Chemical Mechanism and Substrate Specificity of RhlI, an Acylhomoserine Lactone Synthase from *Pseudomonas aeruginosa*†

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ABSTRACT: The enzyme RhlI catalyzes the formation of *N*-butyrylhomoserine lactone from *S*-adenosylmethionine and *N*-butyrylacyl carrier protein. *N*-Butyrylhomoserine lactone serves as a quorum-sensing signal molecule in *Pseudomonas aeruginosa*, and is implicated in the regulation of many processes involved in bacterial virulence and infectivity. The *P. aeruginosa* genome contains three genes encoding acyl carrier proteins. We have cloned all three genes, expressed the acyl carrier proteins, and characterized each as a substrate for RhlI. A continuous, spectrophotometric assay was developed to facilitate kinetic and mechanistic studies of RhlI. Acp1, which has not been characterized previously, was a good substrate for RhlI, with a K_m of 7 μM; the reaction proceeded with a k_{cat} value of 0.35 s^{-1}. AcpP, which supports fatty acid biosynthesis, was also a good substrate in the RhlI reaction, where k_{cat} was 0.46 s^{-1}, and the K_m for AcpP was 6 μM. The third acyl carrier protein, Acp3, was a poor substrate for RhlI, with a K_m of 280 μM; k_{cat} was 0.03 s^{-1}. Taken together with microarray data from the literature which show that expression of the gene encoding Acp1 is under the control of the quorum-sensing system, our data suggest that Acp1 is likely to be the substrate for RhlI in vivo. Isotope labeling studies were conducted to investigate the chemical mechanism of the RhlI-catalyzed lactonization reaction. Solvent deuterons were not incorporated into product, which implicates a direct attack mechanism in which the carboxylate oxygen of the presumptive *N*-butyryl-SAM intermediate attacks the methylene carbon adjacent to the sulfonium ion. Alternative mechanisms, in which *N*-butyrylvinylglycine is formed via elimination of methylthioadenosine, were ruled out on the basis of the observation that RhlI failed to convert authentic *N*-butyrylvinylglycine to *N*-butyryl-L-homoserine lactone.

Cell-to-cell communication in bacteria has recently begun to be appreciated as a common, perhaps universal phenomenon that mediates diverse responses and developmental events (*1*). In *Pseudomonas aeruginosa*, an opportunistic pathogen that causes persistent, often life-threatening infections, cell-to-cell communication, often referred to as quorum-sensing, is involved in the production of virulence factors and the conversion from planktonic growth into a biofilm (*2*). Several different signaling molecules have been identified, but in *P. aeruginosa* the best characterized signaling systems are those that use acylhomoserine lactones.

The Rhl signaling system, which derives its name from its role in rhamnolipid biosynthesis, responds to *N*-butyryl-L-homoserine lactone (C4-HSL[1]), which binds to the receptor RhlR and is produced in the reaction catalyzed by RhlI. The Las signaling system comprises the receptor LasR, which binds *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and LasI, which is the synthase that catalyzes formation of 3-oxo-C12-HSL. The Las and Rhl systems are hierarchical, in that activation of LasR leads to production of RhlR. Microarray studies have identified 353 genes in *P. aeruginosa* that are regulated by 3-oxo-C12-HSL or C4-HSL, many of which are involved in production of virulence factors or secondary metabolites (*3*). Interference with quorum-sensing systems is gaining attention as a means to treat *P. aeruginosa* infections, because it has the potential advantage of decreasing virulence without being bactericidal, thus diminishing the likelihood of resistance arising in response to treatment (*4*).

The acylhomoserine lactones are produced from *S*-adenosylmethionine and acylated acyl carrier protein. In the RhlI reaction, *N*-butyryl-ACP is the acyl group donor, and the products of the reaction are C4-HSL, methylthioadenosine, and apo-ACP (Scheme 1). The kinetic mechanism of the RhlI reaction has been determined (*5*), and random and site-directed mutational studies have identified some residues that are critical for activity (*6*). More detailed studies of the chemical mechanism of the reaction have not been performed; the reactions catalyzed by acyl-HSL synthases are intriguing because they represent a novel use of *S*-adenosylmethionine in which it does not serve as a methyl group donor (*1*). The fact that one of the substrates for the acyl-HSL synthase is itself a protein presents interesting questions about where the determinants of specificity lie, in particular in the case of ACPs, which may serve as substrates for more than one enzyme, fulfilling multiple metabolic functions.

The *P. aeruginosa* genome encodes three putative acyl carrier proteins. To date, most studies of the *Pseudomonas* acyl-HSL synthases have been conducted using *Escherichia*

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[1] Abbreviations: C4-HSL, *N*-butyryl-L-homoserine lactone; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; ACP, acyl carrier protein; AHL, acylhomoserine lactone; DCPIP, 2,6-dichlorophenolindophenol.

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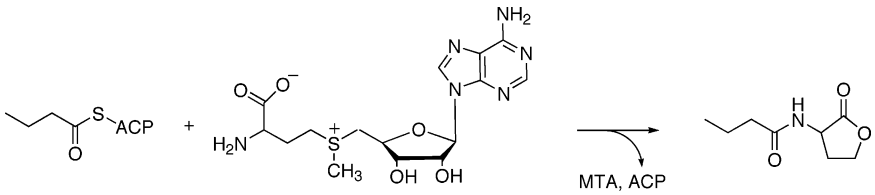
Scheme 1



coli acyl carrier protein, although it has been demonstrated that *P. aeruginosa* AcpP is a substrate for LasI (*7*). Fatty acid biosynthesis relies on acylated AcpP, so if AcpP is the true substrate for the acyl-HSL synthases as well, questions arise about whether quorum-sensing and fatty acid biosynthesis could be controlled in a coordinate manner, and how the synthases could access the pool of fatty acid biosynthetic intermediates. One possibility is that the acyl-HSL synthases have greater specificity for one of the other acyl carrier proteins.

We report here the cloning and expression of all three *P. aeruginosa* acyl carrier proteins, which allows us to explore the acyl carrier protein specificity of RhlI. A colorimetric assay for the RhlI reaction has been developed to facilitate kinetic studies. The rich chemistry available to sulfonium ions suggests several mechanistic possibilities for the RhlI reaction, and we report results that support a direct intermolecular attack mechanism.

MATERIALS AND METHODS

Overexpression and Purification of RhlI. RhlI was purified from an expression system that was the generous gift of Prof. E. Peter Greenberg (University of Iowa). The expression vector, designated pRhlImal, was constructed by subcloning the RhlI coding sequence into the *Eco*RI and *Hin*dIII sites of pMal-c2 (New England Biolabs) (*6*). RhlI is expressed from this vector as a fusion with maltose-binding protein. For expression, XL1-Blue cells were transformed with pRhlImal and grown in LB medium containing 50 μg/mL ampicillin at 30 °C. When the OD at 600 nm reached 0.5, protein expression was induced by the addition of 0.5 mM IPTG. The cells were grown for 3 h and then harvested by centrifugation.

To purify RhlI, 7 g of cell paste was resuspended in 35 mL of 50 mM Tris−HCl, pH 7.5, containing 0.2 M NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK, 0.4 M sucrose, and 2.5% (v/v) glycerol (buffer A). The cells were lysed using a French press operating at a cell pressure of 15000 psi. The cell-free extract was obtained by centrifugation at 31000*g* for 40 min. The nucleic acids were precipitated from the supernatant by the addition of protamine sulfate to a final concentration of 6 mg/g of cell paste. The precipitate was removed by centrifugation at 31000*g* for 20 min and loaded onto a 50 mL amylose column that had been equilibrated in buffer A. The column was washed with 20 bed volumes of buffer A, and RhlI was eluted by washing the column with buffer A supplemented with 10 mM maltose. Column fractions were assayed for RhlI activity and were also analyzed by SDS−PAGE. Active fractions were pooled and concentrated by vacuum dialysis. Purified RhlI was stored in 20% glycerol at −80 °C.

Overexpression and Purification of Holo-ACP Synthase. An *E. coli* holo-ACP synthase expression system was generously provided by Prof. Brian Fox (University of Wisconsin). The coding sequence of the gene for holo-ACP synthase was cloned into the *Nde*I and *Hin*dIII sites of the pET22b expression vector (Novagen). Expression in *E. coli* BL21(DE3)pLysS cells after transformation was achieved by growing the cells in LB Amp medium at 37 °C to an OD at 600 nm of 0.8, and adding IPTG to a final concentration of 0.4 mM. The fermentation temperature was reduced to 30 °C, and the cells were grown for 5 h and then harvested by centrifugation.

To purify holo-ACP synthase, 5 g of cell paste was resuspended in 25 mL of 50 mM potassium phosphate, pH 7.5, containing 0.5 M NaCl and 10 mM imidazole (buffer B). The cells were lysed by passage through a French press operating at a cell pressure of 15000 psi, and the cell debris was removed by centrifugation at 31000*g* for 40 min. Nucleic acids were precipitated from the cell-free extract by the addition of protamine sulfate to a final concentration of 5 mg/g of cell paste. The solution was clarified by centrifugation at 31000*g* for 20 min, and the supernatant was loaded onto a chelating Sepharose fast flow column that had been charged with Ni^{2+} and equilibrated in buffer B. The column was washed with 10 bed volumes of buffer B, and holo-ACP synthase was eluted from the column using a linear gradient from 40 mM imidazole to 0.5 M imidazole in buffer B. Fractions containing holo-ACP synthase were identified by SDS−PAGE, pooled, and stored in 10% glycerol at −80 °C.

PCR Amplification and Cloning of acp Genes. Genomic DNA was isolated from *P. aeruginosa* strain PA01 by the following procedure, which is a modification of published procedures (*8*). Cells were grown in LB medium at 37 °C to mid-log phase, harvested by centrifugation, and washed in 10 mM Tris−HCl, pH 8.0, containing 10 mM EDTA. The cells were resuspended in 10 mM Tris−HCl, pH 8.0, containing 10 mM EDTA, 10% (w/v) Sarkosyl, 5 mg/mL Pronase, and 0.1 mg/mL RNase, and incubated for 30 min at 37 °C. The resulting suspension was extracted with an equal volume of phenol−chloroform; the aqueous phase was recovered, and 0.1 volume of 3 M ammonium acetate was added and mixed well with the solution. Two volumes of 2-propanol was added, the solution was mixed, and genomic DNA was recovered as a pellet following centrifugation for 10 min at 8000*g*. The DNA was dissolved in sterile water and used without further purification for PCR reactions.

Gene-specific primers for each *acp* open reading frame were designed. The *acpP* gene encodes the acyl carrier protein involved in fatty acid biosynthesis; we have designated the other open reading frames *acp1* and *acp3*. PCR amplification reactions were conducted in a volume of 50 μL, and contained 100 ng of genomic DNA, a 200 μM concentration of each dNTP, a 0.2 μM concentration of each primer, 2 U of KlenTaq (Ab Peptides), and the buffer

supplied with the enzyme. Cycling conditions consisted of an initial denaturation step for 2 min at 98 °C, followed by 15 cycles of denaturation for 1 min at 98 °C and annealing and extension for 2.5 min at 70 °C. These cycles were followed by 20 cycles of denaturation for 50 s at 98 °C and annealing and extension for 2.5 min at 70 °C. Following the temperature cycling, Taq DNA polymerase (Amersham Biosciences) was added to each reaction, and the reactions were incubated at 72 °C for 10 min. The amplicons were cloned into the pCR2.1 vector (Invitrogen). Positive clones were digested with *Nde*I and *Bam*HI and subcloned into the pET-14b vector (Novagen), to make constructs that yielded proteins with a His_6-tag at the N-terminus.

Expression of each ACP was achieved by transformation of BL21(DE3)pLysS cells with each expression plasmid; transformed cells were grown to an OD at 600 nm of 0.8. Expression was induced by addition of IPTG to a final concentration of 0.4 mM. After induction, cells expressing Acp1 and AcpP were grown for 3 h at 37 °C; cells expressing Acp3 were grown for 5 h at 30 °C. Purification of each ACP was accomplished using a Ni^{2+}-chelate column, following the same protocol described above for the holo-ACP synthase. Fractions from the metal affinity column were analyzed by SDS−PAGE, and those containing ACP were pooled and stored in 10% glycerol at 4 °C.

Phosphopantetheinylation and Acylation of ACPs. Phosphopantetheinylation of each ACP was performed essentially as described (*9*, *10*). The phosphopantetheinylation reaction contained 50 μM ACP, 100 μM coenzyme A, 10 mM $MgCl_2$, and 4 μM holo-ACP synthase in 50 mM Tris−HCl, pH 8.8. The reaction was gently stirred at 25 °C for 6 h for Acp1 and AcpP, and overnight for Acp3. The reaction was monitored by electrophoresis using native PAGE with 20% gels (*11*, *12*).

Each holo-ACP was acylated chemically using *N*-butyrylimidazole. Synthesis of *N*-butyrylimidazole and acylation of the ACPs were performed as described (*11*).

Synthesis of N-Butyrylvinylglycine The potential intermediate *N*-butyrylvinylglycine was synthesized by acylation of vinylglycine. Butyryl-*O*-succinimide (*13*) (22.2 mg, 0.12 mmol) was dissolved in 1.2 mL of THF and added to a solution of vinylglycine (purchased from Sigma) (10 mg, 0.1 mmol) dissolved in 1 mL of water containing 7.6 mg of $NaHCO_3$. The reaction was stirred at room temperature for 16 h and then acidified to pH 2 with HCl. The solution was extracted with ethyl acetate, and the organic layer was dried over $MgSO_4$. The solvent was removed by rotary evaporation to yield 36 mg of crude product. Analysis by GC/MS revealed that the product contained a small amount of unreacted butyryl-*O*-succinimide, but the crude product was used for experiments with RhlI without further purification.

Assay of RhlI. The enzymatic reaction catalyzed by RhlI was monitored using a colorimetric assay that is sensitive to the free thiol generated upon transfer of the acyl group from either butyryl-CoA or butyryl-ACP. A typical reaction contained 0.1 mM butyryl-CoA, 0.1 mM *S*-adenosylmethionine, and 0.1 mM DCPIP in 50 mM HEPES, pH 7.2. Reactions were initiated by addition of RhlI; the thiol-dependent reduction of DCPIP was monitored at 600 nm (ϵ = 21000 M^{-1} cm^{-1}).

The pH dependence of the RhlI reaction was determined using the following buffers at 50 mM: pH 6.0−6.4, MES; pH 6.4−7.9, HEPES; pH 7.9−8.9, Tris; pH 8.9−9.3, CAPSO. The kinetic parameters determined at each pH were fitted to eq 1, where Y is either V or V/K, C is the pH-independent value of the parameter, and pK_1 and pK_2 are the pK values determined by the kinetic data.

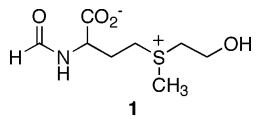
$$Y = \frac{C}{1 + 10^{pK_1 - pH} + 10^{pH - pK_2}} \quad (1)$$

Deuterium Incorporation Studies. The RhlI reaction was run in D_2O to determine whether solvent deuterons were incorporated into the product. In a 1 mL reaction containing 80% (v/v) D_2O, 1.6 mg of RhlI was incubated with 1 mM butyryl-CoA and 1 mM *S*-adenosylmethionine in 50 mM HEPES, pD 7.2. Assay by DCPIP established that the reaction was 85% complete after 12 h. The reaction mixture was extracted with five 2 mL aliquots of ethyl acetate, and the organic fractions were pooled and concentrated to 0.2 mL by rotary evaporation. A sample was subjected to GC/MS analysis using a gas chromatograph (Agilent Technologies, model 6869) equipped with an EI MSD (Agilent Technologies, model 5973N).

Reaction with N-Butyrylvinylglycine. RhlI was incubated with 0.8 mM *N*-butyrylvinylglycine in 50 mM HEPES, pH 7.25, in a total volume of 2 mL. Two samples were prepared, one containing only *N*-butyrylvinylglycine, and the other containing *N*-butyrylvinylglycine and 0.8 mM Acp1. A positive control which contained 1 mM butyryl-CoA and 1 mM SAM was also prepared.

To each sample was added 4.9 mg of purified RhlI; the samples were allowed to incubate for 8 h at 37 °C, and were then extracted four times with two volumes of ethyl acetate. The extracts were pooled and concentrated to ~1 mL by rotary evaporation, and analyzed by GC/MS as described above.

Calculations. Density functional calculations were performed to evaluate the mechanism of the lactonization reaction catalyzed by RhlI. To reduce the computational demands, calculations were conducted on *N*-formyl-*S*-2-hydroxyethylmethionine (**1**), a simplified analogue of *N*-



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butyryl-*S*-adenosylmethionine, and the corresponding transition state and product structures. The structures were energy minimized using the B3LYP hybrid functional and 6-31+G* basis set in the Gaussian 03 program (*14*). Frequency calculations were performed to confirm that the optimized structures lay at energy minima. The calculated structure for the transition state was verified to lie at a saddle point on the energy hypersurface by frequency calculations that showed a single vibrational mode with a negative frequency.

RESULTS

Cloning and Expression of P. aeruginosa ACPs. The *P. aeruginosa* genome contains three open reading frames that encode known or putative acyl carrier proteins. The *acpP* gene, which is involved in fatty acid biosynthesis, is found at locus PA2966; two other open reading frames have been annotated as acyl carrier proteins, one at PA1869, which we

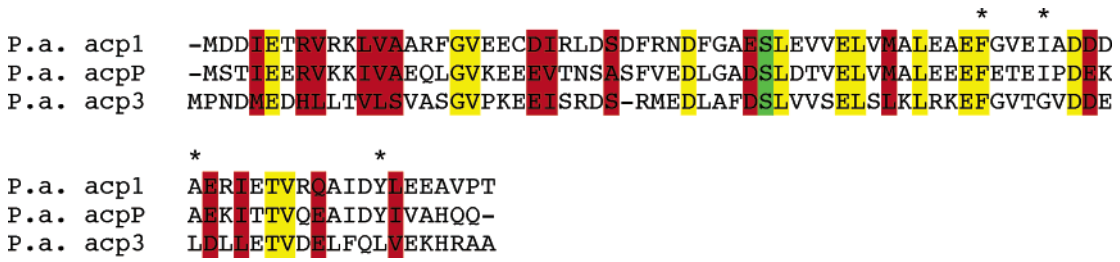


FIGURE 1: Alignment of the primary sequences of the three acyl carrier proteins in *P. aeruginosa*. Residues that are conserved are highlighted in yellow, and similar residues are highlighted in red. The serine residue that is presumed to be phosphopantetheinylated is highlighted in green. The asterisks indicate residues that are conserved among Acp1, *P. aeruginosa* AcpP, and *E. coli* AcpP, which are believed to interact with the acyl chain in acylated ACP.

Table 1: Kinetic Parameters for RhlI-Catalyzed Acylhomoserine Lactone Formation

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Acp1	0.35 ± 0.02	7.4 ± 1.2	0.047 ± 0.008
AcpP	0.46 ± 0.01	5.9 ± 0.4	0.078 ± 0.006
Acp3	0.0264 ± 0.0004	283 ± 9	$(9.3 \pm 0.3) \times 10^{-5}$
butyryl-CoA	0.050 ± 0.002	200 ± 22	$(2.5 \pm 0.3) \times 10^{-4}$

have designated *acp1*, and another at PA3334, which we have designated *acp3*. All three genes were successfully amplified from *P. aeruginosa* PAO1 genomic DNA, and the encoded proteins were expressed and purified as His_6-tagged fusions. The deduced amino acid sequences of the three ACPs are compared in Figure 1. The pairwise identities of the *P. aeruginosa* ACPs were as follows: Acp1 and AcpP, 49%; Acp1 and Acp3, 30%; AcpP and Acp3, 28%. The identities with *E. coli* AcpP were 45%, 87%, and 30%, for *P. aeruginosa* Acp1, AcpP, and Acp3, respectively.

Phosphopantetheinylation and Acylation of P. aeruginosa ACPs. All three acyl carrier proteins were successfully phosphopantetheinylated by *E. coli* holo-ACP synthase. We did not determine kinetic parameters for these reactions, but qualitatively, it was clear that Acp1 and AcpP were better substrates for the holo-ACP synthase than Acp3. Phosphopantetheinylation of Acp1 and AcpP was complete after 6 h, but Acp3 required overnight incubation. The chemical method for acylation of the phosphopantetheinylated acyl carrier proteins was satisfactory for all three proteins.

RhlI Assay and Acyl Carrier Protein Specificity. The colorimetric assay for RhlI activity, which is based on the thiol-dependent reduction of DCPIP, proved to be convenient and robust. Product formation was confirmed by HPLC, GC/MS, and ^1H NMR analysis of the reaction. The assay was applicable both to the reaction using acylated acyl carrier protein and to the reaction using the surrogate substrate, butyryl-CoA.

The steady-state kinetic parameters describing the RhlI reaction with each of the acylated acyl carrier proteins are summarized in Table 1. It is evident that Acp1 and AcpP are good substrates for RhlI. However, Acp3 is a much poorer substrate. Its K_m is 50-fold higher than the K_m values of Acp1 and AcpP, and k_{cat} in the presence of Acp3 is 20-fold slower than the reactions with Acp1 and AcpP. Quantitation of the specificity by comparing values of k_{cat}/K_m reveals that Acp1 and AcpP are equally good substrates for RhlI, but RhlI shows 1000-fold lower specificity toward Acp3. The kinetic parameters that we determined for butyryl-CoA compare favorably with those reported by Parsek et al.

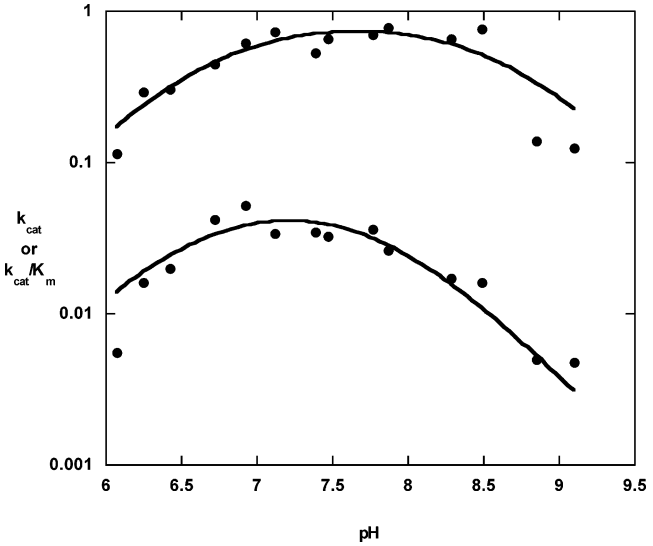


FIGURE 2: Kinetic parameters k_{cat} (○) and k_{cat}/K_m (●) for the RhlI reaction as a function of pH. The reactions were conducted as described in the text, using Acp1 as the butyryl group donor. The lines show the fit to eq 1.

(*5*) ($k_{cat} = 0.03\ s^{-1}$; $K_m = 230\ \mu M$), which provides validation of the DCPIP assay, and also indicates that the maltose-binding domain that is fused to RhlI does not affect its kinetic properties.

pH Studies. The pH dependence of the kinetic parameters for the RhlI reaction using Acp1 was determined. No buffer-specific effects were observed. Both k_{cat} and k_{cat}/K_m described bell-shaped curves with one ionization on the acidic side and one ionization on the basic side (Figure 2). The k_{cat} profile defined pK values of 6.7 ± 0.2 and 8.6 ± 0.2, and the k_{cat}/K_m profile defined pK values of 6.6 ± 0.3 and 7.8 ± 0.3.

Deuterium Labeling Studies. The RhlI reaction was conducted in D_2O to determine if solvent deuterons were incorporated into the product acylhomoserine lactone during the reaction. A sample of authentic *N*-butyryl-L-homoserine lactone was characterized by an M^+ ion of m/z 171.1. The C4-HSL isolated from the RhlI reaction mixture yielded an identical mass spectrum, indicating that no solvent deuterons were incorporated into the product.

Reaction with N-Butyrylvinylglycine. No C4-HSL could be detected by GC/MS upon incubation of RhlI with *N*-butyrylvinylglycine. A similar reaction which contained ACP in addition to *N*-butyrylvinylglycine also did not yield product. A reaction mixture that contained the substrates butyryl-CoA and SAM, which was incubated with RhlI under conditions identical to those used for the reactions with

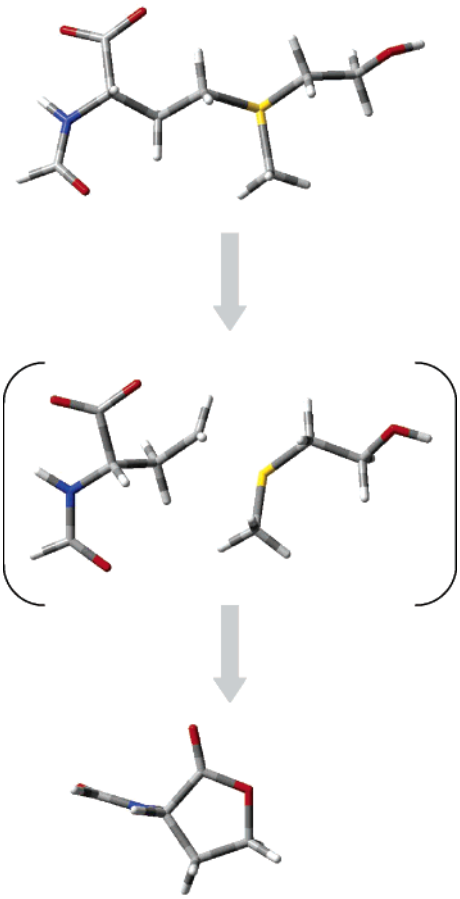


FIGURE 3: Calculated structures for analogues of the RhlI reaction substrate, product, and transition state.

N-butyrylvinylglycine as a positive control, generated C4-HSL that was detected as product eluting from the GC column at 11.07 min with an M^+ ion of *m*/*z* 171.1.

Density Functional Calculations. The optimized structures for analogues of the RhlI reactant, product, and transition state for the cyclization reaction are shown in Figure 3. Consistent with the results of model studies, it appears that nucleophilic attack on the methylene carbon adjacent to the sulfonium center occurs when the attacking atom and leaving group are almost 180° apart. In the substrate structure, the angle among the carboxylate oxygen, the methylene carbon, and the sulfur atom is 128.2°, and the angle increases to 171.0° at the transition state. In the reactant, the distance from the carboxylate oxygen to the methylene carbon is 2.787 Å; at the transition state that distance decreases to 2.287 Å. The C−S bond length is 1.833 Å in the reactant, and the distance between those atoms increases to 2.287 Å at the transition state.

DISCUSSION

Acyl Carrier Protein Specificity. Acylhomoserine lactone formation requires acylated acyl carrier protein as one of the substrates for the synthase reaction; the *P. aeruginosa* genome encodes three acyl carrier proteins, and the possibility that one of them may be a preferred substrate for RhlI has not been addressed. It is known that AcpP is utilized during fatty acid biosynthesis, and if it is also the substrate for RhlI in vivo, interesting questions are raised about how the acylated acyl carrier protein is directed to AHL synthesis or fatty acid synthesis. By considering kinetic data obtained in vitro with purified ACPs and RhlI, and microarray data, which reveal which genes are under the control of quorum-sensing systems, insight can be gained into this issue.

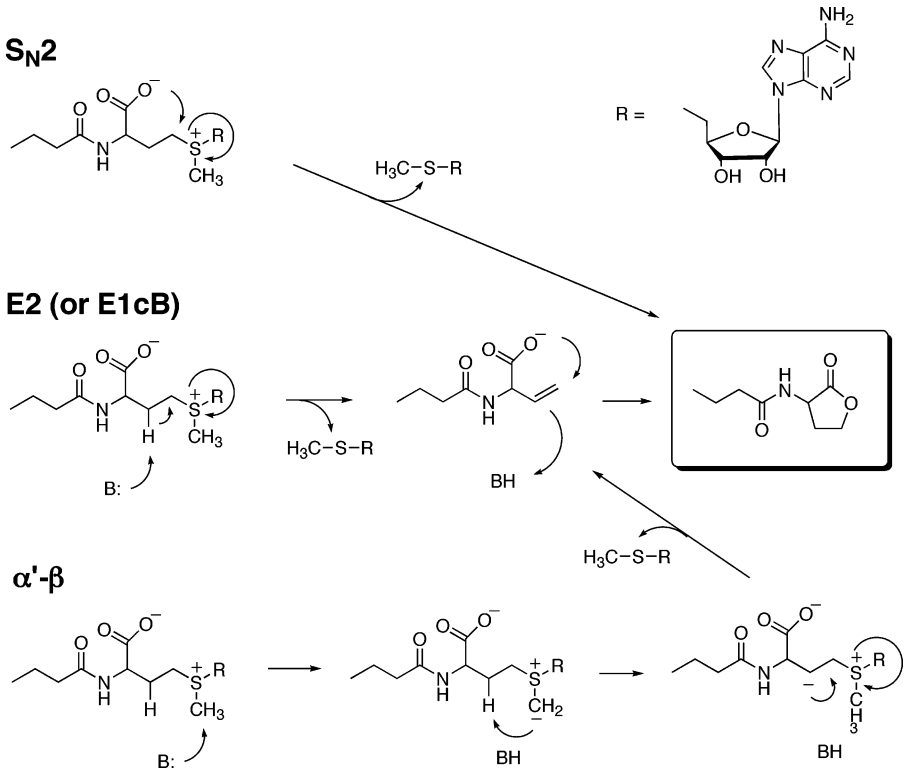
The deduced amino acid sequences of the three ACPs found in *P. aeruginosa* are compared in Figure 1. Acp1 and AcpP are more closely related to each other than either is to Acp3. *P. aeruginosa* AcpP is highly homologous with *E. coli* AcpP (87% identity), so it is not surprising that earlier studies of RhlI conducted with *E. coli* AcpP were successful. Studies of *E. coli* AcpP have identified several amino acid residues that are critical for function, including F50, I54, A59, and Y71 (*E. coli* numbering), which are indicated with asterisks in Figure 1 (*15*). NMR studies have suggested that these residues interact with the acyl chain of acylated ACP (*16*, *17*). All four residues are conserved in *P. aeruginosa* Acp1 and AcpP. Only one of these residues is conserved in Acp3.

The kinetic parameters obtained with all three ACPs in the RhlI reaction are summarized in Table 1. The availability of a continuous colorimetric assay greatly simplified the collection of these data. It is evident that Acp1 and AcpP are good substrates for RhlI, while Acp3 is a poor substrate, with a lower value for k_{cat}/K_m than even the surrogate substrate butyryl-CoA. However, only Acp1 and Acp3 are under the control of quorum-sensing systems. In one microarray study, gene expression in bacteria lacking the AHL synthases was studied, and the gene encoding Acp1 was upregulated 8-fold by the addition of 3-oxo-C12-HSL and 41-fold by the addition of both C4-HSL and 3-oxo-C12-HSL. In wild-type bacteria the Acp1 gene was upregulated 340-fold compared to the expression in the mutant lacking the AHL synthases (*3*). An independent study determined that *acp1* transcription was upregulated 79-fold in response to 3-oxo-C12-HSL and C4-HSL, and a *las* box was identified upstream of the gene encoding Acp1 (*18*). The *las* box is a regulatory element that is proposed to provide the binding site for the complex of the quorum-sensing signal and its cognate receptor. Both microarray studies also found that the gene encoding Acp3 was induced by quorum-sensing signals, although the effect was not as dramatic as that seen for the Acp1 gene. In neither study was induction of the *acpP* gene by quorum-sensing signals observed.

In a third microarray study, it was observed that expression of the genes for Acp1 and Acp3 was decreased by a small-molecule disrupter of the Las and Rhl quorum-sensing systems in *P. aeruginosa* (*4*). Like in the microarray studies cited above, no effect on *acpP* was observed. These data are also consistent with the hypothesis that Acp1, not AcpP, is the preferred in vivo substrate for the AHL synthases. It would be interesting at this point to determine the Acp substrate specificity for LasI. Taken together, the kinetic data and the microarray data suggest that Acp1 is the likely in vivo substrate for RhlI. The kinetic data demonstrate that it is a good substrate, and the genomics data suggest that it is present when required for quorum-sensing. Our results pose another interesting question, however, which is how Acp1 is acylated in vivo.

Chemical Mechanism of the RhlI Reaction. RhlI catalyzes two distinct chemical reactions to form the AHL product, acylation of the amino group of *S*-adenosylmethionine, and lactonization to form the ring system of the product. The

Scheme 2



observation that *N*-butyryl-*S*-adenosylmethionine can be converted to product by RhlI suggests that acylation precedes lactonization (*5*). The lactonization reaction is novel in that *S*-adenosylmethionine is not utilized as a methyl group donor; *S*-methylthioadenosine is expelled as the leaving group, rather than *S*-adenosylhomocysteine. The chemical mechanism of this reaction has not been explored. We considered three potential mechanisms, which are illustrated in Scheme 2.

Lactone formation could proceed by direct nucleophilic attack of the carboxyl oxygen on the methylene group that is α to the sulfonium center in a classic S_N2 reaction. By Baldwin's rules for ring-closure reactions, the RhlI reaction would be a favored 5-exo-tet reaction. Enzymatic SAM-dependent methylations are generally believed to proceed via S_N2 mechanisms. It has been suggested that S_N2 reactions at carbon adjacent to a sulfonium ion have a strict geometrical requirement for the nucleophile to approach 180° from the incipient leaving group. Deviations as small as 20° from this ideal geometry prevented reaction in model studies (*19*). Thus, if the RhlI reaction follows this mechanism, one of the functions of the enzyme must be to control the conformation of the reactant appropriately.

An alternative to the direct nucleophilic attack is an elimination reaction, followed by cyclization. Elimination reactions from sulfonium salts can proceed in a concerted fashion (E2), or in a stepwise manner if the carbanion generated by abstraction of the proton β to the sulfonium center has a finite lifetime (E1cB). Extensive model studies, including kinetic isotope effect measurements, have provided detailed descriptions of elimination reactions from sulfonium ions (*20*), but this mechanism does not yet appear to have been characterized in a SAM-dependent biochemical reaction. According to this mechanism, lactone formation would occur by attack of the carboxylate on the vinyl group that forms upon elimination of methylthioadenosine. In this case the ring closure would be a disfavored 5-endo-trig reaction.

A third mechanistic possibility arises because of the facility with which sulfonium ions form ylides. The $\alpha'-\beta$ mechanism illustrated in Scheme 2 has as its first step the formation of an ylide, arising from deprotonation of the methyl group at the sulfonium ion center. Protonation of the methyl anion by abstraction of a proton from the β-methylene then leads to elimination of methylthioadenosine in a manner similar to the elimination reactions described above, and lactone formation would proceed from the vinyl intermediate. Ylide formation in dilute alkaline solutions is observed for sulfonium salts; for example, trimethylsulfonium ion completely exchanges its methyl protons with D_2O within 3 h (*21*). Presumably, proton exchange of the methyl group protons of *S*-adenosylmethionine is similarly facile, although it is difficult to demonstrate because the glycosidic bond hydrolyzes rapidly under alkaline conditions.

The two elimination mechanisms have in common the intermediacy of *N*-butyrylvinylglycine; thus, we sought to determine whether RhlI could catalyze the conversion of synthetic *N*-butyrylvinylglycine to C4-HSL. Overnight incubations of RhlI with the potential intermediate, in either the presence or the absence of acyl carrier protein, failed to generate detectable levels of C4-HSL. This result, albeit a negative one, strongly argues against either elimination mechanism illustrated in Scheme 2.

As a second test to distinguish between the mechanistic possibilities described above, we sought to determine whether solvent deuterons were incorporated into the AHL product when the RhlI reaction was conducted in D_2O. If product formation occurred via the direct attack mechanism, no solvent deuterium should be incorporated into product. The other two mechanisms involve proton transfers, and unless

the abstracted proton was completely shielded, incorporation of solvent deuterium into product would be expected. Mass spectral data and ^{1}H NMR data (not shown) established that C4-HSL produced by RhlI in D_2O does not contain any deuterium atoms. The lack of incorporation of solvent deuterons strongly suggests that the chemical mechanism for lactone formation proceeds by direct nucleophilic attack of the carboxylate oxygen on the methylene carbon that is adjacent to the sulfur atom. In light of Coward's model studies that suggest that this reaction has strict geometrical requirements, as well as the results of the ab initio calculations presented here, it seems likely that one role of the enzyme in this reaction is to control the conformation of the substrate appropriately. An intriguing possibility that we wish to explore is that the acylation of the SAM amino group serves as a trigger for rearrangement of the *N*-butyryl-SAM intermediate to facilitate the lactonization. Our data do not allow us to rule out another mechanistic possibility, namely, that an active site residue acts as a nucleophile to displace methylthioadenosine and form a covalent intermediate. Attack by the carboxylate oxygen to displace the enzyme residue would then yield the observed product. However, we consider a double displacement reaction to be highly unlikely. Displacement reactions adjacent to sulfonium centers require approach of the incoming nucleophile to be nearly collinear with the incipient leaving group, but the proximity of the SAM carboxyl group seems likely to preclude approach by an enzyme nucleophile.

The results of the ab initio calculations are in agreement with the prediction from model studies (*19*) for the conformation of *N*-acyl-SAM that is required for lactonization. The angle defined by the carboxylate oxygen, the methylene carbon adjacent to the sulfonium center, and the sulfur atom is 128.2° in the reactant state, but the three atoms become nearly collinear at the transition state. The distance between the methylene carbon and the sulfonium center suggests a bond order of 0.35 at the transition state, but the distance between the carboxylate oxygen and the methylene carbon yields a bond order of only 0.06. In other words, cleavage of the C−S bond is farther advanced at the transition state than formation of the C−O bond, and the reaction appears to have considerable S_N1 character, which places carbocationic character at the position where bond cleavage occurs. This result is somewhat at odds with the expectation that formation of primary carbocations is unfavorable. Further studies will be required to address whether the gas-phase calculations that were conducted accurately predict the transition-state structure in the enzyme-catalyzed reaction, and whether additional factors, such as the inclusion of counterions in the calculations, affect the results.

Since the lactonization reaction appears to proceed without the involvement of general-acid−base catalysis, the ionizations that appear in the pH profiles probably arise from residues on the enzyme that participate in the acylation reaction. General-base catalysis would be expected to enhance the nucleophilicity of the SAM α-amino group by deprotonation, and the collapse of the tetrahedral intermediate that is formed upon attack of the acylated ACP thioester would be facilitated by general-acid catalysis to protonate the leaving phosphopantetheine thiol functionality. The basic p*K* that is defined by the k_{cat} profile is displaced relative to that which appears in the k_{cat}/K_m profile, which suggests that binding of acylated ACP perturbs the p*K* of the ionizing residue. The fact that the same ionizations appear in the k_{cat} and k_{cat}/K_m profiles suggests that these residues participate in the catalytic reaction, not just binding (*22*).

The crystal structure of the related enzyme EsaI does not reveal obvious candidates for residues that could act as general-acid−base catalysts for the acylation reaction. However, Glu97 in EsaI, which is conserved as Glu101 in RhlI, has been suggested to operate as a general base to deprotonate the substrate amino group through the intermediacy of water molecules (*23*). The E101K RhlI mutant appears to be devoid of activity (*6*).

In summary, we have established that both Acp1 and AcpP are good substrates for the RhlI reaction, but consideration of microarray data which show that transcription of *acp1* is under the control of the quorum-sensing system suggests that Acp1 is most likely to be the in vivo substrate for the RhlI reaction. The chemical mechanism of the RhlI reaction follows Baldwin's rules for ring closure, and is most likely to occur by direct intramolecular nucleophilic attack on the carbon adjacent to the sulfonium ion center. In contrast to SAM-dependent methylation reactions, which are generally considered to follow S_N2 mechanisms, the lactonization that generates the acylhomoserine lactone appears to have considerable S_N1 character. Isotope effect measurements are in progress to determine whether the transition state for the enzyme-catalyzed reaction is similar to that predicted from calculations for the nonenzymatic reaction.

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