possible to produce proteins for pharmaceutical applications. Consequently, proteins produced via biotechnology now comprise a significant portion of the drugs currently under development. Isolation, purification, formulation, and delivery of proteins represent significant challenges to pharmaceutical scientists, as proteins possess unique chemical and physical properties. These properties pose difficult stability problems. A summary of both chemical and physical decomposition pathways for proteins is given. Chemical instability can include proteolysis, deamidation, oxidation, racemization, and  $\beta$ -elimination. Physical instability refers to processes such as aggregation, precipitation, denaturation, and adsorption to surfaces. Current methodology to stabilize proteins is presented, including additives, excipients, chemical modification, and the use of site-directed mutagenesis to produce a more stable protein species.

---

**KEY WORDS:** protein stability; biotechnology; mutagenesis; denaturation.

## INTRODUCTION

With the recent advances in recombinant DNA technology, the commercial production of proteins for pharmaceutical purposes has become feasible (1,2). As a result, the preparation of proteins as medicinal agents has become an integral part of the pharmaceutical industry. Currently, there are more than 150 recombinant proteins in Phase I clinical trials or beyond, and almost a dozen have received FDA approval. Unfortunately, proteins possess chemical and physical properties which present unique difficulties in the purification, separation, storage, and delivery of these materials. Therefore, formulation of proteins differ greatly from that of rigid small organic molecules. Future pharmaceutical scientists will need to be properly trained to address the various aspects of protein instability. An introduction to these concepts is presented below, with the view that understanding protein stability at a molecular level is essential to solving many of their formulation problems.

Degradation pathways for proteins can be separated into two distinct classes, involving chemical instability and physical instability. First, chemical instability can be defined as any process which involves modification of the protein via bond formation or cleavage, yielding a new chemical entity. Second, physical instability does not involve covalent modification of the protein. Rather, it refers to changes in the higher order structure (secondary and above). These include denaturation, adsorption to surfaces, aggregation, and pre-

cipitation. A summary of the current understanding of each of these processes is presented and illustrated by well-characterized systems. Finally, approaches for retarding or inhibiting these processes and, thereby, increasing protein stability is presented.

## CHEMICAL INSTABILITY

A variety of chemical reactions is known to affect proteins (Fig. 1). These reactions can involve hydrolysis, including both cleavage of peptide bonds as well as deamidation of Asn and Gln side chains.<sup>3</sup> Hydrolysis at Asp-X sites is particularly accelerated. Oxidation of Cys can lead to disulfide bond formation and exchange, whereas oxidation of Met and other amino acids may inactivate or alter the activity of a protein. Other decomposition reactions include beta-elimination and racemization.

### Deamidation

In the deamidation reaction, the side chain amide linkage in a Gln or Asn residue is hydrolyzed to form a free carboxylic acid. Over the past two decades many investigators have observed altered forms of proteins which have been attributed to deamidation. Such a list contains lysozyme (3), bovine growth hormone (bGH) (growth hormone is also known as somatotropin) (4), human growth hormone (hGH) (5,6), insulin (7,8),  $\alpha$ -crystallin (9), cytochrome c (10),  $\gamma$ -immunoglobulin (11), epidermal growth factor (EGF) (12), hemoglobin (13), triosephosphate isomerase (TIM) (14,15), neocarzinostatin (16), prolactin (17), gastrin releasing peptide (18), and adrenocorticotrophic hormone (ACTH) (19,20), suggesting that *in vitro* deamidation is a common phenomenon.

The hydrolysis of Asn and Gln residues for many proteins and peptides has been observed under a variety of

---

<sup>1</sup> Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66045.

<sup>2</sup> To whom correspondence should be addressed.

<sup>3</sup> Unless otherwise noted, all amino acids listed are L-enantiomers of the 20 common amino acids and are referred to by their three-letter abbreviations.

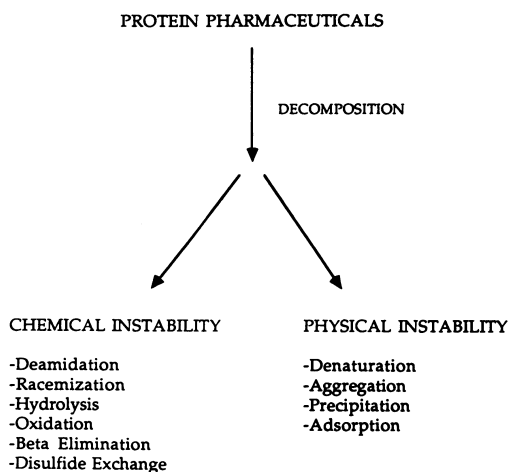


Fig. 1. Summary of the chemical and physical instability processes observed in protein pharmaceuticals.

chemical conditions and has been reviewed by Robinson and Rudd (21). Interestingly, it was realized that the deamidation of Asn residues, which occurs most often at the sequence Asn-Gly, was accelerated at neutral or alkaline conditions (22–24). The rates were also higher relative to the hydrolysis of the amino acid Asn itself (21). An explanation is that deamidation is believed to proceed through a five-membered cyclic imide intermediate formed by intramolecular attack of the succeeding peptide nitrogen at the side chain carbonyl carbon of the Asn residue (see Fig. 2) (25). Subsequently, the cyclic imide spontaneously hydrolyzes to give a mixture of peptides in which the polypeptide backbone is attached via an  $\alpha$ -carboxyl linkage (Asp) or is attached via a  $\beta$ -carboxyl linkage (iso-Asp) (23). Similarly, Gln can also undergo deamidation via formation of a six membered ring (23). Most of the information on the mechanism and rate of deamidation of

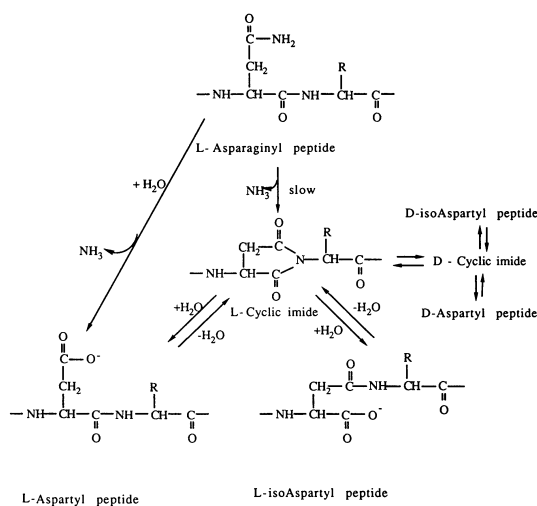


Fig. 2. Pathways for spontaneous deamidation, isomerization, and racemization for aspartyl and asparaginyl peptides.

Asn residues has been obtained from studies on short model peptides (26,27). Clear evidence for a deamidation mechanism involving the cyclic imide intermediate has been obtained by Geiger and Clarke (26). In their study, deamidation of a hexapeptide sequence based on residues 22–27 of ACTH (Val-Tyr-Pro-Asn-Gly-Ala) was studied at 37°C and pH 7.4. Evidence from these studies supporting cyclic imide formation include the appearance of iso-Asp, Asp, and cyclic imide peptides upon deamidation and a ratio of the iso-Asp to Asp peptide formed in the deamidation of this hexapeptide (2.8:1) is the same as that found when purified cyclic imide is hydrolyzed (3.1:1). If there is a significant amount of direct solvent hydrolysis of the amide linkage occurring, the proportion of Asp peptide relative to iso-Asp peptide in the deamidation of a hexapeptide would have increased, which is not the case. The presence of iso-Asp products from the incubations of proteins and peptides implies cyclic imide formation as an intermediate in deamidation reaction (28–32). The Fourier transform infrared photoacoustic spectroscopic measurements (FTIR-PAS) have also provided direct evidence for the formation of a cyclic imide in peptides with Asn-Gly sequences induced by heating in the dry state (33).

Recently, we have shown that both Asp- and iso-Asp-hexapeptides are formed upon deamidation of Val-Tyr-Pro-Asn-Gly-Ala, ACTH<sup>22–27</sup> (Asn-hexapeptide), in the pH range of 5 to 12 at 37°C (34,35). This further confirms the formation of a cyclic imide intermediate in the deamidation process at neutral and alkaline pH's. In the pH range 7 to 12, buffer concentration had significant effect on the rate of deamidation, indicating general acid-base catalysis. No buffer catalysis was observed at pH 5, 6, and 6.5. The ratio of iso-Asp- and Asp-hexapeptides was independent of buffer concentration at all pH's and was approximately 4:1. At acidic pH's (pH 1–2), the deamidation was much slower than at alkaline pH, and only Asp-hexapeptide was produced upon deamidation. Although iso-Asp-hexapeptide was not detected at acidic pH, one new product (Val-Tyr-Pro-Asp) was observed by HPLC. These results suggest that at acidic pH, the probable mechanism of deamidation is direct hydrolysis of the amide side chain of Asn, to form the Asp-hexapeptide, which further degrades in acidic media via peptide bond cleavage at the Asp-Gly bond. Reactions at pH 3 and 4 were very slow at 37°C (degradation of Asn-hexapeptide was not detected for 60 days).

By comparison, when deamidation experiments were carried out with ACTH (1–39), the separation of Asp- and iso-Asp products could not be achieved by either isoelectric focusing or cation-exchange HPLC (20). However, these techniques did separate native ACTH from the deamidated ACTHs (Asp- and iso-Asp-ACTH). The rate constants for the deamidation of both ACTH and Asn-hexapeptide (ACTH<sup>22–27</sup>) at pH 2.0, 7.0, and 9.6 at 37°C were similar. Formation of the iso-Asp product upon deamidation of ACTH at pH 7.0 and 9.6 was verified by the protein carboxymethyltransferase (PCM)-catalyzed methylation of deamidated ACTH. No such methylation was observed when ACTH was incubated at pH 2.0, 37°C. These data indicate the involvement of cyclic imide intermediate at neutral and alkaline pH but not at pH 2.0.

Since the formation of a cyclic imide involves participation of the succeeding amino acid, the size and physico-

chemical properties of neighboring amino acid side chain is expected to play an important role in the rate of formation of cyclic imide. Evidence in support of this conclusion comes from studying the rates of cyclic imide formation in peptides containing Asn (26) or Asp  $\beta$ -benzyl esters (36–45). For example, the rate of cyclic imide formation at pH 7.4 was approximately 50 times slower in the Asn-Leu-hexapeptide than the Asn-Gly-hexapeptide due to steric hinderance by the Leu side chain (26).

Robinson and co-workers (46–49) investigated the non-enzymatic deamidation of Asn residues in synthetic pentapeptides, and the effects of amino acid sequence, pH, temperature, buffer species, and ionic strength. Using synthetic pentapeptides, it has been shown that deamidation is favored by increased pH, temperature, and ionic strength (46,47). These studies showed the importance of primary sequence around the Asn residue, but did not investigate the formation of either iso-Asp or cyclic imide. Similar results are obtained for cytochrome c (50). Similarly, the rate of deamidation of human TIM was facilitated by high temperatures, and was also found to be dependent on the presence of substrate and specific buffers (14). Unlike the hydrolysis of peptides containing esters of Asp, where the cyclic imide intermediate can be trapped (22,24), cyclic imide formation during the deamidation of Asn peptides is the rate determining step (26,27).

The rates of deamidation of Asn residues in proteins are influenced by the secondary and tertiary structures of proteins. Clarke has shown that Asp and Asn residues in native proteins generally exist in conformations where the peptide bond nitrogen atom cannot approach the side chain carbonyl carbon without large scale conformational changes (51). Therefore, certain proteins will not undergo deamidation unless they have been denatured. Cyclic imide could only be formed *in vitro* at Asn<sup>67</sup> of bovine pancreatic ribonuclease in the unfolded state (52). While in the native structure, this residue is poorly positioned for cyclic imide formation. Similarly, it has been shown that urea (a strong denaturant) accelerates the deamidation of bGH, hGH, and prolactin, presumably by unfolding the protein (5). Tertiary structure appears to be the principle determinant for the deamidation of trypsin (53). The study also showed that adjacent Ser residues aid in the formation of the cyclic imide intermediate, consistent with earlier studies on small peptide systems (36,37). Recently, Lura and Schirch (54) have shown that the mechanism of deamidation of Val-Asn-Gly-Ala and N-acetyl-Val-Asn-Gly-Ala varies according to the conformation of the peptide backbone. Above pH 9.0, both peptides have similar conformations and thus deaminate by the same mechanism to give mixture of Asp and iso-Asp peptides. However, at pH 7.0, while the N-acetyl peptide yielded a mixture of Asp and iso-Asp peptides, the non-acetylated peptide gave no detectable amounts of these products, but rather yielded a cyclic peptide believed to be formed by nucleophilic attack of the amide of the Asn residue by the terminal amino group.

It is well known that peptides of Asp esters undergo intramolecular cyclization, under both acidic and basic conditions, leading to a cyclic imide derivative (39,55). However, no reports are available showing the formation of cyclic imide from Asn peptides in acidic media. There are few

examples in the literature which at least indicate that cyclic imide is not involved in the deamidation reaction under acidic conditions. For example, insulin (7,8), neocarzinostatin (16), and ribonuclease A (56), when incubated in acidic media, yield only Asp-containing products from the deamidation of Asn residues. Similar results were obtained by Meinwald and co-workers (27), where Ac-Asn-Gly-NHMe produced only Ac-Asp-Gly-NHMe and the analogous iso-Asn produced only the iso-Asp-containing peptide after 1 day in 1 M HCl.

It has been postulated that deamidation may play a central role as a timer in protein turnover and in aging (21). However, for pharmaceutical preparations, the major concern is the change in protein function upon deamidation. In a few cases, the deamidation of specific Asn residues has been linked to the changes in the protein function, for example, deamidation at two Asn-Gly sequences in TIM resulted in subunit dissociation (15). Deamidation at an Asn-Gly site in a hemoglobin mutant (Hb providence) changed its oxygen affinity (57), and deamidation at an Asn-Asp site in hGH altered its proteolytic cleavage properties (58). Recently, deamidation was shown as one of the major chemical processes responsible for irreversible enzyme inactivation of lysozyme (59) and ribonuclease (60) at 100°C. Deamidation was also responsible for the decrease in biological activity for porcine ACTH (62) and slower rate of refolding after deamidation for ribonuclease (63,64).

With small peptides, the iso-Asp and Asp peptides are separable by chromatographic or electrophoretic methods (65–67). However, with larger proteins similar methodology has not been successful. Chromatofocusing (68) and HPLC (20,29) have been used for separating the native protein from the product mixture, but these techniques do not separate the iso-Asp-peptide from the Asp-peptide. However, there are several indirect ways of showing the presence of iso-Asp residues in proteins. These include (i) NMR methods to distinguish Asp and iso-Asp (27,54,69); (ii) Leu aminopeptidase digestion, since this enzyme will not cleave an iso-Asp peptide bond (67,70); (iii) tryptic peptide mapping and amino acid sequencing (71); and (iv) use of PCM, which is known to methylate selectively the free  $\alpha$ -carboxy group of iso-Asp peptides (72). Recently, Johnson and co-workers have shown the use of this enzyme as a powerful analytical tool for estimating minimum levels of protein deamidation (73). In their work, they monitored the increase in methylation for aldolase, bovine serum albumin, cytochrome c, lysozyme, ovalbumin, ribonuclease A, and TIM upon incubation at pH 11, finding evidence that iso-Asp is formed upon deamidation.

#### Oxidation

The side chains of His, Met, Cys, Trp, and Tyr residues in proteins are potential oxidation sites. Even atmospheric oxygen can oxidize Met residues. Oxidation has been observed in many peptide hormones during their isolation (74–77), synthesis (78), and storage (79). Since the thioether group of Met is a weak nucleophile and is not protonated at low pH, it can be selectively oxidized by certain reagents under acidic conditions (80). For example, hydrogen peroxide can modify indole, sulfhydryl, disulfide, imidazole, phe-

nol and thioether groups of proteins at neutral or slightly alkaline conditions, but under acidic conditions the primary reaction is the oxidation of Met to Met sulfoxide (81). In addition to hydrogen peroxide, a variety of other reagents have been used to oxidize Met to Met sulfoxide. These include periodate, iodine, dimethylsulfoxide, a dye-sensitized photooxidation, chloramine-T, and *N*-chlorosuccinamide (82,83). To oxidize Met to Met sulfone, more drastic conditions and reagents are needed, e.g., 95% performic acid. The structures of the oxidation products of Met, i.e. Met sulfoxide and Met sulfone, are shown in Fig. 3.

Oxidation of Met residues to their corresponding sulfoxides is associated with loss of biological activity for many peptide hormones [e.g., corticotropin (84),  $\alpha$ - and  $\beta$ -melanotropins (85), parathyroid hormone (86), gastrin (87), calcitonin (88), and corticotropin releasing factor (77)] as well as nonhormonal peptides and proteins (81). It has been shown that *E. coli* ribosomal protein L12 loses activity after oxidation of Met residues to Met sulfoxide and that the activity can be restored by incubating the protein with high concentrations of  $\beta$ -mercaptoethanol (89). Restoration of biological activity was found to coincide with the reduction of Met sulfoxide to Met (89). Alpha-1-proteinase inhibitor protein, which is a major serum inhibitor of elastase activity, loses its ability to inactivate elastase when chemically oxidized (90,91). Oxidation by hydrogen peroxide of a single Met residue in subtilisin at pH 8.8 occurs concurrently with changes in kinetic parameters of the enzyme, although it does not abolish enzymatic activity (92). Similar results were obtained with a disulfoxide derivative of  $\alpha$ -chymotrypsin (93,94), and trypsin (95). In many cases, such as parathyroid hormone (86), ribonuclease S-peptide (96), ribonuclease

(97), and lysozyme (98), reduction of Met sulfoxide by thiols results in the recovery of nearly full biological activity.

There are also examples where protein functions are not affected upon Met oxidation. Active monosulfoxide derivatives of pancreatic ribonuclease (99),  $\alpha$ -chymotrypsin (100), and Kunitz trypsin inhibitor (101) have been prepared using mild hydrogen peroxide treatment at low pH (pH 1 to 3). Similarly, EGF (102,103) and glucagon (104) are biologically active when chemically oxidized.

It is also shown that within a given protein, the reactivity of Met residues towards oxidation may be different depending upon their position. For example, in hGH, Met<sup>170</sup> was found to be completely resistant to oxidation by hydrogen peroxide (105). In addition, it was shown that when biosynthetic hGH is chemically oxidized at Met<sup>14</sup>, it exhibits full biological activity and has immunoreactivity identical to that of authentic hGH (6). In human chorionic somatomammotropin (hCS), Met<sup>64</sup>, Met<sup>16</sup>, and Met<sup>179</sup> have markedly different reaction rates (105). The oxidation of Met<sup>64</sup> and/or Met<sup>179</sup> markedly reduced both its affinity for lactogenic receptors and its *in vitro* biological potency (105).

Determination of oxidized Met in proteins is generally a problem, because during conventional amino acid analysis Met sulfoxide is converted to Met during acid hydrolysis. Therefore, Met is commonly determined by using its specific reactions with alkyl halides (106) or cyanogen bromide (107), to which the sulfoxide is resistant. After alkylating the Met residues of the peptide, its Met sulfoxide is oxidized with performic acid to the acid stable sulfones; the sulfone content, determined by amino acid analysis, is then used to correct the Met estimate obtained by conventional amino acid analysis (99). Alternatively, Met containing peptides have been separated from peptides containing oxidized Met residues by ion-exchange chromatography (108), counter-current distribution (109), HPLC (103,110), or affinity chromatography (111). A radioassay for non oxidized Met in peptide hormones based on its specific reaction with iodo[2-<sup>14</sup>C]acetic acid is also developed (112).

The thiol group of Cys (RSH) can be oxidized in steps, successively, to a sulfenic acid (RSOH), a disulfide (RSSH), a sulfinic acid (RSO<sub>2</sub>H), and, finally, a sulfonic acid (RSO<sub>3</sub>H), depending upon reaction conditions. The factors which influence the rate of oxidation include the temperature, pH, and buffer medium used, the type of catalyst (e.g., traces of metal ions), and the oxygen tension (113). An important factor is the spatial positioning of the thiol groups in the proteins. In those cases where contact between thiol groups within the molecule of the protein is hindered, or when the protein contains only a single thiol group, intramolecular disulfide bonds are not formed, but sometimes, under favorable steric conditions, intermolecular disulfide bonds arise, and the protein aggregates (114). Thiol groups are oxidized not only when oxidizing agents (e.g., iodine, ferricyanide, tetrathionate, *O*-iodosobenzoate, and hydrogen peroxide) are added, but also "spontaneously," by oxygen from the air (autooxidation). The oxidation of thiol groups by molecular oxygen takes place at an appreciable rate in the presence of catalytic quantities of metal ions, such as iron and copper ions (115,116). The speed of oxidation of thiol groups is also greatly influenced by the nature of neighboring

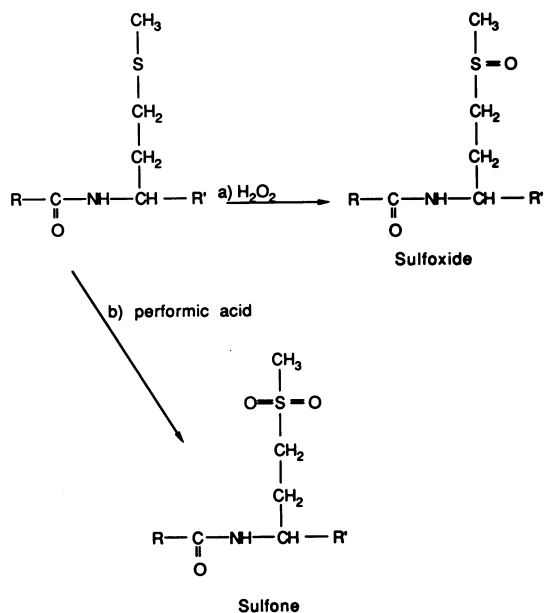


Fig. 3. Mechanism of oxidation of Met-containing peptide under (a) mild and (b) strong conditions.

groups. This was clearly demonstrated by Barron *et al.* (117) and also by Ovabarger and Ferraro (118). From their findings it appears that the rate of oxidation of dithiols is diminished on increasing the distance between the thiol groups in the molecule and also under the influence of neighboring electronegative groups such as carboxyl group (i.e., groups that raise the  $pK_a$  of the thiol group). This fact indicates that the mercaptide ion is oxidized more easily than the undissociated thiol group. Thus, it is shown that usually the oxidation rate increases with increasing pH (119). At 90°C and pH 8.0,  $\alpha$ -amylase from *Bacillus* was shown to undergo irreversible thermostabilization due to air oxidation of the Cys residues along with formation of incorrect or "misfolded" structures (120). Inactivation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by hydrogen peroxide has been shown to result from sulfhydryl group modification to sulfenic acid (114). Various methods for quantitative determination of thiol and disulfide groups in proteins are described by Torchinskii (121).

The side chains of His, Tyr, Met, Cys, and Trp residues can also be oxidized by visible light in the presence of dyes, i.e., via photooxidation. The specificity for the various amino acid side chains is particularly determined by pH. Oxidation of His is a rapid reaction at neutral pH but is quite slow at low pH. At higher pH, Tyr is most reactive (122–124), while Trp and Met are the only amino acids readily oxidized below pH 4. More information on photooxidation is available in a few review articles (125–127). In many cases, loss of enzymatic activity following photooxidation has been attributed to the destruction of critical His residues. For example, the inactivation of rabbit muscle aldolase (128), pig heart aspartate aminotransferase (129,130), cytochrome c (131), renin, and yeast enolase (132) has been attributed to photodegradation of His residues.

#### Proteolysis

It has been established that peptide bonds of Asp residues are cleaved in dilute acid at a rate at least 100 times faster than other peptide bonds (133). Selective hydrolysis is usually achieved by heating for 5–18 hr at 110°C in either 0.03 *N* HCl or 0.25 *N* acetic acid (134). The mechanism of hydrolysis undoubtedly involves intramolecular catalysis by a carboxyl group of the Asp residue. Hydrolysis can take place at either the N-terminal and/or C-terminal peptide bonds adjacent to the Asp residue. Inglis (135) has described the mechanism for such hydrolysis as shown in Fig. 4, where cleavage of the N-terminal peptide bond would proceed via an intermediate containing a six membered ring rather than via a five-membered ring as proposed for C-terminal peptide fission. Such peptide bond cleavage can contribute to the inactivation of proteins. Significant irreversible thermostabilization in lysozyme (59) and ribonuclease A (60) at 90–100°C and pH 4 was found to be due to peptide bond cleavage at Asp-X bonds.

It is now well established that Asp-Pro peptide bonds are particularly labile and are hydrolyzed under conditions where other Asp peptide bonds are stable (136). For example, when rabbit antibody light chain was subjected to 10% acetic acid-pyridine (pH 2.5) in 7 *M* guanidinium hydrochloride

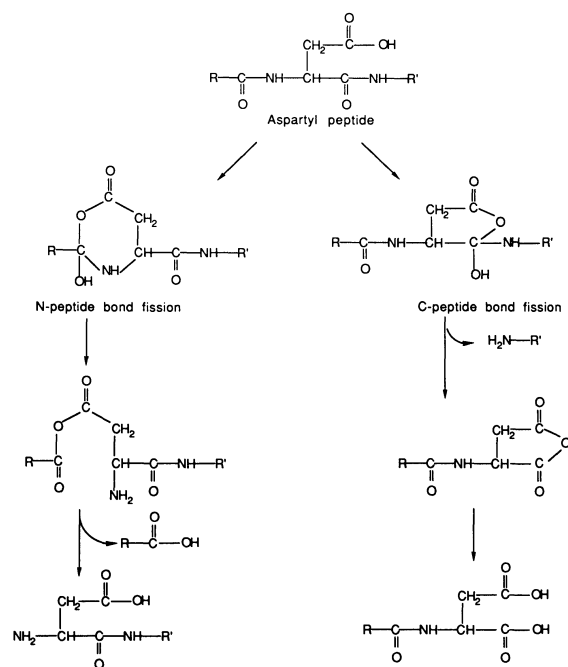


Fig. 4. Mechanism of degradation of aspartyl peptides in acidic media.

ride (GnHCl) for 24–90 hr, selective cleavage was observed at Asp<sup>109</sup>-Pro<sup>110</sup> (137). Piszkiwicz *et al.* have suggested that the hydrolytic reaction proceeds via intramolecular catalysis by carboxylate anion displacement of the protonated nitrogen of the peptide bond and the rate enhancement occurs due to the greater basicity of the Pro nitrogen (136). Marcus has compared the lability of the Asp-Pro bonds to the lability of other peptide bonds, in particular to those of Asp residues (138). In his study, a variety of dipeptides was heated at 110°C in 0.015 *M* HCl. The concentration of amino acid released during the heat treatment was determined by amino acid analysis. The results indicated that Asp-Pro bonds were 8- to 20-fold more labile than other Asp-X or X-Asp peptide bonds. Other peptide bonds that do not involve Asp were found to be stable to hydrolysis under these conditions.

Asp-X peptide bonds also undergo a reversible isomerization between the Asp and the iso-Asp forms via the cyclic imide intermediate as shown in Fig. 3 (139,140). This reaction was first noted by Swallow and Abraham with Asp-Lys derived from hydrolyzates of bacitracin (139). Similar interconversion was also shown for Val-Tyr-Pro-Asp-Gly-Ala (ACTH<sup>22-27</sup>), displaying a half-life of 53 days at pH 7.4 and 37°C (26). Even storage of aqueous solutions of an Asp-containing peptide can result in the formation of cyclic imide derivatives (141). The ring closure is particularly fast when an Asp residue is followed by Gly in the sequence (142). Peptide bonds formed by X-Ser and X-Thr are also labile, but require strong acidic conditions (e.g., 11.6 *M* HCl) (143). The mechanism involves N-O acyl rearrangement (144).

The time course of hydrolysis of amide peptide bonds

can be monitored by gel chromatography or sodium dodecylsulfate polyacrylamide gel electrophoresis (145), and quantitatively assessed by gel scanning densitometry (146). The identities of the amino acids at the new carboxyl and amino termini resulting from peptide chain hydrolysis can be determined by hydrazinolysis/HPLC (147) and dansylation/TLC (148), respectively. Identification of the position of the new termini in the sequences of the protein can be learned from amino acid analysis by HPLC of the sequential digest of the COOH terminus by carboxypeptidases (149) and the NH<sub>2</sub> terminus by the Edman degradation procedure (150,151). Recently, a radioassay was used to study peptide bond hydrolysis at neutral pH and room temperature (152). In this work, a peptide (Phe-Phe-Phe-Gly), radiolabeled with <sup>14</sup>C at the α carbon of the C-terminal residue, was attached to resin and the release of radiolabel due to amide bond hydrolysis was monitored. The half life for this peptide at neutral pH was found to be 7 years (152).

#### Incorrect Disulfide Formation

Sulfhydryl groups and disulfide bonds and their interrelationships are an important factor affecting the properties of the majority of proteins. The interchange of disulfide bonds can result in incorrect pairings, leading to an altered three-dimensional structure and, hence, loss of catalytic activity. The reaction mechanism is different in alkaline and acidic media (153,154). In neutral and alkaline media the reaction is catalyzed by thiols, which, in the form of thiolate ions, carry out nucleophilic attack on a sulfur atom of the disulfide (Fig. 5). Catalytic quantities of thiols can arise by hydrolytic cleavage of disulfides to carry out such disulfide exchange. For example, lysozyme, when heated at 100°C at neutral pH, undergoes beta-elimination of Cys to produce free thiols, which cause disulfide interchange (155). Benesch and Benesch have studied the mechanism of disulfide exchange in acidic media and they proposed that the exchange takes place through a sulfenium cation, which is formed by attack of a proton on the disulfide bond (156). The sulfenium cation carries out an electrophilic displacement on a sulfur atom of the disulfide (Fig. 6). Addition of thiols can inhibit such exchange by scavenging the sulfenium cations.

Disulfide exchange can be prevented if thiol scavengers, such as *p*-mercuribenzoate, *N*-ethylmaleimide, or copper ion, which catalyzes the air oxidation of thiols, are present (157,158). Zale and Klivanov recently reported that the irreversible loss of activity of ribonuclease A at 90°C and pH 8 was significantly lower when incubated in the presence of above-mentioned reagents (60). Proteins with scrambled disulfide bonds can be rearranged to yield native, catalytically active material by incubating the protein with small amounts of thiols, such as mercaptoethanol or Cys (159,160).

#### Racemization

All amino acid residues except Gly are chiral at the car-



Fig. 5. Mechanism of disulfide exchange in neutral and alkaline media.

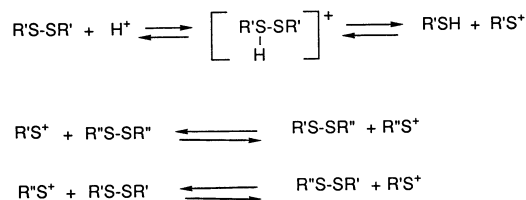
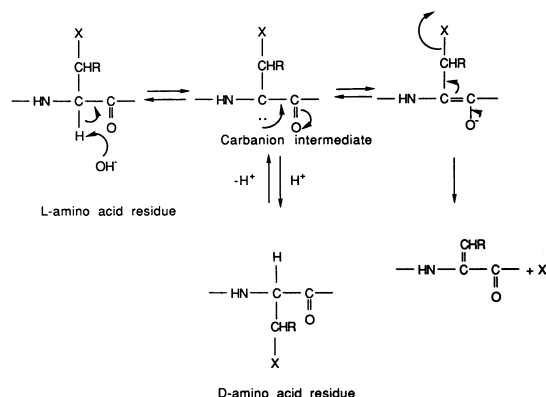


Fig. 6. Mechanism of disulfide exchange in acidic media.

bon bearing the side chain and are subject to base catalyzed racemization. Racemization is generally considered to proceed through the removal of the α-methine hydrogen by base to form a carbanion (see Fig. 7) (161). Stabilization of this carbanion controls the rate of racemization. Racemization of amino acids in protein can generate non-metabolizable forms of amino acids (D-enantiomers) or create peptide bonds inaccessible to proteolytic enzymes.

The relative rates of racemization of amino acid residues in 37 dipeptides was studied at pH 7.5 at 123°C by Smith and Desol (162). They found that the relative rates of racemization of amino acid residues in dipeptides is determined by a delicate balance of factors, including inductive and field effects, intramolecular base action, intramolecular solvation, and steric hindrance to solvation. Racemization of Asp is particularly interesting, because it is shown that Asp residues in proteins racemizes 10<sup>5</sup>-fold faster than the free amino acid (163), in contrast to a 2- to 4-fold increase for all other residues. An explanation for this exceptional behaviour of Asp is contained in the mechanism of its racemization. The mechanism involves the formation of a cyclic imide through nucleophilic attack on the β-carbonyl carbon by the α-nitrogen of the succeeding amino acid (Fig. 2). Resonance structures of the cyclic imide ring involve charge transfer between nitrogen and not only the α-carbonyl but also the β-carbonyl group. The latter resonance allows the peptide carbonyl to intensify resonance with the α-carbanion, formed by proton abstraction as an intermediate in the racemization process (164). Racemization of Asp was shown



X = H, OH, O-glycosyl, O-phosphoryl, SH, SCH<sub>2</sub>-R, aliphatic or aromatic residue  
R = H or CH<sub>3</sub>

Fig. 7. Mechanism of beta-elimination and racemization reactions in alkaline media.

to proceed via cyclic imide at pH 7.4 and 37°C for ACTH<sup>22-27</sup> with a half life of 19.5 hr (26).

### Beta Elimination

High-temperature inactivation of proteins often results from the destruction of disulfide bonds (59,155,158). Chemically, this is the result of  $\beta$ -elimination from the cystine residue. Furthermore, this can occur at lower temperatures at high pH (165,166). Whether this is a general decomposition pathway for protein pharmaceuticals is still unknown, but Volkin and Klibanov have studied more than a dozen proteins at 100°C and found that they all undergo  $\beta$ -elimination of disulfides at similar rates (155). As with previous findings, the rates were greatly accelerated under alkaline conditions. Therefore, under conditions which may lead to rapid deamidation, other chemical instability may also occur. Finally, the resultant thiols from the elimination reaction will certainly contribute to other degradation pathways (aggregation, adsorption, precipitation). Overall, the introduction of disulfides in an effort to increase stability may not always be effective (vide infra).

Other amino acid residues can also undergo  $\beta$ -elimination. For example, Cys, Ser, Thr, Phe, and Lys can be degraded via  $\beta$ -elimination at alkaline conditions as shown in Fig. 7. In many cases, the  $\beta$ -elimination reaction is influenced by pH, temperature and presence of metal ions. For example, the initial rates of  $\beta$ -elimination in phosvitin (167), antifreeze glycoprotein (168), and lysozyme (169) were directly proportional to hydroxide ion concentration and also shown to depend on temperature, while the rate of  $\beta$ -elimination of phosphoserine in phosvitin was markedly enhanced by the addition of calcium chloride (167).

### PHYSICAL INSTABILITY

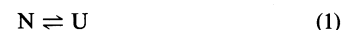
Physical instability is a difficulty rarely encountered for small molecule drugs. However, proteins, because of their polymeric nature and their ability to adopt some form of superstructure (secondary, tertiary, quaternary), can undergo a variety of structural changes independent of chemical modification. Globular proteins fold in a manner in which exposure of hydrophobic groups is minimized (170-174). Loss of globular structure, that is, loss of tertiary structure, is referred to as denaturation. It is possible for a protein to become denatured and still retain some secondary structure (175-188). Once unfolded, the polypeptide chain can undergo further inactivation by association with surfaces (adsorption), aggregation with other protein molecules, or some chemical reaction. Should aggregation lead to macroscopic ensembles, this process is termed precipitation. While the exact interrelationship between all of these pathways is still unclear, each is well documented. Generally, it is believed that denaturation must first occur for other physical instability processes (adsorption, aggregation, precipitation) to proceed.

### Denaturation

Denaturation refers to an alteration of the global fold of a molecule, that is, a disruption of the tertiary and, frequently, secondary structure (172,188-195). Often, denatur-

ation is equated with protein instability, and many review articles refer just to this aspect (189-195). Nevertheless, it is the most widely studied facet of protein inactivation (170,172,189-201). Caused by a variety of conditions (increase in temperature, decrease in temperature, extreme pH, addition of organic solvents or other denaturants), this process can be envisioned as reversible or irreversible. These terms can be misleading, as, in some instances, native structure (and subsequently, activity) can be recovered from irreversibly denatured proteins. Reversible denaturation is defined as unfolding caused by an increase in temperature which can be reversed by subsequent lowering of the temperature. Irreversible denaturation is any unfolding process which does not allow the native structure to be regained simply by lowering the temperature. Although a protein which is irreversibly denatured may still be returned to its native state by addition of denaturant followed by dialysis, the process is still defined as irreversible.

Usually, unfolding is thought to be the cooperative transition between the native (N) and unfolded (U) states of a protein [see Eq. (1)].



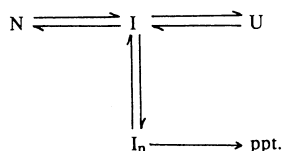
For such a two-state system, an increase in temperature will cause a rapid change in structure from N to U at the melting temperature,  $T_m$ , where  $T_m$  is defined as the temperature at which 50% of the molecules are unfolded (i.e.,  $\Delta G = 0$ ). An increase in  $T_m$  is indicative of a more stable protein structure, even though, strictly speaking, stability should be defined as the Gibbs free energy at a given temperature. It should be noted that  $T_m$  can be quite dependent on pH and concentration. For example, the  $T_m$  of T4 lysozyme is 42°C at pH 2 and 65°C at pH 6.5 (202,203). Enzymes, such as T4 lysozyme, are attractive systems for study as their activity as well as structure can be assayed (190). In any case, recovery of activity or structure upon lowering the temperature is considered reversible denaturation.

Irreversible denaturation or inactivation actually refers to a variety of processes. Such proteins may be simply "misfolded," that is, in a conformation which does not allow them to renature properly (204-206), or they may have undergone some additional process, whether chemical or physical. The ability to recover activity by the addition of denaturants (e.g., GnHCl or urea), followed by dialysis, indicates a structural (aggregation, adsorption, etc.) or misfolding component to the irreversible inactivation.

For mutants of T4 lysozyme, the irreversible denaturation of the protein proceeds through different pathways (202). For the wild type, the inactivation appears to be conformational in nature, whereas the activity of mutants containing a disulfide bridge (e.g., Ile<sup>3</sup> → Cys-Cys<sup>97</sup>/Cys<sup>54</sup> → Val) could not be recovered by the addition of GnHCl. Chemical inactivation was postulated to be the mechanism involved. Interestingly, this seems to be unrelated to the effect of the disulfide bridge on the reversible denaturation of T4 lysozyme. Although less unfolded, mutants containing the disulfide bridge are more susceptible to chemical inactivation. This opposes the usual behavior of proteins, in which they are more likely to undergo a chemical reaction when unfolded (192,207,208).

### Aggregation

The existence of partially unfolded intermediates (Scheme I) has now been shown to exist for many proteins (175,178–183,186–188,209,210). While they may be important in protein folding, their role in protein stability is less evident. However, for bGH and interferon- $\gamma$  (If- $\gamma$ ), these intermediates may lead to inactivation via aggregation. The following scheme (Scheme I) has been proposed for both bGH (182) and If- $\gamma$  (178).



Scheme I. Model of folding phases for proteins which display partially unfolded intermediates.

Moderate amounts of denaturant can generate a partially unfolded intermediate of bGH (180,186,188), whose solubility is less than either the N or the U states of bGH. This species, I, associates or aggregates, as outlined in Scheme I. Retaining much of the native secondary structure, the tertiary structure of I is mostly lost. The site of interaction has been identified as an amphiphilic helix (residues 107–128) which is part of the central four-helix bundle (180,182,183). Increasing the amphiphilicity of this region via mutagenesis produces a protein which associates more readily, precipitates faster, and renatures more slowly. Renaturation is hindered, since the associated intermediate,  $I_n$ , is not directly along the pathway for return to the native conformation.

Interferon- $\gamma$  is inactivated by acid treatment (209,211). Below pH 4.5 and in the absence of NaCl, the dimeric native state is converted into monomers, which are partially denatured (209). Dialysis does lead to the formation of N, but also to large aggregates ( $I_n$ ), which have substantially lower activity (175). By circular dichroism spectroscopy (CD), the aggregates retain a large amount of secondary structure, but the tertiary structure is largely disrupted. Addition of salt also leads to formation of  $I_n$  (178,210). Interferon- $\gamma$  is highly positively charged at pH 4.5, and presumably, the chloride ions mask the charge enough to allow the monomers to associate. This behavior is similar to the salt effects which allow the formation of molten globule-like states in acid- and base-denatured proteins (184,185).

Upon denaturation with GnHCl, antithrombin displays a biphasic denaturation, with midpoints at 0.8 and 2.8 M GnHCl concentrations (as determined by CD and activity measurements) (179). Therefore, at 1.5 M, there is a partially unfolded intermediate, I, which aggregates slowly. Freshly prepared I can be returned to the native state, N, by dialysis. However, once aggregation occurs, the native state cannot be reformed by this approach.

### Surface Adsorption

Adhesion of proteins to surfaces is a well-known phenomenon in the field of biomaterials, as biocompatibility is

essential for artificial limbs, organs, or even contact lenses (212,213). Interaction of plasma proteins with various surfaces has received the most attention. However, while surface adsorption is potentially disastrous in many facets of protein manipulations, reported studies have been fairly limited. One system which has been well studied is insulin. Primarily, this is because it is the one protein which has been on the market for a long period of time and has been the target of numerous delivery devices. Major difficulties have been encountered with insulin adsorbing to the surfaces of delivery pumps (214–218), to glass and plastic containers (219–223), and to the inside of intravenous bags (224–226).

### Precipitation

Precipitation is the macroscopic equivalent of aggregation. Precipitation of proteins has been known to occur for a long time, usually in conjunction with denaturation (195,227,228), but detailed studies of it have been mostly limited to the case of insulin.

Insulin frosting is the formation of a finely divided precipitate on the walls of the insulin container. The proclivity of insulin to precipitate is well established and is particularly troublesome when the insulin is loaded into a long-term infusion device (214–216). Recent work on insulin frosting has shown that the process is accelerated by the presence of a large headspace within the vial (221). Presumably, the insulin is undergoing denaturation at the air–water interface, facilitating the precipitation process. Other factors which can contribute to insulin frosting are the concentration of zinc ion (known to regulate the aggregation state of insulin), pH, and the presence or absence of additives (221). Changes in the types of vials or stoppers did affect the process. Other studies have demonstrated a dependence of insulin precipitation upon the type of materials in contact with the insulin solution (219,222). However, these studies were concerned with much longer time courses (30–120 days).

Upon expression in recombinant organisms, many proteins fail to remain in a soluble form, and often aggregate into macroscopic ensembles termed inclusion bodies (IBs) (229–234). Formation of IBs appears to be a general phenomenon and independent of the degree of overexpression. Thus, it may not simply be a precipitation process. However, the mechanism of IB formation is unknown, and even basic characterization of IBs is lacking (235,236).

Typically, IBs are believed to be comprised of partially or completely denatured protein (232), but evidence is lacking. For proteins containing Cys residues, the possibility of intramolecular disulfide bonds exists. It has been shown that the addition of reducing agents, such as thiols, does aid in the solubilization of IBs (237–239). However, the cytoplasm of *E. coli* is quite reducing, making disulfide formation *in vivo* quite unlikely (240). Consequently, formation of disulfides may be an artifact of IB isolation procedures or the result of localized oxidizing environments (231).

### IMPROVING PROTEIN STABILITY

#### Additives

Additives can be defined as any excipient which is introduced into the formulation in an effort to increase stabil-

ity. Some of the simplest and most effective are salts of other ionic compounds. Salts decrease reversible denaturation via nonspecific binding to the protein (241–247). Specific ion binding sites are also known to exist, even for nonmetalloproteins. Binding of ions to these sites increases thermal stability and has been demonstrated for subtilisin (248–250), ribonuclease (251), thermolysin (252,253), parvalbumin (254), acyl carrier protein (255), alkaline phosphatase (256,257), calbindin D<sub>9k</sub> (258), copper-zinc superoxide dismutase (259,260), and  $\alpha$ -lactalbumin (261–263). Since such a relatively simple process as ion binding can provide a definite increase in thermal stability (i.e., reduced tendency toward denaturation), ion binding sites have been designed into proteins via mutagenesis (see below). In fact, such sites have been constructed in subtilisin (248,250), even without intending to do so. Ion binding can be employed to control physical instability phenomena such as aggregation and precipitation. This has been demonstrated in the case of insulin (264).

Polyalcohol materials, such as glycerol and sugars, are well known to stabilize proteins with respect to denaturation (265–272). Detailed studies by Timasheff and co-workers have established that this occurs through selective solvation of the protein (265–271). At low concentrations of the additive, more water molecules pack around the protein, in order to exclude the more hydrophobic additive. This results in increased stability. At higher concentrations, this is no longer possible, and the more hydrophobic organic solvent begins to denature the protein.

Detergents have often been employed as additives for the stabilization of proteins, including both nonionic (219,222,226) and anionic (273–278) species. Studies on anionic detergents, such as sodium dodecyl sulfate (SDS), have focused on its role in the denaturation of proteins (273–275,277,278). It has also been found to affect the deamidation of food proteins (276). Nonionic detergents, such as Tween and Pluronic, have been evaluated for their ability to prevent adsorption of proteins to surfaces (226,279), to inhibit aggregation and precipitation (219,222), and to hinder denaturation (280,281). These types of additives have the additional advantage of facilitating the delivery of proteins transdermally (282) and intranasally (283).

#### Site-Directed Mutagenesis

Site-directed mutagenesis refers to the methods that provide the ability to make amino acid substitutions at specific sites in a protein. Therefore, it is now possible to alter the primary sequence of a protein in an effort to increase the overall stability (284,285). Certainly, chemical stability can be increased by replacing susceptible functional groups. However, how amino acid substitutions can affect an increase in thermal stability is still uncertain. A number of approaches have been reported, such as attempts

- (i) to improve interior interactions, leading to an increase in thermal stability;
- (ii) to increase  $\alpha$  helix stability via manipulation of the helix dipole;
- (iii) to introduce disulfide bridges in order to stabilize the native conformation and provide stability against reversible thermal denaturation;

(iv) to design ion binding sites which will also increase thermal stability; and

(v) to replace potential sites for chemical degradation, such as deamidation.

#### (i) Stabilization via Increased Efficiency in Packing.

While the overall relationship between amino acid substitutions and stability is not yet clear, some basic principles are emerging. Decreasing conformational flexibility, improving hydrophobic packing, and maximizing hydrogen bonding should all increase stability. Due to its lack of a side chain, Gly possesses the most conformational freedom of all the naturally occurring amino acids. Replacement with Ala should restrict this freedom, thereby decreasing the entropy of unfolding and producing increased stability. This has been demonstrated in two systems. In  $\lambda$  repressor, the substitution of Ala for Gly (mutants Gly<sup>46</sup>  $\rightarrow$  Ala and Gly<sup>48</sup>  $\rightarrow$  Ala) produces a 0.7 to 0.9 kcal/mol increase in stability (286). This relates to an increase in  $T_m$  of 3–5°C. Other substitutions at position 48 (Ser, Asn) also provided increased stability but less than with Ala. The double mutant is more stable by 1.1 kcal/mol. Lack of additivity to the stability of the protein suggests that the conformation for the double mutant has been altered from that of the wild type (287). Similar results have been observed for T4 lysozyme (288), where the Gly<sup>77</sup>  $\rightarrow$  Ala mutant is more stable by 0.4 kcal/mol. Conformational flexibility can be further reduced by introduction of Pro into the polypeptide chain. Identifying an Ala residue with  $\phi$  and  $\psi$  angles (289) which could accommodate replacement with Pro, the Ala<sup>82</sup>  $\rightarrow$  Pro mutant was found to be 0.8 kcal/mol more stable.

Hydrophobic interactions are believed to be important for stability of globular proteins and play a role as nucleation sites in protein folding (170,172,290,291). In barnase, Ile<sup>96</sup> is part of a cluster of hydrophobic amino acids. Replacement with Val destabilizes the protein toward reversible denaturation by 1.2 kcal/mol, whereas substitution with Ala produces a 4.0 kcal/mol decrease (292). Such extensive destabilization does not correlate simply to differences in hydrophobicity. Meanwhile, mutations involving Ile<sup>3</sup> in T4 lysozyme do produce stability changes which correlate with changes in hydrophobicity of the side chain (293).

Hydrogen bonding is certainly an important force in the stabilization of protein structures. Mutagenesis has been used to determine the effect of hydrogen bonding in protein stability. In particular, the hydrogen bond between the side chain of Thr<sup>157</sup> and the amide proton of residue 159 of T4 lysozyme was examined in detail (294). Amino acids which could not accept a hydrogen bond from the amide destabilized the structure. Crystal structures of the mutants indicated that the degree of disruption of the structure did not correlate to the observed instability, suggesting that the structural basis for stability is still not completely understood.

Mutagenesis can be done in a random fashion, as well as in a site-specific manner (284,295–301). For T4 lysozyme, many of the mutants were found to be destabilizing (302,303) or had no effect (304) with respect to reversible denaturation. An investigation of Trp to Tyr mutants found that the Trp<sup>138</sup>  $\rightarrow$  Tyr mutant had a  $T_m$  3°C lower than the wild type, whereas the triple mutant (Trp<sup>126</sup>  $\rightarrow$  Tyr/Trp<sup>138</sup>  $\rightarrow$  Tyr/Trp<sup>158</sup>  $\rightarrow$  Tyr), having every Trp replaced by Tyr, was less

stable by 7°C at pH 2.0 (296). In contrast, the activity and structure (as determined by CD) were comparable for all three proteins. Crystallographic characterization of various mutants of T4 lysozyme (305) lead to the conclusion that the differences in stability arose from effects on the folded state, and not on the unfolded state (305,306).

Random mutations in  $\lambda$  repressor produced little change in stability. Of 12 mutants, 10 showed identical  $T_m$  values to the wild type, while one was lower and one was greater (297). In staphylococcal nuclease (STN), stability was measured by resistance to GnHCl and urea denaturation (298). The mutants seemed to fall into one of two classes. The most interesting were those that displayed greater stability than the wild type at temperatures above 55°C but less than the wild type at 20°C (termed Class I). Unlike in the T4 lysozyme system, the effects were interpreted in terms of their effects on the unfolded state. This was later substantiated by CD studies on large STN fragments (299). Class I mutations produced fragments with a greater degree of structure than the wild type at intermediate concentrations of denaturant.

(ii) *Stabilization of the Helix Dipole.* The  $\alpha$  helix conformation allows the dipoles of the individual peptide groups to add in a constructive manner (307–310), producing a significant dipole moment for extended helices. The helix dipole has been implicated as playing a role in protein folding (310) and the stability of superstructures such as the four-helix bundle (311,312), a common structural motif (313).

Employing the C- and S-peptide fragments from ribonuclease as a model, Baldwin and co-workers have systematically evaluated  $\alpha$  helix stability, particularly with regard to the effect of the helix dipole (314–318). While hydrophobic interactions and salt bridges also play a role (315,316,318–321), the helix dipole appears to be the primary factor involved in helix stability. Reinforcement of the helix dipole is achieved by placing acidic groups near the N-terminus and basic groups near the C-terminus. These principles have now been applied toward stabilization of helices within proteins (300,322,323).

Mutations have been made in T4 lysozyme with the intent to increase the helix dipole and thereby affect an increase in stability (300). Four of the eleven  $\alpha$  helices in this protein have no apparent acidic group near its N-terminus and were evaluated as target sites for mutagenesis. Two such single mutants (Ser<sup>38</sup> → Asp and Asn<sup>144</sup> → Asp) and the corresponding double mutant were constructed. Both single mutants showed an increase in  $T_m$  of 2°C, while the double mutant was approximately 4°C higher. This corresponds to an increase in the free energy of stabilization ( $\Delta\Delta G$ ) of ~1.6 kcal/mol for each mutation (300). The additivity of the effects on stability has been observed previously (193, 202,324).

Similarly, the interaction of the protonated form of His<sup>18</sup> in barnase (a ribonuclease from *Bacillus amyloliquefaciens*) with its  $\alpha$  helix provides ~2 kcal/mol of stabilization relative to various mutants (323). In addition, the importance of charge-helix dipole interactions could be directly assessed by varying the pH so that His<sup>18</sup> was deprotonated, permitting these effects to be studied in the absence of other structural factors introduced by amino acid substitutions.

Replacement of Glu<sup>34</sup> in helix 3 of  $\lambda$  repressor with a Lys residue produced a 2°C drop in  $T_m$  (325). The Glu re-

sides at position 2 of the helix, near the N-terminal end. Removing an acidic group and replacing it with a basic one is in opposition to the helix dipole, thus leading to a decrease in thermal stability.

(iii) *Stabilization via Introduction of a Disulfide Group.* A common approach toward stabilization of a protein is to introduce a disulfide bond, anticipating that this modification would decrease the entropy of unfolding and increase the tendency toward reversible denaturation. In addition, it was hoped that it would produce an unfolded form which would be less susceptible to irreversible inactivation. Disulfides have been introduced into dihydrofolate reductase (326), subtilisin (327,328),  $\lambda$  repressor (329), and T4 lysozyme (202,330–333). Stability in the subtilisin system has been evaluated by the ability to prevent autolytic degradation. Two possible sites were chosen which appeared to be suitable for the placement of an unstrained disulfide bridge. Both the Ser<sup>24</sup> → Cys/Ser<sup>87</sup> → Cys and the Thr<sup>22</sup> → Cys/Ser<sup>87</sup> → Cys mutants displayed faster rates of autolysis than the wild type (327), with Thr<sup>22</sup> → Cys/Ser<sup>87</sup> → Cys being much less stable. Investigations into the effects on reversible denaturation were prevented by aggregation difficulties. However, there are data to suggest that there is a correlation between thermal stability and proteolytic susceptibility (327,328), a phenomenon that has been observed previously (198,297,334). As a final note, it has been suggested that the effects of the single Cys mutations will indicate whether the disulfide will be able to stabilize the protein (327). For example, the Thr<sup>22</sup> → Cys mutation so destabilizes T4 lysozyme that even including it in a disulfide bond will not return the mutant protein to the stability level of the wild type.

Formation of a disulfide bond was engineered into *E. coli* dihydrofolate reductase by replacing Pro<sup>39</sup> with Cys and coupling it to Cys<sup>85</sup> (Pro<sup>39</sup> → Cys/Cys<sup>85</sup>) (326). Loss of stability was observed, as  $T_m$  decreased by 3°C. However, denaturation by GnHCl required a higher concentration to achieve 50% denaturation of the mutant, suggesting that the mutant is more stable. Final analysis of the data did conclude that the mutant was less stable at 25°C than the wild type (284).

Introduction of a disulfide bond into T4 lysozyme also produces differing effects on stability (202,330–333). While the disulfide increase resistance to reversible denaturation, the impact on irreversible denaturation is more complicated (202). Despite being less unfolded at high temperatures, the disulfide-containing mutant is more susceptible to chemical inactivation than the wild type (202), as its activity cannot be recovered by treatment with denaturants. The wild type, while losing activity more rapidly, can be nearly completely reactivated by denaturation with GnHCl followed by dialysis. Again, the effects of mutations may have a quite different impact on reversible and irreversible denaturation of a protein.

(iv) *Stabilization via Introduction of Ion Binding Sites.* As mentioned above, the binding of ions tends to increase the thermal stability of proteins, whether the interaction is specific or not. Engineering such a site into a protein is believed to be relatively simple, compared to designing some other type of stabilizing interaction (251). In fact, secondary calcium binding sites for subtilisin have been produced inadvertently (248,250). Mutagenesis has been employed to

probe the binding of calcium directly at the binding site (258) and at a distance (249). Eliminating a negative charge in the coordination sphere (by substituting Gln for Glu of Asn for Asp) costs approximately 2 kcal/mol in energy (258). For electrostatic interactions over larger distances (5–20 Å), the effect is more modest (248,249) but still can improve stability (248).

(v) *Stabilization by Removing Chemically Susceptible Sites*. Recently, a unique stabilization strategy was reported for triosephosphate isomerase (335). It involved the replacement of potential deamidation sites, Asn<sup>14</sup> and Asn<sup>78</sup>, by Thr and Ile, respectively, producing a genetically engineered protein which was much more stable towards heat inactivation than the native form of the enzyme. Similar results were obtained with recombinant derived human interleukin-1 $\alpha$ , where Asn<sup>36</sup> was replaced with Ser (336). This mutant protein was stabilized against base-catalyzed and temperature-induced deamidation.

#### Chemical Modification of Proteins

While reactive sites can be removed by mutagenesis, that may not always be possible, if a product has already been identified and produced by recombinant techniques. An alternative is to block such groups with chemical agents. One common, though nonspecific, approach has been to couple peptides and proteins to polyethylene glycol (PEG) (337–339) or poly(oxyethylene) (340,341). While these modifications may increase stability, their effect on conformation, activity, and immunogenicity may be quite dramatic or even undesirable. A more promising derivatization is the attachment of a lipid group to the protein or peptide (342–346), a posttranslational modification which occurs *in vivo* (344–346) and appears to facilitate the insertion of the protein into the lipid bilayer. For insulin modified in this manner, the activity is largely retained (342) and delivery problems may be circumvented (343).

Modification of basic residues in proteins is known to increase thermal stability (347–350). Conversion of Lys to homoarginine via guanidination has been shown to stabilize numerous proteins (347–349), although such treatment did not affect the stability of ribonuclease (350). Methylation of basic residues has been observed in heat-shocked prokaryotes (351). Presumably, the methylated amino acids aid in producing a more thermally stable set of proteins.

Replacement of Met by mutagenesis in order to prevent oxidation has been demonstrated. The logical choice for substitution would be the nonnaturally occurring norleucine (Nle), which has a methylene group in place of the sulfur atom. This amino acid has been shown to possess many of the same properties as Met (352). Koide *et al.* have grown recombinant organisms on Nle-enriched medium deficient in Met, leading to the production of EGF with Nle substituted into the single Met site, thus preventing any possible oxidative decomposition (353).

#### CONCLUSIONS

Protein instability encompasses many complicated and interrelated chemical and physical processes. Any of these can occur during the production, isolation, purification, analysis, delivery, and storage of protein pharmaceuticals.

Many of these reactions appear to be ubiquitous, and, therefore, of concern to the pharmaceutical scientist. Deamidation of Asn, oxidation of Met, and interchange of cystine and Cys are relatively rapid reactions and have been observed in a number of compounds. Fortunately, many of the chemical reactions can be retarded or halted by appropriate choice of conditions, yet, many require only mild conditions to proceed.

Physical instability refers to processes in which no change in the chemical nature of the protein occurs. These include denaturation, aggregation, precipitation, and adsorption to surfaces. While the last three have been observed with small organic drug agents, denaturation is unique to this class of compounds. Indeed, it is implicated in all of the other physical phenomenon and influences the chemical susceptibility of proteins as well. While all of the decomposition reactions listed in this article truly define protein instability, it is resistance to denaturation which is commonly equated with protein stability. Since denaturation can be caused by heating, cooling, freezing, denaturants, pH extremes, and organic solvents, proteins are obviously sensitive to solution conditions, requiring proteins to be stored and shipped as solid materials.

#### REFERENCES

1. D. Blohm, C. Bollschweiler, and H. Hillen. *Angew. Chem. Int. Ed. Engl.* 27:207–225 (1988).
2. L. P. Gage. *Am. J. Pharm. Ed.* 50:368–370 (1986).
3. H. H. Tallan and W. H. Stein. *J. Biol. Chem.* 200:507–514 (1953).
4. U. J. Lewis and E. V. Cheever. *J. Biol. Chem.* 240:247–252 (1965).
5. U. J. Lewis, E. V. Cheever, and W. C. Hopkins. *Biochim. Biophys. Acta* 214:498–508 (1970).
6. G. W. Becker, P. M. Tackitt, W. W. Bromer, D. S. Lefebvre, and R. M. Riggan. *Biotech. Appl. Biochem.* 10:326–337 (1988).
7. S. A. Berson and R. S. Yalow. *Diabetes* 15:875–879 (1966).
8. B. V. Fisher and P. B. Porter. *J. Pharm. Pharmacol.* 33:203–206 (1981).
9. F. S. M. Van Kleef, W. W. de Jong, and H. J. Hoenders. *Nature* 258:264–266 (1975).
10. T. Flatmark and K. Sletten. *J. Biol. Chem.* 243:1623–1629 (1968).
11. J. O. Minta and R. H. Painter. *Immunochemistry* 9:821–832 (1972).
12. R. P. Diaugustine, B. W. Gibson, W. Aberth, M. Kelly, C. M. Ferrua, Y. Tomooka, C. F. Brown, and M. Walker. *Anal. Biochem.* 165:420–429 (1987).
13. M. F. Perutz and J. H. Fogg. *J. Mol. Biol.* 138:669–670 (1980).
14. K. U. Yuksel and R. W. Gracy. *Arch. Biochem. Biophys.* 248:452–459 (1986).
15. P. M. Yuan, J. M. Talent, and R. W. Gray. *Mech. Age. Dev.* 17:151–162 (1981).
16. H. Maeda and K. Kuromizu. *J. Biochem. (Tokyo)* 81:25–35 (1977).
17. L. Graf, G. Cseh, I. Nagy, and M. Kurcz. *Acta Biochim. Biophys. Acad. Sci. Hung.* 5:299–303 (1970).
18. T. J. McDonald, H. Jornvall, K. Tatamoto, and V. Mutt. *FEBS Lett.* 156:349–356 (1983).
19. L. Graf, S. Bajusz, A. Patthy, E. Barat, and G. Cseh. *Acta Biochim. Biophys. Acad. Sci. Hung.* 6:415–418 (1971).
20. N. P. Bhatt, K. Patel, and R. T. Borchardt. *Pharm. Res.* 5:572 (1988).
21. A. B. Robinson and C. J. Rudd. In B. L. Horecker and E. R. Stadtman (eds.), *Current Topics in Cellular Regulations*, Vol. 8, Academic Press, New York, 1974, pp. 247–295.
22. E. Sondheimer and R. W. Holley. *J. Am. Chem. Soc.* 76:2467–2470 (1954).

23. E. E. Haley, B. J. Coreoran, F. E. Dorer, and D. L. Buchanan. *Biochemistry* 5:3229-3235 (1966).
24. A. R. Battersby and J. C. Robinson. *J. Chem. Soc.* 259-269 (1955).
25. P. Bornstein and G. Balian. *Methods. Enzymol.* 47:132-145 (1977).
26. T. Geiger and S. Clarke. *J. Biol. Chem.* 262:785-794 (1987).
27. Y. C. Meinwald, E. R. Stimson, and H. A. Scheraga. *Int. J. Peptide Protein Res.* 28:79-84 (1986).
28. D. W. Aswad. *J. Biol. Chem.* 259:10714-10721 (1984).
29. B. A. Johnson and D. W. Aswad. *Biochemistry* 24:2581-2586 (1985).
30. P. Bornstein. *Biochemistry* 9:2408-2421 (1970).
31. A. Di Donato, P. Galletti, and G. D'Alessio. *Biochemistry* 25:8361-8368 (1986).
32. P. Galletti, A. Ciardiello, D. Ingrosso, A. Di Donato, and G. D'Alessio. *Biochemistry* 27:1752-1757 (1988).
33. S. Lou, C. Liao, J. F. McClelland, and D. J. Graves. *Int. J. Peptide Protein Res.* 29:728-733 (1987).
34. K. Patel and R. T. Borchardt. *Pharm. Res.* 5:572 (1988).
35. K. Patel, C. Oliyai, R. T. Borchardt, and M. C. Manning. *J. Cell. Biochem.* 13A:88 (1989).
36. S. A. Bernhard, A. Berger, H. J. Carter, E. Katchalski, M. Sela, and Y. Shalitin. *J. Am. Chem. Soc.* 84:2421-2434 (1962).
37. Y. Shalitin and S. A. Bernhard. *J. Am. Chem. Soc.* 88:4711-4721 (1966).
38. M. A. Ondetti, A. Deer, J. T. Sheeham, J. Pluscec, and O. Kocy. *Biochemistry* 7:4069-4075 (1968).
39. M. Bodanszky and J. Z. Kwei. *Int. J. Peptide Protein Res.* 12:69-74 (1978).
40. S. A. Bernhard. *Ann. N.Y. Acad. Sci.* 421:28-40 (1983).
41. J. K. Blodgett, G. M. Loudon, and K. D. Collins. *J. Am. Chem. Soc.* 107:4305-4313 (1985).
42. S. Capasso, C. A. Mattia, L. Mazzarella, and A. Zagari. *Int. J. Peptide Protein Res.* 23:248-255 (1984).
43. A. J. Alder, G. D. Fasman, and E. R. Blout. *J. Am. Chem. Soc.* 85:90-97 (1963).
44. G. Perseo, R. Furino, M. Galantino, B. Gioia, V. Malatesta, and R. D. Castiglione. *Int. J. Peptide Protein Res.* 27:51-60 (1986).
45. M. Bodanszky and S. Natarajan. *J. Org. Chem.* 40:2495-2499 (1975).
46. A. B. Robinson, J. W. Scotchler, and J. H. McKerow. *J. Am. Chem. Soc.* 95:8156-8159 (1973).
47. J. W. Scotchler and A. B. Robinson. *Anal. Biochem.* 59:319-322 (1974).
48. A. B. Robinson, J. H. McKerow, and P. Cary. *Proc. Natl. Acad. Sci. USA* 66:753-757 (1970).
49. A. B. Robinson, J. H. McKerow, and M. Legaz. *Int. J. Peptide Protein Res.* 6:31-35 (1974).
50. T. Flatmark. *Acta Chem. Scand.* 20:1487-1496 (1966).
51. S. Clarke. *Int. J. Peptide Protein Res.* 30:808-821 (1987).
52. P. Bornstein and G. Balian. *J. Biol. Chem.* 245:4854-4856 (1970).
53. A. A. Kossiakoff. *Science* 240:191-194 (1988).
54. R. Lura and V. Schirch. *Biochemistry* 27:7671-7677 (1988).
55. M. Bodanszky, J. C. Tolle, S. S. Deshmane, and A. Bodanszky. *Int. J. Peptide Protein Res.* 12:57-68 (1978).
56. B. N. Manjula, A. S. Achary, and P. J. Vithayathil. *Int. J. Peptide Protein Res.* 8:275-282 (1976).
57. S. Charache, J. Fox, P. McCurdy, H. Kazazian, Jr., and R. Winslow. *J. Clin. Invest.* 59:652-658 (1977).
58. U. J. Lewis, R. N. P. Singh, L. F. Bonewald, and B. K. Seavey. *J. Biol. Chem.* 256:11645-11650 (1981).
59. T. J. Ahern and A. M. Klibanov. *Science* 228:1280-1284 (1985).
60. S. E. Zale and A. M. Klibanov. *Biochemistry* 25:5432-5444 (1986).
61. C. F. Midelfort and A. H. Mehler. *Proc. Natl. Acad. Sci. USA* 69:1816-1819 (1972).
62. L. Graf, G. Hajos, A. Patthy, and G. Cseh. *Horm. Metab. Res.* 5:142-143 (1973).
63. Y. P. Venkatesh and P. J. Vithayathil. *Int. J. Peptide Protein Res.* 25:27-32 (1985).
64. Y. P. Venkatesh and P. J. Vithayathil. *Int. J. Peptide Protein Res.* 23:494-505 (1984).
65. G. R. Marshall and R. B. Merrifield. *Biochemistry* 4:2394-2401 (1965).
66. C. C. Yang and R. B. Merrifield. *J. Org. Chem.* 41:1032-1041 (1976).
67. E. D. Murray, Jr., and S. Clarke. *J. Biol. Chem.* 259:10722-10732 (1984).
68. K. Bedii Oray, U. Yuksel, and R. W. Gracy. *J. Chromatogr.* 265:126-130 (1983).
69. T. Baba, H. Sugiyama, and S. Seto. *Chem. Pharm. Bull.* 21:207-209 (1973).
70. P. A. Khairallah, F. M. Bumpus, J. H. Page, and R. R. Smeby. *Science* 140:672-674 (1963).
71. C. Secchi, P. A. Biondi, A. Negri, R. Borroni, and S. Ronchi. *Int. J. Peptide Protein Res.* 28:298-306 (1986).
72. S. Clarke. In R. T. Borchardt, C. R. Creveling, and P. M. Ueland (eds.), *Biological Methylation and Drug Design*, Humana Press, Clifton, N.J., 1985, pp. 3-14.
73. B. A. Johnson, J. M. Shirokawa, and D. W. Aswad. *Arch. Biochem. Biophys.* 268:276-286 (1989).
74. H. B. F. Dixon, S. Moore, M. P. Stack-Dunne, and F. G. Young. *Nature* 168:1044-1045 (1951).
75. F. A. Kuehl, Jr., M. A. P. Meisinger, N. G. Brink, and K. Folkers. *J. Am. Chem. Soc.* 75:1955-1959 (1953).
76. H. Rasmussen and L. C. Craig. *Recent Prog. Horm. Res.* 18:269-295 (1962).
77. W. Vale, J. Spiess, C. Rivier, and J. Rivier. *Science* 213:1394-1397 (1981).
78. K. Norris, J. Halstrom, and K. Brunfeldt. *Acta Chem. Scand.* 25:945-954 (1971).
79. M. Coltrera, M. Rosenblatt, and J. T. Potts, Jr. *Biochemistry* 19:4380-4385 (1980).
80. G. E. Means and R. E. Feeney. In *Chemical Modifications of Proteins*, Holden-Day, New York, 1971, pp. 162-165.
81. N. Brot and H. Weissbach. *Trends Biochem. Sci.* 7:137-139 (1982).
82. Y. Shechter, Y. Burstein, and A. Patchornik. *Biochemistry* 14:4497-4503 (1975).
83. Y. Shechter. *J. Biol. Chem.* 261:66-70 (1986).
84. M. L. Dedman, T. H. Farmer, and C. J. O. R. Morris. *Biochem. J.* 78:348-352 (1961).
85. H. B. F. Dixon. *Biochim. Biophys. Acta* 19:392-394 (1956).
86. A. H. Tashjian, D. A. Ontjes, and P. L. Munson. *Biochemistry* 3:1175-1182 (1964).
87. J. S. Morley, H. J. Tracey, and R. A. Gregory. *Nature* 207:1356-1359 (1965).
88. V. B. Riniker, R. Neher, R. Maier, F. W. Kahnt, P. G. H. Byfield, T. V. Gudmundsson, L. Galante, and I. MacIntyre. *Helv. Chem. Acta* 51:1738-1742 (1968).
89. P. Caldwell, D. C. Luk, H. Weissbach, and N. Brot. *Proc. Natl. Acad. Sci. USA* 75:5349-5352 (1978).
90. H. Carp and A. Janoff. *Am. Rev. Resp. Dis.* 118:617-621 (1978).
91. W. R. Abrams, A. Eliraz, P. Kimbel, and G. Weinbaum. *Exp. Lung Res.* 1:211-223 (1980).
92. C. E. Stauffer and D. Etson. *J. Biol. Chem.* 244:5333-5338 (1969).
93. W. J. Ray, Jr., and D. E. Koshland, Jr. *J. Biol. Chem.* 237:2493-2505 (1962).
94. H. Schachter and G. H. Dixon. *J. Biol. Chem.* 239:813-829 (1964).
95. V. Holeysovsky and M. Lazdunski. *Biochim. Biophys. Acta* 154:457-467 (1968).
96. U. W. Kenkare and F. M. Richards. *J. Biol. Chem.* 241:3197-3206 (1966).
97. G. Jori, G. Galiazzo, A. Marzotto, and E. Scoffone. *Biochim. Biophys. Acta* 154:1-9 (1968).
98. G. Jori, G. Galiazzo, A. Marzotto, and E. Scoffone. *J. Biol. Chem.* 243:4272-4278 (1968).
99. N. P. Neumann, S. Moore, and W. H. Stein. *Biochemistry* 1:68-75 (1962).
100. H. Schachter, K. A. Halliday, and G. H. Dixon. *J. Biol. Chem.* 238:PC3134-PC3136 (1963).

101. B. Kassell. *Biochemistry* 3:152-155 (1964).
102. W. F. Heath and R. B. Merrifield. *Proc. Natl. Acad. Sci. USA* 83:6367-6371 (1986).
103. C. George-Nascimento, A. Gyenes, S. M. Halloran, J. Merryweather, P. Valenzuela, K. S. Steimer, F. R. Masiarz, and A. Randolph. *Biochemistry* 27:797-802 (1988).
104. S. A. Coolican, B. N. Jones, R. D. England, K. C. Flanders, J. D. Condit, and R. S. Gurd. *Biochemistry* 21:4974-4981 (1982).
105. L. C. Teh, L. J. Murphy, N. L. Huq, A. S. Surus, H. G. Friesen, L. Lazarus, and G. E. Chapman. *J. Biol. Chem.* 262:6472-6477 (1987).
106. H. G. Gundlach, S. Moore, and W. H. Stein. *J. Biol. Chem.* 234:1761-1764 (1959).
107. E. Gross and B. Witkop. *J. Am. Chem. Soc.* 83:1510-1511 (1961).
108. H. B. F. Dixon and M. P. Stack-Dunne. *Biochem. J.* 61:483-495 (1955).
109. T. B. Lo, J. S. Dixon, and C. H. Li. *Biochim. Biophys. Acta* 53:584-586 (1961).
110. H. P. J. Bennett, A. M. Hudson, C. McMartin, and G. E. Purdon. *Biochem. J.* 168:9-13 (1977).
111. D. C. Shaw and C. E. West. *J. Chromatogr.* 200:185-188 (1980).
112. P. I. Storrington and R. J. Tiplady. *Anal. Biochem.* 141:43-54 (1984).
113. P. C. Jocelyn. *Biochemistry of the SH Groups: The Occurrence, Chemical Properties, Metabolism, and Biological Function of Thiols and Disulfides*, Academic Press, New York, 1972.
114. C. Little and P. J. O'Brien. *Arch. Biochem. Biophys.* 122:406-410 (1967).
115. H. Lamfrom and S. O. Nielson. *J. Am. Chem. Soc.* 79:1966-1970 (1957).
116. D. Cavallini, C. DeMarco, and S. Dupre. *Arch. Biochem. Biophys.* 124:18-26 (1968).
117. E. S. G. Barron, Z. B. Miller, and G. Kalnitsky. *Biochem. J.* 41:62-68 (1947).
118. C. G. Overberger and J. J. Ferraro. *J. Org. Chem.* 27:3539-3545 (1962).
119. L. Philipson. *Biochim. Biophys. Acta* 56:375-377 (1962).
120. S. J. Tomazic and A. M. Klibanov. *J. Biol. Chem.* 263:3086-3091 (1988).
121. Y. M. Torchinsky. In *Sulfhydryl and Disulfide Groups of Proteins*, Consultants Bureau, New York, 1974, pp. 99-124.
122. L. A. Æ. Sluyterman. *Biochim. Biophys. Acta* 60:557-561 (1962).
123. B. R. DasGupta and D. A. Boroff. *Biochim. Biophys. Acta* 97:157-159 (1965).
124. L. Weil. *Arch. Biochem. Biophys.* 110:57-68 (1965).
125. J. D. Spikes and R. Straight. *Annu. Rev. Phys. Chem.* 18:49-436 (1967).
126. C. S. Foote. *Science* 162:963 (1968).
127. W. J. Ray. *Methods Enzymol.* 11:490-497 (1967).
128. P. Hoffee, C. Y. Lai, E. L. Pugh, and B. L. Horecker. *Proc. Natl. Acad. Sci. USA* 57:107-113 (1967).
129. M. Martinez-Carrion, R. Kuczynski, D. C. Tiemeier, and D. L. Peterson. *J. Biol. Chem.* 245:799-805 (1970).
130. M. Martinez-Carrion, C. Turano, F. Riva, and P. Fasella. *J. Biol. Chem.* 242:1426-1430 (1967).
131. M. Nakatani. *J. Biochem. (Tokyo)* 48:633-639 (1960).
132. R. D. Hill and R. R. Laing. *Biochim. Biophys. Acta* 99:352-359 (1965).
133. J. Schulz. *Methods Enzymol.* 11:255-263 (1967).
134. A. Light. *Methods Enzymol.* 11:417-420 (1967).
135. A. S. Inglis. *Methods Enzymol.* 91:324-332 (1983).
136. D. Piszkiwicz, M. Landon, and E. L. Smith. *Biochem. Biophys. Res. Commun.* 40:1173-1178 (1970).
137. K. Poulsen, K. J. Kevin, and E. Haber. *Proc. Natl. Acad. Sci. USA* 69:2495-2499 (1972).
138. F. Marcus. *Int. J. Peptide Protein Res.* 25:542-546 (1985).
139. D. L. Swallow and E. P. Abraham. *Biochem. J.* 70:364-373 (1958).
140. M. A. Naughton, F. Sanger, B. S. Hartley, and D. C. Shaw. *Biochem. J.* 77:149-163 (1960).
141. M. Bodanszky, G. F. Sigler, and A. Bodanszky. *J. Am. Chem. Soc.* 95:2352-2357 (1973).
142. M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams. *J. Am. Chem. Soc.* 89:6753-6757 (1967).
143. K. K. Han, C. Richard, and G. Biserte. *Int. J. Biochem.* 15:875-884 (1983).
144. P. Desnuelle and A. Casal. *Biochim. Biophys. Acta* 2:64-75 (1948).
145. U. K. Laemmli. *Nature* 227:680-685 (1970).
146. J. B. Fleischman. *Immunochemistry* 10:401-407 (1973).
147. V. Braun and W. A. Schroeder. *Arch. Biochem. Biophys.* 118:241-252 (1967).
148. W. R. Gray. *Methods Enzymol.* 25:121-138 (1972).
149. J. L. Meuth. *Biochemistry* 21:3750-3757 (1982).
150. P. Edman and G. Begg. *Eur. J. Biochem.* 1:80-91 (1967).
151. K. A. Walsh, L. H. Ericsson, D. C. Parmelee, and K. Titani. *Annu. Rev. Biochem.* 50:261-284 (1981).
152. D. Kahne and W. C. Still. *J. Am. Chem. Soc.* 110:7529-7534 (1988).
153. R. Cecil and J. R. McPhee. *Adv. Protein Chem.* 14:255-389 (1959).
154. V. L. Lumper and H. Zahn. *Adv. Enzymol. Relat. Areas Mol. Biol.* 27:199-238 (1965).
155. D. B. Volkin and A. M. Klibanov. *J. Biol. Chem.* 262:2945-2950 (1987).
156. R. E. Benesch and R. Benesch. *J. Am. Chem. Soc.* 80:1666-1669 (1958).
157. A. P. Ryle and F. Sanger. *Biochem. J.* 60:535-540 (1955).
158. Y. M. Torchinsky. In *Sulfur in Proteins*, Pergamon Press, New York, 1981, pp. 82-86.
159. E. Haber and C. B. Anfinsen. *J. Biol. Chem.* 237:1839-1844 (1962).
160. A. Galat, T. E. Creighton, R. C. Lord, and E. R. Blout. *Biochemistry* 20:594-601 (1981).
161. A. Neuberger. *Adv. Protein Chem.* 4:297-383 (1948).
162. G. G. Smith and B. S. Desol. *Science* 207:765-767 (1980).
163. M. Friedman and P. M. Masters. *J. Food Sci.* 47:760 (1982).
164. P. J. M. van den Oestelaar and H. J. Hoenders. *Adv. Exp. Med. Biol.* 231:261-267 (1988).
165. T. M. Florence. *Biochem. J.* 189:507-520 (1980).
166. J. R. Whitaker and R. E. Feeney. *CRC Crit. Rev. Food Sci. Nutr.* 19:173-212 (1983).
167. L. C. Sen, E. Gonzalez-Flores, R. E. Feeney, and J. R. Whitaker. *J. Agr. Food Chem.* 25:632-638 (1977).
168. H. S. Lee, D. T. Osuga, A. S. Nashef, A. I. Ahmed, J. R. Whitaker, and R. E. Feeney. *J. Agr. Food Chem.* 25:1153-1158 (1977).
169. A. S. Nashef, D. T. Osuga, H. S. Lee, A. I. Ahmed, J. R. Whitaker, and R. E. Feeney. *J. Agr. Food Chem.* 25:245-251 (1977).
170. T. E. Creighton. *Proteins*, W. H. Freeman, New York, 1983.
171. C. Ghelis and J. Yon. *Protein Folding*, Academic Press, New York, 1982.
172. W. Kauzmann. *Adv. Protein Chem.* 14:1-63 (1959).
173. P. L. Privalov. *Adv. Protein Chem.* 33:167 (1979).
174. K. Dill. *Biochemistry* 24:1501-1509 (1985).
175. Y. R. Hsu and T. Arakawa. *Biochemistry* 24:7959-7963 (1985).
176. T. Arakawa and W. C. Kenney. *Int. J. Peptide Protein Res.* 31:468-473 (1988).
177. T. Arakawa, T. Boone, J. M. Davis, and W. C. Kenney. *Biochemistry* 25:8274-8277 (1986).
178. T. Arakawa, Y.-R. Hsu, and D. A. Yphantis. *Biochemistry* 26:5428-5432 (1987).
179. W. W. Fish, A. Danielsson, K. Nordling, S. H. Miller, C. F. Lam, and I. Björk. *Biochemistry* 24:1510-1517 (1985).
180. D. N. Brems, S. M. Plaisted, E. W. Kauffman, and H. A. Havel. *Biochemistry* 25:6539-6543 (1986).
181. T. F. Holzman, D. N. Brems, and J. J. Dougherty, Jr. *Biochemistry* 25:6907-6917 (1986).
182. D. N. Brems, S. M. Plaisted, H. A. Havel, and C.-S. C. Tomich. *Proc. Natl. Acad. Sci. USA* 85:3367-3371 (1988).
183. D. N. Brems. *Biochemistry* 27:4541-4546 (1988).

184. Y. Goto and A. L. Fink. *Biochemistry* 28:945-952 (1989).
185. J. Baum, C. M. Dobson, P. A. Evans, and C. Hanley. *Biochemistry* 28:7-13 (1989).
186. D. N. Brems, S. M. Plaisted, J. J. Dougerty, Jr., and T. F. Holzman. *J. Biol. Chem.* 262:2590-2594 (1987).
187. D. N. Brems, S. M. Plaisted, H. A. Havel, E. W. Kauffman, J. D. Stodola, L. C. Eaton, and R. C. White. *Biochemistry* 24:7662-7668 (1985).
188. H. A. Havel, E. W. Kauffman, S. M. Plaisted, and D. N. Brems. *Biochemistry* 25:6533-6538 (1986).
189. W. Pfeil. *Mol. Cell. Biochem.* 40:2-38 (1981).
190. M. P. Tombs. *J. Appl. Biochem.* 7:3-24 (1985).
191. W. J. Becktel and J. A. Schellman. *Biopolymers* 26:1859-1877 (1987).
192. C. Tanford. *Adv. Protein Chem.* 23:121-282 (1968).
193. J. A. Schellman. *Annu. Rev. Biophys. Biophys. Chem.* 16:115-137 (1987).
194. C. N. Pace. *CRC Crit. Rev. Biochem.* 3:1-43 (1975).
195. H. Neurath, J. P. Greenstein, F. W. Putnam, and J. O. Erickson. *Chem. Rev.* 34:157-265 (1944).
196. J. M. Thornton. *J. Mol. Biol.* 151:261-287 (1981).
197. T. J. Ahern and A. M. Klibanov. *Meth. Biochem. Anal.* 33:91-127 (1985).
198. C. B. Anfinsen and H. A. Scheraga. *Adv. Protein Chem.* 29:205-300 (1975).
199. T. E. Creighton. *Prog. Biophys. Mol. Biol.* 33:231-297 (1978).
200. P. L. Privalov. *Adv. Protein Chem.* 35:1 (1982).
201. J. F. Brandts. In S. N. Timasheff and G. D. Fasman (eds.), *Biological Macromolecules Series, Vol. 2, Structure and Stability of Biological Macromolecules*, Marcel Dekker, New York, 1967, p. 213.
202. R. Wetzel, L. J. Perry, W. A. Baase, and W. J. Becktel. *Proc. Natl. Acad. Sci. USA* 85:401-405 (1988).
203. W. J. Becktel and W. A. Baase. *Biopolymers* 26:619-623 (1987).
204. J. Novotny, A. A. Rashin, and R. E. Bruccoleri. *Proteins: Structure, Function, Genetics* 4:19-30 (1988).
205. M. H. Zehfus and G. D. Rose. *Biochemistry* 25:5759-5765 (1986).
206. S. H. Bryant and L. M. Anzel. *Int. J. Peptide Protein Res.* 29:46-52 (1986).
207. A. M. Klibanov. *Adv. Appl. Microbiol.* 29:1-28 (1983).
208. R. Lumry and H. Eyring. *J. Phys. Chem.* 58:110-120 (1954).
209. T. Arakawa, N. K. Alton, and Y.-R. Hsu. *J. Biol. Chem.* 260:14435-14439 (1985).
210. D. A. Yphantis and T. Arakawa. *Biochemistry* 26:5422-5427 (1987).
211. T. Hoshino, Y. Mikura, H. Shimidzu, J. Kawai, and H. Toguchi. *Biochim. Biophys. Acta* 916:245-250 (1987).
212. T. A. Horbett. *ACS Adv. Chem. Ser.* 199:233-244 (1982).
213. T. A. Horbett. *ACS Symp. Ser.* 343:239-260 (1987).
214. J. R. Brennan, S. S. P. Gebhart, and W. G. Blackard. *Diabetes* 34:353-359 (1985).
215. D. E. James, A. B. Jenkins, E. W. Kraegen, and D. J. Chisholm. *Diabetologia* 21:554-557 (1981).
216. W. D. Loughheed, H. Woulfe-Flanagan, J. R. Clement, and A. M. Albisser. *Diabetologia* 19:1-9 (1980).
217. L. Peterson, J. Caldwell, and J. Hoffman. *Diabetes* 25:72-74 (1976).
218. M. V. Sefton. *ACS Adv. Chem. Ser.* 199:511-522 (1982).
219. A. S. Chawla, I. Hinberg, P. Blais, and D. Johnson. *Diabetes* 34:420-424 (1985).
220. G. K. Iwamoto, R. A. Van Wagenen, and J. D. Andrade. *J. Colloid Interface Sci.* 86:581-585 (1982).
221. E. H. Massey and T. A. Sheliga. *Pharm. Res.* 5:S34 (1988).
222. W. D. Loughheed, A. M. Albisser, H. M. Martindale, J. C. Chow, and J. R. Clement. *Diabetes* 32:424-432 (1983).
223. S. Sato, C. D. Ebert, and S. W. Kim. *J. Pharm. Sci.* 72:228-232 (1983).
224. Z. J. Twardowski, K. D. Nolph, T. J. McGary, and H. L. Moore. *Am. J. Hosp. Pharm.* 40:583-586 (1983).
225. Z. J. Twardowski, K. D. Nolph, T. J. McGary, H. L. Moore, P. Collin, R. K. Ausman, and W. S. Slimack. *Am. J. Hosp. Pharm.* 40:575-579 (1983).
226. Z. J. Twardowski, K. D. Nolph, T. J. McGary, and H. L. Moore. *Am. J. Hosp. Pharm.* 40:579-581 (1983).
227. M. L. Anson. In C. L. A. Schmidt (ed.), *The Chemistry of the Amino Acids and Proteins*, Charles Thomas, Springfield, Ill., 1938, pp. 407-428.
228. A. E. Mirsky and L. Pauling. *Proc. Natl. Acad. Sci. USA* 22:439-447 (1936).
229. J. K. Krueger, M. H. Kulke, C. Schutt, and J. Stock. *Bio-Pharm. Mar.*:41-45 (1989).
230. F. A. O. Marston. *Biochem. J.* 240:1-12 (1986).
231. J. M. Schoemaker, A. H. Brasnett, and F. A. O. Marston. *EMBO J.* 4:775-780 (1985).
232. D. L. Hartley and J. F. Kane. *Biochem. Soc. Trans.* 16:101-102 (1988).
233. D. C. Williams, R. M. Van Frank, W. L. Muth, and J. P. Burnett. *Science* 215:687 (1982).
234. J. King. *Bio/technology* 4:297-303 (1986).
235. M. Gribskov and R. R. Burgess. *Gene* 26:109-118 (1983).
236. F. A. O. Marston, P. A. Lowe, M. T. Doel, J. M. Schoemaker, S. White, and S. Angal. *Bio/technology* 2:800-804 (1984).
237. S. Cabilly, A. D. Riggs, H. Pande, J. E. Shirely, W. E. Holmes, M. Rey, L. J. Perry, R. Wetzel, and H. L. Heynecker. *Proc. Natl. Acad. Sci. USA* 81:3273-3277 (1984).
238. M. E. Winkler, M. Blabel, G. L. Bennett, W. Holmes, and G. A. Vehar. *Bio/technology* 3:990-1000 (1985).
239. H. J. George, J. J. L'Italien, W. P. Pilancinski, D. L. Glassman, and R. A. Krzyzek. *DNA* 4:273-281 (1985).
240. R. C. Fahey, J. S. Hunt, and G. C. Winham. *J. Mol. Evol.* 10:155-160 (1977).
241. T. Arakawa and S. N. Timasheff. *Biochemistry* 23:5912-5923 (1984).
242. T. Arakawa and S. N. Timasheff. *Biochemistry* 21:6545-6552 (1982).
243. F. Ahmad and C. C. Bigelow. *J. Protein Chem.* 5:355-367 (1986).
244. R. Bhat and J. C. Ahluwalia. *Int. J. Peptide Protein Res.* 30:145-152 (1985).
245. F. Ahmad. *Can J. Biochem. Cell Biol.* 63:1058-1063 (1985).
246. R. Almog. *Biophys. Chem.* 17:111-118 (1983).
247. E. Stellwagen and J. Babul. *Biochemistry* 14:5135-5140 (1975).
248. L. O. Narhi, M. K. Zukowski, and T. Arakawa. *Biophys. J.* 55:23a (1989).
249. M. W. Pantoliano, M. Whitlow, J. F. Wood, M. L. Rollence, B. C. Finzel, G. L. Gilliland, T. L. Poulos, and P. N. Bryant. *Biochemistry* 27:8311-8317 (1988).
250. A. J. Russell and A. R. Fersht. *Nature* 328:496-500 (1987).
251. C. N. Pace and G. R. Grimsley. *Biochemistry* 27:3242-3246 (1988).
252. F. W. Dahlquist, J. W. Long, and W. L. Bigbee. *Biochemistry* 15:1103-1111 (1976).
253. G. Voordouw, C. Milo, and R. S. Roche. *Biochemistry* 15:3716-3724 (1976).
254. V. V. Filimonov, W. Pfeil, T. N. Tsalkova, and P. L. Privalov. *Biophys. Chem.* 8:117-122 (1978).
255. H. Schulz. *FEBS Lett.* 78:303-308 (1977).
256. J. F. Chlebowski, S. Mabrey, and M. C. Falk. *J. Biol. Chem.* 254:5745-5753 (1979).
257. J. F. Chlebowski and S. Mabrey. *J. Biol. Chem.* 252:7042-7050 (1977).
258. S. Linse, P. Brodin, C. Johansson, E. Thulin, T. Grundström, and S. Forsén. *Nature* 335:651-653 (1988).
259. E. Stellwagen and H. Wilgus. In S. M. Friedman (ed.), *Biochemistry of Thermophily*, Academic Press, New York, 1978, pp. 228-232.
260. J. A. Roe, A. Butler, D. M. Scholler, J. S. Valentine, L. Marky, and K. J. Breslauer. *Biochemistry* 27:950-958 (1988).
261. Y. Hiroka, T. Segawa, K. Kawajima, S. Sugai, and N. Murai. *Biochem. Biophys. Res. Commun.* 95:1098-1104 (1980).
262. M. Mitani, Y. Harushima, K. Kawajima, M. Ikeguchi, and S. Sugai. *J. Biol. Chem.* 261:8824-8829 (1986).
263. J. Desmet, I. Hanssens, and F. van Cauwelaert. *Biochim. Biophys. Acta* 912:211-219 (1987).

264. R. Palmieri, R. W.-K. Lee, and M. F. Dunn. *Biochemistry* 27:3387-3397 (1988).
265. K. Gekko and S. N. Timasheff. *Biochemistry* 20:4667-4676 (1981).
266. K. Gekko and S. N. Timasheff. *Biochemistry* 20:4677-4686 (1981).
267. J. C. Lee and S. N. Timasheff. *J. Biol. Chem.* 256:7193-7201 (1981).
268. J. C. Lee and S. N. Timasheff. *Biochemistry* 13:257-265 (1974).
269. J. C. Lee and S. N. Timasheff. *Biochemistry* 14:5183-5187 (1975).
270. S. N. Timasheff, J. C. Lee, E. P. Pittz, and N. Tweedy. *J. Colloid Interface Sci.* 55:658-663 (1976).
271. J. C. Lee and S. N. Timasheff. *Biochemistry* 16:1754-1764 (1977).
272. I. D. Kuntz, Jr., and W. Kauzmann. *Adv. Protein Chem.* 28:239-345 (1974).
273. V. Prakesh, P. K. Nandi, and B. Jirgensons. *Int. J. Peptide Protein Res.* 15:305-313 (1980).
274. B. Jirgensons. *J. Protein Chem.* 1:71 (1982).
275. B. Jirgensons. *Macromol. Chem. Rapid Commun.* 2:213-217 (1981).
276. F. F. Shih and A. D. Kalmar. *J. Agr. Food Chem.* 35:672-675 (1987).
277. K. Takeda, K. Sasa, M. Nagao, and P. P. Batra. *Biochim. Biophys. Acta* 957:340-344 (1988).
278. K. Fukushima, Y. Murata, N. Nishikido, G. Sugihara, and M. Tanaka. *Bull. Chem. Soc. Jap.* 54:3122-3127 (1981).
279. J. L. Bohnert and T. A. Horbett. *J. Colloid Interface Sci.* 111:363-377 (1986).
280. J. Piatigorsky, J. Horwitz, and R. T. Simpson. *Biochim. Biophys. Acta* 490:279-289 (1977).
281. S. Tandon and P. M. Horowitz. *J. Biol. Chem.* 262:4486-4491 (1987).
282. P. S. Banerjee and W. A. Ritschel. *J. Pharm. Sci.* 76:S48 (1987).
283. A. L. Daugherty, H. D. Liggitt, J. G. McCabe, J. A. Moore, and J. S. Patton. *Int. J. Pharm.* 45:197-206 (1988).
284. D. Shortle. *J. Biol. Chem.* 264:5315-5318 (1989).
285. E. Querol and A. Padilla. *Enzyme Microbial Technol.* 9:238-244 (1987).
286. M. H. Hecht, J. M. Sturtevant, and R. T. Sauer. *Proteins Struct. Funct. Genet.* 1:43-46 (1986).
287. P. J. Carter, G. Winter, A. J. Wilkinson, and A. R. Fersht. *Cell* 38:835-840 (1984).
288. B. W. Matthews, H. Nicholson, and W. J. Becktel. *Proc. Natl. Acad. Sci. USA* 84:6663-6667 (1987).
289. G. N. Ramachandran and V. Sasisekharan. *Adv. Protein Chem.* 23:283-437 (1968).
290. S. K. Burley and G. A. Petsko. *Science* 225:23-28 (1985).
291. S. K. Burley and G. A. Petsko. *Adv. Protein Chem.* 39:125-189 (1988).
292. J. T. Kellis, Jr., K. Nyberg, D. Sali, and A. R. Fersht. *Nature* 333:784-786 (1988).
293. M. Matsumura, W. J. Becktel, and B. W. Matthews. *Nature* 334:406-410 (1988).
294. T. Alber, S. Dao-pin, K. Wilson, J. A. Wozniak, S. P. Cook, and B. W. Matthews. *Nature* 330:41-46 (1987).
295. J. F. Reidhaar-Olson and R. T. Sauer. *Science* 241:53-57 (1988).
296. M. L. Elwell and J. A. Schellman. *Biochim. Biophys. Acta* 494:367-383 (1977).
297. M. H. Hecht, J. M. Sturtevant, and R. T. Sauer. *Proc. Natl. Acad. Sci. USA* 81:5685-5689 (1984).
298. D. Shortle and A. K. Meeker. *Proteins Struct. Funct. Genet.* 1:81-89 (1986).
299. D. Shortle and A. K. Meeker. *Biochemistry* 28:936-944 (1989).
300. H. Nicholson, W. J. Becktel, and B. W. Matthews. *Nature* 336:651-656 (1988).
301. R. Hawkes, M. G. Grutter, and J. Schellman. *J. Mol. Biol.* 175:195-212 (1984).
302. M. Grutter, R. Hawkes, and B. Matthews. *Nature* 277:667-669 (1979).
303. W. J. Becktel, W. A. Baase, B. L. Chen, D. C. Muchmore, C. G. Schellman, and J. A. Schellman. *Biophys. J.* 49:572a (1986).
304. M. Grutter and B. Matthews. *J. Mol. Biol.* 154:525-535 (1982).
305. T. Alber, S. Dao-pin, J. A. Nye, D. C. Muchmore, and B. W. Matthews. *Biochemistry* 26:3754-3758 (1987).
306. B. W. Matthews. *Biochemistry* 26:6885-6887 (1987).
307. W. G. J. Hol, P. T. van Duijnen, and H. J. C. Berendsen. *Nature* 273:443-446 (1978).
308. W. G. J. Hol. *Prog. Biophys. Mol. Biol.* 45:149-195 (1985).
309. W. G. J. Hol. *Angew. Chem. Int. Ed. Engl.* 25:767-778 (1986).
310. W. G. J. Hol, L. M. Halie, and C. Sander. *Nature* 294:532-536 (1981).
311. R. P. Sheridan, R. M. Levy, and F. R. Salemme. *Proc. Natl. Acad. Sci. USA* 80:4545-4549 (1982).
312. K.-C. Chou, G. M. Maggiora, G. Nemethy, and H. A. Scheraga. *Proc. Natl. Acad. Sci. USA* 85:4295-4299 (1988).
313. J. S. Richardson. *Adv. Protein Chem.* 34:167-339 (1981).
314. P. S. Kim and R. L. Baldwin. *Nature* 307:329-334 (1984).
315. K. R. Shoemaker, P. S. Kim, D. N. Brems, S. Marqusee, E. J. York, I. M. Chaiken, J. M. Stewart, and R. L. Baldwin. *Proc. Natl. Acad. Sci. USA* 82:2349-2353 (1985).
316. C. Mitchinson and R. L. Baldwin. *Proteins Struct. Funct. Genet.* 1:23-33 (1986).
317. K. G. Strehlow and R. L. Baldwin. *Biochemistry* 28:2130-2133 (1989).
318. S. Marqusee and R. L. Baldwin. *Proc. Natl. Acad. Sci. USA* 84:8898-8902 (1987).
319. H. A. Scheraga. *Proc. Natl. Acad. Sci. USA* 82:5585-5587 (1985).
320. M. F. Perutz and G. Fermi. *Proteins Struct. Funct. Genet.* 4:294-295 (1988).
321. P. C. Lyu, L. A. Markey, and N. R. Kallenbach. *J. Am. Chem. Soc.* 111:2733-2734 (1989).
322. R. J. Abraham, B. D. Hudson, W. A. Thomas, and A. Krohn. *J. Mol. Graph.* 4:28-32 (1986).
323. D. Sali, M. Bycroft, and A. R. Fersht. *Nature* 335:740-743 (1988).
324. W. J. Becktel, W. A. Baase, R. Wetzel, and L. J. Perry. *Biophys. J.* 49:109a (1986).
325. M. H. Hecht, K. M. Hehir, H. C. M. Nelson, J. M. Sturtevant, and R. T. Sauer. *J. Cell. Biochem.* 29:217-224 (1985).
326. J. E. Villafranca, E. E. Howell, D. H. Voet, M. S. Strobel, R. C. Ogden, J. N. Abelson, and J. Kraut. *Science* 222:782-788 (1983).
327. J. A. Wells and D. B. Powers. *J. Biol. Chem.* 261:6564-6570 (1986).
328. M. W. Pantoliano, R. C. Ladner, P. N. Bryan, M. L. Rollence, J. F. Wood, and T. L. Poulos. *Biochemistry* 26:2077-2082 (1987).
329. R. T. Sauer, K. Hehir, R. S. Stearman, M. A. Weiss, A. Jeitler-Nilsson, E. G. Suchanek, and C. O. Pabo. *Biochemistry* 25:5992-5998 (1986).
330. M. Matsumura and B. W. Matthews. *Science* 243:792-794 (1989).
331. L. J. Perry and R. Wetzel. *Science* 226:555-557 (1984).
332. L. J. Perry and R. Wetzel. *Biochemistry* 25:733-739 (1986).
333. M. G. Mulkerrin, L. J. Perry, and R. Wetzel. In D. L. Oxender (ed.), *Protein Structure, Folding, and Design*, Alan Liss, New York, 1986, pp. 297-305.
334. G. McLendon and E. Radany. *J. Biol. Chem.* 253:6335-6337 (1978).
335. T. J. Ahern, J. I. Casal, G. A. Petsko, and A. M. Klibanov. *Proc. Natl. Acad. Sci. USA* 84:675-679 (1987).
336. P. T. Wingfield, R. J. Mattaliano, H. R. MacDonald, S. Craig, G. M. Clore, A. M. Gronenborn, and U. Schmeissner. *Protein Eng.* 1:413-417 (1987).
337. A. Abuchowski. *J. Cell. Biochem.* 11A:174 (1987).
338. P. Koziej, M. Mutter, H.-U. Gremlich, and G. Hölzemann. *Z. Naturforsch.* 40B:1570-1574 (1985).
339. M. Mutter, H. Mutter, R. Uhlmann, and E. Bayer. *Biopolymers* 15:917-927 (1976).
340. P. V. N. Rajasekharan and M. Mutter. *Acc. Chem. Res.* 14:122-130 (1981).

341. A. A. Ribiero, R. P. Saltman, M. Goodman, and M. Mutter. *Biopolymers* 21:2225-2239 (1982).
342. M. Hashimoto, K. Takada, Y. Kiso, and S. Muanishi. *Pharm. Res.* 6:171-176 (1989).
343. D. D. Chow and K. J. Hwang. *J. Pharm. Sci.* 76:S49 (1987).
344. E. R. Jakoi, P. E. Ross, H. P. Ting-Beall, B. Kaufman, and T. C. Vanaman. *J. Biol. Chem.* 262:1300-1304 (1987).
345. D. A. Towler, S. R. Eubanks, D. S. Towery, S. P. Adams, and L. Glaser. *J. Biol. Chem.* 262:1030-1036 (1987).
346. Y. A. Ovchinnikov, N. G. Abdulaev, and A. S. Bogachuk. *FEBS Lett.* 230:1-5 (1988).
347. F. S. Qaw and J. M. Brewer. *Mol. Cell. Biochem.* 71:121-127 (1986).
348. A. T. Fojo, P. L. Whitney, and M. W. Awad, Jr. *Arch. Biochem. Biophys.* 224:636-642 (1983).
349. P. Cujo, W. El-Deiry, P. L. Whitney, and W. M. Awad, Jr. *J. Biol. Chem.* 255:10828-10833 (1980).
350. R. Wolfenden, L. Anderson, P. M. Cullis, and C. C. B. Southgate. *Biochemistry* 20:849-855 (1981).
351. R. Desrosiers and R. M. Tanguay. *J. Biol. Chem.* 263:4686-4692 (1988).
352. R. M. Epan and K. E. Raymer. *Int. J. Peptide Protein Res.* 30:515-521 (1987).
353. H. Koide, S. Yokoyama, G. Kawai, J.-M. Ha, T. Oka, S. Kawai, T. Miyake, T. Fuwa, and T. Miyazawa. *Proc. Natl. Acad. Sci. USA* 85:6237-6241 (1988).