Biochemistry

 Volume 29, Number 9 March 6, 1990

Perspectives in Biochemistry

The Mechanism of Protein Folding. Implications of in Vitro Refolding Models for de Novo Protein Folding and Translocation in the Cell†

Gunter Fischer‡ and Franz X. Schmid*,§

Enzymologie, Sektion Biowissenschaften, Martin-Luther-Universität, Halle-Wittenberg, Domplatz 1, DDR-4020 Halle/Saale, GDR, and Laboratorium für Biochemie, Universität Bayreuth, D-8580 Bayreuth, FRG

Received September 6, 1989

The in vitro refolding of purified, denatured proteins is a spontaneous process. It is driven by a small, but significant, difference in Gibbs free energy under native conditions and generally requires neither the presence of additional factors nor the input of energy (Tanford, 1968, 1970; Kim & Baldwin, 1982; Jaenicke, 1987; Tsou, 1988). In the past few years, however, an increasing number of proteins have been discovered, which were proposed to be involved in the maturation and folding of nascent protein chains in the cell (Pelham, 1986, 1988; Rothman & Kornberg, 1986; Freedman, 1987). These proteins are found in the cytoplasm as well as in the endoplasmic reticulum (ER),[1] the mitochondria, or the chloroplasts. They are thought to be involved in functions such as catalyzing protein folding, keeping protein chains in a nonnative state, or "guiding" proteins to their cellular association partners. When recombinant proteins are overexpressed in foreign hosts, the polypeptide chains frequently do not fold correctly, but they are deposited in the cell in an insoluble, nonnative form (Marston, 1986), which could mean that the correct "folding helpers" are missing. We review here current concepts for the mechanism of in vitro protein folding and evaluate their significance for folding processes in vivo and the role that could be ascribed to cellular components in this process.

Physicochemical Data on the in Vitro Folding and Stability of Proteins

(*1*) *Thermodynamic Stability of Proteins.* Folded proteins are usually stable in a thermodynamic sense at ambient temperature and at neutral pH; however, the difference in Gibbs free energy between the native and the unfolded state is generally small. Frequently, values in the range of −40 kJ/mol are found. Consequently, small shifts in the solvent conditions, such as changes in pH, temperature, or composition, can lead to large changes in the net stability of folded proteins. Also, associations with ligands, other proteins, or subunits may lead to major alterations in the stability of a protein or even promote its unfolding. The thermal or denaturant-induced unfolding transitions of several small proteins are described well by a simple two-state model, which involves only native and unfolded protein at equilibrium (Privalov, 1979). In other words, folding of these proteins is a cooperative process, and molecules where only part of the stabilizing interactions are missing are not populated at equilibrium. Large proteins consist of folding domains, which can behave as independent structural entities with individual unfolding transitions (Privalov, 1982). A number of proteins unfold irreversibly in in vitro experiments. The reasons for the lack of reversibility are not always clear. In some cases posttranslational covalent modifications, such as proteolytic processing, can lead to a loss of reversibility. Generally, small changes of the covalent structure, such as the removal of a few residues from either end of the chain, often result in large changes in the overall stability of a protein (Wetlaufer & Ristow, 1973).

(*2*) *The Unfolded State and Equilibrium Intermediates.* Unfolding of proteins in the presence of high concentrations of "strong" denaturants, such as GdmCl or urea, leads to a state that shows a number of properties that are expected for a randomly coiled polypeptide. The titratable groups are normalized and the aromatic residues are exposed to solvent (Tanford, 1968, 1970). As judged by amide circular dichroism, the secondary structure has disappeared and the NMR spectrum gives no indication for ordered structure. The thermally unfolded state is more difficult to assess. The thermodynamic properties of thermally and denaturant-un-

† Writing of this paper was supported by a grant from the Volkswagenstiftung.

* To whom correspondence should be addressed.
‡ Martin-Luther-Universität.
§ Universität Bayreuth.

[1] Abbreviations: ER, endoplasmic reticulum; BiP, immunoglobulin heavy-chain binding protein; GdmCl, guanidinium chloride; PDI, protein disulfide isomerase; PPIase, peptidyl-prolyl cis–trans isomerase; hsp, heat shock protein.

0006-2960/90/0429-2205$02.50/0

folded proteins are very similar or identical; i.e., in both cases cooperative unfolding transitions with a similar increase in heat capacity are observed for small proteins. However, some residual structure appears to be present in proteins denatured at high temperature. Exposure to solvent of the aromatic residues is not complete, and residual secondary structure is found in some cases (Tanford, 1968).

For a few proteins, such as α-lactalbumin, myoglobin, or carbonic anhydrase, folding intermediates have been found, which are stable in the presence of intermediate concentrations of denaturant and/or in the acid or alkaline pH region (Kuwajima, 1977; Wong & Tanford, 1973; Dolgikh et al., 1984; Brems et al., 1985). Such intermediates are frequently observed for proteins with a low overall stability, i.e., with weak long-range interactions that are abolished under "mildly denaturing" conditions. The addition of extra stabilizing interactions, such as binding of Ca^{2+} ions to α-lactalbumin, leads to an increase in the stability of the native protein and to the disappearance of the partially folded intermediate (Kuwajima et al., 1989). Such intermediates are sometimes called "molten globules". They have some nativelike properties, such as a high amount of secondary structure and a compact shape. On the other hand, there appears to be only little or no well-defined tertiary structure and the aromatic residues are mostly unordered, suggesting that the flexibility of the polypeptide chain is very high in this state. In its enthalpy and heat capacity the molten globule still resembles the unfolded protein (Kuwajima, 1989).

(*3*) *Short Peptides as Models for Local Structure.* Contrary to earlier views that small peptides are essentially devoid of ordered structure in aqueous solution, it has become evident in the last few years that certain peptides as short as four or five residues can adopt significant nonrandom conformations under "native" aqueous conditions. Early evidence for such ordered structure came from circular dichroism measurements by Brown and Klee (1971) on the amino-terminal fragment of ribonuclease A, the S-peptide, which is 20 residues long. A significant amount of helical structure was detected in this peptide in the presence of salt near 0 °C. Extensive studies of the S-peptide and a large number of derivatives by Baldwin and his co-workers demonstrated that the helix in the S-peptide is similar to the helix found in the respective chain region of intact ribonuclease A (Kim & Baldwin, 1984). Furthermore, they showed that the helical content could be increased by amino acid substitutions, which favored salt bridge formation and stabilized the helix dipole (Shoemaker et al., 1987). Also, alanine residues were found to increase the helical content (Marqusee et al., 1989). Dyson et al. (1988a,b) searched for nonrandom structure in a large number of short peptides by NMR. A remarkable finding was that the short pentapeptide YPGDV exists to about 50% in a β-turn conformation at low temperature. Other short peptides contained significant amounts of β-turns in aqueous solution as well, albeit at a lower percentage. They found a correlation between the turn-forming potential of short peptides and the turn probabilities of the same sequences in proteins, as derived from an analysis of known crystal structures. This suggests that formation of some secondary structures can be specified by strictly local sequences. Such chain segments might thus serve as initiation sites for folding (Wright et al., 1988) and lead to a very fast restriction of the conformational space early in the folding process. The results on the sequence variations of the ribonuclease S-peptide show that the naturally occurring sequence is not the one with the highest helical content. This indicates (i) that protein secondary structures have clearly not evolved to maximal stability and (ii) that a high stability of local structures is not desirable for early stages of the folding process. Local ordered structures should indeed be fairly unstable, as required from theoretical considerations. To avoid formation of stable, but incorrect, structures, it is necessary to use the long-range interactions (which are formed late in folding) with their high resolving power to select and further stabilize those early local structures that are on the correct folding pathway. Incorrect local structures are not stabilized and consequently convert back into the ensemble of unfolded conformations (Go, 1983). Thus the finite, but very low, stability of local structures could be the kinetic "proofreading" mechanism of protein folding. In this context it is interesting to note that the stability of ordered structure in natural peptides is always small and that not all turns observed in peptides are also turns in the respective folded proteins, from whence the sequences were derived (Dyson et al., 1988a,b). Introduction of a defined long-range contact, such as a disulfide bond, strongly increases the stability of secondary structure (Oas & Kim, 1988).

(*4*) *Structure and Stability of Protein Fragments.* The question of how much polypeptide chain is necessary for the stability and the correct folding was investigated for a number of proteins. In the case of ribonuclease A, it was found that removal of either 20 residues from the amino terminus or four residues from the carboxy terminus both resulted in the inability of the truncated protein chains to re-form all correct disulfide bonds after denaturation and reduction (Haber & Anfinsen, 1961; Taniuchi, 1970). This defect was "healed" by adding back the S-peptide (1–20) or the C-terminal 110–124 peptide, respectively, to the reoxidation mixture (Kato & Anfinsen, 1969; Andria & Taniuchi, 1978). Large fragments of staphylococcal nuclease were found to be devoid of specific structure; however, folding was possible when overlapping peptides were incubated together (Andria et al., 1971). These examples suggest that for such small single domain proteins essentially the entire chain is necessary for stability and folding. On the other hand, chain extensions, such as a transit peptide, could easily inhibit the formation of correctly folded structures by contributing a few "bad" contacts.

In the case of larger proteins, stable and autonomously folding domains have been detected. Examples include the domains of the immunoglobulin light chain (Tsunenaga et al., 1987), of γ-crystallin (Siebendritt, 1989), and of tryptophan synthase (Zetina & Goldberg, 1980; Matthews & Crisanti, 1981). An interesting system is provided by the carboxy-terminal region of thermolysin. A number of proteolytic fragments of that region can be prepared which show autonomous folding behavior. The smallest of these stable and cooperative fragments, comprising residues 255–316, is highly helical; it is equivalent to a three-helix motif in native thermolysin (Dalzoppo et al., 1985).

(*5*) *Kinetics of Protein Folding Reactions.* The rates of protein folding reactions vary extensively. Folding can be complete within a few milliseconds or can require several hours. To a first approximation, small proteins tend to fold more rapidly than large ones; however, many exceptions from that rule exist. In addition, a single protein can refold on different fast- and slow-folding pathways (Garel & Baldwin, 1973). The occurrence of such fast- and slow-folding species in unfolded proteins is explained by the proline hypothesis (Brandts et al., 1975), which assumes that slow-folding molecules contain incorrect isomers of Xaa–Pro peptide bonds. The presence of such incorrect isomers does not block refolding, as assumed originally; however, it leads to a significant de-

stabilization of folding intermediates and hence to a strong decrease of folding rates. Fast-folding molecules have all important prolyl peptide bonds in the native conformation, and therefore they refold rapidly (in many cases in the millisecond time range). Presumably not all proline residues are important for folding; their role for folding may depend on the location within the polypeptide chain and also on the conditions employed for refolding (Schmid & Blaschek, 1981). Additional slow, rate-determining folding steps exist. Examples are the formation of disulfide bonds (see below), the association of prefolded monomers in the case of oligomeric proteins (Jaenicke, 1987), or slow reshuffling reactions of almost nativelike intermediates (Vaucheret et al., 1987). In general, the slowest, rate-limiting step of unfolding and refolding appears to be close to the native state in terms of ordered structure (Segawa & Sugihara, 1984; Goldenberg & Creighton, 1985). In other words, the activated state of folding with the highest energy is a distorted form of the native protein. The refolding of many proteins is not completely reversible in vitro, and reactivation yields of less than 100% are frequently observed. This holds in particular for experiments at high protein concentration. Competing aggregation of unfolded or partially refolded protein molecules may be the major reason for a lowered reversibility (Jaenicke, 1987). This could reflect simply the lack of success of finding the correct solvent conditions and protein concentrations for reversible refolding, but it could also be a genuine problem of in vitro folding.

The slow rate-limiting steps of folding, such as proline isomerization or other processes, are generally preceded by the rapid formation of folding intermediates. These intermediates are frequently compact molecules and contain most of the secondary structure of the native protein (Kuwajima, 1989). They are still flexible and show little defined tertiary structure. In some aspects they resemble the molten globule intermediates that were found in the equilibrium unfolding transitions of some proteins (see above). High-resolution NMR experiments showed that most of the chain is involved in the formation of these early intermediates and that extensive secondary structure has already formed and is fairly stable (Udgaonkar & Baldwin, 1988; Roder et al., 1988). For cytochrome *c* the interaction of two helices in the amino- and carboxy-terminal regions, respectively, is important for the stabilization of this intermediate. Similar results were obtained for α-lactalbumin, where a sizable part of the molecule is already organized in a nativelike manner in the early intermediate (Kuwajima et al., 1989). These findings demonstrate that information encoded in distant regions of the chain is required already for early steps in folding. These processes are much faster than the biosynthesis of protein chains. On the other hand, chain regions, such as *cis*-proline containing β-turns, can be tolerated in a nonnative state until a late stage in refolding (Lang & Schmid, 1990; Kiefhaber et al., 1990b).

Kinetic intermediates of folding are sometimes sensitive to aggregation (Brems, 1988), because they still have an increased amount of exposed hydrophobic surface. This transient low solubility of incompletely folded chains may also be a problem for the de novo folding of nascent proteins. There is no unique folding mechanism for a particular protein, but the kinetic mechanism may depend strongly on the refolding conditions. The stability of folding intermediates is generally small; therefore, variations in the solvent conditions can strongly affect the formation of critical intermediates. For a review on early steps in protein folding, see Baldwin (1989).

(*6*) *Formation of Disulfide Bonds during Refolding.* Unfolded proteins with reduced disulfide bonds can spontaneously regain the native set of disulfide bonds under suitable conditions. Re-formation of disulfides requires the presence of a redox partner, such as oxygen, glutathione, or dithiothreitol (Creighton, 1978). The redox partner has to be present in both the oxidized and the reduced forms to allow for the reshuffling of incorrect nonnative disulfides, which are generated rapidly at the beginning of reoxidation (Saxena & Wetlaufer, 1970). Incorrect disulfides are generally observed on the in vitro reactivation pathways of reduced proteins. Formation and stabilization of the correct disulfides depend on the local concentrations of the respective sulfhydryl groups, which in turn depend on the conformation of the protein chain (Creighton, 1983). For the complete re-formation of the disulfides, it is essential that the respective sulfhydryl groups remain accessible for the disulfide reagent. Hence partially refolded and reoxidized molecules may not have a compactly folded structure, which could block the access of the redox partner. In the case of pancreatic trypsin inhibitor, a fraction of the refolded molecules contained two buried sulfhydryl groups. They were able to form a disulfide bridge only after heating, which increased the mobility of the chain, thus allowing oxygen to approach and oxidize these two cysteine residues (States et al., 1984).

Proteins That Are Involved in Cellular Protein Folding Events

(*1*) *Protein Disulfide Isomerase.* The first protein that was suggested to be involved in the de novo folding of nascent polypeptide chains is protein disulfide isomerase (PDI). Anfinsen's group showed that reduced and unfolded ribonuclease A can regain its native state with correct disulfide bonds in a spontaneous, but very slow, reoxidation reaction (Sela et al., 1957). Soon after that, an enzyme activity was discovered in liver extracts that was capable of accelerating the reoxidation of reduced proteins, and it was immediately suggested that it might be involved in the formation of disulfide bonds in nascent proteins (Goldberger et al., 1963; Venetianer & Straub, 1963). Generally, only secreted proteins contain disulfide bonds; therefore, PDI should be localized in cell compartments that form the secretory path. Indeed, it is found in the endoplasmic reticulum (ER) at a very high concentration. In rat liver microsomes, PDI amounts to about 2% of the entire protein content (Freedman, 1984, 1989). It carries a carboxy-terminal KDEL sequence, which is typical for proteins resident in the ER (Munro & Pelham, 1987).

PDI catalyzes the in vitro reoxidation of many different proteins. Its activity may depend on the presence of a low molecular weight oxidant, such as molecular oxygen or a mixture of oxidized and reduced glutathione. It is a true catalyst; the product of its action depends on the redox potential of the solution. Accordingly, the formation, reduction, or isomerization of disulfide bonds can be catalyzed by PDI. The conformation of the final product depends on the stability of the target protein under the employed solvent and redox conditions (Freedman, 1984).

Conclusive evidence for an important role of PDI for in vivo folding was provided by Bulleid and Freedman (1988). They depleted dog pancreas microsomes of soluble proteins, including PDI. These microsomal preparations were still able to translocate and process nascent protein chains, but they were defective in the formation of correct disulfide bonds. Addition of purified PDI to these microsomes restored the capacity to generate correctly disulfide-bonded protein.

The role of PDI apears to be fairly clear now. It facilitates the formation of the correct set of disulfide bonds during de novo folding of secreted proteins. It does not determine the

folding pathway (Creighton et al., 1980), but catalyzes slow steps, presumably by rapid reshuffling of incorrect disulfide bonds in the presence of a low molecular weight thiol compound, which may be reduced and oxidized glutathione. The direction of folding and the end product are determined by the protein itself, i.e., by the stable, native set of disulfide bonds and by the suitable solvent and redox conditions. The formation of the correct disulfides can be very rapid. In some cases, such as in the immunoglobulin chains, it is a cotranslational event: as soon as an entire domain is translocated into the lumen of the endoplasmic reticulum, the single intradomain disulfide is formed (Bergman & Kuehl, 1979). Whether PDI is responsible for this particular reaction and whether disulfide bond formation is a cotranslational event for other, more extensively cross-linked proteins as well remain to be elucidated.

PDI is identical with the β-subunit of prolyl-4-hydroxylase (Koivu et al., 1987) and very similar to a protein that is presumably involved in the oligosaccharide transferase system (Geetha-Habib et al., 1988). These recent findings are discussed by Freedman (1989).

(*2*) *Peptidyl-Prolyl Cis–Trans Isomerase.* Some conformational steps in protein folding can be slow; therefore, enzymatic catalysis of folding should be of advantage. An acceleration of crucial folding steps would decrease the risk of proteolytic degradation of partially folded chains, suppress competing unproductive pathways, such as aggregation, and hence "keep the protein on the correct, productive folding pathway". The isomerization of incorrect Xaa–Pro peptide bonds is one of the slow, rate-determining steps in in vitro refolding (Brandts et al., 1975; Schmid & Baldwin, 1978; Lang et al., 1987). In 1984 an enzyme was found by Fischer et al. that accelerates efficiently the cis–trans isomerization of prolyl peptide bonds in short oligopeptides. Accordingly, this protein was named peptidyl-prolyl cis–trans isomerase (PPIase). The catalyzed reaction is a 180° rotation about the C–N linkage of the peptide bond preceding proline, which involves neither net cleavage nor net formation of covalent bonds. Thus, PPIases are "conformases" with a very high efficiency. The porcine 17-kDa PPIase shows a k_{cat}/K_M value that is estimated to be higher than 10^6 M^{-1} s^{-1} for the isomerization of short oligopeptides. PPIase activities are widely distributed; they are found in virtually all tissues and organisms, ranging from mammals to bacteria. They apparently represent a new class of enzymes that are diverse in molecular weight, in cellular location, and in substrate specificity. Surprisingly, it was found that PPIase from porcine kidney is identical with bovine cyclophilin, a protein that binds the immunosuppressant cyclosporin with high affinity (Takahashi et al., 1989; Fischer et al., 1989a).

In addition to their activity on small proline-containing peptides, PPIases also catalyze the slow in vitro refolding of several small proteins for which from independent experiments proline isomerization was assumed to be a rate-limiting step (Lang et al., 1987; Lin et al., 1988). The efficiency of catalysis varies strongly, depending on the target protein. One particular slow step in the folding of ribonuclease T_1 is accelerated more than 100-fold by prolyl isomerase. Catalysis depends on both PPIase and ribonuclease T_1 concentrations (Fischer et al., 1989a; Kiefhaber et al., 1990a,b), and it is abolished in the presence of low concentrations of cyclosporin A, which binds with high affinity to the active site. The folding of some proteins, such as bovine ribonuclease A, chymotrypsinogen, or thioredoxin, is not catalyzed significantly, although good evidence exists that proline isomerization is important for their refolding mechanism (Lang et al., 1987; Lin et al., 1988; Lang, 1988). Apparently, catalysis of in vitro refolding depends on the sequence surrounding the respective proline residues and also on their steric accessibility in partially structured folding intermediates. The importance of accessibility was demonstrated for the folding of ribonuclease T_1, where the efficiency of catalysis depends on the structural environment of two proline peptide bonds (Kiefhaber et al., 1990a,b). The folding of collagen (types III and IV) is also accelerated in the presence of PPIases (Bächinger, 1987; Davis et al., 1989). At this point, however, it is important to keep in mind that proline isomerization is not the only slow process in protein folding. Other conformational steps, unrelated to proline isomerization, can be very slow as well. This may in particular hold for multidomain and oligomeric proteins, which frequently refold in the time range of minutes to hours.

The influence of the flanking sequences around the target proline on the catalysis of protein folding by PPIase has not yet been investigated. Data on small peptides indicate that at least the four amino acids around the rotating bond are important for catalysis (Fischer et al., unpublished).

Little is known at present about the enzymatic mechanism of PPIases. A single thiol group, which is near the active site, appears to be essential for the activity of the porcine 17-kDa PPIase (Fischer et al., 1989a). Inverse secondary kinetic isotope effects in substrates, in which the C_α-H that precedes proline is substituted by deuterium, indicate that a covalent intermediate, possibly a hemi ortho thioamide, is formed transiently (Fischer et al., 1989b).

The role of PPIases for in vivo protein folding or other transconformational reactions in the cell is not yet known, and the effect of the immunosuppressant cyclosporin A is not understood. As shown by immunohistochemical staining, actively growing cells show a higher content of 17-kDa PPIase (cyclophilin) than resting cells and an increased staining of vesicular structures (Harding & Handschumacher, 1988). In *Neurospora crassa* PPIase is found in the cytosol and in the mitochondria; the two respective proteins appear to be encoded by the same gene (Tropschug et al., 1988). The product of the *ninaA* gene of *Drosophila* is homologous with the 17-kDa PPIase. It carries an additional carboxy-terminal hydrophobic extension, which could serve as a membrane anchor. Mutant flies with a defect in the *ninaA* gene show a 10-fold reduction in rhodopsin level, although the opsin mRNA is produced normally. Possibly the *NinaA* protein is important for the maturation of rhodopsin (Shieh et al., 1989; Schneuwly et al., 1989). A nucleotide binding motif was suggested to be present in 17-kDa PPIase (Gschwendt et al., 1988); however, several features typical for such proteins (Wierenga & Hol, 1983) are lacking in the sequence. Also there is no experimental evidence that the activity of PPIases is influenced by the presence of nucleotides.

(*3*) *Proteins Involved in Polypeptide Transport across Membranes.* A number of proteins are translocated during or after biosynthesis across the plasma membrane of procaryotes or across organelle membranes in eukaryotes. It is clear now that these proteins are not transported in a compact globular state but that a more or less unfolded state is required for efficient transfer (Schleyer & Neupert, 1985; Randall & Hardy, 1986; Zimmermann & Meyer, 1986; Chen & Douglas, 1987; Vestweber & Schatz, 1988; Wickner, 1988). Structure formation can be avoided easily by a tight coupling of transport to protein biosynthesis, i.e., by the cotranslational transfer of the nascent chains across membranes, as is observed normally for the transport of proteins into the ER. However, post-translational transport into the ER (Watts et al., 1983), into

the mitochondria (Harmey et al., 1977), and across the procaryotic plasma membrane (Zimmermann & Wickner, 1983; Randall, 1983) is possible as well.

A distinctive feature of proteins that are targeted to different cellular locations is the presence of an amino-terminal leader sequence, which, after transport, is usually removed from the mature protein. The simplest way to keep the preprotein in a "membrane-transport competent" form after the completion of biosynthesis would be to ensure the presence of the leader sequence, thus preventing the formation of stable three-dimensional structure in the preprotein. A few unfavorable interactions of the leader sequence with the remaining polypeptide chain should be sufficient to block or at least decelerate the formation of rigid, folded structure. Such a deceleration of refolding in the presence of the leader sequence was indeed observed for the precursors of maltose- and of ribose-binding protein from *Escherichia coli* (Park et al., 1988). Also, the interaction of the presequence with (negatively charged) membrane lipids may lead to partial unfolding of the precursor prior to transport (Endo & Schatz, 1988; Endo et al., 1989).

Such ways of keeping precursor proteins competent for translocation may not be sufficient in all cases. Also, incompletely folded precursors are presumably sensitive to aggregation. Therefore, the posttranslational import of polypeptide chains to mitochondria or the export from *E. coli* requires the presence of proteins, which, in an ATP-dependent manner, confer or retain transport competence to these polypeptides and keep them in solution (Pfanner et al., 1987; Crooke et al., 1988). The requirement for this ATP-dependent step depends on the folded state of the "passenger" polypeptide. Import of unfolded precursor proteins required less or no ATP (Verner & Schatz, 1987; Pfanner et al., 1988; Crooke et al., 1988). An inverse correlation exists between the stability of the folded state of a precursor and the efficiency of its transport across the mitochondrial membrane. The transfer into the mitochondria of mouse dihydrofolate reductase fused to the signal sequence of yeast cytochrome oxidase subunit IV is facilitated by urea denaturation and impaired by binding the inhibitor methotrexate, which strongly stabilizes the folded state of this protein (Eilers & Schatz, 1986). The implicated ATP-dependent unfolding proteins have not yet been isolated. However, there are indications that members of the "heat shock" protein family such as hsc70 (in cooperation with additional proteins) can stimulate import into microsomes (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988). The precursor of an outer membrane protein from *E. coli*, proOmpA, is held in a membrane assembly competent form by a 63-kDa protein, the trigger factor (Crooke et al., 1988). The difference in Gibbs free energy between the native and the unfolded state of a protein is small, and therefore, the hydrolysis of ATP should be sufficient to drive such a transition to a less ordered conformation.

(*4*) *Proteins That Recognize Unfolded or Incompletely Folded Polypeptide Chains.* Other proteins that have been implicated in folding and unfolding processes in the cell apparently recognize misfolded or aggregated molecules or bind transiently to partially folded or nonassembled polypeptide chains, to prevent them from premature aggregation. Similar to the proteins that facilitate transport through membranes, these proteins belong to the family of the heat shock proteins [for reviews see Pelham (1986, 1988) and Ellis and Hemmingsen (1989)].

The immunoglobulin heavy-chain binding protein (BiP), which is identical with the glucose-regulated protein GRP78, is one of these proteins. BiP was suggested to be a "helper" in the folding and assembly of oligomeric proteins in the ER, such as the immunoglobulins (Munro & Pelham, 1986; Bole et al., 1986; Gething et al., 1986), or alternatively to be a binding protein for misfolded, unassembled, or otherwise aberrant proteins in the endoplasmic reticulum (Kassenbrock et al., 1988; Hurtley et al., 1989). Most of the presently available evidence supports the assumption that the major function of BiP is the recognition of incorrectly folded or aggregated proteins and their ultimate removal from the ER (Kassenbrock et al., 1989; Hurtley et al., 1989). Also, treatments of cells that lead to the accumulation of incomplete or misfolded proteins in the ER induce an increase in BiP–mRNA (Normington et al., 1989; Rose et al., 1989). Whether BiP is also involved in "normal" protein folding and association in the ER is still not entirely clear.

Protein molecules that are necessary for the correct folding of monomeric proteins and for the formation of oligomeric assemblies have been localized in *E. coli* (the GroEL and GroES proteins), in chloroplasts of higher plants (the Rubisco subunit binding protein), and in mitochondria from *Saccharomyces cerevisiae* and *N. crassa* (hsp60). Since these molecules "guide" other proteins to their final native functional state, they were named "molecular chaperones" (Hemmingsen et al., 1988). These chaperones are highly abundant, essential proteins, they show a M_r of about 60 000, and they are strongly homologous to each other in sequence (Hemmingsen et al., 1988; Cheng et al., 1988). Related proteins exist in human leucocytes (Waldinger et al., 1988). All three chaperones occur as multimers of 12–16 identical or different subunits, associated probably with additional proteins such as GroES. Rubisco binding protein is necessary for the correct assembly of the eight large and eight small subunits to form the active complex in the chloroplasts (Roy et al., 1988; Hemmingsen et al., 1988). Rubisco assembly can also be promoted by the GroE proteins, after expression in *E. coli* (Goloubinoff et al., 1989). The mitochondrial hsp60 protein was found to be essential for the correct association of several oligomeric enzymes in the mitochondria (Cheng et al., 1988), as well as for the correct folding of monomeric imported proteins. It is suggested that folding occurs at the surface of the oligomeric hsp60 in an ATP-dependent reaction. Unlike in the case of the BiP protein, it was shown unequivocally that association with hsp60 is transient and is on the pathway of folding. ATP depletion led to an arrest of folding in the hsp60-bound state. When ATP was added back, folding went to completion, and the product dissociated from hsp60 (Ostermann et al., 1989). Hsp60 and GroEL are constitutively expressed (GroEL accounts for about 1% of total cell protein), but the amounts of both proteins increase upon thermal stress. The molecular mechanism of folding assistance by chaperones is unknown. Also, it is not certain whether these proteins are real enzymes. It appears possible that the extended surfaces of the oligomeric chaperones offer sites for the reversible binding of partially folded target molecuels and thereby suppress aggregation. ATP hydrolysis is necessary for the assisted folding. The predominant energy requirement may be related to the dissociation of the folded or nearly folded protein from the chaperone. Whether this is the only energy-requiring step and whether ATP is needed by the chaperone itself or another protein are not yet known [cf. Ostermann et al. (1989)]. The GroEL protein can also interact transiently with newly synthesized unfolded proteins. This association is decreased in the presence of other unfolded proteins, such as heat-denatured apomyoglobin, and it is abolished by ATP hydrolysis (Bochkareva et al., 1988).

Correlation of in Vitro and in Vivo Results

Several key results have emerged from in vitro folding experiments. (i) The structure of the folded state and the folding mechanism are both encoded in the amino acid sequence; hence folding is a spontaneous process under suitable conditions. (ii) The stabilization energy of a native protein is very small. (iii) Partially folded states can be populated rapidly, and the slow rate-determining steps occur late in folding. (iv) Aqueous medium is a poor solvent for unfolded protein chains in the absence of urea or GdmCl. (v) The yields of refolding are frequently smaller than 100% (particularly for large proteins), because aggregation can compete with proper folding. (vi) The structure of prefolded monomers is fairly unstable and different from the structure in the native oligomeric protein.

From point i it is immediately evident that proteins can gain their native structure without any help and most of them (in particular monomeric single-domain proteins) can achieve this in a biologically reasonable time. Times estimated for the in vivo maturation of proteins lie in the range of a few minutes [Goldenberg & King, 1982; for a review, see Wetlaufer (1985)] around 30 °C. Many in vitro folding reactions are similar in rate, or even faster.[2] Nevertheless, assistance from "folding helpers" is useful (i) to accelerate some slow rate-limiting steps, (ii) to suppress competing aggregation reactions, and (iii) if necessary, to reverse or suppress premature and incorrect folding. Examples for all three tasks have been described. Protein disulfide isomerase catalyzes the slow formation of the correct set of disulfide bonds; prolyl isomerase could accelerate the intrinsically slow isomerization of incorrect prolyl peptide bonds. This may be important for prolines that become cis in the native protein, because the product of protein biosynthesis is presumably an all-trans polypeptide chain. Whether catalysis of folding is indeed the in vivo function of the prolyl isomerases is still unknown. Proline isomerization during refolding can also be strongly accelerated by prefolding in the vicinity of the incorrect proline (Schmid & Blaschek, 1981), so that enzymatic catalysis might not be necessary. An alternative in vivo function of prolyl isomerases could be the recognition and/or interconversion of certain surface-exposed turn regions of folded proteins that contain proline residues. Such interactions could be used to trigger and to time slow activation/deactivation processes. PPIases might thus "liquify" the polypeptide backbone in certain regions of folded or partially folded proteins.

The solubility of "unfolded" or partially folded chains in aqueous solvents is poor; therefore, the premature aggregation of proteins prior to the final slow-folding or assembly steps is a major problem in in vitro folding experiments. It can be overcome in some cases by working at high dilution or by following certain time-dependent assembly protocols. By such procedures it was possible to reassemble functional *E. coli* ribosomes in vitro (Nierhaus & Dohme, 1974). In cells, the same problems are apparent, aggravated by the high concentrations of folded and partially folded polypeptide chains. Under cellular conditions newly formed chains will never adopt an extended conformation, but presumably collapse into a nonspecific globule with still a high tendency to aggregate. This state is presumably characterized (i) by packing defects in the hydrophobic core and (ii) by a suboptimal hydrogen bonding of the main chain. Of course, some proteins can complete folding rapidly to the soluble native state. For others, assistance from folding helpers or binding proteins is necessary to cope with the aggregation problem.

The task of keeping polypeptide chains in an incompletely folded state or of converting folded proteins to such a state is managed by several proteins, which are involved in protein transport. These proteins need energy to prevent folding to the native state. The energy requirement is small, since native and unfolded states do not differ strongly in energy. ATP hydrolysis should be sufficient in this respect to convert the binding proteins from a high-affinity state (for incompletely folded proteins) to a state with a low affinity for folded proteins. Little is known about the folding assistance given to cytoplasmic proteins. It is conceivable that the major function of the hsc70-like proteins is the guidance of their folding or even the protection of folded molecules from aggregation. Evidence for such a role is provided by Nguyen et al. (1989).

The marginal stability of folded proteins is the result of a delicate balance between opposing forces. This guarantees a high flexibility of the polypeptide chain, a prerequisite for the proper functioning of a protein, e.g., as a biocatalyst. In the present context it becomes evident that this marginal stability of folded structures is necessary for another vital property as well, i.e., the facile transient unfolding of a protein with a small input of energy to render or to keep it competent for transport or assembly.

As first pointed out by Bychkova et al. (1988), the incompletely folded state that is involved in protein translocation may resemble the "molten globular" state. This state was detected in the folding of several proteins at equilibrium as well as during refolding under native conditions. States such as the molten globule can be regarded as the "unfolded state" under native solvent conditions (as in cells). It is a globular state that is less compact than the native protein with a large amount of secondary and some tertiary structure. However, a number of important long-range interactions are missing and the flexibility of the polypeptide chain is high (Kuwajima, 1989). With respect to cellular protein folding and transport, two properties are important. (i) The difference in stability between the flexible intermediate and the native state is small. Therefore, a small perturbation, such as binding to the ribosome, to the signal-recognition particle, or to a binding protein, will be sufficient either to arrest a folding protein in that state or to convert a folded protein back to a molten globular conformation. This may be particularly easy for precursor proteins, which are additionally destabilized by the presence of the signal peptide. (ii) In addition to this thermodynamic aspect, the kinetic properties of the molten globule are very important. Unfolding of this partially folded intermediate is rapid; further folding to the native state, however, is a slow reaction. These kinetic properties ensure that a passenger protein can rapidly be further destabilized as soon as it leaves the cytoplasm and enters the translocation machinery. In addition, unwanted refolding immediately after dissociation from the binding protein is blocked, since the reaction to the native state is slow.

The last considerations can be described in more general terms. Unfolded molecules, molecules with local structures, and even intermediates with a fairly high amount of ordered structure can occur in very rapid pseudoequilibria at the beginning of folding. This ensemble of structures is separated from the completely folded state by a high activation barrier, which makes the final steps of folding slow (Segawa & Sugihara, 1984; Goldenberg & Creighton, 1985). So the ultimate function of binding proteins and chaperones would be 2-fold. First, they prevent the nascent or prefolded polypeptide chains

[2] The reader should keep in mind that the majority of in vitro folding experiments are carried out at low temperature, such as at 0–10 °C, where folding is slow, in order to resolve individual steps of the folding kinetics.

from crossing the barrier to the native state, e.g., by providing some of their binding energy to stabilize the partially folded state. Second, they protect these incompletely folded, flexible molecules from premature aggregation by binding at their surface. This binding could be to exposed hydrophobic patches. Alternatively, the chaperones might represent a class of "polypeptide binding proteins", which (similar to single-strand DNA binding proteins) recognize sequence-independent properties of their target molecules, i.e., the peptide backbone. They will not bind to completely folded proteins, because the peptide bonds of their backbone are buried in the interior of the molecule. Binding of BiP and hsc70 to various peptides has indeed been observed by Flynn et al. (1989). In summary, these considerations suggest that many of the emerging molecular features of protein folding and transport in the cell and the properties of the involved proteins can be rationalized in the light of the existing models and concepts that resulted from in vitro studies on the stability and folding of mature proteins.

References

Andria, G., & Taniuchi, H. (1978) *J. Biol. Chem. 253*, 2262–2270.

Andria, G., Taniuchi, H., & Cone, J. L. (1971) *J. Biol. Chem. 246*, 7421–7428.

Baechinger, H. P. (1987) *J. Biol. Chem. 262*, 17144–17148.

Baldwin, R. L. (1989) *Trends Biochem. Sci. 14*, 291–294.

Bergman, L. W., & Kuehl, W. M. (1979) *J. Biol. Chem. 254*, 8869–8876.

Bochkareva, E. S., Lissin, N. M., & Girshovich, A. S. (1988) *Nature 336*, 254–257.

Bole, D. G., Hendershot, L. M., & Kearney, J. F. (1986) *J. Cell Biol. 102*, 1558–1566.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Brems, D. N. (1988) *Biochemistry 27*, 4541–4546.

Brems, D. N., Plaisted, S. M., Havel, H. A., Kauffman, E. W., Stodola, J. D., Eaton, L. C., & White, R. D. (1985) *Biochemistry 24*, 7662–7668.

Brown, J. E., & Klee, W. A. (1971) *Biochemistry 10*, 470–476.

Bulleid, N. J., & Freedman, R. B. (1988) *Nature 335*, 649–651.

Bychkova, V. E., Pain, R. H., & Ptitsyn, O. B. (1988) *FEBS Lett. 238*, 231–234.

Chen, W. J., & Douglas, M. G. (1987) *Cell 49*, 651–658.

Cheng, M. J., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., & Horwich, A. L. (1989) *Nature 337*, 620–625.

Chirico, W. J., Waters, M. G., & Blobel, G. (1988) *Nature 332*, 805–809.

Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.

Creighton, T. E. (1983) *Biopolymers 22*, 49–58.

Creighton, T. E., Hillson, D. A., & Freedman, R. B. (1980) *J. Mol. Biol. 142*, 43–62.

Crooke, E., Guthrie, B., Lecker, S., Lill, R., & Wickner, W. (1988) *Cell 54*, 1003–1011.

Dalzoppo, D., Vita, C., & Fontana, A. (1985) *J. Mol. Biol. 182*, 331–340.

Davis, J. M., Boswell, B. A., & Baechinger, H. P. (1989) *J. Biol. Chem. 264*, 8956–8962.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A., & Schekman, R. (1988) *Nature 332*, 800–805.

Dolgikh, D. A., Kolomiets, A. P., Bolotina, I. A., & Ptitsyn, O. B. (1984) *FEBS Lett. 165*, 88–92.

Dyson, H. J., Rance, M., Houghton, R. A., Lerner, R. A., & Wright, P. E. (1988a) *J. Mol. Biol. 201*, 161–200.

Dyson, H. J., Rance, M., Houghton, R. A., Wright, P. E., & Lerner, R. A. (1988b) *J. Mol. Biol. 201*, 201–207.

Eilers, M., & Schatz, G. (1986) *Nature 322*, 228–232.

Ellis, R. J., & Hemmingsen, S. M. (1989) *Trends Biochem. Sci. 14*, 339–342.

Endo, T., & Schatz, G. (1988) *EMBO J. 7*, 1153–1158.

Endo, T., Eilers, M., & Schatz, G. (1989) *J. Biol. Chem. 264*, 2951–2956.

Fischer, G., Bang, H., & Mech, C. (1984) *Biomed. Biochim. Acta 43*, 1101–1111.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989a) *Nature 337*, 268–270.

Fischer, G., Berger, E., & Bang, H. (1989b) *FEBS Lett. 250*, 267–270.

Flynn, G. C., Chappell, T. G., & Rothman, J. E. (1989) *Science 245*, 385–390.

Freedman, R. B. (1984) *Trends Biochem. Sci. 9*, 438–441.

Freedman, R. B. (1987) *Nature 329*, 196–197.

Freedman, R. B. (1989) *Cell 57*, 1069–1072.

Garel, J.-R., & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3347–3351.

Geetha-Habib, M., Noiva, R., Kaplan, H. A., & Lennarz, W. J. (1988) *Cell 54*, 1053–1060.

Gething, M. J., McCammon, K., & Sambrook, J. (1986) *Cell 46*, 939–950.

Go, N. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 183–210.

Goldberger, R. F., Epstein, C. J., & Anfinsen, C. B. (1963) *J. Biol. Chem. 238*, 628–635.

Goldenberg, D. P., & King, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3403–3407.

Goldenberg, D. P., & Creighton, T. E. (1985) *Biopolymers 24*, 167–182.

Goloubinoff, P., Gatenby, A. A., & Lorimer, G. H. (1989) *Nature 337*, 44–47.

Gschwendt, M., Kittstein, W., & Marks, F. (1988) *Biochem. J. 256*, 1061.

Haber, E., & Anfinsen, C. B. (1961) *J. Biol. Chem. 236*, 422–424.

Harding, M. W., & Handschumacher, R. E. (1988) *Adv. Inflammation Res. 12*, 283–293.

Harmey, M. A., Hallermayer, G., Korb, H., & Neupert, W. (1977) *Eur. J. Biochem. 81*, 533–538.

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., & Ellis, J. (1988) *Nature 333*, 330–334.

Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A., & Copeland, C. S. (1989) *J. Cell Biol. 108*, 2117–2126.

Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.

Kassenbrock, C. K., Garcia, P. D., Walter, P., & Kelly, R. B. (1988) *Nature 333*, 90–93.

Kato, I., & Anfinsen, C. B. (1969) *J. Biol. Chem. 244*, 1004–1007.

Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990a) *Biochemistry* (in press).

Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990b) *Biochemistry* (in press).

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Kim, P. S., & Baldwin, R. L. (1984) *Nature 307*, 329–334.

Koivu, J., Mylyllä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K., & Kivirikko, K. I. (1987) *J. Biol. Chem. 262*, 6447–6449.

Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241–258.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.
Kuwajima, K., Mitani, M., & Sugai, S. (1989) *J. Mol. Biol. 206*, 547–561.
Lang, K. (1988) Thesis, Regensburg, West Germany.
Lang, K., & Schmid, F. X. (1990) *J. Mol. Biol.* (in press).
Lang, K., Schmid, F. X., & Fischer, G. (1987) *Nature 329*, 268–270.
Lin, L.-N., Hasumi, H., & Brandts, J. F. (1988) *Biochim. Biophys. Acta 956*, 256–266.
Marqusee, S., Robbins, V. H., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5286–5290.
Marston, F. A. O. (1986) *Biochem. J. 240*, 1–12.
Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20*, 784–792.
Munro, S., & Pelham, H. R. B. (1986) *Cell 46*, 291–300.
Munro, S., & Pelham, H. R. B. (1987) *Cell 48*, 899–907.
Nguyen, V. T., Morange, M., & Bensaude, O. (1989) *J. Biol. Chem. 264*, 10487–10492.
Nierhaus, K. N., & Dohme, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4713–4717.
Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. J., & Sambrook, J. (1989) *Cell 57*, 1223–1236.
Oas, T. G., & Kim, P. S. (1988) *Nature 336*, 42–48.
Ostermann, J., Horwich, A. L., Neupert, W., & Hartl, F.-U. (1989) *Nature 341*, 125–130.
Park, S., Lin, G., Topping, T. B., Cover, W. H., & Randall, L. L. (1988) *Science 239*, 1033–1035.
Pelham, H. R. B. (1986) *Cell 46*, 959–961.
Pelham, H. R. B. (1988) *Nature 332*, 776–777.
Pfanner, N., Tropschug, M., & Neupert, W. (1987) *Cell 49*, 815–823.
Pfanner, N., Hartl, F.-U., & Neupert, W. (1988) *Eur. J. Biochem. 175*, 205–212.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Privalov, P. L. (1982) *Adv. Protein Chem. 35*, 1–104.
Randall, L. L. (1983) *Cell 33*, 231–240.
Randall, L. L., & Hardy, S. J. S. (1986) *Cell 46*, 921–928.
Roder, H., Elöve, G. A., & Englander, S. W. (1988) *Nature 335*, 700–704.
Rose, M. D., Misra, L. M., & Vogel, J. P. (1989) *Cell 57*, 1211–1221.
Rothman, J. E., & Kornberg, R. D. (1986) *Nature 322*, 209–210.
Roy, H., Cannon, S., & Gilson, M. (1988) *Biochim. Biophys. Acta 957*, 323–334.
Saxena, V. D., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015–5023.
Schleyer, M., & Neupert, W. (1985) *Cell 43*, 339–350.
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764–4768.
Schmid, F. X., & Blaschek, H. (1981) *Eur. J. Biochem. 114*, 11–117.
Schneuwly, S., Shortridge, R. D., Laarrivee, D. C., Ono, T., Ozaki, M., & Pak, W. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5390–5394.
Segawa, S.-I., & Sugihara, M. (1984) *Biopolymers 23*, 2473–2488.
Sela, M., White, F. H., & Anfinsen, C. B. (1957) *Science 125*, 691–692.
Shieh, B.-H., Stamnes, M. A., Seavello, S., Harris, G. L., & Zuker, C. S. (1989) *Nature 338*, 67–70.
Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., & Baldwin, R. L. (1987) *Nature 326*, 563–567.
Siebendritt, R. (1989) Thesis, Regensburg, FRG.
States, D. J., Dobson, C. M., Karplus, M., & Creighton, T. E. (1984) *J. Mol. Biol. 174*, 411–418.
Takahashi, N., Hayano, T., & Suzuki, M. (1989) *Nature 337*, 473–475.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Taniuchi, H. (1970) *J. Biol. Chem. 245*, 5459–5468.
Tropschug, M., Nicholson, D. W., Hartl, F.-U., Köhler, H., Pfanner, N., Wachter, E., & Neupert, W. (1988) *J. Biol. Chem. 263*, 14433–14440.
Tsou, C. L. (1988) *Biochemistry 27*, 1809–1812.
Tsunenaga, M., Goto, Y., Kawata, Y., & Hamaguchi, K. (1987) *Biochemistry 26*, 6044–6051.
Udgaonkar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694–699.
Vaucheret, H., Signon, L., Le Bras, G., & Garel, J. R. (1987) *Biochemistry 26*, 2785–2790.
Venetianer, P., & Straub, F. B. (1963) *Biochim. Biophys. Acta 67*, 166–168.
Verner, K., & Schatz, G. (1988) *Science 241*, 1307–1313.
Vestweber, D., & Schatz, G. (1988) *J. Cell Biol. 107*, 2037–2043.
Waldinger, D., Eckerskorn, C., Lottspeich, F., & Cleve, H. (1988) *Biol. Chem. Hoppe-Seyler 369*, 1185–1189.
Watts, C., Wickner, W., & Zimmermann, R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2809–2813.
Wetlaufer, D. B. (1985) *Biopolymers 24*, 251–255.
Wetlaufer, D. B., & Ristow, S. (1973) *Annu. Rev. Biochem. 42*, 135–158.
Wickner, W. (1988) *Biochemistry 27*, 1081–1086.
Wierenga, R. K., & Hol, W. G. J. (1983) *Nature 302*, 842–844.
Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518–8523.
Wright, P. E., Dyson, H. J., & Lerner, R. A. (1988) *Biochemistry 27*, 7167–7175.
Zetina, C., & Goldberg, M. E. (1980) *J. Mol. Biol. 137*, 401–414.
Zimmermann, R., & Wickner, W. (1980) *J. Biol. Chem. 255*, 7973–7977.
Zimmermann, R., & Meyer, D. I. (1986) *Trends Biochem. Sci. 11*, 512–515.
Zimmermann, R., Sagstetter, M., Lewis, J. M., & Pelham, H. R. B. (1988) *EMBO J. 7*, 2875–2880.