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Regeneration of RNase A from the reduced protein: Models of regeneration pathways

(nucleation/folding/growth-type model/rearrangement-type model)

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ABSTRACT Two models of protein-folding pathways are proposed on the basis of equilibrium and kinetic data in the literature. One is a growth-type model—i.e., nucleation of the native-like structure occurs in the folding process, in the rate-limiting step(s), and subsequent folding around the nucleation sites proceeds smoothly to form the native disulfide bonds and conformation. The other is a rearrangement-type model—i.e., proper nucleation does not occur in the folding process; instead, non-native interactions play a significant role in the folding pathways and lead to metastable intermediate species. Such non-native interactions, including incorrect disulfide bonds and proline *cis–trans* isomerization, must be disrupted or rearranged to nucleate the native interactions [a process that is included in the rate-limiting step(s)] for the protein to fold. The rate-limiting steps in the pathways for regeneration of RNase A from the reduced protein are classified as growth- or rearrangement-type pathways. The growth-type pathway is the one accompanying the formation of an intramolecular disulfide bond in the rate-limiting step. The rearrangement-type pathway is the one accompanying the reshuffling or disruption of a disulfide bond in the rate-limiting step. The folding of other proteins, accompanying oxidation of the reduced form, and the folding of denatured proteins with intact disulfide bonds are discussed in terms of the growth- and rearrangement-type models.

On the basis of experimental and theoretical studies, three different models have been proposed for protein-folding pathways. In the first, the dominant near-neighbor interactions lead to the formation of nucleation sites among nearby residues in the amino acid sequence in a rate-limiting step(s) in the *initial* stages of folding; this is followed by a rapid growth phase in which other parts of the chain fold around the nucleation sites (1–19). The existence of ordered structures having some degree of native-like conformation in the so-called unfolded (reduced) form of bovine pancreatic RNase A is indicated by the presence of about 0.04% enzymatic activity (20) and about 6% native conformation [deduced from an immunochemical assay (21)]. Also, interactions in the intermediates lead to nonrandom pairing of half-cystine residues in the formation of disulfide bonds in RNase A (22), and the addition of guanidine hydrochloride, which destroys such nonrandomness, slows the regeneration reaction in RNase A (23).

In a second model, proposed by Creighton (for review, see ref. 24), an incorrect disulfide bond is essential for regeneration [Cys-5—Cys-14 or Cys-5—Cys-38 in the case of bovine pancreatic trypsin inhibitor (BPTI)], and the rate-limiting step occurs at a *late* stage in the reshuffling from the essential incorrect disulfide bond to the correct one (Cys-5—Cys-55) (25), at which point the final native conformation is folded simultaneously (26). Moreover, the species that has two correct disulfide bonds (Cys-14—Cys-38 and Cys-30—Cys-51) cannot fold directly to the native structure; instead, its disulfide bonds must be reshuffled to include the above incorrect one in order that regeneration proceed (27).

In a third model, proposed by Brandts *et al.* (28), the folding pathway is assumed to involve *cis–trans* isomerization of proline residues. In kinetic studies of proteins that have intact disulfide bonds, slow and fast folding processes have been observed (28–33), and the slow phase has been attributed to proline isomerization. This assumption is supported by other investigations (34–40). Recent observations, however, suggest that proline isomerization occurs after most of the conformational folding has taken place (41–43). Also, residual structures were observed in the slowly folding intermediates (39, 44–46). Regardless of whether proline isomerization is involved in the folding pathway, the multiphase kinetics provide evidence that there are several unfolded forms in a denatured protein that can fold by different pathways (41–43).

We have previously characterized the regeneration pathways of RNase A in terms of their rate-limiting steps (47, 48). Here, we propose a general model for protein folding and discuss the observed pathways in the context of the above three models.

DISCUSSION

Slow Steps After the Rate-Limiting Step. In this paper, we define the rate-limiting step as the slowest step in the regeneration of the protein. However, the slow steps from the mostly regenerated protein to the fully regenerated protein, which may occur under certain conditions, are defined as slow steps *after* the rate-limiting step. This definition is adopted because we are interested in how the entire protein molecule is regenerated but not in the local changes of conformation in the passage from the mostly regenerated species to the fully regenerated protein. Here we discuss the factors or conditions that make the process from a mostly regenerated species to the fully regenerated protein a slow one and thereby allow the accumulation of certain *Intermediates**.¶ For example, if one of the *Intermediates** has a native-like conformation with an unreacted and buried cys-

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Abbreviations: GSH, reduced glutathione; GSSG, glutathione; BPTI, bovine pancreatic trypsin inhibitor; *l*S*m*G*n*H and *l*S*m*G*n*H*, intermediates prior to and after the rate-limiting steps, respectively, having *l* cystine residues, *m* mixed disulfide bonds between half-cystines and half-glutathione, and *n* free cysteine residues (if *l*, *m*, or *n* is 0, *l*S, *m*G, or *n*H is omitted—e.g., 8H or 4S).

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¶ The terms *Intermediates* and *Intermediates** represent the intermediates prior to and after, respectively, the rate-limiting steps in the regeneration pathway of RNase A. The fully reduced protein is included in the *Intermediates*. As shown elsewhere (49), there are 7,192 *Intermediates* on purely statistical grounds; undoubtedly, some of them involve steric hindrance and thus cannot exist.

teine residue, then the reactivity of this SH group would be low. This seems to occur in the refolding of the constant fragment of the immunoglobulin light chain, which contains only one disulfide bond, with the reduced form adopting a native-like conformation at pH 7.5 and 25°C (50). Glutathione (GSSG) oxidation of the cysteine residues of the reduced fragment was slower in the native-like conformation than in the conformation of material denatured by 8 M urea (51). From an analysis of their kinetic data, Goto and Hamaguchi (51) proposed that two factors affect the rate of oxidation. One is the concentration of GSSG, and the other is the stability of the reduced native-like conformation. Therefore, the reaction from the *Intermediates** to the native protein will be slow enough for *Intermediates** to accumulate if (*i*) the *Intermediates** take on a stable native-like conformation under the conditions of the regeneration solution, (*ii*) some cysteine residues are buried or have low reactivity in the native-like conformation, and (*iii*) the concentration of GSSG is low. Some examples of accumulated *Intermediates** are presented below.

In the regeneration of reduced RNase A (30 μM) by a low concentration (0.2 mM) of GSSG [in the absence of reduced glutathione (GSH)], an intermediate [designated by Creighton (52) as III_n], containing three correct disulfide bonds (Cys-26—Cys-84, Cys-58—Cys-110, and Cys-65—Cys-72) and two free cysteine residues (cysteines-40 and -95), accumulated (52). Since III_n has a relatively stable native-like conformation (53) and two cysteines are buried in the native conformation, the above three requirements are satisfied.

In the regeneration of reduced BPTI, an *Intermediate** (N^{SH}_{SH} in Creighton's notation) that has two correct disulfide bonds and a native-like conformation (26) accumulated only at low concentrations of GSSG (27).

In the air oxidation of reduced lysozyme, three different intermediates each having three native disulfide bonds but lacking the disulfide bond between cysteines-6 and -127, cysteines-64 and -80, and cysteines-76 and -94 accumulated (54). Since these species exhibited half of the enzymatic activity of the native protein against *Micrococcus lysodeikticus* cells, they may correspond to accumulated *Intermediates**.

According to Cook *et al.* (41), an intermediate, I_n, accumulated at low temperature in a kinetic study of the folding of RNase A (with its disulfide bonds intact) from the guanidine hydrochloride-denatured protein. This intermediate has a native-like conformation except for a local difference at proline-93 (41–43, 55). The local conformational change required to convert I_n to the native structure may be slowed by the rigidity of the native-like conformation of I_n at low temperature; hence, I_n would be an accumulated *Intermediate**.

Since the nucleation sites, or native-like conformations formed in the rate-limiting step, exist in the *Intermediates** but not in the *Intermediates*, comparison of the conformations of those *Intermediates* that participate in the rate-limiting step(s) with the conformations of the accumulated *Intermediates** would provide useful information about the regeneration pathways.

Growth-Type and Rearrangement-Type Pathways. On the basis of investigations of protein folding in this (47–49) and in other laboratories (discussed above), we propose that there are two types of folding pathways, which we designate as growth type and rearrangement type.

In one pathway, the rate-limiting steps involve the folding of the nucleation sites and the formation of correct disulfide bond(s); hence, interactions present in the native structure would play a dominant role in this pathway. [It should be noted that we make no statement as to whether the nucleation sites form before the disulfide bond(s) or vice versa.] Folding of the rest of the protein, and formation or reshuffling of the remaining disulfide bonds, would then occur rapidly (slowly, in some cases, as discussed above). We designate such a pathway as a growth-type one.

On the other hand, if the nucleation sites are not formed in the *Intermediates*, probably because their conformations cannot become native due to the existence of large potential barriers or strong non-native interactions, then the protein would make use of non-native interactions (including incorrect disulfide bonds) to regenerate the native structure. Thus, some specific non-native interactions or incorrect disulfide bonds would bring some residues (or half-cystines) close to each other, and the disruption or rearrangement of such non-native interactions or disulfide bonds to native ones (in the rate-limiting step) would be required to allow the protein to fold to its correct native conformation with correct disulfide bonds. We designate such a pathway as a rearrangement-type one.

Thus, we classify the regeneration pathway as a growth type or rearrangement type depending on whether native or non-native interactions (including disulfide bonds), respectively, play a significant role in the rate-limiting steps. The pathway that is adopted by any given protein would depend on the activation energy for the rate-limiting step, the nature of the *Intermediates*, and the solution conditions (which control the distribution and conformations of the *Intermediates*).

Regeneration of RNase A. Konishi *et al.* (47) reported six possible pathways (each of which constitutes a group of alternative rate-limiting steps) for the regeneration of RNase A (Table 1). We now discuss and classify them as growth- or rearrangement-type pathways. Since 3S1G1H does not seem to be involved in a rate-limiting step (47), we may exclude the reactions 3S1G1H + GSH → 2S2G2H*, 3S1G1H + GSH → 3S2H* + GSSG, 3S1G1H → 3S1G1H*, and 3S1G1H → 4S* + GSH as rate-limiting ones. We also exclude the reaction 3S2H + GSSG → 3S1G1H* + GSH as a rate-limiting step because it is unlikely that the binding of an extra bulky half-glutathione induces the folding of the native conformation. The rate-limiting steps that include the formation of a disulfide bond (which must be a correct one, if the step is a rate-limiting one)—namely, 1S1G5H → 2S4H* + GSH, 2S1G3H → 3S2H*

Table 1. Regeneration pathways of RNase A (47)

Pathway†	Rate-limiting reaction(s)‡
1S6H	1S6H → 1S6H*
1S1G5H	1S1G5H → 1S1G5H* 1S1G5H → 2S4H* + GSH 2S4H + GSH → 1S1G5H*
2S1G3H	2S1G3H → 2S1G3H* 2S1G3H → 3S2H* + GSH 3S2H + GSH → 2S1G3H*
2S2G2H	2S2G2H → 2S2G2H* 2S2G2H → 3S1G1H* + GSH 3S1G1H + GSH → 2S2G2H* 3S1G1H + GSH → 3S2H* + GSSG 3S2H + GSSG → 3S1G1H* + GSH
3S2H	3S2H → 3S2H*
3S1G1H	3S1G1H → 3S1G1H* 3S1G1H → 4S* + GSH 4S + GSH → 3S1G1H*

*l*S*m*G*n*H and *l*S*m*G*n*H*, intermediates prior to and after the rate-limiting step, respectively, having *l* cystine residues, *m* mixed disulfide bonds between half-cystines and half-glutathiones, and *n* free cysteine residues (if *l*, *m*, or *n* is 0, *l*S, *m*G, or *n*H is omitted—e.g., 8H or 4S).

† Each pathway is expressed in terms of the most reduced *Intermediate* involved in the rate-limiting steps.

‡ Possible rate-limiting reactions in each pathway.

+ GSH, and 2S2G2H → 3S1G1H* + GSH—can be classified as growth-type pathways. The rate-limiting steps accompanying the disruption or rearrangement of disulfide bonds—namely, 1S6H → 1S6H*, 1S1G5H → 1S1G5H*, 2S1G3H → 2S1G3H*, 2S2G2H → 2S2G2H*, 3S2H → 3S2H*, 2S4H + GSH → 1S1G5H*, 3S2H + GSH → 2S1G3H*, and 4S + GSH → 3S1G1H*—are classified as rearrangement-type pathways. The preference for any one of these pathways depends on the apparent rate constant of the rate-limiting step, on the relative stabilities of the *Intermediates* that take part in the rate-limiting step, and on the concentrations of GSH and GSSG.

Regeneration of Other Reduced Proteins. We may apply these ideas to the regeneration of other reduced proteins. The regeneration of reduced BPTI has been studied in detail by Creighton (24). Konishi *et al.* (48) examined the regeneration pathways of BPTI from the reduced protein in terms of the conformational chemical potential and pointed out that the conformation of reduced BPTI cannot fold along a growth-type pathway. Instead, the protein must adopt some unstable conformations (relative to that of the native protein) and incorrect disulfide bonds in the *Intermediates* and rearrange its incorrect disulfide bonds to the native ones in the rate-limiting step (24). Thus, the regeneration of BPTI is classified as a rearrangement type; a growth-type pathway does not seem to have been taken because conformational restrictions in reduced BPTI prevent the native interactions from occurring.

The regeneration of lysozyme from its reduced form by GSH and GSSG has been studied by Wetlaufer and co-workers (56, 57). According to these authors, the rapid oxidation of about four cysteine residues by 0.1 mM GSSG and 1 mM GSH probably brings reduced lysozyme to a pre-equilibrium state, and the subsequent slow oxidation and regain of enzymatic activity brings the *Intermediates* to fully regenerated lysozyme (57). Analysis of the disulfide bonds of the *Intermediates* indicated that formation of one or two disulfide bonds among cysteines-64, -76, -80, and -94 and a native disulfide bond between cysteines-30 and -115 occurs before the rate-limiting steps—i.e., in the *Intermediates* (57). Also, a native disulfide bond between cysteines-6 and -127 probably formed at or after the rate-limiting step (57). Thus, if lysozyme is regenerated from its reduced form through a growth-type pathway, the formation of a native disulfide bond (Cys-6—Cys-127, Cys-64—Cys-80, or Cys-76—Cys-94) might be the rate-limiting step. If a rearrangement-type pathway is followed, cleavage or reshuffling of incorrect disulfide bonds among cysteines-64, -76, -80, and -94 to the correct ones might be the rate-limiting step.

Folding from Denatured Proteins that Have Intact Disulfide Bonds. The kinetics of folding of proteins that have intact disulfide bonds involve fast and slow processes (after denaturation has been induced by introduction of guanidine hydrochloride or urea, by pH change, or by heating) and may also be discussed in terms of growth- and rearrangement-type pathways. In this analysis, we rely on the following observations under folding conditions for RNase A: (*i*) There is a slow interconversion between two unfolded species, D_F and D_S; (*ii*) there are both fast and slow folding processes; (*iii*) there appears to be some ordered structure in the slowly folding intermediates (28, 35, 44, 58, 59); and (*iv*) most of the conformational *folding* of the slow-folding species occurs before proline-93 isomerization at low temperature (41–43). We do not, however, make use of a model in which I_S is converted to I_F because no direct observation has been reported for the process from I_S to I_F (Fig. 1). Also, because a minor component in the slow-folding form was detected but not identified (41), it is not included in this analysis. To account for these observations, we make use of the relative conformational chemical potentials of a protein having

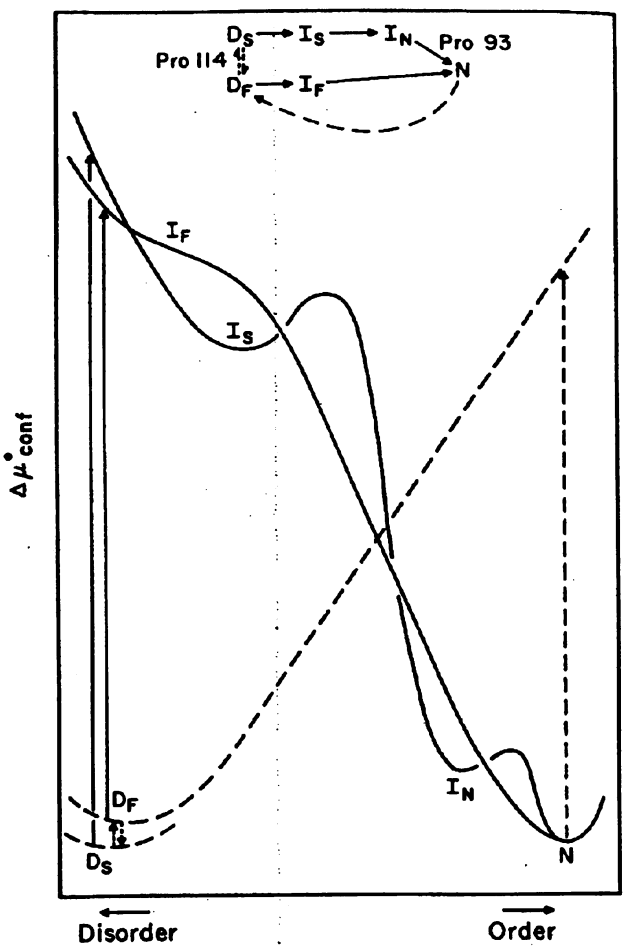

FIG. 1. The kinetics of folding of a protein that has intact disulfide bonds is represented schematically in terms of the conformational standard-state chemical potential ($\Delta\mu^\circ_{conf}$) as a function of the degree of order and disorder of the polypeptide chain. N, native conformation; D_F and D_S, fast- and slow-folding denatured species, respectively; I_F and I_S, fast- and slow-folding *Intermediates*, respectively; I_N, an accumulated *Intermediate** in the slow folding process at low temperature.

intact disulfide bonds, which are identical to the relative standard-state total chemical potentials because there is no chemical reaction during the folding–unfolding process. They are represented *schematically* as a function of the degree of order in Fig. 1, where the full lines represent chemical potentials under folding conditions and the dashed lines pertain to chemical potentials under unfolding conditions.

When a solution of the native protein (N) is rapidly jumped from a folding to a denaturing medium such as 6 M guanidine hydrochloride, the change of solvent increases its chemical potential, as shown by the dashed large vertical arrow and the protein unfolds to conformation D_F along the dashed lines in Fig. 1. Although each of four proline residues is fixed in either the *cis* or the *trans* conformation in the native protein, they might no longer be fixed in D_F and could be *distributed* between *cis* and *trans* conformations. Some proline isomerization may occur locally, but some may accompany significant conformational changes of the entire molecule or in the nucleation sites, producing species D_S slowly. Garel (38) suggested that the isomerization of proline-114, which was predicted to be in the nucleation site, residues 106–118 (16), occurred during *unfolding* in the slow conversion from D_F to D_S. Then, as indicated by the large solid vertical arrows in Fig. 1, the solution is rapidly jumped from unfolding to folding conditions, where D_F and D_S have higher chemical potentials than N. D_F and D_S undergo

very rapid conformational changes (undetectable by experiment) to intermediates I_F and I_S, respectively (33). Species I_F would be formed in a growth-type pathway after the formation of one or more nucleation sites in the folding process; the formation of these nucleation sites is the rate-limiting step. Then the rest of the polypeptide chain folds *smoothly* to N (in terms of conformational chemical potential) around the nucleation sites, as is observed experimentally as *fast* folding (60). Because D_S is produced by a significant conformational change from D_F, the nucleation sites would not form from D_S as they do from D_F. Thus, D_S is converted rapidly to the semistable intermediate I_S. Species I_S has a relatively compact structure with some ordered conformations (44, 46, 59). Some of the interactions in I_S, however, are non-native, and must be rearranged to native ones in the rate-limiting step, as indicated in Fig. 1, involving a chemical potential barrier on the rearrangement-type pathway. After the rate-limiting step, an *Intermediate**, I_N, according to Cook *et al.* (41), is accumulated at low temperature (see the first subsection of the *Discussion*). In the process from I_N to N, some local slow conformational changes, including the isomerization of proline-93, have been suggested (41–43, 55).

While proline isomerization may occur in the folding–unfolding process in proteins, it is, however, not the only possible *slow* process; conformational rearrangements among the fragments might also be slow. Thus, the more general treatment presented here includes proline *cis–trans* isomerization as a kind of native–non-native interaction and enables us to interpret the observed fast and slow folding processes in terms of growth- and rearrangement-type pathways, respectively.

To provide an *a priori* prediction of the type of folding pathway that any given protein would adopt, it would be necessary to compare the native and non-native interactions in the protein to determine which of these would dominate in the pathway, especially in the rate-limiting step.

CONCLUSION

We propose two types of regeneration models for protein folding. One is designated as a growth-type pathway in which the nucleation sites are folded in the rate-limiting step and other parts of the polypeptide chain fold around the nucleation sites. In this pathway, native interactions play a significant role. In the other, the rearrangement-type pathway, some non-native interactions are essential for regeneration and the disruption or rearrangement of these interactions to native ones is the rate-limiting step. The rate-limiting steps in the pathways for regeneration of RNase A from the reduced protein are classified as growth- or rearrangement-type pathways. The regeneration of other proteins can also be characterized similarly. For example, the regeneration of BPTI from its reduced form follows a rearrangement-type pathway, and the fast and slow folding processes of proteins that have intact disulfide bonds follow growth- and rearrangement-type pathways, respectively. The preference for either of these two types of pathways depends on the nature of the protein and the final solution conditions used for the regeneration. We conclude that proteins are regenerated through many pathways, which in general can be characterized as either growth or rearrangement type.

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