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Increased catalytic activity of protein disulfide isomerase using aromatic thiol based redox buffers

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Abstract—PDI is an enzyme that acts as a chaperone, shufflase, and oxidase during the folding of disulfide-containing proteins. The ability of aromatic thiols to increase the activity of PDI-catalyzed protein folding over that of the standard thiol glutathione (GSH) was measured. 4–Mercaptobenzoic acid (ArSH) increased the activity of PDI by a factor of three.

1. Introduction

Although the production of disulfide-containing proteins via recombinant means is routine, several problems still exist.[1] In many cases the proteins aggregate during in vivo folding to form inactive inclusion bodies instead of soluble, active protein.[2–5] The yield-limiting step in obtaining active protein from inclusion bodies is typically the in vitro formation of the correct disulfide bonds. Active protein is obtained by placing resolubilized inclusion bodies in the presence of a redox buffer. Redox buffers, which facilitate the formation of the correct disulfide bonds, typically consist of a small molecule thiol, a small molecule disulfide and, sometimes, denaturants. Unfortunately, in some cases the redox buffers alone can be ineffective.[1,3] To further aid in obtaining active protein, an enzyme, which increases the rate of protein folding is added to the redox buffer.[2,4–10] The most commonly used and best characterized enzyme[11–13] is protein disulfide isomerase (PDI).[2,4–10] However, PDI is expensive and has low catalytic activity.

PDI is a 57 kDa protein and a member of the thioredoxin superfamily of proteins.[14,15] PDI facilitates both in vivo and in vitro folding of disulfide-containing proteins.[14,16–25] The two active sites of PDI each consist of a Cys-Gly-His-Cys motif;[26] one cysteine thiol is solvent-exposed (N-terminal) and the other is buried (C-terminal).[27] The in vitro mechanism of PDI functions through an escape pathway (Fig. 1a): the N-terminal cysteine thiolate nucleophilically attacks a disulfide bond to form a mixed disulfide, then the C-terminal cysteine thiolate attacks the mixed disulfide bond thereby releasing the protein and leaving the active site of PDI in its (oxidized) disulfide form. Site-directed mutagenesis of the cysteine residues confirms that both cysteine residues are necessary for full catalytic activity.[19,28]

Thiols, as a component of the redox buffer, are known to affect the rates of thiol–disulfide interchange reactions involved in protein folding.[29–31] The redox buffer thiols can act as nucleophile, center thiol, or a leaving group (Fig. 1b).[29] Increasing the reactivity of these reactions can increase the overall rate of protein folding. Similarly, in the presence of PDI, the overall rate of protein folding can be increased due to the redox buffer interacting with PDI and the protein being folded.

In vitro protein folding with PDI is accomplished in the presence of a small molecule redox buffer, typically glutathione (a naturally occurring tri-peptide)–glutathione disulfide (GSH–GSSG). A limited number of other redox buffers have been investigated and were found to be less efficient. Optimum conditions for folding a protein in vitro in the presence and absence of PDI using GSH–GSSG were determined by Gilbert and found to be the same.[32,33] GSH–GSSG is also the most

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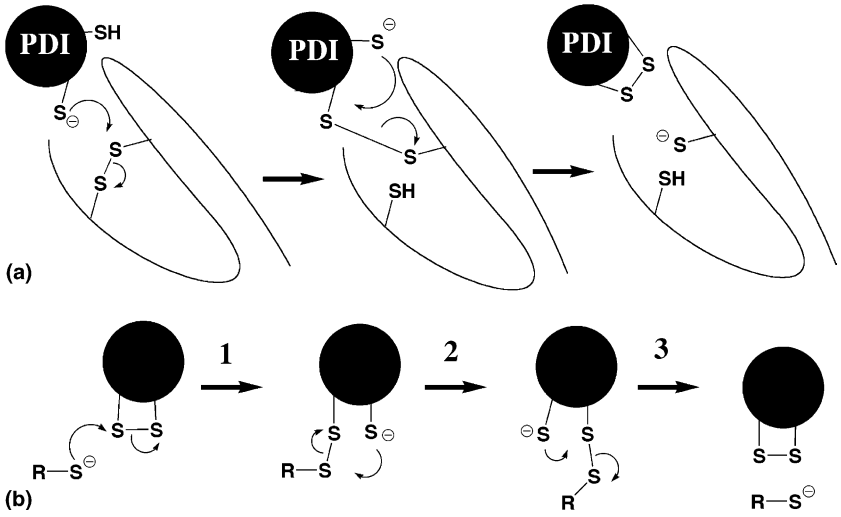


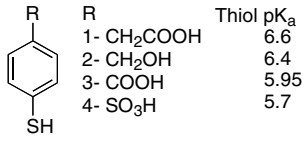
Figure 1. (a) PDI escape pathway as described in the text. (b) Three examples of protein-redox buffer thiol–disulfide interchange reactions, where the redox buffer thiol (R–S) is acting as a 1—nucleophile, 2—a center thiol, and 3—a leaving group.

commonly used redox buffer for folding disulfide-containing proteins in the absence of PDI. Recently, the initial folding velocity of ribonuclease A (RNase A) in the presence of GSH–GSSG without PDI was significantly improved by replacing GSH with an aromatic thiol.[29,30] Thus, aromatic thiols might increase the velocity of protein folding in the presence of PDI. An increase in the activity of PDI would be useful for the production of recombinant disulfide-containing proteins. The ability of aromatic thiols to increase the activity of PDI is investigated herein.

2. Methods and results

A series of *p*-substituted aromatic thiols **1**–**4** (Scheme 1) was tested for its ability to enhance the catalytic activity of protein disulfide isomerase (PDI). The thiols were selected because they increased the velocity of folding scrambled RNase A to native RNase A in the absence of PDI relative to glutathione (GSH).[29,30] Scrambled RNase A (sRNase A), the standard substrate for measuring PDI activity, is fully oxidized RNase A with a relatively random distribution of non-native disulfide bonds. The velocity of sRNase A folding at pH 7.0 was measured at various concentrations of PDI in the presence of each of the thiols.

Protein folding reactions were initiated by simultaneous addition of a concentrated sRNase A solution and PDI (PDI rapidly equilibrates with the redox buffer in situ;[17]) they were performed on a scale of 500 μL at 25 °C, at pH 7.0 (Bis–Tris–propane/AcOH buffer, 10^{-3} M EDTA). All folding reactions contained 25 μM protein, 1 mM EDTA, 0.5–1.5 μM PDI, varying concentrations of each of the four thiols or GSH, and 0.2 mM disulfide (GSSG, or aromatic disulfide). Protein folding was followed by measuring the enzymatic activity using the discontinuous assay developed by Konishi and Scheraga,[34,35] which was modified as previously described.[29,30] The concentration of sRNase A, 0.3 mg/mL, is typical for in vitro protein folding experiments and allows comparison with previously collected data.[1–3] Gilbert observed that the optimal redox buffer conditions (concentrations of GSH and GSSG) for folding RNase A with and without PDI entailed using 0.2 mM glutathione disulfide (GSSG).[32] Optimal redox buffer conditions for folding RNase A using aromatic thiols, in the absence of PDI, are also at 0.2 mM disulfide (aromatic disulfide or GSSG).[29,30] Side-by-side comparison of the PDI-catalyzed folding of sRNase A, using thiol **1** and GSSG or the aromatic disulfide of **1**, revealed that no observable velocity enhancement is gained by using an aromatic disulfide. Therefore, the commercially available GSSG was used as the disulfide in all subsequent folding experiments.



Scheme 1. *para*–Substituted aromatic thiols **1**–**4**.

To maximize the initial folding velocity, V_0, of RNase A the thiol concentration was varied.[29,30] The optimal thiol concentration in the presence of 0.5 μM PDI was similar to that observed in the absence of PDI (Fig. 2a).[29,30] Paralleling experiments performed in the absence of PDI, velocities were measured side-by-side in the presence of 0.5 μM PDI under optimal conditions (Table 1).[29] A significant increase in the initial velocity was observed in the presence of PDI for thiols **1**–**3** and GSH. No significant increase in the initial velocity was observed for thiol **4**, and thus it was not investigated further.

For all the thiols, the optimal folding conditions occurred at approximately the same concentration of protonated thiol, $[RSH]_{prot}$, and not total thiol, (protonated thiol, RSH, plus thiolate, RS^-). Since the concen-