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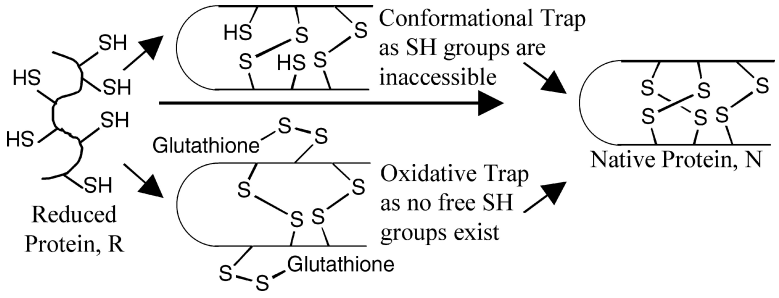

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Balancing Conformational and Oxidative Kinetic Traps during the Folding of Bovine Pancreatic Trypsin Inhibitor (BPTI) with Glutathione and Glutathione Disulfide

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The conditions used to fold proteins in vitro can have a dramatic effect on their folding rate and pathway. For disulfide-containing proteins, which includes most extracellular and almost all pharmaceutically relevant proteins, the composition of the redox buffer can alter in vitro folding rates by orders of magnitude. The redox buffer consists of a disulfide in combination with the corresponding thiol in appropriate proportions. Only two redox buffers have been extensively investigated, glutathione disulfide/glutathione (GSSG/GSH), which is found in vivo, and oxidized/reduced dithiothreitol (DTT^{ox}/DTT^{red}). Even though the folding pathways of several disulfide-containing proteins have been elucidated, the effects of GSSG/GSH redox buffer composition on these pathways has only been studied comprehensively for ribonuclease A (RNase A).[1−4] The seminal work of Scheraga et al. showed that the rate-determining steps varied with the GSH and GSSG concentrations and that they could be classified as either growth type, which involved the net formation of a native disulfide bond, or rearrangement type, which involved the disruption or rearrangement of disulfide bonds. We sought to further understand the effects of redox buffer composition, GSSG/GSH, on protein folding by investigating one of the best characterized systems, BPTI.[5,6] However, only a limited number of redox buffer compositions have been investigated with BPTI. Herein, we report the folding of BPTI using a wide range of redox buffer compositions, demonstrate that the folding rate can be significantly enhanced by the judicious choice of redox buffer composition, and highlight the importance of a growth-type folding pathway for BPTI under in-vivo-like redox conditions.

BPTI contains 58 amino acids of which six are cysteine residues.[5,6] Upon folding to the native structure the cysteine residues form three disulfide bonds between amino acids 5 and 55, 30 and 51, and 14 and 38, [5−55, 30−51, 14−38]. The folding pathway of BPTI was followed by observing the formation and breakage of disulfide bonds. The model proposed by Creighton et al. was based on studies performed at pH 8.7 with 0.125 mM GSSG and no GSH.[5] Subsequent work by Kim et al. demonstrated that only intermediates with native-disulfide bonds accumulated to significant levels at pH 8.7 or 7.3 with 0.15 mM GSSG.[6] Some ensuing work was performed with 0.5 mM GSSG and 2 mM GSH.[7,8] Under all these conditions N′, [30−51;14−38], and N*, [5−55;14−38], are kinetically stable intermediates that rearrange to N^{SH}, [30−51;5−55] intramolecularly prior to being oxidized by GSSG to native protein (N), Figure 1, a rearrangement-type pathway.[9]

The folding of reduced BPTI (0.030 mM)[10] at pH 7.3 was followed in the presence of various concentrations of GSSG (0.125, 0.5, 2, and 5 mM) and/or GSH (0, 1, 2, 5, 10, and 20 mM). The concentrations were selected to encompass those found in vivo. The folding mixture also contained 0.2 M KCl, 0.1 M bis-tris-propane hydrochloride and 1 mM EDTA and was kept at 25 °C under Ar.[10] Aliquots were removed at specific times between 5 min and 7 days, quenched with formic acid,[10] and analyzed by HPLC.[6] The pH was selected to be similar to previous work and close to physiological pH.[5−7]

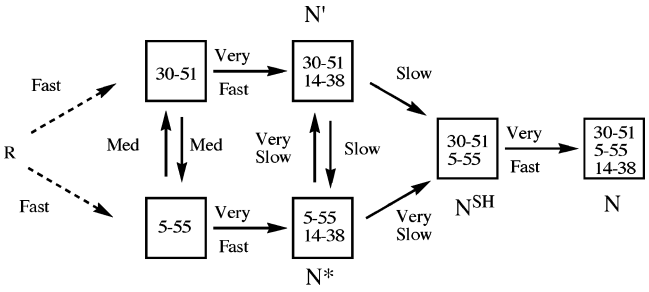

Figure 1. Folding of BPTI from ref 6, where R is reduced BPTI and the disulfide bonds are indicated inside the boxes. The relative rates at pH 7.3 are indicated; Med is medium.[6] R is oxidized to a mixture of one-disulfide intermediates which rapidly rearrange to [5−55] and [30−51].[6]

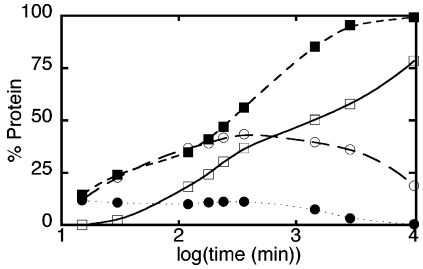

Figure 2. Percent of native protein (squares), and N* (circles), under traditional conditions, 0.125 mM GSSG and no GSH (open symbols) and 5 mM GSSG and 5 mM GSH (closed symbols). Typical absolute errors were 3% for N and 1.5% for N*.

At each of the four GSSG concentrations (0.125, 0.5, 2, and 5 mM), the GSH concentration that produced the most native protein was selected (0, 1, 2, and 5 mM GSH, respectively). At three time points, two for 0.5 mM and one for 0.125 mM GSSG, the selected GSH concentration did not meet this criterion within experimental error. BPTI was then folded multiple times in the presence of each of the four GSSG concentrations and their selected GSH concentrations. Of these four reactions the most native protein was produced at all time points with 5 mM GSSG and 5 mM GSH.

The folding rates obtained with 5 mM GSSG and 5 mM GSH (5/5) were 3−10 fold higher than those obtained using the traditional conditions, 0.125 mM GSSG and no GSH.[5] After 15 min with 5/5, the percentage of N was 10 ± 3%, while after 30 min, under traditional conditions it was only 2.4 ± 0.3%. With 5/5 it took a day to produce 83% N while under traditional conditions after 7 d less than 80% N was obtained, Figure 2.

Ultimately, the folding of BPTI is governed by partitioning between productive and nonproductive routes. Historically, the productive route was via N′ and the only nonproductive route was via the stable kinetic trap N*. The intermediate N* is a conforma-

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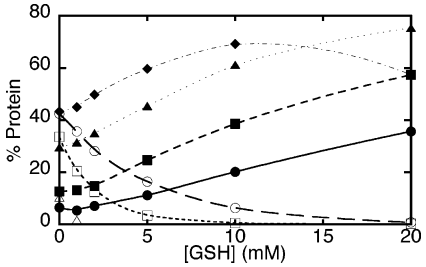
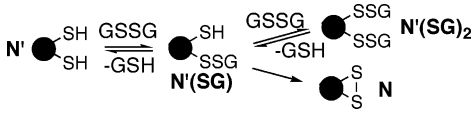


Figure 3. Concentration of N* (closed symbols) and N′$(SG)_2$ (open symbols) at 6 h for 0.125 (diamonds), 0.5 (triangles), 2 (squares), and 5 (circles) mM GSSG. Typical absolute error was 1.5%. The 0.125 mM GSSG and 20 mM GSH point is low because of abnormally slow initial folding under these conditions, 71% N* after 24 h.

Scheme 1. Reactions of N′(SG)[9]



tional kinetic trap since both remaining thiol groups are buried in the protein rendering them unreactive.[5,11] To improve the folding of BPTI, it would be advantageous to decrease the kinetic stability of N* and to produce as little N* as possible. The kinetic stability of N* is decreased by increasing both GSH and GSSG. At 0.125 mM GSSG, increasing the GSH concentration from 0 to 20 mM decreased the kinetic stability of N* slightly, as the reduction rate was increased (half-life from 5 to 3 d).[5] At 5 mM GSSG and all GSH concentrations, <1% N* was observed after a week and the half-life was <1 d, indicating that higher GSSG concentrations also destabilize N*. N* is known to react slowly with alkylating reagents but under traditional conditions reaction of N* with GSSG to transiently form a mixed disulfide was not proposed.[5] The accumulated amount of N* is affected by both GSH and GSSG. The conversion of N′ to the thermodynamically more stable N* is enhanced by high GSH concentrations just as the reverse reaction is, so the amount of N* produced increases with GSH concentration.[5] At a given GSH concentration, the more GSSG present, the less N* produced. Thus, higher GSSG concentrations decrease the kinetic stability of N* and also result in less N* being produced, Figure 3.

Another stable kinetic trap is the doubly mixed disulfide, N′-$(SG)_2$, an oxidative trap. N′$(SG)_2$ is N′ with two mixed disulfide bonds between the protein and glutathione and no free thiols. It thus needs to be reduced before it can react further. N′$(SG)_2$ accumulates at 0.5 mM GSSG and 2 mM GSH but not under traditional conditions. After 2 h, N′$(SG)_2$ forms a preequilibrium mixture with N′(SG), which is N′ with a free thiol group, and one mixed disulfide, $K_{eq} \approx 1.5$, Scheme 1. N′(SG) can react intramolecularly to directly form N, a growth-type pathway, with a rate constant greater than that of N′ to N.[9] Therefore, the kinetic stability of N′$(SG)_2$ will be controlled by the N′$(SG)_2$/N′(SG) ratio and the rate of N′(SG) disappearance. Under strongly oxidizing conditions, such as 5 mM GSSG and no GSH, the rate of conversion of N′-$(SG)_2$ to N will be slow as the N′$(SG)_2$/N′(SG) ratio is high. The amount of N′$(SG)_2$ produced increases with the GSSG/GSH ratio and the GSSG concentration, Figure 3. Thus, the kinetic stability and the accumulation of N′$(SG)_2$ are favored at high GSSG and low GSH concentrations.

Conditions that minimize both nonproductive folding pathways should lead to optimal folding. Since the kinetic stability and production of N* is decreased by high GSSG concentrations and low GSH concentrations but the kinetic stability and production of N′$(SG)_2$ is increased, a single set of conditions cannot be optimal for minimizing both pathways. A balance is achieved at 5 mM GSSG and 5 mM GSH (5/5).

Our most efficient conditions for folding BPTI, 5 mM GSSG, and 5 mM GSH are similar to those found in vivo. The ratio of GSH to GSSG found inside the endoplasmic reticulum (ER) is reported to be between 3:1 and 1:1,[12] but this does not include mixed disulfides between GSH and proteins (approximately 3:1:5 GSH/GSSG/protein mixed disulfides; total [glutathione] in all forms 10 mM).[13] Folding BPTI with 5 mM GSSG and 5 mM GSH also results in the presence of significant amounts of protein mixed disulfides, as is the case in vivo.

The results herein suggest that not only intramolecular rearrangements but also oxidation of N* and N′ to mixed disulfides and subsequent conversion to native protein, a growth-type pathway, is important for the efficient folding of BPTI. More generally, the balance between protein mixed disulfides (oxidative) and conformational kinetic traps may prove to be broadly applicable to the folding of disulfide-containing proteins.

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Supporting Information Available: Assay procedures and sample data. This material is available free of charge via the Internet at http://pubs.acs.org.

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