A small-molecule catalyst of protein folding *in vitro* and *in vivo*

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Background: The formation of native disulfide bonds between cysteine residues often limits the rate and yield of protein folding. The enzyme protein disulfide isomerase (PDI) catalyzes the interchange of disulfide bonds in substrate proteins. The two -Cys–Gly–His–Cys- active sites of PDI provide a thiol that has a low pK_a value and a disulfide bond of high reduction potential ($E°'$).

Results: A synthetic small-molecule dithiol, (±)-*trans*-1,2-bis(2-mercapto-acetamido)cyclohexane (BMC), has a pK_a value of 8.3 and an $E°'$ value of −0.24 V. These values are similar to those of the PDI active sites. BMC catalyzes the activation of scrambled ribonuclease A, an inactive enzyme with non-native disulfide bonds, and doubles the yield of active enzyme. A monothiol analog of BMC, N-methylmercaptoacetamide, is a less efficient catalyst than BMC. BMC in the growth medium of *Saccharomyces cerevisiae* cells increases by > threefold the heterologous secretion of *Schizosaccharomyces pombe* acid phosphatase, which has eight disulfide bonds. This effect is similar to that from the overproduction of PDI in the *S. cerevisiae* cells, indicating that BMC, like PDI, can catalyze protein folding *in vivo*.

Conclusions: A small-molecule dithiol with a low thiol pK_a value and high disulfide $E°'$ value can mimic PDI by catalyzing the formation of native disulfide bonds in proteins, both *in vitro* and *in vivo*.

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Introduction

The formation of native disulfide bonds is the rate-determining step in the folding of many proteins. Reduced, unfolded proteins containing multiple cysteine residues tend to oxidize rapidly and nonspecifically. The non-native disulfide bonds formed during oxidative folding must then be isomerized to achieve the correct cysteine pairings. The greater the number of cysteine residues in an amino acid sequence, the more possibilities arise for incorrect disulfide arrangements. Given that the native structure is required for protein function and that disulfide bonds are often crucial for structural stability, it is not surprising that, *in vivo*, native disulfide bond formation is an enzyme-catalyzed process [1].

Protein disulfide isomerase (PDI; EC 5.3.4.1) is essential for the viability of *Saccharomyces cerevisiae* cells [2]. Although PDI has many known functions [3], its essential role is to catalyze the unscrambling of non-native disulfide bonds in other proteins [4]. PDI is a 57 kDa resident of the endoplasmic reticulum (ER). Each PDI molecule has two active sites that contain the sequence: -Cys–Gly–His–Cys- [5]. Only the amino-terminal cysteine residue in each active site is necessary for catalysis of disulfide bond isomerization [4,6].
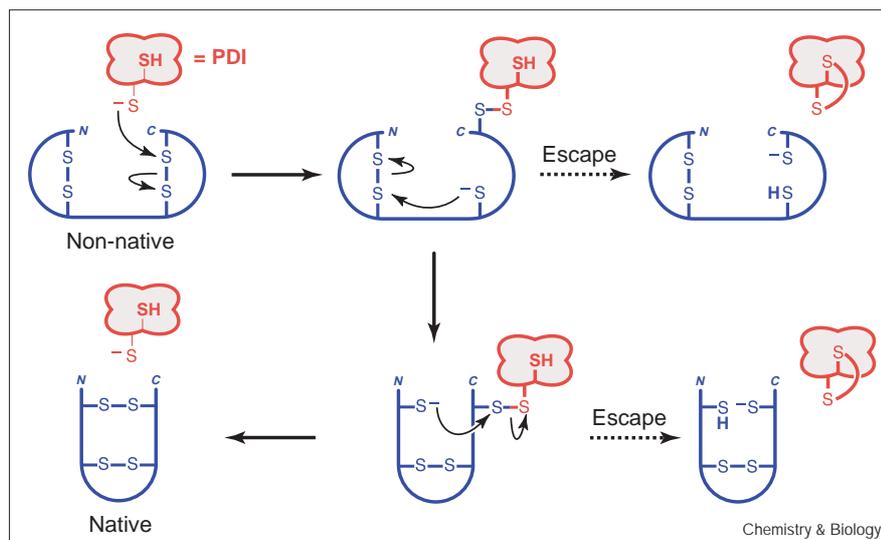
A simple chemical mechanism for catalysis by PDI begins with the nucleophilic attack of its active-site thiolate on a non-native disulfide bond (Figure 1) [7,8]. This attack results in an intermolecular (i.e. mixed) disulfide and a substrate thiolate. Further disulfide rearrangements are then induced by this substrate thiolate. The attainment of the native three-dimensional structure guides the formation of native disulfide bonds and, ultimately, the release of PDI. A striking feature of this mechanism is that it requires the enzyme to provide only a reactive thiolate [7,8].

In a given pH and redox environment, the fraction of PDI active sites that have a thiolate is governed by two factors [9,10]. One is the value of the formal reduction potential ($E°'$) of the disulfide bond that forms between the two cysteine residues in each active site. The other factor is the value of the acid dissociation constant (K_a) of the nucleophilic thiol. In PDI, the active-site disulfide bond has an $E°'$ of −0.18 V, and the thiol of the amino-terminal active-site cysteine residue has a pK_a of 6.7 [11,12]. These two physical parameters can be combined with the properties of the ER ($E_{solution}$ = −0.18 V and pH 7 [13]) to reveal that 33% of the PDI active sites in the ER contain a thiolate [14].

The -Cys–Xaa–Xaa–Cys- (CXXC; where X is any amino acid) active-site motif of PDI is also found in homologous thiol:disulfide oxidoreductases. The best characterized thiol:disulfide oxidoreductase is the *Escherichia coli* thioredoxin (Trx), a 12 kDa cytosolic reducing agent for ribonucleotide reductase and other proteins [15,16]. Trx

Figure 1

Putative chemical mechanism for catalysis of disulfide bond isomerization by protein disulfide isomerase [7,8]. The enzymic active site has two thiols, which enables the enzyme to escape from nonproductive mixed disulfides. After escape, additional redox reactions can lead ultimately to native protein and reduced catalyst.

has a -Cys–Gly–Pro–Cys- active site with an $E^{\circ\prime}$ value of –0.27 V [17] and pK_a value of 7.5 [18]. Wild-type Trx cannot replace PDI in *S. cerevisiae* cells because only 0.021% of Trx active sites would contain a thiolate in the ER. Trx variants with higher fractions of thiolate can, however, substitute for PDI *in vivo* [14]. This finding demonstrates that a small homolog can mimic PDI. Here, we take the next step.

We reasoned that the two active-site thiols of PDI are its most important functional groups, and that a small-molecule dithiol with appropriate $E^{\circ\prime}$ and pKa values could likewise be an effective catalyst of disulfide bond isomerization. The
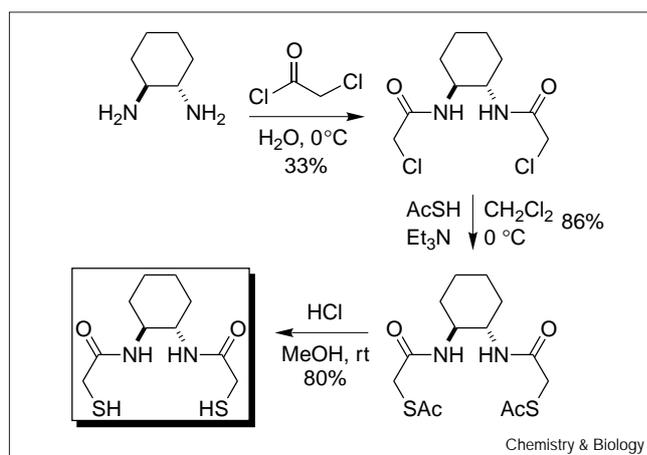
dithiol (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC) had been studied previously as a technetium ligand [19] and as a reducing agent [20]. Here, we synthesize BMC and show that it has $E^{\circ\prime}$ and pK_a values similar to those of PDI. We find that BMC catalyzes disulfide bond isomerization, both *in vitro* and *in vivo*, and is more efficient than its monothiol analog, *N*-methylmercaptoacetamide (NMA).

Results
Synthesis and properties of BMC

We synthesized BMC from (±)-*trans*-1,2-diaminocyclohexane using the three-step route outlined in Figure 2. The overall yield was 23%, or 11 g BMC from 24 ml starting material.
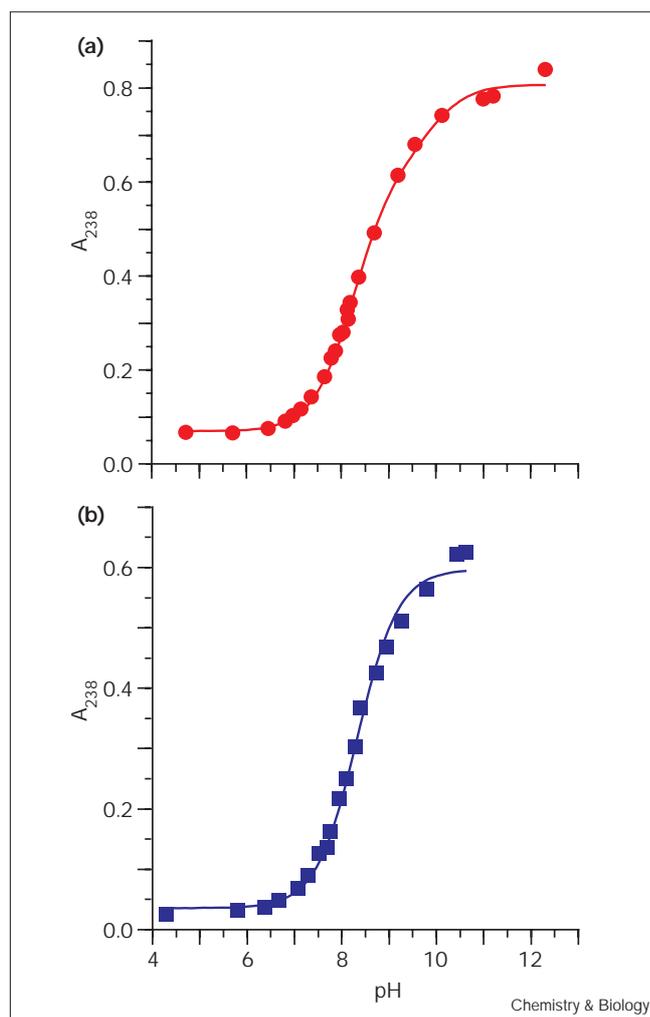
The acid dissociation constant of a titratable group can be determined by measuring the equilibrium concentrations of its protonated and unprotonated forms over a pH range broad enough to encompass the transition from largely protonated to largely unprotonated. The absorbance at 238 nm (A_{238}) of a thiolate exceeds that of its corresponding protonated form. We measured the thiol pK_a values of BMC and NMA using this UV signature. Our analysis of A_{238} as a function of pH indicates that the mercaptoacetamido groups of BMC have thiol $pK_{a1} = 8.3$ and $pK_{a2} = 9.9$ (Figure 3a). The pK_{a1} for BMC is in good agreement with that obtained for NMA, which has $pK_a = 8.3$ (Figure 3b). As a control, a pH titration of glutathione was also performed, giving a thiol pK_a of 9.0 (data not shown).

The reduction potential of a redox-active group can be determined by measuring the equilibrium concentrations of its oxidized and reduced forms in the presence of the

Figure 2

Synthetic route to (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC).

Figure 3

Effect of pH on the absorbance at 238 nm (A_{238}) of **(a)** BMC (0.10 mM) and **(b)** NMA (0.14 mM) in 0.10 M potassium phosphate buffer. Fitting the BMC data to equation 2 ($r^2 > 0.99$) and the NMA data to equation 1 ($r^2 > 0.99$) gives $pK_{a1} = 8.3$ and $pK_{a2} = 9.9$ for BMC and $pK_a = 8.3$ for NMA.

Table 1

Properties of (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane.

Molecular mass (Da)	262
$E°'$ (V)	–0.24
pK_a	8.3; 9.9
Fraction thiolate*	0.16
Specific activity (U/mg)†	0.4

*Fraction thiolate was calculated using the procedure outlined in [8] for a pH 7.6 solution containing 1.0 mM BMC, 1.0 mM reduced glutathione (GSH) and 0.2 mM oxidized glutathione (GSSG) like that used in the *in vitro* assays. †One unit (U) catalyzes the activation of 1 pmol of sRNase A per minute in Tris–HCl buffer (pH 7.6) containing GSH (1.0 mM) and GSSG (0.2 mM).
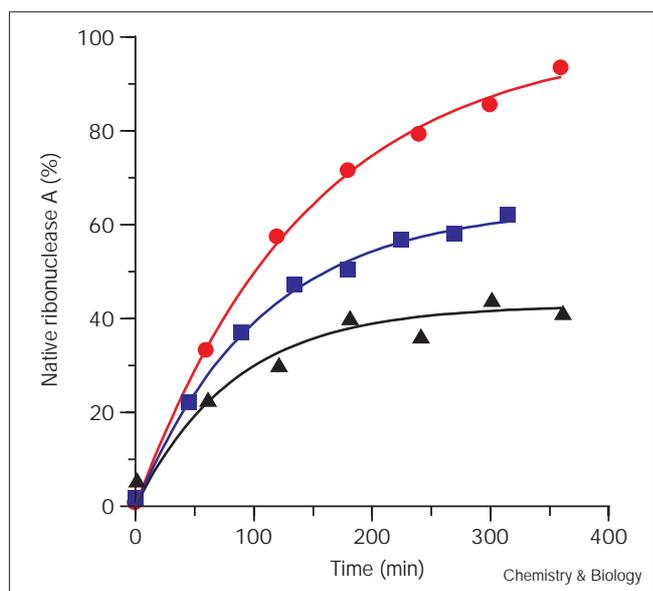
Activity *in vitro*

Bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) has eight cysteine residues, which in the native enzyme form four disulfide bonds. The ability of BMC to catalyze *in vitro* disulfide bond isomerization was assessed by monitoring the activation of scrambled RNase A (sRNase A), which is fully oxidized RNase A with a random distribution of disulfide bonds. sRNase A is a poor catalyst of RNA cleavage, as few of the enzyme molecules are folded properly. An increase in the rate of RNA cleavage is indicative of an increase in the concentration of RNase A molecules with native disulfide bonds.

The presence of BMC increased the rate of activation of sRNase A (Figure 4). Perhaps more significantly, the presence of BMC enhanced by twofold the final yield of native RNase A. NMA also showed unscrambling activity in the *in vitro* assay (Figure 4). Unlike BMC, NMA was not able to activate sRNase A completely. Still, the final concentration of native RNase A produced by NMA was greater than that produced by the glutathione redox buffer alone. Apparently, a small-molecule dithiol is more efficient at unscrambling than is a small-molecule monothiol. Assays with other concentrations of BMC and NMA (or with the glutathione redox buffer alone) gave results similar to those in Figure 4 (data not shown).

Activity *in vivo*

Many of the proteins secreted from eukaryotic cells have multiple disulfide bonds. Those proteins that do not fold quickly and properly are degraded rather than secreted [22,23]. Increasing the endogenous levels of PDI can increase the efficiency of secretion for some of these proteins [24,25]. Presumably, a molecule of small mass and little charge, such as BMC, could gain access to the ER of a yeast cell, where these secretory proteins fold. An increase in heterologous secretion would be consistent with BMC having disulfide isomerization activity inside the cell, because only properly folded proteins are secreted by eukaryotes [26].

oxidized and reduced forms of a reference molecule of known reduction potential. To determine the reduction potential of BMC, we developed a high-performance liquid chromatography (HPLC) method using β-mercaptoethanol (βME; $E°'_{βME} = –0.260$ V [21]) as a reference. As measured by HPLC analysis of the thiol–disulfide exchange equilibrium between BMC and βME, the $E°'$ of BMC is $0.241 ± 0.002$ V. This value is the mean (± SD) of five independent assays starting with different concentrations of BMC and βME.

Having characterized its relevant chemical properties (Table 1), we subjected BMC to two distinct assays for biological catalysis, one *in vitro* and one *in vivo*.

Figure 4

Time course for the activation of scrambled ribonuclease A by various thiols. All unscrambling reactions were performed at 30°C in 50 mM Tris-HCl buffer (pH 7.6) containing glutathione alone (1.0 mM GSH; 0.2 mM GSSG; black triangles) or glutathione (1.0 mM GSH; 0.2 mM GSSG) plus BMC (red circles; 1.0 mM = 0.26 mg/ml) or NMA (blue squares; 2.0 mM). Data points are the average of triplicate measurements, and are fitted to equation 4.

Figure 5
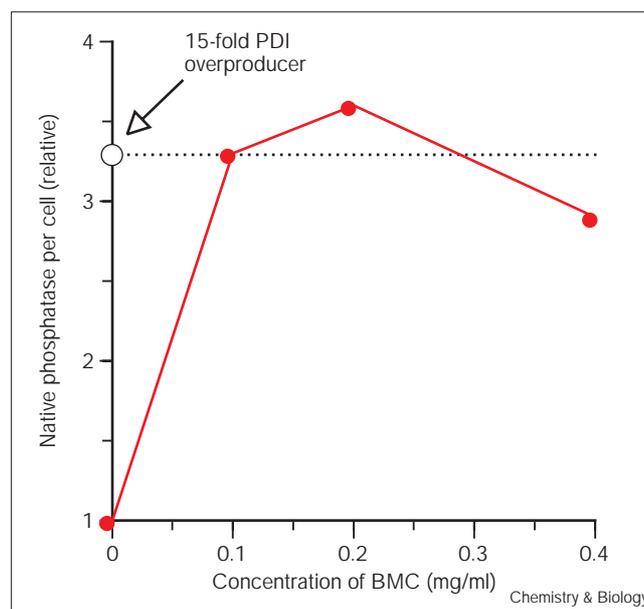
Dependence of *Schizosaccharomyces pombe* acid phosphatase secreted from *S. cerevisiae* on BMC concentration. Cells were grown for 48 h before the assay. In the absence of BMC, A_{435}/OD_{600} = 0.014 (see [24]). Data points are the average of ten replicate measurements. The dotted line represents phosphatase secretion from an isogenic strain in which yeast PDI is overproduced by 15-fold from a single integrated gene [24].

Native *Schizosaccharomyces pombe* acid phosphatase is a 30 kDa homodimer with eight disulfide bonds (six intra-chain and two interchain) and many possibilities for incorrect pairings. Not surprisingly, increased expression of endogenous PDI increases the yield of secreted enzyme [24,27]. The addition of BMC to the growth medium of *S. cerevisiae* cells producing basal levels of PDI increased the yield of secreted *S. pombe* acid phosphatase (Figure 5). The presence of 0.1 mg/ml BMC in the growth medium increases phosphatase secretion by over threefold, equivalent to the increase achieved with 15-fold overproduction of PDI [24]. Higher concentrations of BMC do not lead to higher levels of phosphatase. This accordance of achievable yield suggests that a step other than catalysis of disulfide bond isomerization limits heterologous secretion from *S. cerevisiae* cells treated with BMC or excess PDI. Addition of BMC to cells overproducing PDI produces no incremental increase in phosphatase secretion (data not shown), consistent with a shared mechanism of action between BMC addition and PDI overproduction.

Discussion

Disulfide bond interchange is a facile chemical reaction [28]. The high nucleophilicity of thiolates and high electrophilicity of disulfide bonds allow for rapid thiol–disulfide interchange. Nevertheless, enzymes have evolved to

catalyze this reaction [1]. The enzyme PDI catalyzes the isomerization of non-native disulfide bonds in substrate proteins. The chemical basis for catalysis by PDI results from thiol pK_a and disulfide E°′ values that produce a high fraction thiolate in PDI's native environment — the ER of eukaryotic cells [14,10]. If a high fraction thiolate is the sole requirement for catalysis of disulfide bond isomerization, then it should be possible to design and synthesize an effective small-molecule catalyst of protein folding.

In 1962, Haber and Anfinsen [29] demonstrated that low concentrations of βME could activate sRNase A. Glutathione redox buffers have been used extensively to study *in vitro* oxidative protein folding [30–34]. The use of monothiols to refold proteins is complicated by the observation that the rates and yields of folding reactions do not parallel the redox potential of the solution [33]. The glutathione redox buffer composition for optimum folding of reduced RNase A has been determined empirically by systematic variation of both the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and the total concentration of the two components. At higher total glutathione concentrations, mixed disulfide intermediates accumulate and become trapped. Dithiols have an advantage over monothiols (such as glutathione) in that mixed disulfide intermediates can be cleaved by intramolecular disulfide bond formation.

Previous attempts at using small dithiols to refold proteins have met with only moderate success. Short peptides containing a PDI-like -Cys–Gly–His–Cys- sequence have modest PDI-like activity [35]. The low activity of these peptides relative to PDI probably stems from the loss of the protein context. Specifically, the nucleophilic thiol in the active sites of known thiol:disulfide oxidoreductases is positioned at the amino terminus of an α helix, which provides a depressed thiol pK_a because of the thiol interacting with the helix dipole [36]. Moreover, the CXXC sequence is constrained structurally to provide an active-site disulfide bond of appropriate $E°'$.

Dithiothreitol (DTT) is a poor oxidant of dithiols ($E°' = -0.327$ V [21]). Yet, native RNase A has been produced from the reduced enzyme by using only oxidized DTT [37]. The concentration of DTT used in this experiment was high (0.1 M), and still the refolding rate was slower than that with much lower concentrations of oxidized glutathione. The purpose of using DTT as an oxidant was, however, to demonstrate that a dithiol can oxidize reduced RNase A without the accumulation of mixed disulfide intermediates. Further experiments showed that although DTT oxidizes RNase A more than 100-fold more slowly than does glutathione, DTT is able to rearrange disulfide-containing folding intermediates slightly more quickly than is glutathione [38,39]. It is difficult to create conditions that would favor the isomerization, rather than the reduction, of disulfide bonds by DTT. The stability of its disulfide bond and its high thiol pK_a of 9.2 [20] combine to give an exceedingly low fraction of DTT molecules in the thiolate form. At pH = 7 and $E_{solution} = -0.18$ V, fewer than one in a million DTT molecules would contain a thiolate, severely limiting its efficacy as a catalyst of disulfide bond isomerization.

Thiol pK_a of reduced BMC
Reduced BMC has a low (first) pK_a value. Inductive effects from the electronegative oxygen and nitrogen lower the thiol pK_a from 10.6 (which is the pK_a of propyl mercaptan [40]) to 8.3 (Figure 2a). This pK_a value differs from that reported previously for mercaptoacetamido groups. Using a similar method, other workers [20] reported pK_a values of 7.6–7.8 for the thiols of this group. Their values were determined by fitting the pH-titration data to an equation different from ours and derived after making the assumptions that the extinction coefficient of the fully protonated form is zero and that the extinction coefficient of the doubly unprotonated form is twice that of the singly unprotonated form. Indeed, when using the equation from [20] to fit our pH-titration data, the pK_a value is 7.7. This fit is, however, much worse than that using our equation (Figure 3a). For NMA, a thiol pK_a of 8.05 was determined previously by electrometric titration [41]. The (macroscopic) thiol pK_a value measured here for glutathione is 9.0, which is in the range of those previously reported (8.6–9.2) [41–45], and

precisely between the two microscopic thiol pK_a values of 8.93 and 9.08 determined in a careful study using ^{1}H nuclear magnetic resonance (NMR) spectroscopy [43].

Disulfide reduction potential of oxidized BMC
The disulfide bond of oxidized BMC has a reduction potential of –0.24 V. This value differs from that reported previously. From ^{1}H NMR spectra of solutions containing DTT and BMC, Lamoureux and Whitesides [20] reported that the $E°'$ of BMC was ≥ –0.21 V. This method is imprecise because the reduction potential of BMC is much greater than that of DTT. In a redox equilibrium between equal amounts of BMC ($E°' = -0.24$ V) and DTT ($E°' = -0.327$ V [21]), only 0.1% of the BMC molecules will be in the oxidized form.

Two factors are responsible for this value being higher than that of, say, DTT. The major contributor to the increase is that a ten-membered ring has conformational strain, which is relieved in the reduced molecule. A minor factor arises from the inductive effect — disulfide bonds are more easily reduced if they are formed from thiols with lower pK_a values [10]. As calculated from equation 5 of [10], having mercaptoacetamido groups rather than two propyl mercaptan groups increases the $E°'$ value of BMC by 0.0007 V.

Catalysis *in vitro*
The relevant chemical and catalytic parameters of BMC are listed in Table 1. These parameters highlight the value of the mercaptoacetamido group, which has much merit for small-molecule catalysts of protein folding. In particular, the mercaptoacetamido group will be 100-fold more ionized at pH 7 than a simple alkyl thiol. Moreover, virtually any primary or secondary amine can be elaborated into a mercaptoacetamido group by a synthetic route analogous to that in Figure 2.

Although BMC is an effective catalyst of disulfide bond isomerization, its specific activity is approximately 500-fold less than that of PDI (data not shown). PDI has three attributes that could be responsible for its greater efficacy as a catalyst. First, PDI has a lower thiol pK_a value, a higher disulfide $E°'$ value, and hence a higher fraction of thiolate. Under any solution conditions, the fraction of PDI molecules in the thiolate form would always exceed that for BMC. Second, PDI has a peptide-binding site that enables the formation of a noncovalent complex with a substrate [46,47]. The use of binding energy probably lowers the free energy of the chemical transition state [48,49]. In contrast, BMC collides indiscriminately with other molecules. Third, PDI could interact preferentially with proteins and so could discriminate against glutathione. For example, the glutamate and cysteine residues of glutathione are linked by an irregular (and thus easily distinguishable) amide bond to the γ-carboxyl group of the glutamate residue. BMC cannot discriminate against

glutathione and may be compromised by the formation of mixed disulfides with the redox buffer.

NMA, a small molecule monothiol, is a less efficient catalyst of disulfide bond isomerization than is BMC. A comparison of these two catalysts provides insight into the role of the second thiol group. In the presence of 2.0 mM NMA (which provides the same concentration of thiol as 1.0 mM BMC), full activation of substrate is not achieved (Figure 4). This result is interpretable in terms of the mechanism shown in Figure 1. The product of the first step of this mechanism is a covalent catalyst–substrate complex. Further productive steps in this mechanism rely on the ability of the substrate thiolate to induce further rearrangements. If a disulfide bond isomerization is hindered, the substrate will be slow to rearrange. A monothiol catalyst would then be trapped. In contrast, a dithiol catalyst could escape from the trapped complex via the formation of an intramolecular disulfide bond within the catalyst. This disulfide can then be converted back into a dithiol by reaction with the redox buffer or reduced substrate, enabling another attempt at catalysis. In effect, this second thiol group acts like a clock, providing a set amount of time for the substrate to rearrange and free the catalyst before an intramolecular attack within the catalyst disrupts the complex [6,50]. The difference between the concentration of substrate ultimately activated by a dithiol (BMC) and that activated by a monothiol (NMA or the glutathione redox buffer alone) probably reflects the production of such trapped complexes.

The greater activation rate and increased yield of active RNase A observed for NMA over glutathione alone (Figure 4) may result from the decreased pK_a of the thiol in NMA ($pK_a = 8.3$) relative to that in glutathione ($pK_a = 9.0$). This pK_a difference results in an increased fraction of thiolate in NMA, which increases the population of catalytically active species and thus the activation rate. In addition, the lower pK_a of NMA makes it a better leaving group from mixed disulfides, making it easier for NMA to escape from trapped complexes and thereby increasing the yield of folded protein.

Catalysis *in vivo*
DTT can enter the ER of mammalian cells and prevent the formation of disulfide bonds there [51]. For BMC to exhibit PDI-like activity *in vivo*, it too must enter the ER. The heterologous secretion data suggest that BMC does do so (Figure 5). We suspect that the cell permeability of BMC is enhanced by its lipophilic cyclohexyl ring.

Misfolded proteins with non-native disulfide bonds do occur *in vivo* and are costly to a cell. Co-overproduction of PDI has been a successful strategy for increasing the output efficiency of some overproduced secretory proteins [24]. The *in vivo* activity of BMC suggests that a similar increase

can be obtained simply by adding BMC to the growth medium. Indeed, BMC is the first small-molecule thiol shown to increase the secretion of a heterologous protein.

The pinnacle of biochemical understanding is *de novo* design. To design a minimized mimic of a protein requires a detailed understanding of its function, as a minimal molecule would contain only those chemical groups that are required for function [52]. Some notable success has been attained in creating small mimics of proteins and peptides [53]. For example, a dimer of 20-residue peptides can substitute for erythropoiten, which is a 34 kDa glycoprotein [54,55]. The 28-residue atrial natriuretic peptide has been shrunk to 15 residues with only a moderate loss in activity [56]. A few enzymes have been minimized effectively [57]. A 14-residue peptide can catalyze the decarboxylation of oxaloacetate in place of acetoacetate decarboxylase [58]. Organoselenium compounds can imitate the active-site selenocysteine residue of glutathione peroxidase [59]. To the best of our knowledge, however, BMC is the first small-molecule mimic of an enzyme that has been shown to function both *in vitro* and *in vivo*.

Prospectus
An effective, small-molecule mimic of PDI could have numerous practical applications. BMC is useful for *in vitro* protein folding on a preparative scale. Often, overproduction of proteins in *E. coli* leads to the formation of inclusion bodies [60]. These insoluble aggregates must be solubilized by reduction and denaturation, and then refolded by dilution into an appropriate redox buffer. The most commonly used redox buffer contains reduced and oxidized glutathione, as used here. As a monothiol, glutathione lacks the ability to escape from nonproductive complexes with protein, and can lead to low yields of properly folded protein (Figure 4). Also, the high thiol pK_a of glutathione (9.0) leads to slow disulfide bond isomerization at pH 7. The use of PDI itself for preparative-scale protein folding is hindered by the high cost and instability of enzymic catalysts and by the problem of separating PDI from the target protein. In contrast, a small-molecule catalyst is inexpensive, inert, and readily separable from a target protein by filtration, dialysis or size-exclusion chromatography.

Some proteins cannot be refolded efficiently *in vitro* and are best produced in their native state. Creating a new strain of a microorganism that overproduces PDI (or other enzymic catalyst) can improve the isolated yield of a secreted protein [24], but is tedious to effect. Small molecules such as BMC may obviate the need for such manipulations by achieving a similar effect through addition to the growth medium (Figure 5).

In addition to improving the production of heterologous proteins, small-molecule catalysts of protein folding could have other utility. Several important diseases have been

implicated as arising from defects in protein folding [61–63]. Bioavailable molecules such as BMC could serve as the basis for therapies for such diseases. For example, a small-molecule catalyst of protein folding could act directly by catalyzing the proper folding of the aberrant protein responsible for the etiology of the disease. Alternatively, the catalyst could act indirectly by making the protein-folding machinery of the diseased cells more efficient overall, thereby allowing endogenous processes to fold a higher fraction of the aberrant protein. Finally, a synthetic folding catalyst could ameliorate the symptoms (rather than the cause) of a disease by catalyzing the folding of proteins whose folding is adversely affected by the disease. It is noteworthy that BMC is likely to be well tolerated by human cells because its $E^{\circ\prime}$ value is not extreme, but is between the $E_{solution}$ of the ER and cytosol [13].

Significance

A synthetic small-molecule dithiol, (±)-*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC), has thiol pK_a and disulfide reduction potential ($E^{\circ\prime}$) values similar to those of the essential enzyme protein disulfide isomerase (PDI). Like PDI, BMC catalyzes the unscrambling of non-native protein disulfide bonds *in vitro* and enhances the yield of a protein-folding reaction. Addition of BMC to *Saccharomyces cerevisiae* growth medium increases the heterologous production of a protein with multiple disulfide bonds. BMC is therefore the first small-molecule thiol shown to increase the secretion of a protein. NMA, a monothiol analog of BMC, is a less efficient catalyst than BMC. This observation supports an unscrambling mechanism in which the second thiol of BMC allows escape from nonproductive mixed disulfides. The desirable attributes of PDI can therefore be mimicked in a small-molecule catalyst of significant practical utility.

Materials and methods

General

(±)-*trans*-1,2-Diaminocyclohexane, chloroacetyl chloride, βME and 2-hydroxyethyldisulfide (βME^{ox}) were from Aldrich Chemical (Milwaukee, WI). Thioacetic acid was from Acros Organics (Pittsburgh, PA). NMA was from Fluka (Buchs, Switzerland). sRNase A, GSH and GSSG were from Sigma Chemical (St. Louis, MO). Poly(C) was from Midland Certified Reagents (Midland, TX) and was precipitated from aqueous ethanol (70% v/v) before use. Yeast nitrogen base was from Difco (Detroit, MI). All other chemicals were of reagent grade or better, and were used without further purification.

Thiol concentrations were determined using the method of Ellman [64]. Ultraviolet absorbance measurements were made on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller, from Varian (Sugar Land, TX).

Synthesis of BMC

(±)-*trans*-1,2-Bis(mercaptoacetamido)cyclohexane (BMC) was synthesized by a route similar to those described previously [19,20]. This route is outlined in Figure 2.

(±)-*trans*-1,2-Diaminocyclohexane (22 g, 0.20 mol) and K_2CO_3 (56 g, 0.40 mol) were dissolved in 1 l ice-cold distilled water. Chloroacetyl chloride (34 ml, 0.42 mol) was added dropwise and with stirring to this solution. The resulting cloudy white mixture was stirred at 0°C for 1 h. The precipitate was collected by filtration, washed with cold distilled water, and dried under reduced pressure. The bis(choroacetyl) product (17.5 g, 33%) was obtained as a white powder, which was judged to be pure by ^{1}H NMR analysis [20].

The bis(chloroacetyl) product (5.6 g, 21 mmol) was dissolved in 1 l of methylene chloride, and this solution cooled to 0°C. Thioacetic acid (6 ml, 83 mmol) was added dropwise and with stirring to the cooled solution. Then, triethylamine (12 ml, 83 mmol) was added dropwise and with stirring. The resulting solution was allowed to warm slowly to room temperature and stirred under $N_2(g)$ for 40 h. The reaction was quenched by the addition of 0.5 l of 0.35 M sodium acetate buffer (pH 4). The layers were separated, and the organic layer washed with an ice-cold solution of saturated aqueous sodium bicarbonate. The organic layer was dried, filtered, and concentrated to yield a white powder. This powder was purified by recrystallization from ethyl acetate. The bis(thioacetate) product (6.2 g, 86%) was obtained as a white powder, which was judged to be pure by ^{1}H NMR analysis [20].

The bis(thioacetate) product (3.0 g, 8.75 mmol) was dissolved in 125 ml of 1.2 M HCl in methanol. The resulting clear solution was incubated at room temperature under $N_2(g)$ for 20 h. The excess acid was then removed by bubbling nitrogen gas through the solution, and the solvent was removed under reduced pressure to yield a yellow oil. The oil was dissolved in a minimal amount of methanol, and the resulting solution was cooled in a dry ice/acetone bath. The supernatant was decanted, and the white precipitate was dried under reduced pressure to yield BMC (1.8 g, 80%) as a white solid, which was judged to be pure by spectroscopic analysis: mp 193–194°C; IR (thin film) 3272, 3083, 2917, 2852, 2547, 1650, 1554, 1419, 1328, 1241, 1162, 974, 742, 700 cm^{-1}; ^{1}H NMR (CD$_3$OD, 500 MHz) d 3.66 (br, s, 2 H, NCH), 3.15 and 3.14 (2d, J = 14.5 Hz, 4 H, COCH$_2$S), 1.94 (m, 2 H, NCHCH$_2$ eq.), 1.80 (m, 2 H, NCHCH$_2$ ax.), 1.37 (m, 4 H, NCHCH$_2$CH$_2$); MS (EI) calc'd for C$_{10}$H$_{18}$N$_2$O$_2$S$_2$ *m/e* 262, found 262.

Synthesis of BMC-disulfide (BMC^{ox})

The disulfide form of BMC was synthesized from BMC by oxidation with I$_2$. Briefly, BMC (0.05 g, 0.19 mmol) was dissolved in 0.19 l of ethyl acetate at 0°C. KHCO$_3$ (25 ml of a 10% w/v solution in water) was added. A concentrated solution of I$_2$ (0.088 g in 6 ml ethyl acetate) was added dropwise and with stirring. I$_2$ addition was stopped when the BMC solution turned brown, and the solution was then stirred on ice for 1 h. The reaction was quenched by the dropwise addition of aqueous sodium thiosulfate until the solution became colorless. The organic layer was separated, dried with Na$_2$SO$_4$, filtered, and concentrated to yield a white powder. This powder was purified by recrystallization from ethyl acetate. The BMC^{ox} product (56%) was judged to be pure by spectroscopic analysis: mp 247°C; IR (thin film) 3246, 3066, 2937, 2845, 1633, 1558, 1400, 1326, 1151, 974, 746 cm^{-1}; ^{1}H NMR (d^6-DMSO, 500 MHz) δ 8.0 (NH), 3.8 (NCH), 3.1 (COCH$_2$S), 1.7 (NCHCH$_2$), 1.3 (NCHCH$_2$CH$_2$); MS (EI) calc'd for C$_{10}$H$_{16}$N$_2$O$_2$S$_2$ *m/e* 260, found 260.

Determination of thiol pK_a

Thiol titration curves were obtained by measuring A$_{238}$ as a function of pH. Buffer stock solutions of KH$_2$PO$_4$, K$_2$HPO$_4$ and K$_3$PO$_4$ (0.10 M each) were degassed and flushed with Ar(g). A stock solution of thiol (1.5 mM BMC or 2.1 mM NMA) was prepared using KH$_2$PO$_4$. Different ratios of the buffer stock solutions were combined in duplicate to give two identical sets of 1 ml solutions. These sets of solutions varied in pH according to the ratio of each buffer stock solution added. To each duplicate pair of solutions, 70 µl of KH$_2$PO$_4$ was added to one and used to set the A$_{238}$ at zero. Next, 70 µl of thiol solution was added to the corresponding sample, the A$_{238}$ measured immediately, and the solution recovered. After measuring the A$_{238}$, the pH of each solution was measured using a Beckman (Fullerton, CA) pH meter freshly calibrated with pH 4.00 and pH 10.00 standard solutions from Fisher

(Pittsburgh, PA). This process was repeated for each pair of solutions to generate a plot of A_{238} vs pH.

To determine pK_a values, the A_{238} versus pH data were fitted to either a single (for NMA) or a double (for BMC) titration model (equations 1 and 2, respectively):

$$A_{238} = C_T \left(\frac{\varepsilon_{SH} + \varepsilon_{S^-} 10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \right) \tag{1}$$

$$A_{238} = C_T \left(\frac{\varepsilon_{S^-}^{S^-} 10^{(pH - pK_{a2})} + \varepsilon_{SH}^{S^-} + \varepsilon_{SH}^{SH} 10^{(pK_{a1} - pH)}}{10^{(pH - pK_{a2})} + 1 + 10^{(pK_{a1} - pH)}} \right) \tag{2}$$

derived from Beer's Law and the definition of the acid dissociation constant. C_T is the total thiol concentration, ε_{SH} and ε_{SH}^{SH} are the extinction coefficients of the fully protonated forms, ε_{S^-} and $\varepsilon_{SH}^{S^-}$ are the extinction coefficients of the singly unprotonated forms, and $\varepsilon_{S^-}^{S^-}$ is the extinction coefficient of the doubly unprotonated form. Both the extinction coefficients and pK_as were obtained as parameters of the curve fit.

Determination of disulfide reduction potential
Thiol-disulfide interchange equilibria were established between BMC and βME. In a typical reaction, 25 ml flask containing solid BMC (1 mg) was sealed with a septum and flushed with Ar(g) for 10 min. Potassium phosphate buffer (pH 7.0; 10 ml of a 10 mM solution), that had been saturated with Ar(g) was added, and the resulting mixture was stirred for 10 min to dissolve the solid. Then, βME^{ox} (0.1 ml of a 40 mM stock solution in 10 mM potassium phosphate buffer, pH 7.0) was added, and the ensuing thiol-disulfide interchange reaction was allowed to reach equilibrium at 25°C for 24 h. Similar equilibria were established with different starting concentrations of BMC and βME^{ox}.

The equilibrated mixture was quenched with HCl (1:100 dilution of a 3 M solution) to prevent further reaction. An aliquot (0.1 ml) of the quenched mixture was analyzed immediately by HPLC using a Waters system equipped with a Spectra-Physics integrator and a Vydac analytical C18 reverse phase column. The loaded column was eluted at 1.0 ml/min with water (5.0 ml) followed by a linear gradient (20 ml + 20 ml) of acetonitrile (0–40% v/v). Compounds were detected by their absorption at 205 nm. Four peaks were evident in the chromatograms, and were anticipated to arise from BMC, BMC^{ox}, βME, and βME^{ox}. HPLC analysis of standard solutions revealed that the four peaks did indeed correspond to βME (retention time: 7 min), βME^{ox} (19 min), BMC^{ox} (26 min), and BMC (28 min). No evidence for mixed disulfides was apparent. To correlate peak area with concentration, calibration curves were developed for these compounds and were linear over the concentration range used (data not shown). From these calibration curves, the equilibrium concentration of each component was determined, allowing calculation of $E_{BMC}^{o'}$, from the Nernst equation, here:

$$E_{BMC}^{o'} = E_{\beta ME}^{o'} - \frac{RT}{nF} \ln \frac{[BMC^{OX}][\beta ME]^2}{[BMC][\beta ME^{OX}]} \tag{3}$$

Assay for isomerase activity *in vitro*
The activation of sRNase A was monitored essentially as described previously [65]. Refolding reactions were performed at 30°C in 50 mM Tris-HCl buffer (pH 7.6) containing GSH (1.0 mM), GSSG (0.2 mM), and catalyst (either 1.0 mM BMC or 2.0 mM NMA). BMC was delivered from a 100-fold concentrated stock solution in methanol. Reactions (in triplicate) were initiated by the addition of sRNase A (1.0 μl of a

0.5 mg/ml stock) to a final volume of 0.10 ml. At timed intervals, aliquots were assayed for poly(C) cleavage activity as described until no further increase in activity was observed. Data were fitted to the equation:

$$[\text{active RNase A}] = [\text{sRNase A}]_{t = 0}(1 - e^{-[\text{catalyst}]kt}) \tag{4}$$

to obtain the value of the second-order rate constant, k. The difference in k values for catalyzed and uncatalyzed reactions was used to calculate the specific activity of the catalyst.

Assay for heterologous secretion
S. cerevisiae cells were engineered to overproduce *S. pombe* acid phosphatase. *S. cerevisiae* strain BJ5464 (α *ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) was obtained from the Yeast Genetic Stock Center (Berkeley, CA). BJ5464 was transformed with a multicopy plasmid directing overproduction of *S. pombe* acid phosphatase, as described previously [24]. Yeast cultures (ten replicates) were grown in liquid medium of 50 mM HEPES buffer (pH 6.5) containing BMC (0, 0.1, 0.2, or 0.4 mg/ml from a stock solution in dimethyl sulfoxide), glucose (2% w/v), yeast nitrogen base (0.67% w/v), and synthetic amino acid supplement [arginine (190 mg/l), methionine (108 mg/l), tyrosine (52 mg/l), isoleucine (290 mg/l), lysine (440 mg/l), phenylalanine (200 mg/l), glutamic acid (1260 mg/l), aspartic acid (400 mg/l), valine (380 mg/l), threonine (220 mg/l), glycine (130 mg/l), and the nucleotide adenine (40 mg/l)]. After 48 h of growth at 30°C, the cells were harvested and the concentration of secreted active *S. pombe* acid phosphatase in the supernatant was determined by a colorimetric assay as described previously [24].

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