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Minireview

Mechanism and applications of phosphite dehydrogenase

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Abstract

Phosphite dehydrogenase catalyzes the NAD^+-dependent oxidation of hydrogen phosphonate (common name phosphite) to phosphate in what amounts to a formal phosphoryl transfer reaction from hydride to hydroxide. This review places the enzyme in the context of phosphorus redox metabolism in nature and discusses the results of mechanistic investigations into its reaction mechanism. The potential of the enzyme as a NAD(P)H cofactor regeneration system is discussed as well as efforts to engineer the cofactor specificity of the protein.

Keywords: Dehydrogenase; Phosphite; Cofactor regeneration; Phosphoryl transfer

1. Introduction

Phosphite dehydrogenase (PTDH or PtxD) is a unique, aerobic, NAD^+ dependent enzyme that oxidizes inorganic phosphite (hydrogen phosphonate) to phosphate Eq. (1) [1,2]. This reaction represents an unusual phosphoryl transfer from a hydride donor to a hydroxide acceptor. In the process, the phosphorus atom is

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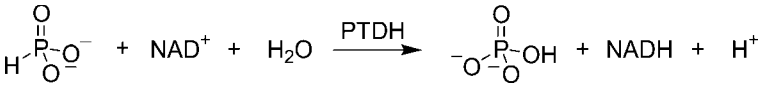
oxidized from a formal oxidation state of +3 to +5, with a redox potential of −0.65 V for the phosphite/phosphate couple [3]. When combined with the redox potential of the NAD^+/NADH couple of −0.32 V at pH 7.0 [4], the equilibrium constant can be calculated as 10^{11} indicating that the oxidation of phosphite by PTDH is essentially irreversible. This review will discuss the current understanding of the mechanism of phosphite dehydrogenase and highlight its application as a cofactor regeneration system.



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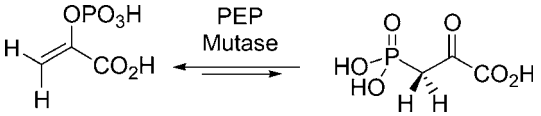
2. Oxidation of reduced phosphorus compounds in nature

2.1. Naturally occurring reduced phosphorus species

Phosphorus in living systems typically exists in the +5 oxidation state in the form of inorganic phosphate, phosphate esters, or phosphate anhydrides. Examples are the ubiquitous nucleotides, RNA, DNA, enzymatic cofactors, and phospholipids. Most enzymes involved in the biosynthesis and breakdown of these compounds catalyze phosphoryl transfer reactions during which the oxidation state on phosphorus is not changed. Less common, and not nearly as well characterized, are enzymes involved in the formation and use of phosphorus containing compounds in which the phosphorus is present in a lower oxidation state. Over the last two decades, many naturally occurring phosphonates ($RP(O)(OR')_2$, +3 formal oxidation state) and phosphinates ($R_2P(O)OR'$, +1) have been reported [5]. A few enzymes involved in P–C bond formation and breakage have been purified and characterized such as phosphoenolpyruvate mutase Eq. (2) [6–11], phosphonoacetaldehyde hydrolase Eq. (3) [12–14], phosphonopyruvate hydrolase [15], and phosphonoacetate hydrolase [16–18]. Although the formal oxidation state on phosphorus changes in the reactions catalyzed by these enzymes, the overall reaction does not represent a redox process.



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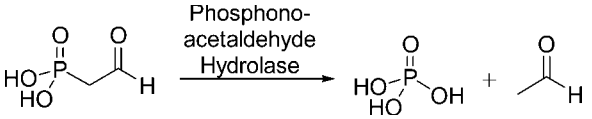


(3)

Aside from transformations involving compounds with P–C bonds, reports of biological chemistry featuring phosphorus in a low oxidation state are rare. Devai and co-workers in 1988 disclosed that a discrepancy in the mass balance of

phosphorus in sewage treatment facilities was accounted for by release of gaseous phosphine (PH_3). This process was shown to involve living organisms as bacterial reduction of inorganic phosphate could be recreated in a lab setting, but no such conversion was observed in the absence of microorganisms [19]. Subsequent reports on the presence of phosphine in the atmosphere suggest that phosphine production may be quite common [20–26].

2.2. Phosphorus-oxidizing bacteria

Several *Bacillus* species can oxidize phosphite and hypophosphite (dihydrogen phosphonate, $H_2PO_2^-$, phosphorus valence +1) to phosphate, including an anaerobic oxidizer which utilizes both reduced phosphorus species [27]. Furthermore, it has been reported that several common laboratory strains, including *Escherichia coli*, are capable of oxidizing phosphite [2]. Whereas these studies have documented oxidation of phosphorus compounds by cell-free extracts [28], in very few instances has a specific protein been implicated in this function. One early study by Malacinski and Konetza [29] identified an orthophosphate-NAD oxidoreductase, but this work was not followed up on. A very recent report shows that *E. coli* can utilize alkaline phosphatase for phosphite oxidation in a process that generates hydrogen [30].

The recently discovered marine bacterium *Desulfotignum phosphitoxidans* uses phosphite as its sole source of electrons [31–33]. This anaerobic oxidation of phosphite in energy metabolism is believed to be coupled to sulfate or carbon dioxide reduction. Expression of a 42 kDa protein was induced by growth on a phosphite-rich medium, but no specific phosphite-oxidizing protein could be detected in cell extracts of this system.

2.3. Genetic analysis of phosphite oxidation in Pseudomonas stutzeri WM88

Pseudomonas stutzeri WM88 was reported by Metcalf and Wolfe in 1998 as an efficient oxidizer of reduced phosphorus compounds [2]. The strain was identified by selection on media containing hypophosphite or phosphite as the sole phosphorus source. Genetic analysis showed that oxidation of hypophosphite to phosphate occurs in two distinct steps with phosphite as an intermediate. The gene clusters responsible for these two steps, the *htx* and *ptx* loci, respectively, have been characterized. The HtxA protein oxidizes hypophosphite to phosphite and is a member of the α-ketoglutarate-dependent dioxygenase family [34]. The functions of other genes in the *htx* cluster have not yet been established but on the basis of sequence homology they are likely involved in hypophosphite uptake and transport as well as carbon–phosphorus lyase activity. Similarly, homology to known *orfs* suggests that *ptxABC* encode phosphite transport proteins and *ptxE* encodes a transcriptional regulator. The gene product PtxD (PTDH) was shown to be directly responsible for phosphite oxidation in a NAD^+-dependent reaction [1] and its expression could be induced by phosphate starvation [35].

3. Potential mechanisms of phosphite oxidation by PTDH

3.1. Sequence and structural homology with the D-hydroxy acid dehydrogenases

PTDH is related to the family of NAD^+-dependent D-hydroxy acid dehydrogenases both by its chemistry and by its amino acid sequence. Sequence alignments reveal that PTDH has 26–35% sequence identity with other members of the family whose functions have been established [1]. Three conserved catalytic residues in this family are also present in PTDH (Fig. 1). The roles of these residues, Arg237, His292, and Glu266 (PTDH numbering), in the oxidation of hydroxy acids has been extensively studied [36–41]. These reports show that arginine binds the carboxylate moiety of the substrates, and that glutamate plays a role in both orientation and modulation of the pK_a of the active site histidine. This latter residue functions as a catalytic acid in the physiological direction of the reaction, the reduction of 2-keto acids, and is present in its protonated state providing a hydrogen bond to the carbonyl group that will be reduced. In the direction of hydroxy acid oxidation, the active site His deprotonates the alcohol to initiate hydride transfer. The catalytic glutamate is not present in formate dehydrogenase (FDH), the only member of the enzyme family that does not require a proton transfer during catalysis.

PTDH has yet to succumb to X-ray crystallographic characterization. A homology model of the enzyme, generated based on existing crystal structures of four D-hydroxy acid dehydrogenases [42], shows that the conserved catalytic residues are in position to participate in the reaction catalyzed by PTDH (Fig. 2). From the homology model, Lys76 was identified as a potential second positively charged residue in the active site to assist in binding the dianionic phosphite substrate [43]. This lysine is not conserved in those members of the D-hydroxy acid dehydrogenase family that utilize a monoanionic carboxylate substrate, but interestingly it is conserved in two *orfs* of unknown function that have much higher homology to PTDH than the other family members identified through a BLAST search of the non-redundant protein Data Bank (Fig. 1) [44]. In fact, a protein from *Klebsiella pneumonia* with essentially identical amino acid sequence to PTDH is retrieved in a nucleotide BLAST on 322 completed and unfinished microbial genomes. Furthermore, a recent report shows that a strain of *Alcaligenes faecalis* that can grow on phosphite contains an ortholog of PTDH [45]. This enzyme also contains the equivalent of Lys76. These searches suggest that phosphite oxidation activity may not be confined to *P. stutzeri* and could be widespread.

3.2. Concerted and covalent mechanisms of catalysis

The hydride and proton transfers during phosphite oxidation and NAD^+ reduction can be accomplished in several ways. Most straightforward would be a concerted mechanism (Fig. 3A), in which an active-site base, possibly the conserved histidine, deprotonates water, and the resulting hydroxide anion attacks the phosphorus center while the hydride leaving group is transferred to NAD^+. The

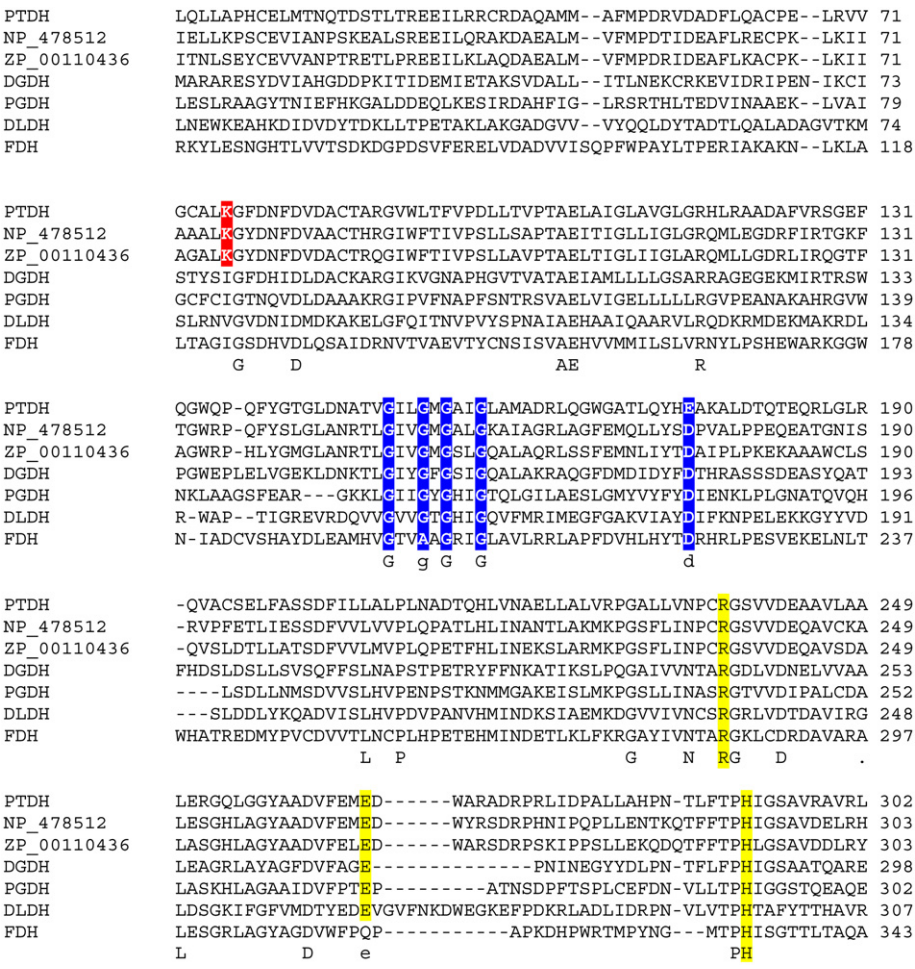


Fig. 1. Partial sequence alignment of PTDH with members of the D-hydroxy acid dehydrogenase family. Conserved residues are indicated in the consensus sequence. In blue are residues of the signature fingerprint motif ($GXGX_2GX_{17}D$) for a Rossmann fold to bind the cofactor [106,107], except that in PTDH the usual Asp is replaced by Glu. In yellow are three residues, Arg237, Glu266, and His292 (PTDH numbering) that have important catalytic functions in the hydroxy acid dehydrogenases [36–38]. A Lys residue (red) that is located in the active site of a homology model of PTDH [42] is conserved in the two BLAST hits with the highest level of identity, the function of which is currently unverified. *Abbreviations* (% identity with PTDH, accession number): DGDH, D-glycerate DH (*Hyphomicrobium methylovorum*, 27%, P36234) [108], DPGDH, D-3-phosphoglycerate DH (*E. coli*, 24%, P08328) [109]; DLDH, D-lactate DH (*Lactobacillus helveticus*, 26%, P30901) [110]; and FDH, formate DH (*Pseudomonas* sp. 101, 25%, P33160) [111]. Accession No. NP_478512 (*Nostoc* sp. PCC 7120, 51%) [112]; Accession No. ZP_00110436 (*Nostoc punctiforme*, 51%). Four of these proteins, DGDH [40]; DLDH (PDB number 2DLD), DPGDH [113], and FDH [39], have been structurally characterized. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

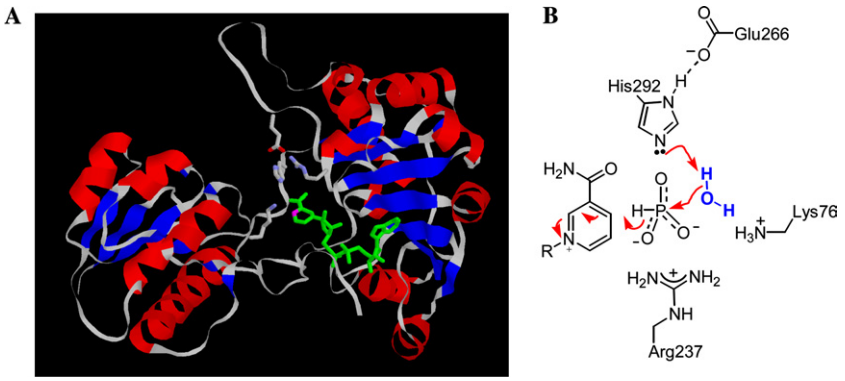


Fig. 2. (A) View of the active site in a homology model of PTDH [42]. (B) Possible roles of the active site residues in substrate binding and catalysis.

transition state in such a concerted process could have a dissociative character as is observed for phosphoryl transfer reactions of phosphate monoesters and monoanhydrides [46–50], species with the same dianionic charge as phosphite. Alternatively, an enzymatic nucleophile such as an active site histidine, cysteine or carboxylate could induce hydride transfer by attack at the phosphorus center. Such covalent catalysis would result in a phosphorylated enzyme intermediate (Fig. 3B), which would subsequently be hydrolyzed.

3.3. Associative mechanism

An associative mechanism, in which the nucleophile forms a bond to phosphorus prior to hydride transfer, necessarily invokes a pentacoordinate phosphorane intermediate (Fig. 3C). There has been much debate in the literature as to the viability of such a structure as a discrete intermediate for phosphate monoester dianions [46,50,51], which has been considered inconsistent with kinetic isotope effects [50,52] and Brønsted LFER studies in intermolecular phosphoryl transfer reactions of phosphate monoesters [48,49,53–55]. However, theoretical studies have questioned whether Brønsted LFER studies have a unique interpretation and rule out phosphorane intermediates [56,57], and a recent structure of the enzyme glucose-1,6-bisphosphatase was believed to contain a pentacoordinate phosphorus species [58]. Re-examination of the latter data by a different group of researchers suggests, however, that the planar four atoms in the bipyramidal species may have been MgF_3^-, an analog that would not differentiate between a bipyramidal intermediate in an associative mechanism and a bipyramidal transition state in a concerted mechanism [59]. As will be discussed below, one of the interpretations of a pH-rate profile on $k_{cat}/K_{m,phosphite}$ of PTDH suggests that the phosphite substrate will be dianionic, which by extrapolation of the existing data on phosphoryl transfer reactions of dianionic phosphate monoesters would suggest that an associative mechanism is less likely.

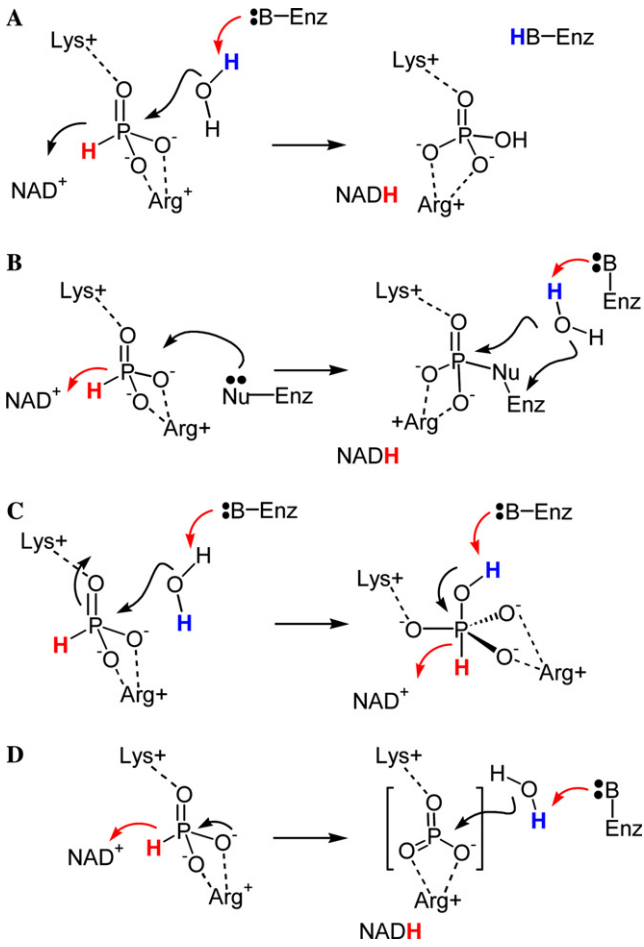


Fig. 3. Potential mechanisms for phosphite oxidation including (A) a concerted process, (B) covalent catalysis, (C) an associative process, and (D) a dissociative process.

3.4. Dissociative mechanism

A third possible mechanism of phosphite oxidation involves a dissociative process in which hydride transfer occurs first, yielding a metaphosphate intermediate, which is subsequently attacked by water (Fig. 3D). Model studies on phosphate ester hydrolysis have generated a wealth of information that suggests that metaphosphate is too unstable to be involved as a discrete intermediate [53,54,60–66]. A recently published structure of fructose-1,6-bisphosphatase, however, was reported to contain a bound metaphosphate-like species [67], leading to speculation that enzyme active sites might be able to sufficiently stabilize the compound to be involved as a true intermediate.

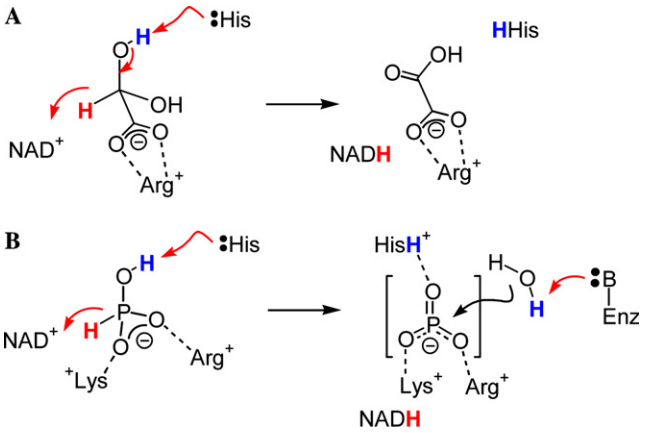


Fig. 4. (A) Proposed mechanism for the oxidation of the hydrated form of glyoxylate to oxalate by L-lactate dehydrogenase. (B) Extrapolation of the mechanism in (A) to the reaction catalyzed by PTDH if the true substrate for the enzyme is monoprotonated phosphite.

A second interpretation of the pH-rate profile on $k_{cat}/K_{m,phosphite}$ for PTDH invokes a reverse protonation model (see Section 4.5). In this mechanism, the substrate for the enzyme would be monoprotonated phosphite. In this context it is interesting to compare the reaction catalyzed by PTDH with the known oxidation of glyoxylate to oxalate by L-lactate dehydrogenase [68–71]. In this process, the active site His is proposed to deprotonate one of the hydroxyl groups of the hydrated aldehyde moiety of glyoxylate to initiate hydride transfer to the cofactor (Fig. 4A). An analogous mechanism could be drawn for PTDH if its substrate is in its monoprotonated form (Fig. 4B), which would lead to a metaphosphate intermediate. Perhaps this high energy intermediate might be accessible in this case due to the high energy phosphite substrate which as noted has a redox potential of −0.65 V. Computational studies on the reaction once a crystal structure is available may provide insight into this issue as will investigations of heavy atom isotope effects that are underway. Until support for a true dissociative mechanism is available, a concerted pathway appears most reasonable for phosphite oxidation by PTDH.

4. Mechanistic studies of the PTDH reaction

4.1. Kinetic parameters, cofactor specificity, and kinetic mechanism

Initial characterization of the enzyme [1] indicated that phosphite dehydrogenase operates via a sequential ordered mechanism (Fig. 5) in which NAD^+ binds first, followed by phosphite. It is assumed that phosphate is released first, followed by NADH, but because the reaction cannot be assayed in the reverse direction as a result of the enormously unfavorable thermodynamics, no experimental support is available for this supposition. This reaction scheme is consistent with that found in formate

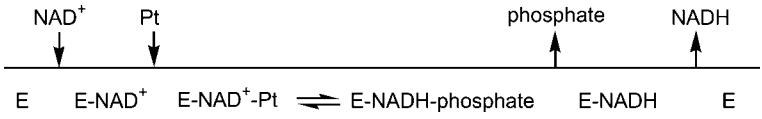


Fig. 5. Sequential ordered bi-bi kinetic mechanism of wt-PTDH.

dehydrogenases [72] and implies that NAD^+ binding induces a conformational change, which allows access to the phosphite binding pocket. This hypothesis is further supported by studies in which the enzyme is protected from thermal inactivation by preincubation with the cofactor but not by preincubation with the substrate [42], and by experiments that show that sulfite, a competitive inhibitor with respect to phosphite, essentially does not bind to the free enzyme but binds very tightly to the PTDH–NAD^+ complex [43]. Kinetic analysis shows that PTDH has K_m values near 50 μM for both phosphite (Pt) and NAD^+, and the V_{max} for the enzyme is approximately 12 μmol min^{-1} mg^{-1} [1]. This latter value is rather small given the significant thermodynamic driving force for the reaction. The low turnover number is not due to physical steps limiting catalysis as discussed in Section 4.4. Interestingly, the two enzymes within the family of D-hydroxy acid dehydrogenases with the smallest substrates and by far the most favorable reaction energetics for substrate oxidation, PTDH ($\Delta G^{0,\prime}$ −15 kcal/mol) and (FDH, $\Delta G^{0,\prime} = -5$ kcal/mol), have the lowest values for k_{cat} (~3–8 s^{-1}), whereas enzymes that catalyze significantly less favorable reactions have much higher k_{cat} values. For instance, D-lactate dehydrogenase from *Lactobacillus bulgaricus* ($\Delta G^{0,\prime} = +6.2$ kcal/mol [73]; $k_{cat} = 150$ s^{-1}) [74], and D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* ($k_{cat} = \sim 56$ s^{-1}) [75], both have significantly higher turnover numbers even in the unfavorable direction of substrate oxidation.

4.2. Substrate specificity and inhibition of phosphite dehydrogenase activity

To date, no alternative substrates have been identified for phosphite dehydrogenase [1,76]. Potential substrates such as thiophosphite, nitrite, methylphosphonate, methylphosphinate, and formate are not oxidized by the enzyme, nor do they act as inhibitors. Furthermore, the substrates utilized by homologous dehydrogenases are neither substrates nor inhibitors of PTDH. This includes the hydrated aldehydes of glyoxylate, glyoxal, or glycol aldehyde. Hydrated glyoxylate is oxidized by L-lactate dehydrogenase to oxalate [68–71].

The only known effective inhibitor of phosphite dehydrogenase is sulfite, SO_3^{2-} [1]. This substrate analog is a competitive inhibitor of phosphite ($K_i = 16$ μM) [1], and acts by forming a covalent adduct to NAD^+ in the active site of the enzyme that resembles the transition state for hydride transfer [76]. Presumably this adduct contains a covalent bond between sulfur and C4 of the cofactor as is observed in non-enzymatic reactions, although the latter require much higher concentrations of both NAD^+ and sulfite [77,78]. When potential alternative nucleophiles such as methanol, trifluoromethanol, and fluoride ion were introduced to compete with water in the reaction, only the native product was observed [76]. The closely related cofactor

$NADP^+$, while not an inhibitor, is a very poor hydride acceptor in the wild type enzyme ($K_{M,NADP+} = 2.51$ mM; $k_{cat}/K_M = 0.034\ \mu M^{-1}\ min^{-1}$) [1,42]. Use of $NADP^+$ also reduces the enzyme's ability to utilize phosphite, as indicated by an increase of $K_{M,Pt}$ to 1.8 mM [42]. Given the very favorable reaction energy of oxidation of phosphite and reduction of NAD^+, other members in the D-hydroxy acid dehydrogenase family have been screened for phosphite oxidation. Those tested to date, D-lactate dehydrogenase, 3-phosphoglycerate dehydrogenase, formate dehydrogenase, and glycerate dehydrogenase did not oxidize phosphite, showing that it is not a general substrate for this class of enzymes [76].

4.3. Stereospecificity of hydride transfer

The stereochemical outcome of hydride transfer to the nicotinamide ring on NAD^+ has been determined by using deuterium-labeled phosphite in the PTDH reaction. Upon incubation with [^{2}H]phosphite, the resulting [^{2}H]NADH was shown by ^{1}H NMR spectroscopy to be labeled at the 4*R* position of the nicotinamide ring establishing *Re* face selectivity and confirming a direct hydride transfer from phosphite to the cofactor [79]. This stereospecificity is the same as found for other members of the D-hydroxy acid dehydrogenase family.

4.4. Kinetic isotope effects

A kinetic isotope effect on phosphite oxidation has been measured with deuterium-labeled phosphite as a substrate [79]. The observed $^DV_{max}$ of 2.1 ± 0.1 indicates that hydride transfer is at least partially rate-limiting or that it becomes rate limiting when deuterated substrate is used. The enzyme displayed a similar effect on $V/K_{m,Pt}$ (1.8 ± 0.3), whereas no isotope effect was observed on $V/K_{m,NAD}$, consistent with an ordered mechanism with NAD^+ binding first [80]. These isotope effects are larger than they appear at first glance because the theoretical maximal classical isotope effect based on infrared stretching frequencies of P–H and P–D bonds can be calculated as 5.0 at 25 °C [79]. FDH, which catalyzes a reaction that may be most analogous to that of PTDH, exhibits comparable kinetic isotope effects of $^D(V/K_m) = 2.8$ and $^D(V_{max}) = 2.3$ at its pH optimum of 7.8 [81]. From heavy atom isotope effect studies, Cleland and co-workers concluded that these effects can be attributed to chemistry being fully rate-limiting with a late transition state explaining their non-maximal values. Whether the chemical step is also fully rate limiting for PTDH remains to be established. The fact that mutants that differ over one order of magnitude in their kinetic parameters produce essentially identical isotope effects as the wt enzyme would be consistent with hydride transfer being fully rate limiting (see Section 4.6) [43].

4.5. pH and temperature dependence

Phosphite dehydrogenase activity is highly dependent on both temperature and pH. The optimal temperature for the enzyme is 35 °C with a sharp decrease in

activity at both high and low temperature [1]. PTDH is quite stable at room temperature, whereas the half-life at 40.5 °C is just 9.6 min [42]. Its k_{cat} is pH independent over a range of 5.5–9.5, but log $k_{cat}/K_{m,phosphite}$ plotted versus pH exhibits a narrow bell shape with a very small pH range (<1 U) centered around pH 7.25 in which the enzyme is most active [76]. Above and below this range, dehydrogenase activity drops off steeply with a slope of unity, indicating that one proton transfer transition is responsible for the observed activity changes in each limb. Using the appropriate equations pK_a values of 6.8 ± 0.1 and 7.8 ± 0.2 were extracted from the pH-rate profile. The acidic pK_a value can be assigned to the second deprotonation of phosphorous acid in which case the dianionic form of phosphite would be the substrate for the enzyme. In this scenario, the basic pK_a would be associated with a residue in the PTDH–NAD^+ complex that must be protonated for phosphite binding. Mutagenesis studies are not consistent with this residue being Lys76, and the residue has been tentatively assigned to Arg237 [43].

An alternative interpretation of the pH-rate profile for $k_{cat}/K_{m,phosphite}$ invokes a reverse protonation model (Fig. 6) in which the group with the higher pK_a must be deprotonated and the group with the lower pK_a must be protonated for activity [82]. When two microscopic pK_as that give rise to a bell shaped pH-rate profile are separated by less than 2 U as is the case for PTDH, one cannot determine from the pH dependence alone whether the reactive enzyme–substrate collision complex is the protonated or the reverse protonated state (for a good graphic presentation of

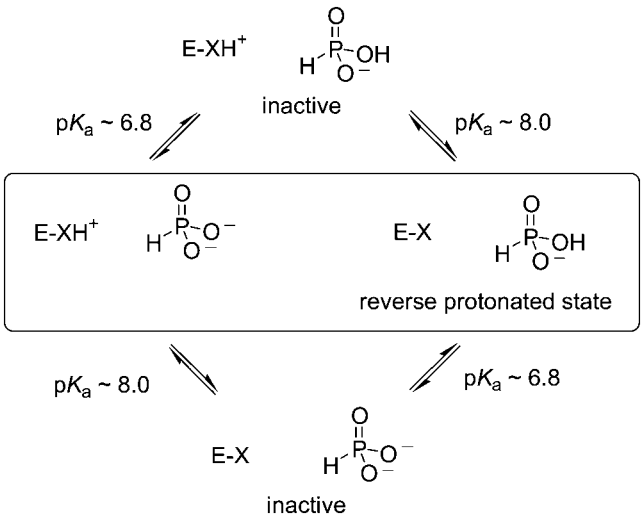


Fig. 6. Possible protonation states of the phosphite substrate and PTDH with the potentially active forms inside the box. In a mechanism that utilizes a regular protonation state (left side), the dianionic form of phosphorous acid is the true substrate and an enzymatic residue must be protonated for binding. In a reverse protonation mechanism, the group with the lower pK_a, here phosphite mono anion, will remain protonated whereas the group with the higher pK_a, here a residue on PTDH, will be deprotonated in the active enzyme–substrate collision complex.

an example, see [83]). In the context of PTDH this would mean that the protonation states of substrate and enzyme that form a complex consist of monoprotonated phosphite and a deprotonated active site enzyme residue (Fig. 6, right side). Such a reverse protonation mechanism would explain the relatively low catalytic efficiency despite the high thermodynamic driving force since only a very small fraction of enzyme and substrate would be in the correct protonation state at the optimal pH. As with most enzymes that utilize a reverse protonation mechanism, one can wonder why PTDH would utilize a reverse protonation mechanism. The answer may lie in the origin of the enzyme. Phosphite has yet to be reported as a constituent of the environment, but it has been used extensively in recent decades in industrial settings. Since phosphite is toxic to microorganisms such as the *Pseudomonas* strain from which PTDH was first isolated, it is conceivable that phosphite oxidation is a relatively recent activity that has not yet been fully optimized. Binding of phosphite in its monoprotonated form may therefore be a remnant of the monoanionic 2-keto acid substrate of its ancestor, although PTDH clearly has evolved to recruit an additional Lys to its active site. Furthermore, binding of the monoprotic form provides a clear avenue for converting an active site set up for oxidation of an alcohol substrate to one that would support oxidation of phosphite. An alternative explanation for the use of a reverse protonation mechanism is that the thermodynamic disadvantage of binding phosphite in its monoprotonated form may be offset by a kinetic advantage in catalysis. Unfortunately, unlike phosphoryl transfer reactions with heteroatom leaving groups [84,85], good model systems are not available to provide insights into advantages that may be conferred onto the reaction by the protonation state of the substrate. Clearly, verification that PTDH uses a reverse protonation mechanism by independently determining the pK_a of the active site residue, possibly using NMR methods, is essential. These studies are currently underway in our laboratory.

4.6. Site-directed mutagenesis

Site-directed mutagenesis studies have provided support for the involvement of the conserved residues shown in Fig. 1 in substrate binding and catalysis. The mutants H292F, H292K, H292Q, and H292N are all inactive [43]. Additionally, when the wild type enzyme is treated with diethyl pyrocarbonate, which selectively modifies histidine residues via nitrogen alkylation, activity is abolished. The enzyme can be protected from this inactivation by adding saturating conditions of NAD^+ and sulfite, a competitive inhibitor of phosphite. These data appear to indicate that His292 is essential for catalysis. Very recent data suggest, however, that the prime reason for loss of activity in the His mutants is the result of abolished NAD^+ binding (H. Relyea, W.A. van der Donk, unpublished results). Hence no firm conclusions can be drawn regarding the importance of His292 in catalysis.

Replacing the conserved Arg with a Leu, His, or Gln residue also led to complete loss of activity. However, the R237K mutant was active albeit with a reduced V_{max} (100-fold), and with a substantial increase in the Michaelis constants of both phosphite and NAD^+ (120- and 20-fold, respectively). Substitution of Glu266 with Gln resulted in a similar increase in $K_{m,Pt}$ but did not show a decreased V_{max} relative

to the wild type. These results rule out Glu266 as a possible nucleophile in covalent catalysis. The pH dependence of the E266Q mutant was essentially unaltered compared to wt-PTDH, in contrast to similar mutations in other D-hydroxy acid dehydrogenases, which resulted in drastic changes [36,74]. The results suggest that unlike the family members that act on hydroxy acids, the role of Glu266 in PTDH is not to increase the pK_a of His292 to which it is hydrogen bonded. This finding is not surprising when taking the physiological roles of these proteins into consideration. In vivo, the D-hydroxy acid dehydrogenases typically are involved in the reduction of 2-keto acids, and hence these proteins have evolved the ability to optimally bind these substrates. This requires a protonated His in the active site that is involved in a hydrogen bond to the carbonyl group of the substrate and that acts as general acid during catalysis [36,74]. For PTDH, its physiological role is phosphite oxidation and in this direction His292 must be deprotonated to act as either a general base or a nucleophile. Hence, the pK_a of His292 is not elevated by Glu266, and ostensibly the glutamate functions to correctly position both His292 and Arg237 for substrate binding. Once substrate is bound, Glu266 does not play an important role in catalysis as shown by the increased k_{cat} when this residue was mutated to Gln [43].

The identification of Lys76 as an additional binding residue in the homology model of PTDH has been supported through mutagenesis studies [43]. Replacement with non-charged residues drastically increased the K_m of phosphite and to a lesser degree also that of NAD^+ and results in a greatly reduced k_{cat}. Substitution with Arg had a much less profound effect on both K_m and k_{cat}. A K76C mutant could be chemically rescued by reaction with 2-bromoethylamine [86,87], resulting in a protein variant with a catalytic efficiency ($k_{cat}/K_{m,phosphite}$) that was almost identical to the wt protein.

4.7. Mechanism of phosphoryl transfer

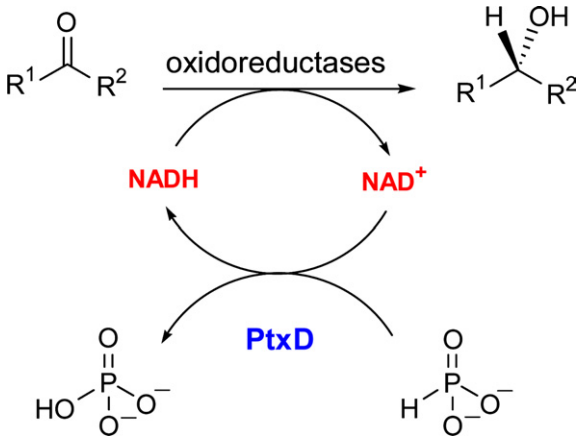
The above sections have discussed the use of isotope effects to study proton and hydride transfer events involved in phosphite oxidation. Furthermore, kinetic analysis of various mutants has provided insight into the conditions necessary to bind phosphite and convert it to phosphate. However, the detailed mechanism of the key step—phosphoryl transfer—remains elusive. Unfortunately, the techniques commonly used to study the course of enzymatic phosphoryl transfer reactions that have provided much of the information discussed in Section 3 have not proven amenable to the PTDH system. The use of chiral phosphorus species to determine the stereochemistry of phosphoryl transfer [88] has not been possible since PTDH accepts neither thiophosphite nor phosphite esters. Moreover, it has not been feasible to use electronically diverse nucleophiles to determine linear free energy relationships [48,49,55]. Hence, the details of the stereochemical and electronic aspects of the reaction have not yet succumbed to investigation. The use of NAD^+ analogs with different redox potentials may allow perturbations of the transition state that will provide insights into the transition state structure [89]. Alternatively, although challenging for the system at hand, the analysis of heavy-atom isotope effects using whole molecule mass spectrometry [90,91] may provide valuable insight into the mechanism of

hydride transfer. Such studies could be performed in several ways. For instance, information regarding the bond order in the transition state of the bonds between the phosphorus atom and the three oxygens derived from phosphite [84,92] may be accessible through a competitive KIE study using [$^{18}O_3$]phosphite and ^{18}O-depleted phosphite. These studies are currently under investigation in our laboratory.

5. Application of PTDH as a cofactor regeneration system

5.1. Regeneration of NAD(P)H and [^{2}H]NAD(P)H

Enzymes that stereoselectively reduce carbonyl and imine moieties are widely used in the preparation of enantiomerically enriched products [93]. Many of these proteins utilize NADH or its phosphorylated analog NADPH as stoichiometric hydride donors. While the enzymatic reactions are efficient and selective, the cofactors utilized are very expensive. The cost of NADH is upwards of $25/mmol while the price of NADPH is greater than $500/mmol (Sigma 2004–2005 catalog). Several regeneration systems have been developed which convert the oxidized cofactor back to its reduced form, rendering the cofactor catalytic. For example, NAD(P)H can be recycled electrochemically via a rhodium mediator with a turnover rate of 36 h^{-1} [94–98]. Alternatively, a second enzyme can be used as a cofactor reducing agent (Scheme 1) [73,99]. This second enzyme needs a sacrificial reductant that must be cheap and innocuous. The current standard for enzymatic cofactor regeneration is formate dehydrogenase. FDH catalyzes the oxidation of formate to carbon dioxide in an essentially irreversible process at a rate of about 8000 h^{-1}. Additionally, both substrate and product are relatively inert. This system has been used in NADH regeneration on an industrial scale for a number of years [100,101], but still has drawbacks in that the enzyme has low specific activity and is very sensitive to organic solvents.



Scheme 1. Cofactor regeneration.

5.2. Phosphite dehydrogenase as an NADH regeneration system

PTDH and phosphite constitute a very promising system due to the great thermodynamic driving force for catalysis ($\Delta G^0 = -15$ kcal/mol compared to -5 kcal/mol for FDH) and the low expense of the substrate. During NADH regeneration with PTDH, a phosphite buffer is essentially converted to a phosphate buffer at a turnover rate of approximately 15,000 h^{-1}, and reaction progress can be easily and non-invasively monitored via ^{31}P NMR spectroscopy [102]. Furthermore, deuterium and tritium labeled phosphorous acid can be cost-effectively produced by incubation with D_2O or tritiated water. Fortuitously, the exchange reaction that readily takes place at acidic pH essentially does not occur once the two oxygens are deprotonated at pH 7, and hence labeled phosphite can be utilized for the production of high-value deuterium and tritium labeled products (Fig. 7) [102]. In contrast, the labeling of formate with heavy hydrogen atoms is much less facile, making the FDH system less amenable for preparing labeled products for use in basic research and medicine.

5.3. Rational design to alter cofactor specificity

PTDH can be used efficiently to render NADH catalytic in a synthetic process, but the inability of the wild type enzyme to utilize $NADP^+$ prevents the enzyme from being a universal nicotinamide regeneration system. The prospect of making cofactor binding less specific was addressed by analysis of a homology model of PTDH, and subsequent manipulation of the enzyme by site directed mutagenesis to remove steric and electronic constraints for NADP binding [42]. Docking of NAD^+ and $NADP^+$ into the homology model revealed a glutamate residue (Glu175) in the cofactor binding site that would result in steric and electronic repulsive interactions with the phosphate group of $NADP^+$. With NAD^+, this Glu forms productive hydrogen bonds with the 2′- and 3′-hydroxyl groups on the ribose. In an effort to remove the phosphate–carboxylate interaction, the double mutant E175A/A176R was generated.

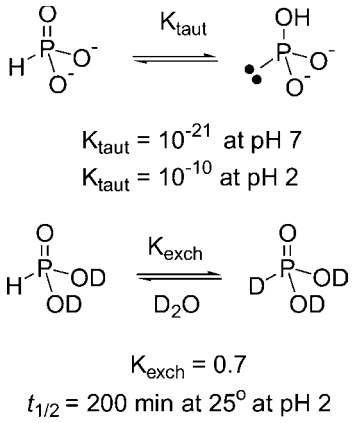


Fig. 7. Exchange of the hydrogen bound to phosphorus with solvent is much less favorable at pH 7 than at pH 2 [114,115].

Kinetic analysis of this protein revealed that the K_m for $NADP^+$ was much improved relative to the wild type ($K_{M,NADP^+} = 3.5$ μM). Somewhat surprisingly given the loss of two hydrogen bonds to the ribose hydroxyl groups, the K_m for NAD^+ was also improved in this mutant ($K_{M,NAD^+} = 20$ μM) resulting in a protein that has an increased k_{cat}/K_M for both cofactors and phosphite relative to wild type [42]. In the case of $NADP^+$, the improvement was three orders of magnitude. Hence, this double mutant is currently the protein of choice when utilizing PTDH for cofactor regeneration.

5.4. Directed evolution to improve regeneration efficiency

The effectiveness of phosphite dehydrogenase as a cofactor regeneration system is somewhat limited by the relatively low value for k_{cat} ($\sim 4\ s^{-1}$). Given the large thermodynamic driving force and the fact that chemistry is at least partially rate limiting as shown by the kinetic isotope effect studies, efforts are ongoing to increase the enzyme's turnover number. The approach of choice in this case is the use of directed evolution, in which PTDH mutants generated via error-prone polymerase chain reaction (PCR) are selected by the ability of the organism that expresses these genes to survive on phosphite as the only source of phosphorus [103]. Using this selection method both k_{cat} and K_m were improved as well as the level of soluble expression. The latter is as important as the enzyme's activity since for an economically viable process the regenerative enzyme must be relatively inexpensive in terms of cost per unit, making optimization of enzyme production very important. A further improvement on the wt protein has been achieved by screening for mutants with a higher thermal stability [104]. The best mutant emerging from three rounds of random mutagenesis and high throughput screening contained 12 mutations and is 7000-fold more stable at 45 °C than the wild type without jeopardizing the catalytic activity. These enhancements have provided a robust biocatalyst whose performance is currently being evaluated in the reactor-scale production of *tert*-leucine, a process that is used industrially [105].

6. Conclusion

PTDH is an unusual NAD^+-dependent oxidoreductase, which specifically converts phosphite to phosphate. The enzyme has sequence homology with the D-hydroxy acid dehydrogenases although it differs greatly in the type of chemistry it catalyzes. Nevertheless, the active site residues Arg237, Glu266, and His292 are conserved in PTDH and are important for phosphite binding and/or catalysis. His292 is the likely active site base that deprotonates the water nucleophile, although a role as a nucleophilic catalyst cannot be ruled out. Given the unstable nature of a phosphohistidine intermediate and the fact that thiophosphite is not a substrate preventing stereochemical studies, it will be challenging to address the possibility of covalent catalysis. Arg237 is important for phosphite binding and Glu266 positions both His292 and Arg237. Lys76 may provide an additional binding interaction to accommodate a dianionic phosphite substrate, and Glu175 and Ala176 play a role

in binding of the cofactor and determine cofactor specificity. The hydride of phosphite is directly transferred to the nicotinamide cofactor and this step is at least partially rate limiting. The individual rates of the various steps involved in the overall catalytic process still need to be elucidated. Unfortunately, the lack of alternative substrates and the practical irreversibility of the reaction pose impediments for these mechanistic studies. On the other hand, the strong exergonic nature of the reaction make the system particularly adaptable for use as a cofactor regeneration enzyme.

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