



# Thiols and Selenols as Electron-Relay Catalysts for Disulfide-Bond Reduction\*\*

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For the proper function of many proteins, sulfhydryl groups need to be maintained in a reduced state or disulfide bonds need to be maintained in an oxidized state.<sup>[1]</sup> In cells, this maintenance entails thiol–disulfide interchange reactions, often initiated by a membrane-associated protein and mediated by a soluble protein or peptide (e.g., glutathione).<sup>[2]</sup> In vitro, small-molecule thiols and disulfides, such as those in Scheme 1, can accomplish this task.<sup>[3]</sup>

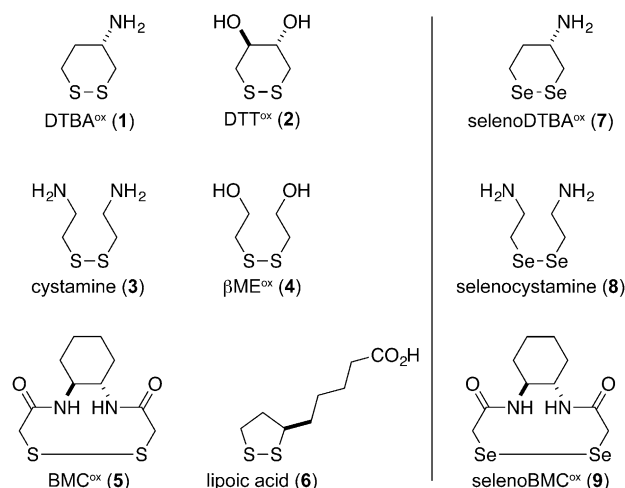
Recently, we reported on a novel disulfide-reducing agent, (2*S*)-2-amino-1,4-dimercaptobutane (dithiobutylamine or

DTBA; reduced **1**), derived from L-aspartic acid.<sup>[4]</sup> As dithiothreitol (DTT; reduced **2**), DTBA is a dithiol capable of adopting an unstrained ring upon oxidation.<sup>[5]</sup> A distinct and untapped attribute of DTBA is the ability of its amino group to act as a handle for facile conjugation. Small-molecule reducing agents typically need to be maintained at millimolar concentrations, and their removal diminishes process efficiency and economy. We reasoned that attaching DTBA to a solid support would enable its removal after disulfide reduction by either filtration or centrifugation.<sup>[6]</sup>

To test our hypothesis, we choose TentaGel resin as the solid support. This resin consists of hydrophilic poly(ethylene glycol) units grafted onto low-cross-linked polystyrene.<sup>[7]</sup> We found DTBA immobilized on TentaGel to be a potent disulfide-reducing agent with  $E^\circ = (-0.316 \pm 0.002)$  V (see Figures S1 and S2 in the Supporting Information), a value similar to that of soluble DTBA.<sup>[4]</sup> Immobilized DTBA (10 equiv) was able to reduce cystamine (**3**) and oxidized  $\beta$ -mercaptoethanol (**4**) completely (Figures S3 and S4). Immobilized DTBA (10 equiv) was even able to reduce highly stable disulfides, such as oxidized DTBA (**1**) and oxidized DTT (**2**) with yields of 76 % and 68 %, respectively (Figures S5 and S6). After each procedure, the resin was easily isolated, regenerated, and reused without any observable loss in activity. The latter are not attributes of immobilized reducing agents derived from phosphines, which form recalcitrant phosphine oxides.

Next, we assessed the ability of immobilized DTBA to reduce a disulfide bond in a folded protein, which can be a challenging task.<sup>[8]</sup> As the target protein, we choose papain, a cysteine protease.<sup>[9]</sup> Upon treatment with *S*-methyl methanethiosulfonate, the active-site cysteine of papain (Cys25) forms a mixed disulfide that has no detectable enzymatic activity.<sup>[10]</sup> When we incubated the oxidized enzyme with 100 equivalents of immobilized DTBA, we found that less than half of papain-Cys25-S-S-CH<sub>3</sub> had been reduced after 30 minutes (Figure 1). Moreover, the rate of reduction for this heterogeneous reaction was slow, approximately 0.1 % of that provided by typical solution-phase reagents,<sup>[4,11]</sup> and activation ceased after 10 minutes. When papain was treated with 1000 equivalents of immobilized DTBA, full generation of activity was observed within 10 minutes (Figure 1). These data indicate that the inefficiency is likely due to a diminished ability of the protein disulfide—in comparison to small-molecule disulfides—to access the sulfhydryl groups of immobilized DTBA.<sup>[8a,c]</sup>

Taking inspiration from cellular thiol–disulfide interchange reactions,<sup>[2,12]</sup> we reasoned that the utility of immobilized DTBA could be enhanced by a soluble molecule that could “relay” electrons from the resin to the protein

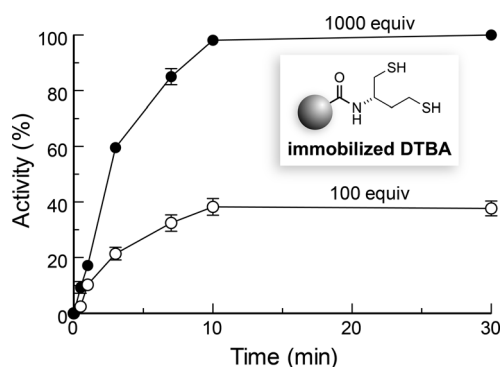


**Scheme 1.** Disulfides (**1–6**) and diselenides (**7–9**) used in this work. Compounds **2**, **5**, **6**, and **9** are racemic mixtures.

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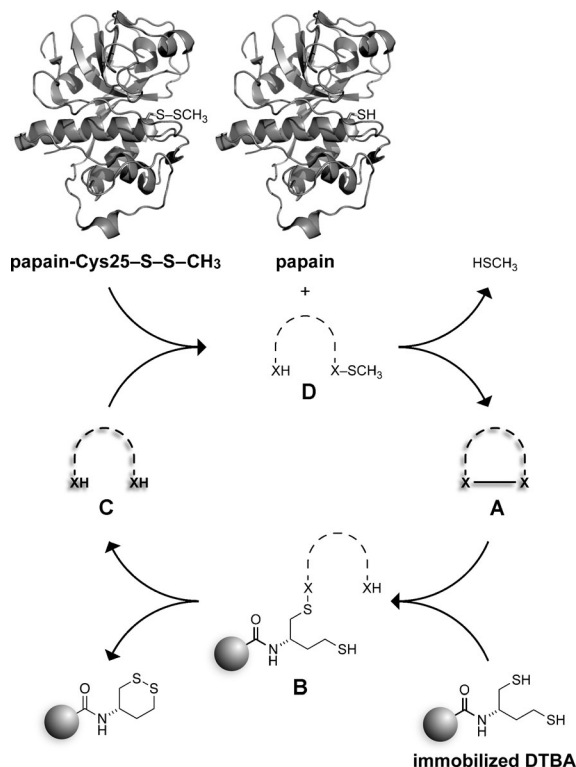
Supporting information for this article (including experimental details) is available on the WWW under <http://dx.doi.org/10.1002/anie.201307481>.



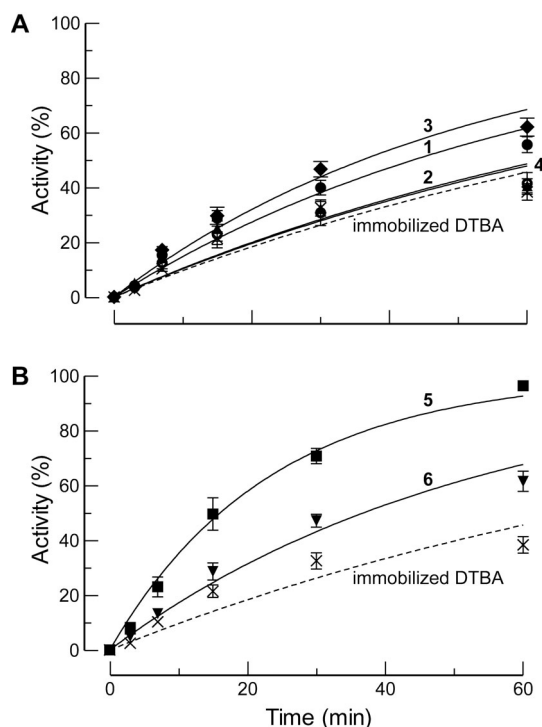
**Figure 1.** Time course for the reactivation of papain-Cys25-S-S-CH<sub>3</sub> by immobilized DTBA (100 or 1000 equiv) in imidazole-HCl buffer (0.10 M, pH 7.0) containing EDTA (2 mM).

(Scheme 2).<sup>[13]</sup> To test this hypothesis, we incubated papain-Cys25-S-S-CH<sub>3</sub> with 100 equivalents of immobilized DTBA and 30 mol % of disulfides **1–4** (relative to oxidized protein). Unfortunately, we observed only a slight rate enhancement (Figure 2A).

Suspecting that the rate of the heterogeneous reaction between immobilized DTBA and unstrained disulfides **1–4** was slow (**A**→**B** in Scheme 2),<sup>[15]</sup> we turned to disulfides **5** and **6**, believing that their incipient strain would accelerate the turnover of the soluble catalyst. BMC<sup>ox</sup> (**5**) is a ten-membered cyclic disulfide. Rings of this size suffer trans-



**Scheme 2.** Cycle for electron-relay catalysis of disulfide-bond reduction by soluble thiols (**C**, X=S) or selenols (**C**, X=Se). Papain was depicted with the program PyMOL (Schrodinger, Portland, OR) using PDB entry 1ppn.<sup>[14]</sup>



**Figure 2.** Time course for the reactivation of papain-Cys25-S-S-CH<sub>3</sub> by immobilized DTBA (100 equiv) and a solution-phase disulfide catalyst (30 mol %). Reactions were performed in imidazole-HCl buffer (0.10 M, pH 7.0) containing EDTA (2 mM). A) Unstrained disulfide catalysts. Cystamine (**3**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 1.9$ ; DTBA<sup>ox</sup> (**1**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 1.6$ ; DTT<sup>ox</sup> (**2**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} \approx 1.0$ ;  $\beta$ ME<sup>ox</sup> (**4**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} \approx 1.0$ . B) Strained disulfide catalysts. BMC<sup>ox</sup> (**5**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 4.3$ ; lipoic acid (**6**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 1.9$ . Data for immobilized DTBA alone are shown in both panels.

annular strain.<sup>[5b,16a,b]</sup> Similarly, cyclic five-membered disulfides (i.e., 1,2-dithiolanes), such as **6**, place significant distortion on the preferred CSSC dihedral angle.<sup>[5b,17]</sup> Hence, the rate constant for the reaction between 1,3-propanedithiol and 1,2-dithiolane is around 650 times greater than that for the homologated exchange reaction between 1,4-butanedithiol and 1,2-dithiane.<sup>[18]</sup>

Consistent with our expectations, we found that disulfide **5** provided a significant enhancement in the rate of papain-Cys25-S-S-CH<sub>3</sub> reduction. Disulfide **6** was somewhat less effective, as its mixed disulfide (**B** in Scheme 2) has a higher tendency to partition back to the disulfide (**A**).<sup>[5b]</sup> Moreover, in the absence of immobilized DTBA, we found that the reduced form of DTBA regenerates activity faster than does the reduced form of BMC (Figure S8), affirming that the reduction of the soluble disulfide catalyst (**A**→**C** in Scheme 2) limits the rate of electron-relay catalysis.

To improve catalytic efficiency further, we considered the use of selenium, which has physicochemical properties similar to those of sulfur. Yet, selenols manifest several desirable attributes as reducing agents in aqueous solution.<sup>[19]</sup> For example, selenols have pK<sub>a</sub> values that are typically three units lower than those of analogous thiols, significantly enhancing their nucleophilicity near neutral pH and their ability to act as a leaving group.<sup>[20]</sup> Diselenides also have *E*<sup>o</sup> values that are typically 0.15 V lower than those of analogous

disulfides, making selenols more potent reducing agents. In addition, reactions with selenium as the electrophile can be  $10^4$  times faster than those with sulfur as the electrophile, and might not require strain for efficient turnover. Indeed, there are numerous reports of small-molecule diselenides being used as catalysts for biochemical oxidation reactions.<sup>[21]</sup> Enzymes, such as thioredoxin reductase,<sup>[19b]</sup> are known to employ a selenol as a reducing agent. Yet, reported in vitro reduction reactions rarely employ small-molecule selenols, and never diselenols. A practical problem is the high reactivity of selenols with molecular oxygen. We recognized that this problem would be averted in our system, which would generate catalytic selenols in situ (Scheme 2). Because of the efficacy of disulfide **5** (Figure 2B), we were motivated to investigate its seleno congener. Accordingly, we synthesized selenoBMC<sup>ox</sup> (**9**) as well as selenoDTBA<sup>ox</sup> (**7**), and we obtained selenocystamine (**8**), which is available commercially and has demonstrated marked success in mediating thiol–disulfide interchange reactions.<sup>[20,21a,b]</sup>

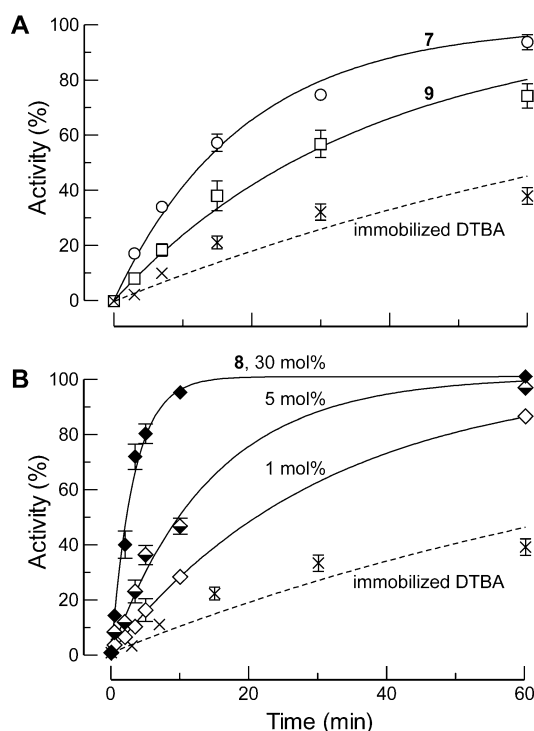
We found that diselenide **7** is superior to its congener **1**, and that diselenide **9** performs comparably to its congener **5** (Figure 3A). These two cyclic diselenides were, however, worse catalysts than was acyclic diselenide **8** (Figure 3B). This finding is attributable to the selenylsulfide (**B** in Scheme 2) generated by the reaction of **7** and **9** (but not **8**) with immobilized DTBA tending to partition back to the diselenide (**A**) rather than to the diselenol (**C**) needed for catalysis.<sup>[18,22]</sup> Notably, diselenide **8** led to significant rate enhancements even at low loadings of catalyst.

In summary, we have established that the amino group of DTBA allows for its facile conjugation to a resin. This supported reagent was effective at reducing disulfide bonds in small molecules. Unlike soluble reducing agents, immobilized DTBA was easy to recover and reuse. We also demonstrated that the rate of reducing a disulfide bond in a protein can be enhanced markedly when the reduced resin is used in conjunction with a “relay”. In this biomimetic strategy, the resin acts as a repository of electrons that are relayed to a macromolecule via a small-molecule catalyst. The optimal catalysts are strained cyclic disulfides and acyclic diselenides, both of which react with excess immobilized DTBA to form a covalent intermediate that partitions toward reduced catalyst and oxidized resin. Finally, we note that a vast excess of soluble reducing agent is typically used to preserve proteins in a reduced state.<sup>[23]</sup> Instead, maintenance could require a minute (e.g., sub-micromolar) amount of a soluble catalyst along with immobilized DTBA. We anticipate that the low level of soluble reducing agent would be advantageous in common bioconjugation reactions entailing the *S*-alkylation of cysteine residues,<sup>[24]</sup> as well as in many other experimental procedures.

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**Figure 3.** Time course for the reactivation of papain-Cys25-S-S-CH<sub>3</sub> by immobilized DTBA (100 equiv) and a solution-phase diselenide catalyst. Reactions were performed in imidazole-HCl buffer (0.10 M, pH 7.0) containing EDTA (2 mM). A) Cyclic diselenide catalysts (30 mol%). SelenoDTBA<sup>ox</sup> (**7**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 5.3$ ; selenoBMC<sup>ox</sup> (**9**):  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 2.7$ . B) Selenocystamine (**8**) as a catalyst. 30 mol%:  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 30$ ; 5 mol%:  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 6.8$ ; 1 mol%:  $k_{\text{cat}}^{\text{obs}}/k_{\text{uncat}}^{\text{obs}} = 3.2$ . Data for immobilized DTBA alone are shown in both panels.

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