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Disulphide-coupled protein folding pathways

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SUMMARY

Protein folding pathways that involve disulphide bond formation can be determined in great detail. Those of bovine pancreatic trypsin inhibitor, α-lactalbumin and ribonucleases A and T_1 are compared and contrasted. In each species, whatever conformation favours one disulphide bond over another is stabilized to the same extent by the presence of that disulphide bond in the disulphide intermediates. The pathways differ markedly in the nature of that conformation: in bovine pancreatic trypsin inhibitor a crucial intermediate is partly folded, in α-lactalbumin the intermediates tend to adopt to varying extents the molten globule conformation, while in the ribonucleases the early disulphide intermediates are largely unfolded, and none predominate. In each case, however, the slowest step is formation of a disulphide bond that will be buried in a stable folded conformation; the most rapid step is formation of an accessible disulphide bond on the surface of a folded conformation. Quasi-native species with the native conformation, but incomplete disulphide bonds, can either increase or decrease the rate of further disulphide formation.

1. INTRODUCTION

Determining the mechanism by which a protein folds remains a very difficult and complex task. Small model proteins can fold on a timescale as short as 10^{-2} s, which is very much faster than would be possible by random searching of all conformations, but much slower than the time taken for unfolded polypeptides to interconvert conformations (approximately 10^{-9} s). Therefore, each molecule of an unfolded protein could be sampling up to 10^7 conformations during even very rapid folding. The situation is more complicated for experimental studies, which deal with populations of between 10^{14}–10^{17} protein molecules. Each of these unfolded molecules is likely to have a unique conformation at the start of folding, when the unfolded protein is transferred to refolding conditions, so it is possible that up to 10^{24} conformations could be sampled during folding of all these molecules. Fortunately, the kinetics of folding are relatively simple (Creighton 1988, 1990), suggesting that many molecules adopt the same or similar conformations, following the same pathway, and that relatively few conformations are sampled during much of the timescale of folding. Nevertheless, the partly folded intermediates that are believed to produce rapid folding are difficult to detect, and even more difficult to characterize. In particular, the kinetic roles of the intermediates that are detected are uncertain in most cases, and those intermediates that are kinetically most important are probably not detectable because of their instability (Creighton 1994).

2. DISULPHIDE BONDS AS PROBES OF PROTEIN FOLDING

Many of the difficulties associated with determining protein folding pathways can be overcome if folding is coupled to disulphide bond formation (Creighton 1978, 1986). This is the only type of protein-stabilizing interaction that is susceptible to specific experimental control, due to its oxidation/reduction nature. Folding is coupled to disulphide formation if the folded conformation requires the correct disulphide bonds. The reduced protein is unfolded, and folding accompanies disulphide formation.

To study disulphide-coupled folding of a reduced protein, a disulphide reagent RSSR is added, with or without its reduced form RSH. The spontaneous thiol-disulphide exchange reaction between reagent and protein is monitored as a function of time by quenching the reaction, then separating and identifying the trapped protein species with different disulphide bonds (both those between cysteine residues of the protein and mixed disulphides with the reagent). The kinetics of the folding reaction are measured by varying the concentrations of both RSSR and RSH and by following both folding and unfolding. A plausible kinetic scheme must be able to account quantitatively for the rates of appearance and disappearance of all the protein species, in both directions and with all concentrations of RSSR and RSH. The rate constant that is most pertinent to protein folding is that of the intramolecular step in forming each disulphide bond, in which the mixed disulphide of the reagent with one cysteine residue of the protein is displaced by a second cysteine residue.

It is not sufficient to concentrate on just the most prominent intermediates: an intermediate can be

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crucial for a kinetic step, yet not accumulate to a significant quantity. Fortunately, the existence of such crucial but ephemeral intermediates can be detected by examining the effects of removing the various cysteine thiol groups (Creighton 1977*a*). It is necessary, therefore, to confirm the pathway by studying both the isolated intermediates and the effects of deleting the various cysteine thiol groups of the protein. Finally, the conformations of the trapped intermediates should explain why certain disulphide bonds are made and not others.

3. KNOWN DISULPHIDE FOLDING PATHWAYS

(*a*) *Bovine pancreatic trypsin inhibitor* (*BPTI*)

The most extensively characterized disulphide folding transition is that of BPTI (Creighton 1978, 1990, 1992*c*), which has been conserved during evolution (Hollecker & Creighton 1983) and is summarized in figure 1*a*. The kinetic pathway was elucidated by normal kinetic analysis of both unfolding and refolding (Creighton & Goldenberg 1984) and confirmed by removing the various cysteine thiol groups (Creighton 1977*a*, 1978; Kosen *et al.* 1992; Darby & Creighton 1993; Darby *et al.* 1993, 1995). The pathway and the rate constants for all the steps account quantitatively for the rates of appearance and disappearance of all the disulphide species during both unfolding and refolding over a range of redox conditions. The conformational basis of the pathway is now largely understood, because the three-dimensional structures of all the most important intermediates have been characterized in detail (Kosen *et al.* 1983; States *et al.* 1987; Darby *et al.* 1991, 1992; van Mierlo *et al.* 1991*a*, *b*, 1992, 1993, 1994; Hurle *et al.* 1992; Kemmink & Creighton 1993). These results are incompatible with revisions of the pathway proposed by Weissman & Kim (1991); their results were not inconsistent with any of the important aspects of the original pathway (Goldenberg & Creighton 1984), but largely confirmed it (e.g. Weissman & Kim 1992) and do not require its revision or reinterpretation in any way (Creighton 1992*a*, *b*).

Certain intermediates containing solely native disulphide bonds accumulate primarily because they tend to adopt the native conformation; they are indicated by the subscript N. The folded conformation of native BPTI is so stable that it is still populated when any one of the native disulphide bonds is missing. It is remarkable that in these structures essentially all of the residues adopt their native conformations, indicating that the native conformation is a single cooperative structure and that it is only stabilized, not specified, by the disulphide bonds. The fully folded native conformation is still detectable with only the 5–55 disulphide bond (van Mierlo *et al.* 1991*b*), which stabilizes the native conformation most (Creighton & Goldenberg 1984). The occurrence of these quasi-native states is decreased by studying disulphide formation at relatively high pH, where it is more closely coupled to folding because the thiol groups tend to ionize, decreasing their tendency to be buried in folded structures.

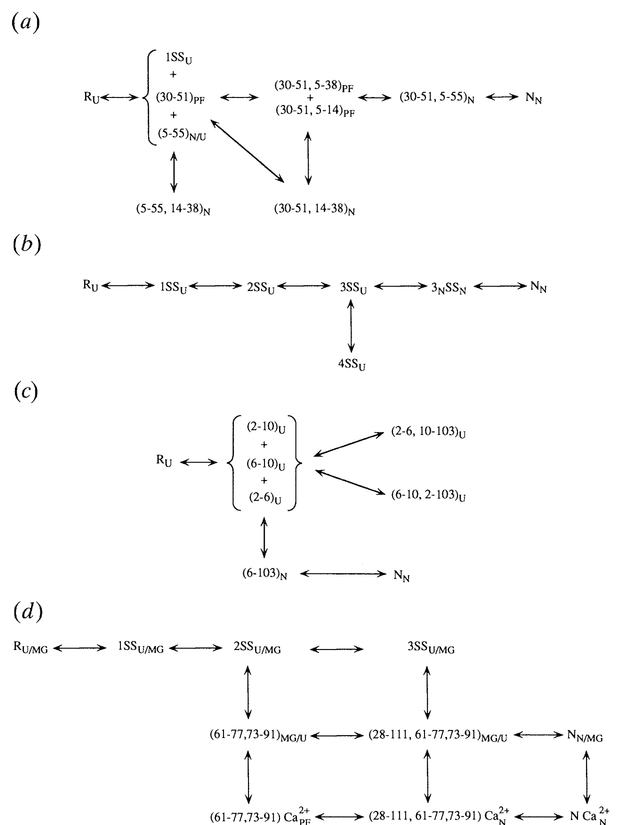

Figure 1. The disulphide folding pathways of: (*a*) BPTI; (*b*) RNase A; (*c*) RNase T_1 and; (*d*) α-LA. In each case, R is the fully reduced protein. Intermediates with specific disulphide bonds are indicated by the residue numbers of the cysteine residues paired. Mixtures of many isomers with different disulphide bonds (which are usually interconverting by intramolecular disulphide rearrangements when thiol groups are also present) are depicted by the number present, i.e. 1SS, 2SS, 3SS and 4SS. Arrows to and from such species represent the creation, destruction or rearrangement of many different disulphide bonds. The designation 3_NSS indicates that all the disulphide bonds are native-like. N is the protein with all the native disulphide bonds: (30–51,14–38,5–55) in BPTI; (26–84,40–95,58–110,65–72) in RNase A; (2–10,6–103) in RNase T_1 and; (6–120,28–111,61–77,73–91) in α-LA. The arrows indicate the most important steps, in kinetic terms, and can involve either disulphide creation, destruction or rearrangement. The predominant conformations of the various species are indicated by the subscripts U (unfolded), MG (molten globule), PF (partly folded), and N (native-like). Where two or more conformations are present in equilibrium, they are separated by /. The brackets in (*a*) and (*c*) indicate that the one-disulphide intermediates included are in equilibrium; the '+' between two or more species indicates that they have the same kinetic roles. Taken in part from Creighton *et al.* (1995); used with permission.

(i) *Forming the first disulphide bonds*

Reduced BPTI is largely unfolded (Goldenberg & Zhang 1993), although there are local elements of non-random conformation that have small effects on the folding process (Kemmink & Creighton 1993). Being unfolded, the protein forms first disulphide bonds between any of the 15 possible pairs of the six cysteine residues on an almost random basis, varying only approximately by the distance between the pair of residues (Darby & Creighton 1993). Formation of the

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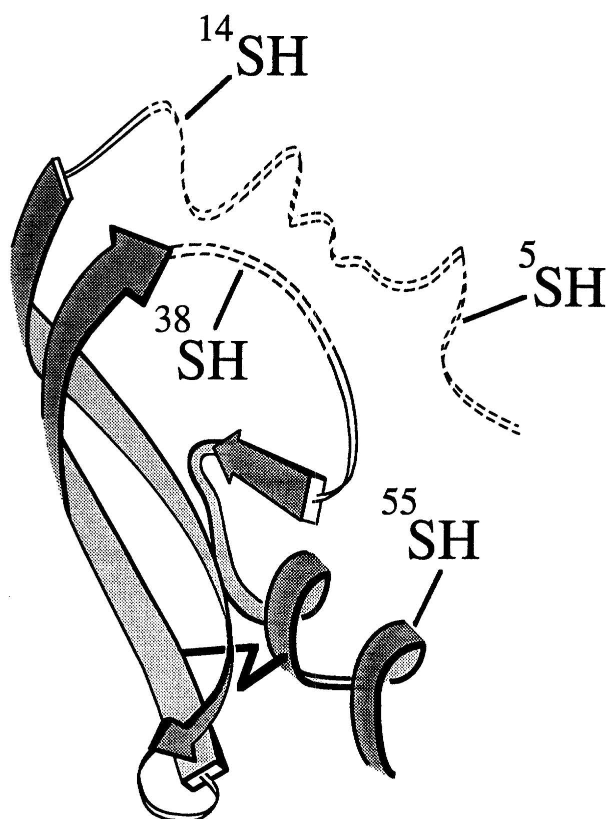

Figure 2. The partly folded conformation of intermediate (30–51) of the BPTI disulphide folding pathway. The portion of the polypeptide chain that is folded into a stable, native-like conformation is indicated in solid lines. The solid cross link is the 30–51 disulphide bond; it is linking the major α-helix of the protein to the β-sheet, which comprise the major hydrophobic core of the native conformation. The portions indicated by broken lines are unfolded or very flexible. The positions of the free cysteine residues are indicated by 'SH'. Taken from van Mierlo *et al.* (1993); used with permission.

three native disulphide bonds directly accounts for only about 5% of the total observed rate.

The resulting one-disulphide intermediates are in relatively rapid equilibrium by intramolecular thiol-disulphide interchange and accumulate according to their relative free energies. They are largely unfolded (Darby *et al.* 1992), except for the quasi-native $(5–55)_N$ and the partly folded (30–51). Intermediate $(5–55)_N$ accumulates to substantial levels at neutral pH, but not at pH 8.7, because the thiol groups of Cys30 and Cys51 are buried in its quasi-native conformation. The kinetically crucial intermediate (30–51) predominates at both pH values and has a partly folded conformation in which about two thirds of the residues have native-like conformations and the remainder are unfolded or very flexible (see figure 2).

(ii) *Forming the second disulphide bonds*

The tendency of intermediate $(5–55)_N$ to adopt a quasi-native conformation allows it to form the 14–38 disulphide bond rapidly. This is because these two cysteine residues are held in proximity on the surface of the protein, where they can readily react with disulphide reagents and with each other. The resulting $(5–55,14–38)_N$ has an even more stable quasi-native conformation (States *et al.* 1984; Eigenbrot *et al.* 1992) and completes refolding most readily by first reducing the 14–38 disulphide bond (Creighton & Goldenberg 1984).

The predominance of the (30–51) intermediate is important for increasing the rate of formation of the second disulphide bonds, as the observed rate is 12-fold lower in the presence of 8 M urea, where all the one-disulphide intermediates are unfolded and populated to similar extents (Creighton 1977*b*). The partly folded conformation of intermediate (30–51) accounts for its tendency to form any of the three possible disulphide bonds between the three cysteine residues that are in the unfolded or flexible regions: Cys5, Cys14 and Cys38 (see figure 2). Of the resulting two-disulphide intermediates, (30–51,5–14), (30–51,5–38) and $(30–51,14–38)_N$, the first two have non-native second disulphide bonds and conformations like that of (30–51), but with the flexible parts of the polypeptide chain tethered by the second disulphide bond (van Mierlo *et al.* 1994). The third intermediate has a quasi-native conformation of virtually all the residues, even in the absence of the 5–55 disulphide bond linking the ends of the polypeptide chain (van Mierlo *et al.* 1991*a*).

These three two-disulphide intermediates are normally in relatively rapid equilibrium by intramolecular disulphide rearrangements. They do not readily form third disulphide bonds, and they complete refolding most rapidly under usual experimental conditions by rearranging to the native-like N^{SH}_{SH}. This is the slowest intramolecular transition on the normal pathway, with an apparent half-time of about 140 s. It occurs directly, in a single step and at similar rates in both (30–51,5–14) and (30–51,5–38). Intermediate $(30–51,14–38)_N$ probably rearranges via these particular non-native disulphide intermediates, although the more rapid equilibration between these intermediates precludes direct demonstration of this; it also rearranges to $(5–55,14–38)_N$ (Creighton & Goldenberg 1984).

The importance of the pathway via the non-native intermediates is demonstrated by the rapid rates of folding to N^{SH}_{SH} in the absence of just Cys14 or Cys38. This had been demonstrated with chemically blocked thiol groups (Creighton 1977*a*), but virtually identical results have been obtained with replacement of each of the cysteine residues by serines (Darby *et al.* 1995). One folds through (30–51,5–14), the other through (30–51, 5–38). The rate through this pathway is the same as that observed with normal BPTI. These non-native two-disulphide intermediates are the most productive intermediates preceding N^{SH}_{SH}; in their absence, as when both Cys14 and Cys38 thiol groups are absent, folding is slowed remarkably (Creighton 1977*a*; Goldenberg 1988). In contrast, the absence of the quasi-native $(5–55,14–38)_N$ and $(30–51,14–38)_N$ does not slow the folding process, indicating that they are not productive intermediates. They accumulate to substantial levels during folding of normal BPTI because of their relative stabilities and because they are blocked in forming the final native disulphide bonds.

Forming the 5–55 or 30–51 disulphide bonds is slow when the stable native conformation will result; this is most evident in (30–51), $(30–51,14–38)_N$, and (5–55,

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14–38)$_N$. It does not result from inaccessibility of the thiol groups of the relevant intermediates (Creighton 1981). The reason for the kinetic blockage is apparent upon considering the reverse process, reduction of these disulphide bonds (Creighton 1978). Both the 30–51 and 5–55 disulphide bonds are buried in native BPTI and in N_{SH}^{SH}, and consequently both are reduced directly only very slowly; the transition states for both steps have very high free energies. All the steps in the BPTI pathway are observed to exhibit microscopic reversibility, so the same transition state is encountered in both directions for each step. Therefore, forming the 30–51 or 5–55 disulphide bonds last, to generate three-disulphide native BPTI or N_{SH}^{SH}, will encounter the same high energy transition states, and each step will be correspondingly slow. Consequently, instead of forming the 5–55 disulphide intermediate directly, (30–51) much more rapidly forms the 5–14, 5–38 and 14–38 disulphide bonds and intermediate (30–51,14–38)$_N$ tends to rearrange intramolecularly to N_{SH}^{SH}. A disulphide bond will always be formed slowly if, once formed, it is buried in a stable folded conformation. This is a general phenomenon and is observed in the other protein disulphide folding transitions that have been studied (see below).

The disulphide rearrangements occur in BPTI because the energetic barrier to forming the disulphide bonds that will be buried is overcome in a solely intramolecular transition, rather than in a bimolecular reaction involving a disulphide reagent to form the disulphide bond directly. In fact, the free energy barriers to the intra- and intermolecular reactions are similar in magnitude under normal conditions, suggesting that the same conformational transitions are occurring in each, and that both are likely to involve at least some unfolding of the conformations present (Creighton 1978; Mendoza *et al.* 1994). The rearrangement pathway is not a consequence of the occurrence of the quasi-native species, as it predominates even when they are not populated (Zhang & Goldenberg 1993).

(iii) *Forming the third disulphide bond*

The native-like (30–51,5–55)$_N$, or N_{SH}^{SH}, produced by the disulphide rearrangements has the Cys14 and Cys38 thiol groups in proximity and on the surface of the protein. Consequently, it can rapidly form the third, 14–38 disulphide bond, to complete refolding.

Ribonuclease A (*RNase A*) RNase A is the classic subject of protein folding (Anfinsen 1973). Nevertheless, its disulphide folding pathway (see Figure 1*b*) has been elucidated only in outline, due to its complexity (Creighton 1977*c*, 1979; Wearne & Creighton 1988; Talluri *et al.* 1994). Disulphide formation between the eight cysteine residues of reduced RNase A is initially random, as in BPTI, but none of the disulphide bonds substantially stabilize non-random conformations of the polypeptide chain. The initial intermediates are largely unfolded, none predominate, and further disulphide formation remains statistical in nature. The conformational tendencies of the RNase A polypeptide chain appear to be particularly weak; as a consequence, the disulphide intermediates are unstable. These intermediates become increasingly unstable with further disulphide formation, which tends to be slowed, rather than increased, by any disulphide bonds present.

Formation of correctly refolded RNase A is relatively slow. The rate-limiting intramolecular step is formation of the fourth disulphide bond in one or more intermediates with three native disulphide bonds, which undoubtedly adopt the native-like conformation. Two such quasi-native species have been identified: one lacks the 40–95 disulphide bond (Creighton 1980), the other the 65–72 disulphide bond (Talluri *et al.* 1994). The former appeared not to be a productive intermediate, whereas the latter may be (Talluri *et al.* 1994). All four disulphide bonds in native RNase A are buried and inaccessible, and so making any one of them to form all those species with either three or four native disulphide bonds is slow. There is no equivalent of N_{SH}^{SH} of BPTI. Which particular disulphide bonds are reduced first in native RNase A, and formed last upon refolding, depends upon their detailed accessibilities and reactivities in the native conformation, and not folding *per se*.

Ribonuclease T_1 (*RNase T_1*) Reduced RNase T_1 is unfolded at low salt concentrations, but adopts a quasi-native conformation at high concentrations (Mücke & Schmid 1994). Under conditions where it is unfolded, reduced RNase T_1 forms disulphide bonds readily between any pair of the nearby Cys2, Cys6 and Cys10 and the protein remains unfolded (see figure 1*c*). Forming a disulphide bond between any of these residues and the much more distant Cys103 is much slower, as expected for a random polypeptide chain. Forming the native 6–103 disulphide bond is especially slow, as in this case the protein will adopt the native conformation, in which this disulphide bond will be buried. Instead of being formed directly, the 6–103 disulphide bond is formed most rapidly under the usual experimental conditions by intramolecular rearrangement of the other one-disulphide intermediates (Pace & Creighton 1986).

The (6–103)$_N$ intermediate is comparable to N_{SH}^{SH} of BPTI (see figure 1) because in its native-like conformation the Cys2 and Cys10 residues are proximate and accessible, so that they can readily form the second native disulphide bond and complete refolding.

α-Lactalbumin (*αLA*) αLA differs from the previous proteins in that it tends to adopt a molten globule type of conformation, and it binds specifically one Ca^{2+} ion (Kuwajima 1989). The molten globule conformation is stabilized only slightly by any disulphide bonds between its eight cysteine residues (Ewbank & Creighton 1991), so reduced αLA adopts this compact conformation about 30% of the time (Ikeguchi & Sugai 1989; Ewbank & Creighton 1993*b*). Otherwise, reduced αLA is unfolded, and it initially forms disulphide bonds randomly, which interconvert readily (see figure 1*d*). The compactness of the molten globule conformation increases the frequency of disulphide formation between cysteine residues distant in the poly-

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peptide chain (Ewbank & Creighton 1993*a*). None of the reduced and one-disulphide αLA species bind Ca^{2+} significantly, so the presence of the ion makes little difference at this stage.

At the two-disulphide stage, an intermediate with two native disulphide bonds (61–77,73–91) is populated significantly in the presence of high concentrations of Ca^{2+} because it can adopt a partly native-like conformation with the Ca^{2+}-binding site. The remainder of the polypeptide chain is disordered but compact, and can readily form non-native disulphide bonds between the four free cysteine residues.

In the absence of Ca^{2+}, the three-disulphide intermediates have nearly random disulphide bonds, although biased by the presence of the 80% molten globule conformation (Creighton & Ewbank 1994). One of these species has three native disulphide bonds (28–111,61–77,73–91) and can tightly bind Ca^{2+} when it adopts a quasi-native conformation (Kuwajima *et al.* 1990; Ewbank & Creighton 1993*a*, *b*). The Cys6 and Cys120 cysteine residues are in proximity and readily form the fourth disulphide bond, even though it is strained and super-reactive. The precise pathway followed by αLA in forming and breaking its four disulphide bonds depends upon the Ca^{2+} concentration.

4. LESSONS FROM THE DISULPHIDE FOLDING PATHWAYS

The protein folding process is accelerated if only a few productive intermediates predominate. This is illustrated most clearly with BPTI, as its rate of folding could be measured in 8 M urea, where initial intermediates are unfolded, but the final N_{SH}^{SH} and native protein are not; the rate was decreased 14-fold (Creighton 1977*b*). This effect is relatively modest because just crosslinking the polypeptide chain with disulphide bonds limits the conformational flexibility markedly; consequently, proteins such as RNase A can refold on a reasonable timescale without any productive intermediates predominating. However, completion of disulphide folding can be slowed if the conformations adopted by the intermediates are too stable. The BPTI pathway demonstrates the advantages of having an intermediate (30–51) with a conformation that is sufficiently stable to cause it to predominate, yet sufficiently flexible to permit it to complete folding (see figure 2). It is also advantageous to have a disulphide bond on the surface, such as 14–38 of BPTI, which can be formed rapidly in the final step, after its cysteine residues have participated in intramolecular disulphide rearrangements. *In vivo*, of course, all of these steps are catalysed (Creighton *et al.* 1994).

The disulphide intermediates demonstrate how a folded conformation is stabilized by the effect of multiple interactions stabilizing each other. Whatever conformation is stabilized by a disulphide bond in turn stabilizes that disulphide bond to the same extent (Creighton 1986). This fundamental relation is not specific to disulphide bonds but applies to all other interactions that stabilize folded conformations; it is probably the key to understanding protein folding and stability. A major challenge then, is to understand why the proteins described here adopt such different conformations in their disulphide intermediates.

Disulphide folding is influenced by the covalent nature of the disulphide bond, its strict geometric requirements, and its formation and breakage only by thiol-disulphide exchange – frequently with an external reagent – and this makes accessibility of thiols and disulphides to the solvent of great importance. These considerations are not so severe with other types of interactions, but they should also apply (although to a lesser extent) to hydrogen bonding: other things being equal, a protein hydrogen bond will be made and broken more rapidly by interchange, perhaps with the aqueous solvent, than in isolation (Creighton 1978).

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