Reactivity and Reaction Order in Acylhomoserine Lactone Formation by *Pseudomonas aeruginosa* RhlI[†]

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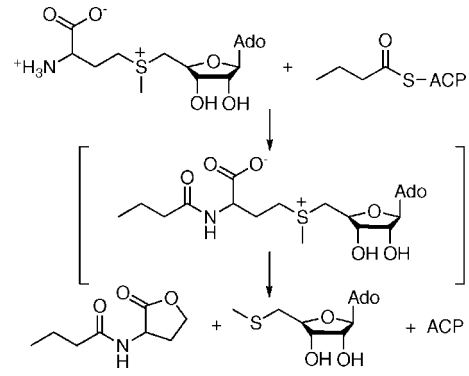
ABSTRACT: The formation of *N*-butyrylhomoserine lactone catalyzed by RhlI has been investigated by transient-state kinetic methods. A single intermediate, assigned to *N*-butyryl-*S*-adenosylmethionine, was observed. Under single-turnover conditions, the intermediate formed with a rate constant of 4.0 ± 0.2 s^{-1} and decayed with a rate constant of 3.7 ± 0.2 s^{-1}. No other intermediates were detected, demonstrating that the RhlI reaction proceeds via acylation of *S*-adenosylmethionine, followed by lactonization. *S*-Adenosylhomocysteine acted as a pseudosubstrate, in that it did not undergo either acylation or lactonization but did induce the deacylation of butyryl-acyl carrier protein. The K_m for *S*-adenosylhomocysteine was approximately 15-fold higher than the K_m for *S*-adenosylmethionine. The reactivities of acylated and unacylated sulfonium ions that were analogues of *S*-adenosylmethionine were investigated by computational methods. The calculations indicated that acylation of the substrate amino group activated the substrate for lactonization, by allowing the carboxyl group oxygen to approach more closely the methylene carbon to which it adds. This observation provides a satisfying chemical rationale for the order of the individual reactions in the catalytic cycle.

Social interactions among bacteria, such as those required for launching an infectious attack or growing in a biofilm, are enabled by small molecule communication signals termed quorum sensing signals. In Gram-negative bacteria, acylhomoserine lactones serve as one class of quorum sensing signal. *Pseudomonas aeruginosa*, an opportunistic human pathogen that poses particular risks for cystic fibrosis patients, utilizes two AHLs,[1] *N*-butyrylhomoserine lactone and *N*-3-oxododecanoylhomoserine lactone. C4-HSL and 3-oxo-C12-HSL bind to their cognate transcription factors to regulate the expression of a wide variety of genes. In fact, more than 350 genes are under the control of the AHL quorum sensing system in *P. aeruginosa* (*1, 2*).

C4-HSL is synthesized from butyryl-acyl carrier protein and *S*-adenosylmethionine in a reaction catalyzed by the enzyme RhlI. Two separate chemical events occur during the reaction, transfer of an acyl group from butyryl-ACP to the amino group of SAM and lactonization, concomitant with expulsion of methylthioadenosine (Scheme 1). The steady-state kinetic mechanism of RhlI has been examined (*3*), as well as the acyl carrier protein specificity and the chemical mechanism of the lactonization reaction (*4*). Initial velocity kinetic experiments in which the substrate concentrations were varied yielded a family of parallel lines, but a ping-pong mechanism was ruled out by product and dead-end inhibition studies (*3*). In addition, a presumptive intermediate in the reaction, *N*-butyryl-*S*-adenosylmethionine, was shown to be chemically competent, although its reaction was slow.

Scheme 1



We report here transient-state kinetic studies to further characterize the nature of the intermediate in the reaction. We see evidence of an obligatory order of chemical events, first acylation and then lactonization. We have also revisited the steady-state kinetics, using the slow substrate butyryl-CoA in place of butyryl-ACP.

MATERIALS AND METHODS

The RhlI used in these studies was a fusion protein with maltose-binding protein and was purified as described previously. The plasmid encoding the fusion protein was the generous gift of E. Peter Greenberg (University of Washington, Seattle, WA). *P. aeruginosa* Acp1 was used in experiments requiring acyl carrier protein. Acp1 was phosphopantetheinylated enzymatically and acylated chemically, as described previously (*4, 5*). Common chemicals and

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[1] Abbreviations: AHL, acylhomoserine lactone; C4-HSL, *N*-butyrylhomoserine lactone; 3-oxo-C12-HSL, *N*-3-oxododecanoylhomoserine lactone; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; ACP, acyl carrier protein.

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biochemicals were obtained from Sigma and Aldrich and were used without further purification.

The preparation of [^{14}C]butyryl-acyl carrier protein was accomplished using [^{14}C]butyryl imidazole. Sodium [1-^{14}C]butyrate (50 μCi, 54 mCi/mmol, Moravek Biochemicals) was converted to the free acid by dilution of the stock solution into 20 mL of 0.1 N HCl. The solution was extracted with six 20 mL aliquots of $CHCl_3$, and the organic extracts were combined and dried over $MgSO_4$, before removal of the solvent under reduced pressure. The [^{14}C]butyric acid was dissolved in 3 mL of tetrahydrofuran, and carbonyldiimidazole (3 mmol, 0.49 g) was added. Unlabeled butyric acid (2 mmol, 0.18 mL) was added, and the solution was heated to reflux for 15 min. The solvent was removed under reduced pressure, and the product [^{14}C]butyrylimidazole was stored, without purification, at −20 °C. Acylation of holo-Acp1 was conducted in the same manner in which unlabeled material was prepared.

Steady-State Kinetic Studies. The RhlI reaction was routinely monitored by coupling the formation of the free sulfhydryl group on either the phosphopantetheine prosthetic group of Acp1 or CoA to the reduction of dichlorophenolindophenol. The free sulfhydryl group forms as a consequence of transfer of an acyl group from either acylated Acp1 or butyryl-CoA. The reduction of DCPIP caused a decrease in the absorbance of the solution at 600 nm ($\epsilon = 21000\ M^{-1}\ cm^{-1}$). Typical assays included 0.1 mM acylated Acp1 or butyryl-CoA, 0.1 mM SAM, and 0.1 mM DCPIP in 50 mM HEPES (pH 7.2). The DCPIP assay was also used to assess whether RhlI catalyzed the acylation of *S*-adenosylhomocysteine. All steady-state kinetic assays were conducted at 25 °C.

Initial velocity kinetic data in which a single substrate was varied were fitted to the Michaelis–Menten equation. The data from the experiment in which SAM and butyryl-CoA were varied were fitted to eq 1 using Grafit.

$$v = \frac{V_{max}[A][B]}{[A][B] + K_a[B] + K_b[A] + K_{i_a}K_b} \quad (1)$$

Rapid-Mixing Chemical Quench Studies. Single-turnover experiments were performed using a KinTek RQF-3 instrument. The reactants and reaction chamber were maintained at 25 °C. One sample loop was loaded with RhlI and [^{14}C-*carboxy*]SAM (5 mCi/mmol, American Radiolabeled Chemicals) and the other with butyryl-Acp1. The reactions were conducted in 50 mM HEPES (pH 7.2), and the concentrations after mixing were 150 μM RhlI, 180 μM SAM, and 67 μM butyryl-Acp1. Before the experiment, an aliquot was removed from the syringe containing the [^{14}C]SAM and its radioactivity was measured by liquid scintillation counting. The catalytic reactions were quenched by the addition of 0.1 N HCl after periods of time ranging from 0.005 to 50 s. An aliquot was removed from each sample and counted, to determine the dilution that occurred in the experiment. The quenched samples were then extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate solutions, which contained *N*-butyrylhomoserine lactone, and the aqueous layer, which contained residual SAM and the reaction intermediate (vide infra), were analyzed separately by HPLC. The aqueous samples were analyzed with a Thermo Electron Corp. BioBasic-18 C_{18} reverse phase column (250 mm × 4.8 mm, 5 μm particle) using a binary solvent system that combined an ion-pairing mobile phase and an organic mobile phase (*6*). Solvent A consisted of 8 mM sodium octanesulfonate and 50 mM NaH_2PO_4, adjusted to pH 8.0 with H_3PO_4, and solvent B was methanol. The column was equilibrated in 20% solvent B, and after injection of the sample, it was developed with a gradient to 40% solvent B over 40 min at a flow rate of 1 mL/min. The absorption of the effluent at 260 nm was monitored, and the radioactivity was monitored by scintillation counting with a β-RAM model 3 (IN/US Inc.) flow-through radiodetector. The ethyl acetate extracts were analyzed with the same column, equilibrated in 20% methanol, and developed with a gradient to 100% methanol over 30 min.

The molar concentrations of the SAM and reaction intermediate were determined from the radioactivity in the peaks eluting from the HPLC column, the specific radioactivity of the starting material, and the dilution factor. The molar concentration of C4-HSL was also determined from the radioactivity in the peak on the HPLC chromatogram, after correction for the efficiency of the ethyl acetate extraction, which was determined experimentally to be 80%. The reaction terminated when the limiting reagent, butyryl-Acp1, was exhausted. The amount of unreacted SAM that remained was subtracted from the amount measured at each time point before the data were analyzed. The data were then normalized to the concentration of the limiting reagent so that during the course of the reaction the level of SAM varied from 1 to 0 and the level of C4-HSL varied from 0 to 1.

Analysis of the transient-state kinetic data was accomplished using DYNAFIT (*7*). DYNAFIT allows the user to define a model that consists of a sequence of chemical reactions. The program derives the differential equations that describe the time dependence of each chemical species in the model. Data are fitted to the model by determining the values of rate constants that minimize the differences between the experimental and calculated data. The data were fitted to three different models in which the substrate was converted to an intermediate and then to the product. In the simplest model, the elementary steps were irreversible; in the second model, the first step was reversible and the second step was irreversible, and in the third model, both steps were reversible. The *F*-test was used to evaluate whether the inclusion of the reversible steps in the models was justified by the data (*8*). Experiments in which the substrate and enzyme concentrations were varied were not conducted, so the models did not include substrate association steps. Product dissociation steps were not included in the models because free product and enzyme-bound product were not distinguishable in these chemical quench experiments.

A second set of rapid quench experiments was conducted using [^{14}C-*butyryl*]Acp1. The concentrations of the reactants after mixing were 10 μM [^{14}C-*butyryl*]Acp1, 150 μM SAM, and 75 μM RhlI. The raw data were corrected for dilution, normalized to the concentration of the limiting reagent, [^{14}C-*butyryl*]Acp1, and fitted to eq 2.

$$y = \frac{k_1}{k_1 - k_2}(e^{-k_2 t} - e^{-k_1 t}) \quad (2)$$

Calculations. Ab initio calculations were performed for two model compounds, *S*-methylmethionine and *N*-acetyl-

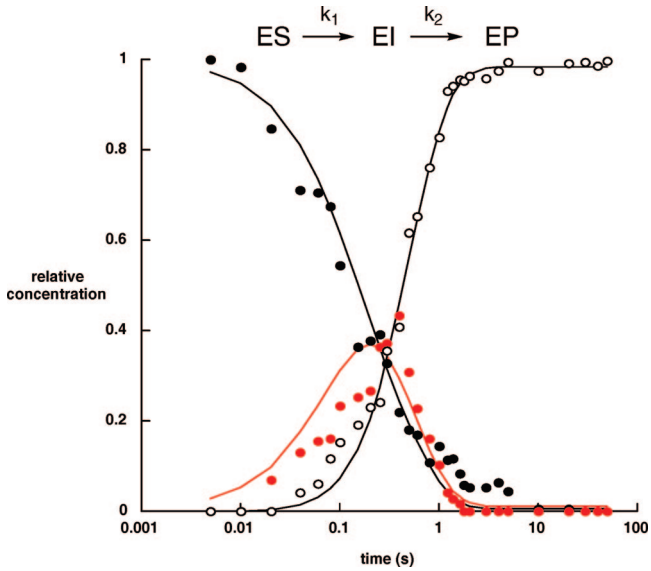

FIGURE 1: Time course for the RhlI reaction under single-turnover conditions. The reaction was conducted with [^{14}C-*carboxy*]SAM under conditions described in the text. The C4-HSL (○) in each quenched sample was extracted with ethyl acetate. Residual SAM (●) and the reaction intermediate (red circles) were separated by HPLC. The experimental data were corrected as described in the text and fitted to the model indicated.

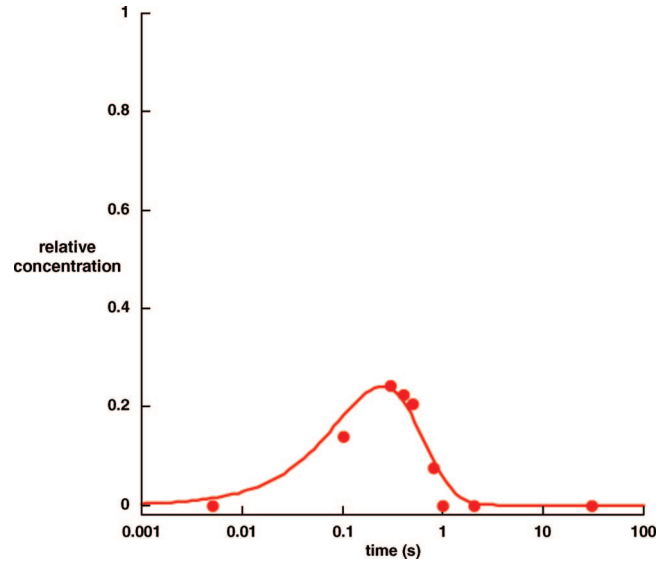

FIGURE 2: Time course using [^{14}C]butyryl-ACP as the substrate for RhlI. The reaction conditions and the conditions for separating the intermediate in the quenched samples are described in the text.

S-methylmethionine. Density functional theory calculations using the B3LYP hybrid functional and the 6–31 + G(d,p) basis set were carried out using Gaussian 03 (*9*). Relaxed potential energy scans were performed for each compound where the orientation of the carboxyl group was rotated through 360°. The alkyl sulfonium chain was locked in an extended conformation appropriate for lactone formation, and the zwitterionic character of *S*-methylmethionine was preserved by constraining the N−H bonds to remain at 1.208 Å.

RESULTS

Under single-turnover conditions, a catalytic intermediate in the RhlI reaction was readily detected (Figure 1). The intermediate did not partition into ethyl acetate, indicating that it is more polar than the product C4-HSL. It was detectable by its absorbance at 260 nm, and the fact that it was radioactive when generated from [^{14}C-*carboxy*]SAM indicated that the amino acid and nucleoside portions of SAM were still covalently linked. When the reaction was conducted using [^{14}C]butyryl-acyl carrier protein, the intermediate was labeled as well. These observations provide very strong evidence that the intermediate is *N*-butyryl-*S*-adenosyl-L-methionine.

The kinetic data were fitted to a two-step, irreversible model (two rate constants), a two-step model in which the first step was reversible (three rate constants), and a fully reversible two-step model (four rate constants). The improvement in the fit with the addition of reversible steps was not significant. The fitted values for rate constants k_1 and k_2 were 4.0 ± 0.2 and 3.7 ± 0.2 s^{-1}, respectively. From steady-state kinetic studies, k_{cat} for the RhlI reaction was found to be 0.35 s^{-1} (*4*), so neither formation nor decomposition of the intermediate is rate-limiting.

The time course for the reaction conducted with [^{14}C]butyryl-ACP and unlabeled SAM was similar to the one observed using [^{14}C-*carboxy*]SAM. A labeled species was detected which appeared with a rate constant of 2.7 ± 0.4 s^{-1} and disappeared with a rate constant of 5.8 ± 0.5 s^{-1}. Within the limitations of the errors in the data introduced by the complicated sample workup, these values are consistent with the assignment of *N*-butyryl-*S*-adenosyl-L-methionine as the intermediate in the reaction (Figure 2).

Initial velocity kinetic studies in which SAM and butyryl-CoA were both varied yielded data that fit a sequential model. The Michaelis constant for SAM was 7 ± 1 μM, and K_{iSAM}, the constant for dissociation of SAM from the RhlI·SAM complex, was 4 ± 2 μM. The K_m for butyryl-CoA was 170 ± 20 μM (Table 1).

SAH was a pseudosubstrate for RhlI; it was neither acylated nor lactonized, but it caused RhlI to catalyze the deacylation of both butyryl-ACP and butyryl-CoA. The K_m for butyryl-ACP was unchanged, regardless of whether SAH or SAM was the other substrate. Similarly, the K_m for butyryl-CoA was the same in the presence of SAH and SAM. The apparent K_m for SAH in the presence of 0.5 mM butyryl-CoA was 340 ± 46 μM, considerably higher than the K_m for SAM under the same conditions (20 ± 8 μM). Evidence for production of *N*-butyryl-SAH was sought using [^{14}C]butyryl-ACP as the cosubstrate, but no acylated SAH was detected, either after extended incubation or as a transient intermediate.

Representative stuctures of energy-minimized conformers of *S*-methylmethionine and *N*-acetyl-*S*-methylmethionine are given in Figure 3. Because of the conformational constraints that were placed on the molecules, these are local minima, not global minima. However, the extended conformations in which the molecules were constrained to remain are consistent with those observed experimentally and in calculations that take into account the influence of solvent (*10*).

DISCUSSION

Parsek et al. established that RhlI catalyzes the formation of C4-HSL from an authentic sample of *N*-butyryl-*S*-adenosyl-L-methionine, but with a V_{max} that was slower than

Table 1: Apparent Steady-State Kinetic Parameters for the RhlI Reaction[a]

	SAM		SAH	
acyl substrate	V_{max} (rel)	$K_{m(acyl\ substrate)}$ (μM)	V_{max} (rel)	$K_{m(acyl\ substrate)}$ (μM)
butyryl-ACP	1.0	7 ± 1	0.22 ± 0.02	5 ± 1
butyryl-CoA	0.27 ± 0.02	260 ± 30	0.28 ± 0.03	260 ± 60

[a] Reactions were conducted in the presence of 100 μM SAM and 500 μM SAH. These concentrations are subsaturating, so the kinetic parameters that are reported are apparent values.

for the reaction with SAM and butyryl-ACP (*3*). This suggests that *N*-butyryl-*S*-adenosyl-L-methionine does not meet the criterion for a kinetically competent intermediate; however, it is not unusual for an exogenously added intermediate to react slowly because of slow formation of a productive complex with the enzyme (*11*). Transient-state kinetic studies are an independent method for detecting and characterizing reaction intermediates. Our rapid-mixing chemical quench data indicate that *N*-butyryl-*S*-adenosyl-L-methionine is formed during the RhlI reaction, and that it lies on the catalytic pathway.

The evidence for the chemical identity of the intermediate comes from the observations that it is an acylated species (it contains ^{14}C when [^{14}C]butyryl-ACP is the substrate), and it contains both the nucleotide and amino acid portions of SAM (it absorbs at 260 nm, and it contains ^{14}C when [^{14}C-*carboxy*]SAM is the substrate). The transient-state kinetic data are fitted adequately by a simple, unbranched model in which the intermediate lies on the catalytic pathway. Under the conditions of our experiments, the intermediate accumulated to a level of 30–40% of the concentration of the ternary enzyme complex. The slow turnover of exogenously added *N*-butyryl-SAM that was observed (*3*) must arise from slow binding to the enzyme.

The observation that SAH is not acylated by RhlI was somewhat surprising and sheds some light on the interactions, or lack thereof, that coordinate the chemical reactions at the RhlI active site. The transient-state kinetic data make it clear that acylation occurs before lactonization in the normal RhlI reaction. Even though SAH is not reactive toward lactonization, it is unclear why it should not be a substrate for acylation. RhlI follows an ordered kinetic mechanism with SAM binding before acyl-ACP, which suggests that the presence of SAM is required to form the binding site for acyl-ACP. (Alternatively, the ordered binding could arise because SAM cannot bind to the acyl-ACP•enzyme complex, but we have no evidence that acyl-ACP can form a dead-end complex with the free enzyme.) The K_m for acyl-ACP does not change whether SAM or SAH is the other substrate, so it would appear that both SAM and SAH are equally competent to induce or allow the binding of acyl-ACP. When the SAM binding site is occupied, transfer of an acyl group from acyl-ACP is induced. In the presence of SAM, this leads to formation of the observed intermediate, *N*-butyryl-SAM. When SAH occupies the SAM binding site, the *N*-butyryl group is transferred to water. It seems most likely that SAH does not bind in exactly the same manner as SAM so that its amino group is not positioned appropriately for attack on the acyl-ACP thioester and is replaced by water at the active site, which implies that the methylated sulfonium of SAM is a key determinant for binding in the proper conformation for acylation.

The fact that a single intermediate species is detected makes it clear that there is a required order in which the chemical transformations of the RhlI reaction take place, with acylation preceding lactonization. The chemical logic underlying this sequence is nicely illustrated by consideration of the properties of different conformers of an acylated sulfonium ion and the corresponding unacylated compound. Model studies and calculations demonstrate that the sulfide product formed from nucleophilic attack at a sulfonium ion center leaves along a trajectory that is 180° from the approach of the incoming nucleophile (*4, 12*). In the case of the lactonization catalyzed by RhlI, nucleophilic attack at the methylene carbon adjacent to the sulfonium center can be accomplished only when the attacking carboxyl group rotates into the proper position (Figure 3).

The energetic and structural consequences of rotation of the carboxyl group around the C−C bond were explored using calculations. The results that we report here on the conformation of a model sulfonium ion are in agreement with the extensive findings described by Markham et al. (*10*). In addition to computational studies, those authors surveyed the conformations of crystallographically observed protein-bound SAM and compared them with the energy-minimized structures and found few differences. Thus, although we have no structural information about how SAM binds to RhlI, precedent suggests that SAM tends to bind at active sites in conformations very similar to the minimum-energy conformation in solution.

For the acylated and unacylated compounds examined in this work, the conformations that position the carboxyl group appropriately for nucleophilic attack are at energy minima. However, the difference in the energies of the least stable and most stable conformers of the acylated compound was 6 kcal/mol, while it was 12 kcal/mol for the unacylated compound (Figure 4). The larger difference in energy for the conformers of the unacylated compound presumably arises from the strong electrostatic interaction between the negatively charged carboxylate and the positively charged amino group, which is not present in the acylated compound.

The structural consequences of the electrostatic interaction are shown in Figure 4. The angles defined by the α carbon, the nitrogen atom, and the carboxyl group carbon differ significantly depending on whether the nitrogen is acylated. In the unacylated compound, the angle varies between 100° and 103°, depending on the orientation of the carboxyl group. This angle is considerably smaller than that expected for a tetrahedral carbon, reflecting the attractive interaction between the amino and carboxylate groups. As a result, the carboxylate oxygen of SAM is limited in how closely it can approach the methylene carbon adjacent to the sulfonium center. In the acylated compound, the attractive interaction between the carboxylate and amine is greatly weakened, and the angle between them varies between 109° and 110°. As a consequence, the closest approach of the carboxylate oxygen to the methylene carbon in the acylated species is 0.2 Å closer than in the unacylated compound.

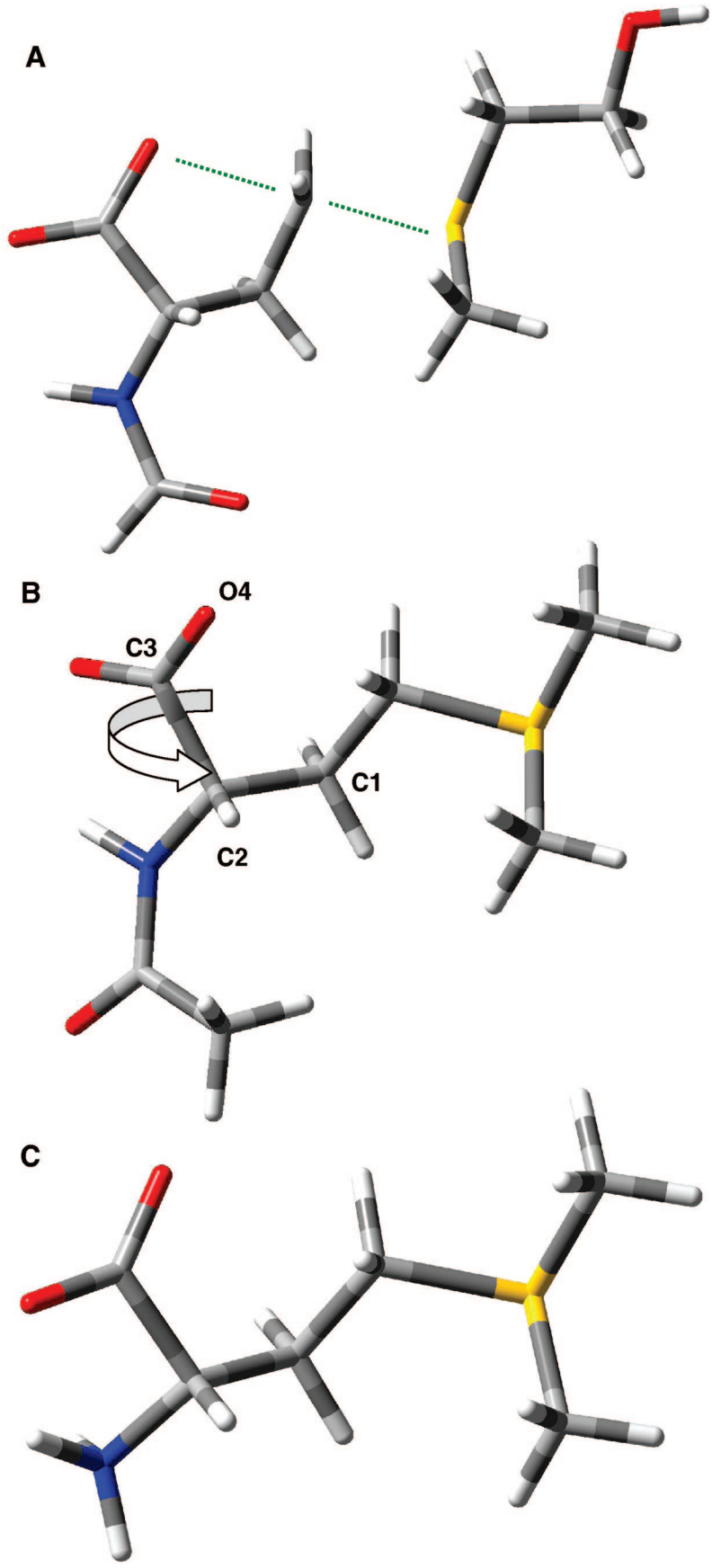

FIGURE 3: Calculated structures for model compounds in the RhlI reaction. (A) Calculated transition-state structure for lactonization. This structure was described in ref *4*. (B) Minimized structure of *N*-acetyl-*S*-methylmethionine. The dihedral angle that was varied in the calculations to rotate the carboxyl group is indicated. (C) Minimized structure of *S*-methylmethionine.

The most dramatic effect of acylation is seen in the bond length between the methylene carbon and the sulfur atom. The C−S bond length varies by 0.025 Å in the unacylated compound as the carboxylate oxygen rotates into and out of

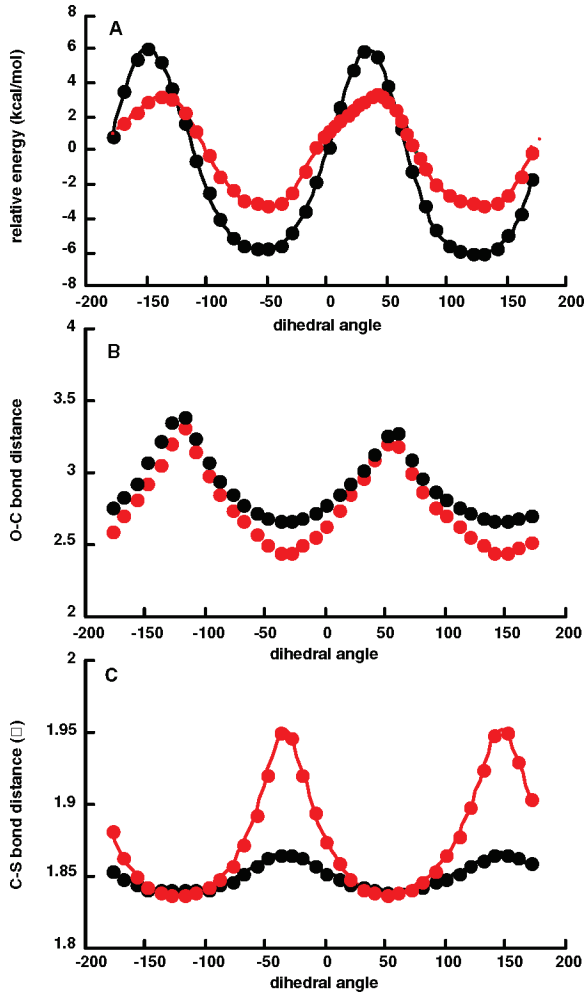

FIGURE 4: Energies and key structural descriptors for *N*-acetyl-*S*-methylmethionine (red circles) and *S*-methylmethionine (●). Two maxima and two minima are observed because the carboxylate oxygens are equivalent. (A) Relative energies of the conformers as a function of the carboxyl group position. The O4−C3−C2−C1 dihedral angle is indicated in Figure 3B. The zero point for the energy was arbitrarily chosen. The asymmetry in the energies of *N*-acetyl-*S*-methylmethionine arises from a change in the orientation of the *N*-acetyl group, which does not influence the other structural parameters considered here. (B) Distance between the carboxylate oxygen and the methylene carbon that is adjacent to the sulfonium center, as a function of carboxylate orientation. (C) Distance between the methylene carbon and the sulfonium sulfur, as a function of carboxylate orientation.

position. The range over which the C−S bond length in the acylated compound varies, however, is >0.1 Å. In the conformation that is appropriate for lactonization, the calculated C−S bond length is 1.95 Å; at the transition state, the C−S bond length is 2.15 Å, so the increase that occurs upon rotation of the carboxylate group constitutes approximately one-third of the total change.

Our data suggest that the linkage between acylation and lactonization catalyzed by RhlI arises through interdependencies of the chemistry of the two reactions. Although there may be a conformation change that occurs after acylation which facilitates lactonization, we have been unable to observe any change in the intrinsic fluorescence of the protein during turnover in stopped-flow experiments (unpublished observations). Acylation clearly activates the substrate for lactonization; it therefore makes sense that it occurs first in the sequence of reactions. This supposition is borne out experimentally by the observation of a single intermediate, *N*-butyryl-SAM, and rationalized by the calculations. Lactonization, which can occur only with the activated sulfonium ion substrate, does not activate the substrate for acylation. However, lactonization is coupled to acylation by virtue of the fact that the sulfonium center is required for SAM to bind in the proper position for acylation. Without a sulfonium methyl group, SAH binds in such a manner that the acyl group from ACP is transferred to solvent.

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