New Water-Soluble Phosphines as Reductants of Peptide and Protein Disulfide Bonds: Reactivity and Membrane Permeability†

Daniel J. Cline, Sarah E. Redding, Stephen G. Brohawn, James N. Psathas, Joel P. Schneider, and Colin Thorpe*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

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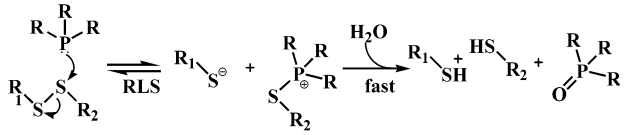
ABSTRACT: Tris(2-carboxyethyl)phosphine (TCEP) is a widely used substitute for dithiothreitol (DTT) in the reduction of disulfide bonds in biochemical systems. Although TCEP has been recently shown to be a substrate of the flavin-dependent sulfhydryl oxidases, there is little quantitative information concerning the rate by which TCEP reduces other peptidic disulfide bonds. In this study, mono-, di-, and trimethyl ester analogues of TCEP were synthesized to evaluate the role of carboxylate anions in the reduction mechanism, and to expand the range of phosphine reductants. The effectiveness of all four phosphines relative to DTT has been determined using model disulfides, including a fluorescent disulfide-containing peptide (H_3N^+-VTWCGACKM-NH_2), and with protein disulfide bonds in thioredoxin and sulfhydryl oxidase. Mono-, di-, and trimethyl esters exhibit phosphorus p*K* values of 6.8, 5.8, and 4.7, respectively, extending their reactivity with the model peptide to correspondingly lower pH values relative to that of TCEP (p*K* = 7.6). At pH 5.0, the order of reactivity is as follows: trimethyl- > dimethyl- > monomethyl- > TCEP ≫ DTT; tmTCEP is 35-fold more reactive than TCEP, and DTT is essentially unreactive. Esterification also increases lipophilicity, allowing tmTCEP to penetrate phospholipid bilayers rapidly (>30-fold faster than DTT), whereas the parent TCEP is impermeant. Although more reactive than DTT toward small-molecule disulfides at pH 7.5, all phosphines are markedly less reactive toward protein disulfides at this pH. Molecular modeling suggests that the nucleophilic phosphorus of TCEP is more sterically crowded than the thiolate of DTT, contributing to the lower reactivity of the phosphine with protein disulfides. In sum, these data suggest that there is considerable scope for the synthesis of phosphine analogues tailored for specific applications in biological systems.

The water-soluble phosphine, tris(2-carboxyethyl)phosphine (TCEP)[1] (*1*, *2*), is now widely employed as a reductant of disulfide bonds in a variety of peptide, protein, and cellular systems (*3*−*7*). Model studies show that reduction of disulfides by phosphines is initiated by rate-limiting formation of a thiophosphonium salt (*1*, *8*−*11*). Subsequent, rapid, hydrolysis releases the second thiol fragment and the phosphine oxide (Scheme 1).

TCEP provides a particularly useful complement to dithiothreitol (DTT) (*1*, *3*−*5*) because the phosphine is comparatively air-stable, and an essentially irreversible reductant of disulfide bonds. Unlike oxidized DTT, oxidized TCEP cannot directly catalyze thiol−disulfide exchange reactions, and this, together with its superior reactivity at low pH values, has made it the reagent of choice for a number of biochemical applications. In addition to its use in protein chemistry, TCEP is finding more prevalent application as a reductant in cellular systems (*6*, *7*, *12*−*15*).

Scheme 1: Disulfide Reduction by Phosphines



We recently observed that TCEP is a substrate of avian sulfhydryl oxidase (*16*). To place the rate constant for reduction of the enzyme by TCEP in context, we needed comparable values for other disulfide-containing peptides and proteins. However, we could not find relevant literature values for second-order rate constants for any peptide or protein disulfides. Thus, the first aim of this paper was to obtain kinetic data for the reduction of model disulfides, peptides, and proteins by this increasingly important biochemical reductant. Since TCEP has sometimes been viewed as a reductant kinetically superior to DTT, we have included this dithiol for comparison. Second, we wanted to evaluate whether the carboxylate moieties of TCEP were important for reduction efficacy. This led us to synthesize and characterize three methyl ester derivatives of TCEP (Figure 1). These new water-soluble phosphines show enhanced reactivity at low pH values compared to TCEP and may, therefore, be useful when additional suppression of dithiol reoxidation and disulfide exchange is desired. Finally, this

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* To whom correspondence should be addressed. Telephone: (302) 831-2689. Fax: (302) 831-6335. E-mail: cthorpe@udel.edu.

[1] Abbreviations: DTNB, 5,5′-dithio(2-nitrobenzoic acid); DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; GSH, glutathione; QSOX, flavin-dependent sulfhydryl oxidases homologous to Quiescin Q6; Trx, thioredoxin; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; mm-, dm-, and tmTCEP, mono-, di-, and trimethyl esters of TCEP, respectively; TCNP, tris(2-cyanoethyl)phosphine.

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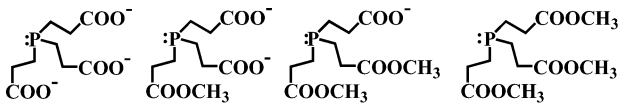


FIGURE 1: Water-soluble phosphines used in this work. The predominant ionic form for TCEP and its mono-, di-, and trimethyl analogues is shown for pH 8.0.

paper shows that while TCEP is essentially impermeant, tmTCEP crosses phospholipid bilayers considerably faster than DTT, a reagent widely used as a permeant disulfide reductant in cellular systems. Thus, TCEP esters may provide additional reagents for the modulation of intracellular redox poise. Overall, this study shows that there is considerable scope for the design of new water-soluble phosphines for specific applications in biochemistry and cell biology.

EXPERIMENTAL PROCEDURES

Materials. TCEP-HCl was purchased from Pierce, Fluka, and Molecular Probes and, when necessary, recrystallized from concentrated hydrochloric acid prior to use. Tris-(hydroxypropyl)phosphine was from Calbiochem. All other chemicals were obtained from Sigma-Aldrich. Solvents were from Fisher Scientific. The oxidized fluorescent probe peptide (H_3N^+-VTWCGACKM-NH_2) was synthesized and purified as described previously (*17*). Dowex 50W cation-exchange resin (Bio-Rad) was soaked in methanol for 1 h. Smaller beads were discarded by decantation, and the remaining resin was treated with 5 M HCl for 20 min. The resin was washed on a Buchner funnel with 10 volumes of water followed by 10 volumes of methanol. The resin was then used immediately or stored capped for later use. Avian sulfhydryl oxidase was purified as described previously (*18*, *19*).

Synthesis of TCEP Esters. TCEP-HCl (50 mg in 1 mL of methanol) was stirred at room temperature with 50 mg of treated Dowex 50W cation-exchange resin. Esterification was monitored by TLC using a $CHCl_3$/CH_3OH/CH_3COOH mixture (85:15:0.1) and visualized with a DTNB stain [10 mM DTNB in 50 mM phosphate and 0.3 mM EDTA (pH 7.5), diluted 1:1 with ethanol]. Reaction times varied from 1 to 24 h, depending on the desired extent of esterification; longer reaction times favor the trimethyl ester. When the desired ratio of esterified products was obtained, the resin was removed by filtration and the filtrate was concentrated by rotary evaporation to afford a pale yellow oil. The oil was dissolved in 0.1% TFA in water and purified using a Varian Pro-Star automated HPLC system equipped with a C-18 semipreparative column (Vydac). Products were separated using a 40 min linear gradient from 0 to 18% acetonitrile in 0.1% TFA following absorbance at 220 nm. TCEP and its mono-, di-, and trimethyl esters elute at 10, 15, 17, and 20 min, respectively. Pure esters were recovered by lyophilization and stored at −20 °C under nitrogen. The esters are oils at room temperature. TCEP and its methyl esters were characterized by NMR and ESI-MS.

TCEP: ^{1}H NMR (D_2O, 400 MHz) δ 2.44 (dt, J = 14.00 and 7.02 Hz, 6H), 2.73 (dt, J = 18.27 and 7.08 Hz, 6H); ^{13}C NMR (D_2O, 100 MHz) δ 13.77 (d, J = 52.9 Hz), 27.19 (d, J = 5.4 Hz), 175.15 (d, J = 8.3 Hz); ESI-MS m/z 251.06734 [$(M + H)^+$, calcd for $H^+P(CH_2CH_2COOH)_3$, 251.06845].

mmTCEP: ^{1}H NMR (D_2O, 360 MHz) δ 2.58 (dt, J = 13.68 and 7.08 Hz, 4H), 2.62 (dt, J = 13.32 and 7.08 Hz, 2H), 2.86 (dt, J = 18.36 and 7.08 Hz, 4H), 2.91 (dt, J = 17.64 and 7.08 Hz, 2H), 3.74 (s, 3H); ^{13}C NMR (D_2O, 91 MHz) δ 16.50 (d, J = 51.8 Hz), 16.63 (d, J = 52.5 Hz), 29.74 (d, J = 4.8 Hz), 30.18 (d, J = 4.8 Hz), 55.26, 176.42 (d, J = 9.0 Hz), 178.27 (d, J = 8.0 Hz); ESI-MS m/z 265.08445 [$(M + H)^+$, calcd for $H^+PCH_2CH_2COOCH_3(CH_2CH_2COOH)_2$, 265.08410].

dmTCEP: ^{1}H NMR (D_2O, 360 MHz) δ 2.54 (dt, J = 13.32 and 7.08 Hz, 2H), 2.58 (dt, J = 13.32 and 7.08 Hz, 4H), 2.78 (dt, J = 18.78 and 7.08 Hz, 2H), 2.90 (dt, J = 17.55 and 7.08 Hz, 4H), 3.74 (s, 6H); ^{13}C NMR (D_2O, 91 MHz) δ 16.76 (d, J = 49.7 Hz), 17.14 (d, J = 50.4 Hz), 29.85 (d, J = 4.1 Hz), 30.90 (d, J = 4.1 Hz), 55.239, 176.55 (d, J = 9.0 Hz), 179.24 (d, J = 7.6 Hz); ESI-MS m/z 279.10048 [$(M + H)^+$, calcd for $H^+P(CH_2CH_2COOCH_3)_2CH_2CH_2$-COOH, 279.09975].

tmTCEP: ^{1}H NMR (D_2O and 1% TFA, pH ∼2.0, 360 MHz) δ 2.63 (dt, J = 13.86 and 7.08 Hz, 6H), 2.93 (dt, J = 17.86 and 7.08 Hz, 6H), 3.75 (s, 9H); ^{13}C NMR (D_2O and 1% TFA, pH ∼2.0, 91 MHz) δ 16.23 (d, J = 51.1 Hz), 29.63 (d, J = 4.1 Hz), 55.30, 176.44 (d, J = 8.3 Hz); ESI-MS m/z 293.11527 [$(M + H)^+$, calcd for $H^+P(CH_2CH_2COOCH_3)_3$, 293.11540].

^{31}P NMR Measurements. Phosphorus NMR data were obtained on a Bruker AMX 360 MHz spectrometer equipped with a QNP probe and interpreted using Xwinnmr software on a Silicon Graphics platform. Samples were dissolved in degassed H_2O to concentrations of 4−10 mM, and the pH was carefully adjusted with concentrated KOH or HCl prior to NMR experiments. The spectrometer was first locked using a separate standard of 10% D_2O in H_2O before phosphine samples were analyzed. Using the phosphorus channel at 145 MHz, the deprotonated and protonated resonances were approximately 20 and −24 ppm, respectively. Phosphorus pK values were determined from the pH dependence of the integrated areas using the equation (deprotonated phosphine area)/(total phosphine area) = $10^{pH-pK_a}/(1 + 10^{pH-pK_a})$ using GraphPad Prism 3.0. All fits showed R^2 values of ≥0.99.

Rapid Mixing Kinetics. Stopped-flow absorbance and fluorescence experiments were performed at 20 °C in a model SF-61 DX2 stopped-flow instrument (Hi-Tech Scientific) equipped with a 75 W superquiet xenon lamp. Entrance and exit slits were set to 5 nm. Apparent first-order rate constants were calculated using KinetAsyst 3 software (Hi-Tech Scientific) fitting to $Y = Ae^{-kx} + C$.

For second-order rate constant determinations at constant pH, one syringe contained disulfides dissolved in degassed buffer [100 mM potassium phosphate and 0.3 mM EDTA (pH 7.5)] at concentrations of 20 μM for DTNB and peptide and 1 mM for DTT_{ox}. The other syringe contained 0.5, 1, 3, 5, and 7 mM phosphine for the DTNB and peptide experiments and 3, 5, 7, and 10 mM phosphine for the DTT_{ox} reduction. The instrument was used in absorbance mode to follow DTNB reduction at 412 nm [ϵ = 14 150 M^{-1} cm^{-1} (*20*)] and DTT_{ox} reduction at 300 nm [ϵ = 190 M^{-1} cm^{-1} (*21*)]. Reduction of the peptide was monitored by fluorescence using 290 nm as the excitation wavelength following

the increase in emission using a 320 nm cutoff filter. Second-order rate constants were obtained from the linear dependence of pseudo-first-order values versus reductant concentration (fits gave R^2 values of ≥ 0.99).

The pH dependence of reduction by the phosphine derivatives was investigated under pseudo-first-order conditions. The fluorescent peptide [20 μM in unbuffered 0.5 M KCl (pH ~6)] was mixed with an equal volume of a 5 mM phosphine solution in 0.5 M KCl, 50 mM KP_i, 50 mM citrate, and 0.3 mM EDTA adjusted between pH 2.7 and 8.0 with KOH or HCl. After the solution had been mixed, pH values were found to deviate by less than 0.05 pH unit. Concentrations of phosphine in the reductant syringe were routinely redetermined after each set of experiments. Data were analyzed as described above using the equation observed rate constant = (high-pH limit rate constant)$(10^{pH-pK_a})/(1 + 10^{pH-pK_a})$. Because decreased peptide solubility prevented attainment of the high-pH limit for reduction by TCEP and mmTCEP, limiting rate constants for these phosphines were estimated using the p*K* determined from ^{31}P NMR experiments (see the text).

Reduction of Thioredoxin. Oxidized *Escherichia coli* thioredoxin was quantitated by the absorbance at 280 nm [ϵ = 13 700 M^{-1} cm^{-1} (*22*)] in an Agilent 8453 UV−vis spectrophotometer. For reduction with phosphines, oxidized thioredoxin [7 μM in 1 mL of 100 mM phosphate buffer containing 0.3 mM EDTA (pH 7.5)] was placed in a fluorescence cuvette with a path length of 1.0 cm. Where needed, the pH of this solution was adjusted with small volumes of 1 M KOH to compensate for the protons introduced later upon addition of 1−10 mM TCEP, mmTCEP, or dmTCEP. Reduction of thioredoxin was monitored by following the increase in tryptophan fluorescence (*23*) (λ_{ex} = 290 nm, λ_{em} = 350 nm, 4 nm excitation and emission slits) using an SLM Aminco-Bowman Series 2 luminescence spectrometer. To allow absolute comparison between data on different days, the instrument photomultiplier voltage was set to give a sensitivity of 3% of full scale using the Raman water band as an internal and common reference (λ_{ex} = 350 nm, λ_{em} = 397 nm, 4 nm excitation and emission slits). Pseudo-first-order rate constants were calculated using GraphPad Prism 3.0.

The more rapid reduction of thioredoxin by DTT required the use of the stopped-flow fluorimeter (exciting at 290 nm and with emission above 320 nm, 5 nm slit widths). Final concentrations were 10 μM protein and 1.25−5 mM DTT in 50 mM phosphate buffer and 0.65 mM EDTA (pH 7.5) at 20 °C. The fluorescence increase was first-order and was analyzed as described previously.

Phospholipid Vesicle Construction and Permeability Assay. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC, 20 mg; Avanti Polar Lipids) was added to 1 mL of a 20 mM solution of DTNB in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA (*24*). The suspension was forced 21 times through a 0.1 μm Corning Nuclepore track-etch membrane using an Avanti mini-extruder. The resulting, uniform, unilamellar vesicles emerged in the excluded volume of a 15 cm × 1.5 cm Sepharose CL-4B column well separated from free DTNB. Vesicles were prepared fresh daily. Aliquots of stock vesicle suspensions (typically ~30 μL) were diluted to 150 μL with buffer to give an aggregate concentration of 25 μM DTNB in the assay mixture. Thus, the addition of excess permeant reductant (1 mM DTT) to the assay mixture would give a net increase in absorbance of 0.7 at 412 nm after 5 min in self-masking microcells with a path length of 1 cm (corresponding to the reduction of all encapsulated DTNB). Where necessary, small amounts of DTNB that leak out of the vesicle stock could be reduced by the addition of an external insoluble reductant prior to standardization. Each disulfide reductant was assessed for permeability using either 12.5 μM (for phosphines and DTT) or 25 μM (for glutathione) in the spectrophotometric assay (see Figure 5). To assess the possibility that tmTCEP was disrupting vesicles (see later), 90° light scattering experiments were performed using 0.2 mL of a suspension of DTNB-loaded vesicles prepared as described above. After the scattering had been recorded for 2 min using 4 nm entry and exits slits of the luminescence spectrometer set at 600 nm, 12.5 μM tmTCEP was added and no change was seen over the course of 3 min. Then 0.1% (v/v) Triton X-100 was added to show the marked decrease in scattering accompanying disruption of the vesicles.

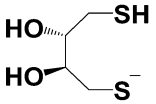
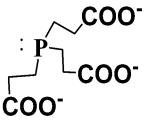
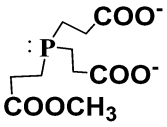
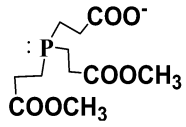
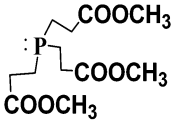
Water−Octanol Partitioning. Solutions (1 mM) of the reductant were made in octanol-saturated buffer [100 mM KP_i and 1 mM EDTA (pH 7.5)]. A 500 μL portion of this stock was mixed with an equal volume of buffer-saturated octanol, capped tightly, and mixed end over end for 1 h at 4 °C. After centrifugation, the reductant concentration remaining in the aqueous phase was determined with the DTNB assay, and compared to the corresponding concentration in the stock buffer. Measurements were taken in triplicate.

Reduction of DTNB in the Presence of Acetone. To assess the selectivity of TCEP as a reductant of disulfides in the presence of carbonyl groups, 50 μM phosphine was added to 1 mM DTNB in phosphate buffer in the presence and absence of 1 M acetone and the absorbance change determined at 412 nm. Under these conditions, acetone is an insignificant oxidant of TCEP in competition with DTNB. Controls, replacing TCEP with DTT, showed no appreciable effect of 1 M acetone on the spectrum of the TNB anion.

O_2 Electrode Assay. QSOX activity with the phosphines was assayed using an oxygen electrode as described previously (*25*, *26*) in 2 mL of air-saturated 50 mM potassium phosphate buffer and 0.3 mM EDTA (pH 7.5) at 25 °C. Concentrated solutions of phosphines were prepared immediately prior to assays in distilled water and adjusted to pH 7.5 before standardization with DTNB. The final concentration of each phosphine in the assay was varied from 0 to 9 mM. A background trace was taken before each reaction to account for nonenzymatic oxygen depletion, and assays were initiated by the addition of 100 nM avian QSOX. Turnover numbers (moles of oxygen consumed per mole of enzyme subunit per minute) were calculated from duplicate initial rate measurements. Second-order rate constants were derived from the linear dependence of turnover number over this range of phosphine concentrations.

Docking Experiments. E. coli thioredoxin (PDB entry 2TRX) was first energy-minimized with a molecule of either deprotonated DTT or TCEP placed in the vicinity of C32. This surface-accessible cysteine sulfur atom was then tethered to the nucleophilic center of the reductant with a modest spring constant of 1.5 kcal mol^{-1} $Å^{-2}$. A molecular dynamic simulation was then performed for each complex by raising

Table 1: Second-Order Rate Constants (M^{-1} s^{-1}) for the Reduction of Disulfides by DTT, TCEP, and Derivatives at pH 7.5[a]

	DTT 	TCEP 	mmTCEP 	dmTCEP 	tmTCEP 
DTNB	**2,900**	**18,000**	**32,000**	**40,000**	**56,000**
DTT_{ox}	**n.d.**	**43**	**24**	**18**	**7**
$peptide_{ox}$	**56**	**650**	**968**	**461**	**155**
TRX_{ox}	**280**	**2.4**	**4.3**	**1.7**	**0.3**
QSOX	**100,000**	**813**	**1,327**	**1,001**	**152**

[a] Data were obtained in 100 mM phosphate and 0.3 mM EDTA at 20 °C (but 50 mM KP_i and 0.3 mM EDTA at 25 °C for QSOX). The structures are shown in their active nucleophilic forms for consistency (see the text). n.d. means not done.

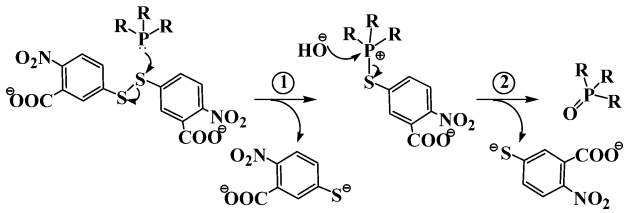
the temperature from 100 to 500 K in 4 ps, and then lowering the temperature to 300 K in 6 ps and to 100 K in a further 10 ps. Throughout, harmonic restraints for every atom were set at 1 kcal mol^{-1} $Å^{-2}$, and a generalized Born solvent model was used to mimic solvation effects.

RESULTS

Synthesis and Characterization of TCEP Esters. The mono-, di-, and trimethyl esters of TCEP can be prepared by incubating the phosphine in methanol in the presence of a cation-exchange resin in its protonated form. Esterification was followed by TLC or analytical reverse phase HPLC. When the desired extent of esterification was obtained, the acid catalyst was filtered off and the mixture of esters purified by preparative HPLC (see Experimental Procedures). As expected, longer reaction times favor the trimethyl ester (not shown). The pure esters were recovered by lyophilization, analyzed for purity by analytical HPLC, and characterized by NMR and mass spectroscopy. All three esters are soluble in water to concentrations of at least 50 mM. Mono-, di-, and trimethyl esters lost less than 5% of reducing ability after aerobic storage for 24 h at 4 °C in water or phosphate buffer (pH 7.5). Although not the focus of this work, this simple esterification procedure can be readily adapted to longer chain alcohols. For example, di- and tributyl esters were formed by heating a suspension of TCEP and an equal weight of ion-exchange resin in refluxing 1-butanol overnight. Synthesis of additional phosphine analogues for more specific applications is in progress.

Choice of Disulfides for Probing Phosphine Reactivity. Table 1 compares the reactivity of TCEP and its methyl esters toward a range of disulfides at pH 7.5. First, two model disulfides were chosen with widely different redox potentials: one highly oxidizing, DTNB, and the other being notably thermodynamically stable, DTT_{ox} [$E'° = -0.33$ V (*21*)]. The three other entries in Table 1 contain peptide or protein disulfides in CXXC motifs, where X is any amino acid. These motifs are utilized in a wide range of redox proteins and enzymes, including thioredoxins, glutaredoxins, protein disulfide isomerases, DsbA, and flavin-dependent sulfhydryl oxidases (*27*−*43*). The oxidized nonapeptide (H_3N^+-VTW**C**GA**C**KM-NH_2) was iteratively designed from the thioredoxin sequence and gave a 40% increase in tryptophan fluorescence upon reduction (*17*). Reduction of full-length *E. coli* thioredoxin, the next entry in Table 1, was similarly followed by tryptophan fluorescence (*23*).

Scheme 2: Reaction of Phosphines with DTNB



Finally, Table 1 summarizes second-order rate constants for the interaction between the phosphines and avian sulfhydryl oxidase. Flavin-dependent sulfhydryl oxidases of the QSOX family have three CXXC motifs that are critical for the efficient oxidation of protein sulfhydryl groups (*43*, *44*). In the avian QSOX sequence, these motifs are CGHC, CQEC, and CPQC. The first of these, located in a PDI-like thioredoxin domain, likely represents the entry point for reducing equivalents from normal thiol substrates (*43*, *44*).

Reduction of Disulfides at pH 7.5. DTNB is often used to quantitate TCEP because the reaction is rapid and stoichiometrically yields the strongly absorbing thionitrobenzoate (TNB) anion (*3*). Reduction, followed in the stopped-flow spectrophotometer at 412 nm, is monophasic (not shown), indicating that even with this highly activated disulfide, hydrolysis of the phosphonium ion is much faster than the initial nucleophilic cleavage of the disulfide by TCEP (steps 2 and 1, respectively, Scheme 2).

If this were not the case, the subsequent release of the second TNB thiolate (step 2) would lead to a biphasic increase in 412 nm absorbance. Reduction of DTNB is first-order in both disulfide and all phosphine reductants that were studied, leading to the second-order rate constants shown in Table 1. A small increase in reactivity is observed at pH 7.5

(amounting to ~3-fold) with progressive methylation of TCEP. Under these conditions, the phosphine esters are between ~6- and ~18-fold more reactive than DTT toward DTNB (Table 1).

It should be stressed such comparisons of reactivity are strongly pH-dependent. Only the deprotonated form of DTT is a significant nucleophile, and one would similarly expect (*1*, *11*) protonation of TCEP at phosphorus [reported p*K* of 7.6−7.7 (*45*, *46*)] to suppress drastically, or completely, the reactivity of the phosphine as a nucleophile and disulfide reductant (see later). Given the p*K* values:

$$DTT \leftrightarrow DTT^- + H^+ \; [pK = 9.2\ (21)]$$

$$R_3PH^+ \leftrightarrow R_3P: + H^+ \; [pK = 7.6\ (45, 46)]$$

at pH 7.5 only ~2% of DTT is in a reactive thiolate form whereas nearly half of TCEP would be unprotonated at phosphorus. Thus, the limiting rates at high pH would reverse the order of reactivity of these reductants: DTT thiolate would be some 3-fold more reactive than the deprotonated phosphine center of TCEP. The pH dependence of the rate of reduction of a peptidic disulfide by phosphines will be presented later. Here, we continue with a survey of the reactivity of these phosphine reductants at pH 7.5, a value appropriate for many physiological conditions.

Reduction of the disulfide in DTT_{ox} can be conveniently followed by the decrease in absorbance at 300 nm (*21*; see Experimental Procedures). This disulfide is of considerable stability (*21*), and this is consistent with the approximately 400-fold decrease in the rate of reduction by TCEP when compared to that of the strongly oxidizing DTNB disulfide (Table 1). Again, reduction shows monophasic kinetics. The second-order rate constants in Table 1 show small decreases with each methylation amounting to an aggregate 6-fold decrease with tmTCEP.

As mentioned earlier, there has been little quantitative information about the reduction of peptide disulfides with TCEP. Peptides containing a CXXC motif of the type VTW**C**GA**C**KM-NH_2 typically have redox potentials $E^{o\prime}$ of approximately −200 mV (*47*, *48*) and are thus considerably more oxidizing than DTT_{ox}. Pseudo-first-order rate constants were collected from the increase in tryptophan fluorescence at a range of reductant concentrations as described in Experimental Procedures and yielded the second-order values in Table 1. Here mmTCEP is the best and tmTCEP the worst kinetic reductant at pH 7.5, although there is again an only 6-fold range of second-order rate constants between them. Clearly, the carboxylate functions of TCEP are not essential for reduction of this nonapeptide, although they exert a significant effect on the p*K* of the phosphonium center and the intrinsic nucleophilicity of the phosphine at the high-pH limit (see later).

The reaction between phosphines and *E. coli* thioredoxin can be conveniently monitored using the approximately 2.5-fold increase in protein tryptophan fluorescence upon reduction of the redox-active disulfide (*23*). At pH 7.5, this reaction was sufficiently slow to allow the increase in tryptophan fluorescence to be monitored by manual mixing in a conventional fluorimeter. Reactions were first-order in both phosphine and thioredoxin and yielded second-order rate constants shown in Table 1. As observed with the peptide, mmTCEP is the best kinetic reductant among the phosphines, although there is an only 13-fold range between it and tmTCEP. Unlike the prior examples in Table 1, DTT is a much better reductant of oxidized thioredoxin, and measurement in the stopped flow gave a bimolecular rate constant of 280 M^{-1} s^{-1}, some 100-fold better than that of TCEP under the same conditions. Holmgren and co-workers (*49*) observed even faster rates of reduction of thioredoxin (1650 M^{-1} s^{-1}) using DTT at pH 7.2.

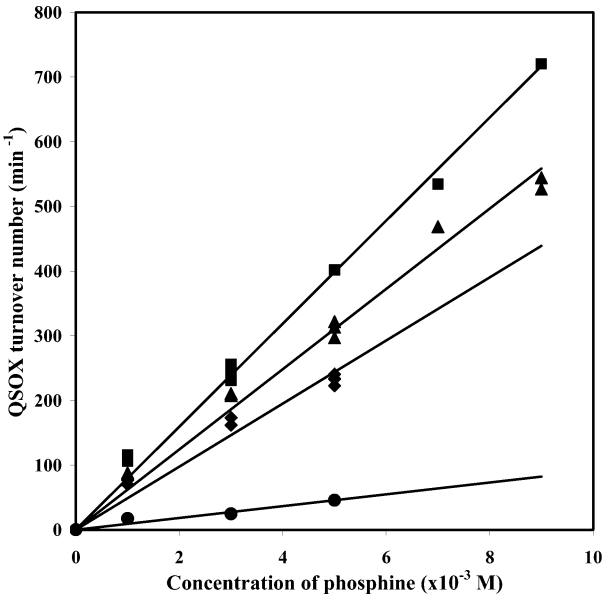


FIGURE 2: Water-soluble phosphines as substrates of QSOX. Enzyme assays were conducted in the oxygen electrode in 50 mM phosphate buffer at pH 7.5 and 25 °C: (◆) TCEP, (■) mmTCEP, (▲) dmTCEP, and (●) tmTCEP. Where necessary, the pH was readjusted to pH 7.5 after the addition of acidic phosphine reagents. The assay was started by adding enzyme. Turnover numbers are expressed as moles of oxygen reduced per moles of flavin per minute.

Preliminary observations showed that TCEP is a significant substrate of avian QSOX, although we do not know which of the three CXXC motifs of the enzyme (CGHC, CQEC, and CPQC) serves as the entry point for reducing equivalents in this case (*16*, *43*). TCEP, mmTCEP, and dmTCEP are all similar in reactivity (800−1300 M^{-1} s^{-1}), with the trimethyl analogue significantly lower in efficacy (150 M^{-1} s^{-1}). Here, we evaluated the kinetics from the linear dependence of the turnover number of the enzyme from 0 to 9 mM phosphine (see Experimental Procedures). Over this range, there was no evidence of the saturation observed for thiol substrates (Figure 2). Clearly, the corresponding rate constants with the phosphines far exceed those for the model peptide substrate and for thioredoxin itself. However as with thioredoxin, DTT is a dramatically better kinetic reductant of QSOX (~100-fold, Table 1). Factors influencing the differences in the effectiveness of phosphines and DTT are discussed later.

pH Dependence of the Rate of Reduction of a Model CXXC Peptide by Phosphines. Esterification of the carboxyl functions of TCEP [pK_{app} = 4.7, 5.9, and 6.6 (*45*)] would be expected to lower the p*K* at phosphorus [7.6−7.66 for TCEP (*45*, *46*)] by progressive loss of the favorable electrostatic interactions between phosphonium and carboxylate centers (Figure 1). Hence, we reasoned that the methyl esters of TCEP might show enhanced reactivity toward disulfides at

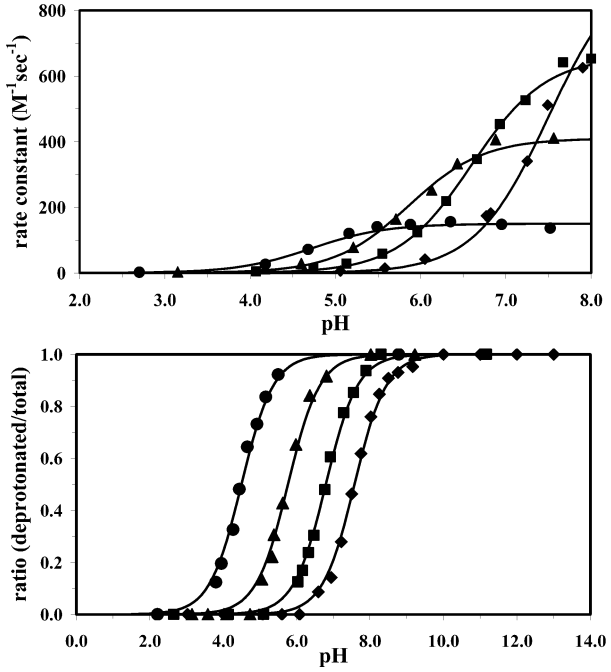


FIGURE 3: p*K* determination for phosphines using reactivity and ^{31}P NMR. (A) pH dependence of reduction of the CXXC peptide determined in the stopped flow by mixing the oxidized peptide (20 μM in 500 mM KCl) with an equal volume of 5 mM phosphines in 100 mM citrate/phosphate buffers in 500 mM KCl at 20 °C (see Experimental Procedures). Reduction of the peptide was followed by the increase in tryptophan fluorescence exciting at 290 nm. The solid lines are theoretical fits calculated for a monoprotic process with a low-pH limit of 0 and high-pH limits of 950, 660, 410, and 150 M^{-1} s^{-1} for TCEP (◆), mmTCEP (■), dmTCEP (▲), and tmTCEP (●), respectively (see Experimental Procedures). (B) ^{31}P integrated intensities were determined as a function of pH (4−10 mM phosphine in water adjusted to the desired pH values with HCl or KOH). Data were fit to a single proton slope as described in the text.

lower pH values when compared to TCEP. Figure 3A illustrates this effect with the model CXXC peptide used in Table 1. These experiments included 0.5 M KCl and used relatively low buffer concentrations to minimize changes in ionic strength with pH. We found that the oxidized peptide was insufficiently soluble above pH 8 for accurate measurement of the limiting reactivity of TCEP and mmTCEP at the high-pH limit (see Experimental Procedures). The fits in Figure 3A correspond to monoprotic ionizations (see the legend) and show the expected steady decrease in apparent p*K*: 7.5 for TCEP, 6.6 for mmTCEP, 5.9 for dmTCEP, and 4.7 for tmTCEP. The p*K* at phosphorus for each derivative was then measured independently by ^{31}P NMR as described in Experimental Procedures (Figure 3B, yielding p*K* values of 7.6, 6.8, 5.8, and 4.7). Our data for TCEP are in good agreement with previous determinations by NMR [7.6 (*46*)] and titrimetry [7.66 (*45*)].

The agreement between kinetic and NMR determinations of p*K* and the very low apparent reactivity of the protonated phosphine at the low-p*K* limit (Figure 3) indicates that the major determinant in the reactivity of these reagents is the p*K* at phosphorus. The high-pH limiting reactivity for these phosphines follows the expected trend: the lower the p*K*, the lower the intrinsic nucleophilicity of a series of related compounds (*50*). Thus, the high-pH limiting reactivity is as follows: TCEP > mmTCEP > dmTCEP > tmTCEP (with the extrapolated value for TCEP being some 6-fold more

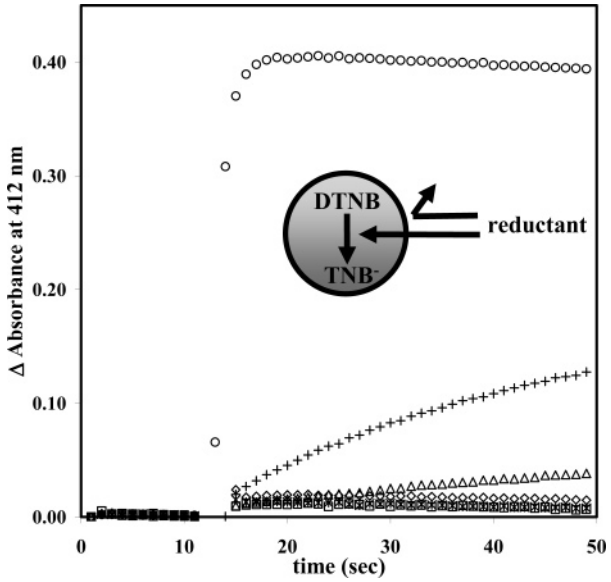


FIGURE 4: Permeability of phosphines across POPC lipid bilayers. Unilamellar vesicles were made by extrusion in 50 mM phosphate buffer containing 20 mM DTNB (see Experimental Procedures). The vesicles were purified by gel filtration and then mixed with 12.5 μM phosphine or DTT or 25 μM glutathione in phosphate buffer at pH 7.5 and 25 °C. The rate of increase in absorbance at 412 nm is a reflection of the permeability of the reductants. tmTCEP (○) crosses the model membrane at least 30-fold faster than DTT (+) and 250-fold faster than dmTCEP (△). TCEP (◇), mmTCEP (□), and GSH (*) were virtually impermeant.

reactive than that for tmTCEP). At pH <5.5, the observed order of reactivity is reversed; e.g., at pH 5, tmTCEP is 35-fold more reactive toward the peptide than TCEP, 6-fold more reactive than mmTCEP, and 2-fold more reactive than dmTCEP. At pH 2.5, tmTCEP is 100-fold more reactive than TCEP in reduction of the peptide.

Cell Permeant Water-Soluble Phosphine Reductants. DTT has been used widely as a cell permeant reductant to modulate the cellular redox poise and for the identification of proteins catalyzing disulfide bond formation in *E. coli* (*51*, *52*), yeast (*33*, *34*, *53*), and mammalian cells (*54*). DTT has also been used widely in studies of the unfolded protein response (*55*−*61*). There have been reports that TCEP is also a permeant reductant (*6*, *12*), although this seems *a priori* surprising given its charge density at neutral pH (three carboxylates and a partial positive charge on phosphorus; see above). Since phosphines have potential advantages in the modulation of redox poise under certain situations (see the Discussion), we investigated the ability of the phosphines used in this work to traverse a widely used model for lipid bilayers (*24*).

Figure 4 shows a simple protocol for evaluating the permeability of any reductant that can react with DTNB. Unilamellar vesicles encapsulating 20 mM DTNB in 50 mM phosphate at pH 7.5 were purified by gel filtration to remove excess DTNB (see Experimental Procedures). An aliquot of a vesicle suspension was placed in 50 mM phosphate buffer, and a background absorbance at 412 nm was recorded prior to addition of the reductant. Figure 4 shows that the rate of appearance of the TNB anion is too fast to accurately measure using 12.5 μM tmTCEP. (We were unable to use the stopped-flow spectrophotometer here because rapid mixing led to shearing of vesicles.) Control experiments,

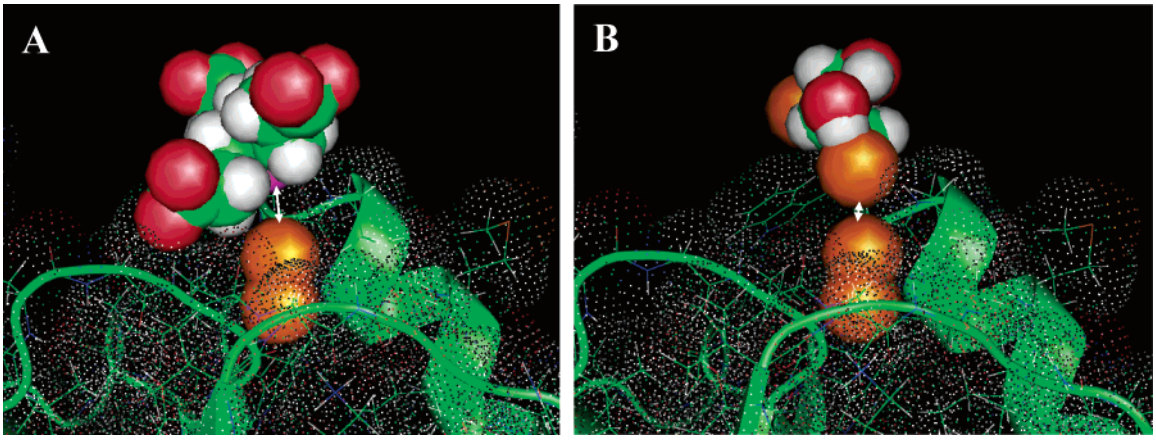


FIGURE 5: Modeling an encounter between *E. coli* thioredoxin and TCEP and DTT prior to reduction of C32−C35. Panel A shows the closest approach of the P atom in TCEP (purple) to the sulfur atom of C32 in oxidized *E. coli* thioredoxin (the arrow represents an internuclear distance of 4.8 Å). Panel B shows the comparable closest approach (3.7 Å) of the thiolate sulfur of the DTT monoanion.

using light scattering (not shown; see Experimental Procedures), demonstrated that tmTCEP does not disrupt these stable phospholipid vesicles under the conditions described in the legend of Figure 4 (*24*). In contrast to tmTCEP, the highly polar TCEP was virtually impermeant, as was a sample of reduced glutathione (GSH) that we used as a control (Figure 4). DTT, a widely used permeant reductant, penetrates these vesicles at least 30-fold slower than tmTCEP. We have also synthesized more lipophilic analogues of these phosphines (e.g., butyl esters), and they, too, are rapidly permeant (not shown). These results correlate with a simple evaluation of water−octanol partitioning (see Experimental Procedures) where the percentages of reductant that remained in buffer were as follows: 100.5 ± 0.8 for TCEP, 95.5 ± 4.2 for mmTCEP, 98.9 ± 1.2 for dmTCEP, 18.6 ± 0.4 for tmTCEP, 48.7 ± 1.1 for DTT, and 97.7 ± 3.8 for GSH.

DISCUSSION

Phosphines have been suggested as superior reductants for peptides and protein disulfides, but it is clear that the widely differing p*K* values for the nucleophilic center in TCEP and DTT makes such comparisons highly pH-dependent. This work shows that while TCEP is an excellent reductant for small disulfides and peptides at pH 7.5, it is noticeably inferior to DTT for CXXC motifs in thioredoxin and sulfhydryl oxidase at this pH. This difference is some 100-fold at pH 7.5 (Table 1) but increases to ∼2000-fold when comparing fully deprotonated TCEP with the DTT monoanion at the high-pH limit. An obvious contributor to this difference is the marked steric crowding of the three carboxyethyl substituents around the central phosphorus atom. Figure 5 shows this clearly for thioredoxin reduction by both TCEP and DTT. A docking calculation allowing full conformational flexibility to the reductants and limited movement of thioredoxin (see Experimental Procedures) shows that the phosphorus atom of TCEP could only move within 4.8 Å of the exposed (interchange) sulfur atom of C32. In contrast, DTT thiolate can move within van der Waals contact of C32 (∼3.7 Å), leading to mixed disulfide formation and eventual liberation of reduced thioredoxin. Similarly, steric factors are likely to play an important role in the considerably lower reactivity of TCEP toward QSOX because an N-terminal thioredoxin domain is the likely entry point for reducing equivalents in this multidomain oxidase (*43*, *44*). While differences in the steric bulk of TCEP and DTT have not been generally considered, the larger footprint of these phosphines is likely to make them more sterically demanding and more generally selective reagents for solvent-accessible protein disulfides.

Although it lacks the potential versatility of the TCEP ester approach described in this paper, an additional water-soluble reagent, tris(hydroxypropyl)phosphine (THP), in which the carboxyl functions of TCEP are replaced with hydroxymethylene groups is commercially available. A limited series of experiments show that THP is a very good reductant of the model peptide at pH 7.5 (1930 M^{-1} s^{-1} compared to 650 M^{-1} s^{-1} for TCEP). The p*K* at phosphorus was determined by ^{31}P NMR to be 7.22 (not shown). These data provide further evidence that the carboxylate functions of TCEP are not essential for its effectiveness as a reductant. However, despite the predominant electroneutrality of THP at pH 7.5, this phosphine penetrates membranes rather slowly (at a rate comparable to that of DTT; not shown).

Finally, tris(2-cyanoethyl)phosphine (TCNP) has been proposed in the commercial literature to be a potentially useful reducing agent for buried disulfides. However, it proves to be a surprisingly slow kinetic reductant for the systems examined here. Thus, second-order rate constants for TCNP are 30-fold slower than those of TCEP toward DTNB, 80-fold for DTT_{ox}, ∼300-fold for the model peptide, and >300-fold for QSOX (it is an undetectable substrate up to its solubility limit of ∼3 mM). Part of the sluggish reactivity of TCNP is a reflection of its very low p*K* at phosphorus [∼1.4 (*62*); see above]. In addition, TCNP penetrates lipid vesicles poorly, under the conditions of Figure 4 at rates which are even slower than dmTCEP.

In summary, this study shows that there is considerable scope for tailoring phosphine reductants to make them more selective reductants of disulfide bonds in biochemical systems or more effective reductants at lower pH values. Further, permeant phosphine esters deserve consideration as potential reductants of intracellular peptide and protein disulfide bonds. Reduction is essentially irreversible (*1*, *4*, *8*), yielding the thermodynamically very stable phosphine oxide (Scheme 1). These permeant phosphines are likely to be particularly suited for time-resolved studies since their effect could not be perpetuated by the sort of intracellular

redox cycling that would occur when oxidized DTT is re-reduced by a range of cellular reductants, including thioredoxin and pyridine nucleotide-dependent disulfide oxidoreductases. Development of additional phosphine reductants is in progress.

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