Structural and Functional Evidence for Bacillus subtilis PaiA as a Novel N¹-Spermidine/Spermine Acetyltransferase*

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Bacillus subtilis PaiA has been implicated in the negative control of sporulation as well as production of degradative enzymes. PaiA shares recognizable sequence homology with N-acetyltransferases, including those that can acetylate spermidine/spermine substrates. We have determined the crystal structure of PaiA in complex with CoA at 1.9 Å resolution and found that PaiA is a member of the N-acetyltransferase superfamily of enzymes. Unexpectedly, we observed the binding of an oxidized CoA dimer in the active site of PaiA, and the structural information suggests the substrates of the enzyme could be linear, positively charged compounds. Our biochemical characterization is also consistent with this possibility, since purified PaiA possesses N¹-acetyltransferase activity toward polyamine substrates including spermidine and spermine. Further, conditional overexpression of PaiA in bacteria results in increased acetylation of endogenous spermidine pools. Thus, our structural and biochemical analyses indicate that PaiA is a novel N-acetyltransferase capable of acetylating both spermidine and spermine. In this way, the pai operon may function in regulating intracellular polyamine concentrations and/or binding capabilities. In addition to preventing toxicity due to polyamine excess, this function may also serve to regulate expression of certain bacterial gene products such as those involved in sporulation.

In Bacillus subtilis, the pai operon is involved in negative control of sporulation as well as the production of extracellular and cell-associated proteases and other enzymes such as α-amylase and alkaline phosphatase (1). The pai operon contains two genes, paiA and paiB, encoding proteins of 172 (PaiA) and 207 (PaiB) amino acid residues, respectively. Chromosomal disruption of the paiA gene abolishes repression of protease synthesis in glucose-enriched medium, whereas disruption of paiB is lethal to the bacteria, suggestive of its importance for growth. On the other hand, overexpression of the two genes down-regulates the amount of transcripts for the extracellular metalloprotease, suggesting that this operon negatively controls the protease levels through transcriptional repression (1). A putative helix-turn-helix motif was recognized based on the amino acid sequence of the protein PaiA, and it was suggested that PaiA may be a DNA-binding protein (1).

However, PaiA also contains motifs that are conserved among the members of the N-acetyltransferase superfamily (2) (Fig. 1), suggesting that PaiA may actually be an enzyme. N-Acetyltransferases perform a variety of cellular functions by transferring an acetyl group from the cofactor acetyl-CoA to a nitrogen atom in their protein or small molecule substrate (2–4). Protein acetylation may be a covalent modification that is as important as phosphorylation for functional regulation (5). Acetylation of small molecules such as serotonin, polyamines, and aminoglycoside also has crucial roles in the function and metabolism of these compounds. In B. subtilis, the BltD gene product was found to be a membrane-bound spermidine/spermine N¹-acetyltransferase (SSAT), which participates in the export of polyamines (6, 7). The multidrug transporter function of the Blt-containing operon may be secondary to the more physiological role of preventing toxicity due to polyamine excess.

To understand the molecular mechanism for the biological functions of PaiA, we have determined the crystal structure of this protein in complex with CoA at 1.9 Å resolution. The structure confirms that PaiA is a member of the N-acetyltransferase superfamily. Unexpectedly, the structure contains an oxidized CoA dimer in the active site, linked by a disulfide bond. Whereas one of the CoA molecules is fully ordered and mimics the CoA substrate, the adenine nucleotide portion of the other CoA molecule is completely disordered. The presence of the pantetheine and β-mercaptoethyamine portion of this CoA molecule in the active site suggested that the substrate of PaiA could be a long, linear molecule. We therefore examined the activity of PaiA with respect to polyamine substrates, including spermine and spermidine. Our biochemical experiments demonstrate that PaiA has N¹-acetyltransferase activity toward spermine and spermidine. Our structural and biochemical analyses therefore assign a function to the PaiA protein as a non-membrane-bound SSAT.

MATERIALS AND METHODS

Expression and Purification of PaiA — The production of PaiA protein was carried out as part of the high throughput protein production process of the Northeast Structural Genomics Consortium (8). PaiA corresponds to NSEQ Target SR64. The full-length paiA gene from B. subtilis was cloned into a plpT21d (Novagen) derivative, generating plasmid pSR64-21. The resulting recombinant protein contains eight nonnative residues (LEHHHHHH) at the C terminus. The construct was

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‡ The abbreviations used are: SSAT, spermidine/spermine N¹-acetyltransferase; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside.

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sequence-verified by standard DNA sequence analysis. Escherichia coli BL21 (DE3) pMGK cells, a rare codon enhanced strain, were transformed with pSR64-21. A single isolate was cultured in M9 minimal medium (9) supplemented with selenomethionine, lysine, phenylalanine, threonine, isoleucine, leucine, and valine for the production of selenomethionine-labeled PaiA (10). Initial growth was carried out at 37 °C until the A_{600} of the culture reached 0.6–0.8 units. The incubation temperature was then decreased to 17 °C, and protein expression was monitored by analytical gel filtration detected by static light scattering, as pooled, and buffer conditions providing monomeric samples were optimized. The purified PaiA protein was concentrated to 10 mg/ml, flash-frozen in aliquots, and used for crystallization screening. Sample purity (97%) and molecular mass (21.2 kDa) were confirmed by SDS-PAGE and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry, respectively. The yield of purified PaiA as a Novel Spermidine/Spermine N-Acetyltransferase

Structure Determination—A single wavelength anomalous diffraction data set to 1.9 Å resolution was collected at the peak absorption wavelength of selenium at the X4A beamline of the National Synchrotron Light Source. The diffraction images were processed with the HKL package (11), and the selenium sites were located with the program SnB (12). SOLVE/RESOLVE (13) was used for phasing the reflections and automated model building, which correctly placed 85% of the residues in the two monomers. A complete model of each monomer, except the initiating (seleno)methionine and residues 27–34 of the second monomer, was built with the program XtalView (14) and refined by CNS (15). Noncrystallographic symmetry restraint was applied in the early stages of the refinement but released at the final stage, since the two monomers differ in their active site conformation. The data processing and refinement statistics are summarized in TABLE ONE.

Polyamine N-Acetyltransferase Assay and Enzyme Kinetics—N-acetyltransferase activity was determined as previously described for SSAT and expressed as nmol/min/mg (16). Briefly, the reaction mixture (final volume 50 μl) included 10 μl of 0.5× Tris buffer, 5 μl of 30 mM substrate (final concentration, 3.0 mM), 10 μl of double distilled H_2O, 5 μl of 1 mM [14C]acetetyl-CoA (60 mCi/mmol; PerkinElmer Life Sciences; final concentration, 100 μM) and 20 μl containing 125 ng of purified PaiA protein. The assay was optimized for pH (9.0), enzyme concentration (2.5 μg/ml), and time (5 min) so as to be linear in nature.

Substrates tested included the polyamines, agmatine, aminopropyl-leadaverine, N^1-acetyl spermine, N^1-acetyl spermidine, N^8-acetyl spermidine, cadaverine homospermidine, norspermidine, putrescine, spermine, and spermidine, all at a final concentrations of 3.0 mM. In addition, the polyamine analog N^1,N^11-diethylnorspermine was examined as a substrate in order to evaluate the ability of the enzyme to...
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acetate secondary amines. Following a 5-min incubation at 37 °C, the enzyme reaction mixture was terminated by the addition of 20 μl of 0.5 M NH₄OH·HCl and heating in boiling water for 3 min. The mixture was microcentrifuged, and 50 μl of the supernatant was spotted onto a p-81 phosphocellulose disc and counted in a scintillation counter. A detailed kinetic analysis was performed by varying the concentration of potential polyamine substrates from 10 μM to 3.0 mM in the presence of saturating levels (100 μM) of acetyl-CoA (17). Acetyl-CoA kinetic analyses were performed in the presence of saturating spermine concentrations (3 mM), and the coenzyme was varied from 3 to 400 μM. Estimates of the kinetic constants \( V_{\text{max}} \) and \( K_M \) were obtained from Michaelis-Menten plots (18) using Sigma Plot-Enzyme Kinetics Module software (Systat Software Inc., Richmond, CA).

RESULTS AND DISCUSSION

Structure Determination—The crystal structure of \( B. subtilis \) PaiA has been determined at 1.9 Å resolution by the selenomethionyl single wavelength anomalous diffraction method (20). The protein is enriched in methionine residues, with 8 such residues in each molecule of 172 amino acids. The asymmetric unit of the crystal contains two PaiA molecules, A and B, but they do not have strong interactions with each other. This is consistent with static light scattering data demonstrating that PaiA is monomeric in solution (data not shown).

The current atomic model for the enzyme contains residues 2–172 for monomer A and residues 2–26 and 35–172 of monomer B. An oxidized CoA dimer and two oxidized DTT molecules are bound to monomer A, whereas only one CoA and a sulfate ion are bound to monomer B. The atomic model has low \( R \) values and excellent agreement with expected geometric parameters (TABLE ONE). None of the residues of the protein is located in the disallowed region, whereas 92% of the residues are located in the most favored region of the Ramachandran plot (data not shown).

Overall Structure of PaiA—The structure of PaiA contains seven \( \beta \)-strands (named \( \beta_1 \) through \( \beta_7 \)) and 5 \( \alpha \)-helices (\( \alpha A \) through \( \alpha E \)) (Fig. 2A). The \( \beta \)-strands are mostly antiparallel to each other and form a semicylindrical \( \beta \)-sheet. The N-terminal helices (\( \alpha A \), \( \alpha B \), and \( \alpha C \)) connect strands \( \beta_1 \) and \( \beta_2 \) and are arranged on the convex face of this \( \beta \)-sheet, whereas helices \( \alpha D \) and \( \alpha E \) are located on the concave face (Fig. 2A). The C-terminal ends of the neighboring strands \( \beta_3 \) and \( \beta_5 \), the only two parallel \( \beta \)-strands in the sheet, are splayed apart from each other (Fig. 2B). This splits the \( \beta \)-sheet into two groups of antiparallel \( \beta \)-strands composed of \( \beta_1–\beta_4 \) and \( \beta_5–\beta_7 \). More importantly, this creates a tunnel in the center of the \( \beta \)-sheet for the binding of the CoA cofactor and the substrate (Fig. 2C; see “Binding Mode of CoA”).

Our structural information confirms that PaiA has a similar overall backbone fold as that of \( N \)-acetyltransferases and that PaiA is a member of this superfamily of enzymes (3, 21, 22). Based on the program Dali (23), the closest structural homologs include phosphoinothrin acetyltransferase (Protein Data Bank accession code 1VHS), Hpa2 histone \( N \)-acetyltransferase (22), aminoglycoside 6'-N-acetyltransferase (24), and serotonin \( N \)-acetyltransferase (Fig. 3) (25). However, the amino acid sequence conservation with these other proteins is very low, in the 10–20% identity range.

PaiA also possesses several unique structural features. Most notably, helix \( \alpha B \) is unique to the PaiA structure (Fig. 3). This helix may be involved in binding the substrate of the enzyme (see below), suggesting that the acetylated target of PaiA is different from those of other \( N \)-acetyltransferases whose structures are already available in the Protein Data Bank. Besides helix \( \alpha B \), the loop connecting strands \( \beta_3 \) and \( \beta_4 \) as well as the C-terminal region comprising \( \beta_6 \) and \( \beta_7 \) show significant structural differences from other \( N \)-acetyltransferases (Fig. 3). Strands \( \beta_6 \) and \( \beta_7 \) may also be involved in substrate binding.

The helix-turn-helix motif proposed earlier for DNA binding corresponds to residues 98–121 of PaiA (1). In the structure, these residues form helix \( \alpha D \) and the loop connecting strand \( \beta_4 \) and this helix (Figs. 1 and 2A). Therefore, these residues do not form a helix-turn-helix motif in the structure of PaiA. Moreover, these residues actually contain the motif A of \( N \)-acetyltransferases (2), and they interact with the phosphoryl group of CoA (see below). PaiA is unlikely to be a DNA-binding protein, and its effect on the regulation of gene transcription is indirect.

Binding Mode of CoA—The CoA cofactor in each monomer has well-defined electron density (Fig. 4A). However, the CoA molecule bound to monomer A has much lower temperature factor values as compared with that bound to monomer B. In fact, the overall average temperature factor value of monomer A of PaiA (16 Å²) is lower than that of monomer B (26 Å²), implying that monomer A is more ordered than monomer B. This may be related to the fact that monomer A contains an oxidized CoA dimer in the active site and has a different conformation compared with monomer B.

The oxidized CoA dimer in the active site of molecule A contains a disulfide bond between the sulfur atoms of the two CoA molecules (Fig. 4A). One of the CoA molecules of this dimer (CoA\(_A\)) is fully ordered and assumes a conformation that is similar to the CoA molecule in monomer B. Therefore, the binding mode of this CoA molecule probably corresponds to the CoA/acyt-CoA substrate for catalysis and will be described in more detail here. The binding mode of the second CoA molecule (CoA\(_B\)) of the oxidized dimer will be described under “Binding Mode of Second CoA and DTT.”

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**TABLE ONE**

| Summary of crystallographic information | Parameters | Values |
|----------------------------------------|------------|-------|
| Maximum resolution (Å)                 | 1.9        |       |
| No. of observations                    | 424,802    |       |
| \( R_{merge} \) (%)                    | 7.0 (24.2) |       |
| No. of reflections                     | 54,707     |       |
| Resolution range used in refinement    | 30–1.9     |       |
| Completeness (%)                       | 99 (98)    |       |
| \( R \) factor (%)                     | 20.6 (20.2)|       |
| \( R \) free factor (%)                | 24.1 (24.1)|       |
| Root mean square deviation in bond lengths (Å) | 0.006 |       |
| Root mean square deviation in bond angles (degrees) | 1.1 |       |

* \( R_{merge} \) = \( \sum_{i} \sum_{j} \frac{|I_{ij} - \langle I_{ij} \rangle|}{\sum_{i} \sum_{j} I_{ij}} \), The numbers in parentheses are for the highest resolution shell.  
* \( R = \sum_{i} F_{o} - F_{c} \sqrt{\sum_{i} F_{o}^2} \).
The adenine moiety of the CoA molecule (CoA1) is situated on the surface of PaiA, having interactions with residues Ala140 and Phe141 in helix H9251 (Fig. 4B). The N6 amino group of the adenine base is hydrogen-bonded to the side chain of Asn137, but the N1 atom is not recognized by PaiA. The CoA molecule assumes a folded conformation, such that the N7 atom of adenine interacts with one of the oxygen atoms on the β-phosphate group through a water molecule (Fig. 4B). The 3’-phosphate group on the ribose ring is recognized by the side chains of Lys109 and Lys144. The - and -phosphates of CoA are placed near the N-terminal end of helix αD and hydrogen-bonded to the amide groups at the end of the helix as well as having favorable interactions with the dipole of this helix. The phosphates are also hydrogen-bonded to the main chain amide groups of residues in the loop connecting strand β5 and helix αD. This loop and the beginning of helix αD correspond to motif A that is conserved among all canonical N-acetyltransferases (2, 26), and this motif appears to be crucial for the recognition of the pyrophosphates of CoA.

The pantothenate and the -mercaptoethylamine moieties of CoA are inserted into the tunnel that is present between strands β4 and β5 (Fig. 2C). The two amide groups in this region are hydrogen-bonded to the open segment of strand β4, mimicking a β-strand (Fig. 4B). This mode of interaction has also been observed in canonical N-acetyltransferases (3, 21, 22, 26).

**Binding Mode of Second CoA and DTT**—Molecule A of PaiA in the crystallographic asymmetric unit contains an oxidized CoA dimer and an oxidized DTT in the active site (Fig. 2A). The second CoA molecule (CoA2) of this oxidized dimer as well as the DTT molecule (Fig. 4C) are bound in the other end of the tunnel in the enzyme (Fig. 2B), which is probably where the substrate of this enzyme is bound for catalysis (see below).

In contrast to the first CoA molecule (CoA1), only the -mercaptoethylamine, pantothenate, and -phosphate moieties of the second CoA molecule (CoA2) are ordered in the structure (Fig. 2A). Half of the protein surface in this binding tunnel is highly hydrophobic and is
formed by the side chains of residues from helices H9251A and H9251B (Phe24, Phe28, Asn32, Asn36, Met37, Tyr40) and strands H92526 and H92527 (Trp132, Phe155, Met157) (Fig. 5). The disulfide bond of the oxidized DTT molecule is placed in this hydrophobic portion of the tunnel, whereas its two hydroxyl groups are projected into the solvent (Fig. 5).

The binding site for the CoA2 molecule is more hydrophilic in nature. There are two ion pairs in this region, between Glu53 and Arg95 and between Lys75 and Glu94 (Fig. 5). In addition, the side chain of Glu92 is hydrogen-bonded to the main chain amide and side chain hydroxyl of Ser83, which may help stabilize the conformation of the β3-β4 loop. These polar groups do not have specific recognition of the CoA2 molecule, since there is only one hydrogen bond between the molecule and this binding site.

Conformational Changes Associated with Substrate Binding—The two molecules of PaiA in the asymmetric unit have different ligands bound in the active site. A comparison of the structures of the two PaiA molecules showed that there are major conformational differences between them in the binding site for CoA2 and oxidized DTT. Since this is the expected binding site for the substrate of PaiA, our structural information suggests that there might be major conformational changes in the enzyme upon substrate binding as well. In comparison, binding of the CoA1 molecule does not seem to induce significant conformational changes in the enzyme, since the structure of the free enzyme of PaiA (data not shown) is similar to that of the complex with CoA only (molecule B).

Remarkably, the conformational changes in PaiA lead to an expanded active site for the binding of CoA2 and DTT and possibly the substrate as well. This is in contrast to most other enzymes, which adopt a more closed conformation upon substrate binding. In the binary complex with CoA only (molecule B), helix αB is in contact with strands β6 and β7 and their connecting loop, whereas residues 27–34 in the αA-αB loop are disordered (Fig. 6A). In the ternary complex with oxidized CoA dimer and oxidized DTT, helix αB and strands β6 and β7 retract from each other, each moving by about 3 Å (Fig. 6A), thereby creating an enlarged binding site that is sufficient for the binding of CoA2 and DTT (Fig. 6B). Coupled to this change, helices αB and αC and the loop connecting αB and αC also move by about 3 Å. Residues 27–34 become ordered in the ternary complex, since they interact with the disulfide bond in the oxidized DTT molecule. In contrast, the binding site in the

FIGURE 5. The binding mode of the second CoA molecule and DTT. Shown is a stereographic drawing of the interactions among CoA2, the oxidized DTT molecule, and PaiA. These polar groups do not have specific recognition of the CoA2 molecule, since there is only one hydrogen bond between the molecule and this binding site.
binary complex with CoA only (molecule B) is too small to accommodate the CoA$_2$ and the DTT molecules (Fig. 6C). In fact, the binding site for DTT does not exist in this binary complex with CoA.

**PaiA as a Possible SSAT**—We suspected that the observed binding modes of the CoA$_2$ and the oxidized DTT molecules might mimic that of the genuine substrate of the PaiA enzyme. The tunnel has only a small opening toward the CoA$_2$ molecule, and only the linear portion (pantothenate and β-mercaptoethyamine) of the CoA$_2$ molecule is located in this part of the tunnel. This suggested that the substrate could be a linear molecule. Second, the β-phosphate of CoA$_2$ is placed on the surface of this binding site, suggesting that a negatively charged species may not be favored in this binding pocket. This led to the hypothesis that the substrates of PaiA could be polyamines, such as spermine and spermidine, which are linear, positively charged molecules. The structural observations are supported by the fact that PaiA shares weak but recognizable sequence homology with characterized SSATs (Fig. 1; see below).

Our biochemical studies confirm that PaiA possesses N-acetyltransferase activity toward polyamine substrates. At the optimized enzyme concentration that we used (125 ng/reaction), PaiA readily acetylates the tetra-amine spermine and, to a lesser extent, the trimines, spermidine and aminopropylcadaverine (Fig. 7). Structure-activity analysis with various polyamines and polyamine analogs indicates that the enzyme prefers the aminopropyl moiety of the molecule, since (a) spermine, which contains two terminal aminopropyl moieties, is more active as a substrate than spermidine, which contains one; (b) norspermidine, which contains two terminal aminopropyl moieties, is more active than homospermidine, which contains two terminal aminobutyl moieties; and (c) N$_1$-acetylspermidine, which is preacetylated on the aminobutyl moiety, is a more effective substrate than N$_4$-acetylspermidine, which is preacetylated on the aminopropyl moiety. PaiA is also capable of diacetyling spermine, as indicated by the finding that N$_1$-acetylspermine is a slightly better substrate than spermidine based on enzyme activity (Fig. 7), the product being N$_1$,N$_1$-diethylnorspermine. The analysis also indicated that the enzyme is incapable of acetylating secondary amines, since N$_3$,N$_3$-diethylnorspermine had no measurable substrate activity. To exclude the possibility that the enzyme was inhibited by the analog, we showed that activity toward spermine was retained in the presence of the analog (Fig. 7, last column). We also found that the diamine diaminopropane had substrate activity, whereas putrescine and cadavarine did not (Fig. 7). Last, we note that the arginine metabolite, agmatine, showed substrate activity similar to that of diaminopropane in the radioactivity assay. We considered the possibility that the acetylated diamines and agmatine may not be detected in our radioactivity-based enzyme assay due to lack of sufficient charge for adherence to the cellulose phosphate filter paper. To exclude this possibility, we used a spectrophotometric assay (27) to quantitate the conversion of acetyl-CoA to CoA by PaiA in the presence of agmatine or spermidine. In this assay, the substrate activity of agmatine (777 nmol/min/mg) was found to be similar to that for spermidine (762 nmol/min/mg). N1AcSpd, N$_1$-acetylspermidine; N8AcSpd, N$_8$-acetylspermidine; N1AcSpm, N$_1$-acetylspermine; ApCad, aminopropylcadaverine; Cad, cadaverine; Dap, 1,3-diaminopropane; DENSPM, N$_1$,N$_1$-diethylnorspermine; hSpd, homospermidine; nSpd, nonspermidine; Spd, spermidine; Spm, spermine.

**Kinetic characterization** was performed with those polyamines having physiological substrate potential (TABLE TWO). Analysis of spermine as a substrate showed that the enzyme follows Michaelis-Menten kinetics with an apparent $K_m$ of 76 μM, a $V_{max}$ of 480 nmol/min/mg enzyme, and a $k_{cat}$ of 19.1 min$^{-1}$ (TABLE TWO). As found with the
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**TABLE TWO**

| Substrate                     | Kinetic parameters $^a$ | Polyamine pools $^b$ |
|-------------------------------|-------------------------|----------------------|
|                               | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ | Put | N$^2$-AcSpd | Spd | Spm $^c$ |
| Spermidine                    | 323 ± 80 | 130 ± 10 | 5.2 ± 0.40 | 270 |  |
| Aminopropylcadaverine         | 410 ± 75 | 154 ± 34 | 6.1 ± 1.4 | 250 |  |
| Spermine                      | 76 ± 2 | 481 ± 90 | 19.1 ± 3.6 | 4200 |  |
| N$^2$-Acetyspermine           | 295 ± 6 | 178 ± 12 | 7.1 ± 0.5 | 400 |  |
| Acetyl-CoA$^e$                | 31 ± 3 | 550 ± 60 | 21.9 ± 2.4 | 11,800 |  |

$^a$ Mean ± S.D. based on a minimum of four radioactivity determinations in triplicate except for N$^2$-acetyspermine, which was performed twice in triplicate.

$^b$ Determined spectrophotometrically in the presence of saturating levels (3 mM) of spermine.

$^c$ Mean nmol/min/mg.

$^e$ Determined spectrophotometrically in the presence of saturating levels (3 mM) of spermine.

$^f$ Put, putrescine; N$^2$-AcSpd, N$^2$-acetyl spermidine; Spd, spermidine; Spm, spermine.

**TABLE THREE**

| Induction$^a$ (IPTG) | Treatment (1 mM, 3 h) | Put | N$^2$-AcSpd | Spd | Spm$^c$ |
|-----------------------|-----------------------|-----|--------------|-----|--------|
| PaiA-Off              | None                  | 87.1 ± 7.1 | <0.01 | 15.5 ± 0.7 | <0.01 |
| PaiA-On               | None                  | 72.4 ± 4.2 | 4.0 ± 0.2 | 11.3 ± 0.5 | <0.01 |
| PaiA-Off              | Spd                   | 65.2 ± 4.6 | <0.01 | 32.7 ± 3.3 | <0.01 |
| PaiA-On               | Spd                   | 53.4 ± 6.1 | 6.4 ± 0.8 | 20.4 ± 2.5 | <0.01 |

$^a$ Bacterial cells containing PaiA under an IPTG-inducible promoter were incubated in the presence (PaiA-On) or absence (PaiA-Off) of 1 mM IPTG with or without 1 mM spermidine for 3 h and then extracted for polyamine pool analysis.

$^b$ Put, putrescine; N$^2$-AcSpd, N$^2$-acetyl spermidine; Spd, spermidine; Spm, spermine.

$^c$ In addition to spermine, agmatine, aminopropylcadaverine, and cadaverine were also not detectable (i.e. <0.05 nmol/mg of protein) under any treatment condition.

Initial activity determinations (Fig. 7B), the substrate rank order based on $k_{cat}/K_m$ values was as follows: spermine $>$ N$^2$-acetyl spermine $>$ spermidine $=$ aminopropylcadaverine (TABLE TWO). Other bacterial acetyltransferases have also been found to have similar $K_m$ values as PaiA. Thus, the $K_m$ of 76 μM toward spermine compares favorably with 61 μM for another B. subtilis SSAT known as BltD (7) and is somewhat lower than the 220 μM for an E. coli SSAT known as SpeG (28). Similarly, the $K_m$ value of 363 μM for spermidine is somewhat lower than the 1290 μM reported for SpeG (28). It is interesting to note that SpeG is capable of acetylation either end of the spermidine molecule (29), which may account for its higher $K_m$ value. As a generality, the bacterial $K_m$ values are much higher than eukaryotic SSAT values, which for spermine and spermidine range in the low micromolar concentrations (30). Despite similarities in $K_m$ between PaiA and other bacterial acetylases, the PaiA $V_{max}$ for spermidine and spermine is ≈4 and 40 times lower, respectively, than the values for BltD. Thus, although PaiA binds polyamines similarly to other acetyltransferases, the enzyme appears to be less efficient in substrate turnover. Finally, the apparent $K_m$ of 31 μM for acetyl-CoA by PaiA is 3 times lower than that of BltD (95 μM) (7).

The possibility that spermidine is a natural substrate for PaiA is supported by the fact that the $K_m$ value for spermidine compares favorably with the known intracellular polyamine concentration of ~6 mM found in bacteria (17). Although the $K_m$ values suggest that spermidine is favored over spermine, the intracellular levels of spermine were found to be negligible in B. subtilis (TABLE THREE). It is relevant to consider, however, that under certain growth conditions and environments, intracellular spermine could rise to levels of biological significance. It is also possible that acetyltransferases such as PaiA and BltD (7) export spermine as soon as it is taken up or synthesized. As further evidence for the suitability of spermidine as a substrate for acetylation by PaiA, we examined the enzyme reaction in the context of intact cells. Namely, bacterial cells conditionally overexpressing PaiA were incubated for 3 h in the presence or absence of 1 mM spermidine and then analyzed for polyamine pool differences. As shown in TABLE THREE, there was no N$^2$-acetyl spermidine present in PaiA-Off cells either in the presence or absence of exogenous spermidine. However, in PaiA-On cells, N$^2$-acetyl spermidine was observed in significant quantities in both the presence and absence of spermidine, and correspondingly, there was a 37 and 28% decrease, respectively, in spermine over that seen in PaiA-Off cells. The presumption is that PaiA converted intracellular spermidine to N$^2$-acetyl spermidine. Somewhat unexpectedly, putrescine pools also decreased, suggesting that the N$^2$-acetyl spermidine was being exported out of the cell instead of being back-converted to putrescine via a polyamine oxidase, in which case putrescine pools would have increased. Taken together with the above biochemical data, these findings support the concept that PaiA is capable of acetylating spermine and spermidine in cells. It remains possible, however, that PaiA might show greater preference for some yet to be identified endogenous molecule as a physiological substrate.

The side chain of Tyr$^{142}$ interacts with the sulfur atom of CoA in the active site of PaiA (Fig. 4B) and may have an important role in the acetylation of the polyamine substrates by this enzyme. The catalytic mechanism of PaiA may be similar to that of serotonin N-acetyltransferase, which also contains a Tyr residue (Tyr$^{168}$) in the active site (25). Moreover, structural superimposition of PaiA and serotonin N-acetyltransferase reveals that Tyr$^{142}$ of PaiA perfectly overlays onto Tyr$^{168}$ of serotonin N-acetyltransferase (Fig. 3). In the acetylation reaction, a water molecule functions as the general base to extract a proton from the terminal primary ammonium ion of the polyamine substrate. The amino group then attacks the carbonyl carbon of the acetyl group in the acetyl-CoA substrate, forming the tetrahedral intermediate. Collapse of this intermediate produces the acetylated polyamine product, and the Tyr$^{142}$ residue functions as a general acid to protonate the thiolate anion of the CoA product.
Interestingly, our crystallization solution actually contained 15 mM spermidine and 5 mM acetyl-CoA, and this may explain why we observed CoA instead of acetyl-CoA in the structure. The enzyme catalyzed the transfer of the acetyl group to spermidine, producing a large amount of CoA in the solution. It took 1–3 months to produce diffraction quality crystals, during which time the CoA and the DTT molecules were oxidized. After the characterization of PaiA as a polyamine N-acetyltransferase, we attempted to determine the binding mode of the polyamine substrate to the enzyme but were not successful.

Possible Function of the pai Operon—Based on our structural information, we hypothesized that PaiA is an N-acetyltransferase for polyamines, and our biochemical analyses tend to confirm this prediction. Therefore, we have been able to assign a probable biochemical function to the first gene of the pai operon in B. subtilis; paiA appears to encode a novel SSAT that is distinct from BltD (7). The original proposal that PaiA may be a transcriptional regulator (1) is unlikely to be correct, since the helix-turn-helix motif was not found to exist in the structure of PaiA.

Polyamines are small, aliphatic cations that have important roles in many biological processes (17), including DNA binding and stability, chromatin condensation, RNA binding and conformation, mRNA translation, protein binding, and other processes (31–34). The intracellular levels of these cations are tightly regulated, through biosynthesis, import, degradation, and export pathways (35). SSAT catalyzes the first reaction in both the degradation and the export pathways for polyamines. For the degradation pathway (also known as the back-conversion pathway), acetylated spermine and spermidine serve as substrates for polyamine oxidase, which converts them to spermidine and putrescine, respectively, through the removal of an acetamidopropanal group. For the export pathway, acetylated polyamines are excreted out of the cell through transporters located in the plasma membrane.

An operon containing an SSAT and a transporter has been characterized in B. subtilis (7). The SSAT gene is downstream of the transporter gene in this operon, and the encoded protein (BltD) is a homolog of the E. coli SSAT known as SpeG (28). Our structural and biochemical studies suggest that PaiA is a unique SSAT, since it shares only weak (although recognizable) homology with both BltD and SpeG (Fig. 1). The Tyr residue that may play an important role in the catalytic mechanism of PaiA is also conserved in BltD and SpeG (Fig. 1).

In contrast to the Blt operon, the pai operon has a different organization and composition. The paiA gene for the putative SSAT is located at the 5’ end of the operon, and the paiB gene is unlikely to encode a transporter, since its sequence does not appear to have hydrophobic, transmembrane segments. These are the only two genes in this operon. We examined the possibility that paiB might encode a novel polyamine oxidase. Our preliminary bioinformatic analysis of the PaiB protein suggests that it might be a split barrel flavin-binding protein, which is consistent with the fact that polyamine oxidases bind flavin (32, 36). However, we have been unable to detect any oxidase activity against acetylated polyamines using purified PaiB. The fact that we can purify PaiB from the cytosol further reduces the possibility that PaiB can be a membrane-associated polyamine transporter.

Polyamine acetylation in bacteria has been associated with a variety of chemical and physical stresses (37). As a regulator of sporulation and degradative enzymes (1), PaiA may also be stress-sensitive. Despite longstanding awareness of polyamine acetylation and its relationship to stress in both prokaryotes (37) and eukaryotes (38), the physiological purpose of this response remains uncertain. It is well known that intracellular polyamine excess can be toxic to cells (29), and acetylation can counter such toxicity by targeting polyamines for export or back-conversion. BltD has been especially implicated in such an activity, since the Blt operon contains an export protein. As noted above, PaiA does not seem to be functionally linked to an exporter in a similar manner, and in fact, the function of PaiB remains uncertain. In addition to affecting polyamine concentrations, PaiA undoubtedly affects the binding properties of polyamines, since acetylation reduces the net molecular charge by 1. Since the existence of a polyamine-responsive transcription factor has been reported in eukaryotic systems (39), it is possible that PaiA regulates expression of other genes by controlling polyamine levels and binding. Our structural and biochemical evidence implicate PaiA as a novel SSAT, suggesting that the pai operon in B. subtilis may be involved in polyamine homeostasis.

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