Cautionary Tale of Using Tris(alkyl)phosphine Reducing Agents with NAD\(^+\)-Dependent Enzymes

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**ABSTRACT:** Protein biochemistry protocols typically include disulfide bond reducing agents to guard against unwanted thiol oxidation and protein aggregation. Commonly used disulfide bond reducing agents include dithiothreitol, \(\beta\)-mercaptoethanol, glutathione, and the tris(alkyl)phosphine compounds tris(2-carboxyethyl)phosphine (TCEP) and tris(3-hydroxypropyl)phosphine (THPP). While studying the catalytic activity of the NAD(P)H-dependent enzyme \(\Delta^1\)-pyrroline-5-carboxylate reductase, we unexpectedly observed a rapid non-enzymatic chemical reaction between NAD\(^+\) and the reducing agents TCEP and THPP. The product of the reaction exhibits a maximum ultraviolet absorbance peak at 334 nm and forms with an apparent association rate constant of \(2.3 \times 10^9 \text{M}^{-1}\text{s}^{-1}\). The reaction is reversible, and nuclear magnetic resonance characterization (\(^1\text{H}, ^{13}\text{C}, \text{and} ^{31}\text{P}\)) of the product revealed a covalent adduct between the phosphorus of the tris(alkyl)phosphine reducing agent and the C4 atom of the nicotinamide ring of NAD\(^+\). We also report a 1.45 Å resolution crystal structure of short-chain dehydrogenase/reductase with the NAD\(^+\)-dependent oxidoreductases are widespread in nature, our results may be broadly relevant. These findings serve to caution researchers when using TCEP or THPP in experimental protocols with NAD(P)\(^+\). Because NAD(P)\(^+\)-dependent oxidoreductases are widespread in nature, our results may be broadly relevant.
in the range of 30 dissociation (absorbance at 334 nm in the insets along with the observed rate constant (kobs). The dependence of kobs on THPP and TCEP concentration was determined by holding the NAD⁺ concentration at a fixed value (0.5 mM) and varying the THPP and TCEP concentration (5–150 mM). kobs values were determined from single-exponential fits of single-wavelength data collected at 334 nm. The apparent forward association (k⁺app) and reverse dissociation (k⁻app) rate constants were determined from the plot of kobs vs (c) THPP and (d) TCEP concentration. Data are plotted as the mean ± the standard deviation of three technical replicates and fit to a linear equation. All concentrations are after mixing.

TCEP (Figure 1d) concentration was then plotted to determine the apparent forward association (k⁺app) and reverse dissociation (k⁻app) rate constants. SI Table 1 summarizes the rate constants and shows that both the forward (491 M⁻¹ s⁻¹) and reverse (24 s⁻¹) rate constants with TCEP are faster than with THPP. The equilibrium association constant (K⁺app), however, is slightly higher with THPP (35 M⁻¹) than with TCEP (20 M⁻¹). The reaction between NAD⁺ and TCEP (25 mM) was repeated in different buffers, and similar kobs values in the range of 30–35 s⁻¹ for the increase at 334 nm were obtained, indicating product formation is independent of the buffer composition (SI Figure 3).

We next considered the possibility that TCEP and THPP had reduced NAD⁺ to NADH. L-Lactate dehydrogenase (LDH) and pyruvate were added to the TCEP/THPP–NAD⁺ reaction product, but no decrease in absorbance (330–340 nm) was observed, indicating that the product was not used by L-lactate dehydrogenase to catalyze the reduction of pyruvate. We also did not find evidence for NADH as the reaction product by mass spectrometry analysis.

Nuclear magnetic resonance (NMR) was then used to characterize and identify the product of the reaction between TCEP or THPP and NAD⁺. Initial NMR experiments included one-dimensional (¹H and ³¹P) and two-dimensional (¹H–³¹P HSQC and ¹H–³¹P HSQC-TOCSY) analyses (Figure 2 and SI Figure 5; corresponding spectral data summarized in SI Table 4). The resulting data showed through-bond coupling of the phosphorus nucleus of THPP to protons of the nicotinamide ring of NAD⁺. Additionally, the ¹H–³¹P HSQC-TOCSY data in SI Figure 5 indicate the reaction was reversible as there were ³¹P correlations for both the covalent adduct nicotinamide ring protons and the lone NAD⁺ nicotinamide ring protons. ¹H–³¹P HSQC-TOCSY correlations to NAD⁺ nicotinamide ring protons meant the tris(alkyl)phosphine compound dissociated from the nicotinamide ring during the TOCSY spin lock.

Both one-dimensional (³¹P) and two-dimensional (¹H–³¹P HSQC and ¹H–³¹P HSQC-TOCSY) NMR analyses showed phosphorus signals at 34 and −13 ppm for the NAD⁺ and THPP reaction mixture. The phosphorus nuclei at 34 ppm show through-bond coupling to the nicotinamide ring protons of NAD⁺, as previously described. The phosphorus signals at −13 ppm are attributed to the phosphates in ADP; as expected, these phosphorus nuclei show through-bond coupling to ribose ring protons.

To further confirm the structural assignment, additional NMR experiments were conducted on the NAD⁺ and THPP reaction mixture. The sample had been stored at 4 °C for 3 weeks and showed minor decomposition; however, the reaction product was still the main component. These additional experiments included one-dimensional (¹³C) and two-dimensional (¹H–¹H COSY, ¹H–¹H TOCSY, ¹H–³¹C HSQC, and ¹H–³¹C HMBC) analyses (SI Figures 6 and 7 and SI Table 5). The additional data indicated that THPP attacked C4 of the nicotinamide ring of NAD⁺, the same position at which hydride transfer occurs, resulting in two diastereomers in an ∼1:0.6 ratio (Figure 3a). NMR analyses of NAD⁺ alone and NADH alone (spectral data summarized in SI Tables 2 and 3, respectively) also support these structural assignments.

Lastly, the one-dimensional proton spectra of the reaction product of NAD⁺ with THPP and TCEP were very similar (SI Figure 4), indicating a similar covalent adduct is formed with both tris(alkyl)phosphine compounds (Figure 3a,b).

The covalent adduct between NAD⁺ and TCEP was also characterized in the context of an oxidoreductase active site using X-ray crystallography. The 1.45 Å resolution structure of a short-chain dehydrogenase/reductase (SDR) from Burkholderia ambifaria was determined from a crystal grown in the presence of 5 mM NAD⁺ and 1 mM TCEP (Protein Data Bank entry 1SVPS). Whereas NAD⁺ was explicitly added to the reaction mixture, the covalent adduct with TCEP formed upon mixing NAD⁺ with TCEP. This is the first reported structure of a covalent adduct with a trisphosphine compound, and it provides insights into the mechanism of action of such compounds.

Figure 1. Stopped-flow kinetics of the NAD⁺–tris(alkyl)phosphine reaction. NAD⁺ (0.5 mM) was rapidly mixed with 50 mM (a) THPP and (b) TCEP in 100 mM HEPES (pH 7.5) buffer. Plots of absorbance at 334 nm fit to a single-exponential equation are shown in the insets along with the observed rate constant (kobs). The dependence of kobs on THPP and TCEP concentration was determined by holding the NAD⁺ concentration at a fixed value (0.5 mM) and varying the THPP and TCEP concentration (5–150 mM). kobs values were determined from single-exponential fits of single-wavelength data collected at 334 nm. The apparent forward association (k⁺app) and reverse dissociation (k⁻app) rate constants were determined from the plot of kobs vs (c) THPP and (d) TCEP concentration. Data are plotted as the mean ± the standard deviation of three technical replicates and fit to a linear equation. All concentrations are after mixing.

Figure 2. Two-dimensional ¹H–³¹P HSQC spectrum of the NAD⁺ and THPP reaction mixture. The spectrum was acquired with a J(HP) coupling constant of 11 Hz.
strong electron density indicated that the NADP⁺ cofactor had been covalently modified at the C4 atom of the nicotinamide ring (Figure 4b). The electron density is consistent with the structure of the adduct determined from solution NMR. The structure shows that several active site residues form hydrogen bonds and ion pairs with the carboxyethyl groups of the TCEP (Figure 4b). These results suggest that the reaction of tris(alkyl)phosphine reducing agents with NAD(P)⁺ inactivates the biological cofactor and that the species generated can potentially inhibit enzymes.

To the best of our knowledge, this is the first characterization of a covalent adduct between NAD(P)⁺ and TCEP or THPP. The reaction occurs at physiological pH within a range of concentrations of TCEP and THPP (0.5−50 mM) typically used in protein biochemistry and structural biology experiments. The reaction is reversible and occurs on a time scale comparable to that of TCEP reduction of disulfide bonds in DTTP (43 M⁻¹ s⁻¹), peptide substrates containing a CXXC motif (650 M⁻¹ s⁻¹), and oxidized cysteines in proteins (1.5−813 M⁻¹ s⁻¹). The reaction between NAD⁺ and TCEP or THPP most likely proceeds through a nucleophilic attack by the phosphine at the C4 atom of the nicotinamide ring similar to that of a tertiary phosphine attacking an alkyl halide to form a phosphonium ion. The nicotinamide ring of NAD⁺ has electrophilic character and is susceptible to modifications by nucleophiles. Biologically relevant is the fact that sulfhydryl compounds such as cysteine and glutathione can form adducts at the C4 atom of the nicotinamide with absorbance maxima at 330−335 nm. In urocanase, a covalent intermediate between imidazolepropionate and NAD⁺ exhibited an absorbance peak at 335 nm. In that case, NMR showed that the imidazole nitrogen formed a covalent adduct at the C4 atom of the nicotinamide ring. The absorbance peak of 334 nm observed here for the covalent NAD−phosphine adduct is similar to that previously reported for various covalent NAD species. NAD addition reactions are also known for cyanide, bisulfite, and dihydroxyacetone. LDH has been shown to catalyze NAD adduct formation with cyanide and pyruvate. The NAD−pyruvate adduct inhibits LDH at high concentrations of pyruvate. Other examples include isoniazid−NAD/NADP adducts found in Mycobacterium tuberculosis that inhibit different enzymes such as dihydrofolate reductase.

We showed that tris(alkyl)phosphine reducing agents inactivate the biological cofactor NAD(P)⁺, and the inactivated cofactor can, at least in one case, bind the active site of an enzyme. These results suggest that including TCEP or THPP in crystallization trials of NAD(P)⁺-dependent oxidoreductases could have unintended consequences. In the SDR structure, the active site is large enough to accommodate the bulky TCEP group, and several residues serendipitously stabilize the carboxyethyl groups (Figure 4). In other enzymes, the adduct may be too large to fit in the active site, which could result in weak or no electron density for NAD(P)⁺ despite the inclusion of the cofactor in the crystallization setup. One can also imagine cases in which the covalent modification causes the cofactor to bind in an atypical pose, making it difficult to infer the biochemical significance of the structure. Finally, even if the adduct does bind to the enzyme in crystallo, if the active site cannot stabilize the conformation of the tris(alkyl)phosphine molecule, the nicotinamide may appear disordered, which would complicate the interpretation of the structure.

TCEP and THPP could also cause problems with enzyme assays and binding studies. To illustrate how TCEP could

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**Figure 3.** Chemical structures of the reaction products. Resolved covalent adduct products from the reaction of (a) NAD⁺ with THPP and (b) NAD⁺ with TCEP.

**Figure 4.** Structure of *B. ambifaria* SDR with a NADP⁺−TCEP adduct in the active site. (a) Cartoon drawing of the protein with the adduct colored pink. The inset shows electron density for the adduct (Polder omit, 3.0σ). (b) Close-ups of the adduct showing electron density for the P−C4 bond (left) and interactions with the carboxyethyl groups (right).
interfere with the assay of a dehydrogenase, we measured the activity of glucose-6-phosphate dehydrogenase (G6PDH) in the presence of 1–50 mM TCEP. With the glucose-6-phosphate concentration fixed at 5 mM and the NAD⁺ concentration varying from 0.25 to 0.4 mM, the apparent reaction velocity steadily decreased with an increase in TCEP concentration at all NAD⁺ concentrations tested (SI Figure 8). The effect of TCEP was more pronounced at the lower NAD⁺ concentrations and was significant even at the lowest TCEP concentration tested. For example, the inclusion of 1 mM TCEP decreased the apparent rate by ~20% when NAD⁺ was used in the range of 0.025–0.2 mM. The artifactual underestimation of the catalytic activity is presumably due to the reaction of TCEP with NAD⁺ decreasing the effective concentration of the latter. Another potential problem is that adding TCEP or THPP to NAD(P)⁺-dependent assays can generate a rapid absorbance increase around 340 nm that, without proper controls, could be misinterpreted as enzyme activity. This particularly could be a problem in enzyme-coupled reactions using NAD⁺/NADP⁺ and high-throughput assays that use only end point measurements. Also, the adduct may inhibit the enzyme, as implied by the SDR structure (Figure 4). With regard to binding studies, the addition of tris(alkyl)phosphine compounds would potentially lead to lower concentrations of free NAD(P)⁺, as in the glucose-6-phosphate dehydrogenase example (SI Figure 8), resulting in anomalous errors in the determined binding constant. In addition, data analysis could be complicated by unknowingly having two species present, NAD(P)⁺ and the NAD(P)⁺–tris(alkyl)phosphate covalent adduct. Methods that measure changes in heat to quantify binding events, such as isothermal titration calorimetry, would have complications due to the heat of the reaction between NAD(P)⁺ and TCEP or THPP. 31

In summary, we characterized the chemical reaction kinetics of NAD⁺ and tris(alkyl)phosphines. Evidence from stopped-flow spectrophotometry, multidimensional NMR, and X-ray crystallography indicates that the product is a reversible covalent adduct between the phosphorus of the tris(alkyl)-phosphine and the C4 atom of the NAD⁺ nicotinamide. Altogether, our findings here serve as a cautionary note when using TCEP and THPP in biological assays and structural studies of NAD(P)⁺-dependent oxidoreductases.

### ASSOCIATED CONTENT

* Supporting Information The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00490.

Methods, SI Tables 1–6, and SI Figures 1–8 (PDF)

### Accession Codes

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### References


