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Effects of redox buffer properties on the folding of a disulfide-containing protein: dependence upon pH, thiol pK_a, and thiol concentration

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Abstract

Aliphatic thiols are effective as redox buffers for folding non-native disulfide-containing proteins into their native state at high pH values (8.0–8.5) but not at neutral pH values (6–7.5). In developing more efficient and flexible redox buffers, a series of aromatic thiols was analyzed for its ability to fold scrambled ribonuclease A (sRNase A). At equivalent pH values, the aromatic thiols folded sRNase A 10–23 times faster at pH 6.0, 7–12 times faster at pH 7.0, and 5–8 times faster at pH 7.7 than the standard aliphatic thiol glutathione. Similar correlations between thiol pK_a values and folding rates at each pH value suggest that the apparent folding rate constants (k^{app}) are a function of the redox buffer properties (pH, thiol pK_a and [RSH]). Fitting the observed data to a three-variable model ($\log k^{app} = -4.216(\pm 0.030) + 0.5816(\pm 0.0036)\text{pH} - 0.233(\pm 0.004)\text{p}K_a + \log(1 - e^{-0.98\,(\pm 0.02)[\text{RSH}]})$) gave good statistics: $r^2 = 0.915$, $s = 0.10$.

Keywords: Protein folding; RNase A; Aromatic thiol; Redox buffer; pH

1. Introduction

Disulfide bonds provide stability to the tertiary structure of many extracellular proteins and almost all protein-based pharmaceuticals. Typically, native disulfide bonds are required for native structure and activity. The production of disulfide-containing proteins is undertaken using several different strategies including recombinant and synthetic methods (Lilie et al., 1998). The overexpression of recombinant disulfide-containing proteins often results in the formation of protein aggregates known as inclusion bodies (Guise et al., 1996; De Bernardez Clark, 1998). To obtain native protein, inclusion bodies need to be resolublized before undergoing in vitro protein folding to form native protein (De Bernardez Clark, 2001). For many

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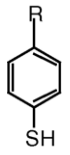
synthetic preparations of disulfide-containing proteins, the penultimate step is the deprotection of the cysteine residues, and the final step is the in vitro protein folding to obtain native structure (Moroder et al., 1996).

Commonly, the slow step in the in vitro folding of disulfide-containing proteins is the formation of native disulfide bonds (Creighton et al., 1995). Disulfide bonds rearrange into their native state via thiol–disulfide interchange reactions (Creighton et al., 1995). Statistically, proteins with more than one disulfide bond have many possible combinations of disulfide bonds, only one of which corresponds to native protein. The breaking of non-native disulfide bonds and the subsequent formation of native disulfide bonds are typically the rate-determining steps in protein folding.

In vitro, the formation of native disulfide bonds is typically facilitated by the addition of a small molecule thiol and small molecule disulfide, which is also called a redox buffer (Woycechowsky et al., 1999, 2003; Woycechowsky and Raines, 2003; Annis et al., 1998; Winter et al., 2002; Wedemeyer et al., 2000; Konishi et al., 1981; Gough et al., 2002, 2003). In some cases, in vivo catalysts, such as protein disulfide isomerase (PDI) (Kersteen and Raines, 2003; Winter et al., 2002; Robinson et al., 1994), are added to the redox buffer to improve the in vitro folding (Gilbert, 1997; Puig and Gilbert, 1994). However, PDI is not typically used due to its high cost and low catalytic activity.

A limited number of small molecule thiols have been investigated for their ability to fold disulfide-containing proteins. Traditionally, aliphatic thiols such as glutathione, β-mercaptoethanol, or dithiothreitol (DTT) have been used for folding disulfide-containing proteins (Konishi and Scheraga, 1980a; Rothwarf and Scheraga, 1993b). More recently, new aliphatic thiol redox buffers have been developed for increasing the overall yield of active protein, but they do not significantly increase the rate of protein folding (Woycechowsky et al., 1999, 2003; Woycechowsky and Raines, 2003; Annis et al., 1998). Aromatic thiols have been shown to significantly increase the folding rate of disulfide-containing proteins over those obtained using glutathione (Gough et al., 2002, 2003). Previously, thiols **1**–**5** (Scheme 1) were analyzed for their ability to fold scrambled RNase A at pH 6.0 (Gough et al., 2003). The redox buffer pH was chosen so that the effect of thiol pK_a on the apparent folding rate could be analyzed; two of the thiols had pK_a values



R	Thiol pK_a
1- CH_2COOH	6.6
2- CH_2OH	6.4
3- $COOH$	5.95
4- SO_3H	5.7
5- SO_2NHCH_2COOH	5.2

Scheme 1.

lower than redox buffer pH, two higher than, and one equal to the redox buffer pH. The results demonstrated that the concentration of protonated thiol (thiol in the SH form) was more important for optimizing the folding reactions than the total concentration of aromatic thiol (protonated thiol (SH) plus thiolate (S^-)). The optimal total thiol concentration for aromatic thiols **1**–**5** varied considerably, 2.5–11 mM, but the optimal concentration of protonated thiol varied minimally, 1.8–2.6 mM. Since RNase A folds by initially forming a pre-equilibrium mixture which then forms native protein via rate-determining steps (Narayan et al., 2000), it was proposed that the concentration of protonated thiol affects the composition of the pre-equilibrium mixture through an equilibrium process. The rate of the rate-determining steps then may be affected by the concentration of thiolate. For each aromatic thiol, the concentration of thiolate and protonated thiol are linked by a single thiol pK_a value; however, within the series of aromatic thiols, the thiol pK_a values vary thus allowing a distinction between protonated thiol and thiolate.

To fully depict and quantify the nature of folding RNase A with aromatic thiol based redox buffers, it is necessary to examine the impact that solution pH has upon folding rates; the consequences of changing the solution pH of the redox buffer has not been thoroughly investigated for any redox buffer. To ascertain the processes that pH influences, it was necessary to determine if the conclusions reached at pH 6.0 were valid over a wide range of pH values, including those most relevant for protein folding. Practically, it was important to establish an empirical understanding of the effect that aromatic thiols have on protein folding so that this technology could be applied to other disulfide-containing proteins. Herein, the effect of redox buffer pH on the folding of RNase A using aromatic thiols is evaluated. A group of aromatic thiols with different thiol pK_a values was analyzed for their ability to fold RNase A at pH 7.0 and 7.7. Based upon these results, a general equation for the folding of RNase A is proposed.