Characterization of the Covalent Binding of Thiostrepton to a Thiostrepton-Induced Protein from *Streptomyces lividans*†

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ABSTRACT: Thiostrepton is a highly modified multicyclic peptide antibiotic synthesized by diverse bacteria. Although best known as an inhibitor of protein synthesis, thiostrepton is also a potent activator of gene expression in *Streptomyces lividans*. In these studies, we characterize the nature of the interaction between thiostrepton and two proteins that it induces, TipAL and TipAS. In the absence of added cofactors, thiostrepton formed a complex with either TipAL or TipAS in aqueous solution. The TipA−thiostrepton complex was not dissociated by denaturants such as SDS, urea, or disulfide reducing agents. The mass of the TipAS−thiostrepton complex as determined by both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and mass spectrometry (MS) was equivalent to the sum of TipAS and thiostrepton. Thiostrepton also reacted spontaneously with free cysteine (but not with other amino acids tested) to generate stable compounds having masses equivalent to thiostrepton plus 3 or 4 cysteines. Blocking experiments indicated that complex formation required dehydroalanine residues on thiostrepton and cysteine residues on TipAS. When the TipAS−thiostrepton complex was digested with trypsin and analyzed by MS, the thiostrepton adduct was found bound only to the unique cysteine-containing TipAS peptide fragment. Amino acid analysis confirmed that the TipAS−thiostrepton complex contained lanthionine, the product of a reaction between dehydroalanine and cysteine. Together, these data document a covalent attachment of thiostrepton to TipA proteins mediated by bond formation between dehydroalanine of thiostrepton and cysteine of TipAS. Implications regarding the function of TipAS as a thiostrepton (electrophile)-sequestering protein and thiostrepton-mediated activation of TipAL as a model of irreversible transcriptional activation are discussed.

Structurally heterogeneous small molecular weight compounds generated during the secondary metabolism of bacteria and fungi are sometimes classified as antibiotics because they inhibit growth of microorganisms. Antibiotics may provide competitive advantage in soil ecosystems for producing organisms. However, alternative biological activities for antibiotics as triggers of gene expression (Murakami et al., 1989), differentiation (Sarkar & Paulus, 1972), and sporulation (Andrés et al., 1990; Özcengiz & Alaeddinoglu, 1991; Ristow et al., 1975) have been suggested. The mechanism by which antibiotics elicit these alternate responses or influence the metabolism of producing or resistant bacteria is only partly understood (Demain, 1995; Luckner, 1990). These alternate biological activities can be rationalized by the premise that antibiotics represent "molecular fossils" that exert their biological activities through interaction with ancient conserved sites in macromolecules (Davies, 1992). However, some of these interesting effects on bacterial metabolism may be uncovered only when the primary mechanism of antibiotic activity is blocked.

Thiostrepton is a member of a family of highly modified multicyclic peptide antibiotics synthesized by diverse bacteria including *Streptomyces*, *Bacillus*, and *Micrococcus* (Abraham et al., 1956; Cundliffe, 1980; Pagano et al., 1956; Petska & Bodley, 1975; Steinberg et al., 1956; Su, 1948; Van Deputte & Dutcher, 1956) (Figure 1). These compounds are employed as antibiotics, growth promotants, and prophylactic agents for livestock (Mine et al., 1972; Muir et al., 1980). Thiostrepton is well-known for its ability to bind to prokaryotic ribosomes, thereby inhibiting protein synthesis, and has been used widely as a tool to study ribosome function (Cundliffe, 1987). Some thiostrepton-producing organisms possess a specific methylase that modifies a particular nucleotide in the 50S ribosomal subunit, thereby preventing thiostrepton from binding to the ribosome (Thompson et al., 1982b). Ribosomes that are methylated *in vitro* are not thiostrepton-sensitive and do not bind detectable amounts of the antibiotic (Cundliffe, 1980). The gene encoding this methylase, *tsr*, has been cloned from *Streptomyces azureus* (Thompson et al., 1982a) and is routinely employed as a selectable marker in most streptomycete cloning vectors (Bibb et al., 1985; Kuhstoss & Rao, 1991; Smokvina et al., 1990). Surprisingly, the addition of thiostrepton to these *Streptomyces* strains uncovers another biological phenomenon: Thiostrepton induces the synthesis of more than ten proteins (Murakami et al., 1989, Puglia et al., 1994).

The gene encoding the most abundant thiostrepton-induced protein, TipAS, has been cloned and sequenced (Murakami et al., 1989). TipAS does not have statistically significant similarity to any other protein in the sequence data banks. TipAL, the protein that activates transcription from the *tipA*

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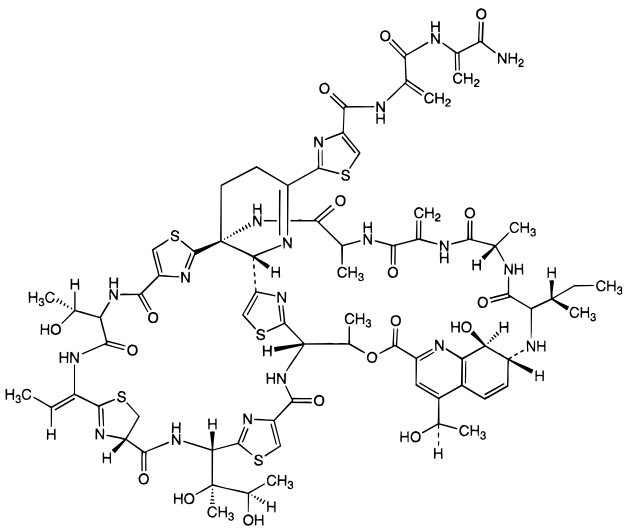

FIGURE 1: Structure of thiostrepton. Thiostrepton is a multicyclic peptide antibiotic with modified amino acids. There are three dehydroalanines and one dehydrobutyrine. A dehydroalanine and the dehydrobutyrine are found in the multicyclic domain. The other two dehydroalanines are found on the tail that comes off from the pyridyl moiety. The quinaldic acid group is the source of UV luminescence of thiostrepton.

promoter corresponds to TipAS with an additional amino-terminal DNA-binding domain (Holmes et al., 1993b). This domain contains a putative helix−turn−helix motif similar to a family of known transcriptional activators such as MerR (Barrineau et al., 1984; Summers, 1992), SoxR (Amabile-Cuevas & Demple, 1991), BmrR (Ahmed et al., 1994), and NolA (Sadowsky et al., 1991). Transcription from the *tipA* promoter is induced by thiostrepton *in vivo* (Murakami et al., 1989) or *in vitro* (Holmes et al., 1993a) through interactions with TipAL. Similarly, other transcriptional activators are believed to be activated by different effector molecules: MerR with mercuric ion (Ansari et al., 1992, Helmann et al., 1990); SoxR with superoxide anion (Hidalgo & Demple, 1994); BmrR with rhodamine 6G (Ahmed et al., 1994), and NolA with genistein (Sadowsky et al., 1991).

Transcriptional activation from *tipA* promoter by the TipAL−thiostrepton complex results in the synthesis of TipAL and large amounts of TipAS. TipAS sequestration of thiostrepton may confer antibiotic resistance and limit TipAL transcriptional activation. TipAL may also act as a control for an antibiotic resistance regulon, since thiostrepton induces resistance to antibiotics that are structurally unrelated (Holmes et al., 1993a).

In this paper, we show that the binding of thiostrepton to TipAS results from a novel covalent interaction involving a dehydroalanyl group on thiostrepton and a cysteinyl group on TipAS.

MATERIALS AND METHODS

Chemicals and Reagents. Thiostrepton, lanthionine, cysteine, and duramycin were obtained from Sigma Chemical Co. Dimethyl sulfoxide (DMSO)[1] was purified by passing through dry alumina and dry silica columns. The ninhydrin reagent was an aqueous solution of 0.3% ninhydrin, 3% acetic acid, and 20% *n*-butanol. HPLC-grade water, acetonitrile, and trifluoroacetic acid were obtained from Fluka. Sequence-grade trypsin was obtained from Boehringer Mannheim, Germany. 2,5-Dihydroxybenzoic acid was obtained from Aldrich.

Bacterial Strains, Plasmids, and Growth Conditions. These studies were carried out using *Streptomyces lividans* 1326 (Lomovskaya et al., 1972) transformed with a vector (pIJ486) (Wart et al., 1986) containing the thiostrepton resistance gene (*tsr*) (Thompson et al., 1982a). YEME liquid medium (Hopwood et al., 1985) with thiostrepton (50 μg/μL) was inoculated with a spore suspension of *S. lividans*/pIJ486 (100 μL of 10^3 CFU/μL in 20% glycerol) and grown for 52 h at 30 °C.

Escherichia coli W3110 containing the cI857 bacteriophage λ repressor gene (W3110*cI*) was transformed with a TipAS expression vector (pNB2 for TipAL and pNB3 for TipAS) (Campbell et al., 1978; Holmes et al., 1991). W3110*cI*/pNB2 or pNB3 was grown at 30 °C in Lysogeny Broth medium (10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 100 μg/μL ampicillin (Sambrook et al., 1989). The cultures were grown to late log phase at 30 °C, and then the production of TipAS/AL was induced by heating quickly and incubating at 42 °C for 2 h.

Purification of TipAS and the TipAS−Thiostrepton Complex. Thiostrepton-induced *S. lividans*/pIJ486 mycelia were collected by centrifugation at 10 000*g* on a GSA rotor for 30 min, washed with cold lysis buffer (50 mM Tris-HCl at pH 8.0, 1 mM EDTA, at pH 8.0, 20 μM phenylmethanesulfonyl fluoride, and 1 mM 2-mercaptoethanol), resuspended in the same buffer, and sonicated twice at 30 Watts for 30 s. The cell debris was removed by centrifugation at 22 000*g* on a SS34 rotor for 30 min at 4 °C to yield a crude extract. All further TipAS purification steps were conducted at 4 °C. TipA proteins were precipitated with 0.4% polyethyleneimine, and the precipitate was collected by centrifugation at 22 000*g* on a SS34 rotor for 30 min at 4 °C and monitored by SDS−PAGE. The pellet was resuspended in 1 M ammonium sulfate and centrifuged for 30 min at 4 °C. The supernatant was dialyzed against MA1 buffer (20 mM Tris-HCl of pH 8.0, 1 mM EDTA at pH 8.0, and 1 mM 2-mercaptoethanol), applied to a DEAE−Sepharose column and eluted in MA1 buffer in a gradient of 0−0.5 M NaCl. The fractions were collected and concentrated with an Amicon Ultrafiltration cell with a YM10 membrane. The concentrated solution was loaded on a Superose 12 HR 10/30 column (Pharmacia Biotech) and eluted isocratically with MA1 buffer. The fractions containing TipAS were concentrated with Centricon 10 concentrators (Amicon).

Mass spectrometric studies were performed on recombinant TipAS from *E. coli* purified using a thiostrepton-affinity column as previously described (Holmes et al., 1993b).

TipAS used in thiostrepton and thiostrepton-derivative binding experiments and amino acid analysis studies was purified from *E. coli* W3110*cI*/pNB3 cultures. After induction, the cultures were centrifuged for 30 min at 5000*g*. After the cells were washed with MA1 buffer, the cell pellet was resuspended in MA1 buffer and sonicated at 30 W for 5 min. The solution was centrifuged for 15 min at 10 000*g*. The purification protocol was the same as that described above

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; ESI, electrospray ionization; MALDI−TOF, matrix assisted laser desorption ionization time of flight; DTT, dithiothreitol; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; UV, ultraviolet; TLC, thin layer chromatography; LC, liquid chromatography; RP, reversed phase; NMR, nuclear magnetic resonance; CFU, colony forming units.

for TipAS−thiostrepton isolated from *S. lividans*/pIJ486 cultures.

Protein and Antibiotic Reactions. The following protocol was used to react thiostrepton (or its derivatives) with TipAS (or its derivatives). TipAS (10 μg in 50 mM Tris at pH 8.0, 1 mM EDTA at pH 8.0) was reduced by the addition of sodium cyanoborohydride (2 μL of freshly made, 1 mM in water). After 5 min at room temperature, excess sodium cyanoborohydride was removed by the addition of acetone (1 μL). Thiostrepton (10 μL of a 50 μg/μL in DMSO solution) was then added, and the reaction was incubated at room temperature for 15 min. Estimation of TipAS concentrations were based on 280 nm absorption using the molar absorptivities of the tryptophans.

N-Ethyl maleimide (10 μL of a 10 μg/μL DMSO solution) was added to sodium cyanoborohydride-treated TipAS (10 μg) to alkylate the cysteines in TipAS.

Thiostrepton−cysteine complex was made by reacting thiostrepton (10 μL of 20 μg/μL in DMSO) with cysteine (50 μL of freshly made 50 μg/μL in 50 mM potassium phosphate buffer at pH 7.0) at room temperature for 1 h. The thiostrepton and thiostrepton−cysteine complexes were purified by HPLC using an E. Merck Lichrosorb 4.6 × 250 mm RP-18 (5 μm) column employing a gradient of 80% water/0.1% TFA, 20% acetonitrile/0.1% TFA to 100% acetonitrile/0.1% TFA.

TipAS and TipAS−antibiotic complex formation was analyzed using SDS−PAGE gels (Schagger & von Jagow, 1987). All protein samples were boiled for 3 min in a SDS−PAGE sample buffer containing 20% (w/v) glycerol, 6% SDS, 10% 2-mercaptoethanol, and 0.05% bromophenol blue in 0.25 M Tris at pH 6.8. The gels were stained with Coomassie-blue (0.1% in 10% acetic acid).

TLC Conditions. Amino acids (glycine, cysteine, cystine, alanine, lysine, histidine, arginine, glutamine, glutamic acid, asparagine, aspartic acid, leucine, isoleucine, serine, threonine, methionine, tryptophan, tyrosine, phenylalanine, and proline), thiols (2-mercaptoethanol, dithiothreitol, and glutathione), and ribonucleotides (adenosine, uridine, guanosine, thymidine, and cytidine) were tested for their ability to react with thiostrepton. Solutions of amino acids and ribonucleotides (100 μL of 0.1 M amino acid or ribonucleotide in either 0.1 M sodium acetate at pH 5.0 or 0.1 M sodium phosphate at pH 8.0) were added to thiostrepton (10 μL of 10 mM in DMSO). After 30 min at room temperature, the reaction mixture (5 μL) was applied to a Silica G60 thin-layer chromatography (TLC) plate. After the sample was dried and the chromatogram developed in 90% chloroform, 10% methanol, and 0.1% acetic acid, the thiostrepton-related compounds were visualized in UV light. Free amine groups were detected by spraying the TLC plate with ninhydrin reagent.

Mass Spectrometry. Samples used for mass spectrometry were first lyophilized and then resuspended in 50 mM ammonium bicarbonate containing 100 μM dithiothreitol (DTT) at pH 8.5. Purified TipAS, TipAS−thiostrepton complex, thiostrepton, and thiostrepton−amino acid adducts were analyzed by liquid chromatography−electrospray ionization mass spectrometry (LC−ESI−MS). Samples were introduced into a Finnigan MAT (San Jose, CA) TSQ-700 triple-stage quadrupole mass spectrometer using an ABI 130 syringe pump HPLC. After injection, samples were loaded onto a microbore 1 × 100 mm reversed-phase octyl column and eluted directly into the mass spectrometer using a linear gradient of acetonitrile. Spectra were recorded as described elsewhere (Griffin et al., 1991).

Peptide maps were analyzed by matrix-assisted, laser desorption ionization−time-of-flight mass spectrometry (MALDI−TOF−MS). In general, samples were prepared by mixing a 0.5 μL aliquot of the peptide mixture with 0.5 μL of a solution of dihydroxybenzoic acid (saturated, ca. 50 mM in distilled−deionized H_2O) on a stainless-steel sample target surface. Mass spectra were obtained using a Finnigan MAT Vision 2000 MALDI−TOF mass spectrometer operated in the normal reflection mode. Ionization was accomplished with a 337 nm beam from a LSI VSL-337 ND nitrogen laser (5 ns pulse width, 20 Hz). Ions were accelerated to 5 kV, reflected using a high-resolution gridless ion mirror and detected with a secondary electron multiplier. Signal from the detector was digitized at a sampling rate of 100 MHz using an 8 bit/64K high-speed static RAM transient recorder. Data processing was accomplished using a Gateway 386 PC running Finnigan Vision 2000 software. Typically, spectra were generated from the sum of 5−15 laser shots. Spectra were calibrated externally using a mixture of angiotensin I, oxidized insulin β chain, cytochrome *c*, and myoglobin (all from Sigma, 1 mM).

Peptide Mapping. Samples used for peptide mapping were first lyophilized and then resuspended in 50 mM ammonium bicarbonate containing 100 μM dithiothreitol (DTT) at pH 8.5. Peptide maps were generated by exposing TipAS and TipAS−thiostrepton complex to trypsin [1:100 (w/w) enzyme/substrate] for 24 h at room temperature. All reactions were stopped by the addition of 0.1% TFA.

Amino Acid Analysis. For amino acid analysis, all protein samples were first purified on E. Merck RP C-18 4 × 250 mm HPLC column using a gradient of 100% water/0.1% TFA to 100% acetonitrile/0.1% TFA at room temperature. The HPLC-purified protein solutions (containing 1−3 μg) were dried down in a speed-vac at room temperature and resuspended in 20 μL of water. The samples were hydrolyzed in 6 N HCl for 24 h at 110 °C in an argon gas environment. The sample hydrolysate was resuspended in 50 mM sodium bicarbonate, pH 8.3. Dabsyl chloride was added and the reaction took place at 70 °C for 12 min. The dabsylated hydrolysate was separated on a Merck LiChrospher 100 CH-18/2 using a gradient starting from 25 mM sodium acetate, pH 6.4/4% dimethyl formamide buffer to acetonitrile. Peaks were detected at 436 nm.

RESULTS

Detection of the TipAS−Thiostrepton Complex Using SDS−PAGE. Following incubation of TipAS with thiostrepton, its apparent molecular mass on SDS−PAGE gels increased from 17 to 20 kDa (Figure 2). This complex was stable to boiling in a standard protein sample solubilization buffer containing SDS and 2-mercaptoethanol. In addition, neither buffers containing 7 M urea nor 6 M guanidine hydrochloride dissociated the complex. This complex was also stable during passage through a reversed-phase C18 HPLC column using 0.1% TFA/water to acetonitrile solutions. This increase in mass corresponded roughly to the addition of thiostrepton (1664 Da) to TipAS (17 kDa). Mass spectrometry of TipAS and TipAS−thiostrepton was used to obtain a more accurate determination of this mass increase.

Mass Spectrometry of TipAS and TipAS−Thiostrepton Complex. Data obtained from the analysis of thiostrepton,

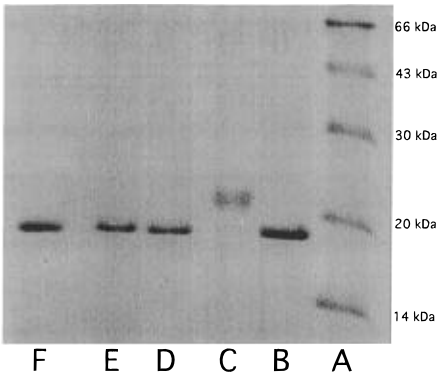

FIGURE 2: SDS−PAGE of TipAS and TipAS−thiostrepton complex. Reactions were carried out in aqueous buffers at room temperature as described in Materials and Methods, separated by discontinuous tricine−SDS−PAGE, and visualized by Coomassie blue staining. (A) Molecular mass markers (66, 43, 30, 20, 14 kDa); (B) TipAS; (C) TipAS−thiostrepton; (D) TipAS after cysteine alkylation with *N*-ethyl maleimide. (NEM); (E) TipAS alkylated with NEM and then treated with thiostrepton; (F) TipAS treated with thiostrepton−cysteine complex. The thiostrepton−cysteine reaction product was purified by HPLC (see Materials and Methods). Although the band corresponding to the TipAS−thiostrepton complex was more diffuse than that of TipAS, the predicted range of masses for the heterogeneity was not observed by ESI or MALDI−TOF mass spectrometry.

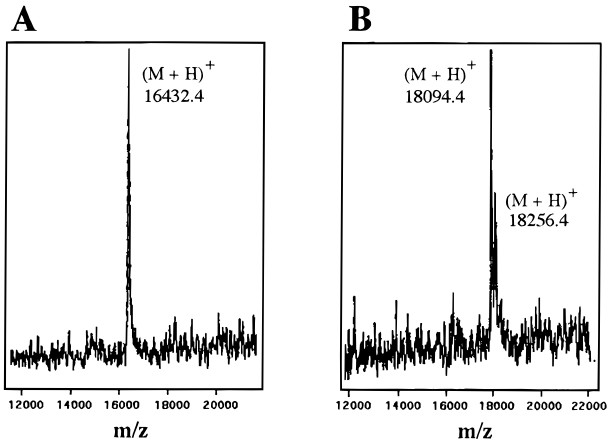

FIGURE 3: LC−ESI−MS of TipAS and TipAS−thiostrepton complex. LC−ESI−MS was used to determine the masses of *E. coli* purified TipAS and TuipAS−thiostrepton complex. (A) A deconvoluted mass spectrum of TipAS analyzed by LC−ESI−MS. The observed $(M + H)^+$ molecular ion mass of TipAS was 16 432.4 Da which was in excellent agreement with the predicted molecular mass of *des*Met TipAS, 16 432.6 Da. (B) A deconvoluted mass spectrum of TipAS complexed with thiostrepton. TipAS−thiostrepton complex was made by incubating thiostrepton with reduced TipAS in aqueous solution at room temperature. The observed $(M + H)^+$ molecular mass for the complex was 18 094.4 Da. This mass corresponded to the mass of one molecule of *des*Met TipAS and one molecule of thiostrepton. In addition, a molecular $(M + H)^+$ ion at 18 256.4 Da was observed which corresponds well with the mass of a DTT−*des*Met TipAS−thiostrepton complex.

TipAS, and TipAS−thiostrepton complexes by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) are summarized in Table 1. The predicted protonated molecular mass for TipAS, based on the protein's amino acid sequence, was calculated to be 16 563.8 Da. Analysis of TipAS from *E. coli* by LC−ESI−MS resulted in the deconvoluted spectrum shown in Figure 3A. The protonated molecular mass of TipAS was experimentally determined to be 16 432.4 Da, which was in excellent agreement with the *des*-Met form of TipAS (predicted mass of 16 432 Da, delta mass of −0.2 Da). The loss of an N-terminal methionine from TipAS isolated from *E. coli* is often catalyzed by an amino-terminal methionine aminopeptidase found in *E. coli* (Hirel et al., 1989). Most *E. coli* proteins (97%) having a penultimate glycine, such as that found in TipAS, lack the amino-terminal methionine (Hirel et al., 1989).

LC−ESI−MS analysis of reaction mixtures of TipAS and thiostrepton generated a complex mass spectrum, presumably due to oxidation of thiostrepton and to the binding of thiols, such as DTT, to the complex. Analysis of some reaction mixtures showed clearly the addition of DTT to complexes of TipAS and thiostrepton (see Figure 3B). TipAS−thiostrepton complexes that were fractionated off-line by RP-HPLC prior to LC−ESI−MS analysis gave the least complex mass spectrum (Figure 3B). The predicted mass for a complex of *des*-Met TipAS and thiostrepton, the sum of 16 432.6 and 1664.5 Da, was 18 096.1 Da. The observed mass for the TipAS−thiostrepton complex was 18 094 Da with a delta mass of −1.7 Da. The mass of the complex indicated a 1:1 molar ratio of TipAS and thiostrepton. No other complexes were found. The ability to measure the TipAS−thiostrepton complex through mass spectrometry gave additional indication that this complex was extremely stable.

Analysis of TipAS−thiostrepton complex from *S. lividans* yielded a slightly higher mass of 18 661.6 Da, which was 567.2 Da larger than the observed mass of TipAS from *E. coli* (see Table 1). By analogy to the reactivity of DTT and other thiols, this additional 567.2 Da may correspond to other

Table 1: Summary of Mass Spectrometry Analysis of Thiostrepton, Thiostrepton−Cysteine, TipAS, and TipAS−Thiostrepton Complex by LC−ESI−MS

protonated mass (in daltons)	predicted	observed	delta
TipAS (no formylation)	16 563.8	16 432.4	−131.4
TipAS (*des*Met)	16 432.6	16 432.4	−0.2
TipAS−thiostrepton (*des*Met)	18 096.1	18 094.4	−1.7
increase in mass	+1663.5[a]	+1662.0	−1.5
TipAS−thiostrepton (desMet)[b]	18 096.1	18 661.6	+565.5
thiostrepton	1664.5	1664.4	−0.1
thiostrepton + cysteine	20 27.5 (+3 Cys)	2027.4	−0.1
thiostrepton + cysteine	2148.5 (+4 Cys)	2148.4	−0.1
thiostrepton + cysteine	2066.6 (+3 Cys + K^+)	2064.6	−2.0
thiostrepton + cysteine	2182.6 (+4 Cys + K^+)	2187.0	−0.6

[a] The increase in mass for TipAS−thiostrepton complex was calculated as follows. The predicted protonated molecular mass for TipAS−thiostrepton complex was determined to be 18 096.1 Da based on a predicted molecular mass of TipAS of 16 432.6 Da, plus the neutral molecular mass of thiostrepton, 1663.5 Da, or 16 432.6 + 1663.5 = 18 096.1 Da. Thus, 18 096.1 Da − 16 432.6 Da = 1663.5 Da, the predicted increase in mass for the TipAS−thiostrepton complex from *E. coli*. [b] Denotes TipAS−thiostrepton complex isolated from *S. lividans*. All other protein samples were isolated from *E. coli*.

cellular thiol(s) of *S. lividans* bound to the TipAS−thiostrepton complex.

Although the multivalent nature of thiostrepton (three dehydroalanines and one dehydrobutyrine) and TipAS (two cysteines) could have generated multiple TipAS−thiostrepton complexes, such complexes were not detected. Molecular species corresponding to 2, 3, or 4 TipAS bound to 1 thiostrepton were not observed by SDS−PAGE, LC−ESI−MS, or MALDI−TOF−MS. The molecular recognition of TipAS for thiostrepton during complex formation presumably excluded the possibility of generating any of these multiple complexes.

Detection of Thiostrepton−Cysteine Complexes. Different amino acids (glycine, cysteine, cystine, alanine, lysine, histidine, arginine, glutamine, glutamic acid, asparagine, aspartic acid, leucine, isoleucine, serine, threonine, methionine, tryptophan, tyrosine, phenylalanine, and proline), nucleotides (adenosine, uridine, guanosine, thymidine, and cytidine), and thiols (2-mercaptoethanol, dithiothreitol, and glutathione) were screened for binding to thiostrepton in low and high pH buffers. None of these compounds except for cysteine bound to thiostrepton in aqueous conditions at room temperature. Ultraviolet light excited the quinaldic acid group of thiostrepton, generating luminescent spots on the silica TLC plates. When cysteine was added to thiostrepton, a new luminescent spot was seen at the origin, suggesting that thiostrepton was chemically altered. Similarly, thiostrepton reacted to 2-mercaptoethanol, dithiothreitol, and glutathione. The reaction took place in less than 1 min at room temperature. Ninhydrin analysis of the TLC plates showed that cysteine and the thiostrepton−cysteine complexes remained at the origin. Since thiostrepton did not react with ninhydrin, only the free amines in cysteine and thiostrepton−cysteine complexes were detected.

LC−ESI−MS was used to determine the stoichiometry of cysteine binding to thiostrepton. The spectrum of thiostrepton was dominated by ions at 1664.4 Da, with less abundant ions at higher mass presumably due to oxidation or to the presence of $(M + Na)^+$ and $(M + K)^+$ ions (Figure 4A). The observed mass was in excellent agreement with the predicted mass of 1664.5 Da. Analysis of thiostrepton−cysteine reaction mixtures by LC−ESI−MS exhibited the complex mass spectrum shown in Figure 4B. Abundant ions observed at 2027.4 and 2148.5 Da corresponded to thiostrepton with three bound cysteines and thiostrepton with four bound cysteines, respectively.

^{1}H NMR studies revealed that the thiostrepton−$(cysteine)_3$ complex was a mixture of different species and suggested that the cysteine thiol reacted with the three dehydroalanine groups and the one dehydrobutyrine group of thiostrepton (data not shown). While most of the NMR signals of thiostrepton did not change, there were changes in the chemical shifts of the protons of dehydroalanine groups and nearby protons and formation of new chemical shift peaks corresponding to methylene protons.

Role of the Cysteine of TipAS and Dehydroalanine of Thiostrepton in Covalent Binding. To investigate potential covalent interactions between the cysteines of TipAS and thiostrepton, the cysteine thiol groups of TipAS were chemically blocked with *N*-ethyl maleimide. When thiostrepton was added to this complex, no TipAS−thiostrepton complex was observed by SDS−PAGE (Figure 2E). TLC and HPLC experiments demonstrated that thiostrepton did not bind to *N*-ethyl maleimide. Thus the alkylation of TipAS cysteines prevented the covalent binding of TipAS to thiostrepton.

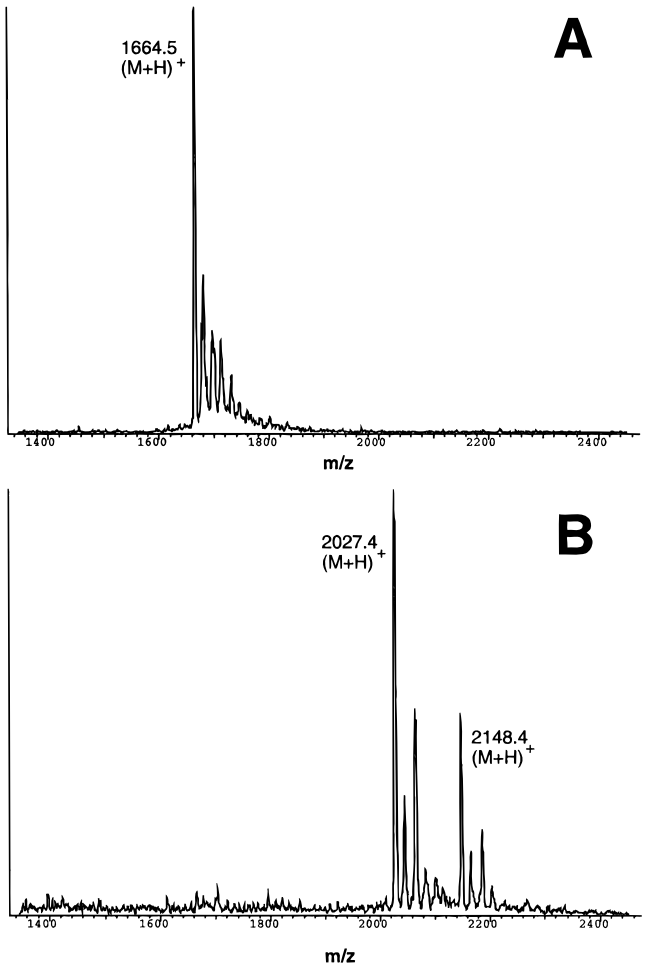

FIGURE 4: LC−ESI−MS of thiostrepton−cysteine complex. (A) The mass spectrum of thiostrepton obtained by LC−ESI−MS. Abundant protonated molecular ions $(M + H)^+$ were observed at 1664.5 Da. Also ions for $(M + Na)^+$ and $(M + K)^+$ were detected. (B) The mass spectrum of thiostrepton−cysteine complexes generated from analysis by LC−ESI−MS. Thiostrepton−cysteine complex was obtained from a HPLC purification of a thiostrepton reaction with cysteine in a DMSO/phosphate buffer at room temperature for 30 min. The complex spectrum resulted from the addition of three $[(M + H)^+ = 2027.4]$ and four $[(M + H)^+ = 2148.4]$ molecules of cysteine per molecule of thiostrepton. Also ions for $(M + Na)^+$ and $(M + K)^+$ were detected.

In a complementary experiment, the dehydroalanine and dehydrobutyrine groups of thiostrepton were blocked by reaction with cysteines. Mass spectrometry demonstrated that one molecule of thiostrepton was bound to three molecules of cysteine. When this thiostrepton−cysteine complex was added to TipAS, no TipAS−thiostrepton complex was seen on SDS−PAGE (Figure 2F). Similarly, covalent complex formation was not detected when TipAS was mixed with the thiostrepton−glutathione and thiostrepton-2-mercaptoethanol derivatives (data not shown). Hence the blocking of dehydroalanine and dehydrobutyrine groups prevented the formation of a covalent bond between TipAS and the thiostrepton−cysteine complex.

Analyses of Tryptic Maps of TipAS and TipAS−Thiostrepton Complexes. Tryptic peptide mixtures generated from *E. coli* purified TipAS and *E. coli* TipAS−thiostrepton complexes were analyzed by MALDI−TOF−MS (Table 2). The tryptic map of purified TipAS from *E. coli* spanned most of the protein's primary structure. Only seven amino acid

Table 2: Summary of MALDI−TOF−MS Analysis of Peptides Generated by Trypsin Digestion of Purified TipAS and TipAS−Thiostrepton Complexes

		TipAS		TipAS−thiostrepton complex	
sequence	predicted mass[a]	observed mass[a]	delta	observed mass[a]	delta
2−10	1001.1	N/O[c]		1002.0	0.9
2−27	3104.3	3103.5	−0.8	3105.8	1.5
11−27	2122.2	2122.6	0.4	2123.2	1.0
28−29	304.3	N/O		N/O	
30−37	983.0	983.9	0.9	982.7	−0.3
38−40	362.4	362.9	0.5	N/O	
41−42	276.3	N/O		N/O	
42−48	670.7	671.5	0.8	670.3	−0.4
49−53	733.8	734.4	0.6	733.1	−0.7
54−63	1190.3	1190.6	0.3	1190.3	0.0
64	175.2	N/O		N/O	
65−88	2506.7	2506.6	−0.1	2507.8	1.1
89−93	544.6	545.2	0.6	543.8	−0.8
94−115	2653.9	2652.2	1.7	N/O	
94−115+ thiostrepton[a,b]	4218.4	N/O		4883.1	564.8
116−118	423.5	N/O		422.5	−1.0
119−132	1491.8	1492.4	0.6	1491.9	0.1
133−141	943.1	943.7	0.6	942.6	−0.5
142−144	354.4	354.9	0.5	N/O	

[a] Protonated mass in Daltons. [b] Predicted mass for residues 94−115 complexed with thiostrepton was based on the mass of the fragment with one molecule of thiostrepton. An additional mass of 564.8 Da was found in the TipAS−thiostrepton complex purified from *S. lividans*. [c] N/O, not observed in experiment.

residues were not identified in this analysis. Most of these were associated with one or two amino acid tryptic digestion products. All TipAS peptides observed had recorded masses within 1.0 Da of their corresponding predicted masses, with the exception of one peptide, residues 94−115, which deviated by 1.7 Da.

Peptide mixtures generated from tryptic digestion of purified TipAS−thiostrepton complexes from *S. lividans* yielded results similar to those described above (Table 2). Mass spectral analysis afforded near complete coverage of the protein's primary structure. Only the fragment containing residues 94−115 had an observed mass that was significantly different from the predicted mass. As anticipated from the thiostrepton−cysteine complex mass spectrometric data, only the TipAS cysteine-containing tryptic peptide, residues 94−115, showed an increase in mass. The mass of this peptide predicted by the gene sequence was 2653.9 Da; its experimentally determined mass was 4883.1 Da. The difference between the observed mass (4883.1 kDa) and the sum of the masses of the predicted segment and thiostrepton (2653.8 + 1664.5) corresponded to an additional unidentified chemical species bound to the TipAS−thiostrepton complex. The difference in mass between this tryptic peptide of TipAS from *E. coli* and TipAS−thiostrepton complex from *S. lividans* (564.8 Da) corresponded well to the difference in mass between the two undigested protein species (565.5 Da). Hence the additional chemical species of 564.8 Da was associated only with the thiostrepton-containing tryptic peptide fragment covering residues 94−115.

Amino Acid Analyses of TipAS and TipAS−Thiostrepton. The blocking studies indicated that the covalent bond could involve the reaction of the dehydroalanine or dehydrobutyrine groups of thiostrepton with the cysteine groups of TipAS. If the binding of TipAS to thiostrepton involved the reaction of dehydroalanine with cysteine, a new amino acid, lanthionine, would be formed (Schoberl & Wagner, 1947) (Figure 5). Likewise, cysteine reacting with butyl dehydroalanine or dehydrobutyrine would generate methyl-lanthionine (Belitz, 1967). During amino acid analysis, these nonstandard dabsyl−amino-acid derivatives eluted at positions different from those of the standard dabsyl−amino-acid derivatives. Amino acid analysis of lanthionine and duramycin was used to establish the elution positions of lanthionine, methyl lanthionine, and lysinoalanine (data not shown). The dabsyl derivative of lanthionine had an elution time distinct from other dabsyl amino acid derivatives.

Amino acid analysis of thiostrepton−cysteine complexes revealed a lanthionine residue that was not present in thiostrepton or in TipAS. No methyl lanthionine or lysinoalanine residues were detected in the amino acid analysis of the thiostrepton−cysteine complex. Comparison of TipAS and TipAS−thiostrepton showed that the latter had a new lanthionine residue (Figure 6). The integrated area of the dabsyl−lanthionine derivative of the TipAS−thiostrepton

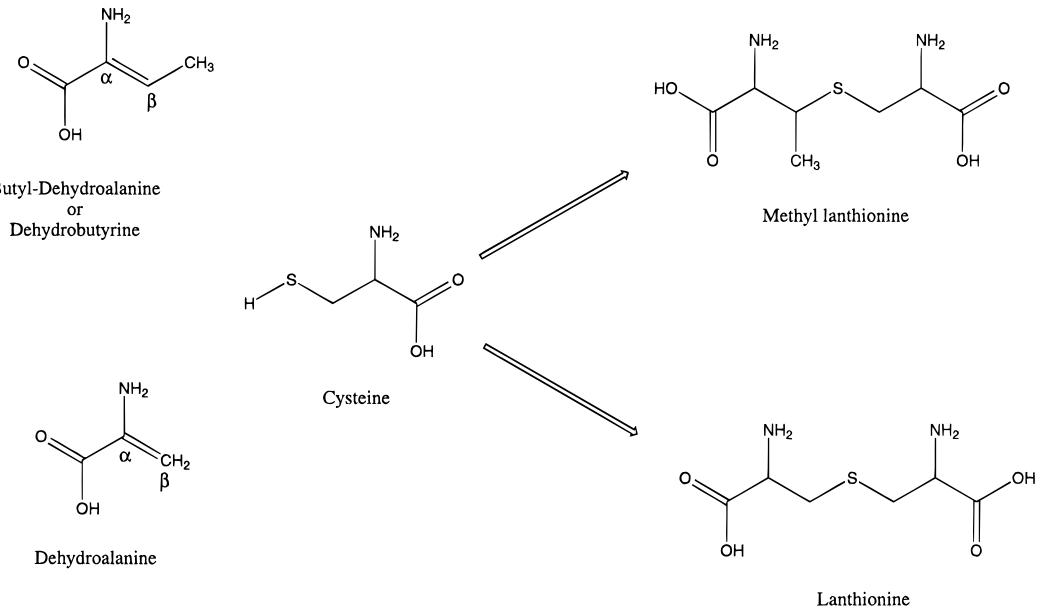

FIGURE 5: Reaction of cysteine with dehydroalanine and dehydrobutyrine. Cysteine can react with either dehydroalanine or dehydrobutyrine to form either lanthionine or methyl lanthionine, respectively, in aqueous solution.

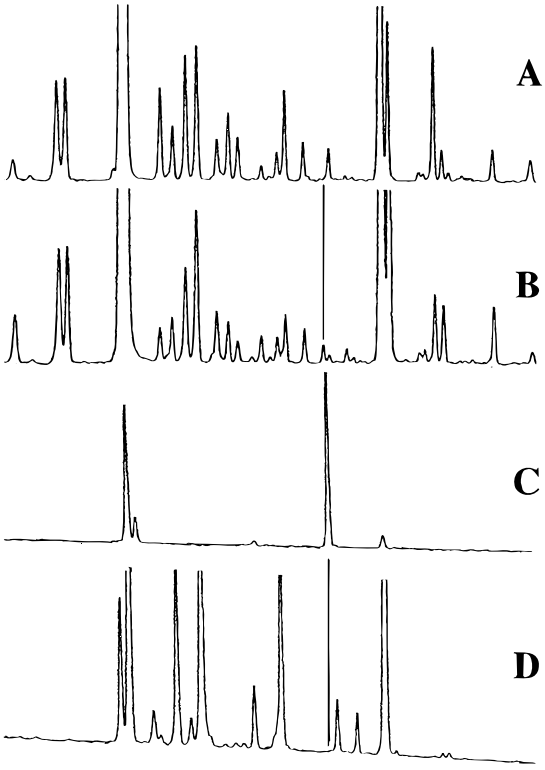

FIGURE 6: Amino acid analysis detection of lanthionine in the TipAS−thiostrepton complex. Amino acid analysis detected lanthionine in (A) TipAS isolated from *E. coli*; (B) TipAS−thiostrepton isolated from *E. coli*; (C) lanthionine; (D) thiostrepton. The proteins were hydrolyzed with HCl in argon for 24 h at 110 °C. The hydrolyzate was treated with dabsyl chloride and then separated on a HPLC reversed-phase C18 column. All peaks, monitored at 436 nm, corresponded to dabsyl derivatives of amino acids. A line was drawn to show the position of the dabsyl derivative of lanthionine. Its elution position was distinct from other dabsyl−amino acid derivatives. This residue was seen only in the TipAS−thiostrepton complex. No other new dabsyl amino acid derivative was seen in this assay. The presence of the lanthionine in the TipAS−thiostrepton complex suggested that the linkage was a covalent one involving a TipAS cysteine and a thiostrepton dehydroalanine.

complex corresponded approximately to the addition of one lanthionine residue per complex. This ratio suggested that the complex was composed of one thiostrepton molecule attached to one molecule of TipAS. Besides the only new peak in the amino acid analysis of the TipAS−thiostrepton complex corresponded to the dabsyl−lanthionine derivative. Therefore, TipAS bound to thiostrepton via a dehydroalanine on thiostrepton and a cysteine on TipAS. These data reaffirmed the stoichiometry of the TipAS−thiostrepton complex obtained from mass spectrometry.

DISCUSSION

Diverse biosynthetic conversions and detoxifications involve nucleophilic attack of thiol groups on electrophilic substrates. Uncontrolled reactions of this type could be detrimental for biological systems, and therefore, regulated enzyme catalysis is usually required. Nevertheless, we observed autocatalytic covalent reactions that occur, both *in vivo* and under physiological conditions *in vitro*, between an electrophilic dehydroalanine of thiostrepton and either free thiols or a cysteine of the TipA proteins. Dehydro-amino acids found in highly modified peptides of the thiostrepton family such as thiopeptin, nosiheptide, berninamycin, and promothiocins undergo similar reactions (Chiu et al., manuscript in preparation). We were interested in these unexpected reactions especially as to how they might relate to the role of thiostrepton as both an inhibitor of ribosome function and as an activator of transcription.

Reactions of cysteine with specific dehydro-amino acids are believed to occur spontaneously to form particular lanthionine thioether rings in lantibiotics such as nisin, subtilin, epidermin, gallidermin, pep5, SA-FF2, and lacticin 481 (Allgaier et al., 1986; Gross & Morell, 1967; Gross et al., 1969; Jack et al., 1994; Kellner et al., 1988; Piard et al., 1993; Weil et al., 1990). Some dehydro-amino acids of these lantibiotics are not involved in ring formation and thus retain their vinyl groups. In the cases of nisin and subtilin, a certain dehydroalanine is essential for biological activity and reacts with free thiols and unidentified macromolecular sulfhydryl groups in the cell envelope of susceptible bacteria (Liu & Hansen, 1990, 1993).

Our understanding of thiostrepton's antibiotic activity is based largely on *in vitro* studies which have demonstrated that it affects various partial reactions of protein synthesis that rely on EF-Tu and EF-G (Cundliffe, 1990). However, our data showed that thiostrepton−thiol conjugates were formed rapidly and spontaneously in standard buffers containing millimolar concentrations of dithiothreitol or 2-mercaptoethanol (Bhuta & Chladek, 1982; Cundliffe et al., 1979; Egebjerg et al., 1989, 1990; Highland et al., 1975; Rosendahl & Douthwaite, 1993, 1994; Thompson & Cundliffe, 1991). Therefore, it is difficult to know to what extent these earlier studies reflect the activity of thiostrepton itself, a specific thiostrepton−thiol conjugate, or a combined effect of the mixture of thiostrepton species. Reported studies of thiostrepton−ribosome interactions could reflect neutral, negative, or positive effects of dehydroalanines (or their thiol derivatives) and should therefore be reassessed individually.

Thiostrepton forms a very tight association with the ribosome. The association constant of this complex (greater than 10^9 M^{-1}) is larger than that of other ribosome-binding antibiotics (Cundliffe, 1990). Although it can bind relatively weakly to a 59 bp region of the 23S rRNA, its binding is significantly enhanced by L11 and L10$(L12)_4$ proteins (Cundliffe & Thompson, 1981). Although thiostrepton "does not appear" to bind L11 by itself (Cundliffe, 1990) and does not react with ribonucleotides, one must consider nevertheless the possibility that this complex is fixed to the ribosome by covalent binding of thiostrepton to ribosomal protein(s).

Thiostrepton also has an activity that is apparently independent of its interactions with ribosomes. Synthesis of more than ten proteins is induced by thiostrepton in *S. lividans* (Murakami et al., 1989; A. M. Puglia and J. Vohradsky, personal communication). A similar response has been subsequently observed in other species (Kumar & Martin, 1994). Two proteins with molecular masses of 17 or 20 kDa accumulate to very high levels, representing greater than 10% of the total soluble cellular protein (Murakami et al., 1989). Data presented here as well as immunoblot experiments showed that the 17-kDa protein corresponded to TipAS and the 20-kDa protein to the TipAS−thiostrepton complex. TipAL also bound to thiostrepton and formed a complex that was as stable to chemical denaturants as the TipAS−thiostrepton complex (data not shown).

Thiostrepton did not react spontaneously with the vast majority of *S. lividans* proteins (data not shown) or any amino acid except cysteine. Either of the two cysteines in

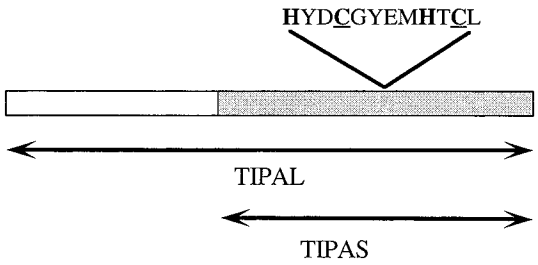

FIGURE 7: The primary sequence of TipAL and TipAS. Both TipAL and TipAS share a carboxy-terminal domain that was involved in the covalent bond to thiostrepton. This domain contained two cysteines, which are underlined and in boldface. Histidine residues, in boldface, are four or three residues away from each cysteine. The amino-terminal domain of TipAL has sequence similarity to other transcriptional regulators with putative helix−turn−helix domains.

the carboxyl-terminal domain of TipAS and TipAL was the primary target of thiostrepton (Figure 7). The only different amino acid that was detected in the TipAS−thiostrepton complex was lanthionine. Chemical reactions involving noncysteine amino acids with dehydro-amino acids have been reported (Greenstein & Winitz, 1961, Schmidt et al., 1979). For example, histidine and lysine can react with dehydro-amino acids at elevated temperature in strong base or in the presence of free radicals. However, none of these reactions are expected to occur under physiological conditions (Asquith & Carthew, 1972, Asquith et al., 1977; Snyder & MacDonald, 1955; Su & Greenstein, 1955). Although NMR and mass spectrometry results indicated that free cysteine could react with the dehydrobutryl group in thiostrepton (data not shown), the absence of a methyl-lanthionine in the TipAS−thiostrepton complex implied either that the TipAS cysteine or the thiostrepton dehydrobutyrine was not available for reaction with each other. In addition, the unusual reactivity of the TipA cysteine for thiostrepton may be related to the proximity of a histidine to either one of the cysteines. A nearby histidine might modulate the sulfhydryl group reactivity to electrophiles (Friedman, 1973; Snow et al., 1975a,b, 1976).

Indeed, eukaryotic glutathione transferase employs such a mechanism to detoxify xenobiotic compounds. A histidine in glutathionine transferase acts as a Lewis base to activate the thiol group of glutathione, which allows it to react covalently with an electrophilic center in the toxic compound (Friedman, 1973).

It is possible that similar detoxification systems involving such thiol-labeling systems are present in *S. lividans*. However, glutathione is absent from *Streptomyces* and other Gram-positive bacteria (Fahey et al., 1978; Fahey & Newton, 1983). Actinomycetes do have millimolar concentrations of cytoplasmic low molecular weight thiols including ovothiol A (497.4 Da), ergothionine (222.7 Da), and U17 (497.4 Da) (Genghof, 1970; Newton et al., 1993, 1995; Spies & Steenkamp, 1994). The additional mass of 564.8 Da that was associated with the TipAS−thiostrepton complex purified from *S. lividans* may correspond to one or a combination of these or other unidentified compounds.

The antibiotic activity of thiostrepton can be inactivated by thiol conjugation. Three thiostrepton−thiol conjugates (thiostrepton−cysteine, thiostrepton−2-mercaptoethanol, and thiostrepton−dithiothreitol) showed drastically reduced (up to 1000-fold lower) antibiotic activity against indicator organisms *Micrococcus luteus* and *S. lividans* 1326 (data not shown). Hence, the dehydro-amino acids of thiostrepton are important for antibiotic activity *in vivo*. Nonetheless these activities could be a result of either changes in uptake or altered antibiotic−ribosome interactions.

Proteins, rather than small molecular weight compounds, are also known to sequester and thus inactivate toxic compounds. These include bleomycin, β-lactam, and fusidic acid binding proteins (Davies, 1994; Glauner et al., 1984; Hayes & Wolf, 1990; Nicholas et al., 1985). Metallothioneins, cysteine-rich proteins that are present in a wide variety of eukaryotes and constitute a major fraction of the intracellular protein thiols, can bind intracellularly to electrophilic drug compounds such as cisdiaminedichloroplatinum, melphalan, and chlorambucil (Kelley et al., 1988).

Although it is clear that thiostrepton might activate such resistance systems, its ability to interact with TipA proteins and induce major changes in gene expression may reflect molecular mimicry involving the dehydroalanine residues. Many enzymes employ transitory association with cofactors or substrates having dehydroalanines or dehydroalanine-like intermediates. These include urocanase (Orum & Rasmussen, 1992; Retey, 1994), tryptophan synthetase (Parkhust & Hodgins, 1972), serine and threonine dehydratase (Christen & Metzler, 1985), proline racemase (Cardinale & Abeles, 1968), alanine racemase (Wasserman et al., 1984), acetylserine sulfhydrolase (Becker et al., 1969), pyruvoyl enzymes (Recsei & Snell, 1984), and many other aminotransferases and decarboxylases (Hayashi et al., 1990). However, stable covalent attachment of the enzymatic product to the protein as seen for TipA proteins has not been reported. Thiostrepton binding to one of these enzymes could interfere with their activity and elicit unpredictable effects.

The reactivity of thiostrepton to thiols or the TipAL proteins may provide some clues to explain the bewildering variety of thiostrepton-induced physiological effects that can be observed in *S. lividans*. Thiostrepton and related compounds may titrate cytoplasmic thiol compounds needed for maintaining native protein conformations (Bardwell, 1994), scavenging of oxygen free radicals (Fahey, 1991), and modulating enzyme activity (Perham, 1991, Reed, 1974), and lead to changes in reduced levels of NADP or NAD (Thomas, 1995). This stress response could be related to regulation of the multiple drug resistance phenotype induced by thiostrepton (Holmes et al., 1993b) or the *Streptomyces* developmental cycle. An 18 amino acid modified peptide (SapB) produced by *S. coelicolor* may act as a pheromone to stimulate genes involved in aerial mycelium formation (Willey et al., 1991, 1993). Covalent binding between a transcriptional regulatory protein and its inducer would be well suited to activate irreversible developmental switches displayed by this group of organisms.

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