In Vivo and in Vitro Analyses of Single-amino Acid Variants of the Salmonella enterica Phosphotransacetylase Enzyme Provide Insights into the Function of Its N-terminal Domain*

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The function of the N-terminal domain (~350 residues) of the Pta (phosphotransacetylase) enzyme of Salmonella enterica is unclear. Results from in vivo genetic and in vitro studies suggest that the N-terminal domain of Pta is a sensor for NADH and pyruvate. We isolated 10 single-amino acid variants of Pta that, unlike the wild-type protein, supported growth of a strain of S. enterica devoid of Acs (acetyl-CoA synthetase; AMP-forming) activity on 10 mM acetate. All mutations were mapped within the N-terminal domain of the protein. Kinetic analyses of the wild type and three variant Pta proteins showed that two of the variant proteins were faster enzymes ($k_{cat}$ 2.5–3-fold > $k_{cat}$ PtaWT). Results from sedimentation equilibrium experiments are consistent with PtaWT being a trimer. Pta variants formed more hexamer than the PtaWT protein. NADH inhibited PtaWT activity by inducing a conformational change detectable by limited trypsin proteolysis; NADH did not inhibit variant protein PtaR252H. Pyruvate stimulated PtaWT activity, and its effect was potentiated in the variants, being most pronounced on PtaR252H.

Short-chain fatty acids, including acetate, are present at substantial concentrations (i.e. up to 130 mM) in the human gut (2–4), a niche of Salmonella enterica. Hence, it is not surprising that this bacterium has evolved efficient ways of utilizing this compound as a source of carbon and energy (5).

Acetate enters central metabolism as Ac-CoA, a building block for lipid and amino acid biosynthesis (6–8), cell wall synthesis (9), and is broadly used as an acetylating agent in antibiotic inactivation (10–12), to control the activity of central metabolism enzymes (13), to control ribosome activity (14–17), and as a precursor during the synthesis of secondary metabolites (18, 19). Ac-CoA is synthesized through either one of two distinct pathways. The high affinity pathway (Reaction 1) is catalyzed by Acs (Ac-CoA synthetase; EC 6.2.1.1).

The low affinity pathway is catalyzed by AckA (acetate kinase, EC 2.7.2.1) (Reaction 2),

\[
\text{Acetate} + \text{ATP} \leftrightarrow \text{Acetyl-AMP + PP}_i
\]

\[
\text{Acetyl-AMP + CoASH} \leftrightarrow \text{Ac-CoA + AMP}
\]

where $\Delta G^{\text{p}}_{\text{obs}} = -13$ kJ/mol (1), and by Pta (phosphotransacetylase, EC 2.3.1.8) (Reaction 3),

\[
\text{Ac-P + CoASH} \leftrightarrow \text{Ac-CoA + P}_i
\]

where $\Delta G^{\text{p}}_{\text{obs}} = +9$ kJ/mol (1).

When the concentration of acetate in the environment is low (i.e. $\leq$10 mM), S. enterica uses Acs (AMP-forming) to catalyze the synthesis of Ac-CoA from acetate and ATP via an Ac-AMP intermediate (20). Acs is the high affinity system for Ac-CoA synthesis, and the enzyme is present in all forms of life (20).

In contrast, when the concentration of acetate in the environment is high (i.e. $\geq$25 mM), S. enterica uses AckA to activate acetate to acetate-phosphate (Ac-P)3 and Pta to convert Ac-P to Ac-CoA; the AckA and Pta reactions are both reversible (21) and together comprise the low affinity pathway of acetate metabolism. Until recently, the AckA-Pta pathway was thought to exist only in prokaryotes. A recent report suggested that the AckA-Pta pathway is intact and functional in some lower eukaryotes (22). In prokaryotes, the AckA-Pta pathway is considered to function primarily as a dissimilatory, carbon overflow pathway (23).

In S. enterica, acetate/propanoate kinases and Pta activities are relevant to threonine, odd-numbered fatty acid, ethanolamine, and 1,2-propanediol catabolisms (5, 24–26). Acetate/ Ac-CoA homeostasis is of great importance to all cells, and Pta plays a central role in maintaining a balanced flux between bio-

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 4 and 5 and Figs. 8–11.

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3 The abbreviations used are: Ac-P, acetyl-phosphate; Tc, tetracycline; Ap, ampicillin; NB, nutrient broth; LB, lysogeny broth; MCS, multiple cloning site; His$_{6}$-Pta, N-terminally tagged Pta; MES, 2-morpholinoethanesulfonic acid; CHES, 2-(N-cyclohexylamino)ethane sulfonic acid; rTEV, recombinant tobacco etch virus; LDH, lactate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DTBS, dithiobiotin synthase.
Insights into the Role of the N-terminal Domain of Pta

synthesis and energy generation (27). Likewise, Ac-P is an important phosphorylating agent used by the cell to modulate gene expression (28–31).

Surveys of sequenced microbial genomes reveal two classes of Pta enzymes. Class I enzymes (Pta\(^{\text{I}}\)) are ∼350 residues in length, whereas class II enzymes (Pta\(^{\text{II}}\)) are twice as long as Pta\(^{\text{I}}\) (∼700 residues) (26, 32). The pduL gene of S. enterica was recently shown to encode an additional, evolutionarily distinct class of phosphotransacetylase enzymes (33).

Pta\(^{\text{I}}\) enzymes share end-to-end homology with the C-terminal domain of Pta\(^{\text{II}}\) enzymes; hence, it is inferred that the active site of Pta\(^{\text{II}}\) enzymes is located within their C-terminal domain. This assignment leaves open the question of what the function of the N-terminal domain of Pta\(^{\text{II}}\) might be.

Earlier biochemical characterizations of the Pta enzyme of Escherichia coli (a class II enzyme) showed that its forward ACCoA-forming activity was allosterically regulated by nucleotides, including ADP, ATP, and NADH (negative effectors), and by pyruvate (positive effector) (34). These authors hypothesized that the N-terminal of the Pta\(^{\text{II}}\) enzyme had a regulatory role. However, this idea was not pursued any further.

We set out to investigate the function of the N-terminal domain of the Pta\(^{\text{II}}\) enzyme of S. enterica (encoded by pta\(^{\text{II}}\)). Our initial approach was genetic. A positive selection was used to isolate alleles of the pta gene that encoded variant proteins with improved catalytic properties. We isolated 10 single-amino acid variants, all of which had the mutation within the N-terminal domain of the protein. We report here the kinetic analysis of three variant Pta proteins and discuss the implications of these findings.

EXPERIMENTAL PROCEDURES

Microbiological Techniques: Bacterial Strains and Growth Conditions

All strains used in this study were derivatives of S. enterica serovar Typhimurium strain LT2 (hereafter referred to as S. enterica). The genotypes of bacterial strains and plasmids used in this work are listed in supplemental Table 4. S. enterica strains were grown in no-carbon essential minimal medium (35) supplemented with potassium acetate (10 mM), MgSO\(_4\) (1 mM), L-methionine (0.5 mM), and trace metals (36). Nutrient broth (NB) (37) and lysogeny broth (LB) (38, 39) were used as rich media to cultivate strains. Working concentrations of antibiotics were 100 μg/ml for ampicillin (Ap) and 20 μg/ml for tetracycline (Tc) in rich medium and 2 μg/ml for tetracycline in minimal medium.

Growth behavior was analyzed using a 96-well microtiter dish (BD Biosciences) format using a computer-controlled Ultra Microplate Reader (Bio-Tek Instruments) equipped with the KC4 software package. The temperature of the incubation chamber was set at 37 °C. Each well of the plate contained 195 μl of fresh minimal medium supplemented with 10 mM potassium acetate, pH 7. Each well was inoculated with 5 μl of a culture of S. enterica grown in NB medium for ∼24 h. Growth was monitored as the increase in the optical density at 650 nm (OD\(_{650}\)). Cultures were shaken for 870 s between readings, pausing for 10 s before each reading. Each experiment consisted of seven replicates per strain, and the experiment was performed at least four times from four independent sets of cultures to ensure reproducibility and statistical significance.

Genetic Techniques

Transductions

All P22-mediated transduction crosses were performed using established protocols (37) using phage P22 HT105/1 int-210 (40, 41). Transductants were freed of phage as described (42).

Isolation of pta Alleles Encoding Variant Pta Proteins That Support Growth on Low Acetate

Localized mutagenesis (43) was performed to isolate chromosomal pta alleles encoding variant Pta proteins that would support growth on 10 mM acetate. Briefly, phage was propagated on strain JE7807, which harbored a mini-Tn10 element (44) in an open reading frame proximal to pta (i.e. open reading frame strm2340).

Phage P22 was concentrated by centrifugation at 39,191 × g for 2 h at 4 °C using a Beckman Coulter Avanti J-25 centrifuge equipped with a JA-25.50 rotor. Hydroxylamine mutagenesis of the phage was monitored by a plaque assay (37) using strain TR6583 as an indicator strain. Recipient strain JE4312 (Δacs) was transduced to Tc\(^{\text{R}}\) using mutagenized phage as donor. Tc\(^{\text{R}}\) recombinants were replica-printed onto minimal medium plates containing 10 mM acetate as the sole source of carbon and energy, tetracycline, and the calcium chelator EGTA. Tc\(^{\text{R}}\) recombinants that grew on 10 mM acetate were purified on the selective minimal medium and reconstructed using P22 phage grown on them as donor and strain JE4312 as recipient.

Recombinant DNA Techniques

Identification of the Causative Mutations in pta Alleles

Strains carrying pta alleles encoding Pta variants capable of supporting growth on low acetate (10 mM) were grown overnight in 2 ml of NB. A 10⁻¹ dilution of cells was boiled for 5 min at 95 °C, and debris was removed by centrifugation for 1 min at 18,000 × g in a Microfuge\(^{\text{R}}\) 18 centrifuge (Beckman-Coulter). The resulting DNA preparation was used as template during PCR amplification using forward primer 5'-TGTAAAAACG-GGCCCAAAAGACTGTAACGA-3' (pta\(^{\text{I}}\)) and reverse primer 5'-TGTAACC-GCGGCCCCAAAAGACTGTAACGA-3' (pta\(^{\text{II}}\)). The resulting 2.2-kb fragment was purified using a QiaQuick gel extraction kit (Qiagen) and was used as template for DNA sequencing using nonradioactive BigDye\(^{\text{R}}\) (ABI PRISM) protocols (University of Wisconsin-Madison Biotechnology Center).

Plasmid Constructions

Primers used to generate site-directed mutant alleles are shown in supplemental Table 5. The Internet-based program PrimerX (available on the World Wide Web at bioinformatics.org/primersx/) was used to help design mutagenic primers.

Plasmid pPTA21—Allele pta\(^{\text{II}}\) from S. enterica was amplified from strain TR6583 using the forward primer 5'-TGTAAACC-GCGGCCCCAAAAGACTGTAACGA-3' and reverse primer 5'-
The underlined bases denote the engineered 5′ Smal and 3′ XbaI restriction sites, respectively. The 2.2-kb DNA fragment was blunt-ended, phosphorylated, and ligated into the multiple cloning site (MCS) of plasmid pCC1 (Epicenter). The presence and orientation of the insert (opposing the PT7 promoter) was verified by restriction analysis. This plasmid (named pPTA3) was cut with restriction enzymes Smal and XbaI. The released pta+ fragment was extracted from the gel and ligated into the same sites of plasmid pBAD30 (45), yielding plasmid pPTA11. The sequence encoding N-terminal domain amino residues 1–401 was amplified from pPTA11 using the forward primer 5′-GAGG-ATAAACCATTGGCCCGTATTATTATGCTG-3′ and reverse primer 5′-ATTTCGGGTTTACGCAGCTGCTAGTCA-3′. Underlined bases denote the engineered 5′ Ncol and 3′ Smal restriction sites, respectively. Construction of the 5′ Ncol site incorporated the amino acid substitution S2A. The 1.1-kb DNA fragment was A-tailed with Vent (exo-). (New England Biolabs), gel-extracted, and ligated into the MCS of pGEM-T-Easy (Promega). The presence of the insert was confirmed by restriction enzyme analysis, and this plasmid was named pPTA19. The 1.1-kb DNA containing the Pta N-terminal domain coding sequence was released from pPTA19 using Ncol and Smal. Following gel extraction, the fragment was ligated into the same restriction sites of cloning vector pT7Blue (New England Biolabs). The resulting plasmid encoded the N-terminal 401 amino acid residues of Pta fused to a chitin binding domain tag at the C terminus of the protein. We used restriction enzyme analysis and DNA sequencing to verify that the plasmid was constructed correctly. The resulting plasmid was 8.6 kb long and was named pPTA21.

Plasmid pPTA69—The pta+ gene was amplified from pPTA11 using the forward primer 5′-GTTACACAGGAGGAGG-GCTAGCATGTCCCGTA-3′ and the reverse primer 5′-TCA-CCTCTAGACCTGACAAGCGGTCCATCAC-3′. The underlined bases denote the engineered 5′ Ncol and 3′ XbaI restriction sites, respectively. The 2.2-kb DNA fragment was A-tailed with Vent (exo-), gel-extracted, and ligated into the MCS of plasmid pGEM-T-Easy, opposite to the direction of transcription of P lacZ. This 2.2-kb pta+ DNA fragment was released by cutting with restriction enzyme NheI and was ligated into the MCS of plasmid pGEM-T-Easy (Promega). The presence of the insert was confirmed by restriction enzyme analysis, and this plasmid was named pPTA19. The 1.1-kb DNA containing the Pta N-terminal domain coding sequence was released from pPTA19 using Ncol and Smal. Following gel extraction, the fragment was ligated into the same restriction sites of cloning vector pT7Blue (New England Biolabs). The resulting plasmid encoded the N-terminal 401 amino acid residues of Pta fused to a chitin binding domain tag at the C terminus of the protein. We used restriction enzyme analysis and DNA sequencing to verify that the plasmid was constructed correctly. The resulting plasmid was 8.6 kb long and was named pPTA21.

Plasmids pPTA73-75 and pPTA83-84—Plasmid pPTA69 was subjected to site-directed mutagenesis using the QuikChange® XL kit (Stratagene) to construct allele pta105 encoding variant protein Pta5′G74D (plasmid pPTA73), allele pta104 encoding variant Pta5′M294I (plasmid pPTA74), allele pta106 encoding variant Pta5′R252H (plasmid pPTA75), allele pta107 encoding Pta5′G311D (plasmid pPTA83), and allele pta108 encoding Pta5′R113H (plasmid pPTA84); all plasmids were 7.7 kb long. The presence of the mutations was individually verified by DNA sequencing.
of protein was determined before freezing and storage at −80 °C. Protein purity was ≥90%.

Cleavage of the Hexahistidine Tag of His<sub>6</sub>-Pta Proteins by rTEV Protease—We used rTEV protease isolated in house (>60% homogeneity; data not shown) as described elsewhere (see, on the World Wide Web, www.cfac.ac.uk/biosi/staff/ehrmann/tools/TEVprot.html). Extended incubation of His<sub>6</sub>-Pta proteins with rTEV protease (2 days at 4 °C) was needed to remove the His<sub>6</sub> tag of 40% of the Pta proteins. After elution of His<sub>6</sub>-Pta proteins from the affinity column, they were mixed with rTEV (1:50 rTEV:His<sub>6</sub>-Pta (w/w)) and extensively dialyzed, first against buffer E (Tris-HCl (50 mM, pH 8.0, at 4 °C) containing NaCl (250 mM) and EDTA (10 mM)) for 2 h, followed by a 4-h dialysis against buffer F (buffer containing EDTA at 0.5 mM). His<sub>6</sub>-Pta/rTEV mixtures were further dialyzed overnight against buffer G (buffer F plus dithiothreitol (3 mM)). Dialysis was then performed using buffer H (Tris-HCl (50 mM, pH 8.0, at 4 °C) containing 0.5 mM EDTA, 250 mM NaCl, and dithiothreitol (2 mM) for 8 h, with a second overnight dialysis against the same buffer.

After cleavage, the protein mixtures were dialyzed into buffer A and passed over the His-Trap FF 5-ml column following the protocol described above. Protein that did not bind to the column was analyzed by 8% SDS-PAGE. Fractions containing Pta protein were pooled and dialyzed overnight against buffer B (containing 250 mM NaCl). A second dialysis was performed twice against buffer C (containing 250 mM NaCl) for at least 2 h each. Tagless Pta protein was quantified via Bradford assay, frozen in liquid nitrogen, and stored at −80 °C until used.

Overproduction and Purification of the N-terminal Domain of S. enterica Pta<sup>4</sup> Enzyme—The N-terminal domain of the S. enterica Pta<sup>1</sup> enzyme (residues 1–401) was overproduced in E. coli strain BL21(ADE3) and purified using the IMPACT<sup>®</sup> system (New England Biolabs). After transforming plasmid pPTA21 pta<sup>+</sup> into the host, a single colony was used to inoculate 10 ml of LB broth supplemented with Ap and grown overnight at 30 °C. The culture was subcultured 1:100 (v/v) into 1 liter of LB supplemented with Ap. Cultures were grown to an OD<sub>600</sub> of ~0.6–0.8 and placed on ice for 10 min. pta gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (250 μM), followed by overnight incubation (~16 h) at 15 °C. Cells were harvested by centrifugation for 10 min at 10,543 × g at 4 °C in a Beckman-Coulter Avanti J-20 XPI centrifuge equipped with a JLA-8.100 rotor. The cell pellet was resuspended in 35 ml of cold buffer I (HEPES (20 mM, pH 7.9, at 4 °C) containing NaCl (500 mM), EDTA (1 mM)) and passed three times through a chilled French pressure cell at 1.05 × 10<sup>6</sup> kilopascals. Cell debris was removed by centrifugation at 39,191 × g for 30 min at 4 °C. Extract was loaded onto a chitin column (20-ml bed volume) equilibrated with buffer I and developed according to the manufacturer’s instructions with a flow rate of 200 ml h<sup>−1</sup>. Overnight, on-column cleavage of the tag was achieved with DL-dithiothreitol (dithiothreitol, 50 mM). Fractions containing Pta N-terminal domain purified to ≥97% homogeneity (data not shown) were pooled and dialyzed against buffer I lacking EDTA and at pH 7.5 (4 °C) overnight. Protein was dialyzed extensively against buffer J (Tris-HCl (10 mM, pH 7.5, at 4 °C) containing 10% glycerol (v/v)). Protein was quantified and flash-frozen as ~50-μl drops in liquid nitrogen before storage at −80 °C.

Rabbit Polyclonal Antibodies against the Pta N-terminal Domain and Western Blot Analysis

Polyclonal antibodies were elicited by subcutaneous injection of 270 μg of purified N-terminal domain as primary antigen into a New Zealand White female rabbit (Laboratory Animal Resources, University of Wisconsin, Madison, WI). Subsequent antigen boosts were performed with ~250 μg of protein. Western blots were performed using standard protocols (49) with a 1:5,000 dilution of antiserum as primary antibody and a 1:70,000 dilution of Immunopure<sup>®</sup> donkey anti-rabbit immunoglobulin G (IgG, heavy and light chains) conjugated to horseradish peroxidase (Pierce) as secondary antibody. Signal was detected using the SuperSignal<sup>®</sup> West Dura trial chemiluminescence kit (Pierce) and a computer-controlled Cyclone phosphor imager furnished with OptiQuant version 4.00 imaging software (Packard Instruments).

In Vitro Activity Assays

Phosphotransacetylase Activity Assays

Pta in vitro activity assays of the forward reaction (Reaction 3, Ac-CoA-forming direction) were performed by monitoring the formation of the thioester bond as the increase in absorbance at 333 nm. The amount of product formed was quantified using the extinction coefficient (ε) of 5.55 mm<sup>−1</sup> cm<sup>−1</sup> (26, 32). The only modification to the reported protocol was the final volume of the reaction mixture, which was increased to 1 ml; dithiothreitol was not required for Pta activity.

Assays of the back reaction (Reaction 3, Ac-P-forming direction) monitored the release of free CoASH as the increase in absorbance at 412 nm observed when the thiolate anion of 5,5′-dithiobis(2-nitrobenzoic acid) (ε<sub>412</sub> = 13,600 mm<sup>−1</sup> cm<sup>−1</sup>) was formed upon reaction of 5,5′-dithiobis(2-nitrobenzoic acid) and the sulfhydryl group of CoA (50–53). Reaction mixtures contained Tris-HCl buffer (50 mM, pH 7.5, at 37 °C), NAD<sub>4</sub>Cl or KCl, Pta protein, and one substrate (plus allosteric effector, if added). In experiments that involved allosteric effectors, we used HEPES buffer (50 mM, pH 7.5) containing 100 mM KCl to prevent destruction of pyruvate by Tris buffer (54). Preincubation times were 1 min for the forward reaction and 2 min for the back reaction. Reactions were initiated by the addition of the second substrate. Progress of the reaction was monitored using a computer-controlled PerkinElmer Lambda 40 spectrophotometer (PerkinElmer Life Sciences) furnished with the UV KinLab software package.

Lactate Dehydrogenase (LDH) Activity Assays

LDH activity was determined by monitoring the oxidation of reduced nicotinamide adenine dinucleotide (β-NADH) (decrease in absorbance at 340 nm; ε<sub>340</sub> = 6.3 mm<sup>−1</sup> cm<sup>−1</sup>) (55). Lyophilized rabbit muscle lactate LDH (Calbiochem) was used as a positive control. Briefly, LDH was dissolved in HEPES buffer (50 mM, pH 7.5, at 37 °C) containing KCl (100 mM) and mixed with glycerol to 10% (v/v). The protein concentration was determined, and the resulting preparation was stored in
100-μl samples at −80 °C until used. Reactions were incubated for 2 min with LDH or His₆-Pta proteins, buffer, and pyruvate (0.7 μmol); reactions were initiated by the addition of β-NADH (0.2 μmol).

**Kinetic Analysis**

Reactions were performed under conditions that ensured that the Pta enzyme did not consume more than 10% of the substrate. Pseudo-first order kinetic parameters were calculated using Prism version 4.0a software (GraphPad Software), fitting the data to Equation 1,

\[ V_o = V \times [S] / (K_m + [S]) \]  
(Eq. 1)

where \( V_o \) is initial velocity, \([S]\) is substrate concentration, \(V\) is maximum velocity, and \(K_m\) is the Michaelis constant (56).

For Ac-P titrations that exhibited positive cooperativity, data were fitted to Equation 2, where \( h \) represents the Hill constant and \( K_{0.5} \) is the concentration of substrate yielding half-maximal velocity to account for positive cooperativity.

\[ V_o = V \times [S]^h / (K_{0.5} + [S])^h \]  
(Eq. 2)

\( K_i \) and \( K_e \) values were calculated by plotting \( V_o^{-1} \) against effector concentration; data were fitted to Equation 3, where \( E \) represents the effector concentration. In the absence of effector, \( V_o = a \), whereas at infinite effector concentration \( V_o = b \). \( K_e \) is the level of effector that gives \((a + b)/2\).

\[ V_o = (a + bE/K_e)/(1 + E/K_e) \]  
(Eq. 3)

When determining \( K_i \) for NADH, \( K_e = K_a \) and when determining \( K_i \) for pyruvate, \( K_e = K_a^{-1} \).

**Oligomeric State Analysis of Pta**

**Gel Filtration**—Gel filtration was performed using a ÄKTA fast protein liquid chromatography Explorer system equipped with a Superdex 200 HR 10/30 column (Amersham Biosciences). The column was equilibrium with sodium phosphate buffer (50 mM, pH 7.4, at 4 °C) containing NaCl (150 mM). Pta protein (~100 μg) was applied onto the column, which was developed isocratically at a rate of 0.5 ml min⁻¹. Molecular weight calibration was performed by using bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa), all components of the Bio-Rad gel filtration standards kit. The exclusion limit of the column matrix was defined using blue dextran.

**Equilibrium Sedimentation**—Experiments were performed at the Biophysics Instrumentation Facility (University of Wisconsin, Madison, WI) in a refrigerated (4 °C) computer-controlled XL-A analytical ultracentrifuge (Beckman-Coulter). Tagless PtaWT protein was extensively dialyzed against HEPES buffer (50 mM, pH 7.5, at 4 °C) containing KCl (100 mM) and then diluted to A₂₇₈S of ~0.8, ~0.4, and ~0.2 using the final dialysate as diluent. Run speeds ranged from 3000 to 11,000 rpm with a run at 32,000 rpm to deplete out macromolecular species at the conclusion of the experiment. A reversal was performed at 5400 rpm to investigate slowly changing aggregation over time. Data were analyzed using software developed in house.

**Analysis of the NADH-induced Conformation Change of Pta**

Limited trypsin proteolysis of His₆-Pta proteins was performed as described (57), except diphenylcarbamyl chloride-treated trypsin (Sigma) was used in 20-μl reactions performed in 50 mM Tris-HCl buffer (pH 7.5, at 24 °C). Reactions were allowed to proceed after a 10-min preincubation with the effector molecule present at the indicated concentration. Protein composition of the reaction mixture was analyzed by 12% SDS-PAGE. Tryptic fragments were excised from the gel and subjected to in-gel tryptic digestion followed by MALDI-TOF mass spectrometry (University of Wisconsin-Madison Biotechnology Center). Peptide fingerprint data were analyzed using Mascot (available on the World Wide Web at www.matrixscience.com/) with the following modifications: carbamidomethyl (atyon) of (cysteine), deamidation of asparagine and glutamine, and oxidation of methionine.

**RESULTS**

**Single-amin o Acid Variants of Pta Support Growth of S. enterica on Low Acetate**

Growth of strains of *S. enterica* lacking functional Acs protein was not supported by low concentrations of acetate (≤10 mM) in the medium (Fig. 1, compare acs⁺ pta⁺ versus acs pta⁻). We isolated 10 *S. enterica* strains carrying chromosomal alleles of *pta* that encoded variant Pta proteins capable of supporting growth on 10 mM acetate in the absence of a functional Acs protein. Alleles of *pta* encoding these variants were obtained after hydroxylamine mutagenesis of a P22 lysate grown on strain JE7807 (prpC114::MudJ) pta⁺ stm2340::Tn10(dTet⁺). Mutagenized P22 phage was used as donor in a cross with strain JE4312 (Δacs231) as recipient. Tetracycline-resistant transductants were screened for the ability to grow on minimal medium containing 10 mM acetate as sole source of carbon and energy. Colonies displaying robust growth were freed of phage, P22 phage was grown on strains that grew on 10 mM acetate, and the resulting phage lysate was used as donor in a cross with strain JE4312 (Δacs231) as recipient. Transductants that were resistant to tetracycline and grew on 10 mM acetate were further analyzed. Analysis of *pta* coding sequences in 10 strains identified the following single-amino acid changes: G31D, R113H, G140S, P177S, T184I, R252H, R252C, G273D, M294I, and G296S. Growth behavior analysis of four strains demonstrated that variant Pta proteins could support growth on 10 mM acetate compared with the acs pta⁺ strain. Strains harboring mutant *pta* alleles achieved similar final optical densities in liquid culture as the strain containing a functional Acs protein but reached these densities at a faster rate (Fig. 1, alleles *pta*104–pta107).

**Gene Dosage Effect**

To determine whether high levels of variant proteins had any deleterious effect on growth on 10 mM acetate, five mutant *pta* alleles were individually cloned into an overexpression vector. Resulting plasmids (supplemental Table 4) were introduced...
into strain JE8086 (Δacs pta recA supplemental Table 4), and growth on 10 mM acetate was assessed subculturing from overnight cultures grown in LB broth supplemented with Ap. Alleles pta104–pta107 supported growth of strain JE4312 on 10 mM acetate, but allele pta108 (encodes PtaR113H) did not. Interestingly, allele pta108 did support growth on 50 mM acetate. No additional mutations were present in pta108 or in the promoter region, as determined by DNA sequencing (data not shown). Variant PtaR113H was not isolated or analyzed in vitro.

Optimal in Vitro Conditions for Pta Activity

To understand the effect of the mutations described above on Pta activity, it was necessary to kinetically characterize the wild type enzyme first. For this purpose, the pta+ allele was cloned into plasmid pKLD37, fusing a tobacco etch virus-cleavable hexahistidine tag to the N terminus of the Pta polypeptide. This plasmid was moved into E. coli strain BL21 (DE3), the pta+ gene was overexpressed, and the His6–PtaWT protein was overproduced and purified (>70% homogeneity; data not shown). Activity assays with MES, Tris-HCl, and CHES buffers identified a pH optimum of 7.5 for Pta (Fig. 2A).

Inclusion of salts in the reaction mixture indicated that NH4Cl was most efficient at stimulating Pta enzyme activity when present at 40 mM (3-fold; Fig. 2B, squares). No significant increase in activity was observed at NH4Cl > 40 mM. KCl also stimulated Pta activity but to a slightly lesser degree (2.5-fold; Fig. 2B, triangles), whereas NaCl did not stimulate Pta activity at all (Fig. 2B, circles).

Oligomeric State of Native PtaWT

We performed gel filtration and equilibrium sedimentation studies to gain insights into the oligomeric state of native and variant forms of Pta enzyme from S. enterica. Results of equilibrium sedimentation experiments suggested an average minimum species of a trimer above a protein concentration of 0.9 μM. However, when present at 40 mM (3-fold; Fig. 2B, squares). No significant increase in activity was observed at NH4Cl ≥ 40 mM. KCl also stimulated Pta activity but to a slightly lesser degree (2.5-fold; Fig. 2B, triangles), whereas NaCl did not stimulate Pta activity at all (Fig. 2B, circles).

FIGURE 1. Growth behavior of S. enterica strains harboring mutant pta alleles. All strains contain a deletion of the acs gene encoding Ac-CoA synthetase (AMP-forming) and the stated pta allele in the genome. The bars denote final density of cultures grown in minimal medium containing 10 mM acetate as the sole source of carbon and energy. Data were collected during a 24-h incubation at 37 °C using a microplate reader. The numbers above the bars represent the doubling time (h) of each culture.

FIGURE 2. Optimal pH and salt for Pta activity. Profiles were generated for the forward (Ac-CoA-forming) reaction, using CoASH (0.6 μmol) and Ac-P (6 μmol). A, pH profile with activities normalized to pH 7.5, the optimal pH in the forward reaction. B, effect of various salts on phosphotransacetylase activity; Tris-HCl (50 mM, pH 7.5, at 37 °C) was used as buffer. Data are presented as -fold change over no added salt.

FIGURE 3. Equilibrium sedimentation of S. enterica PtaWT enzyme. Data were acquired at 4 °C, monitoring changes in absorbance at 278 nm. Shown above are data from runs at 5,400 and 8,200 rpm with cell loading concentrations of 0.8, 0.4, and 0.2 absorbance units (AU; closed squares, open circles, and closed triangles, respectively). Fitting the data to a two-independent-species model suggests a trimer as the minimum weight average species above 0.9 μM.
higher ratio of the observed mass to calculated Pta\textsuperscript{II} polypeptide mass (M<sub>o</sub>/M<sub>p</sub>), consistent with a loss of mass due to aggregation (data not shown). In contrast, the behavior of Pta on a gel permeation column was consistent with a mass of ~490 kDa, suggesting a hexameric protein (supplemental Fig. 8). It is possible that native Pta is a dimer of trimers.

**Kinetic Analysis of His\textsubscript{6}-Pta\textsubscript{WT}**

Forward Reaction (Ac-CoA-forming)—The His\textsubscript{6}-Pta\textsubscript{WT} enzyme displayed measurably higher affinity for CoASH (K<sub>m</sub> = 162 \mu M) than for Ac-P (K<sub>m</sub> = 1 mM) (Table 1), and the enzyme was very active (V<sub>max</sub> = 142.2 \mu mol min\textsuperscript{-1} mg\textsuperscript{-1}) (Table 1). The forward reaction displayed positive cooperativity with regard to Ac-P concentration. No phosphotransacetylase activity was detected in a preparation of protein purified in a strain harboring the empty pKLD37 vector.

Back Reaction (Ac-P-forming)—The reaction was slower, with product (CoASH) release occurring at a rate ~7-fold slower than that of the forward reaction (V<sub>max</sub> = 20.6 \mu mol min\textsuperscript{-1} mg\textsuperscript{-1}; Table 2). Under the conditions used, the His\textsubscript{6}-Pta\textsubscript{WT} enzyme displayed higher affinity for Ac-CoA (K<sub>m</sub> = 329.3 \mu M) than for P<sub>i</sub> (1.5 mM) (Table 1), and the enzyme was very active (V<sub>max</sub> = 142.2 \mu mol min\textsuperscript{-1} mg\textsuperscript{-1}) (Table 1). The forward reaction displayed positive cooperativity with respect to Ac-P concentration. No phosphotransacetylase activity was detected in a preparation of protein purified in a strain harboring the empty pKLD37 vector.

**Pta\textsubscript{R252H}, Pta\textsubscript{G273D}, and Pta\textsubscript{M294I} Variants Are Faster than the Pta\textsubscript{WT} Enzyme**

To investigate the effect of changes in residues Met\textsuperscript{294}, Glu\textsuperscript{273}, and Arg\textsuperscript{252} on Pta activity, pta alleles encoding Pta\textsubscript{R252H}, Pta\textsubscript{G273D}, and Pta\textsubscript{M294I} variants were introduced into plasmid pPTA69 by site-directed mutagenesis. The resulting His\textsubscript{6}-tagged proteins supported growth on low acetate (10 mM) of an acs pta recA strain (data not shown); the same strain carrying the empty vector or the plasmid containing the pta<sup>−</sup> allele did not grow on acetate. Once the phenotype was confirmed, each plasmid was transformed into E. coli strain BL21(ARTH21). E. coli strain BL21(ARTH21) was used for overexpression. Under the conditions used, the purification of Pta\textsuperscript{WT} and variant proteins yielded 0.6–2 mg of Pta protein from 500 ml of culture, with a calculated purity of ~70% (data not shown).

In the forward (Ac-CoA-forming) (Table 1) and back (Ac-P-forming) reaction (Table 2), variant proteins Pta\textsuperscript{R252H} and Pta\textsuperscript{G273D} were 2–3-fold faster than Pta\textsuperscript{WT}, whereas Pta\textsuperscript{M294I} was slightly slower (0.7-fold) than Pta\textsuperscript{WT}. In the forward and back reactions, variant and wild-type Pta proteins displayed similar affinities for their substrates (Tables 1 and 2).

**NADH Inhibits Pta Activity by Changing Its Conformation**

To learn more about how NADH inhibited Pta activity, His\textsubscript{6}-Pta\textsuperscript{WT} protein was subjected to limited trypsin proteolysis, and changes in its structure were assessed by SDS-PAGE followed by MALDI-TOF mass spectrometry. Exposure of Pta proteins to trypsin in the presence of NADH resulted in appearance of two prominent fragments as a function of NADH concentration. The size of each fragment and their location within the wild-type protein was established by MALDI-TOF mass spectrometry. One fragment was the result of tryptic cleavage after residues Arg<sup>115</sup> and Arg<sup>415</sup> (42 kDa), and the second fragment resulted from cleavage after Lys<sup>270</sup> and Arg<sup>415</sup> (38 kDa) (data not shown). It should be noted that the 38-kDa peptide ran abnormally during SDS-PAGE analysis (supplemental Fig. 9). Polyclonal antibodies against the N-terminal domain of Pta reacted with both fragments. Although the trypsin cleavage site needed to generate the 38-kDa fragment (site A) was accessible in the absence of NADH, the trypsin cleavage site for the generation of the 42-kDa fragment (site B) was detected only when the concentration of NADH was 0.2–0.5 mM (Fig. 4 (closed inverted triangles), supplemental Fig. 9A).

Similar experiments were performed with variant Pta proteins. (Fig. 4, supplemental Fig. 9). Results from experiments with variant Pta<sub>R252H</sub> showed that the mutation affected exposure of site B, showing 2-fold decrease in the band intensity ratio when the concentration of NADH reached 5 mM. (Fig. 4, circles). The effect of the M294I or G273D substitution on the β-NADH-induced
conformational change was not significantly different than the PtaWT protein (Fig. 4, open triangles and diamonds, respectively).

**Variants PtaR252H, PtaG273D, and PtaM294I Have Altered Responses to at Least One Allosteric Effector**

To determine the effect of the amino acid substitutions on NADH inhibition and pyruvate stimulation, \( K_i \) and \( K_a \) values were determined using fast protein liquid chromatography-purified His\(_6\)-PtaWT. To calculate \( K_i \) and \( K_a \) values, we plotted the reciprocal of the initial velocity (\( V^{-1} \)) of the reaction versus the concentration of the effector; data were fitted to Equation 3, where \( E \) was the effector concentration.

His\(_6\)-PtaWT was found to have an apparent \( K_i \) of 1.1 ± 0.2 mM for NADH and an apparent \( K_a \) of 2.6 ± 0.2 mM for pyruvate (Fig. 5, A and B, respectively). Under conditions of subsaturating levels of substrates (at the \( K_m \)), NADH inhibition of the back reaction catalyzed by His\(_6\)-PtaWT was incomplete, with a maximum reached at 70–80% inhibition (Fig. 6, black bars). Under the same conditions, His\(_6\)-PtaWT enzyme activity in the presence of pyruvate was 120–140% higher than in the absence of pyruvate (Fig. 6, hatched bars). Pyruvate overcame the inhibition by NADH. Inclusion of pyruvate (30 mM) in the reaction mixture was optimal for counteracting the inhibitory effect of NADH (Figs. 5C and 6, checkered bars).

Comparison of the response of wild-type and variant Pta proteins with allosteric effectors showed that variants PtaG273D and PtaM294I were equally sensitive to NADH inhibition as PtaWT (Fig. 6, black bars). Strikingly, NADH did not inhibit the PtaR252H variant protein.

The stimulatory effect of pyruvate was stronger on the variant proteins than on wild-type protein. Pyruvate stimulated the activity of variant proteins by at least 3-fold over the no-addition control, whereas pyruvate stimulated PtaWT activity 0.2-fold over the no-addition control.

Pyruvate counteracted the negative effect of NADH more efficiently in variant proteins (Fig. 6, checkered bars). The strongest effect of pyruvate was measured with the PtaR252H protein in the presence of NADH. Under the same conditions, oxidation of NADH was not detected in the presence of pyruvate for the WT or variant Pta proteins. This indicated that Pta proteins did not have LDH activity and that the effect of pyruvate and NADH was a result of direct allosteric activation and inhibition, respectively. Rabbit muscle LDH was used as the positive control, yielding a specific activity of 531.6 ± 29.1 \( \mu \)mol of NADH oxidized min\(^{-1} \) mg\(^{-1} \).

**Variants PtaR252H, PtaG273D, and PtaM294I Variants Show Less Aggregation than PtaWT**

Results from gel filtration analysis of PtaR252H, PtaG273D, and PtaM294I variant proteins revealed a lower proportion of large
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The Pta<sup>R252H</sup> Variant Is Different from Other Variants in Its Response to Allosteric Effectors

Pyruvate effect—The increased responsiveness of Pta<sup>R252H</sup> to pyruvate when NADH is present in the reaction mixture (Fig. 6) suggests that pyruvate and NADH may bind to the protein at different sites. Clearly, the conformation of Pta<sup>R252H</sup> is more responsive to the synergistic positive effect on the effectors than Pta<sup>WT</sup>. Interestingly, substitution of residue Arg<sup>252</sup> with cysteine yields a variant protein capable of supporting growth on low acetate. A three-dimensional structure of the Pta protein would help rationalize the effect of this mutation and clarify the role of this important residue.

The bioinformatics analysis by Galperin and Grishin (62) suggested that the N-terminal domain of Pta<sup>II</sup> evolved from a common ancestor of the family of enzymes that includes dethiobiotin synthase (DTBS), the cell division protein MinD, and the amidotransferases CbiA and CbiP, involved in coenzyme B<sub>12</sub> biosynthesis. To help us visualize the effects of the mutations we have isolated, we placed them within the reported three-dimensional structure of DTBS complexed with ADP, 7-(carboxyamino)-8-aminononanoic acid, and calcium (Protein Data Bank code 1DAF) (63) (supplemental Fig. 11). In the DTBS structure, residue Gly<sup>31</sup> is equivalent to Pta Gly<sup>31</sup>, residue Phe<sup>121</sup> in DTBS is equivalent to Pta Arg<sup>113</sup>, and residue Lys<sup>148</sup> in DTBS is equivalent to Pta Gly<sup>140</sup>. Using the DTBS structure as a model for the N-terminal domain of Pta, we hypothesize that residues Gly<sup>31</sup> and Gly<sup>140</sup> of Pta may affect the positioning of the P-loop, perhaps playing a role in binding NADH. On the other hand, residue Arg<sup>113</sup> may be involved in binding pyruvate, since in the DTBS structure the analogous residue is near the 7-(carboxyamino)-8-aminononanoic acid substrate (supplemental Fig. 11).

It is not clear whether pyruvate exerts its positive effect through a conformational change of the protein. If it does, the change is not as large as the one induced by NADH. Tryptic digestion of Pta<sup>WT</sup> protein in the presence of pyruvate did not reveal any differences compared with the tryptic digest obtained in the absence of pyruvate (data not shown). The effect of pyruvate can be amplified by concentrating the protein using polyethylene glycol 400. We have measured up to 54% stimulation of Pta<sup>WT</sup> activity by pyruvate in the presence of 5% polyethylene glycol 400, relative to the condition when polyethylene glycol 400 is present but pyruvate is not (data not shown). The nature of the effect is not known and may be a consequence of molecular crowding. Alternatively, the effect could be the result of reducing water activity through dehydration of the reaction components.

NADH Effect—Unlike the E. coli Pta enzyme (25), we measured positive cooperativity in the forward reaction in the absence of NADH (Table 1, Hill constants). At present, we do not know what the cause of the cooperativity differences between the E. coli and the S. enterica Pta enzyme could be.
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The S. enterica Pta Protein Is Likely to Have an Elongated Shape

The large discrepancy between gel filtration data and data obtained from equilibrium sedimentation experiments suggests a somewhat nonglobular shape for the S. enterica Pta protein. This is not surprising, since characterization of the E. coli enzyme also showed a large difference in estimated mass (64). Although the proportions of aggregate and hexamer were significantly different in the variants with respect to the WT protein, each His6-Pta protein eluted from the column with a retention time consistent with a hexamer, suggesting that the altered kinetic properties of the variants is not a result of a decrease in oligomeric state. Further insights into the shape and oligomeric state of the PtaII enzyme will be obtained when a crystal structure of the protein becomes available. Although protein structure data bases contain multiple structures of PtaI proteins (Methanosarcina thermophila, Protein Data Bank code 1QZT; Streptococcus pyogenes, Protein Data Bank code 1R5J; Bacillus subtilis, Protein Data Bank code 1TD9; E. coli, Protein Data Bank code 1VMJ), no PtaII structure has been reported to date. Attempts to crystallize the full-length PtaII protein of S. enterica are ongoing.

PtaI Versus PtaII

The environmental or physiological conditions that dictate whether a prokaryote employs a class I or class II Pta enzyme remain unclear. Perhaps in some habitats the use of nonregulated PtaI enzyme is favored over the allosterically controlled PtaII enzyme, because the concentration of acetate in the environment is low. This idea is supported by reported kinetic analyses of PtaI enzymes, whose substrate affinity constants are at least 1 order of magnitude lower than PtaII enzymes and are also faster than PtaII enzymes (26, 65).

Whether a prokaryote uses a PtaI (not allosterically regulated) or a PtaII (allosterically regulated) class enzyme may hinge on the existence of an alternative mechanism for modulating the level of Pta activity (e.g. pta gene expression control). The alluded pta expression control system would use pyruvate as the metabolite that signals the presence of high levels of glucose in the environment.

The Gram-positive bacterium B. subtilis is one example where this level of control of Pta activity does occur. A recent report by Sonenshein and co-workers (66) showed that the global regulatory protein CodY activates ackA transcription. In this paper, the authors allude to unpublished results of transcription microarray analyses that suggest that CodY also activates pta gene expression. Three facts are relevant to this discussion. First, in B. subtilis, the pta gene encodes a PtaI class enzyme not subject to allosteric control. Second, branched-chain amino acid biosynthesis serves as an overflow pathway for pyruvate (67) (i.e. the higher the intracellular level of pyruvate, the more branched-chain amino acids are synthesized), and third, high branched-chain amino acid levels stimulate CodY activity (68), which in turn activates pta gene expression, increasing the level of PtaI activity in the cell.

Impact of Allosteric Regulation of Pta Activity on S. enterica Physiology

The analysis of PtaII activity reported here reveals the expanded capability of this central metabolic enzyme of prokaryotes. Allosteric control of Pta activity allows the cell to rapidly adjust to changing concentrations of critical metabolites that report the energy state of the cell and the quality of carbon source available in the environment.

In Fig. 7, we depict the role of the Pta protein in S. enterica with our findings incorporated into the model. Pta can generate Ac-CoA for biosynthesis or act in concert with acetate kinase to convert Ac-CoA to Ac-P and acetate with concomitant generation of ATP (21).

Conditions that lead to the accumulation of high levels of reducing power (i.e. high NADH) would inhibit Pta activity, resulting in acetate excretion via PoxB or an as yet unidentified mechanism (69, 70). This inhibition would persist until NADH is oxidized and the inhibition is lifted.

Pyruvate may serve as a nutritional signal that carbon is abundant (e.g. excess glucose in the environment), accelerating the Ac-CoA-forming reaction to increase amino acid biosynthesis or the Ac-P-forming reaction to conserve energy while excreting excess carbon as acetate (27). Increasing flux toward Ac-P could also help modulate gene expression by increasing the intracellular level of Ac-P.

Stimulation of activity by pyruvate appears to be the dominant effect, since NADH inhibition can be countered as levels of pyruvate rise intracellularly. Since the allosteric effects affect the forward (Ac-CoA-forming) reaction (34) and the back (Ac-P-forming) reaction (this work), we suggest that that PtaII enzymes sense changes in the carbon and energy status of the cell, triggering physiological changes by altering the level of Pta II in the cell (23, 30, 31, 71).

Although more efficient variants of PtaII can be obtained by single-amino acid changes in the N-terminal regulatory domain, such changes can lead to somewhat less stable proteins.
For example, the G31D variant lost activity upon dilution to an appropriate concentration for kinetic analyses. It appears that the extant form of Pta\textsuperscript{II} may be a compromise between tunable activity and stability.

**Do Other Allosteric Effectors of Pta\textsuperscript{II} Exist?**

The fact that phosphotransacetylase activity can be regulated by nucleotides including NADH and ATP and by pyruvate leads us to believe that the N-terminal domain of Pta acts as an energy and nutritional sensor. Of additional interest is the homology of the N-terminal domain of Pta\textsuperscript{II} to coenzyme B\textsubscript{12} biosynthetic enzymes (62). It is possible that the N-terminal domain may bind a *de novo* precursor of coenzyme B\textsubscript{12}, a signal of an anoxic environment (72). It is also possible that other cyclic tetrapyroles (e.g. heme) may bind to the N-terminal domain of Pta. These possibilities need to be explored, and their effects (if any) should be analyzed within the context of the back and forward reactions. This work is currently in progress.

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