Two-component signal transduction systems allow bacteria to sense and respond rapidly to changes in their environment leading to specific gene activation or repression. These two-component systems are integral in the ability of pathogenic bacteria to mount and establish a successful infection within the host and, consequently, have been recognized as targets for new antimicrobial agents. In this paper, we define the site and mechanism of action of several previously identified inhibitors of bacterial two-component systems. We show that the most potent inhibitors target the carboxy-terminal catalytic domain of the sensor kinase and exert their effect by causing structural alterations of the kinase leading to aggregation. Recognition of this phenomenon has important implications for the development of novel inhibitors of two-component systems and should facilitate the rapid identification and elimination of compounds with nonspecific affects from medicinal chemistry drug discovery programs.

Bacteria are highly adaptive organisms capable of growth in an extreme variety of carbon and nitrogen sources and environmental conditions. The key to adaptivity is the large reservoir of genes and pathways encoded in the genome that can be turned on and off at will to reflect the composition of the environment and the available carbon and energy sources. Signals specific for each potential pathway must be sensed and converted to transcription activation or repression by signal transduction systems to facilitate responses to environmental change. One of the major means by which this is accomplished is through two-component signal transduction systems (1). In general, such systems consist of a signal ligand responsive histidine kinase and a response regulator transcription factor. Specific signal ligand binding by the kinase activates an auto-phosphorylation reaction to a histidine residue. This phosphoryl group is transferred to an aspartate of a response regulator domain, which serves to activate its transcriptional properties. In some cases, notably NRII kinase of Escherichia coli, signal ligands work in the opposite manner and activate the phosphatase properties of the kinase (2). Regardless of their mechanism of action such systems serve to interpret the condition of the cellular environment and make adjustments to the repertoire of genes being expressed.

Virulence and pathogenicity are properties of certain bacteria that find themselves in a suitable host environment. Genes and pathways specific for invasion and growth in the host need to be activated in response to signals emanating from that environment. Two-component signal transduction systems play a key role in controlling virulence responses of a wide variety of bacterial pathogens (3). For example, many of the two-component systems of Streptococcus pneumoniae when mutated affect the ability of this organism to mount a successful infection in mouse models without having deleterious effects on growth in laboratory media (4). Two-component signal transduction systems are woven in the fabric of cellular regulation and play important roles in the complex cellular processes required for virulence and pathogenicity (5). Recognition of this role has led the pharmaceutical industry to spend untold millions of dollars on the search for suitable inhibitors of such systems as potential anti-infective agents. The results of several inhibitor screens and structure activity relationship (SAR)1 programs of medicinal chemistry have been published (6–10). While many different compounds have been described with inhibitory activity on these systems, the mechanism(s) of inhibition is largely unknown. In this paper studies to define the means by which several of such inhibitors function are presented.

EXPERIMENTAL PROCEDURES

Reagents—RWJ-49815 was provided by R. W. Johnson Pharmaceuticals. Closantel (N-[5-chloro-4-[(R,S)-4-chlorophenyl]cyclohexylmethyl]-2-methylphenyl-2-hydroxy-3,5-diiodobenzamide) was obtained from Wake Pure Chemical Industries Ltd. 3,3′,4′-4′-Tetrachlorosalicylanilide was purchased from Acrros Organics. Compound-A and AMP-PNP (adenosine 5′-β,γ-imidotriphosphate) were provided by Dr. Bernard Weisblum and Dr. James Zapf, respectively. Ofloxacin and all other reagents were purchased from Sigma.

Biochemical Assay of Kinase Activity—Bacillus subtilis KinA, the carboxy-terminal domain of KinA (KinA-C, KinA amino acids 389–606) and SpoOF were purified according to published protocols (11, 12). The E. coli histidine kinase, NRII (GlnL), was purified as described previously (13). Biochemical assays for the kinases were carried out in 20-μl volumes containing 50 mM K-EPPS buffer (pH 8.5), 20 mM MgCl2, 0.1 mM EDTA and 5% (v/v) glycerol (11).

For autophosphorylation reactions, KinA and NRII were used at final concentration of 0.2 μM and KinA-C was used at 2.5 μM. For KinA-mediated phosphotransfer to SpoOF the proteins were used at final concentrations of 0.2 and 2 μM, respectively. In all cases reactions were initiated by the addition of 100 μCi of γ32P/ATP (PerkinElmer Life Sciences) diluted to a final concentration of 300 μM with cold ATP. All compounds tested as inhibitors were dissolved in Me2SO, and to

1 The abbreviations used are: SAR, structure activity relationship; AMP-PNP, adenosine 5′-β,γ-imidotriphosphate; EPPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Inhibitors of Two-component Signal Transduction Systems

RESULTS AND DISCUSSION

Inhibition of the Activity of Two-component Systems by Antimicrobials—The kinase used to investigate the effects of inhibitors on two-component signal transduction systems is part of the multicomponent phosphorelay controlling cellular differentiation in B. subtilis (16). This kinase, KinA, is responsible for

Inhibitors of Two-component Signal Transduction Systems

reflect the presence of this solvent in the test reactions, it was also included in control reactions at an equivalent concentration (10% v/v). Unless stated otherwise, the inhibitors were used at final concentrations of 100 μM for KinA and NRII and 200 μM for KinA-C. Prior to the initiation of reactions with [γ-32P]ATP, kinases were preincubated with the inhibitors at 25 °C for 30 min. To determine the affect of inhibitors on the total KinA/Spo0F reaction (i.e. KinA autophosphorylation and subsequent phosphotransfer to Spo0F), KinA alone was preincubated for 30 min with inhibitors as described above, and Spo0F was added immediately before initiation of the reaction with [γ-32P]ATP.

Reactions were incubated at 25 °C, stopped by the addition of 0.2 volume of 5× SDS-PAGE sample buffer (250 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 280 mM β-mercaptoethanol, and 0.01% bromphenol blue), flash-frozen in a dry ice/ethanol bath, and stored at −20 °C until required. In the case of KinA, NRII and KinA-mediated phosphotransfer to Spo0F the reactions were allowed to proceed for 4 min. When compared with full-length KinA, the autophosphorylation of KinA-C occurs at a significantly reduced rate (14), and consequently, 2.5 μM of this protein was phosphorylated with [γ-32P]ATP for 60 min at 25 °C to obtain a discernible phosphorylation signal.

To determine whether the prior interaction of KinA and Spo0F could protect this kinase from inhibition by RWJ-49815, KinA was preincubated with Spo0F for 5 min at 25 °C, after which RWJ-49815 (or Me2SO for controls) was added to a final concentration of 16 μM. Incubation was continued for a further 30 min at 25 °C, after which time the reactions were initiated with [γ-32P]ATP. Controls were also included in which KinA was incubated with RWJ-49815 (or Me2SO) for 5 min at 25 °C before the addition of Spo0F and a further incubation for 30 min.

Aggregation of KinA—To investigate aggregation of the kinases by the test compounds, the kinases were first phosphorylated with [γ-32P]ATP. KinA and NRII were incubated for 10 min and KinA-C for 60 min at 25 °C. Phosphorylated kinases were then reacted with each compound for 30 min at 25 °C followed by the addition of glutaraldehyde to a final concentration of 0.22% with incubation at 25 °C for 30 min. Cross-linking was terminated by the addition of SDS-PAGE sample buffer containing glycine to produce a final concentration of 400 mM in the reaction mix. Samples were flash-frozen as before and stored at −20 °C.

SDS-PAGE and Autoradiography—Radiolabeled KinA–P, KinA-C–P, and NRII–P were separated on 10% (w/v) acrylamide electrophoresis gels using the Tricine SDS-PAGE buffer system (15). Spo0F–P was separated on 15% (w/v) gels. Following electrophoresis, the bottom portions of gels were removed to reduce the background from unincorporated [γ-32P]ATP, and the gels were used to expose x-ray film (Kodak, X-Omat XR-1) at −80 °C. The amounts of the phosphorylated forms of the proteins produced were routinely quantified by phosphorimaging (Molecular Dynamics PhosphorImager SF) and analyzed with the associated software (ImageQuant).

Inhibitors of Two-component Signal Transduction Systems

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FIG. 1. Two-component signal transduction inhibitors.

FIG. 2. Inhibition of autokinase and phosphotransfer activities of purified components of bacterial two-component signal transduction systems. A, autoradiograph of a 10% SDS-PAGE gel separating the products of autophosphorylation and phosphotransfer. KinA (lane 1), KinA/Spo0F (lane 2), KinA-C (lane 3), and NRII (lane 4) were labeled with [γ-32P]ATP as described under “Experimental Procedures.” The labeled band in lane 2 that ran between KinA and Spo0F is Spo0F dimer. Label in the Spo0F position in reactions not containing Spo0F is due to spillover from [γ-32P]ATP, which runs at the bottom of the gel. B–E, autophosphorylation and phosphotransfer activities in the presence of potential inhibitors. Reactions and SDS-PAGE gels were run as in A with the label in each protein compared with and without inhibitor. Inhibition of autophosphorylation and phosphotransfer activities of KinA (B), Spo0F (C), KinA-C (D), and NRII (E) in the control (lane 1) or in the presence of RWJ-49815 (lane 2), Ofloxacin (lane 3), Clofazimine (lane 4), tetra-chorosalicylanilide (lane 5), or compound-A (lane 6). Inhibitors were used at 100 μM with KinA and NRII and 200 μM for KinA-C. Spo0F results were obtained using a 15% SDS-PAGE gel to prevent labeled ATP contamination.
the majority of phosphate input into the phosphorelay and transfers phosphate to its cognate response regulator Spo0F. KinA is a dimeric molecule composed of an amino-terminal “sensing” domain and a carboxyl-terminal “kinase” domain containing the catalytic histidine and four-helix bundle characteristic of sensor kinases (17, 18).

The potential inhibitors used in this study are shown in Fig. 1. Of the compounds selected, RWJ-49815, Closantel, tetrachlorosalicylanilide, and compound-A have been shown previously to be active against various bacterial two-component signal transduction systems (6, 8, 19). Ofloxacin, a quinolone antimicrobial that acts via DNA gyrase/topoisomerase (20), was included in this study because the ATP binding site of histidine kinases resemble that of gyrase (21).

The inhibitory activity of the compounds on the in vitro autophosphorylation and phosphotransfer activities of purified components of two bacterial two-component signal transduction systems was tested using a phosphorylation assay with [γ-32P]ATP. Autophosphorylation and phosphotransfer activities of the kinases used in this study are shown in Fig. 2A. When compared with control reactions addition of the inhibitor compounds resulted in varying levels of inhibition of autokinase and phosphotransfer activities of the purified components (Figs. 2, B–E). Of the compounds tested, the most potent inhibitors of the B. subtilis KinA/Spo0F system were RWJ-49815 and Closantel, consistent with previous observations (6, 8). Both RWJ-49815 and Closantel resulted in the accumulation of less than 3% of the KinA–P formed in the control reaction (Fig. 2B) with a comparable reduction in the amount of phosphorylated Spo0F (Fig. 2C).

To determine whether these compounds targeted the signal input domain or the autokinase domain of KinA, the autokinase domain was produced free of the 388-amino acid input domain. RWJ-49815 and Closantel drastically affected the residual autophosphorylation of the KinA carboxyl-terminal autokinase domain, KinA-C (Fig. 2D), indicating that the site of action of these two compounds is located within this domain of KinA. Furthermore, RWJ-49815 and Closantel almost completely (<3% of the control) inhibited the autokinase activity of NRII (Fig. 2E), suggesting a common mode of action of these inhibitors on the two-component systems from B. subtilis and E. coli used in this study.

As was observed previously (8), tetrachlorosalicylanilide had a significant inhibitory affect on the autokinase activity of KinA and phosphotransfer to Spo0F, resulting in the accumulation of 50 or 25% of the amount of KinA–P and Spo0F–P formed, respectively, in control reactions (Fig. 2, B and C). However, tetrachlorosalicylanilide did not cause a corresponding reduction in the residual autokinase activity of KinA-C (Fig. 2D), suggesting that the site of action of this inhibitor on KinA is located at a site distinct from the carboxyl terminus, or truncation of KinA affects the conformation of the autokinase domain. The autophosphorylation of NRII was not as severely affected by tetrachlorosalicylanilide as it was by RWJ-49815 and Closantel; the level of NRII–P formed in the presence of tetrachlorosalicylanilide was reduced to 70% of the control (Fig. 2E).

Compound-A, which has been shown to be active against certain bacterial two-component systems (19), inhibited the autophosphorylation of KinA (60% of the control, Fig. 2B) but, surprisingly, did not appear to cause a corresponding reduction in the amount of Spo0F–P formed (Fig. 2C). KinA-C was largely unaffected by compound-A (Fig. 2D), and the amount of NRII–P formed was reduced to 70% of the control reaction (Fig. 2E). Finally, Ofloxacin showed slight inhibition of the autophosphorylation of KinA and NRII so that the amounts of the phosphorylated forms of the proteins were reduced to ~80% of the control reactions (Figs. 2, B and E).

Thus for the autophosphorylation and phosphotransfer activities, RWJ-49815 and Closantel were found to be potent inhibitors of the B. subtilis KinA/Spo0F system, in agreement with previous observations (6, 8). Furthermore, these data define the major site of action of these compounds on KinA, since both RWJ-49815 and Closantel completely inhibit the autokinase activity of the isolated carboxyl-terminal domain as well as the whole kinase.

The Mechanism of Inhibition—At this point it was unclear how the active compounds inhibited the kinases other than by targeting the COOH-terminal kinase domain. Kinetic experiments with RWJ-49815 and KinA showed uncompetitive kinetics with ATP, suggesting the compounds do not mimic ATP. In view of the hemolytic activity of RWJ-49815 (22), the possibility was entertained that the active compounds may cause structural perturbations in the kinase. One means to test for this possibility is to use cross-linking agents whose activity may be affected by conformational changes in the kinase.

In the autophosphorylation reaction with [γ-32P]ATP and KinA, the addition of SDS-loading buffer dissociates the la-

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K. Stephenson, Y. Yamaguchi, and J. A. Hoch unpublished data.
beled KinA dimer to monomer (Fig. 3A, lane 1). When KinA–P was cross-linked with glutaraldehyde in the absence of any inhibitor, the expected higher molecular mass form corresponding to the kinase dimer (~138 kDa) was produced (Fig. 3A, lane 2) and did not dissociate in SDS-loading buffer. However, when KinA–P was incubated with RWJ-49815 or Closantel with subsequent cross-linking, no kinase dimer could be detected on the gel (Fig. 3A, lanes 3 and 5). In both cases the failure to detect the dimer form was accompanied by a large amount of aggregated kinase remaining in the stacking gel. Tetrachlorosalicylanilide also caused a slight reduction (~40%) in the amount of KinA–P dimer, and a small amount of aggregated protein remained in the stacking gel (Fig. 3A, lane 6). In contrast, neither Ofloxacin or compound-A caused detectable aggregation of the kinase. Furthermore, the nonhydrolyzable ATP analogue AMP-PNP, which inhibits kinases by binding irreversibly to the active site, did not cause aggregation of KinA–P (Fig. 3A, lane 8). Although the affect on the phosphorylated kinase is reported here, RWJ-49815 and Closantel also cause aggregation of unphosphorylated KinA3.

The site of action of the inhibitors on the KinA molecule was further investigated using the isolated carboxyl-terminal domain. In the absence of inhibitors, phosphorylated KinA-C was cross-linked to a higher molecular mass form the size of the KinA-C dimer (~48 kDa, Fig. 3B, lane 2). In a manner identical to the complete KinA protein (Fig. 3A), RWJ-49815 and Closantel caused aggregation of the isolated carboxyl-terminal domain indicated by an absence of KinA-C–P dimer and a concomitant increase in the amount of material remaining in the stacking gel (Fig. 3B, lanes 3 and 5). None of the other compounds tested caused a significant reduction in the amount of KinA-C–P dimer (Fig. 3B). Moreover, when RWJ-49815 and Closantel were tested for their ability to aggregate NRII–P, a similar affect was observed (Fig. 3C, lanes 3 and 5); a reduction in the amount of dimerized kinase with a corresponding increase in aggregated protein that was retained within the stacking gel. Additionally, these two compounds appeared to affect the dimerization of NRII–P, since an increase in the monomer form was also observed that was not present in the cross-linked control reaction (Fig. 3C). Ofloxacin did not cause significant aggregation of NRII–P (Fig. 3C).

The relationship between RWJ-49815 concentration and the relative degree of KinA aggregation was investigated. As expected, increasing the concentration of RWJ-49815 caused a corresponding increase in the amount of aggregated KinA–P that remained in the wells of the stacking gel with a concomitant reduction in the amount of kinase dimer (Fig. 4). A concentration of RWJ-49815 as low as 16 μM caused considerable aggregation of the kinase, and it should be noted that we have previously reported that this concentration is sufficient to cause almost complete inhibition of autophosphorylation of KinA, when assayed under standard conditions (6).

Since the majority of the inhibition of the activity of KinA by RWJ-49815 occurs by protein aggregation, it was of interest to determine whether Spo0F could protect the kinase from the activity of this inhibitor. KinA was incubated with RWJ-49815 before or after the kinase and Spo0F were allowed to interact for 5 min. Prior interaction with Spo0F protected KinA from the inhibitory affects of RWJ-49815, as indicated by the presence of the response regulator drastically reduced the amount of Spo0F–P (Fig. 5, lane 3), indicating that Spo0F cannot reverse the inhibition of the kinase. These data demonstrate that the interaction of KinA and Spo0F protects the kinase from the inhibitory activity of RWJ-49815, suggesting that the response regulator reduces the accessibility of this compound to its site of action on KinA. However, Spo0F cannot protect at high levels of RWJ-49815. This is in agreement with

3 K. Stephenson, Y. Yamaguchi, and J. A. Hoch, unpublished observations.
our observation that RWJ-49815 targets the carboxyl-terminal domain of KinA.

The data described here show that the major mechanism of action of RWJ-49815 and Closantel on the autokinase activity of KinA is structural alteration of the kinase leading to aggregation that results in inhibition of the catalytic activity of the enzyme and that this effect is mediated through the carboxyl-terminal kinase domain. Cross-linking was required to detect large aggregates of the kinases, since when tested in the standard biochemical assay only apparent inhibition of autophosphorylation may be observed. Under these assay conditions (i.e., without cross-linking) aggregates were not visible, due to a combination of solubilization in sample buffer and the inability of the denatured kinase to autophosphorylate. Finally, RWJ-49815 and Closantel strongly inhibit the autokinase activity of NRII (Fig. 2E) by aggregation (Fig. 3C), indicating a common mode of action on this unrelated kinase from E. coli.

Previous studies with RWJ-49815 showed this compound to have hemolytic activity suggesting RWJ-49815 acts as a non-specific detergent (22). Closantel, on the other hand, did not hemolyze red cells, indicating, perhaps, a different mechanism of inhibition. The studies reported have shown that this is unlikely, since both compounds result in the same effects on both KinA and NRII. It seems possible that the medicinal chemistry SAR that generated RWJ-49815 and other inhibitors results in the production of enzyme denaturants with limited specificity. Thus RWJ-49815 finds a target in the red cell membrane, and Closantel does not but both interact similarly with the kinases. It is therefore not surprising that SAR studies in a number of instances find hydrophobic planar aromatic molecules where minor substituent groups play a major role in specificity.

Structural studies on fragments of histidine kinases have revealed that the phosphotransferase four-helix bundle of the autokinase domain is likely to be the dimerization surface of this domain (17, 18, 23). The observed effects of molecules, such as RWJ-49815 on KinA and NRII, suggest these compounds may intercalate in the hydrophobic core of the four-helix bundle driving the bundle apart and exposing hydrophobic surfaces that interact incorrectly, causing aggregation (Fig. 6). The multiplicity of compounds that can cause inhibition by aggregation generates a surfeit of lead compounds that result in SAR programs around the wrong compounds. Recognition of this phenomenon should lead to rapid elimination of spurious lead compounds.
The Mechanism of Action of Inhibitors of Bacterial Two-component Signal Transduction Systems
Keith Stephenson, Yasuchika Yamaguchi and James A. Hoch

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