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Pyrazine-derived disulfide-reducing agent for chemical biology†

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For fifty years, dithiothreitol (DTT) has been the preferred reagent for the reduction of disulfide bonds in proteins and other biomolecules. Herein we report on the synthesis and characterization of 2,3-bis(mercaptomethyl)pyrazine (BMMP), a readily accessible disulfide-reducing agent with reactivity under biological conditions that is markedly superior to DTT and other known reagents.

The redox state of cysteine residues can have a profound effect on protein structure and function.[1–4] Consequently, reagents that reduce disulfide bonds to thiols can be crucial to progress in chemical biology.[1,5,6] Necessarily, the reduction of disulfide bonds within biomolecules must be accomplished under mild conditions: in water, at neutral pH, and at room temperature.[1,7–10] Thiols can accomplish these goal and do so (unlike phosphines) in a reversible manner. Their reduction mechanism entails thiol–disulfide interchange initiated by the attack of a thiolate.[11–18] The use of monothiols such as L-glutathione or β-mercaptoethanol (βME) can lead to the trapping of the resulting intermediate as a mixed disulfide. In 1964, Cleland reported that dithiothreitol (DTT or Cleland's reagent; Fig. 1), a racemic dithiol, readily completes the reduction reaction by forming a stable six-membered ring.[7] The potency of DTT is evident from the low reduction potential ($E^{\circ\prime} = -0.327$ V) of its oxidized form.[19] As a result, DTT has achieved widespread use for the quantitative reduction of disulfide bonds in proteins and other biomolecules.

DTT has, however, a serious limitation. As thiolates but not thiols are nucleophilic in aqueous solution,[20] the observed rate of disulfide reduction is dependent on the thiol pK_a of the reducing agent. With thiol pK_a values of 9.2 and 10.1, <1% of DTT resides in the reactive thiolate form at neutral pH.[8] As a result, several attempts have been made to create water-soluble reducing agents that sport depressed thiol pK_a values.[9,21,22]

	Thiol pK_a	Disulfide $E^{\circ\prime}$
DTT (racemate)	9.2 (10.1)[a]	–0.327 V[b]
DMH	8.0 ± 0.2 (9.1 ± 0.1)[c]	(–0.262 ± 0.004) V[c]
DTBA	8.2 ± 0.2 (9.3 ± 0.1)[d]	(–0.317 ± 0.002) V[d]
BMMP	7.6 ± 0.1 (9.0 ± 0.1)[c]	(–0.301 ± 0.003) V[c]

Fig. 1 Physicochemical properties of dithiol reducing agents. [a] Values are from ref. 11. [b] Value is from ref. 19. [c] Values are from this work. [d] Values are from ref. 23.

Recently, we reported on dithiobutylamine (DTBA; Fig. 1), a dithiol reducing agent derived from L-aspartic acid.[23] Like DTT, DTBA is a potent disulfide-reducing agent. Moreover, the amino group of DTBA confers depressed thiol pK_a values of 8.2 and 9.3 and facile functionalization.[23,24] That amino group, however, appeared to deter the ability of the molecule to reduce certain disulfide bonds due to unfavorable Coulombic interactions.[23]

We were determined to improve upon DTBA. Dithiols (like DTBA and DTT) that form six-membered cyclic disulfides are potent reducing agents, reflecting a balance between the high enthalpic stability of the incipient ring and the low entropic loss for its formation.[19,25] We reasoned that the entropic loss

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could be diminished further by limiting rotation around one of the bonds between the two sulfhydryl groups. We also sought an electron-withdrawing group that could lower a thiol pK_a to a value close to physiological pH (which is pH 7.365 in human blood). Then, an optimal balance is achieved between the concentration of the thiolate nucleophile (low pK_a is better) and its incipient nucleophilicity (high pK_a is better).[11,26] Accordingly, we suspected that incorporating a highly electron-deficient moiety (*e.g.*, a pyrazine ring) that also serves to preorganize the reagent for disulfide-bond formation could be advantageous. A compound that fits this description is 2,3-bis(mercaptomethyl)pyrazine (BMMP; Fig. 1).

Prior to designing a synthetic route to BMMP, we calculated the free energies for the optimized geometry of reduced BMMP and oxidized BMMP and compared them to those of reduced DTT and oxidized DTT. We were aware of a prior study that predicted the stability of cyclic disulfides using molecular mechanics calculations,[25] and sought to assess our design at a higher level of theory (B3LYP/6-311+G(2d,p),[27] see ESI†). The thiol pK_a values of BMMP were calculated to be substantially lower than those of DTT. Moreover, the oxidation of BMMP was calculated to be slightly more favorable than that of DTT.

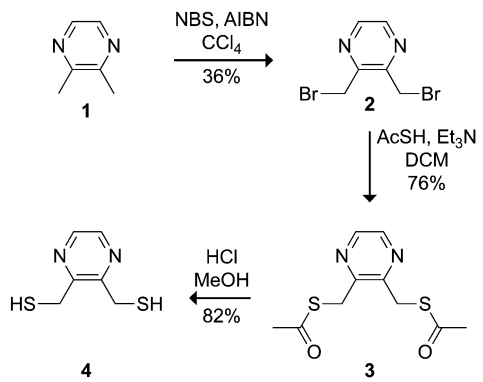
Because of these encouraging computational results, we synthesized BMMP from 2,3-dimethylpyrazine (**1**) *via* a simple three-step route (Scheme 1). We isolated BMMP as an off-white/yellow powder in 22% overall yield. The compound has low odor and aqueous solubility (64 mM reduced; 8 mM oxidized) that is adequate for typical applications in chemical biology.

As predicted, BMMP has low thiol pK_a values. A pH titration monitored by UV spectroscopy revealed these values to be 7.6 ± 0.1 and 9.0 ± 0.1. These pK_a values are significantly lower than those of DTT and DTBA (Fig. 1).[23,28] Moreover, the lower of its two thiol pK_a values is closer to physiological pH than any known dithiol-based reducing agent (*vide infra*).[8,21,29]

BMMP was also found to be a potent reducing agent, albeit slightly less than predicted by our calculations. We found that the equilibrium reaction between reduced BMMP and oxidized DTT favors those species (rather than oxidized BMMP and reduced DTT) by ~1.2 kcal mol^{-1}, which corresponds to a reduction potential of $E^{\circ\prime}$ = (−0.301 ± 0.003) V (Fig. 1 and Fig. S2, ESI†) for oxidized BMMP. This $E^{\circ\prime}$ value is slightly less negative than both DTT and DTBA, and presumably results from the decreased enthalpic stability imparted by the two sp^2-hybridized carbons in its six-membered ring.[25] BMMP is, however, a much more potent reducing agent than common monothiols such as β-mercaptoethanol (βME), cysteamine, and L-glutathione.[16,30] To probe this difference, we equilibrated oxidized βME (βME^{ox}) with a slight excess of reduced BMMP for 24 h. Analysis by HPLC revealed the complete reduction of βME^{ox} (Fig. S3, ESI†).

Singh and Whitesides put forth *N*,*N*′-dimethyl-*N*,*N*′-bis(mercaptoacetyl)hydrazine (DMH; Fig. 1) as a faster disulfide-reducing agent than DTT.[21] Notably, their reported pK_a = 7.6 (8.9) and $E^{\circ\prime}$ = −0.300 V values for DMH are indistinguishable from those of BMMP (Fig. 1). The $E^{\circ\prime}$ value of DMH was corrected subsequently by Lees and Whitesides to be −0.272 V,[19] which is more consistent with its forming an 8-membered ring upon oxidation. To our knowledge, the pK_a value of DMH had not been examined again. Accordingly, we synthesized DMH so as to reexamine its properties and utility. Our observed value of $E^{\circ\prime}$ = (−0.262 ± 0.003) V for DMH was even higher than that of Lees and Whitesides, and confirms that DMH is a markedly weaker reducing agent than is BMMP, DTBA, or DTT (Fig. 1). Likewise, our value of pK_a = 8.0 ± 0.1 (Fig. 1) is higher than that reported by Singh and Whitesides, but consistent with values reported for mercaptoacetamido groups.[9,22,31]

Next, we analyzed the reactivity of BMMP with relevant disulfide bonds. At pH 7.0, BMMP reduced the disulfide bond



Scheme 1 Synthetic route to BMMP (**4**).

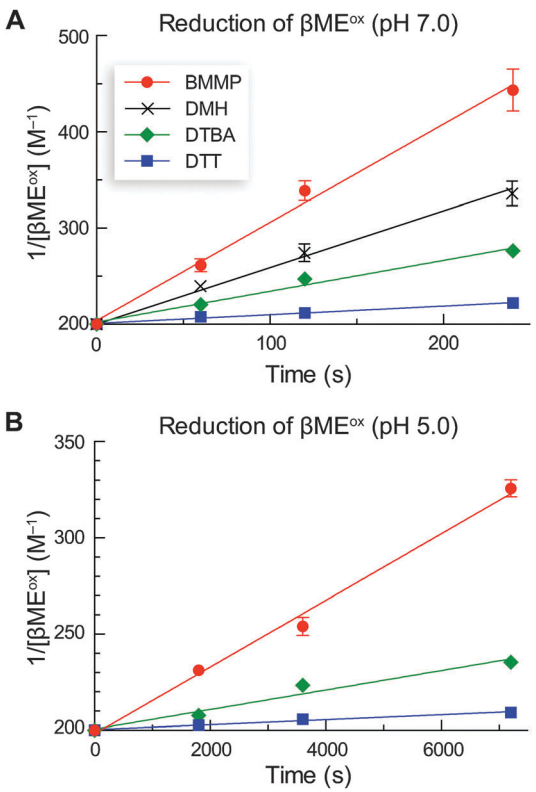


Fig. 2 Time-course for the reduction of βME^{ox} (5 mM) by BMMP, DMH, DTBA, or DTT (5 mM) in buffered water. (A) In 50 mM potassium phosphate buffer, pH 7.0: $k_{obs}^{BMMP}/k_{obs}^{DMH}$ = 1.8, $k_{obs}^{BMMP}/k_{obs}^{DTBA}$ = 3.2, $k_{obs}^{BMMP}/k_{obs}^{DTT}$ = 11.4. (B) In 50 mM sodium acetate buffer, pH 5.0: $k_{obs}^{BMMP}/k_{obs}^{DTBA}$ = 3.6, $k_{obs}^{BMMP}/k_{obs}^{DTT}$ = 14.1.

in βME^{ox} 11-fold faster than did DTT and 3-fold faster than did DTBA (Fig. 2A; Table S1, ESI†). Commensurate with their pK_a values, DMH reduced βME^{ox} faster than did DTT or DTBA but slower than did BMMP. At pH 5.0, BMMP reduced βME^{ox} 14-fold faster than DTT and 4-fold faster than did DTBA (Fig. 2B; Table S1, ESI†). These data highlight the broad pH-range at which BMMP can be utilized effectively.

Finally, we assessed the ability of BMMP to reduce disulfide bonds in two proteins. Papain is a cysteine protease that contains an active-site sulfhydryl group (Cys25) that needs to be in a reduced state for catalysis.[32] Treatment with *S*-methyl methanethiosulfonate generates an active-site mixed disulfide that results in complete loss of enzymatic activity.[33] This loss in activity, however, is reversible upon treatment with a disulfide-reducing agent. We found that BMMP reduced the mixed disulfide in papain 13-fold faster than did DTT and at a rate comparable to that of DTBA (Fig. 3A; Table S1, ESI†).[23]

Creatine kinase, like papain, is an enzyme that contains a thiol group (Cys283) that needs to be in a reduced state for catalytic function.[34–37] When treated with oxidized L-glutathione, the resulting mixed disulfide eliminates its enzymatic activity. Previously, we reported that the ability of DTBA to reduce this disulfide bond was compromised-presumably by unfavorable Coulombic interactions-resulting in a low reaction rate comparable to that of DTT.[23] In contrast to DTBA, BMMP is not cationic near neutral pH. (For example, the pK_a of the conjugate acid of 2,5-dimethylpyrazine is 2.1.[38]) Indeed, unfavorable Coulombic interactions were not apparent with BMMP, which was found to reduce the mixed disulfide in creatine kinase 6-fold faster than did DTT and 7-fold faster than did DTBA (Fig. 3B; Table S1, ESI†).

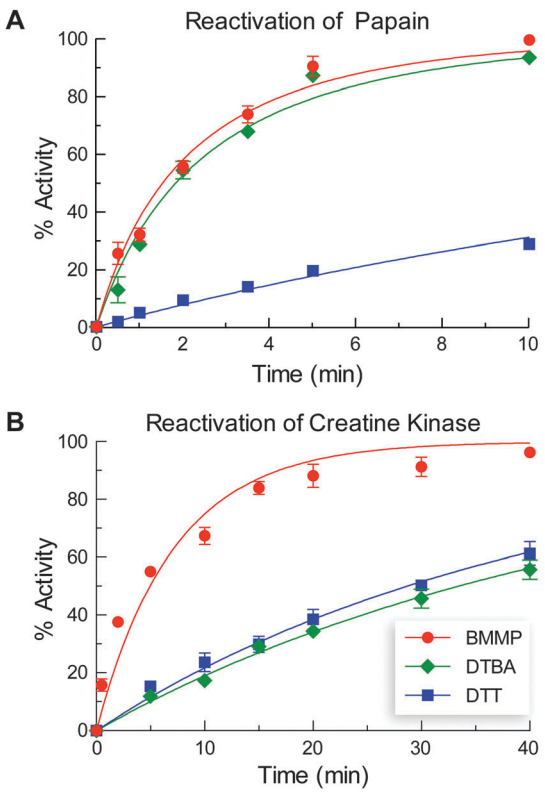


Fig. 3 Time-course for the reduction of a mixed disulfide in proteins by BMMP, DTBA, or DTT (7.8 μM) in 0.10 M imidazole–HCl buffer, pH 7.0, containing EDTA (2 mM). (A) Papain Cys25–S–S–CH_3 (4.4 μM): $k_{obs}^{BMMP}/k_{obs}^{DTBA}$ = 1.2, $k_{obs}^{BMMP}/k_{obs}^{DTT}$ = 13.1. (B) Creatine kinase Cys283–S–S–L-glutathione (0.34 μM): $k_{obs}^{BMMP}/k_{obs}^{DTBA}$ = 6.8, $k_{obs}^{BMMP}/k_{obs}^{DTT}$ = 5.8.

In conclusion, we have designed, synthesized, and characterized BMMP, a novel disulfide-reducing agent with high reactivity under biological conditions. The pyrazine ring of BMMP fuels its enhanced performance without Coulombic consequences. In a variety of relevant assays, BMMP reduces disulfide bonds ~10-fold faster than does DTT. Notably, the depressed thiol pK_a values of BMMP extended the pH range at which disulfide bonds can be reduced efficiently. These attributes render BMMP as an attractive reagent for the reduction of the disulfide bonds encountered in chemical biology.

This paper is dedicated to the memory of our colleague, W. W. (Mo) Cleland (1930–2013), on the 50th anniversary of his seminal publication on DTT (ref. 7). We are grateful to Robert W. Newberry for advice on computations. This work was supported by grant R01 GM044783 (NIH), and made use of the National Magnetic Resonance Facility at Madison, which is supported by grant P41 GM103399 (NIH).

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