Characterization of a novel spermidine/spermine acetyltransferase, BltD, from *Bacillus subtilis*

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Overexpression of the *BltD* gene in *Bacillus subtilis* causes acetylation of the polyamines spermidine and spermine. *BltD* is co-regulated with another gene, *Blt*, which encodes a multidrug export protein whose overexpression facilitates spermidine export [Woolridge, Vazquez-Laslop, Markham, Chevalier, Gerner and Neyfakh (1997) J. Biol. Chem. **272**, 8864–8866]. Here we show that BltD acetylates both spermidine and spermine at primary propyl amine moieties, with spermine being the preferred substrate. In the presence of saturating concentrations of acetyl CoA, BltD rapidly acetylates spermine at both the N^1 and N^{12} positions. The K_m (app) values for spermine, spermidine and N^1-acetylspermine are $\leqslant 67$, 200 and 1200 μM, respectively. Diamines ranging from 1,3-diaminopropane to 1,12-diaminododecane, monoacetylputrescine and N^8-acetylspermidine were not substrates for BltD. Putrescine (1,4-diaminobutane) and N^8-acetylspermidine were competitive inhibitors of spermidine acetylation by BltD, with K_i values of 0.25 and 5.76 mM, respectively. CoA competitively inhibited both spermidine and acetyl-CoA interactions with BltD. These data and other results indicate that the mechanism of spermidine and spermine acetylation by BltD is a random-order mechanism of bi-molecular kinetics.

Key words: bacteria, enzyme kinetics, polyamine.

INTRODUCTION

A novel operon, encoding the two genes *Blt* and *BltD*, has been identified in *Bacillus subtilis* [1]. *Blt* is a member of the multidrug exporter family of genes. *BltD* was identified as a gene downstream of *Blt* and is co-regulated with it. Sequence analysis indicated that BltD shared sequence similarities with several known bacterial and mammalian acetyltransferases. Recently, we have shown that *B. subtilis* overexpressing *Blt* actively export spermidine, whereas overexpression of *BltD* results in formation of N^1-acetylspermidine [2]. These findings indicate that Blt and BltD act in a concerted fashion on spermidine, which is the predominant polyamine in *B. subtilis*.

The polyamines spermidine and spermine, and their precursor putrescine, are ubiquitous organic cations found in near-millimolar concentrations in most cells. These molecules are essential for normal cell growth and differentiation [3,4]. Polyamine contents fluctuate during bacterial cell growth [5] and the mammalian cell cycle [4]. The contents of these amines are elevated in some human cancers, and their biosynthetic enzymes are targets for human-cancer-prevention strategies [6]. Their metabolism is highly regulated, which further emphasizes the importance of polyamines in biological systems [3–6].

In eukaryotes, acetylation is the rate-limiting step in polyamine catabolism [7–9]. The mammalian spermidine/spermine N^1-acetyltransferase (SSAT) preferentially recognizes and acetylates the primary propyl amines of spermidine or spermine [10,11]. SSAT activity is transcriptionally and post-transcriptionally regulated, and is stress inducible [12–14]. In *Escherichia coli*, speG acetylates spermidine at the N^1 or N^8 positions [15,16]. The activity of the speG gene product is expressed constitutively in *E. coli* grown in nutrient-rich medium, increases when these cells are grown in nutrient-poor medium [16], but is not stress inducible [15]. Acetylation acts to reduce positive charges in polyamines, thus changing the physical properties of these molecules. Overexpression of SSAT in bacteria suppresses bacterial cell growth [17,18]. Re-expression of speG in *E. coli* mutants that do not express this gene product affords protection against the toxic effects of excess spermidine when cells are grown for extended periods in stationary phase [19].

In this study, we have characterized the polyamine-acetylating activity of the *B. subtilis BltD* gene product. Our results indicate that BltD is unique amongst the polyamine acetyltransferases in terms of its mechanism of action and substrate-binding properties.

EXPERIMENTAL

Bacterial strains, media, growth conditions and chemicals

Two strains of *B. subtilis* were used, including the multidrug-resistant AcfA cells, which overexpress *Blt*/*BltD* through a mutation that constitutively activates the promoter for this operon, and the parental BD170 cells [1]. The *E. coli* strain CAG2242 was kindly provided by Carol Gross and shown by us to be deficient in spermidine-acetylating activity [15]. These cells were transformed with the pTrc99A expression vector (Pharmacia) containing either the *B. subtilis BltD* gene [1], as described elsewhere [2], or the human *SSAT* gene [18]. BltD protein was approximately 10% of total cellular protein in bacteria overexpressing the *BltD* gene [2]. *SSAT* expression was induced at the upstream *lac* promoter by the addition of 5 mM isopropyl β-D-thiogalactoside. Bacteria were grown at 37 °C under aerobic conditions in Luria–Bertani broth or a minimal medium consisting of Mops medium, as described elsewhere [15,18]. The AcfA mutant phenotype was maintained by the addition of 5 μg/ml ethidium bromide. The CAG2242/SSAT transformants were grown regularly in medium containing tetracycline (20 μg/ml) and ampicillin (50 μg/ml) to maintain the acetylation-deficient phenotype and plasmid, respectively. All polyamines and their acetyl derivatives were from Sigma.

BD170 and AcfA cells in exponential growth (attenuance

Abbreviation used: SSAT, spermidine/spermine N^1-acetyltransferase.

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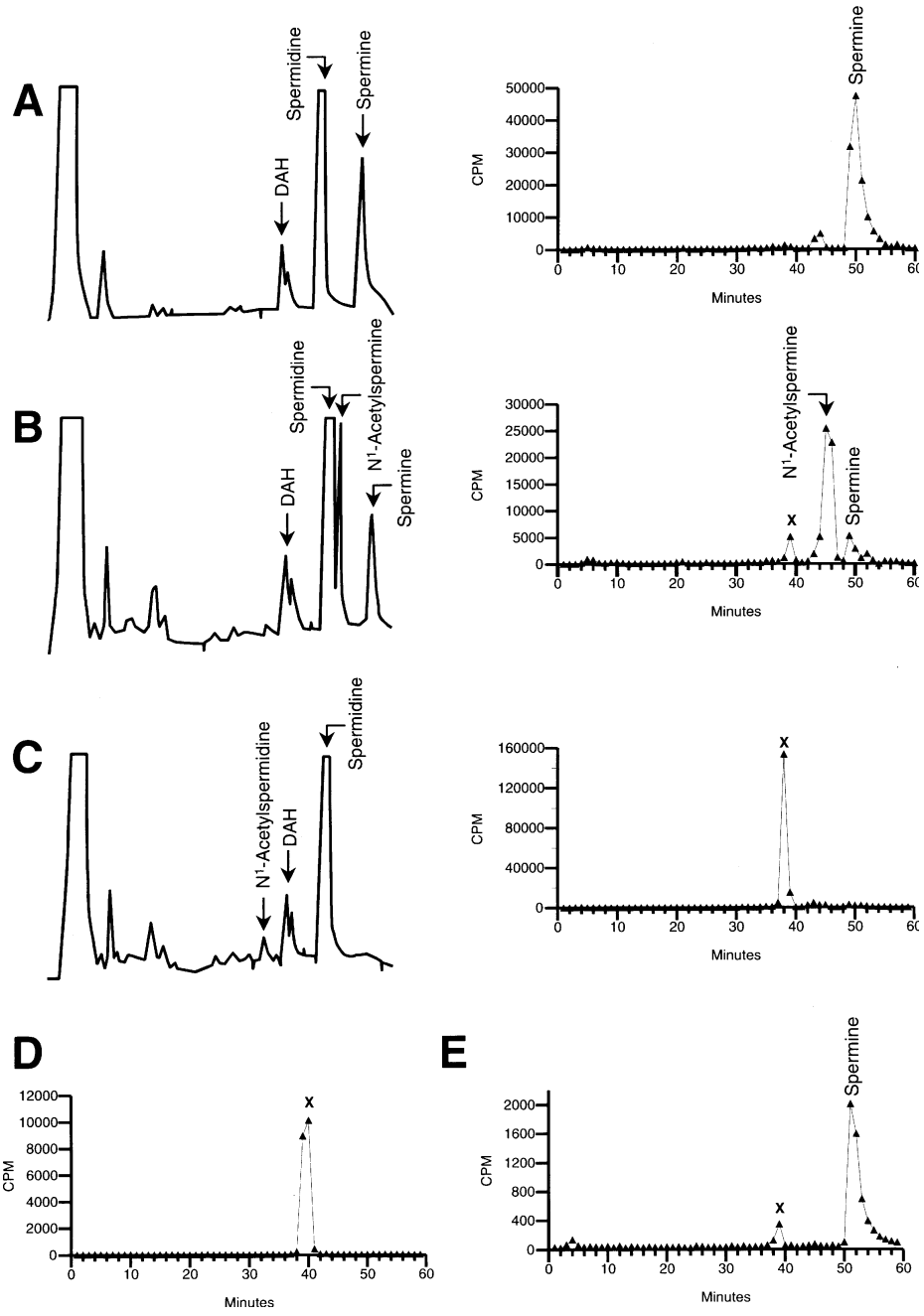

Figure 1 Acetylation of spermine by BltD

Radiolabelled spermine was added to extracts of AcfA cells, which overexpressed *BltD*. Reactions (1000 μl final volume) were carried out at 37 °C in the presence of: 1 μCi [^{14}C]spermine (75 μCi/μmol) and stopped immediately (**A**); 1 μCi [^{14}C]spermine for 1 h (**B**); or 1 μCi [^{14}C]spermine with 200 μM acetyl CoA for 1 h (**C**). (**D**) and (**E**) represent repeat HPLC separations of the isolated reaction product before (**D**) and after (**E**) hydrolysis in 6 M HCl for 18 h at 118 °C. The left-hand panels in (**A**), (**B**) and (**C**) represent detection of HPLC-separated amines by post-column derivatization methods, whereas the right-hand panels of (**A**), (**B**) and (**C**), and panels (**D**) and (**E**), show detection of radiolabel in HPLC fractions. Results shown are for 100-μl injections of perchloric acid extracts (95% lysates). Specific absorbance for spermidine was greater than for spermine. The results in (**A**) indicate that the spermine concentration was $\approx$ 6 μM, whereas the spermidine concentration was $\approx$ 10 μM, based on comparison with authentic standards. The peaks marked X are discussed in the text. DAH, diaminoheptane.

values at 540 nm, $\approx$ 1.2) were pelleted and washed in ice-cold PBS (137 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM K_2HPO_4). All subsequent purification procedures were performed at 4 °C. Cell pellets were then resuspended in 1 × culture volume with 50 mM Tris buffer (pH 7.5)/1 mM dithiothreitol/40 μg/ml PMSF. The resuspension was then treated with 10 μg/ml lysozyme and sonicated to lyse cells.

Acetylation assay

Varying amounts of acetyl-CoA and the individual polyamines were added as indicated prior to thawing cell lysates. Incubations were then performed immediately upon thawing at 37 °C for the duration of the reaction. Adjusting to 0.2 M $HClO_4$ and cooling to 4 °C halted the reactions.

Polyamine analysis

Reaction samples were separated into 0.2 M $HClO_4$-soluble and -insoluble fractions. Polyamine contents were determined from the acid-soluble fraction and protein content was determined from the insoluble fraction. The acid-soluble fraction was separated by reversed-phase ion-pair HPLC as described elsewhere [15,18,20]. Polyamine detection and analysis involved fluorescence measurements of primary amines derivatized after separation by HPLC. The protein-containing pellet was dissolved in 0.5 M NaOH and detection was carried out using the bicinchoninic acid protein assay reagent (Pierce). Radiolabelled polyamines were detected by a direct collection of column eluents without derivatization. Column eluate fractions were diluted in Ecolite scintillation cocktail and counted on a Beckman scintillation counter.

RESULTS

BltD acetylation characteristics

We examined the activity of BltD in extracts of *B. subtilis* overexpressing BltD (AcfA cells). Radiolabelled spermine and unlabelled acetyl-CoA were added to extracts of AcfA cells and incubated for 1 h at 37 °C. Polyamine products in acid-soluble fractions were then analysed as described in the Experimental section (Figure 1). Figure 1(A) shows the result of adding spermine to AcfA extracts and immediately terminating the reaction by the addition of 0.2 M $HClO_4$. Figure 1(B) shows that incubating spermine in this extract for 1 h, without exogenous acetyl-CoA, lead to the formation of N^1-acetylspermine, as detected by both fluorescence and radiolabel assays. Addition of both exogenous acetyl-CoA and spermine to the extract caused formation of N^1-acetylspermidine and complete disappearance of spermine (Figure 1C, left column) and a shift to a shorter retention time of the radiolabelled amine (Figure 1C, right column). The radiolabelled peak (marked 'X') at 39 min did not correspond to any peaks observed by fluorescence detection of post-column derivatives.

No acetylation was detected by either of these methods in extracts of BD170 cells. Since the fluorescence-detection method used here only detects unmodified primary amines, the most likely explanation for the lack of detection of radiolabelled peak X by our fluorescence-detection method is that radiolabelled peak X is the di-acetylated product of spermine. To confirm this possibility, a portion of this material was acid hydrolysed (6 M HCl, 118 °C, 18 h) to non-enzymically remove acetyl groups.

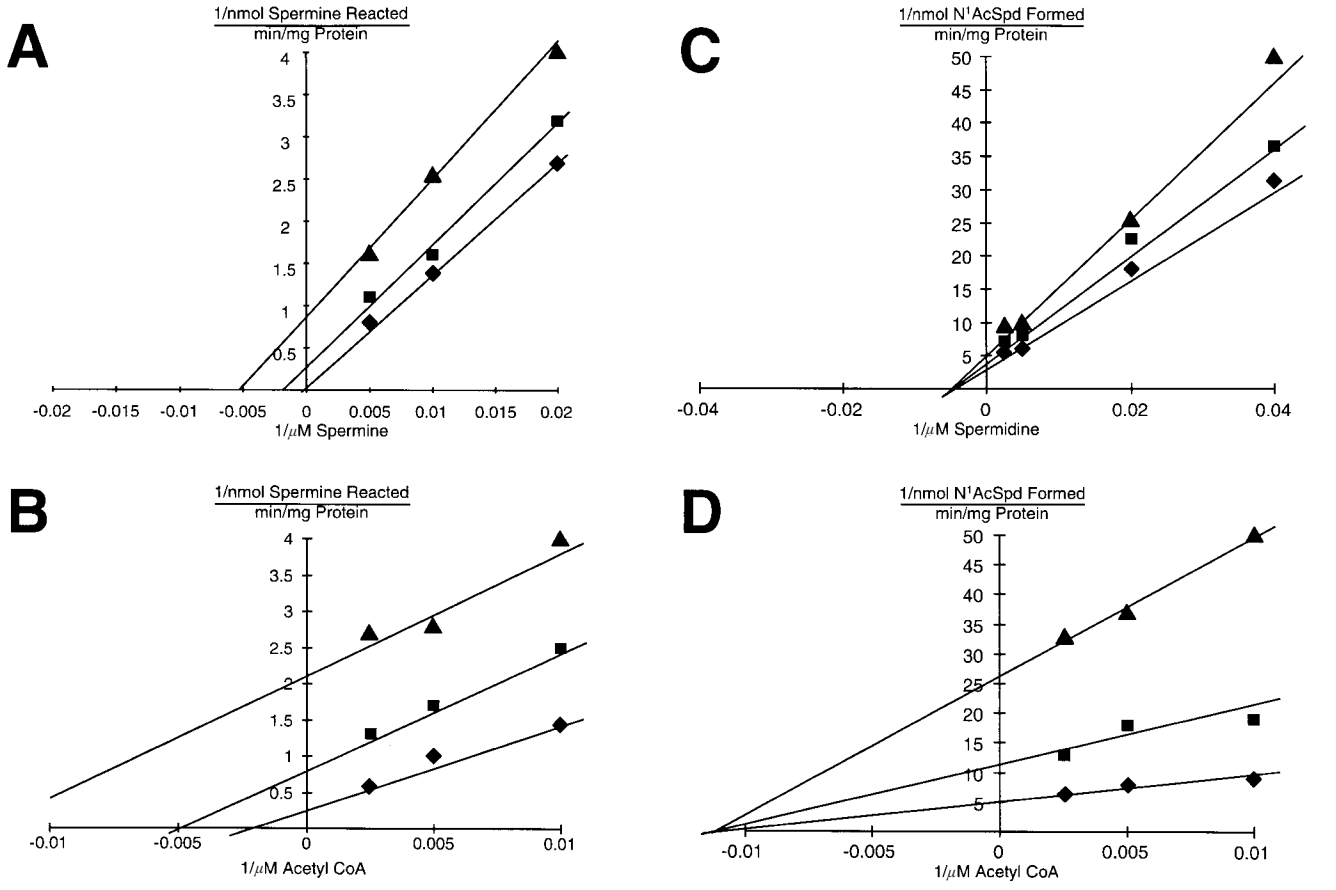

Figure 2 Double-reciprocal plots for the acetylation of spermine and spermidine

Graphs show inverse of the initial reaction velocities of spermine acetylation taken from 10-min reactions (**A**, **B**) and spermidine acetylation taken from 20-min reactions (**C**, **D**) at 37 °C. (**A**) Inverse rates of spermine (nmol) reacted, as a measure of formation of N^1,N^{12}-diacetylspermine, are plotted as a function of 1/μM spermine at 400 (◆), 200 (■) and 100 (▲) μM acetyl-CoA. (**B**) Inverse rates of spermine reacted, as a measure of spermine di-acetylation, versus 1/μM acetyl-CoA at 200 (◆), 100 (■) and 50 (▲) μM spermine. (**C**) Inverse spermidine N^1-acetylation (N^1AcSpd) values are plotted as a function of 1/μM spermidine at 400 (◆), 200 (■) and 100 (▲) μM acetyl CoA. (**D**) Inverse spermidine N^1-acetylation versus 1/μM acetyl-CoA at 200 (◆), 100 (■) and 25 (▲) μM spermidine. Velocities in (**C**) and (**D**) represent nmols of N^1-acetylspermidine produced/min per mg of protein.

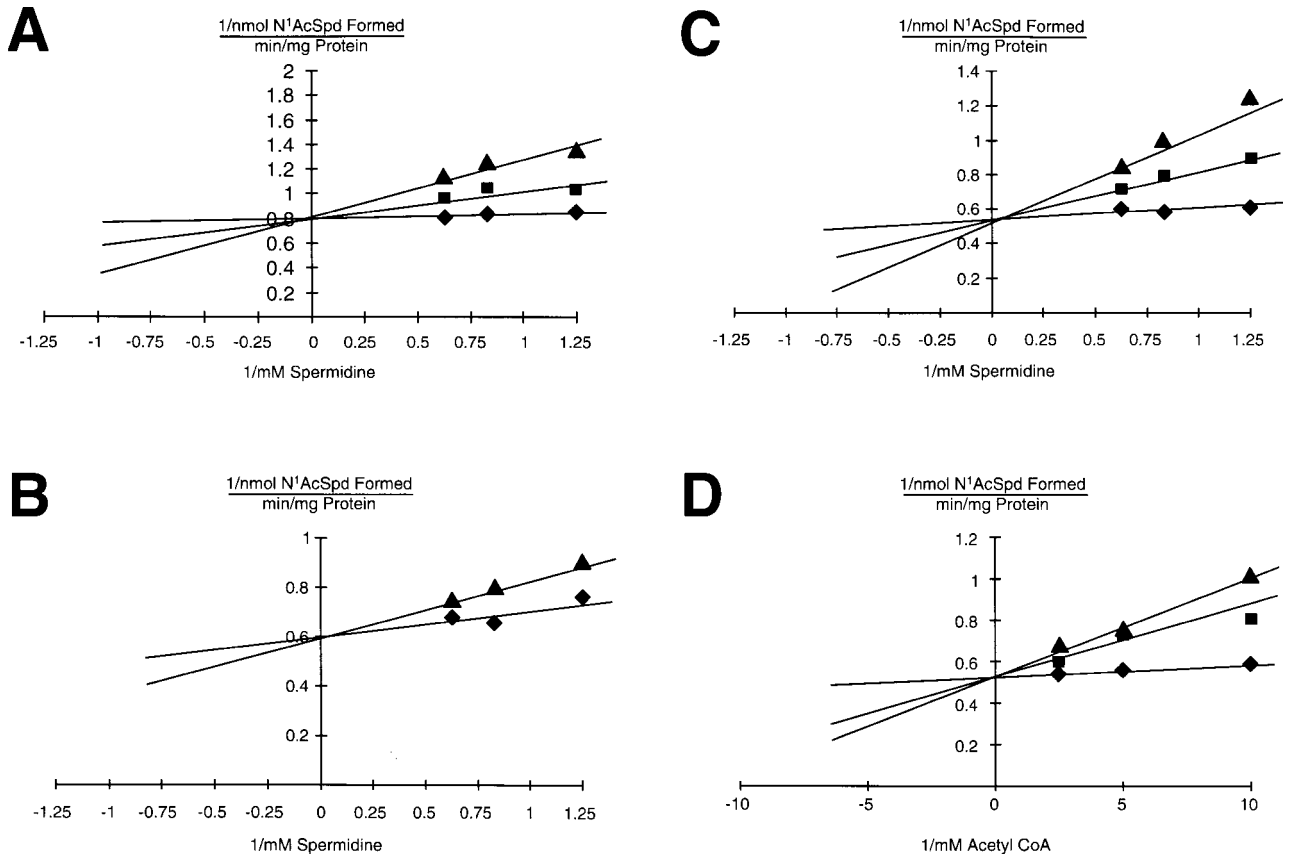

Figure 3 Product inhibition of spermidine acetylation by BltD

Double-reciprocal plots of spermidine acetylation at various inhibitor concentrations. In these experiments, reaction conditions were controlled by keeping either acetyl-CoA (400 μM; **A**, **B** and **C**) or spermidine (200 μM; **D**) concentrations constant and in excess in each case. Velocities represent nmol of N^1-acetylspermidine (N^1AcSpd) produced/min per mg and were plotted as a function of inverse spermidine (**A**, **B** and **C**) or inverse acetyl-CoA (**D**) concentrations. Reactions were conducted at 37 °C for 20 min. Reaction velocities are shown at: (**A**) 1.6 (▲), 0.8 (■) and 0 (◆) mM putrescine; (**B**) 1.6 (▲) and 0 (◆) mM N^8-acetylspermidine; and (**C**, **D**) 800 (▲), 400 (■) and 0 (◆) μM CoA.

Table 1 Apparent kinetic constants for BltD acetyltransferase activity for different substrates and inhibitors

Apparent kinetic constants were determined from data shown in Figures 2 and 3. K_m values for amines were determined at a saturating concentration of acetyl CoA (400 μM). The K_m for acetyl CoA was determined with spermidine (400 μM) as the amine substrate. The units for V_{max} (app) were nmol of amine acetylated per min per mg of protein of bacterial extract.

Compound	Activity	K_m (app) (μM)	K_i (app) (μM)	V_{max}(app)
Spermine	Substrate	$\leqslant$ 67		19.5
N^1-Acetylspermine	Substrate	1200		7.4
Spermidine	Substrate	200		0.6
Acetyl-CoA	Substrate	95		
N^8-Acetylspermidine	Inhibitor		5760	
Putrescine	Inhibitor		248	
CoA	Inhibitor		196	

This treatment causes the reappearance of authentic spermine, as detected both by radiolabelling (Figure 1E) and post-column derivatization methods (results not shown). To confirm that this was indeed a di-acetylated product, a reaction was performed using [^{14}C]acetyl-CoA and N^1-acetylspermine to determine the relative amounts of each molecule. N^1-Acetylspermine also serves as a substrate of BltD and reacted to form peak X (Figure 1C). The ratio of radiolabelled acetate/N^1-acetylspermidine in peak X was 1:1. Radiolabelled peak X in Figure 1 was not detected using our post-column derivatization method (indicating loss of unmodified primary amines), had a ratio of two molecules of acetate per molecule of spermine and yielded authentic spermine upon acid hydrolysis. We therefore conclude that radiolabelled peak X is N^1,N^{12}-diacetylspermine.

Determination of BltD steady-state kinetics

We determined the relative binding affinities for amines that serve as substrates for BltD. Using extracts of BltD over-expressing cells to measure BltD activity *in vitro*, reactions were performed at varying substrate concentrations. Incubation times used were within the linear range for initial product formation. Figure 2 shows double-reciprocal plots for reactions incubated in the presence of various concentrations of a polyamine substrate, either spermine or spermidine as indicated, and acetyl CoA. When spermine was used as the polyamine substrate (Figures 2A and 2B), the double-reciprocal plots showed alterations in intercepts with constant slope when the concentration of either substrate was varied. In these panels, velocity data are plotted as 1/nmol of spermine reacted per min per mg of protein. These data were corroborated by radiolabel detection of N^1-acetyl-

spermine and N^1,N^{12}-diacetylspermine. Due to the complex nature of the di-acetylation reaction, an accurate estimation of the Michaelis–Menten binding affinity, K_m, could not be given. At the highest substrate concentrations used, the K_m (assoc) was determined as 67 μM. Since linearity is obtained, it is therefore reasonable to assume that the K_m (app) for spermine is less than 67 μM.

When spermidine was used as the polyamine substrate (Figures 2C and 2D), the slopes of the double-reciprocal plots were not constant, as was the case with spermine. In these panels, velocity is plotted as 1/nmol of N^1-acetylspermidine produced per min per mg of protein. We were unable to detect the formation of either N^8-acetylspermidine or N^1,N^8-diacetylspermidine when spermidine served as the polyamine substrate for BltD. Interpretations of these double-reciprocal plots are presented in the Discussion section.

Product-inhibition studies

Due to the complexity of the di-acetylation reaction, we chose to focus on the single acetylation reaction of the less-preferred polyamine substrate spermidine. Inhibitors used were not substrates for acetylation by BltD. Reactions were performed with varying concentrations of each inhibitor while holding one substrate (spermidine or acetyl-CoA) constant and varying the concentration of the other substrate. Figures 3(A) and 3(B) show double-reciprocal plots in which acetyl-CoA was held at constant levels and spermidine was varied. Each line represents different concentrations of putrescine (Figure 3A) and N^8-acetylspermidine (Figure 3B).

Results show a series of lines with alterations in slope but common *y*-axis-intercept values, consistent with competitive-type inhibition [21,22]. The product-inhibition patterns for CoA were then investigated (Figures 3C and 3D). Figure 3(C) shows a double-reciprocal plot that represents reactions at various CoA concentrations while spermidine was varied and acetyl-CoA kept constant. Figure 3(D) represents reactions similar to those shown in Figure 3(C), but with varying acetyl-CoA and constant spermidine. Both plots show variations in slope with common intercept values, consistent with competitive inhibition [21,22].

Table 1 summarizes the quantitative results of the cell-free studies, which were qualitatively confirmed in intact cells. Spermine is the preferred substrate for BltD, with a K_m (app) of $\leqslant$ 67 μM. Both mono- and di-acetylated spermine are products of this reaction, depending on the concentration of acetyl CoA. Spermidine and N^1-acetylspermine are also substrates of BltD, with K_m (app) values of 200 and 1200 μM, respectively (results for N^1-acetylspermine not shown). Acetyl-CoA is the acetyl-group donor for this reaction, with a K_m (app) of 95 μM. This value was determined using spermidine as the polyamine substrate, since spermidine is the major polyamine in *B. subtilis*. A variety of polyamines and acetyl derivatives were evaluated as potential substrates for BltD, both in intact *B. subtilis* and in extracts of AcfA cells overexpressing BltD. The diamines 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diamino-pentane (cadaverine), 1,7-diaminoheptane, 1,8-diamino-octane, 1,12-diaminododecane and N^8-acetylspermidine were not substrates for BltD, either in intact cells or in cell extracts (results not shown).

DISCUSSION

Previous work has shown that *BltD* shares sequence similarities with genes encoding a variety of acetyltransferases [1] and that overexpression of *BltD* causes an increase in the accumulation of N^1-acetylspermidine and spermine in both *B. subtilis* and *E. coli* [2]. Here we have characterized the polyamine-acetylating activity of BltD in extracts of bacteria overexpressing *BltD*. Several important features of this system facilitated our analyses. BltD was highly stable both at 37 and -80 °C. Polyamine and acetyl-CoA concentrations added to these extracts (up to 400 μM) were well in excess ($>$ 25 times) of endogenous concentrations (see Figure 1 legend for details). No polyamine oxidase activity was detectable in the extracts. In addition, substrate affinities for the human SSAT measured in this system were similar to those reported by others [23] using the purified enzyme protein (results not shown). Thus the substrate affinities measured using this system can be considered to be relatively accurate approximations of actual values. Maximal velocity values, on the other hand, are normalized to the amount of cellular protein for each sample. Here we used a cell-lysate system in which BltD, although highly expressed, accounted for only a fraction ($\approx 10\,\%$) of the total protein content [2]. Therefore, these values can be used to qualitatively compare substrates, but cannot be used to quantify actual enzyme activity.

Levels of acetylated spermidine were very low in these extracts. Under the growth conditions used here, AcfA cells export much of the acetylated spermidine produced (results not shown). Even considering export, spermine was clearly acetylated to a greater degree than spermidine, even though the K_m (app) values were not dramatically different ($\leqslant$ 67 versus 200 μM for spermine versus spermidine). This could indicate that our estimate of K_m(app) for spermine was high, or that other parameters including reaction velocity or substrate localization could be important factors in this reaction.

All product-inhibition studies demonstrated a competitive type of product inhibition, suggesting that in each scenario the inhibitor associates with either the same form of the enzyme or a form that is separated by a series of reversible steps [21,22]. In addition, linearity was seen in all primary and secondary plots generated. This argues against the possibility of abortive complex formation or multiple forms of the bound enzyme. These results imply a random type of substrate addition, as is seen with the human liver L-glutamate *N*-acetyltransferase [24]. Since an ordered type of substrate-addition mechanism demands the existence of different forms of the enzyme (thus determining the order), such a mechanism cannot be the case here. Ordered additions such as is seen with SSAT [23] would manifest either non-competitive or mixed inhibition patterns due either to the inhibitor binding a different form of the enzyme or to the presence of non-reversible steps.

Spermine is the preferred substrate for BltD. The evidence for this conclusion is based upon results from experiments with intact cells and cell extracts. BltD has both a higher affinity and maximal velocity of reaction when spermine is used as the substrate. One possible explanation for the increased affinity may be the symmetry of the molecule itself. Spermine has two possible orientations in which it can bind the active site compared with a single orientation for spermidine. This increases the probability of association. The higher velocity of the di-acetylation reaction of spermine is not as simple to explain. Double-reciprocal plots derived from varying concentrations of both substrates, as shown in Figures 2(A) and 2(B), have parallel slopes. This type of behaviour suggests Ping Pong-type molecular kinetics, and implies the existence of a stable intermediate in the reaction [21,22]. In this situation, two types of mechanism are possible. One mechanism could involve two entirely separate acetylation reactions, one in which the mono-acetylated product is completely released and then a second in which the mono-acetylated product is then reacted upon again to produce the di-acetylated

product. An alternative mechanism could involve a reaction in which the mono-acetylated product is not released and is maintained in a stable enzyme–substrate form. Progression would then follow by the addition of acetyl-CoA and subsequent acetylation. If the first of these possibilities were true, di-acetylation of spermine would progress at a rate comparable with the slowest acetylation reaction. Here we find that the rate of the di-acetylation of spermine is higher than that of N^1-acetylspermine acetylation alone. N^1-Acetylspermine is a low-affinity substrate and therefore must not be released, but remains bound to the enzyme for the second acetylation to occur. Under saturating acetyl-CoA conditions (Figure 1C), this appears to be true. However, under limiting acetyl-CoA conditions (Figure 1B), mono-acetylspermine is released and recovered in the acid-soluble fraction.

Putrescine was found to be a stronger inhibitor than N^8-acetylspermidine. Competitive inhibition suggests that the inhibitor and substrates bind the same form of the enzyme, implying that the diaminobutyl moiety is the domain being recognized. In addition, weaker inhibition by N^8-acetylspermidine suggests that the presence of either a positive charge or a free primary amine is optimal for binding. CoA inhibition patterns showed competitive-type inhibition with respect to the polyamine substrate and acetyl-CoA. This result, in combination with the finding that putrescine and N^8-acetylspermidine are competitive inhibitors, is highly suggestive of a random-type order of substrate addition. We therefore propose a model for BltD acetylation in which the polyamine substrate and acetyl-CoA can add in either order. Isomerization and formation of the acetylated polyamine would then occur and the products would be released. Another feature of this proposed mechanism for acetylation by BltD is the rapid di-acetylation step. The possibility of an artificial elevation in binding affinities and diminished reaction velocities due to endogenous polyamines or acetyl-CoA-binding proteins must be considered. Since kinetic parameters were determined under conditions in which contaminating endogenous polyamines in the cell extracts were present in very low concentrations (< 25 times the concentration of either added substrates or added inhibitors), errors due to endogenous amines can be considered negligible. Definitive determination of enzyme mechanisms and kinetic parameters, however, will require future confirmation using purified BltD protein rather than the simple overexpression system used here. In spite of these shortcomings, the results obtained allow definitive assignment of spermidine and spermine acetyltransferase activities to the *BltD* gene product and useful comparisons with other acetyltransferases.

BltD and SSAT are similar in their mechanism of action in that they both acetylate spermidine and spermine at primary aminopropyl groups, and prefer spermine to mono-acetylspermine as a substrate ([11] and this study). The *E. coli speG* gene product acetylates spermidine at either N^1 or N^8 with apparently equal frequencies [15,16]. Amino acid sequence analysis shows that SSAT and BltD have 51 % similarity and 29 % identity, whereas speG and BltD have 51 % similarity and 24 % identity.

These data confirm and extend the results in our initial report [2] identifying BltD as an SSAT in *B. subtilis*. Current experiments are being carried out that address the physiological conditions that induce expression of the *Blt/BltD* operon, in order that the consequences of co-ordinate expression of a polyamine acetyltransferase (BltD) and a polyamine exporter (Blt) can be understood.

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