Phosphite Dehydrogenase: An Unusual Phosphoryl Transfer Reaction

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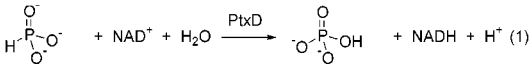
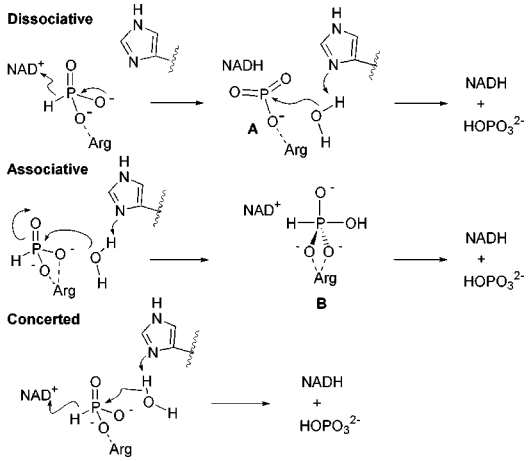
Phosphoryl transfer constitutes one of the most important reactions in biology. These transformations are involved in as diverse processes as DNA/RNA synthesis and maintenance, energy metabolism, and signal transduction. We report here mechanistic studies on a fundamentally different phosphoryl transfer reaction, the unprecedented enzymatic oxidation of inorganic phosphite[1] to phosphate (eq 1).[2,3]



The enzyme phosphite-NAD^+ oxidoreductase (PtxD) has recently been isolated from *Pseudomonas stutzeri* WM88,[2] and represents the first protein that catalyzes redox chemistry on inorganic phosphorus compounds. Initial studies have shown the enzyme to have low but significant sequence homology (23−34%) with the class of D-hydroxy acid dehydrogenases (DHs).[3] Among the conserved residues are three proposed active site residues, Arg237, Glu266, and His292.[4] On the basis of biochemical[4c,5] and crystallographic studies,[6] the roles of these residues in D-hydroxy acid DHs are believed to involve binding of the carboxylate of the substrate (Arg), deprotonation of the substrate alcohol (His), and stabilization of the protonated histidine via a catalytic diad with the active site glutamate.

The mechanisms of phosphoryl transfer reactions in solution and catalyzed by enzymes have been investigated in great detail.[7] The most straightforward extrapolation of the mechanism of D-hydroxy acid DHs to the reaction catalyzed by PtxD would invoke a metaphosphate intermediate **A** (Scheme 1).[8] This species

Scheme 1



has been the subject of extensive investigation, and its viability as a free reaction intermediate in aqueous solution or enzyme active sites has been ruled out in all systems investigated thus far.[7,9−11] Alternatively, an associative mechanism could generate a pentacoordinate phosphorane intermediate **B** that could subsequently transfer a hydride to the cofactor. However, such phosphorane intermediates have been considered inconsistent with kinetic isotope effects[7d,e] and Brønsted LFER studies[11,12] in intermolecular phosphoryl transfer reactions of phosphate monoesters. Most researchers currently favor concerted mechanisms for this type of reaction.[13] The transition states for these concerted mechanisms are believed to be dissociative,[7d−f,11] with a larger degree of bond cleavage than bond formation. While the phosphite dehydrogenation reaction described here has similarities with phosphoryl transfer involving phosphate monoesters, the structure of the substrate and leaving group are very different. Thus, experimental studies are required to provide insight into the transition state structure for phosphite oxidation.[14]

All mechanisms in Scheme 1 invoke direct transfer of the phosphorus-bound hydrogen to the cofactor. To verify this, we prepared deuterium-labeled phosphite by repeated lyophilization of phosphorous acid in D_2O. The reaction of the enzyme with this labeled substrate was monitored at 340 nm to ensure complete conversion of the substrate.[15] The reduced cofactor was purified by anion exchange chromatography, and the ^1H NMR spectrum

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(1) While we use the common name phosphite for $HP(O)O_2^{2-}$, the correct nomenclature for this compound would be hydrogenphosphonate.

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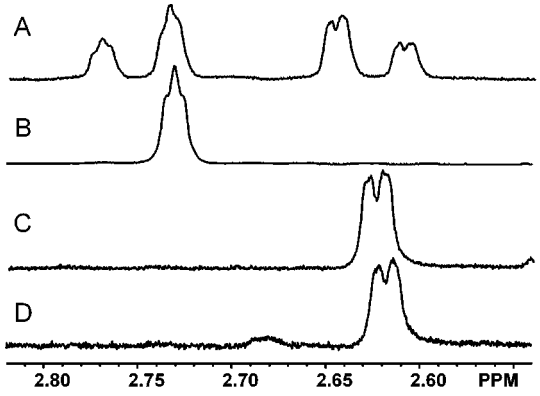

Figure 1. ^{1}H NMR spectra of (A) commercial NADH, (B) (4*S*)-[4-^{2}H]-NADH, (C) (4*R*)-[4-^{2}H]-NADH, and (D) the product formed by incubation of PtxD with ^{2}H-phosphite.

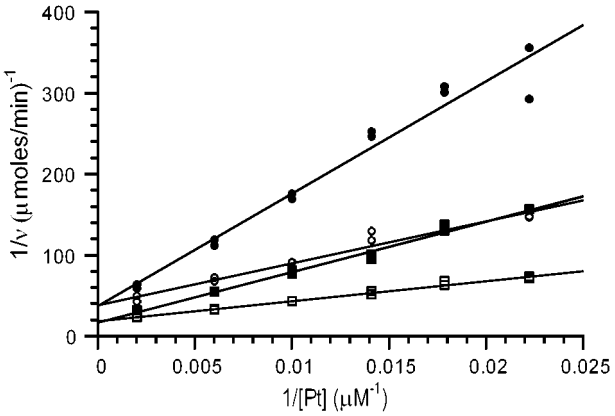

Figure 2. Reciprocal plots of the initial rates in the reaction of PtxD with unlabeled phosphite (squares) and deuterium-labeled phosphite (circles) at fixed NAD^+ concentrations of 165 (closed symbols) and 500 μM (open symbols).

was recorded and compared with that of commercial NADH (Figure 1, panels D and A, respectively). Inspection of the spectral region containing the proton resonances at position 4 of the nicotinamide ring clearly shows that the product is stereoselectively deuterium labeled. To determine the configuration of the product, authentic (4*R*)-[4-^{2}H]-NAD^{2}H and (4*S*)-[4-^{2}H]-NAD^{2}H were prepared by incubation of glucose dehydrogenase with [1-^{2}H]-glucose and formate dehydrogenase with [1-^{2}H]-formate, respectively (Figure 1B,C).[16] By comparison, the NMR spectra show that PtxD transfers a hydride from phosphite to the *Re*-face of NAD^+.[17]

The mechanisms in Scheme 1 differ in the timing of the P−H bond cleavage. For enzymatic reactions, kinetic isotope effects can provide valuable information regarding the relative contribution of the rate constant for a certain chemical step to the overall kinetic process, and/or the extent of X−H bond cleavage in the transition state of this step. Given the support for direct hydride transfer (Figure 1), the deuterium-labeled phosphite was used to determine whether PtxD displays a kinetic isotope effect on phosphite oxidation. Initial rates were determined at six fixed concentrations of NAD^+ and six varying concentrations of either labeled or unlabeled phosphite.[18] As shown in Figure 2 for a subset of these kinetic experiments at two fixed NAD^+ concentrations, a steady-state kinetic isotope effect of 2.1 ± 0.1 was observed on V_{max}. Using previously reported stretching frequencies for P−H and P−D bonds in phosphorous acid,[19] we estimate the theoretical maximum for a classical kinetic isotope effect for the cleavage of these bonds to be around 5.0 at 25 °C. Therefore, the observed isotope effect on V_{max} suggests that the hydride transfer step is partially rate limiting, or that deuterium substitution renders this step rate limiting for labeled phosphite.[20] As expected for a steady-state ordered mechanism with the cofactor binding first, essentially no isotope effect (1.0 ± 0.2) was observed on $V_{max}/K_{m,NAD^+}$.[21] The isotope effect on $V_{max}/K_{m,phosphite}$, 1.8 ± 0.3, was within experimental error of that for V_{max}.[22] These findings are similar to the observed isotope effects for yeast formate dehydrogenase from *C. boidini* ($^DV = 2.7$, $^D[V/K_{formate}] = 2.8$),[23,24] a member of the D-hydroxy acid dehydrogenase family that has 27% sequence identity with PtxD. For formate dehydrogenase, a large body of evidence suggests that the hydride transfer is entirely rate limiting and that the observed steady-state isotope effect represents the intrinsic isotope effect on the transformation. This allowed a detailed description of the transition state geometry of this step.[23,25] Pre-steady state and tritium isotopic effect studies[24] are in progress to determine if hydride transfer is also rate limiting for PtxD or whether other steps may be kinetically significant in phosphite oxidation.

In summary, we report the first mechanistic studies on a highly unusual phosphoryl transfer reaction. Our results indicate stereoselective transfer of hydride from phosphite to the *Re*-face of NAD^+ with observed steady-state kinetic isotope effects of 2.1 on V_{max} and 1.8 on V_{max}/K_m.

Acknowledgment. This work was supported by the University of Illinois and the Arnold and Mabel Beckman Foundation (W.A.V.) and the National Institutes of Health (grant GM59334 to W.W.M.).

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(15) Given the redox potentials at pH 7.0 for NAD^+/NADH (−0.32 V: Kaplan, N. O. *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1960; Vol. 3, p 105) and phosphite/phosphate (−0.65 V: In *Standard Potentials in Aqueous solutions*; Bard, A. J., Parsons, R., Jordan, J., Eds.; Marcel Dekker: New York, 1985), the equilibrium constant for the oxidation of phosphite by NAD^+ can be estimated as 10^{11}.

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