Kinetic and mechanistic analyses of new classes of inhibitors of two-component signal transduction systems using a coupled assay containing HpkA–DrrA from *Thermotoga maritima*

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Two-component signal transduction systems (TCSs) play fundamental roles in bacterial survival and pathogenesis and have been proposed as targets for the development of novel classes of antibiotics. A new coupled assay was developed and applied to analyse the kinetic mechanisms of three new kinds of inhibitors of TCS function. The assay exploits the biochemical properties of the cognate HpkA–DrrA histidine kinase–response regulator pair from *Thermotoga maritima* and allows multiple turnovers of HpkA, linear formation of phosphorylated DrrA, and Michaelis–Menten analysis of inhibitors. The assay was validated in several ways, including confirmation of competitive inhibition by adenosine 5′-β,γ-imidotriphosphate (AMP-PNP). The coupled assay, autophosphorylation and chemical cross-linking were used to determine the mechanisms by which several compounds inhibit TCS function. A cyanoacetoacetamide showed non-competitive inhibition with respect to ATP concentration in the coupled assay. The cyanoacetoacetamide also inhibited autophosphorylation of histidine kinases from other bacteria, indicating that the coupled assay could detect general inhibitors of histidine kinase function. Inhibition of HpkA autophosphorylation by this compound was probably caused by aggregation of HpkA, consistent with a previous model for other hydrophobic compounds. In contrast, ethdin was a potent inhibitor of the combined assay, did not inhibit HpkA autophosphorylation, but still led to aggregation of HpkA. These data suggest that ethdin bound to the HpkA kinase and inhibited transfer of the phosphoryl group to DrrA. A peptide corresponding to the phosphorylation site of DrrA appeared to inhibit TCS function by a mechanism similar to that of ethdin, except that autophosphorylation was inhibited at high peptide concentrations. The latter mechanism of inhibition of TCS function is unusual and its analysis demonstrates the utility of these approaches to the kinetic analyses of additional new classes of inhibitors of TCS function.

INTRODUCTION

Bacteria have the ability to sense and respond to changes in their environment. This capability is necessary for growth and survival of many bacteria and for virulence of pathogens. Much of this ability to sense and adapt to environmental stimuli is mediated through two-component signal transduction systems (TCSs) (reviewed by Hoch & Varughese, 2001; Inouye & Dutta, 2003; Stock et al., 2000; West & Stock, 2001). These TCSs typically consist of a sensor histidine kinase and a cognate response regulator (Fig. 1). The histidine kinase is often an integral membrane protein in which the sensor domain is extracellular and the catalytic kinase domain is intracellular (Inouye & Dutta, 2003). Upon binding of a ligand or sensing a change in the...
Pathogenic bacteria face a number of challenges during invasion of a host, especially the changing environment experienced by the bacterium during the pathogenic process. Many pathogenic bacteria utilize TCSs to sense and adapt to these environmental changes (Groisman, 2001; Hubbard et al., 2003; Kallipolitis & Ingmer, 2001; Stephenson & Hoch, 2002; Throup et al., 2000). The importance of these TCSs to the pathogenic process is emphasized by the many examples of attenuated virulence observed with pathogenic strains in which one or more TCS have been deleted (Groisman, 2001; Hubbard et al., 2003; Kallipolitis & Ingmer, 2001; Stephenson & Hoch, 2002; Throup et al., 2000). In addition, many pathogenic bacteria contain TCSs that are essential for viability. For example, Staph. aureus and Strept. pneumoniae contain single TCSs essential for viability, whereas Helicobacter pylori contains four (Beier & Frank, 2000; Fabret & Hoch, 1998; Lange et al., 1999; Martin et al., 1999; Stephenson & Hoch, 2002; Throup et al., 2000).

Besides their role in virulence, TCSs have been implicated in the antibiotic resistance of a number of clinically important pathogenic strains (Walsh, 2003; Wright et al., 1993). Vancomycin is considered one of the last lines of defence in antibiotic therapy. However, vancomycin resistance has now become common in enterococci. The resistance to vancomycin in these strains is mediated by the VanRS TCS, which responds to cell wall damage by turning on expression of a set of genes that results in the synthesis of a peptidoglycan containing a modified pentapeptide chain (reviewed by Walsh, 2003). Vancomycin-binding to strains carrying this modification is significantly reduced, rendering the antibiotic ineffective. A TCS designated temporarily as VncRS was reported to mediate vancomycin tolerance in Strept. pneumoniae, possibly through the action of a toxic peptide (Novak & Tuomanen, 2003; Novak et al., 1999). However, later work showed that this tolerance to vancomycin depended in part on reduced growth caused by erythromycin in growth media (Robertson et al., 2002) and not on the VncRS TCS per se.

The roles of TCSs in viability, pathogenesis and antibiotic resistance have led to numerous efforts by industrial and academic laboratories to develop small molecules that inhibit these systems (Hilliard et al., 1999; Hubbard et al., 2003; Matsushita & Janda, 2002; Stephenson & Hoch, 2002). Some approaches have targeted specific TCSs, such as AgrCA (Lyon et al., 2000), whereas other approaches have been aimed at identifying inhibitors that target a broad collection of TCSs (reviewed by Matsushita & Janda, 2002; Stephenson & Hoch, 2002). In the latter case, the presence of conserved domains within histidine kinases and response regulators (Hoch & Varughes, 2001; Stock et al., 2000; West & Stock, 2001) has provided the rationale that a single molecule could inhibit multiple TCSs. Most searches for small-molecule inhibitors have been based on assays of either autophosphorylation of histidine kinases or phosphotransfer from histidine kinases to response regulators (Matsushita & Janda, 2002; Stephenson & Hoch, 2002). Each of these assays has certain limitations, and kinetic analyses of inhibitors have not been widely reported, partly because of these limitations (e.g. see Stephenson & Hoch, 2002). Here we report a new coupled assay using the HpkA histidine kinase and DrrA response regulator of Thermotoga maritima, first characterized by Stock and coworkers (Goudreau et al., 1998; Lee & Stock, 1996). The coupled assay, which allows analysis of the steady-state environment, the kinase undergoes autophosphorylation on a conserved histidine residue. The signal is then propagated by transfer of the phosphoryl group from the histidine kinase to a conserved aspartate residue in the response regulator. The phosphotransfer activates the response regulator, which typically acts to change gene expression. See text for further details.

**Fig. 1.** Typical TCS pathway in bacteria. The histidine kinases consist of a sensor domain exposed to the outside of the cell membrane, a membrane-spanning domain, and a cytoplasmic histidine kinase domain. Upon stimulation of the sensor domain, the histidine kinase autophosphorylates specific histidine residues located in a helix bundle in its cytoplasmic domain. The cognate response regulator catalyses the transfer of the phosphoryl group from the histidine of the kinase to a conserved aspartate residue in the response regulator. The phosphotransfer activates the response regulator, which typically acts to change gene expression. See text for further details.
turnover of histidine kinase activity, was used to determine the kinetic properties of several new classes of compounds that inhibit autophosphorylation or phosphotransfer of TCs.

METHODS

Reagents. Unless otherwise noted, all chemicals and biochemicals, including adenosine 5'β,γ-imidotriphosphate (AMP-PNP), ATP and ethidin (6,9-diamino-2-oxetyl acidine lactate) were obtained from Sigma. Cyaanoacetamide (CAMA) was obtained from the archive compound library at Lilly. Closantel (N-[5-chloro-4-[[R,S]-4-chlorophenyl]cyclohexylmethyl]-2-hydroxy-3,5-dioxobenzamide) was obtained from Wako Pure Chemical Industries. [γ-32P]ATP and [γ-32P]ATP were purchased from Perkin Elmer-NEN. The DrRa peptide MFIWAL was purchased from ResGen (Invitrogen). FPLC HiTrap chelating columns were from Amersham Pharmacia Biotech and were run using a Pharmacia FPLC system. Wide-range protein standards, electrophoresis reagents, SDS-polyacrylamide gels (4–20%), and Colloidal Blue staining kit were from Invitrogen. MultiScreen-Immobilon-P hydrophobic membranes. 96-well filter plates were from Millipore. Ecoscint 20 scintillation fluid was from Packard.

Construction of plasmids expressing His-tagged HpkA and DrRa. Expression vectors were constructed to allow His-tagged versions of HpkA and DrRa to be purified from Escherichia coli (Novagen technical literature, 2003; www.novagen.com). A DNA fragment corresponding to a truncated HpkA, lacking 77 N-terminal amino acids (HpkA77), was amplified from chromosomal DNA of Thermotoga maritima [American Type Culture Collection (ATCC); product no. 43589] by PCR with oligonucleotides primers hpkA5' (5'-AAGTCTCTCAGGCAATATGAGCCTGACGAGAAC-3') and hpkA3' (5'-GGTATCGTTATGCGACTTTACCTGCTGATGACTGAGT-3') and cloned into the Ndel and BamHI restriction sites of pET-16B (Novagen), producing N-terminally His-tagged HpkA77. The same procedure was used to produce a full-length, N-terminally His-tagged HpkA77. The resulting constructs were confirmed by DNA sequencing.

Expression and purification of His-HpkA77 and DrRa. His-HpkA77 was expressed in E. coli BL21(DE3)pLYS3 (Novagen). Cells were grown to mid-exponential phase (OD600 ~ 0.5) in LB medium containing 100 μg ampicillin ml−1 and expression was induced by the addition of IPTG to a final concentration of 0.4 mM. Induction was continued at 25°C for 4–5 h. For purification, cells were lysed by sonication on ice (6 min total, 30 s pulse on, 45 s off). All remaining steps were performed at 4°C. Membranes and cell debris were removed by ultracentrifugation (Beckman Ti70 rotor, 40,000 r.p.m., 60 min). Supernates were applied to a 5 ml HiTrap chelating Sepharose column (previously charged with NiCl2 in water) equilibrated in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4, containing protease inhibitor cocktail III lacking EDTA (Calbiochem). After washing the 5 ml HiTrap chelating column with 10× the column volume of binding buffer, His-HpkA77 was eluted with a gradient of 0–0.5 M imidazole in the binding buffer. His-HpkA77 fractions were pooled, dialysed extensively against buffer (20 mM sodium phosphate, 0.2 M NaCl, pH 7.4), concentrated using an Ultra-15 centrifugal filter unit (Amicon), and stored at −80°C. Protein concentrations were determined using a Coomassie Plus Protein Assay Kit (Pierce), and purity was assessed by Coomassie staining of SDS-PAGE gels (see Fig. S1, available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). Approximately 5–10 mg of His-HpkA77 was obtained from 1 litre of cells. His-HpkA77 at a concentration of 2 μM was shown to exist primarily as a dimer by chemical cross-linking experiments (see below). Dimerization was also likely at concentrations of 80 nM and 20 nM, because His-HpkA77 was active in autophosphorylation and coupled reactions, respectively, at these lower concentrations (see below).

Expression and purification of His-DrRa. His-DrRa was expressed in E. coli BL21(DE3)pLYS3 and purified as described above for His-HpkA, except that the following buffers were used. The binding buffer for DrRa was 50 mM Tris/HCl 0.1 M KCl, pH 8–9, and the dialysis buffer was 20 mM Tris/HCl, pH 8–9, containing 0.5 M arginine to retard protein aggregation (see supplementary Fig. S1).

Initial velocity of autophosphorylation. The rate of autophosphorylation was determined by incubating 80 nM His-HpkA77 in autophosphorylation buffer (50 mM Tris/HCl, pH 8–5, 50 mM KCl, 5 mM MgCl2, 0.5 mM EDTA and 1 mM DTT) (Goudreau et al., 1998) containing ATP concentrations ranging from 1 to 400 μM with 90–180 μCi [γ-32P]ATP, in the presence of 10% DMSO. Following equilibration at 37°C, reactions were initiated by addition of ATP. For kinetic analyses, 6 μl aliquots were removed at various times over 5 min and quenched with 60 mM EDTA, pH 8–9, which was shown to stop the reaction in control experiments. SDS-PAGE sample buffer was added to the quenched reactions, and samples were applied without heating to 4–20% (w/v) Tris-glycine SDS-polyacrylamide gels. After electrophoresis, the bottoms of the gels were removed to lower the background from the unincorporated radiolabelled ATP. Gels were dried without staining on a BioRad GelAir drying system and exposed to a PhosphorImager screen, which was analysed using a PhosphorImager SF and ImageQuant software (Molecular Dynamics). The amount of [γ-32P]-His-HpkA77 in each lane was quantified by comparison with a standard curve of spotted [γ-32P]ATP. Initial rates of autophosphorylation of His-HpkA77 were calculated from linear regression analysis of plots of phosphorylated His-HpkA77 (HpkA-P) formed versus time.

HpkA77-DrRa combined assay. Assays were performed at 37°C, either in 96-well microtitre plates or in microcentrifuge tubes. Two assay formats were employed for determination of enzyme activity: SDS-PAGE gels and Immobilon-P 96 PVDF filters contained in a 96-well multiscan vacuum manifold (Millipore). In each well or tube, 20 nM His-HpkA77, 4 μM His-DrRa and 5% DMSO in reaction buffer (50 mM Tris/HCl, pH 8–5, 50 mM KCl, 5 mM MgCl2 and 1 mM DTT) were pre-incubated at 37°C for 15 min. Reactions were initiated by the addition of ATP, with concentrations ranging from 1 to 512 μM [γ-32P]ATP [4–245 Ci mmol−1 (148–9065 GBq mmol−1), gel format] or from 1 to 512 μM [γ-32P]ATP [3–121 Ci mmol−1 (110–4480 GBq mmol−1), filter format]. Reactions were stopped at various times (see figure legends) by the addition of equal volumes of SDS sample buffer (gel format) or 60 mM EDTA, pH 8–9 (filter format). For the gel format, samples were applied directly to SDS-PAGE gels without heating and analysed as described above. For the filter format, samples were transferred to a pre-wetted 96-well filter plate connected to a vacuum line at room temperature and washed with buffer (50 mM Tris/HCl, pH 8–5, 500 mM NaCl), and then water. Filters were dried for 2 h at 37°C. Microscint 20 scintillation fluid (Packard) was dispensed into each well and the radioactivity was determined using a Packard Top Count liquid scintillation counter.

Temperature dependence of HpkA77-DrRa combined assay. The HpkA77-DrRa combined assay was performed as described above except that 100 μM [γ-32P]ATP (45 Ci mmol−1, 1665 GBq mmol−1) was used at a range of temperatures. The samples were pre-incubated
DC50 values, defined as the concentration of an inhibitor at which bands, and percentage activity or percentage dimer were calculated equal areas were subtracted from the intensities of the His-HpkA scanned using ImageQuant software. Backgrounds of approximately cross-linked fractions were silver-stained (Invitrogen), dried, and cross-linked fractions) were analysed as described above. Gels of were applied to denaturing polyacrylamide gels. Activity gels (non-glycine to a final concentration of 400 mM. The reaction products were terminated by addition of SDS-PAGE sample buffer containing

Inhibitor studies. Unless otherwise indicated, inhibitors were dissolved in 100 % DMSO. When testing inhibitors, the final DMSO concentration in the assays was either 5 % or 10 % (v/v), as indicated in the figure legends. Controls lacking inhibitors contained an equal concentration of DMSO. Inhibitor–response curves were analysed by three-parameter fits using GraphPad Prism 3.0 software to determine IC50 values, defined as the concentration of compound giving 50 % inhibition of enzyme activity (Copeland, 2000). Inhibitor studies. The effect of enzyme inhibitors on the oligomeric state of His-HpkA77 was investigated by a modification of the method reported by Stephenson et al. (2000). Inhibitors at a range of concentrations were incubated with 2 μM His-HpkA77 in a pH 8.5 assay buffer (5 mM K-EPPS, pH 8.5, 20 mM MgCl2, 0.1 mM EDTA, 5 %, v/v, glycerol) (Stephenson et al., 2000). After a 2 min pre-incubation at 42 °C, autophosphorylation reactions were initiated by the addition of 45 μCi [γ-32P]ATP diluted to a final concentration of 20 μM with unlabelled ATP. After 30 min the sample was split into two equal parts. One fraction was cross-linked for 30 min by the addition of glutaraldehyde to a final concentration of 22 % (v/v). The autophosphorylation and cross-linking reactions were terminated by addition of SDS-PAGE sample buffer containing glycine to a final concentration of 400 mM. The reaction products were applied to denaturing polyacrylamide gels. Activity gels (non-cross-linked fractions) were analysed as described above. Gels of cross-linked fractions were silver-stained (Invitrogen), dried, and scanned using ImageQuant software. Backgrounds of approximately equal areas were subtracted from the intensities of the His-HpkA bands, and percentage activity or percentage dimer were calculated relative to control samples that had not been treated with inhibitor. DC50 values, defined as the concentration of an inhibitor at which only 50 % of His-HpkA was present as a dimer, were determined by three-parameter fits using GraphPad Prism 3.0 software.

Results

Enzymic activities of His-HpkA77 and His-DrrA

The histidine kinase–response regulator pair HpkA–DrrA was purified and characterized from T. maritima previously (Goudreau et al., 1998; Lee & Stock, 1996). We used a modification of the published protocols to clone and purify His-tagged versions of HpkA77 and DrrA (see Methods and supplementary Fig. S1). Purified His-HpkA77 autophosphorylated in the presence of ATP (Fig. 2). Addition of the His-DrrA response regulator resulted in loss of the phosphoryl group from His-HpkA77 with concomitant gain of phosphate by His-DrrA, suggesting phosphotransfer between the two proteins (Fig. 2). Both of these activities (autophosphorylation and phosphotransfer) were consistent with previous data that established HpkA–DrrA as a cognate TCS (Goudreau et al., 1998).

The time-course of autophosphorylation of HpkA was non-linear and displayed an exponential curve typical of first-order reactions (data not shown). A first-order reaction mechanism was expected, because HpkA is effectively consumed during the reaction as it becomes autophosphorylated and is then unable to turn over again without loss of the phosphoryl group. Despite the non-linear nature of the reaction, the initial rates of autophosphorylation were estimated by following the reaction over a very short time after addition of ATP. Such an analysis yielded a hyperbolic dependence of the initial rate on the ATP concentration (see Fig. S2, available as supplementary data with the online version of this paper at http://mic.sgmjournals.org), with an estimated apparent Km for ATP of 25 μM for His-HpkA77 (Table 1).

Development of a combined autophosphorylation and phosphotransfer assay

To investigate the kinetics of steady-state turnover of HpkA, we developed an assay format that combines both autophosphorylation and phosphotransfer. In this assay, His-HpkA77 was present in catalytic amounts, whereas ATP and His-DrrA were treated as substrates and were added in vast excess (see Methods). The assay was based on the prior finding that the product of the reaction, phosphorylated DrrA (DrrA-P), is relatively stable at 37 °C (t1/2 ~ 4.7 h in the presence of Mg2+; Goudreau et al., 1998). Under these conditions, the assay displayed linear kinetics, and the rate of appearance of DrrA-P was linear with time (see Fig. S3, available as supplementary data with the online version of this paper at http://mic.sgmjournals.org).
Table 1. Kinetic parameters for the HpkA–DrrA combined and HpkA autophosphorylation assays determined at 37 °C and 67 °C

See Methods for details of the assays.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Combined assay</th>
<th>Autophosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>DrrA</td>
</tr>
<tr>
<td></td>
<td>$V_{\text{max}}$ (nM min$^{-1}$)</td>
<td>$K_m$ (μM)</td>
</tr>
<tr>
<td>37</td>
<td>0.287 ± 0.017 n = 8</td>
<td>260 ± 5.2 n = 8</td>
</tr>
<tr>
<td>67</td>
<td>2.19 ± 0.32 n = 5</td>
<td>216 ± 69 n = 5</td>
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In addition, the rate of the combined reaction was dependent on the HpkA concentration. Doubling the concentration of HpkA doubled the rate of the reaction (supplementary Fig. S3). The above observations are consistent with a steady-state kinetic mechanism in which the HpkA is turning over multiple times. Using this assay format, we determined an apparent $V_{\text{max}}$ of His-HpkA77 and an apparent $K_m$ for both ATP and DrrA (Table 1; see also Fig. S4, available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). The $K_m$ of 26.0 μM determined for ATP was similar to that determined for the autophosphorylation assay (25.4 μM; supplementary Fig. S2, Table 1). The $K_m$ for DrrA was 0.175 μM.

The combined assay was temperature dependent between 27 and 82 °C. The initial rate increased sharply from 42 °C, reached an optimum at 67 °C, and declined at temperatures greater than 67 °C (data not shown). The high temperature for optimum activity is consistent with the source of the enzyme from a thermophilic organism and with the maximum activities of the individual autophosphorylation and phosphotransfer reactions described previously (Goudreau et al., 1998). The $V_{\text{max}}$(ATP) at 67 °C was calculated to be 2.19 nM DrrA-P formed min$^{-1}$, which was 10-fold greater than that at 37 °C (Table 1). The $K_m$(ATP) also increased significantly with temperature, from 26.0 μM at 37 °C to 216 μM at 67 °C (Table 1). Although the enzymes were more active at 67 °C, the DrrA-P product was less stable. The His-DrrA-P was stable for up to 2 h at 37 °C, but only for 30 min at 60 °C (data not shown). The decreasing stability of DrrA-P at increasing temperatures confirmed the results of Goudreau et al. (1998).

Validation of the combined assay with AMP-PNP

We validated the combined assay format for the determination of the kinetic mechanisms of action of inhibitor compounds by using AMP-PNP, which is a non-hydrolysable analogue of ATP (Fig. 3A). AMP-PNP often inhibits ATP-dependent reactions by competing for the active site of enzymes (e.g. Stephenson et al., 2000). The experiments were done at 37 °C. Besides the inherent stability of DrrA-P, another advantage of performing the combined assay at 37 °C was preservation of potential inhibitor compounds, which potentially would degrade at higher temperatures. AMP-PNP inhibited the combined reaction (Fig. 4). The degree of inhibition was dependent on ATP concentration, and a mechanistic analysis indicated that the AMP-PNP was competitive with ATP (Fig. 4). The $K_i$ for AMP-PNP was calculated as 100 μM. The concentration of DrrA in the reaction had no effect on the inhibition by AMP-PNP (data not shown), suggesting that this compound did not affect the phosphotransfer reaction. Thus the combined assay was a valid format to study mechanisms of action of inhibitor compounds.

Validation of aggregation analyses

A recent study by Stephenson et al. (2000) concluded that certain inhibitors of histidine kinases exert their action through aggregation of the enzyme. In that analysis, the phosphorylated histidine kinase was formed before compounds were added. We were concerned that this protocol...
separated autophosphorylation from the effect of inhibitors on aggregation. Therefore, in our experiments, the inhibitor compound was added to the histidine kinase at the same time as ATP (see Methods). This allowed a direct comparison to be made between inhibition of autophosphorylation and level of aggregation. Using this approach, we studied the effects of AMP-PNP on the autophosphorylation and aggregation state of His-HpkA77. In the absence of inhibitors, cross-linked His-HpkA77 ran as a dimer on SDS-PAGE, consistent with the native form of the enzyme. As expected, the autophosphorylation activity of His-HpkA77 decreased as the concentration of AMP-PNP increased (Fig. 5a). Within the error of these experiments, increasing concentrations of AMP-PNP did not have a significant effect on the oligomeric state of His-HpkA77 determined on silver-stained protein gels (Fig. 5b). These results are consistent with those obtained by Stephenson et al. (2000) for inhibition of KinA by AMP-PNP.

In final validation experiments, we tested the effect of the known inhibitor closantel (Fig. 3B) on His-HpkA77 autophosphorylation activity and cross-linking. Stephenson et al. (2000) reported that closantel strongly inhibited the autophosphorylation activities of two different histidine kinases and led to aggregation of phosphorylated kinases. In agreement with that report, increasing concentrations of

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**Fig. 4.** Competitive inhibition of the HpkA–DrrA combined assay by AMP-PNP. (a) Lineweaver–Burk plot of His-DrrA-P formation in the presence of varying concentrations of AMP-PNP (■, 0 µM; ▲, 50 µM; ▼, 100 µM; ◆, 200 µM). Lines were drawn based on linear regression analysis. (b) IC₅₀ determinations of AMP-PNP at different ATP concentrations. IC₅₀: 67.3 µM and 530.4 µM at ATP concentrations of 1 µM (■) and 100 µM (▲), respectively.

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**Fig. 5.** Inhibition of autophosphorylation and lack of aggregation of His-HpkA77 in the presence of AMP-PNP. Inhibition of His-HpkA autophosphorylation and percentage His-HpkA dimer remaining following cross-linking with glutaraldehyde were determined as described in Methods, in reactions containing 2 µM His-HpkA77 and 20 µM ATP that were incubated at 42°C for 30 min. Reactions were then divided into two aliquots, one of which was stopped while the other was cross-linked by the addition of glutaraldehyde for 30 min longer before quenching. (a) PhosphorImage of SDS-PAGE gel showing inhibition of HpkA-P formation with increasing concentration of AMP-PNP. Reactions contained the following AMP-PNP concentrations: lane 1, none; lane 2, 250 µM; lane 3, 500 µM; lane 4, 750 µM; lane 5, 1 mM; lane 6, 2.5 mM; lane 7, 5 mM; lane 8, 7.5 mM; lane 9, 10 mM. (b) Silver-stained protein gel of cross-linked His-HpkA in the presence of the following AMP-PNP concentrations: lane 1, none, lane 2, 500 µM; lane 3, 750 µM; lane 4, 1 mM; lane 5, 2.5 mM; lane 6, 5 mM; lane 7, 7.5 mM; lane 8, 10 mM; lane 9, none, without cross-linking reagent. (c) Graphic representation of the data in (a) and (b) relative to the samples lacking AMP-PNP (defined as 100% activity or dimer amount). ■, Percentage autophosphorylation activity; ▲, dimer amount.
closantel resulted in a nearly concomitant loss of His-HpkA77 autophosphorylation activity (IC\textsubscript{50} 28 \mu M) and disappearance of His-HpkA77 dimer (DC\textsubscript{50} 25 \mu M) in the assay format in which closantel was added to reactions at the same time as ATP (see Methods; data not shown).

**New inhibitors**

We carried out a programme to target TCSs for antibiotic drug discovery. In the course of these studies, we identified a number of novel inhibitor compounds from high-throughput screening of compound libraries and molecular modelling, including a cyanoacetoacetamide compound (CAA), ethodin and a DrrA peptide (Fig. 3). The assays described above were applied to characterize the mechanisms of action of these inhibitors.

**Cyanoacetoacetamide (CAA).** The compound designated CAA (Fig. 3C) is the parent compound of a structure–activity relationship (SAR) chemistry effort that was developed around the cyanoacetoacetamide group. The cyanoacetoacetamide series inhibited the autophosphorylation activities of several bacterial histidine kinases with IC\textsubscript{50} values as low as \(~\text{30}~\mu \text{M}\) (see below), did not strongly inhibit other classes of kinases, such as the mammalian Raf serine/threonine kinase, showed MICs against Gram-positive and Gram-negative bacteria as low as \(~\text{1}~\mu \text{g}~\text{ml}^{-1}\), did not exhibit acute cytotoxicity of mammalian cell lines in 24 h assays, and did not disrupt cellular membranes at 4 \times \text{MIC} (W. Luo, D. Mullen, A. Riley, J. Zhao, T. I. Nicas, R. Gilmour & M. E. Winkler, unpublished data). Unfortunately, the MICs and inhibition of autophosphorylation were abolished by the addition of serum and purified serum albumin (W. Luo, D. Mullen, A. Riley, J. Zhao, T. I. Nicas, R. Gilmour & M. E. Winkler, unpublished data), and the SAR was not able to separate binding from inhibition of autophosphorylation.

CAA inhibited both the combined autophosphorylation–phosphotransfer and the autophosphorylation assays (Figs 6 and 7a). In the combined assay, the CAA compound acted as a non-competitive inhibitor with ATP (Fig. 6). For CAA, there appeared to be a direct relationship between inhibition of autophosphorylation and the oligomeric state of the enzyme. Activity gels revealed that the autophosphorylation activity of His-HpkA77 decreased with increasing amount of CAA (Fig. 7a). Silver-stained protein gels of His-HpkA77 cross-linked in the presence of CAA clearly demonstrated that the dimer disappeared as the compound concentration increased (Fig. 7b). The disappearance of His-HpkA77 in lanes 6–9 of Fig. 7(b) was attributed to formation of cross-linked aggregates that were unable to enter these PAGE gels. Control experiments demonstrated that samples that were not cross-linked contained the same amount of protein in each lane (data not shown). Similar to the case for closantel (above), the data displayed a strong correlation between the loss of autophosphorylation activity (IC\textsubscript{50} 129 \mu M) and the loss of protein dimer through aggregation (DC\textsubscript{50} 102 \mu M) with increasing CAA concentrations (Fig. 7c). Finally, CAA inhibited autophosphorylation of His-HpkA77 less strongly (IC\textsubscript{50} 129 \mu M; Fig. 7c) than it inhibited the autophosphorylation of truncated versions of the *Strep. pneumoniae* VicK (YycG) and *E. faecium* VanS histidine kinases (IC\textsubscript{50} 50 \mu M and 76 \mu M, respectively; data not shown). This result indicates that the coupled assay can detect general histidine kinase inhibitors that are not specific for the HpkA histidine kinase (see Discussion).

**Ethodin.** Ethodin (ethacridine lactate) (Fig. 3D) was identified from virtual screening of structures of response regulators. The compound is an antiseptic and intercalator that is known to cause uterine contractions (Rudolph et al., 1997). Therefore, it was never a candidate for the discovery of a new antibiotic. In contrast to CAA, ethodin inhibited the combined assay with an IC\textsubscript{50} that was dependent on the concentration of DrrA (IC\textsubscript{50} 29-5 or 201-4 \mu M at 0-5 \mu M or 4 \mu M His-DrrA, respectively; Fig. 8a). The dependence of IC\textsubscript{50} on DrrA concentration suggests that this compound has a different mechanism of inhibition from that of closantel or CAA (see above; Fig. 6). Although ethodin inhibited the combined assay, it did not inhibit the autophosphorylation assay (Fig. 8b). Unexpectedly, despite the lack of an effect on autophosphorylation activity, ethodin still induced some level of aggregation of His-HpkA, although the number of data points was limited in these experiments (Fig. 8b).

**DrrA peptide.** Based on the DrrA response regulator, a hydrophobic 8-mer peptide was synthesized in which the aspartate residue that accepts the phosphoryl group was replaced by alanine (Fig. 3E). The DrrA peptide inhibited the combined assay with an IC\textsubscript{50} that was again dependent on the concentration of DrrA (IC\textsubscript{50} 7-04 or 54-7 \mu M at 0-5 \mu M or 4 \mu M His-DrrA, respectively; Fig. 9a). This
strong inhibition was consistent with competition between the peptide and His-DrrA for the phosphorylated histidine site of His-HpkA77. Consistent with an interaction between the peptide and the histidine site of His-HpkA77, the DrrA peptide inhibited autophosphorylation of His-HpkA77 (IC50 = 8 μM; Fig. 9b). The DrrA peptide also caused loss of His-HpkA77 dimer and presumably aggregation in the cross-linking assay at what appeared to be a considerably lower concentration of DrrA peptide than that needed for inhibition of His-HpkA77 autophosphorylation (Fig. 9b).

We do not know whether the DrrA peptide is structured, but we think it more likely that this unusual pattern of inhibition may be caused by fortuitous hydrophobic interactions.

**DISCUSSION**

Several properties of TCSs have made them attractive targets for the discovery of new antibacterial agents. They are widespread in Gram-positive and Gram-negative species, as well as some atypical bacteria (see Introduction). They are not present in mammals and they play roles in survival, pathogenesis and antibiotic resistance. Histidine kinases contain highly conserved structural domains that are unlike those found in mammalian tyrosine or serine/threonine kinases.
New kinds of inhibitors of two-component systems

kinases, and the structure of a histidine kinase interacting with its cognate response regulator has been determined (reviewed by Hoch & Varughese, 2001; Inouye & Dutta, 2003; Stock et al., 2000; West & Stock, 2001). Each bacterial species contains multiple TCSs, so there is the possibility that resistance would not rapidly develop to a general inhibitor of TCS function (Hubbard et al., 2002; Matsushita & Janda, 2002; Stephenson et al., 2000). How-

ever, in most of these reports, the kinetic mechanisms of action of the inhibitors were not reported (see Stephenson et al., 2000).

One of the challenges in determining the kinetic mechanism of action of histidine kinase inhibitors using autophosphorylation is that the reaction kinetics are nonlinear (see Results). Once phosphorylated, the histidine kinase forms a dead-end complex and cannot turn over unless the phosphoryl group is removed by the response regulator, a phosphatase, spontaneous dissociation, or the back reaction. Moreover, the autophosphorylation reaction consumes a considerable amount of enzyme, which reduces the sensitivity of detecting moderate inhibitors in high-throughput screens. Using the DrrA response regulator as a substrate in the assay, we developed a coupled assay format that allows analysis of multiple turnover events of the HpkA histidine kinase and linear formation of the DrrA-P product (supplementary Figs S3 and S4). This format depended on the stability at 37°C of DrrA-P from the thermophile T. maritima (Results; Goudreau et al., 1998; Lee & Stock, 1996). Coupled assay formats linking receptor function to phosphorylation of CheY protein have previously been used extensively to study the signal transduction pathway of bacterial chemotaxis (e.g. see Borkovich et al., 1989; Bornhorst & Falke, 2000; Ninfa et al., 1991).

The HpkA–DrrA coupled assay was validated in several ways. The $K_m$ (ATP) for the coupled reaction (26–0 μM) was comparable to that determined for the HpkA autophosphorylation reaction (25–4 μM) (Results; Table 1). The coupled format allowed variation of either the ATP or DrrA substrate. Although not identical, the $V_{max}$ (ATP) and $V_{max}$ (DrrA) for the coupled reaction (0–287 nM min$^{-1}$ and 0–165 nM min$^{-1}$, respectively) were comparable (Table 1). The $K_m$ (ATP) of the HpkA–DrrA coupled reaction increased significantly with temperature compared to that of the HpkA autophosphorylation reaction (Table 1). This unexpected result suggests that the presence of DrrA may somehow destabilize the HpkA–ATP complex at higher temperatures (e.g. see Copeland, 2000).

The linear rates of product formation and multiple turnover events allowed us to characterize inhibitors using traditional Michaelis–Menten analysis. As validation for the assay, we demonstrated that AMP-PNP was an ATP-competitive inhibitor in the coupled assay, with a $K_i$ of 100 μM (Fig. 4). Previously, Stephenson et al. (2000) used a cross-linking approach to demonstrate that AMP-PNP did not cause aggregation of histidine kinase dimers. We confirmed this result using a protocol in which ATP and the AMP-PNP inhibitor were added to reactions simultaneously (Methods; Fig. 5). In contrast, the compound closantel, which is a known toxin that inhibits histidine kinases (Sachsenmaier & Schachtele, 2002; Stephenson et al., 2000), inhibited HpkA autophosphorylation and caused concomitant aggregation of HpkA, as expected from previous results (Stephenson et al., 2000).

As part of drug discovery efforts, we identified three new kinds of compounds (Fig. 3) that inhibited TCS function and determined their mechanisms by the HpkA–DrrA coupled assay, HpkA autophosphorylation, and cross-linking of HpkA. Each compound showed differences in
its mechanism of inhibition of TCS function. The CAA compound, which had promising properties as an antibiotic but bound strongly to serum, showed non-competitive inhibition with respect to ATP concentration in the HpkA–DrrA coupled assay (Fig. 6). Similar to closantel and other hydrophobic inhibitors of histidine kinases analysed before (Stephenson et al., 2000), CAA led to aggregation of the HpkA kinase (Fig. 7). Furthermore, the inhibition of HpkA kinase activity followed the same curve as aggregation of the HpkA dimer, consistent with inhibition being caused by aggregation. The inhibition patterns of CAA fit the model proposed by Stephenson and coworkers in which some hydrophobic inhibitors intercalate into the hydrophobic core of the four-helix bundle near the C-terminus of histidine kinases and thereby drive the bundle apart, which leads to aggregation and inhibition of kinase activity (Stephenson & Hoch, 2002; Stephenson et al., 2000).

One concern about the coupled assay might be whether it would detect only compounds that specifically inhibit autophosphorylation of the HpkA histidine kinase or phosphoryl group transfer from HpkA-P to DrrA. This concern can be dispelled for the autophosphorylation reaction. In the coupled assay, AMP-PNP and CAA showed different inhibition patterns (Figs 4 and 6) that were consistent with different mechanisms of inhibition of autophosphorylation (Figs 5 and 7). Moreover, CAA inhibited the autophosphorylation of the E. faecium VanS and Strep. pneumoniae VicK histidine kinases even more strongly than it inhibited T. maritima HpkA (see Results).

Finally, other classes of compounds that were competitive inhibitors of autophosphorylation of several different bacterial kinases showed competitive inhibition in the coupled HpkA–DrrA assay, similar to that of AMP-PNP (Fig. 4; data not shown). Kinetic analysis of inhibition of phosphoryl group transfer has generally been hampered by the fast rates of these reactions and instability of the phosphorylated response regulators. The HpkA–DrrA coupled assay allows a starting point for the detection and detailed kinetic analysis of putative inhibitors of phosphoryl group transfer. The generality of putative inhibitors in the coupled assay can then be tested in other more challenging assay formats.

Our studies identified two compounds that are putative inhibitors of phosphoryl group transfer. Ethodin is a small hydrophobic intercalator-like molecule that inhibited TCS function by a different mechanism from that of CAA or closantel. Ethodin was a potent inhibitor of the HpkA–DrrA combined assay (Fig. 8) and was shown to have antibacterial activity (data not shown). Due to its known toxic properties, ethodin was not pursued further as a possible antibiotic. Unlike CAA and closantel, ethodin appeared to compete with DrrA for binding to HpkA, and addition of excess DrrA eliminated inhibition of the coupled assay by ethodin (Fig. 8). Interestingly, ethodin did not inhibit the HpkA autokinase activity, suggesting that the compound binds to DrrA or binds to HpkA, but only inhibits phosphoryl group transfer. Evidence for the binding of ethodin directly to the HpkA histidine kinase came from aggregation studies. Even though ethodin failed to inhibit HpkA autophosphorylation, it still caused loss of the HpkA dimer and presumably aggregation in crosslinking reactions (Fig. 8). This result is consistent with ethodin acting by binding to the HpkA histidine kinase and inhibiting phosphoryl group transfer from the kinase to the DrrA response regulator. The ability of this compound to cause a histidine kinase to aggregate without inhibition is unusual and indicates a different mechanism of inhibition from CAA, closantel and the compounds studied by Stephenson et al. (2000).

A hydrophobic peptide corresponding to the phosphorylation site in DrrA (Fig. 3) inhibited the HpkA–DrrA coupled assay by a mechanism that was similar to that of ethodin, with one important difference (Fig. 9). Like ethodin, the DrrA peptide appeared to compete with DrrA for binding to HpkA in the HpkA–DrrA coupled assay (Fig. 9). Similar to ethodin, the DrrA peptide bound to HpkA and led to loss of dimer and presumed aggregation in the crosslinking assay without significant loss of HpkA autoprophosphorylation activity. However, unlike ethodin, higher concentrations of the DrrA peptide inhibited the HpkA autophosphorylation activity. Given the similarity of the inhibition patterns of ethodin and the DrrA peptide, the peptide may also act by binding to the HpkA kinase and inhibiting phosphoryl group transfer from the kinase to DrrA. However, we do not have evidence for folding of this peptide or for specific interactions with HpkA, analogous to those of DrrA.

Together, these results demonstrate the utility of the coupled assay reported here to elucidate rapidly the kinetic mechanisms of inhibition of TCSs by new compounds. Using this assay and approaches developed previously by other laboratories, we identified inhibitors, such as ethodin and DrrA peptide, with an unusual mechanism of inhibition of TCS function. To date, no TCS inhibitor has progressed to a clinical candidate antibiotic, despite considerable efforts by several companies and academic laboratories (e.g. see Barrett et al., 1998; Hilliard et al., 1999; Matsushita & Janda, 2002; Stephenson & Hoch, 2002). Some inhibitors, such as the CAA reported here, probably cause aggregation of histidine kinases that leads to inhibition. Despite some favourable biological properties, CAA failed to progress as a drug candidate because of strong serum binding. Other histidine kinase inhibitors that cause aggregation kill bacterial cells by mechanisms other than TCS inhibition, such as RWJ-49815, which disrupts membranes (Hilliard et al., 1999; Stephenson & Hoch, 2002). The inability to develop high-quality drug candidates that target a broad spectrum of histidine kinases might relate to the fact that all of the compounds identified so far appear to act outside of the ATP-binding site. An effective, less toxic broad-spectrum histidine kinase inhibitor might come from identification of a potent, specific ATP-competitive inhibitor. Such a compound would have a novel mechanism and would probably
not cause protein aggregation on the basis of the precedent set by our results with the analogue AMP-PNP. On the other hand, structure-based design of compounds targeted to the interaction between histidine kinases and response regulators may lead to new classes of TCS inhibitors (Hubbard et al., 2003; Matsushita & Janda, 2002; Stephenson & Hoch, 2002). The coupled assay reported herein could be used to distinguish rapidly between these two different kinds of inhibitors.

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