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A Comparison between the Sulfhydryl Reductants Tris(2-carboxyethyl)phosphine and Dithiothreitol for Use in Protein Biochemistry[1]

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The newly introduced sulfhydryl reductant tris(2-carboxyethyl)phosphine (TCEP) is a potentially attractive alternative to commonly used dithiothreitol (DTT). We compare properties of DTT and TCEP important in protein biochemistry, using the motor enzyme myosin as an example protein. The reductants equally preserve myosin's enzymatic activity, which is sensitive to sulfhydryl oxidation. When labeling with extrinsic probes, DTT inhibits maleimide attachment to myosin and must be removed before labeling. In contrast, maleimide attachment to myosin was achieved in the presence of TCEP, although with less efficiency than no reductant. Surprisingly, iodoacetamide attachment to myosin was nearly unaffected by either reductant at low (0.1 mM) concentrations. In electron paramagnetic resonance (EPR) spectroscopy utilizing nitroxide spin labels, TCEP is highly advantageous: spin labels are two to four times more stable in TCEP than DTT, thereby alleviating a long-standing problem in EPR. During protein purification, Ni^{2+} concentrations contaminating proteins eluted from Ni^{2+} affinity columns cause rapid oxidation of DTT without affecting TCEP. For long-term storage of proteins, TCEP is significantly more stable than DTT without metal chelates such as EGTA in the buffer, whereas DTT is more stable if metal chelates are present. Thus TCEP has advantages over DTT, although the choice of reductant is application specific.

Preserving the reactive sulfhydryls of a protein in a reduced state is critical to the maintenance of function of many proteins. The most commonly used disulfide reductants are thiols themselves (1). The mechanism of disulfide reduction by thiols is an exchange of the thiolate anion (XS^-), as shown in Reactions [1] and [2].

$$XS^- + RSSR \rightarrow RSSX + RS^- \quad [1]$$

$$XS^- + RSSX \rightarrow XSSX + RS^- \quad [2]$$

The two most commonly used thiol reductants are 2-mercaptoethanol and dithiothreitol (DTT)[3] (Fig. 1). In the case of DTT, Reaction [2] is intramolecular and so involves the formation of two products from one reactant, with the DTT being converted to a stable cyclic disulfide. As a result, reduction of disulfide by DTT has an equilibrium constant of 1.3×10^4 (2), compared to an equilibrium constant close to unity for monothiol reductants such as 2-mercaptoethanol.

However, disulfide reduction by thiols can be inconvenient when reacting protein sulfhydryls with extrinsic probes. The –SH groups of the reductant compete directly with those of the protein for attachment of thiol-reactive labels such as maleimide and iodoacetamide derivatives. Therefore, thiol-based reductants are typically removed before the protein is labeled. In addition, the sulfhydryls of DTT readily reduce the nitroxide spin probes used in electron paramagnetic resonance (EPR) spectroscopy, thus eliminating the

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[3] Abbreviations used: DTT, dithiothreitol; TCEP, tris(2-caroxythyl)phosphine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoate; HMM, heavy meromyosin; TMRIA, tetramethylrhodamine-5-iodoacetamide; TMRM, tetramethylrhodamine-5-maleimide; SL, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperdinyl) maleimide; TBP, tributylphosphine; EPR, electron paramagnetic resonance.

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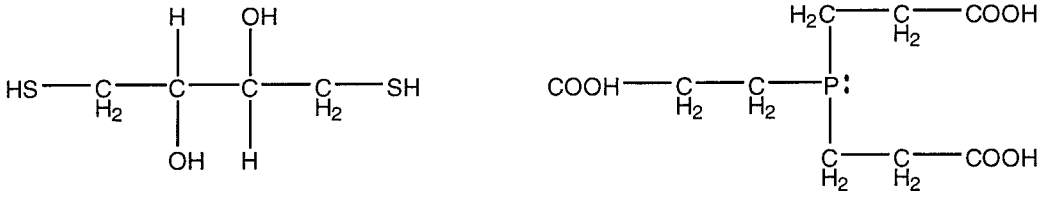

dithiothreitol (DTT) tris(2-carboxyethyl)phosphine (TCEP)

FIG. 1. Sulfhydryl reducing agents.

free radical that allows detection of probe orientation and mobility. A third problem is that DTT oxidation is catalyzed by ubiquitous metal ions, such as Fe^{3+} and Ni^{2+} (1, 3, 4), and so DTT is not stable in the reduced form for long times.

It may be possible to circumvent these problems by using trialkylphosphines as the reducing agent. In 1991, Burns *et al.* (5) described a convenient and large-scale synthesis of tris(2-carboxyethyl)phosphine (TCEP) (Fig. 1), and TCEP has been commercially available since 1992. In aqueous solutions, TCEP stoichiometrically and irreversibly reduces disulfides according to Reaction [3] (6, 7).

$$(CH_2CH_2COOH)_3P: + RSSR + H_2O \rightarrow (CH_2CH_2COOH)_3P{=}O + 2RSH \quad [3]$$

TCEP has been shown to be significantly more stable than DTT at pH values above 7.5, and a faster and stronger reductant than DTT at pH values below 8.0 (8). Thus TCEP is a useful reductant over a much wider pH range (1.5–8.5 (8)) than is DTT, although the buffer composition, including the presence of phosphates, can deleteriously affect TCEP stability (4, 5, 8). In addition, TCEP has been advertised as being unreactive with thiol-reactive compounds, thereby eliminating the need to remove it before labeling (9).

To quantify the advantages, if any, of TCEP over DTT, we compared these two reductants in several applications related to protein biochemistry: (1) stability at neutral pH, including in the presence of trace Ni^{2+} at concentrations expected to contaminate proteins eluted from Ni^{2+}-affinity columns; (2) the ability to preserve enzymatic activity, tested over a range of reductant concentrations which we find stabilizes enzymatic activity and which is widely used in biochemistry, 0.1–5.0 mM; (3) interference with attachment of labels to protein thiols; (4) reduction of nitroxide spin probes; and (5) the ability to cause unwanted protein degradation at elevated temperatures used in gel electrophoresis preparations. The second and third of these assays were performed using heavy meromyosin (HMM), a proteolytic fragment of the motor protein myosin. HMM is a good test case because its enzymatic activity is affected by oxidation or modification of its two most reactive sulfhydrals, Cys-697 and Cys-707, and there is a simple assay to determine its enzymatic activity (reviewed by Crowder and Cooke (10)). Specifically, modification of either of these sulfhydryls attenuates HMM's K^+-ATPase hydrolysis rate (11).

METHODS

Materials. DTT was purchased from Sigma (St. Louis, MO), and TCEP was purchased from Molecular Probes (Eugene, OR). Single isomers of both tetramethylrhodamine-5-iodoacetamide (TMRIA) and tetramethylrhodamine-5-maleimide (TMRM) were purchased from Molecular Probes (Eugene, OR). Stock solutions of these dyes were dissolved at mM levels in anhydrous dimethyl sulfoxide (Aldrich, Milwaukee, WI). 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperdinyl) maleimide (SL) were purchased from Aldrich.

Protein preparation. Myosin was prepared from rabbit back and leg muscles (12) and stored at −30°C in 0.3 M NaCl, 10 mM TES, 0.25 mM DTT, 50% glycerol. Heavy meromyosin (HMM) was prepared from skeletal myosin by standard methods (13, 14). Protein concentrations were determined using extinction coefficients of $2.39 \times 10^5\ M^{-1}\ cm^{-1}$ (myosin) or $2.35 \times 10^5\ M^{-1}\ cm^{-1}$ (HMM) at 280 nm.

ATPase assays. For K^+-ATPase assays, HMM (10.0 μM) was stored at room temperature in 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 50 mM Mops, pH 7.0. Samples were prepared with TCEP or DTT or no reductant. ATPase activities were measured by determining the rate of release of inorganic phosphate at 25°C (15). K^+-ATPase was assayed in 0.6 M KCl, 1.0 mM EDTA, 50 mM Mops, pH 7.0. The reaction was initiated by addition of 1.0 mM ATP. At 20, 60, 120, 180, 240, and 300 s, aliquots were quenched with 3.1% perchloric

acid. The rate of ATP hydrolysis was constant during this time.

Maleimide and iodoacetamide labeling. In the absence of nucleotide, only one of myosin's reactive sulfhydryls, Cys-707, is easily modified (11, 16, 17). Because HMM is a dimer, there are two Cys-707's per HMM molecule. HMM at 10 μM in 50 mM KCl, 2 mM $MgCl_2$, 50 mM Mops, pH 7.0 was reacted overnight on ice with TMRIA or TMRM. Labeling ratios (dye:Cys-707) ranged from 1.0 to 3.9 for TMRM (20 to 78 μM TMRM), and from 1.0 to 2.0 for TMRIA (20 to 40 μM TMRIA). In all cases, reductant concentration is in excess of label concentration so as to best detect inhibition of labeling by reductant. For data analysis, comparisons are made only between samples paired by labeling ratio and reductant concentration. Unattached TMR was removed using a G-75 Sephadex size-exclusion column (Amersham Pharmacia Biotech, Piscataway, NJ), and the molar ratio of bound TMR to HMM was determined by absorbance at 280 nm (primarily HMM absorbance with some TMR absorbance), 330 nm (scattering and TMR absorbance), 555 nm (TMR absorbance), and 650 nm (baseline) (18).

Reductant stability. Samples of TCEP and DTT were prepared at different temperatures (4 and 25°C), reductant concentrations (0.1, 0.5, and 1.0 mM) and solution compositions. Solutions consisted of metal ion (Mg^{2+}, Fe^{3+}, or Ni^{2+}) in the absence of chelating agent (50 mM KCl, 50 mM Mops, 2 mM $MgCl_2$ or various concentrations of $FeCl_3$ or $NiCl_2$: "Mg^{2+} buffer," "Fe^{3+} buffer" or "Ni^{2+} buffer," respectively), chelating agent in the absence of metal ion (50 mM KCl, 50 mM Mops, 1 mM EGTA: "EGTA buffer"), or both metal ion and chelating agent (50 mM KCl, 50 mM Mops, 1 mM EGTA, 2 mM $MgCl_2$ or various concentrations of $FeCl_3$ or $NiCl_2$: "Mg^{2+}/EGTA buffer," "Fe^{3+}/EGTA buffer," or "Ni^{2+}/EGTA buffer"). The pH was adjusted to 7.2 at the temperature appropriate for each sample. All samples were incubated in tubes with closed lids. Concentrations of the various chemical species existing in the different buffers were determined by solving the full nonlinear system of balance equations by a Newton iteration technique using the binding constants of Sillen and Martell (19).

The concentration of reduced TCEP or DTT remaining in each sample at various times was determined by reduction of DTNB (8). At each time point, an aliquot was removed from the reductant solution and added to a solution of 0.513 mM DTNB in 50 mM Tris–HCl, pH 9.0, prepared immediately before use. Final concentrations of reductant and DTNB were 20–30 and 410–500 μM, respectively. Both TCEP and DTT reduce DTNB rapidly and stoichiometrically at pH 9.0 to liberate two equivalents of 2-nitro-5-thiobenzoate (NTB) (8). Liberated NTB was quantified by the increase in absorption at 412 nm using a molar extinction coefficient of 14,150 M^{-1} cm^{-1}. Since DTNB is not stable at high pH, autoreducing to yield the colored NTB it was necessary to eliminate the contribution of DTNB autoreduction to 412 nm absorption. A blank (no TCEP or DTT) measured immediately prior to each of the TCEP or DTT readings was subtracted from the reading. It was routinely confirmed that the extent of DTNB autoreduction did not change during the (short) course of a TCEP or DTT reading by comparing the blank's absorption measured immediately prior to and following a TCEP or DTT reading.

The percentages of reductant oxidation presented in Table 1 and in Fig. 2 were determined by first fitting either a linear or exponential time course to data collected at 10 different time points (from 0 to 13 days), using the average of three measurements per time point. The percentage of reductant oxidized in 1 day (24 h) or 1 week (168 h) was then calculated from the best fit line.

EPR spectroscopy. EPR absorbance spectra were collected with an ER/200D spectrometer (Bruker, Inc., Billerica, MA) interfaced to a PC-AT computer. The incident microwave power in the TM cavity was 25 mW; peak-to-peak modulation amplitude, 2.0 Gauss; center field, 3460 Gauss; time constant, 500 ms; sweep time, 50 s; frequency modulation, 100 kHz; sweep width, 120 Gauss. Samples were prepared in which spin label (SL), free in a solution of 50 mM Mops, pH 7.0, was mixed with concentrations of TCEP or DTT varying from 0.1 to 0.2 to 1.0 mM. In all samples, the final SL concentration was 0.1 mM. The first-derivative EPR spectrum was collected for each sample at 0, 1, 2, 3, 24, 48, and 170 h after mixing, and samples were stored on ice in the dark between measurements. For each sample, the three peaks of the first-derivative spectrum were averaged to provide a value proportional to the total EPR signal intensity. This parameter was compared to a standard of SL with no added reductant to provide a measure of SL reduction by TCEP or DTT with time.

Proteolysis by reductants. To test the capacity of TCEP to break peptide bonds under conditions commonly used in gel electrophoresis, pairs of samples were prepared which contained 10 μM myosin in 0.2 M 2-mercaptoethanol, 1 M urea, 1.3% SDS, 60 mM Tris–HCl, pH 6.8, plus TCEP varying in concentration from 0.1 to 50 mM. A pair of samples was also prepared without added TCEP. After a 60-min incubation on ice, one sample of each pair was boiled for 10 minutes while the other sample was left on ice. All samples were run on both 10 and 12.5% SDS gels.

Linear regression. All curve fits were determined by least squares using Kaleidagraph (Abelbeck Software, Reading, PA). All statistics are reported as means $\pm$ standard error, unless otherwise specified.

TABLE 1

Reductant Stability[a]

Temperature (Initial reductant concentration)	% DTT or TCEP (oxidized in 1 week) Mg^{2+} buffer (mean ± SD)	Mg^{2+}/EGTA buffer (mean ± SD)	EGTA buffer (mean ± SD)
4°C			
1.0 mM DTT	28 ± 2		
0.5 mM DTT	41 ± 3	12 ± <1	12 ± 1
0.1 mM DTT	76 ± 5	4 ± <1	3 ± 2
1.0 mM TCEP	3 ± 1		
0.5 mM TCEP	4 ± 1	10 ± 2	14 ± 3
0.1 mM TCEP	5 ± 3	12 ± 3	14 ± 4
25°C			
1.0 mM DTT	100 ± 3		
0.5 mM DTT	100 ± <1	39 ± 4	38 ± 3
0.1 mM DTT	100 ± <1	11 ± 3	13 ± 3
1.0 mM TCEP	9 ± 2		
0.5 mM TCEP	8 ± 2	72 ± 6	72 ± 7
0.1 mM TCEP	11 ± 4	68 ± 6	70 ± 5

[a] The concentration of reduced DTT or TCEP remaining in each sample at various times was determined by reduction of DTNB as described under Methods. Oxidation of DTT was linear with time under all conditions (Mg^{2+}, Mg^{2+}/EGTA, and EGTA buffers). Oxidation of TCEP was exponential with time under all conditions. To allow comparison between the rates of reductant oxidation, results are presented as the percentage of reductant oxidized after 1 week. All values are reported as means ± standard deviation (mean ± SD). Buffer compositions are given under Methods.

RESULTS

Reductant Stability

Both TCEP and DTT will auto-oxidize under certain conditions. To characterize reductant stability over a range of commonly used conditions, and to determine the mechanism of oxidation, we measured the stability of the reduced form of both DTT and TCEP as a function of solution composition, reductant concentration, and temperature. Under some conditions, reductant oxidation is linear with time (zero-order kinetics with respect to DTT concentration), while under other conditions reductant oxidation proceeds exponentially, as discussed further, below. Because zero-order and first-order rate constants cannot be directly compared, and since what is of practical interest is the amount of reductant remaining after a given period of time, Table 1 and Fig. 2 present the results of these experiments as a percentage of reductant oxidized after 1 day or after 1 week, so as to facilitate comparison of results obtained under the various conditions.

We found that decreased temperature increases the stability of both DTT and TCEP. The results in Table 1 indicate that TCEP is reasonably stable at 4°C (less than 15% oxidation in one week), under all conditions, an important result for long-term storage of proteins in the presence of reductant. DTT is also stable at 4°C (less than 15% oxidation in 1 week), but only in the presence of a chelating agent such as EGTA.

The presence of a metal ion oxidizing agent, such as Fe^{3+} or Ni^{2+}, greatly decreases DTT stability. Figure 2A illustrates that even small amounts of added Fe^{3+} or Ni^{2+} (1 nM to 200 μM) cause oxidation of more than half of a 0.5 mM DTT stock in 1 day at 25°C. Thus both Fe^{3+} and Ni^{2+} appear to act as catalysts for DTT oxidation. As shown in Fig. 2A, the rate of DTT oxidation

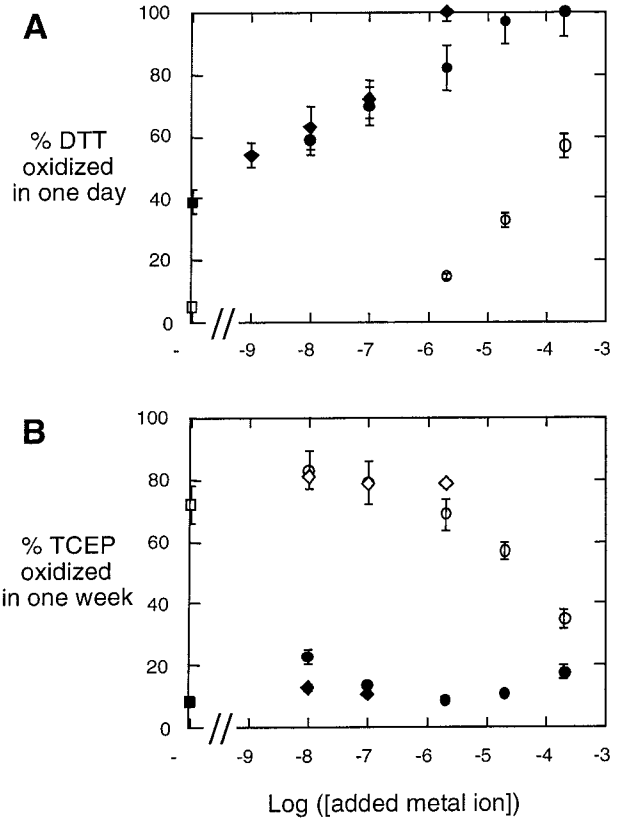

FIG. 2. Reductant oxidation in the presence of added Fe^{3+} or Ni^{+2}. Filled squares, Mg^{2+} buffer (no added Fe^{3+} or Ni^{2+}—see data on *Y* axis); filled circles, Fe^{3+} buffer; filled diamonds, Ni^{2+} buffer. Open squares, Mg^{2+}/EGTA buffer (no added Fe^{3+} or Ni^{2+}—see data on *Y* axis); open circles, Fe^{3+}/EGTA buffer; open diamonds, Ni^{2+}/EGTA buffer. All points are shown as means ± standard deviation. Abscissa indicates the log of the concentration of added Fe^{3+} or Ni^{2+}. (A) Percentage of DTT oxidized in 1 day at 25°C. The rate of DTT oxidation increased with increasing concentrations of added Fe^{3+} or Ni^{2+}. The presence of the metal chelator EGTA significantly improved DTT stability. The time course of DTT oxidation was exponential with time in the presence of added Fe^{3+} and Ni^{2+} (Fe^{3+}, Ni^{2+}, Fe^{3+}/EGTA, Ni^{2+}/EGTA buffers) but linear with time without added Fe^{3+} or Ni^{2+} (Mg^{2+} and Mg^{2+}/EGTA buffers). (B) Percentage of TCEP oxidized in 1 week at 25°C. TCEP is generally more stable than DTT: note that the percentage of TCEP oxidized in 1 week in (B) is compared to the percentage of DTT oxidized in 1 day in (A). The presence of the metal chelator EGTA significantly reduced TCEP stability. The time course of TCEP oxidation was exponential with time under all conditions. Buffer compositions are as given under Methods. Concentrations of reduced DTT or TCEP were determined as in Table 1.

increases approximately linearly with the log of the added Fe^{3+} or Ni^{2+} concentration. Without added Fe^{3+} or Ni^{2+} (in Mg^{2+} buffer; Fig. 2A, filled square), a 0.5 mM DTT stock is 40% oxidized in 1 day at 25°C, presumably because the glass distilled water used in these experiments contains trace amounts of metal ion oxidizing agents. Addition of chelating agent (see below) greatly increases stability, as would be expected if DTT oxidation is due to free metal ions. Mg^{2+}, however, does not significantly oxidize DTT, even at the high Mg^{2+} concentrations used here: DTT stability is equal in the Mg^{2+}/EGTA and EGTA buffers (Table 1). (The Mg/EGTA buffer contains 1.8 mM free Mg^{2+} because in both Mg/EGTA and EGTA buffers EGTA exists predominantly in the $H_2 \cdot$ EGTA form.)

DTT stability is greatly increased by the presence of EGTA (Table 1, cf. DTT stability in Mg^{2+} vs Mg^{2+}/EGTA buffers; Fig. 2A, cf. Fe^{3+} vs Fe^{3+}/EGTA buffers), presumably because EGTA is a strong chelating agent for free metal ions (EGTA–Fe^{3+}: log K = 20.5; EGTA–Ni^{2+}: log K = 13.5 (19)).

In the presence of added Fe^{3+} or Ni^{2+}, DTT oxidation is exponential, indicating that it is the frequency of DTT–catalyst collisions that dictates the oxidation rate. Without added Fe^{3+} or Ni^{2+}, DTT oxidation is linear with time, in agreement with previous measurements of DTT oxidation (3, 4, 20). The zero-order kinetics of this case indicate that a step separate from DTT–catalyst collision is rate-limiting to oxidation. Taken together, these data suggesting that the catalysis rate is dependent on catalyst concentration, and that the catalyst may be more than one metal ion. In the presence of EGTA, DTT oxidation appears to depend on the square of the DTT concentration, perhaps due to disulfide formation between two DTT molecules.

TCEP is generally much more stable than DTT: Fig. 2B plots the percentage of TCEP oxidized in one week compared to the percent of DTT oxidized in one day plotted in Fig. 2A. In contrast to DTT, the stability of TCEP is greatly reduced by the presence of EGTA (Table 1, cf. TCEP stability in Mg^{2+} vs Mg^{2+}/EGTA and EGTA buffers; Fig. 2B, cf. Fe^{3+} vs Fe^{3+}/EGTA buffers, and Ni^{2+} vs Ni^{2+}/EGTA buffers). In the absence of a chelating agent, TCEP is quite stable even at 25°C, and neither Fe^{3+} nor Mg^{2+} has a deleterious effect on TCEP stability (Fig. 2B, filled symbols). In the presence of EGTA, however, the majority of a 0.5 mM TCEP sample is oxidized within 1 week at 25°C (Fig. 2B, open symbols). Thus, a metal chelate, as opposed to a free metal, appears to be involved in the catalytic oxidation of TCEP. Indeed, oxidation by metal-chelate complexes are well known (21, 22).

To determine in what form EGTA can act as an oxidation catalyst, TCEP was incubated with EGTA alone, and with EGTA in the presence of Mg^{2+} (2 mM), Fe^{3+} (10 nM to 200 μM), or Ni^{2+} (10 nM to 2 μM). The results indicate that neither Fe^{3+} nor Ni^{2+} nor Mg^{2+}, complexed with EGTA, participates in the catalytic oxidation of TCEP. In fact, as the Fe^{3+} concentration is raised from 10 nM to 200 μM in the presence of 1 mM EGTA, the rate of TCEP oxidation is decreased by more than a factor of two, suggesting that the higher Fe^{3+} concentrations remove more EGTA from a form in which it can catalyze TCEP oxidation (Fig. 2B, open circles). An increase in the Ni^{2+} concentration from 10 nM to 2 μM caused no change in the rate of TCEP oxidation (Fig. 2B, open diamonds). The Mg/EGTA buffer contains approximately 50 μM Mg · EGTA and another 50 μM H · Mg · EGTA. However, TCEP stability in the Mg/EGTA buffer is identical to TCEP stability in EGTA buffer. Thus Mg complexed with EGTA does not appear to catalyze TCEP oxidation. From the present experiments, the most likely catalyst for TCEP oxidation appears to be EGTA in one of its ionized acid forms (EGTA, H · EGTA, H_2 · EGTA). Under all conditions, TCEP oxidation is exponential with time and the amount of TCEP oxidized per unit time is linear in TCEP concentration (reflected as a constant percentage of TCEP oxidized per unit time in Table 1). Both observations support first-order reaction kinetics.

Protection of Enzymatic Activity

The K^+-ATPase activity of the motor enzyme myosin, and of its proteolytic fragment HMM, is sensitive to sulfhydryl oxidation. The presence of a reducing agent significantly stabilized this activity compared to no reductant, and we find that TCEP and DTT preserved this activity equally well. HMM at 10 μM (3.5 mg/mL) was stored at room temperature in the presence of 0, 0.1, or 1.0 mM reductant, and the K^+-ATPase activity of each sample was monitored over time. Although storage at room temperature and a reduced protein concentration are not optimal for preserving enzymatic activity, these conditions are frequently used in spectroscopic studies. For all conditions, the ATPase rate declined linearly with time. Reductant concentrations of 0.1 and 1.0 mM were equally effective at preserving the enzymatic activity of HMM. (In both cases reductant was in excess of reactive protein sulfhydryls.) The presence of (excess) reductant slowed the loss of ATPase activity by a factor of 1.8 ± 0.2 (n = 4) relative to no reductant. Although the rate of decline of ATPase activity varied significantly from one protein batch to another, ranging from 2.5 to 8.6% per hour for HMM stored without reductant, the presence of reductant consistently improved the active lifetime of the enzyme. Additional studies performed using whole myosin stored at 4°C support the conclusion that reductants preserve the activity of myosin, although rates of ATPase decline for samples stored at 4°C were significantly slower than for samples stored at room temperature (0.2% per hour for 8.4 μM (4.4 mg/mL) myosin stored on ice without reductant). At 4°C, reductant

prolonged ATPase activity by a factor of 8.0 ± < 0.1 (n = 2). Under all conditions, the enzymatic activity of samples stored with TCEP was indistinguishable from that of samples stored with an equal amount of DTT.

Effect on Protein Labeling

One of the disadvantages of reductants is that they may interfere with the attachment of thiol-reactive probes to proteins. Here we use the extent of covalent attachment of both a maleimide (–M) and an iodoacetamide (–IA) tetramethylrhodamine dye (TMR) to HMM's reactive sulfhydryls in the presence of reductant concentrations shown above to preserve HMM's enzymatic activity. These reductant concentrations, 0.1–5.0 mM, are commonly used in protein biochemistry, and mean that reductant is generally in significant excess of both protein and label.

Figures 3A and 3B show representative labeling of HMM with TMRM and TMRIA, respectively, in the presence of 0.1 mM TCEP, 0.1 mM DTT, and no reductant. While absolute levels of labeling varied significantly between protein preparations and dye lots, the ratio of labeling with TCEP vs DTT vs no reductant was very reproducible.

When labeling with maleimide (Fig. 3A), both TCEP and DTT substantially decreased labeling efficiency compared to no reductant. This inhibition was more pronounced with DTT; at all dye concentrations tested, DTT allowed only 20–30% of the labeling observed with TCEP. For example, at a labeling stoichiometry of 2 TMRM:1 Cys-707, the presence of 0.1 mM TCEP allowed 35% labeling and 0.1 mM DTT allowed 9% labeling, while 95% labeling was achieved in the absence of reductant. Higher reductant concentrations further inhibited maleimide attachment to HMM. For example, labeling efficiencies at 1.0 mM reductant were decreased twofold for both TCEP and DTT compared to 0.1 mM reductant. Over all reductant concentrations tested (0.1–5.0 mM), TCEP proved less deleterious to maleimide attachment than TCEP; TCEP allowed 3.6 ± 0.2 (n = 7) times greater labeling of HMM than did an equal amount of DTT.

When labeling with iodoacetamide (Fig. 3B), the presence of either 0.1 mM TCEP or 0.1 mM DTT had little effect on labeling efficiency. For example, at 1 TMRIA:1 Cys-707, labeling efficiency was 83, 86, and 90% in the presence of 0.1 mM TCEP, 0.1 mM DTT, and no reductant, respectively. The lack of interference from DTT is quite surprising, given that DTT contains a free thiol, but was very reproducible. Higher reductant concentrations reduced labeling efficiency considerably; labeling in the presence of 1.0 mM DTT or TCEP, for example, was two- to threefold less efficient than without reductant. Furthermore, higher reductant concentrations demonstrated that TCEP is in fact more deleterious to iodoacetamide attachment than is DTT; labeling in the presence of 1.0 or 5.0 mM TCEP was 0.71 ± 0.01 (n = 3) times as efficient as labeling in the presence of an equal amount of DTT.

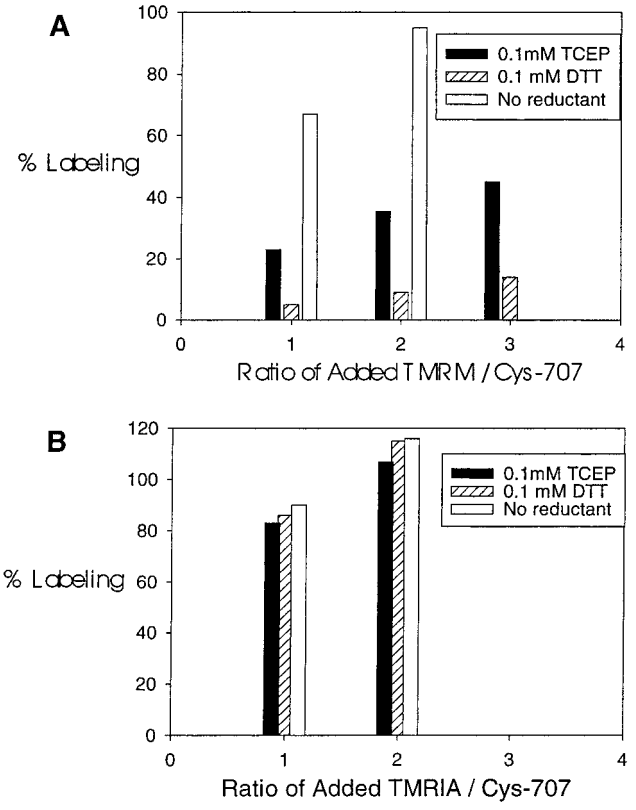

FIG. 3. Cysteine labeling by TMR dye in the presence of 0.1 mM reductant. The percentage modification of the reactive cysteine, Cys-707, of HMM with (A) TMRM or (B) TMRIA is determined at various ratios of added dye/Cys-707. For both dyes, the labeling reaction proceeded with 20 μM Cys-707 for 2 h on ice in the presence of 0.1 mM TCEP (filled bars), 0.1 mM DTT (hatched bars) or no reductant (open bars). Unattached dye was removed by size-exclusion column, and final dye and protein concentrations were determined by absorption as described under Methods. Both DTT and TCEP interfered significantly with maleimide attachment, DTT more so than TCEP (A). At 0.1 mM, neither reductant significantly inhibited iodoacetamide attachment (B).

Reduction of Nitroxide Spin Labels

Nitroxide spin labels contain an unpaired electron spin. In the presence of a strong magnetic field, this free radical gives rise to an absorption spectrum consisting of two or three sharp peaks. The shape of this spectrum yields information on the orientation and mobility of the protein to which the spin label is attached. The amplitude of this EPR spectrum is directly proportional to the number of unpaired spins in the sample. Reduced spin label does not contain an unpaired electron and so will not contribute to EPR signal intensity. Thus spin label reduction by sulfhydryl reductants included in the protein sample has been a long-standing problem in EPR spectroscopy.

TABLE 2

Effect of Reductant on Spin Label Intensity[a]

Reductant concentration	% Reduction in EPR signal intensity 3 h (mean ± SD)	48 h (mean ± SD)
0.1 mM DTT	10 ± 3	22 ± 5
0.1 mM TCEP	5 ± 2	3 ± 2
1.0 mM DTT	30 ± 5	94 ± 10
1.0 mM TCEP	14 ± 4	47 ± 10

[a] The intensity of the electron paramagnetic resonance (EPR) signal was measured for samples of spin label incubated with DTT or TCEP. As the spin label is reduced by DTT or TCEP, the intensity of the EPR signal declines: reduced spin label does not contribute to EPR signal intensity. EPR signal intensity declined nonlinearly with time, with a rapid initial decline followed by a slower loss of signal. Total EPR signal intensity was measured as the average of the three peaks of the first-derivative spectrum. All values are reported as means ± standard deviation (mean ± SD).

Samples were prepared in which spin label (SL), free in solution, was mixed with TCEP or DTT at reductant concentrations of 0.1 or 1.0 mM and stored on ice. At 1.0 mM, TCEP reduced SL about half as quickly as an equal amount of DTT (Table 2). After 2 days, 1.0 mM TCEP decreased EPR signal intensity to about half its initial value, while an equal amount of DTT almost completely eliminated the EPR signal in this time. Reduction of SL decreased with decreasing reductant concentration. At 0.1 mM, TCEP did not significantly reduce SL over the course of 7 days (<10% reduction in signal intensity), whereas DTT at this concentration reduced EPR signal intensity by 35% of its initial value in this time. During EPR experiments in which spin label is attached to myosin light chains in muscle cells, we routinely use 0.1 mM TCEP, and find that this amount of TCEP preserves the enzymatic activity of the myosin and has a negligible deleterious effect on the spin label (data not shown).

Due to DTT auto-oxidation, the results presented in Table 2 slightly underestimate the true reduction of SL by a fixed amount of DTT. Like the HMM labeling buffers, SL samples were maintained at 4°C and contained no EGTA. Therefore, from Table 1, we expect ~20% oxidation of 0.1 mM DTT, ~8% oxidation of 1.0 mM DTT, and ~1% oxidation of TCEP over the course of 48 h. These minor corrections emphasize the fact that TCEP reduces SL more slowly than DTT for the same reductant concentration.

Ability of Reductants to Break Peptide Bonds

Some investigators have suggested that TCEP can break peptide bonds at very high temperatures. It is known that DTT will not cause proteolysis, and SDS–PAGE protein samples, which are commonly heated to 95°C for 5 min, are usually prepared with high concentrations of DTT. To determine if TCEP would cause protein cleavage, samples containing 10 μM myosin and up to 50 mM TCEP were boiled for 10 min. No effect of TCEP concentration on protein integrity could be detected on 10 or 12.5% SDS gels (data not shown).

DISCUSSION

In 1991, Burns *et al.* (5) presented a straightforward and convenient synthesis of TCEP, and showed TCEP to be a nonvolatile and water-soluble reducing agent. Thus, TCEP avoided two of the most undesirable qualities of tributylphosphine (TBP), which was, previous to TCEP, the most commonly used trialkylphosphine reducing agent (7). TCEP is now commercially available. Despite its potential value, the use of TCEP in biochemical applications has been limited (22, 23), with most studies employing the thiol reductant DTT instead. Here, we present a comparison between DTT and TCEP in terms of their (1) stability at neutral pH, (2) ability to preserve enzymatic activity, (3) interference with attachment of labels to protein thiols, (4) reduction of nitroxide spin probes, and (5) ability to cause unwanted protein degradation at elevated temperatures used in gel electrophoresis preparations.

Our intent is to provide information that will help researchers decide on a reductant protocol appropriate for their specific application. These experiments show that reductant stability, an important consideration at room temperatures, can be optimized by the proper choice of reductant and buffer. Both reductants effectively protect protein sulfhydryls, and so TCEP is preferred over DTT if working with spin labels or labeling with maleimides, although TCEP is not completely benign in labeling reactions. If purchased commercially, however, TCEP is two to three times more expensive than DTT (9, 24, 25).

Previous studies have found that the stability of TCEP and DTT is affected by the presence or absence of metal chelates, although the mechanism and even direction of the effect is controversial (3, 4, 8, 20, 26). We find that TCEP is significantly more stable than DTT in the absence of a metal chelator, in agreement with previous results (8). Our experiments were conducted using glass distilled water, which likely contains trace amounts of metal ions. For this reason, we and others commonly include a chelating agent for free metals in the storage buffer of sensitive proteins. We find that the presence of EGTA increases the stability of DTT but decreases TCEP stability because DTT oxidation is catalyzed by a free metal oxidizing agent, such as Fe^{3+} or Ni^{2+}, while TCEP oxidation is catalyzed by a metal chelator such as EGTA. However, neither $Mg^{2+} \cdot$ EGTA nor $Fe^{3+} \cdot$ EGTA nor $Ni^{2+} \cdot$ EGTA is the chemical species responsible for TCEP oxidation.

The specific effect of Ni^{2+} on the stability of DTT and TCEP was tested because Ni^{2+} is used in affinity col-

umns employed in protein purification. The Qiagen (Valencia, CA) system, for example, uses nickel–nitriloacetic acid (Ni–NTA) metal-ion affinity chromatography to purify biomolecules tagged with six consecutive histidine residues (6× His tag). The affinity of the NTA matrix for Ni^{2+} is on the order of 10^9 M^{-1}. Therefore, a column 90% saturated with Ni^{2+} will contain about 10 nM free Ni^{2+} that will elute with the protein. Proteins purified by metal-ion chromatography are thus expected to contain trace amounts of the metal ion. Here we have shown that such trace metal contaminants are very harmful to DTT, but not to TCEP.

In vivo, proteins are in a reducing environment, so sulfhydryl groups are maintained in their reduced form. In solution, reducing agents must be added to mimic *in vivo* conditions. We find that both TCEP and DTT at concentrations ranging from 0.1 to 5.0 mM significantly preserve the enzymatic activity of myosin, particularly at room temperature, and are therefore useful additions to protein storage and assay buffers.

TCEP has been advertised as being noncompetitive with protein sulfhydryls for attachment of thiol-reactive dyes (9). This property would give TCEP a great advantage over DTT, and so we compared reductant interference with cysteine labeling using reductant concentrations shown above to protect enzymatic activity (0.1–5.0 mM). We found that while DTT significantly inhibits the reaction of maleimide probes with myosin, maleimide attachment to myosin could be achieved in the presence of TCEP, although with significantly reduced efficiency compared to no reductant: threefold less labeling in the presence of 0.1 mM TCEP than with no reductant. At low concentrations (0.1 mM), both reductants interfered very slightly with iodoacetamide labeling of myosin, although higher concentrations of both DTT and TCEP (≥1.0 mM) reduced labeling efficiency substantially (two- to threefold). Competition of TCEP for reaction with iodoacetamides is expected, as the reaction of trialkylphosphines with alkyl halides is well characterized (as in the Wittig reaction (27)).

Thiol reductants added to reduce protein sulfhydryls have the unfortunate consequence of also reducing nitroxide spin probes. This has been a continuing problem in the field of EPR spectroscopy. TCEP alleviates this problem by reducing nitroxide spin probes half as quickly as DTT. At 0.1 mM, a concentration shown to have a protective effect on myosin ATPase activity, TCEP caused no significant reduction of spin label in 1 week.

In summary, TCEP has a number of advantages over DTT. TCEP is clearly the superior reductant when Ni^{2+}-column purification or EPR spin probes are used, or when labeling proteins with maleimides if reductant is not removed prior to labeling. However, the choice of reductant will depend on the chemical environment, duration, and nature of the specific experiment.

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