Isolation and Biochemical Characterization of Hypophosphite/2-Oxoglutarate Dioxygenase

A NOVEL PHOSPHORUS-OXIDIZING ENZYME FROM PSEUDOMONAS STUTZERI WM88*

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The htxA gene is required for the oxidation of hypophosphate in Pseudomonas stutzeri WM88 (Metcalf, W. W., and Wolfe, R. S. (1998) J. Bacteriol. 180, 5547–5558). Amino acid sequence comparisons suggest that hypophosphate:2-oxoglutarate dioxygenase (HtxA) is a novel member of the 2-oxoglutarate-dependent dioxygenase enzyme family. To provide experimental support for this hypothesis, HtxA was overproduced in Escherichia coli and purified to apparent homogeneity. Recombinant HtxA is identical to the native enzyme based on amino terminus sequencing and mass spectral analysis, and it catalyzes the oxidation of hypophosphate to phosphite in a process strictly dependent on 2-oxoglutarate, ferrous ions, and oxygen. Succinate and phosphate are stoichiometrically produced, indicating a strict coupling of the reaction. Size exclusion analysis suggests that HtxA is active as a homodimer, and maximal activity is observed at pH 7.0 and at 27 °C. The apparent $K_m$ values for hypophosphate and 2-oxoglutarate were 0.58 ± 0.04 mM and 10.6 ± 1.4 μM, respectively. $V_{max}$ and $k_{cat}$ values were determined to be 10.9 ± 0.30 μmol min$^{-1}$ mg$^{-1}$ and 355 min$^{-1}$, respectively. 2-Oxoadipate and pyruvate substitute poorly for 2-oxoglutarate as a cosubstrate. The highest specific activity is observed with hypophosphate as substrate, but HtxA is also able to oxidize formate and arsenite at significant rates. The substrate analog inhibitors, formate and nitrate, significantly reduce HtxA activity.

The current view of phosphorus metabolism dictates that, unlike other elements essential for growth, phosphorus does not undergo a biologically catalyzed oxidation-reduction cycle in nature. The biochemistry involving this essential and often limiting nutrient is typically thought to be restricted to the formation and hydrolysis of phosphate esters, in which phosphorus exists in its most oxidized state ($P^{5+}$). However, the number of microorganisms capable of using reduced phosphorus compounds as the sole source of phosphorus or able to synthesize reduced phosphorus compounds clearly demonstrates that this is not the case (1–5). Although microbial metabolism of reduced phosphorus compounds has been documented in the literature for several decades, it has remained unexplored in any detail on either the biochemical or genetic levels until recently. This is especially true with respect to the microbial oxidation of the reduced $P_1$ compounds, phosphate ($P^{3+}$) and hypophosphate ($P^{2+}$).

Microbial growth on hypophosphate or phosphate as the sole source of phosphorus has been reported in such microorganisms as Escherichia coli, Bacillus spp., Pseudomonas fluorescens, Klebsiella aerogenes, and Erwinia spp. (2, 4, 6–9); however, little is known about the biochemistry of hypophosphate or phosphate oxidation in these organisms. In P. fluorescens, activity of partially purified phosphorus-oxidizing enzyme was demonstrated to be NAD$^+$-dependent and specific for phosphate; however, a more detailed analysis was not completed (10). Similarly, hypophosphate oxidation was detected in cell extracts of Bacillus caldolyticus, which was demonstrated to grow on hypophosphate as the sole source of phosphorus (11). This enzyme was also partially purified, but nothing is known about the reaction beyond the requirement for NAD$^+$; neither the responsible enzyme nor the mechanism of hypophosphate oxidation was determined.

Recently we isolated Pseudomonas stutzeri WM88, an organism with the ability to oxidize hypophosphate and phosphate (12). Genetic analysis of hypophosphate and phosphate oxidation in P. stutzeri WM88 led to the identification of two regions of the chromosome involved in utilization of these compounds as the sole phosphorus sources, one region for the oxidation of hypophosphate and the other for phosphate oxidation. Furthermore, these studies showed that the genes involved in phosphate oxidation were also required for growth on hypophosphate, suggesting that hypophosphate oxidation to phosphate proceeds via a phosphate intermediate. Sequence analysis of the chromosomal region for hypophosphate oxidation revealed five open reading frames, htxABCDE, which appear to form a transcriptional unit. Analysis of the predicted amino acid sequence of the htxA gene product indicates that it is most similar to members of the 2-oxoglutarate-dependent dioxygenase family, having 28% identity to proline 4-hydroxylase from DacryostilbNewtoniun (13). The putative htxBCDE products likely comprise a binding protein-dependent hypophosphate transporter. Of the five open reading frames identified, only the htxA gene was required for hypophosphate oxidation in P. stutzeri WM88. This, in addition to the amino acid sequence similarities of hypophosphate:2-oxoglutarate dioxygenase (HtxA) to

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1 The abbreviations used are: HtxA, hypophosphate:2-oxoglutarate dioxygenase; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methylene; CAPS, 3-(cyclohexylamino)propanesulfonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MES, 2-(N-morpholino)ethanesulfonic acid; MBF, maltose-binding protein; MOPS, 3-(N-morpholino)propanesulfonic acid; PtxD, NAD:phosphite oxidoreductase.
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FIG. 1. Proposed biochemical pathway for the oxidation of hypophosphite to phosphate in P. stutzeri WM888. HtxA is a novel member of the 2-oxoglutarate-dependent dioxygenase superfamily of enzymes, strongly suggests that htxA encodes a novel enzyme responsible for hypophosphite oxidation, HtxA. Sequence analysis of the phosphite oxidation region suggested the presence of a binding protein-dependent phosphite transporter, encoded by ptxB, and an NAD-phosphate oxidoreductase (PtX), encoded by ptxD (12).

Based on these data, a biochemical pathway for the oxidation of hypophosphite to phosphate was proposed (Fig. 1) (12). In this pathway hypophosphite is first oxidized to phosphite by the HtxA protein. The phosphite produced in this reaction is subsequently oxidized to phosphate by the PtX protein. Strong biochemical evidence has been provided for the second step in this putative pathway (14, 15). The responsible enzyme, PtX, has been characterized in pure form. This enzyme is highly specific for its substrates and stoichiometrically produces phosphate and NADH from phosphite and NAD+. However, biochemical support for the initial step, oxidation of hypophosphite to phosphite, has yet to be provided. In this paper we address this deficiency by reporting the purification and initial biochemical characterization of HtxA, thus completing the first biochemical characterization of a pathway for the oxidation of the reduced inorganic phosphorus compounds.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—E. coli DH5α (16) was used as a host for cloning experiments and as a host for vector pMAL-c2X (New England Biolabs Inc., Beverly, MA) and its derivatives. E. coli BL21(DE3) (17) was used as a host for expression vector pET11a (Novagen, Madison, WI) and its derivatives. E. coli strains were grown in Luria-Bertani medium with either 100 μg/ml ampicillin or 50 μg/ml carbenicillin when appropriate. P. stutzeri strain WM536 is a spontaneous smooth colony mutant of the original phosphite- and hypophosphite-oxidizing isolate, WM88, and P. stutzeri WM567 is a spontaneous streptomycin-resistant derivative of WM536 (12). P. stutzeri strains were grown in either Luria-Bertani medium or MOPS minimal medium with 0.4% glucose and the appropriate phosphorus source (18). Phosphorus solutions were made fresh and sterilized by filtration immediately before use. For large scale expression of native or recombinant proteins, cultures were grown in a 30-liter stainless steel bioreactor (model P30A, B. Braun Biotech, Allentown, PA) at 30 °C. To remove residual phosphate remaining after the cleaning processes, all glassware for growth and media preparation was soaked in ultrapure deionized water with several changes. The bioreactor vessel was washed additionally with 0.1 m nitric acid and rinsed with ultrapure deionized water. For the preparation of phosphate-free solid glucose MOPS minimal medium, the agar was rinsed before use with several changes of ultrapure deionized water.

Construction of HtxA Expression Vector pAG4—Standard methods for DNA manipulation and cloning were used throughout (19). Plasmid pAG4, which carries HtxA under control of the T7 promoter, was constructed by PCR amplification of the htxA gene from P. stutzeri WM888 chromosomal DNA using Taq polymerase (Invitrogen) and the following primers: 5′-GGGGGGGGCCATATGGTTGCAGCAGCAAGCC-3′, which introduces an Ndel site at the translational start codon, and 5′-GGATCCCTCGAGTACGATAAGC-3′, which introduces a BamHI site just past the translational stop codon of htxA (20). The resulting PCR product was digested with Ndel and BamHI and ligated into the same sites of vector pET11a (Novagen). The sequence of the cloned htxA gene was determined using standard T7 promoter and terminator primers and was identical to the sequence determined previously.

Construction of Htx Promoter-lacZ Fusion Strain, WM2940—The 1.6-kb region of DNA directly upstream from the htxA translational start site was amplified by PCR using Taq polymerase (Invitrogen) and the following primers: 5′-GGGGGGGGCCATATGGTTGCAGCAGCAAGCC-3′, which introduces a SpeI site, and 5′-GGATCCCGGCGCGCGCATGTCGCGC-3′, which introduces a NotI restriction site (underlined) immediately after the htxA translational stop codon. The PCR fragment was digested with NotI and cloned into the NotI site of pJAML-c2X (New England Biolabs), creating pAW32. Creation of the correct fusion was verified by sequencing using the malE and M13/pUC sequencing primers (New England Biolabs).

Construction of Htx Promoter-lacZ Fusion Strain, WM2940—The 1.6-kb region of DNA directly upstream from the htxA translational start site was amplified by PCR using Taq polymerase (Invitrogen) and the following primers: 5′-GGGGGGGGCCATATGGTTGCAGCAGCAAGCC-3′, which introduces a SpeI site, and 5′-GGATCCCGGCGCGCGCATGTCGCGC-3′, which introduces a NotI restriction site (underlined) immediately after the htxA translational stop codon. The PCR fragment was digested with NotI and inserted into the same site of pET11a to create pAW36. Construction of the correct transcriptional htx promoter-lacZ fusion was verified by DNA sequencing. Plasmid pAW36 was transformed into the transfer-competent E. coli strain BW20767 (20) with selection for ampicillin resistance, followed by mating of the transformants with P. stutzeri strain WM567 with selection on glucose MOPS minimal medium containing ampicillin at 100 μg/ml. The resulting transformant was digested with NotI and inserted into the same site of pJAML-c2X to create pJAML-c2X. Construction of a htx promoter-lacZ fusion, a P. stutzeri exconjugate (WM2940) harboring pAW36 was grown on glucose MOPS minimal medium containing carbenicillin and either 2 mM Pi and 0.15% glucose (excess phosphorus) or 0.1 mM phosphate, phosphate, or hypophosphite and 1% glucose (limiting phosphorus). Cells were harvested at stationary phase (A560 about 1.0) by centrifugation, and extracts were made as described above, using β-galactosidase assay buffer (50 mM Tris-Cl, pH 8.0, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol) to resuspend the cells. Continuous β-galactosidase assays were performed in a 1-ml volume of β-galactosidase buffer with the addition of 2.7 mM o-nitrophenyl-β-D-galactoside and 0.05 ml of extract containing about 0.1 mg of protein. Activity was monitored as an increase in absorption at 420 nm.

Expression and Purification of MBP-PtxD—WM2921 (E. coli DH5α transformant harboring pAW32) was grown in 30 liters of Luria-Bertani medium, 0.2% glucose medium with carbenicillin in a 30-liter stainless steel bioreactor at 25 °C. The culture was induced for expression of the htxA gene in P. stutzeri WM888 chromosomal DNA using Taq polymerase (Invitrogen) and the following primers: 5′-GGGGGGGGCCATATGGTTGCAGCAGCAAGCC-3′, which introduces an Ndel site at the translational start codon, and 5′-GGATCCCTCGAGTACGATAAGC-3′, which introduces a BamHI site just past the translational stop codon of htxA (20). The resulting PCR product was digested with Ndel and BamHI and ligated into the same sites of vector pET11a (Novagen). The sequence of the cloned htxA gene was determined using standard T7 promoter and terminator primers and was identical to the sequence determined previously.

High speed extract containing about 300 mg of protein at 3 mg/ml was loaded at 0.75 ml/min onto a 2.5-cm (inner diameter) × 20-cm amylose/agarose column (New England Biolabs) preequilibrated in col...
umn buffer. Unbound protein was removed with about 10 column volumes of column buffer at 1.5 ml/min until protein was no longer detected in the eluant by UV absorption at 280 nm. MBP-PtxD was eluted with column buffer containing 20 mM maltose at a flow rate of 1.5 ml/min. The purest fractions determined by SDS-PAGE analysis and spectrometry were pooled, desalted, and concentrated using a Centriprep 30 concentrator (Amicon). The concentrated pools of three such purifications were then loaded onto a 4.6-mm (inner diameter) × 300-mm POROS NH2 anion exchange column (PerSeptive Biosystems, Inc., Framingham, MA) preequilibrated in buffer A. Unbound sample was removed with about 10 column volumes of buffer B. HtxA was eluted with a linear 0–100 mM NaCl, pH 7.0, as the mobile phase.

Expression and Purification of Recombinant HtxA—WM580 (E. coli BL21 (DE3) harboring pAG4) was grown at 37 °C in a 30-ml stainless steel bioreactor in Luria-Buranti medium containing carbenicillin. Protein expression was induced when the culture reached mid-log phase (A600, about 0.6) by the addition of IPTG to a final concentration of 1 mM. The cells were incubated for an additional 1.5 h and harvested by centrifugation.

Crude extracts were prepared by resuspending about 8 g of cells (wet weight) in 20 ml of buffer A (20 mM MOPS, 10% glycerol, 75 mM NaCl, pH 8.0). Approximately 10 mg of DNase I was added, and the cells were broken by passage through a French pressure cell twice at 12,000 p.s.i. High speed extracts were obtained as described above.

Chromatography for the purification of both recombinant and native HtxA was performed using AKTA fast protein liquid chromatography (Amersham Biosciences) at 4 °C. Recombinant HtxA was purified on a 10-mm (inner diameter) × 100-mm POROS® HQ anion exchange column (PerSeptive Biosystems, Inc, Framingham, MA), preequilibrated in buffer A, by applying high speed extract containing about 200 mg of protein at 24 mg/ml at 0.5 ml/min. Unbound protein was removed with 40 column volumes of buffer A at 3.0 ml/min. Recombinant HtxA was eluted with a 30-column volume linear gradient of 75–300 mM NaCl in 20 mM MOPS, 10% glycerol, pH 8.0, collecting 2.0-ml fractions. The purest fractions determined by HtxA specific activity and SDS-PAGE analysis were pooled, desalted, and concentrated using the ultrafiltration cell and membrane described above. The purest pools from five such purifications were combined and stored at −70 °C in 20 mM MOPS, 15% glycerol, pH 7.25, for use in the studies described below.

Expression and Purification of Native HtxA—The expression of HtxA in its native host, 30 liters of P. stutzeri WM567, was grown in a 30-liter bioreactor at 30 °C in MOPS minimal medium containing 0.4% glucose, 2 mM hypophosphite, and Antifoam 289 (Sigma). Cells were harvested at stationary phase (A600, about 1.4) by centrifugation. Approximately 25 g of cells was resuspended in 60 ml of buffer B and centrifuged at 10,000 × g. Unbound sample was removed with about 10 column volumes of buffer B. HtxA was eluted with a linear 0–200 mM NaCl, 1 mM FeCl3, 20 µM FeCl2, and 0.4–100 mM 2-oxoglutarate gradient over 40 column volumes. The purest fractions determined by HtxA specific activity and SDS-PAGE analysis were pooled, desalted, and concentrated using the ultrafiltration cell and membrane described above. The purest pools from five such purifications were combined and stored at −70 °C in 20 mM MOPS, 10% glycerol, 1 mM 2-oxoglutarate, 2 mM hypophosphite, and 1 mM NaCl, pH 7.0, as the mobile phase.

Amino Terminus Sequencing and Mass Spectrometry—Partially purified recombinant and native HtxA were separated from contaminating proteins on a 12% Tris-HCl precast polyacrylamide gel (Bio-Rad) under denaturing conditions. The protein was transferred to a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) in 10 mM CAPS, 10% methanol buffer, pH 11.0. Protein bands were visualized with Coomasie Blue, and the membranes were submitted to the University of Illinois Protein Sciences Facility for amino terminus sequencing by Edman degradation. Matrix-assisted laser desorption ionization mass spectrometry was performed at the University of Illinois Mass Spectrometry facility using a Voyager-DE STR mass spectrometer (PerSeptive Biosystems).

Enzyme Activity Assays—For purification and characterization of HtxA, a continuous coupled spectrophotometric assay using MBP-PtxD as a reporter enzyme was used. Activity was measured by NADH production monitored as an increase in absorbance at 340 nm. The extinction coefficient of 6,220 M−1 cm−1 was used to calculate NADH production, and enzyme activities are given in standard enzyme units (µmol of NADH produced/min). The standard assay mixture contained 20 mM MOPS, pH 7.0, 1 mM 2-oxoglutarate, 2 mM hypophosphite, 1 mM NaCl, 200 µM FeCl3, 0.2–1 mg of purified PtxD, and 5–10 µg of purified HtxA in a 1.0-ml volume. All activity assays were done at room temperature unless otherwise stated. Because HtxA activity rapidly diminishes in assay buffer (significant loss is observed within first 2 min at low pH), spectrophotometric data were collected once every 2 min. Only the linear portion of the curve, which was typically about 20 s, was used in activity calculations. Anaerobic activity assays were done using substrate and enzyme solutions, which were made anaerobic with multiple cycles of alternating vacuum and nitrogen gas exchange; the assays were carried out in gas-tight cuvettes. For the determination of pH optimum, 100 mM Tris, 50 mM glacial acetic acid, 50 mM sodium acetate, and the pH was adjusted appropriately with HCl or NaOH. Theionic strength of this buffer remained constant over the pH range tested (22). Stoichiometry studies and analysis of the use of alternative substrates were done by measuring succinate production in an end point assay using a commercially available kit (Roche Molecular Biochemicals).

31P NMR Assays—31P NMR spectra were acquired with a Varian Unity 500 spectrometer equipped with a 5-mm Nalorac QUAD probe at the Varian Oxford Instruments Center for Excellence in NMR Laboratory at the University of Illinois. The HtxA assay solution contained 20 mM MOPS, pH 7.0, 5 mM hypophosphite, 3 mM 2-oxoglutarate, 100 µM FeCl3, and 0.5 mg/ml HtxA in a 3-ml volume. A similar assay without HtxA was used as the enzyme-free control. Both mixtures were incubated with constant mixing at room temperature for about 2 h, after which samples were removed, D2O was added to 10% final concentration, and the samples were analyzed with 31P NMR. The phosphate only standard contained 5 mM phosphate in 20 mM MOPS buffer. Proton-coupled 31P spectra were acquired with an acquisition time of 0.655 s with 2,000 transients and using a pulse width of 5.2 µs. An external reference of 85% phosphoric acid set at 0 ppm was used.

RESULTS

Hypophosphite Oxidation Is Catalyzed by HtxA Protein—To demonstrate that HtxA encodes a protein that catalyzes the oxidation of hypophosphite to phosphate in a 2-oxoglutarate-dependent manner, a coupled continuous HtxA activity assay...
was developed using MBP-PtxD as a reporter enzyme. Accordingly, enzymatic oxidation of hypophosphite to phosphite is coupled to phosphite-dependent NAD+ reduction, which is monitored at 340 nm (see Fig. 1). However, using this assay, hypophosphite oxidation could not consistently be detected in crude extract of *P. stutzeri* WM88 grown on hypophosphite as the sole source of phosphorus; therefore, HtxA was overproduced in *E. coli*. Cell extract from *E. coli* WM804, in which HtxA is overexpressed, catalyzes hypophosphite oxidation with a specific activity of 2.40 units/mg. This activity is strictly dependent on the addition of 2-oxoglutarate and ferrous ions and was not detected in extract from *E. coli* BL21(DE3) harboring the pET11a expression vector only. These data strongly support the hypothesis that HtxA is a 2-oxoglutarate-dependent hypophosphite dioxygenase.

**Purification of Recombinant and Native HtxA—Overexpression in E. coli BL21(DE3) produces very high levels of soluble HtxA in the IPTG-induced extracts allowing about 95% homogeneous protein to be obtained after a single anion exchange chromatography step (Table I and Fig. 2). Further purification using 2-oxoglutarate cosubstrate affinity chromatography was not helpful and resulted in significant loss in activity accompanied by an insignificant increase in purity. Additional purification attempts, including anaerobic purification, also resulted in a significant and irreversible loss of activity.

The nucleotide sequence of *htxA* revealed the presence of two putative translation start sites for HtxA which would change the size of the protein by 14 amino acids. To ensure that the recombinant form of HtxA was expressed from the correct putative translation start site and that it is identical to the native enzyme isolated from *P. stutzeri* WM536, attempts to purify native HtxA were made. Extracts of *P. stutzeri* WM536 grown with 2 mM hypophosphite as the sole source of phosphorus were fractionated with tandem anion exchange chromatography followed by 2-oxoglutarate affinity chromatography. Partially purified HtxA was obtained (about 30% homogeneity) and subjected to amino terminus sequencing and mass spectral analysis. The amino-terminal sequence of both the native and recombinant forms of the enzyme was determined to be MFAEQQREYLDKGYT, which is in complete agreement with the predicted amino acid sequence of *htxA* (as annotated in GenBank, accession no. AFO61267). Mass spectral analysis yielded peaks at 32,485 ± 40 and 32,475 ± 40 Da for the native and recombinant forms of HtxA, respectively, which are consistent with the predicted molecular mass of the monomer of 32,503 Da. Based on these analyses, the native and recombinant forms of the enzyme are indistinguishable, and recombinant HtxA was used in all further studies.

**HtxA Is a 2-Oxoglutarate-dependent Hypophosphite Dioxygenase—**Purified HtxA demonstrates a strict requirement for ferrous ions, oxygen, 2-oxoglutarate, and hypophosphite for activity (Fig. 3). Only in the presence of all of the components required for the continuous coupled assay was an increase in absorbance at 340 nm observed. The end products succinate and phosphate were produced in equimolar quantities, demonstrating a 1:1 stoichiometry for the reaction, with 0.524 ± 0.032 mol of succinate/0.510 ± 0.010 mol of phosphate (as measured by PtxD-catalyzed NADH production) produced after a 20-min incubation time. Given the strict substrate specificity of PtxD determined in a previous study (15), it seems evident that phosphite is the phosphorus product of the HtxA reaction. However, to identify the product of the HtxA reaction unequivocally, 31P NMR was used to analyze the reaction products (Fig. 4). With the addition of 3 mM 2-oxoglutarate in the assay, 2.85 mM phosphite was produced in the complete assay mixture (Fig. 4C). These data show that phosphite is the only phosphorus product made upon incubation of hypophosphite with HtxA and that phosphite production is stoichiometric with 2-oxoglutarate consumption.

**Biochemical Characterization of Purified HtxA—**HtxA was rapidly inactivated in assay buffer (see below); however, purified HtxA was stable upon storage in 20 mM MOPS, pH 7.0, 0.5 mM NAD+, 2 mM hypophosphite, 0.5 mM 2-oxoglutarate, 20 μM FeCl₃, 1.4 units of PtxD, and 0.048 unit of HtxA.

**Fig. 2. SDS-PAGE analysis of HtxA purification.** One-step anion exchange chromatography results in about 95% pure HtxA. Lanes 1 and 7, low range molecular mass markers (kDa). Lane 2, extract from cells before induction with IPTG. Lane 3, 10 μg of crude extract after IPTG induction. Lane 4, 10 μg of cell-free crude extract. Lane 5, 5 μg of high speed extract. Lane 6, 5 μg of the purest fraction from POROS HQ chromatography.

**Fig. 3. HtxA is a 2-oxoglutarate-dependent dioxygenase.** Detection of HtxA activity is dependent on the presence of all HtxA and PtxD assay components. The complete assay consists of 20 mM MOPS, pH 7.0, 0.5 mM NAD+, 2 mM hypophosphite, 0.5 mM 2-oxoglutarate, 20 μM FeCl₃, 1.4 units of PtxD, and 0.048 unit of HtxA.


**Kinetic Analysis of Hypophosphite Oxidation Catalyzed by HtxA**—The oxidation of hypophosphite to phosphate by HtxA follows Henri-Michaelis-Menten kinetics. The kinetic constants were determined using the continuous coupled assay with MBP-PtxD as the reporter enzyme. $V_{\text{max}}$ and $k_{\text{cat}}$ were determined to be $10.9 \pm 0.30 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and $355 \text{ min}^{-1}$, respectively (Fig. 6). The apparent $K_{\text{m}}$ values were determined to be $0.58 \pm 0.04 \mu\text{M}$ for hypophosphite (Fig. 6A) and $10.6 \pm 1.4 \mu\text{M}$ for 2-oxoglutarate (Fig. 6B). To determine the very low $K_{\text{m}}$ of 2-oxoglutarate, a 5-cm path length cuvette was used to increase the sensitivity of the assay.

Substrate and Cosubstrate Specificity of HtxA—The substrate specificity of HtxA was examined using an end point assay in which accumulation of succinate was measured using a highly sensitive enzyme assay for the detection of succinate (Table III). Both inorganic and organic compounds that are structurally analogous or that have similar oxidation states to hypophosphite were examined. Of the alternative substrates tested, sulfate, nitrite, methylphosphonate, methylphosphinate, dimethylphosphinate, taurine, and formaldehyde were not oxidized by HtxA. Formate and arsenite, and to a lesser extent, phosphate, each yielded succinate after a prolonged incubation with 66 $\mu$g/ml HtxA and 4 mM substrate. Surprisingly, the amount of succinate produced with formate and arsenite as substrates exceeded that produced with hypophosphite as substrate. To address this further, the specific activity of HtxA with each substrate supporting activity was determined under conditions identical to those above with the exception of the enzyme concentration, which was reduced to 19.3 $\mu$g/ml to slow the reaction enough to make the intermediate measurements possible (Table III). With hypophosphite as the substrate, the specific activity of HtxA was 11.0 units mg$^{-1}$. The specific activities acquired with formate and arsenite were 10.0 and 5.3 units mg$^{-1}$, respectively. Activity was not detected with phosphate as the substrate using this assay. Thus, HtxA demonstrates relaxed substrate specificity, being able to oxidize several substrate analogs, a common characteristic among the members of the 2-oxoglutarate-dependent dioxygenase enzyme family (26–28).

The cosubstrate range of HtxA was also examined (Table IV). The continuous coupled assay was used to determine whether other 2-oxoacids could support the oxidation of hypophosphite. Among the alternative cosubstrates tested, oxaloacetate, 2-oxovalerate, 2-oxocaprate, and 2-oxobutyrate could not substitute for 2-oxoglutarate as cosubstrate, even when provided at 5 mM. At this concentration, only 2-oxoadipate, and to a much lesser extent, pyruvate, resulted in the oxidation of hypophosphite. When provided at 0.5 mM, only 2-oxoadipate supported HtxA activity, resulting in about 37% of the activity observed with 2-oxoglutarate. Effects of Substrate Analogs and Reaction Products on the Activity of HtxA—Inhibition by substrate analogs was examined using the continuous coupled HtxA activity assay (Table V). The resulting specific activities were compared with the activity observed with no inhibitor present. Of the substrate analogs tested, nitrate and formate severely inhibited HtxA activity, resulting in only 28.5 and 9.7% activity, respectively. HtxA activity was only mildly inhibited by the presence of nitrite, arsenite, sulfate, methylphosphonate, and aminoethylphosphonate. Product inhibition was examined by adding succinate or phosphate to the assay. The presence of phosphate slightly enhanced activity, whereas succinate resulted in about 50% inhibition of HtxA activity.

The Expression of HtxA Is Phosphate Starvation-inducible—Because of the relaxed substrate specificity of HtxA shown above, a genetic approach was taken to elucidate further the in vivo role of this enzyme with regard to its substrate. If the in vivo substrate of HtxA is an alternative phosphorus source such as hypophosphite or phosphate, then expression of HtxA might be induced under conditions of phosphate starvation. To examine the regulation of expression of the htx locus with
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![Graph](image_url)

**Table II**

| Divalent cation** | Specific activity | % Activity | Divalent cation in addition to FeCl₂ | % Activity |
|------------------|-------------------|------------|--------------------------------------|------------|
| FeCl₂            | 8.6               | 100.0      | FeCl₂                                | 100.0      |
| FeSO₄            | 8.7               | 100.9      | NiCl₂                                | 88.4       |
| Fe(NH₄)₂SO₄      | 8.7               | 100.3      | CoCl₂                                | 72.2       |
| NiCl₂            | ND*               | NA*        | MnCl₂                                | 80.0       |
| CoCl₂            | ND                | NA         | CaCl₂                                | 84.1       |
| MnCl₂            | ND                | NA         | MgCl₂                                | 89.2       |
| CaCl₂            | ND                | NA         |                                      |            |
| MgCl₂            | ND                | NA         |                                      |            |
| None             | 0.2               | 2.2        |                                      |            |

* Divalent cations were added at 100 μM final concentration.
* FeCl₂ was held constant at 100 μM with the addition of 100 μM alternative divalent cation.
* ND, not detected; the limit of detection is ~0.04 μmol/min/mg.
* NA, not applicable.

The activity of HtxA was determined using the PtxD-coupled assay with 2 mM hypophosphite and 1 mM 2-oxoglutarate. The results shown are the average of two experiments.

**DISCUSSION**

Although there have been numerous accounts of microorganisms that can grow on hypophosphite as the sole source of phosphorus with excess carbon, the expression of β-galactosidase from this fusion is induced 10–15-fold, relative to expression with growth on excess phosphate. These data strongly suggest a role for HtxA in acquiring an alternative source of phosphorus from hypophosphite and phosphate and further support that hypophosphite is the in vivo substrate of this enzyme.

**TABLE II**

Effect of divalent cations on the activity of 2-oxoglutarate-dependent dioxygenase

The activity of HtxA was determined using the PtxD-coupled assay with 2 mM hypophosphite and 1 mM 2-oxoglutarate. The results shown are the average of two experiments.

| Divalent cation | Specific activity | % Activity | Divalent cation in addition to FeCl₂ | % Activity |
|----------------|-------------------|------------|--------------------------------------|------------|
| FeCl₂          | 8.6               | 100.0      | FeCl₂                                | 100.0      |
| FeSO₄          | 8.7               | 100.9      | NiCl₂                                | 88.4       |
| Fe(NH₄)₂SO₄    | 8.7               | 100.3      | CoCl₂                                | 72.2       |
| NiCl₂          | ND*               | NA*        | MnCl₂                                | 80.0       |
| CoCl₂          | ND                | NA         | CaCl₂                                | 84.1       |
| MnCl₂          | ND                | NA         | MgCl₂                                | 89.2       |
| CaCl₂          | ND                | NA         |                                      |            |
| MgCl₂          | ND                | NA         |                                      |            |
| None           | 0.2               | 2.2        |                                      |            |

* Divalent cations were added at 100 μM final concentration.
* FeCl₂ was held constant at 100 μM with the addition of 100 μM alternative divalent cation.
* ND, not detected; the limit of detection is ~0.04 μmol/min/mg.
* NA, not applicable.

growth on various phosphorus sources, expression analysis from the htx promoter was performed in *P. stutzeri* WM2940, which carries a plasmid-borne htx promoter-lacZ transcriptional fusion. With growth on limiting phosphate, or on hypophosphite or phosphate as the sole source of phosphorus with excess carbon, the expression of β-galactosidase from this fusion is induced 10–15-fold, relative to expression with growth on excess phosphate. These data strongly suggest a role for HtxA in acquiring an alternative source of phosphorus from hypophosphite and phosphate and further support that hypophosphite is the in vivo substrate of this enzyme.

**DISCUSSION**

Although there have been numerous accounts of microorganisms that can grow on hypophosphite as the sole source of phosphorus, the biochemical process by which this occurs had not been examined in detail, leaving a novel and significant area of phosphorus metabolism largely unexplored. The isolation and characterization of HtxA presented in this paper represent the first detailed biochemical analysis of an enzyme in this family. The biochemical characterization of HtxA presented here classifies this enzyme as a novel 2-oxoglutarate-dependent dioxygenase. This family of enzymes is remarkably diverse in the reactions its members catalyze, which include amino acid hydroxylations, secondary metabolite biosynthesis, and degradation of alternative carbon and sulfur sources (for review, see Refs. 29 and 30). The commonality among all of the members of this family is that they all require activation of a molecule of dioxygen by enzyme-bound ferrous ions to generate a highly reactive ferryl oxidant. The formation of the ferryl species is linked to the oxidative decarboxylation of 2-oxoglutarate, giving rise to succinate and CO₂, and mediates hydroxylation of the substrate (31, 32). Thus, all members of this family are dependent on ferrous ions, oxygen, and 2-oxoglutarate (or a similar 2-oxoacid) for activity. Although the substrates acted upon by the members of this family are diverse, all that have been characterized, to our knowledge, act on organic substrates (29, 30). In contrast, of the substrates tested in this study, HtxA showed the highest activity with the inorganic substrate hypophosphite, making it the first enzyme in this family to have an inorganic substrate. Several organic phosphorus compounds thought to be possible alternative substrates for HtxA (methylphosphinate, dimethylphosphinate, and methylphosphonate) were not oxidized by this enzyme.

The low degree of amino acid sequence identity among 2-oxoglutarate-dependent dioxygenases mirrors their catalytic diversity. Among the few highly conserved residues found in many members of this family are those that have been identified to be involved in binding of the ferrous ion. These residues form a conserved motif, designated the 2-His-1-carboxylate facial triad, typical of non-heme Fe(II) enzymes (33). Advanced...
Hypophosphite/2-Oxoglutarate Dioxygenase

Determination of HtxA activity with alternate substrates was done by detection of succinate in an end point assay as described under "Experimental procedures." The results shown are the average of two experiments.

| Substrates | Succinate produced | Specific activity |
|------------|-------------------|------------------|
| Hypophosphite | 0.275             | 11.0             |
| Phosphate   | 0.079             | ND               |
| Arsenite    | 0.307             | 5.3              |
| Formate     | 0.412             | 10.0             |
| Sulfit      | ND                | NA               |
| Nitrite     | ND                | NA               |
| Methylphosphinate | ND        | NA               |
| Dimethylphosphinate | ND      | NA               |
| Methylphosphonate | ND      | NA               |
| Taurine     | ND                | NA               |
| Formaldehyde | ND                | NA               |

a Substrates were present at 4 mM with the exception of formaldehyde, which was added at 50 μM. 2-Oxoglutarate was added at 1 mM.

b Succinate accumulation was detected after a 20-min incubation period with substrate and 66 μg/ml HtxA.

c Specific activity was determined from the averages of duplicate end point assays taken at 6-s intervals over 24 s, using 4 mM substrate and 19.3 μg/ml HtxA.

d ND, not detected; the minimum level of detection is ~0.005 μmol of succinate.

e NA, not applicable.

Effect of product and substrate analog inhibitors on HtxA activity

HtxA activity was detected using the PtxD-coupled assay as described. Hypophosphite was added at 2 mM, and the cosubstrates were added at the indicated concentrations. The results shown are the average of two experiments.

| Cosubstrate | Specific activity | % Activity | Specific activity | % Activity |
|-------------|------------------|------------|------------------|------------|
| 5.0 mM      | units/mg         | 0.5 mM     | units/mg         |           |
| 2-Oxoglutarate | 7.7             | 100        | 9.1              | 100        |
| 2-Oxoadipate | 1.8             | 23.1       | 3.4              | 36.9       |
| Oxaloacetate | ND               | NA         | ND               | ND         |
| Oxalacetate  | ND               | NA         | ND               | ND         |
| 2-Oxocaproate | ND              | NA         | ND               | ND         |
| 2-Oxobutyrate | ND              | NA         | ND               | ND         |
| Pyruvate     | 0.04             | 0.005      | 0.00             | 0.00       |

a ND, not detected; the detection limit is ~0.04 μmol/min/mg.

b NA, not applicable.

d Determination of HtxA activity was done using the PtxD-coupled assay as described. Hypophosphite was added at 2 mM, and the cosubstrates were added at the indicated concentrations. The results shown are the average of two experiments.

| Inhibitor | Specific activity | % Activity |
|-----------|------------------|------------|
| None      | 6.7              | 100        |
| Phosphate | 8.6              | 128.8      |
| Succinate | 3.4              | 51.5       |
| Arsenite  | 7.0              | 104.5      |
| Nitrate   | 1.9              | 28.5       |
| Nitrite   | 3.8              | 56.9       |
| Sulfate   | 5.7              | 85.7       |
| Formate   | 0.6              | 9.7        |
| Methylphosphonate | 6.0          | 90.1       |
| Aminoethylphosphonate | 6.4          | 95.7       |

a Inhibitors were added at a final concentration of 4 mM.

spectroscopic techniques (34), site-directed mutagenesis studies (35–40), and examination of the crystal structures of cephalosporin synthase and the mechanistically related, isopenicillin N synthase (41, 42), have further elucidated the role of these residues in binding Fe(II), oxygen, and 2-oxoglutarate. The 2-His-1-carboxylate motif is also present in HtxA and its closest sequence homologs (Fig. 7). Prolyl 4-hydroxylase shows the highest degree of sequence similarity to HtxA, sharing 26% identity with the kinase analysis program KINSIM (33).
HtxA catalyzes a strictly coupled oxidation of hypophosphite, producing equimolar amounts of succinate and phosphite, demonstrating the expected stoichiometry of the 2-oxoglutarate-dependent dioxygenase reaction. Interestingly, an uncoupled reaction of proline 4-hydroxylase has been well characterized, in which 2-oxoglutarate is oxidatively decarboxylated to succinate and CO₂, without the concomitant hydroxylation of the substrate (24, 25, 43). However, HtxA showed no such uncoupled reaction because succinate was not produced upon incubation of the enzyme with 2-oxoglutarate in the absence of hypophosphite.

The effect of ascorbate on HtxA activity was explored because it has been reported to stabilize the activity of numerous members of this family of enzymes during the reaction. This effect is attributed to the role of ascorbate as a reducing agent that has been shown to counteract Fe(II) oxidation to inactive Fe(III), believed to occur as a side reaction during the hydroxylation of the substrate (24, 25, 43). However, HtxA showed no such uncoupled reaction because succinate was not produced upon incubation of the enzyme with 2-oxoglutarate in the absence of hypophosphite.

The effect of ascorbate on HtxA activity was explored because it has been reported to stabilize the activity of numerous members of this family of enzymes during the reaction. This effect is attributed to the role of ascorbate as a reducing agent that has been shown to counteract Fe(II) oxidation to inactive Fe(III), believed to occur as a side reaction during the hydroxylation of the substrate (24, 25, 43). HtxA was able to catalyze the oxidation of both arsenite and formate, although when comparing specific activities, hypophosphite was a slightly better substrate. Arsenite and formate were among those chosen as alternative substrates to examine based on their chemical and/or similarity to hypophosphite. Given these similarities and the relaxed substrate specificity observed in many of the members of this enzyme family (27, 45, 46), it is not surprising that these compounds could be oxidized. This is especially true in light of the highly reactive dioxygen-derived ferryl species generated upon binding of 2-oxoglutarate and oxygen at the ferrous ion binding site (for review, see Refs. 29 and 47). To be oxidized via this reaction, the only requirement for the compound would be that it fit into the active site in juxtaposition with this highly reactive ferryl species.
phosphosphate, this seems the most likely explanation for the relaxed substrate specificity of HtxA. Because formate can act as a substrate, it is also not surprising that formate significantly inhibits HtxA activity, probably because of competitive binding with hypophosphite at the active site. This further supports the conclusion that formate acts simply as a structural analog to hypophosphite. The structural similarity between these two compounds has been demonstrated previously by the ability of hypophosphite to act as a substrate analog to both pyruvate formate lyase (48) and formate hydrogenlyase and regulatory proteins involved in their expression (49). Although the specific activity of HtxA with arsenite as a substrate is significant, arsenite is a poor inhibitor of HtxA activity, suggesting weak binding of arsenite in the active site (relative to hypophosphite). Thus, arsenite is also probably not the true substrate of HtxA. Considering both the biochemical evidence presented here and the regulation of the htx and ptx loci by phosphate starvation (this report and Ref. 15), hypophosphite is almost certainly the in vivo substrate for HtxA, with the role of providing P. stutzeri WM88 with an alternate phosphorus source via oxidation of inorganic reduced phosphorus compounds.

Compared with other members of the 2-oxoglutarate-dependent dioxygenase enzyme family, HtxA shows somewhat strict cosubstrate specificity. Similar to taurine dioxygenase, HtxA was able to use only 2-oxoapdate to a significant degree in the place of 2-oxoglutarate (27). This is in contrast to most members of this family, which are able to use a broad range of 2-oxoacids, with or without a second carboxyl group (45,46). Both the cosubstrate specificity and the apparent K_m for 2-oxoglutarate fall within the ranges observed for other members of this family.

Because of the dearth of knowledge regarding biological oxidation of reduced phosphorus compounds and of the presence of these compounds in the soil, examining the substrate specificity of HtxA was essential to understanding the physiological role of this enzyme in phosphorus metabolism. It is clear from the genetic analysis that the htxA gene allows P. stutzeri WM88 to grow on hypophosphite as a sole phosphorus source and at a rate similar to growth on phosphate (12). In addition, examination of the regulation of expression of the ptx and htx operons indicates that both are highly expressed under conditions of phosphate starvation, including growth on both limiting phosphate and on hypophosphite or phosphate as the sole phosphorus sources. Sequence analysis of the region upstream from the htxA translation start site revealed the presence of a Pho box (data not shown), a conserved binding sequence for transcriptional activation via PhoB (50). In numerous microorganisms, PhoB is the response regulator responsible for activating transcription of the Pho regulon, which is comprised of numerous phosphate starvation-inducible loci, all involved in the assimilation of phosphorus in response to phosphate starvation. These genetic data strongly suggest that the in vivo role of these genes is to allow use of an alternative phosphorus source, such as phosphate and hypophosphite.

What is less clear is the extent to which hypophosphite is present in nature. Although it is widely used industrially as a reducing agent, and microbe-mediated reduction of phosphate to phospohite, hypophosphite, and phosphate has been reported (3,51), no direct measurements of inorganic reduced phosphorus compounds in the soil have been documented. The apparent K_m of HtxA for hypophosphite of 0.58 mM is quite likely to be sufficiently low to support growth of this organism on even the very low concentrations of hypophosphite one might expect to find in the environment. This is particularly true given that htxA appears to be cotranscribed with a binding protein-dependent transporter that is required, in addition to htxA, for growth on hypophosphite in the heterologous hosts, E. coli and Pseudomonas aeruginosa (12). Such transporters are known to be able to accumulate very high intracellular levels of their substrate, with concentration gradients of up to 100,000-fold (52), which would put intracellular hypophosphite concentrations well within the range allowing HtxA to catalyze in vivo hypophosphite oxidation.

Finally, hypophosphite oxidation has been studied previously in B. caldolyticus, and activity was found to be dependent on NAD+ (11), and anaerobic oxidation of hypophosphite has been documented in uncharacterized Bacillus isolate (8). In contrast to these findings, HtxA does not require NAD+ for oxidation of hypophosphite, and oxygen is absolutely required for the HtxA reaction. This indicates that HtxA is a very different enzyme from those studied in two Bacillus species, supporting the possible existence of multiple pathways for the oxidation of hypophosphite in diverse microorganisms and the idea that hypophosphite oxidation is an important activity for survival in the environment. Given the large number and diversity of microorganisms reported to oxidize hypophosphite or phosphate, it seems clear that oxidation of reduced phosphorus compounds is not an uncommon activity and should be considered a significant aspect of phosphorus biochemistry. A more profound understanding of the environmental significance of reduced phosphorus biochemistry and of the reaction mechanisms involved awaits additional detailed analyses of the enzymes catalyzing these reactions and of the genes encoding them.

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Isolation and Biochemical Characterization of Hypophosphite/2-Oxoglutarate Dioxygenase: A NOVEL PHOSPHORUS-OXIDIZING ENZYME FROM PSEUDOMONAS STUTZERI WM88
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