Rapid Reports

Catalysis of Oxidative Protein Folding by Small-Molecule Diselenides†

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ABSTRACT: The production of recombinant, disulfide-containing proteins often requires oxidative folding in vitro. Here, we show that diselenides, such as selenoglutathione, catalyze oxidative protein folding by O_2. Substantially lower concentrations of a redox buffer composed of selenoglutathione and the thiol form of glutathione can consequently be used to achieve the same rate and yield of folding as a standard glutathione redox buffer. Further, the low pK_a of selenols extends the pH range for folding by selenoglutathione to acidic conditions, where glutathione is inactive. Harnessing the catalytic power of diselenides may thus pave the way for more efficient oxidative protein folding.

Numerous pharmaceutically interesting proteins contain multiple disulfide bonds in unique native arrangements. However, production of recombinant proteins in bacteria often leads to insoluble aggregates consisting of relatively pure material with scrambled disulfide bonds. Such inclusion bodies need to be reduced, solubilized, and then oxidatively folded to produce a functional protein (*1, 2*). Redox buffers consisting of mixtures of oxidized and reduced glutathione (GSSG and GSH, respectively) are frequently used to facilitate correct disulfide bond formation during in vitro folding. GSSG acts as a stoichiometric thiol oxidant, and GSH catalyzes the rearrangement of disulfide bonds. Detailed empirical studies have shown that a distinct optimal concentration exists for both components of this redox pair (*3, 4*). These concentrations, usually 0.2 mM GSSG and 1 mM GSH, represent a large excess over protein in a typical folding reaction. Thiol-disulfide oxidoreductases, such as protein disulfide isomerase (PDI), DsbA, and glutaredoxin (*5, 6*), can improve folding rates when added to the standard redox buffer, but these enzymes are expensive, are not particularly efficient, and can be difficult to remove after folding. Consequently, there is a demand for novel, small-molecule reagents that improve the efficiency of the oxidative folding process.

We previously reported that selenoglutathione (GSeSeG), a diselenide bond-containing variant of glutathione, can serve as an oxidant during protein folding (Figure 1) (*7*). Stoichiometric concentrations of GSeSeG were shown to fold different proteins, at pH ≥8, either in the presence or in the absence of oxygen, and with small rate enhancements compared to GSSG. Here, we examine the effects of different redox buffer conditions on the rate and yield of GSeSeG-mediated folding.

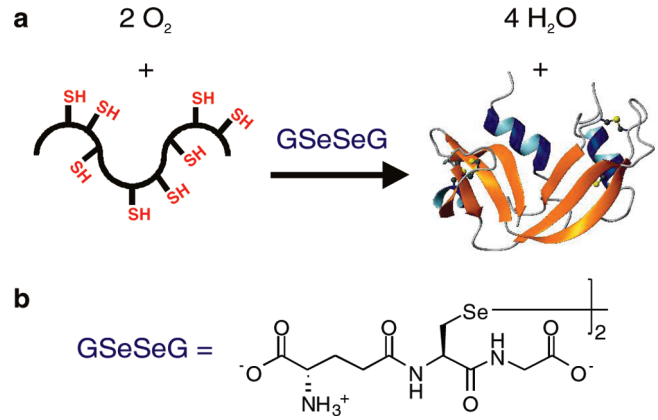

FIGURE 1: Oxidative folding of RNase A by selenoglutathione. (a) Reaction of reduced RNase A with O_2 catalyzed by selenoglutathione. (b) Structure of oxidized selenoglutathione.

As noted above, the optimal redox buffer for RNase A folding contains both oxidant and reductant. Using a discontinuous spectrophotometric assay based on the ability of native RNase A to hydrolyze cCMP (*4*), we find that GSeSeG can directly replace GSSG in the standard disulfide-containing mixture, giving a comparable yield and a slightly faster rate (see Figure S1 of the Supporting Information). Interestingly, the GSeSeG/GSH pair can still match the rate and yield of the best GSSG/GSH pair using 10-fold less redox buffer (Figure 2a), suggesting a broader optimum for the diselenide compared to the disulfide. Even lower concentrations of the GSeSeG/GSH pair are effective, although the rate of native RNase A formation decreases a few fold when the concentrations of the redox reagents are 1% of the best GSSG/GSH conditions (see Figure S2 of the Supporting Information).

The low concentrations of GSeSeG that suffice to fold RNase A indicate that the diselenide actually catalyzes protein oxidation. In fact, reduced RNase A can also be folded by substoichiometric amounts of GSeSeG in the presence of air and the absence of reductant. At a starting ratio of one diselenide to 20 protein thiols, quantitative yields of the native enzyme are obtained as judged by the RNase

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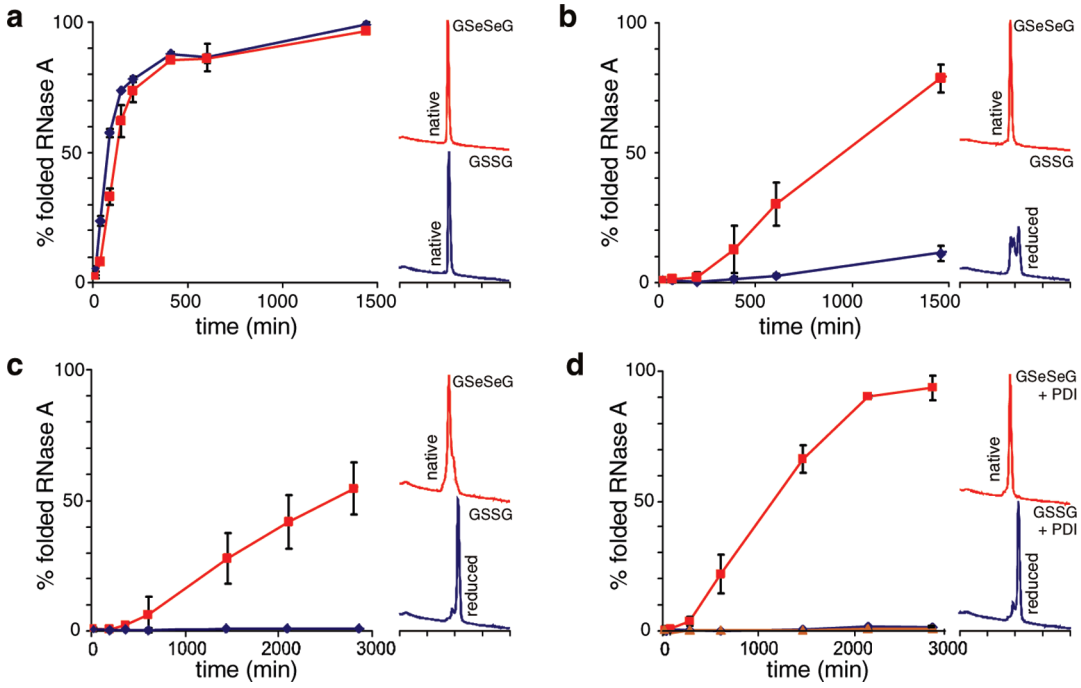

FIGURE 2: Kinetics of RNase A folding. The folding reaction was initiated by adding fully reduced RNase A (5 μM) which has eight cysteine residues. Aliquots were withdrawn at various time points and directly assayed for cCMP hydrolysis activity, which is specific to native RNase A containing four disulfide bonds. In each panel, time traces of folding at room temperature are shown at the left, and HPLC chromatograms corresponding to the end points of the reactions are at the right. (a) Comparison of redox buffers at pH 8.0: 0.2 mM GSSG and 1 mM GSH (blue) and 0.02 mM GSeSeG and 0.1 mM GSH (red). (b) Substoichiometric amounts of oxidant in the absence of a thiol reductant at pH 8.0: 2 μM GSSG (blue) and (red) GSeSeG 2 μM. The end point, at 2820 min (100 $\pm$ 1% yield), is not shown in the time trace. (c) Substoichiometric amounts of oxidant at pH 5.0: 2 μM GSSG (blue) and 2 μM GSeSeG (red). The end point, at 4080 min (75 $\pm$ 8% yield), is not shown in the time trace. (d) Influence of 100 nM PDI at pH 5.0: 2 μM GSSG (blue), 2 μM GSeSeG (red), and PDI alone (brown).

A activity assay and analytical HPLC (Figure 2b). In contrast, the same ratio of GSSG to protein thiols did not result in appreciable folding. Substantial, albeit slower, folding was seen at even lower concentrations of GSeSeG (see Figure S3 of the Supporting Information). These observations are in line with previous reports that small diselenides catalyze reactions such as air oxidation of GSH to GSSG (*8*), reduction of disulfides by $NaBH_4$ (*9*), and thiol-disulfide exchange between a small molecule and a protein (*10*).

Oxidative folding by atmospheric O_2 is normally slow and affords relatively small amounts of properly folded protein (*11*). GSSG has emerged as a (super)stoichiometric protein oxidant to provide a suitable pathway from the reduced to the native state. With excess GSSG, the mechanism of protein folding involves the rapid initial formation of protein disulfide bonds and their subsequent (rate-limiting) isomerization. At substoichiometric concentrations of GSSG, protein oxidation becomes rate-limiting, which is exacerbated by the inherently slow reaction of GSH with molecular oxygen (*3*). In contrast, a large excess of GSeSeG is not required for efficient folding because diselenides are more susceptible to nucleophlic attack by protein thiols than are disulfides (*12*), which makes GSeSeG a kinetically superior oxidant to GSSG. The catalytic behavior of GSeSeG arises from its rapid regeneration by molecular oxygen. As atmospheric O_2 is abundant and cheap, its use as the actual protein oxidant represents a significant benefit of GSeSeG-mediated folding.

The advantageous kinetic properties of GSeSeG extend to acidic pH, expanding the practical range of solution conditions for oxidative protein folding. At pH 5, protein thiols are fully protonated and therefore much less reactive than at pH 8. As a consequence, reduced proteins are often stored under acidic conditions to prevent their oxidation. Selenols have much lower pK_a values than thiols (e.g., 5.2 for selenocysteine vs 8.3 for cysteine). The greater reactivity of diselenides relative to disulfides and the higher rate of protein disulfide isomerization seen with selenium-containing compounds are both derived, at least in part, from this pK_a difference. Accordingly, aromatic thiols with depressed pK_a values have previously been shown to promote protein disulfide bond isomerization at pH 6 (*13*). As expected, we find that GSSG is completely unable to fold RNase A at pH 5. However, under the same conditions, GSeSeG provides a 75% yield of native RNase A in 68 h (Figure 2c).

While GSeSeG displays substantial oxidative folding activity for RNase A at pH 5, a fraction of the protein appears to be kinetically trapped. Combining PDI with GSeSeG increases the yield to 100%, showing that the isomerase can rescue misfolded species under these conditions (Figure 2d). PDI also enhances the folding rate, although no activity was seen for the isomerase either by itself or in combination with GSSG. Elucidating the mechanistic details of this dual-catalyst system will require further study, but it is worth noting that a cysteine in the PDI active site also possesses a depressed pK_a value (*14*), which may be the key to its function. The related oxidoreductase DsbA, which has an active-site cysteine with an extraordinarily low pK_a, has also been used for acidic oxidative protein folding in a glutathione redox buffer with varying degrees of success (*15, 16*). In our hands, substoichiometric amounts of both GSSG and PDI are unable to fold RNase A under acidic conditions, but

GSeSeG and PDI effectively work together, presumably because the diselenide promotes initial protein oxidation.

The catalytic function of GSeSeG resides primarily in its diselenide bond. Preliminary experiments show that selenocystamine (*17*), which is a commercially available and inexpensive diselenide, can also catalyze the aerobic folding of reduced RNase A (see Figure S4 of the Supporting Information). At pH 8, selenocystamine, which has (up to) two positive charges per diselenide, exhibits a faster initial rate but an ~25% lower yield of RNase A folding compared to GSeSeG, which has a net charge of −2. The difference in rate suggests that the charge present on the diselenide catalyst helps to determine its protein folding activity, perhaps by influencing the selenol pK_a or through interactions with the protein substrate [RNase A has a pI of 9.3 (*18*)]. The origin of the difference in yield remains unclear but might be due to partial partitioning of the protein into a kinetic trap. At pH 5, the differences between selenocystamine and GSeSeG are even more pronounced, as the former compound affords a much lower rate and yield. However, at both pH values examined here, PDI can enhance folding efficiency with selenocystamine somewhat, perhaps by rescuing kinetically trapped, misfolded intermediates. Since the best choice of folding reagent is likely to be case-dependent, engineering of the electrostatic environment around the diselenide bond should allow tuning of reactivity, leading to new molecules tailored for improved activities.

Diselenide reagents, such as GSeSeG, have high potential utility for oxidative protein folding applications. The greater flexibility in the choice of solution conditions could be useful for in vitro folding of traditionally troublesome proteins, such as antibodies (*19, 20*) and growth factors (*21*). More detailed investigations into the mechanism of GSeSeG-catalyzed folding should aid the design of new and useful diselenides and might productively focus on protein disulfide bond isomerization, which is often the rate-limiting step in oxidative folding. Other modifications, such as immobilization (*22, 23*), may also enhance the practicality of GSeSeG by facilitating catalyst recycling in batch-wise or chromatographic protein folding formats.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures and additional kinetic data for the experiments described here. This material is available free of charge via the Internet at http://pubs.acs.org.

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