Stabilization of the Phospho-aspartyl Residue in a Two-Component Signal Transduction System in *Thermotoga maritima*†

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ABSTRACT: The central signaling pathway in many bacterial regulatory systems involves phosphotransfer between two conserved proteins, a histidine protein kinase, and a response regulator. The occurrence of two-component signaling systems in thermophilic bacteria raises questions of how both the proteins and the labile acyl phosphate of the response regulator are adapted to function at elevated temperatures. *Thermotoga maritima* HpkA is a transmembrane histidine kinase, and DrrA is its cognate response regulator. Both DrrA and the cytoplasmic region of HpkA (HpkA57) have been expressed in *Escherichia coli*, purified, and characterized. HpkA57 and DrrA have apparent T_m's of 75 and 90 °C, respectively. HpkA57 exhibits ATP-dependent autophosphorylation activity similar to that of histidine kinases from mesophiles, with maximum activity at 70 °C. DrrA catalyzes transfer of phosphoryl groups from HpkA57 and exhibits Mg^{2+}-dependent autophosphatase activity, with maximum activity at approximately 80 °C. At this temperature, the half-life for phospho-DrrA is approximately 3 min. In the absence of Mg^{2+}, the half-life is 26 min, significantly greater than the half-life of a typical acyl phosphate at 80 °C. In the absence of Mg^{2+}, at all temperatures examined, phospho-DrrA exhibits much greater stability than acetyl phosphate. This suggests that the active site of this hyperthermophilic response regulator is designed to protect the phospho-aspartyl residue from hydrolysis.

Stimulus-response coupling in prokaryotes commonly proceeds through a phosphotransfer pathway involving a histidine protein kinase and a response regulator protein (for reviews see ref *1*). In the numerous systems that have been characterized to date, there are many variations on the basic pathway. There are differences in the arrangement of histidine protein kinase and response regulator domains, and in the way these conserved components are integrated and utilized within different signaling systems. However, the basic biochemical activities of the two protein components are conserved. The histidine protein kinase is phosphorylated at a conserved histidine residue in a trans-phosphorylation reaction between monomers of a dimer. The response regulator protein catalyzes transfer of the phosphoryl group from the phospho-histidine of the protein kinase to an aspartic acid residue in its own regulatory domain. Phosphorylation is thought to induce structural changes in the regulatory domain that, in most cases, result in activation of an associated effector domain.

The regulatory domains can be considered to be phosphorylation-activated switches that control the activities of either attached domains or other proteins. Thus a major and important point of regulation in the two-component signaling pathways is the lifetime of the phosphorylated state of the response regulator protein. In different systems, the lifetime is determined by the intrinsic autophosphatase activity of the response regulator itself, phosphatase activity of the histidine protein kinase, dephosphorylating activity of auxiliary proteins, or a combination of these factors. Half-lives of phosphorylated response regulators, determined in vitro in the absence of auxiliary proteins, vary from seconds (*2*), to minutes (*3*, *4*), to hours (*5*). The lifetimes observed for different response regulators span a range of approximately 4 orders of magnitude, and the relatively long or short lifetimes can be rationalized in terms of the nature of the system controlled by the response regulator.

Genes encoding histidine protein kinases and response regulators have been cloned from *Thermotoga maritima*, a hyperthermophilic bacterium that has an optimal growth temperature of 80 °C (*6*−*8*). *T. maritima* HpkA is a 48.0 kDa transmembrane histidine protein kinase and DrrA is a 27.6 kDa response regulator protein belonging to the OmpR/PhoB subfamily of DNA-binding proteins (*8*). HpkA and DrrA are encoded in a single operon and presumably function together to regulate gene expression. The pair exhibits highest sequence similarity to two-component proteins involved in phosphate assimilation (*Escherichia coli* PhoR/PhoB and *Bacillus subtilis* PhoR/PhoP), but the genes regulated by HpkA and DrrA are presently unknown. The *T. maritima* two-component proteins might be expected to exhibit both similarities and differences when compared with

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their mesophilic homologues. To the extent that the proteins are similar, the *T. maritima* proteins may be used as model proteins whose enhanced stability may offer advantages for biophysical and structural studies. Conversely, the differences may provide insight into adaptation of the ubiquitous two-component phosphotransfer signaling system for function at elevated temperatures.

To address these issues, we have used the *T. maritima* two-component proteins for biophysical and biochemical studies. The cytoplasmic region of the histidine protein kinase HpkA and the intact response regulator DrrA have been expressed in *E. coli* and purified to homogeneity. Phosphorylation and dephosphorylation activities were examined in vitro. HpkA57, a soluble form of HpkA lacking the N-terminal 57 residues, autophosphorylates at a histidine residue with maximum activity at 70 °C, just slightly below its thermal denaturation point. DrrA exhibits both phosphotransfer and autophosphatase activities with maximum dephosphorylating activity at approximately 80 °C, close to its denaturation point. In the absence of phosphatase activity, the lifetime of the phospho-aspartyl residue in DrrA is significantly greater than that of a typical acyl phosphate. Thus the lifetime of the phosphorylated state of DrrA, like those of mesophilic response regulators, appears to be highly regulated. In the case of the hyperthermophilic response regulator DrrA, the lifetime of the phosphorylated state results from a combination of both stabilizing and destabilizing influences of the protein.

EXPERIMENTAL PROCEDURES

Materials. Ammonium sulfate from ICN and Tris from United States Biochemical were ultrapure grade. [γ-^{32}P]-ATP[1] (3000 Ci/mmol) was from Amersham Corp. Ecoscint A scintillation fluid was from National Diagnostics, and Biomax X-ray film was from Kodak. Low-range molecular weight standards, electrophoresis reagents, and Bradford protein assay solution were from Bio-Rad. SDS−polyacrylamide gels (15%) were run under reducing conditions using a Mini-PROTEAN II system (Bio-Rad) and were stained with Coomassie blue. Native gels (10% Tris-glycine polyacrylamide gels) obtained from Novex were run according to the manufacturer's instructions under nonreducing conditions with samples applied without prior heating. Immobilon-P PVDF membranes were from Millipore, and nitrocellulose membranes were from Schleicher and Schuell. Medi-Prep size exclusion spin columns were from 5 Prime → 3 Prime, Inc. FPLC columns (5-mL HiTrap-Blue, 5-mL and 1-mL HiTrap-Q, Superdex 75, and Superdex 200 Hiload 26/60) were from Pharmacia and were run using a Pharmacia FPLC system. *E. coli* PhoR1084, a soluble form of PhoR lacking residues 1−83, was purified by ammonium sulfate fractionation and chromatography on cellulose phosphate P-11 (Whatman), Orange A dye-affinity agarose (Amicon), and CM52 cation-exchange cellulose (Whatman) columns using modifications of previously described procedures (*4*). *E. coli* PhoB was purified using ammonium sulfate fractionation, cellulose phosphate P-11, DE52 anion-exchange cellulose, and Bio-Gel HT hydroxyl apatite (Bio-Rad) chromatography using modifications of previously described procedures (*9*). All other materials were reagent grade from standard commercial sources. Liquid scintillation counting was performed with a Beckman scintillation spectrometer (model LS650), phosphoimaging was performed with a Molecular Dynamics system, and densitometry was performed using a Bio-Rad densitometer (model GS-679) and the associated Molecular Analyst Software (Bio-Rad). Ultraviolet and visible spectroscopy was done on a Beckman spectrophotometer (model DU 650), and CD data were collected on an Aviv spectropolarimeter (model 60DS).

Purification of Recombinant T. maritima HpkA57 and DrrA Expressed in E. coli. All purification steps were performed at 4 °C, unless indicated otherwise. The heat-treated cell lysate from 3 L of cells, prepared as described previously (*8*), was brought to 75% saturation with ammonium sulfate by adding 47.5 g of ammonium sulfate/100 mL of solution. The solution was stirred for 1 h and then centrifuged at 12000*g* for 30 min. The pellet was resuspended in 50 mM Tris-HCl, 100 mM KCl, pH 8.0, and dialyzed against 50 mM Tris-HCl, 100 mM KCl, pH 8.0, overnight with 2 × 4 L of buffer. The dialyzed ammonium sulfate fraction was diluted with 2 volumes of 50 mM Tris-HCl, pH 8.0, filtered through a 0.22-μm filter, and subjected to affinity chromatography on a 5-mL HiTrap-Blue column equilibrated with 50 mM Tris-HCl, pH 8.0. Bound proteins were eluted with a linear gradient of 0−2.0 M KCl in the column equilibration buffer, and fractions containing HpkA57 were identified by native polyacrylamide gel electrophoresis and pooled. In the case of DrrA, the column was washed with a linear gradient of 0−1.0 M KCl in the column equilibration buffer followed by an isocratic elution of DrrA with 1.0 M KCl. Fractions containing DrrA were identified by SDS−PAGE analysis and pooled. The pooled fractions from the HiTrap-Blue column were then subjected to size exclusion chromatography. Superdex 200 and Superdex 75 (Hiload 26/60) were used for HpkA57 and DrrA, respectively. The chromatography was performed with 50 mM Tris-HCl, 100 mM KCl, pH 8.0, and peak fractions (assayed by absorbance at 280 nm) were pooled and stored at −20 °C. The DrrA-containing fractions obtained from the Superdex 75 chromatography were diluted with an equal volume of 10 mM Tris-HCl, pH 8.0, and loaded onto a 5-mL HiTrap-Q anion-exchange column equilibrated in 25 mM Tris-HCl, 50 mM KCl, pH 8.0. The protein was eluted with a linear gradient of 50−400 mM KCl in column equilibration buffer, and fractions containing DrrA were pooled and stored at −20 °C. Protein concentrations were estimated using the Bradford dye-binding method (*10*).

Far-UV Circular Dichroic Spectral Analysis. CD spectroscopy was conducted on degassed protein solutions in 1-mm-path-length quartz cuvettes. Spectra collected in the far-UV region were acquired every 1 nm with a 1-nm bandpass. Thermal denaturation data were monitored by observing changes in ellipticity acquired at 222 nm for HpkA57 and 220 nm for DrrA. To demonstrate the reversibility of thermal denaturation, a complete CD spectrum was obtained at 25 °C before heating. After the thermal denaturation was carried out, the sample was cooled back to 25 °C and another

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate.

complete spectrum was collected.

Preparation of Phospho-HpkA57 and Phospho-DrrA for Determination of Chemical Stabilities. [^{32}P]Phospho-HpkA57 was prepared by incubating 2.0 μM HpkA57 with 0.1 mM [γ-^{32}P]ATP (5 Ci/mmol) in 10 μL of TKMED buffer (50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM DTT, pH 8.5) for 15 min at 50 °C. Free ATP was removed using a size exclusion spin column. Two-microliter aliquots of column eluate were applied to 1 cm^2 PVDF membranes that had been pre-wetted in methanol and rinsed in water. [^{32}P]Phospho-DrrA was prepared by phosphoryl transfer from [^{32}P]phospho-HpkA57, prepared by incubating 10 μM HpkA57 with 1.0 mM [γ-^{32}P]ATP (1 Ci/mmol) in 100 μL of TKMED buffer for 30 min at 55 °C. The 100-μL [^{32}P]phospho-HpkA57 reaction mixture was added to 300 μL of 24 μM DrrA in 100 mM Tris-HCl, 110 mM KCl, 17 mM $MgCl_2$, pH 7.5, and incubated for 15 min at 55 °C. The mixture was applied to a 1 mL HiTrap-Q anion-exchange column equilibrated in 25 mM Tris-HCl, pH 8.0. DrrA was eluted using a 5-mL linear gradient of 0−400 mM KCl in Tris-HCl buffer. Fractions containing [^{32}P]-phospho-DrrA were identified by SDS−PAGE followed by autoradiography and were then pooled. Five-microliter aliquots of [^{32}P]phospho-DrrA (approximately 5000 cpm) were applied to prewetted 1 cm^2 PVDF membranes.

Determination of Phospho-Amino Acid Chemical Stabilities. [^{32}P]Phospho-HpkA57 and [^{32}P]phospho-DrrA were prepared and applied to 1 cm^2 PVDF membranes as described above. Membranes were dried, incubated in 12.5 mM sodium phosphate, 200 mM NaCl, 0.1% Triton X-100, pH 7.5, for 10 min, and then rinsed successively with 50 mM Tris-HCl, pH 7.0, and then deionized water. Membranes were then incubated in 0.6 mL of different hydrolysis solutions (0.1 N HCl, pH 1; 0.2 M sodium citrate, pH 2.4; 0.2 M sodium citrate, pH 7.5; 0.1 M pyridine, pH 8.9; and 2 N NaOH, pH 14). Incubations in pyridine were performed at 25 °C; all other incubations for HpkA57 and DrrA were performed at 40 and 45 °C, respectively. At 10, 30, 60, 90, and 120 min, single PVDF membranes were transferred successively to fresh solutions, 2 mL of Ecoscint scintillation fluid was added to each incubation solution, and radioactivity was determined by liquid scintillation spectrometry. Total radioactivity was calculated as the sum of radioactivity released at each timepoint and that remaining on the membrane after 120 min of incubation. The phospho-protein remaining at each time was calculated as the difference between total and released radioactivity (the sum of all measurements up to that time), and first-order rate constants were calculated from the linear region of semilogarithmic plots.

Autophosphorylation of HpkA57. All reactions were performed in TKMED buffer. The salt concentration, buffer, pH, and temperature were varied as indicated. Autophosphorylation reactions were performed with 2 μM HpkA57. Samples were pre-equilibrated at the designated temperatures for 2 min prior to initiating the reaction by addition of ATP at a final concentration of 80 μM [γ-^{32}P]ATP (2.5 Ci/mmol). At the indicated times, 5-μL aliquots were removed and the reactions were quenched by transfer to 2.5 μL of a stop solution containing 250 mM NaOH, 125 mM EDTA. Five-microliter aliquots of the quenched reactions were applied to 1 cm^2 nitrocellulose filters. The filter squares were dried, washed three times in 250 mL of buffer (100 mM Tris-HCl, pH 8.5), and then assayed for radioactivity using a scintillation spectrometer. Initial rates of autophosphorylation of HpkA57 were calculated from linear regression analysis of plots of phospho-HpkA57 generated versus time.

Temperature Dependence of Phosphotransfer between HpkA57 and DrrA. [^{32}P]Phospho-HpkA57 free of ATP was prepared as described above. The phosphotransfer reaction was performed in TKMED buffer with each protein at a final concentration of 10 μM. Phosphotransfer reactions were initiated by mixing [^{32}P]phospho-HpkA57 and DrrA that had been pre-equilibrated at the indicated temperatures. At indicated times, aliquots were removed from the reaction and quenched in an equal volume of 2X SDS Laemmli sample buffer. Following electrophoresis on 15% SDS−polyacrylamide gels, gels were dried without staining and exposed to a phosphoimager screen for 5 h. The amount of [^{32}P]-phospho-HpkA57 was determined by phosphoimaging.

Stability of the Acyl Phosphate in Phospho-DrrA. Native phospho-DrrA, isolated from HpkA57 and ATP by ion-exchange chromatography, was prepared as described above for the determination of chemical stabilities. Hydrolysis of phospho-DrrA was assayed in the presence of 20 mM $MgCl_2$ or 20 mM EDTA. The reaction mixtures contained 2.7 μM [^{32}P]phospho-DrrA (~3000 cpm/pmol) in 25 mM Tris-HCl, 100 mM KCl, pH 8.0. A 10-μL aliquot was removed and applied to a 1 cm^2 PVDF membrane, pre-wetted in methanol, and rinsed in water, to serve as the zero time point. The reaction mixtures were then incubated at the indicated temperatures and, at appropriate intervals, 10-μL aliquots were removed and applied to pre-wetted 1 cm^2 PVDF filters. The filters were dried, washed three times in 50 mM Tris-HCl, pH 8.0, at 4 °C, transferred to scintillation vials containing 2 mL of Ecoscint scintillation fluid, and assayed for radioactivity by liquid scintillation spectrometry. Hydrolysis under denaturing conditions was assayed as described above by incubating phospho-DrrA in the presence of 1% SDS and 20 mM EDTA. A correction for the radioactive decay of ^{32}P was applied to determinations at 4 °C. Each value represents the average of two independent experiments in which a minimum of 8 time points were taken at appropriate intervals during a time period that extended for at least one half-life, except where noted.

Stability of Acetyl Phosphate. A modification of the spectrophotometric method of Lipmann and Tuttle (*11*) was used to quantitate acetyl phosphate. Solutions of 100 mM acetyl phosphate, 50 mM Tris-HCl, 100 mM KCl, pH 8.0, with either 20 mM $MgCl_2$ or 20 mM EDTA as indicated, were preincubated at the designated temperatures for 2 min. At intervals appropriate for the temperature, 10-μL aliquots were removed, added to a 0.5-mL reaction mixture containing 0.2 mL of hydroxylamine solution (freshly prepared 14% hydroxylamine hydrochloride, 8% sodium hydroxide, pH ~6.4) and 0.3 mL of acetate buffer (67 mM sodium acetate, pH 5.4), and then incubated at 25 °C for 20 min. The colorimetric reaction was developed by addition of 0.5 mL of 10% $FeCl_3 \cdot 6H_2O$ in 0.7 N HCl. After a 5-min incubation at 25 °C, the absorbance at 505 nm was measured relative to a control reaction without acetyl phosphate. Each rate is calculated from a minimum of six measurements taken during a time period that extended for at least one half-life.

Effect of HpkA57 on the Stability of the Acyl Phosphate in Phospho-DrrA. Native phospho-DrrA, isolated from HpkA57 and ATP by ion-exchange chromatography, was prepared as described above for the determination of chemical stabilities. Hydrolysis of phospho-DrrA was assayed at 55 °C. Reaction mixtures contained 10 μM [^{32}P]phospho-DrrA (~1500 cpm/pmol) in 25 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, pH 8.0. To determine their effects on the stability of phospho-DrrA, either HpkA57 (1 μM), ATP (1 mM), or ADP (1 mM) was added to separate reactions. During a 2-h time course, 10-μL aliquots were removed from the reaction mixtures and were quenched by the addition of 10 μL of 2X SDS Laemmli sample buffer. Following electrophoresis in 15% SDS−polyacrylamide gels, wet gels were exposed to film. The developed films were quantified by densitometric analysis.

RESULTS

Purification of Histidine Protein Kinase HpkA57 and Response Regulator DrrA. The differences in thermostability between hyperthermophilic proteins and mesophilic expression host proteins have led to the utilization of heat treatment as an efficient step in the purification of hyperthermophilic proteins prior to the application of further purification steps (*12*−*14*). The purification of the *T. maritima* two-component proteins relied on the fact that heating caused denaturation and insoluble aggregation of the majority of the *E. coli* host proteins while HpkA57 and DrrA remained soluble. HpkA57 was purified from the heat-treated cell lysate by application of only two chromatographic steps, dye affinity and size exclusion (Figure 1A). The mobility of HpkA57 during size-exclusion chromatography is consistent with a dimeric state (data not shown), similar to that reported for many other mesophilic histidine protein kinases (*15*−*17*). An approach similar to that described for HpkA57 was used for the purification of DrrA. After removal of precipitated host proteins from heat-treated cell lysates, three chromatographic steps, dye affinity, size exclusion, and anion exchange, were utilized to obtain purified DrrA (Figure 1B).

Thermal Stabilities of HpkA57 and DrrA. Thermal denaturation of HpkA57, monitored by far-UV circular dichroism (CD) spectroscopy, was used to study the effect of temperature on the folded state of HpkA57. A CD spectrum (200−300 nm) of HpkA57 at 25 °C exhibits a typical profile with a minimum at 212 nm (data not shown). Far-UV circular dichroism at 222 nm was measured for HpkA57 as a function of temperature (Figure 2A). A gradual decrease in the magnitude of the negative ellipticity at 222 nm was observed with increasing temperature. A minor thermal transition was observed between 30 and 45 °C, and this transition was found to be reversible (data not shown). Within this lower temperature range, only small-scale structural changes appear to be occurring. A large thermal transition was observed over a broad temperature range between 65 and 85 °C with the midpoint of this temperature-induced denaturation (T_m) at approximately 75 °C. The thermal denaturation did not reach an end point, as seen by incomplete leveling off of the curve above 100 °C. Protein denatured to this point could be reversibly refolded as evidenced by reacquisition of negative ellipticity when subjected to cooling (data not shown).

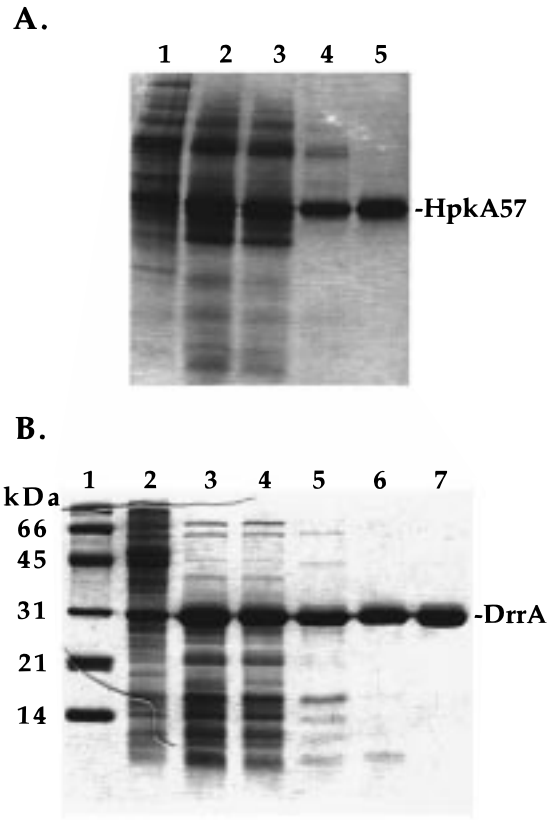

FIGURE 1: Polyacrylamide gel electrophoretic analysis of the purification of HpkA57 and DrrA. (A) Native polyacrylamide gel electrophoresis of HpkA57. Samples from each of the purification steps of HpkA57 were analyzed on a 10% native Tris-glycine polyacrylamide gel and visualized by staining with Coomassie blue. Approximately 5 μg of total protein was loaded in each of the following lanes: cell-free crude extract, lane 1; heat-treated cell lysate, lane 2; ammonium sulfate-precipitated fraction, lane 3; HiTrap-Blue pooled fractions, lane 4; and Superdex 200 pooled fractions, lane 5. (B) SDS−PAGE analysis of DrrA. Samples were analyzed on a 15% SDS−polyacrylamide gel and stained with Coomassie blue. Approximately 5 μg of total protein from each of the purification steps was loaded in the following lanes: molecular weight standards with approximate masses indicated at the left, lane 1; cell-free crude extract, lane 2; heat-treated cell lysate, lane 3; ammonium sulfate-precipitated fraction, lane 4; HiTrap-Blue pooled fractions, lane 5; Superdex 75 pooled fractions, lane 6; and HiTrap-Q pooled fractions, lane 7.

Like HpkA57, DrrA exhibited a typical CD spectrum (200−300 nm) at 25 °C, with a minimum at 212 nm (data not shown). The thermal denaturation curve of DrrA (Figure 2B), monitored at 220 nm, exhibited a minor and reversible transition from 40 to 65 °C and a major transition from 90 to above 100 °C, with an apparent T_m at approximately 95 °C. The thermal transition of DrrA above 95 °C was irreversible.

Chemical Stabilities of Phosphorylated Residues in HpkA57 and DrrA. It has been demonstrated that the phosphohistidine in mesophilic histidine protein kinases exhibits a characteristic instability under acidic conditions but is stable in alkaline conditions (*18*, *19*). We compared the relative hydrolysis rates of denatured PVDF membrane-bound phospho-HpkA57 in buffers at various pH values to assess the stability of the phospho-amino acid. Hydrolysis rates were obtained by linear regression analysis of semi-logarithmic plots of the percentage of phospho-HpkA57 remaining on the membranes as a function of time. The first-

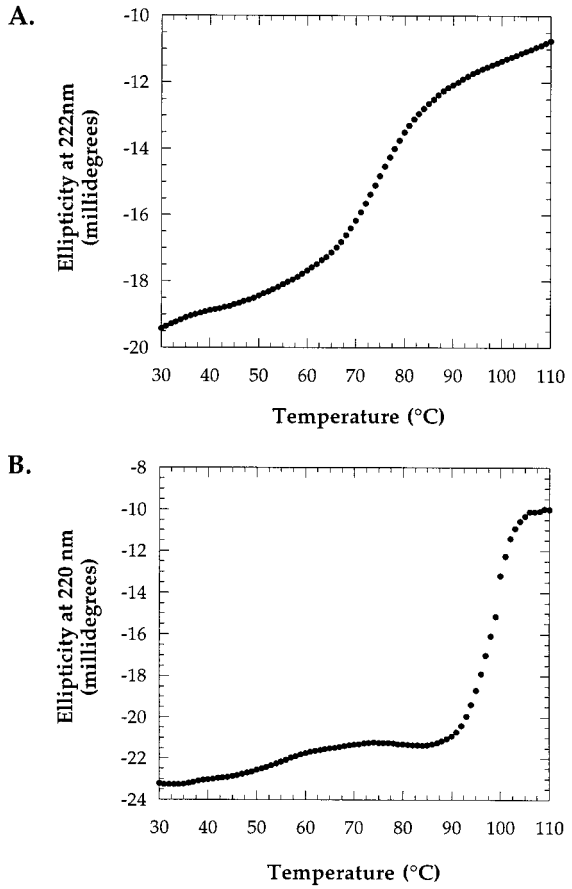

FIGURE 2: Circular dichroism analysis of the thermal denaturation of HpkA57 and DrrA. CD data were collected from samples containing 0.2 mg/mL protein in 50 mM Tris-HCl, 100 mM KCl, pH 8.0, at 1 °C increments from 25 to 110 °C with a 1-min temperature equilibration time and a 30-s averaging time per data point. (A) Thermal denaturation of *T. maritima* HpkA57 monitored at 222 nm. (B) Thermal denaturation of DrrA monitored at 220 nm.

Table 1: Chemical Stability of Phosphorylated Two-Component Proteins[a]

	rate of hydrolysis, k_1 (min^{-1})					
condition[b]	HpkA57	CheA	NtrB	Enzyme I	DrrA	CheY
pH 1	0.016				0.025	
pH 2.4	0.014	0.021	0.017	0.025	0.018	0.012
pH 6.5−7.5[c]	0.007	0.000	0.001	0.008	0.009	0.016
pH 14	0.002	0.000	0.003	0.008	0.11	>0.2
pyridine	0.009	0.009	0.020	0.031	0.009	0.009

[a] Values are compared to those previously obtained under similar conditions for the phosphohistidine containing proteins *S. typhimurium* Enzyme I (*20*), *S. typhimurium* CheA (*18*), and *E. coli* NtrB (*19*) and for the phosphoaspartate containing protein *S. typhimurium* CheY (*22*). [b] Incubations at pH 2.4, 6.5−7.5, and 14 were at 45 °C for CheA, NtrB, and CheY and at 46 °C for Enzyme I. Other conditions were as described for HpkA57 and DrrA. [c] The values for Enzyme I were determined in 50 mM potassium phosphate, pH 6.5, and the values for CheA, NtrB, and CheY were determined in 50 mM potassium phosphate at pH 7.0.

order rate constants (Table 1) were similar to those obtained for mesophilic histidine protein kinases.

The phosphoryl group of denatured phospho-HpkA57 was relatively stable to hydrolysis at neutral and alkaline pH but rather unstable at acidic pH. The relative rates of hydrolysis in the presence of pyridine also resemble the values obtained for hydrolysis of the phosphohistidine in Enzyme I of the sugar transport system (*20*) and in other mesophilic histidine protein kinases (*4*, *18*, *19*). These observations are consistent with phosphorylation at a histidine residue in phospho-HpkA57.

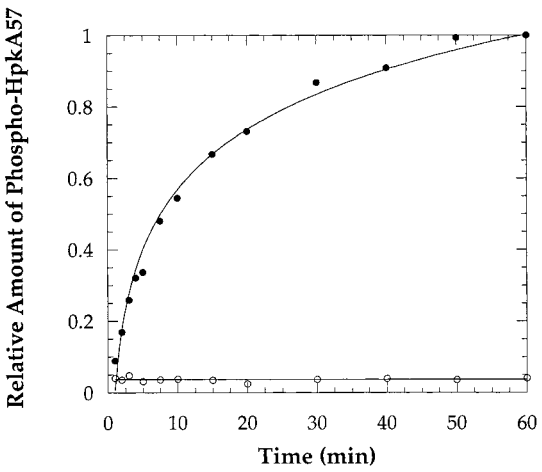

FIGURE 3: Autophosphorylation of HpkA57. HpkA57 at a final concentration of 2 μM in TKMED buffer (see Experimental Procedures) both with (○) and without (●) 20 mM EDTA was incubated with 80 μM [γ-^{32}P]ATP (2.5 Ci/mmol) at 45 °C. Phospho-HpkA57 measured at the indicated times is expressed as a fraction of the phospho-HpkA57 obtained at 60 min (approximately 0.9 mol of phosphate/mol of HpkA57).

It has been previously demonstrated that response regulators from mesophiles are phosphorylated at aspartate side chains (*3*, *21*). The chemical stability of the phosphorylated amino acid residue in DrrA was examined by hydrolysis of denatured [^{32}P]phospho-DrrA at various pH conditions. [^{32}P]-Phospho-DrrA, immobilized on PVDF membranes, was exposed to different pH conditions, and the subsequent hydrolysis was monitored. The first-order rate constants exhibit a pattern of hydrolysis characteristic of an acyl phosphate, which is relatively unstable in both acid and alkaline conditions (Table 1). This pattern is similar to that reported for the mesophilic response regulator CheY (*22*). Additionally, a mutant DrrA protein, in which Asp54, the residue corresponding to the phosphorylation site in other response regulators, is substituted with cysteine, is incapable of catalyzing phosphotransfer from phospho-HpkA57 (R. Saxl, and A. Stock, unpublished observations).

Autophosphorylation of HpkA57. Phosphoryl transfer signal transduction pathways that utilize a single histidine protein kinase and response regulator pair involve three phosphotransfer reactions. The γ-phosphoryl group in ATP is first transferred to a histidine side chain of the histidine protein kinase. The phosphoryl group is then transferred from the histidine residue to an aspartate side chain of the response regulator. Finally, the phosphoryl group is transferred from the phosphoaspartate residue to water. The kinetics of each reaction can be specifically characterized by measurement of the rates of formation or hydrolysis of the two phosphorylated intermediates, phospho-histidine protein kinase and phospho-response regulator. The autophosphorylation activity of HpkA57 was examined by measuring the initial rate of formation of phospho-HpkA57. The rate of autophosphorylation exhibited simple exponential behavior (Figure 3). An initial rate of 0.1 mol of phosphate/

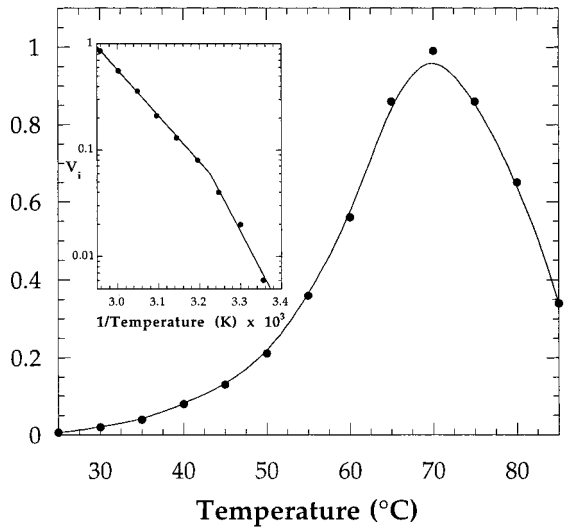

FIGURE 4: Temperature dependence of the initial rate of HpkA57 autophosphorylation. Initial rates of autophosphorylation of 2 μM HpkA57 were determined at the indicated temperatures in the presence of 80 μM [γ-^{32}P]ATP as described in Experimental Procedures. The inset shows an Arrhenius plot of the same data.

mol of HpkA57/min was observed at 45 °C. The incorporation of phosphate from ATP into HpkA57 reached a maximum of 0.9 mol of phosphate/mol of HpkA57.

Addition of EDTA to the reaction solution abolished autophosphorylation, demonstrating the requirement of divalent cations for the autophosphorylation activity of HpkA57. The initial rate of autophosphorylation of HpkA57 did not exhibit any significant change over the range from pH 7.0 to 9.0 (data not shown). The effect of ionic strength on the initial rate of autophosphorylation of HpkA57 was examined by varying the concentration of KCl (5−500 mM) in the reaction buffer. No significant changes were observed over a wide range of KCl concentrations above 25 mM. As a result of these studies, further characterization of HpkA57 was carried out in solutions containing 50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 8.5.

Effect of Temperature on HpkA57 Autophosphorylation. Autophosphorylation activity of HpkA57 was examined at temperatures ranging from 25 to 85 °C. Over this temperature range the pH of the reaction solution varied from approximately 9 to 7, a pH range throughout which HpkA57 autophosphorylation activity was found to be constant. The autophosphorylation of HpkA57 was strongly temperature-dependent (Figure 4). The initial rate showed a rather rapid increase at temperatures above 40 °C and dropped abruptly beyond 70 °C. The maximal autophosphorylation activity of HpkA57, observed at 70 °C, was approximately 50-fold greater than that observed at 30 °C.

An Arrhenius plot (Figure 4, inset) of the autophosphorylation reaction exhibits two regions of linearity, with a break occurring at approximately 36 °C. The calculated values of the corresponding activation energies for the protein are 10.4 kcal/mol for the lower temperature range (from 25 to 36 °C) and 6.5 kcal/mol for the upper temperature range (from 36 to 65 °C).

Phosphotransfer between HpkA57 and DrrA and between T. maritima and Mesophilic Two-Component Proteins. Like all other activities of HpkA57 and DrrA, phosphotransfer was observed to be temperature-dependent within the range

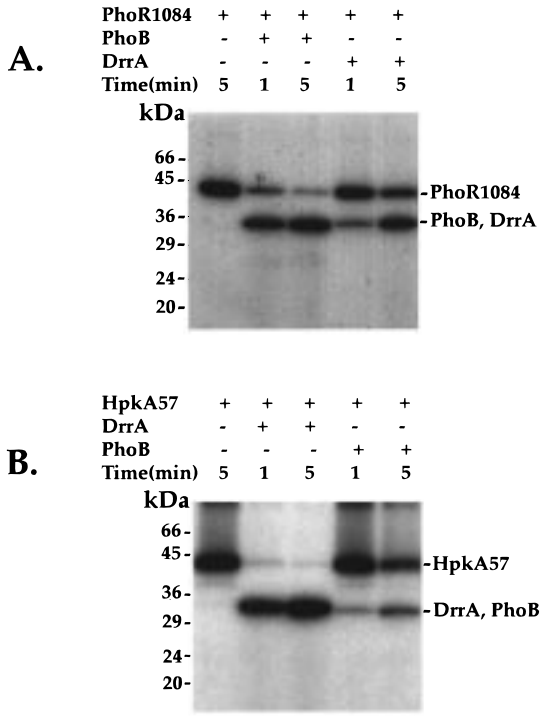

FIGURE 5: Phosphotransfer between thermophilic and mesophilic histidine protein kinases and response regulators. Histidine protein kinases (2 μM) were incubated in 20 μL of 100 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 1.0 mM EDTA, 0.3 mM DTT, 0.1 mM [γ-^{32}P]ATP (5 Ci/mmol), pH 8.5, for 30 min at 35 °C for *E. coli* PhoR1084 and 50 °C for *T. maritima* HpkA57. To initiate the reactions, 20-μL aliquots of *E. coli* PhoB (15 μM in 50 mM potassium phosphate, pH 7.2), *T. maritima* DrrA (15 μM in 25 mM Tris-HCl, 250 mM KCl, pH 8.0), or TKMED buffer were added to the kinase mixtures as indicated. After 1- and 5-min incubation of the reaction mixtures at 35 °C, 10-μL aliquots were removed, added to an equal volume of 2X SDS Laemmli sample buffer, and analyzed by SDS−PAGE on 15% polyacrylamide gels. Autoradiographs of the gels are shown. (A) Phosphotransfer from *E. coli* PhoR1084. (B) Phosphotransfer from *T. maritima* HpkA57.

of 4−70 °C, with the highest rates of transfer at 70 °C (data not shown). As has been observed for other two-component proteins, the phosphotransfer required a divalent cation. At 55 °C in the presence of EDTA, phosphotransfer was undetected (data not shown).

Phosphotransfer has been observed in vitro between mesophilic histidine protein kinases and response regulators of different two-component systems (*19*, *23*−*26*). However, there is significant selectivity. Different pairs show different efficiencies of phosphotransfer, possibly reflecting different degrees of similarity and differences in tertiary structure. Phosphoryl transfer between *T. maritima* and mesophilic two-component proteins was examined using *E. coli* PhoR/PhoB proteins, a pair of two-component proteins that exhibit the highest sequence similarity to HpkA/DrrA (*8*). In consideration of the stability of the mesophilic proteins, the phosphotransfer reactions were examined at 35 °C. To eliminate complications resulting from different autophosphatase activities of the response regulators, phosphotransfer was assessed by following depletion of [^{32}P]phosphoryl groups from the phospho-histidine protein kinases. Using phospho-PhoR as the phosphoryl donor, transfer was observed with PhoB and to a lesser extent with DrrA (Figure 5A). Using phospho-HpkA57 as the phosphoryl donor, transfer was observed with DrrA and to a lesser extent with

Table 2: Stabilities of Acyl Phosphates in Phospho-DrrA and Acetyl Phosphate[a]

	DrrA-P half-life			acetyl phosphate half-life	
temp (°C)	+$MgCl_2$	+EDTA	denatured in 1% SDS	+$MgCl_2$	+EDTA
4	≫15 days[b]	≫15 days[b]	14 ± 1.4 days[c]	11 days	14 days
25	24 ± 0.4 h	7.1 ± 0.4 days	17 ± 2.1 h	17 h	21 h
37	4.7 ± 0.2 h	1.8 ± 0.2 days	3.2 ± 0.1h	2.7 h	3.2 h
55	37 ± 8.1 min	5.0 ± 0.2 h	30 ± 10 min	23 min	24 min
70	9.1 ± 1.0 min	1.7 ± 0.1 h	4.2 ± 0.9 min	4.5 min	5.0 min
80	3.0 ± 0.1 min	29 ± 3.8 min	2.3 ± 0.3 min	2.1 min	2.8 min

[a] Half-lives were determined from semilogarithmic plots of phospho-DrrA or acetyl phosphate remaining versus time, assayed as described in Experimental Procedures. Each value represents the average of two independent experiments. [b] At this time >90% of phospho-DrrA remained. [c] The 4 °C determinations were performed in the presence of 0.5% SDS.

PhoB (Figure 5B). It is worth noting that these reactions were examined at a temperature close to the optimum for the mesophilic response regulators, but significantly below the optimal temperature for DrrA phosphotransfer activity.

Effect of Temperature on the Rate of Dephosphorylation of Phospho-DrrA. Mesophilic response regulators exhibit varying degrees of divalent cation-dependent autophosphatase activity that result in phospho-response regulator half-lives ranging from seconds to hours (*2*−*5*). Hydrolysis of phospho-DrrA was examined at a variety of temperatures and conditions. The intrinsic hydrolysis rates of phospho-DrrA-exhibited first-order kinetics and half-lives were calculated from semilogarithmic plots. In the presence of Mg^{2+}, dephosphorylation exhibited temperature dependence, with the half-life of phospho-DrrA ranging from 3 min at 80 °C to >15 days at 4 °C (Table 2). It is intriguing that the half-life of phospho-DrrA at 80 °C, the growth temperature for *T. maritima*, is similar to the half-life of *E. coli* phospho-PhoB at 37 °C (*4*). In the presence of EDTA, dephosphorylation of DrrA was significantly slower, with half-lives approximately 10-fold greater at each temperature for which values were determined. Thus, DrrA exhibits Mg^{2+}-dependent autophosphatase activity as has been observed for other response regulators (*21*, *27*).

The stability of phospho-DrrA in the presence of EDTA is substantially greater than that of a typical acyl phosphate (*28*, *29*), suggesting that the folded protein stabilizes the phosphoaspartate. To further pursue this hypothesis, the half-lives of acyl phosphates in denatured phospho-DrrA protein and in the small molecule, acetyl phosphate, were determined at temperatures from 4 to 80 °C in the presence of 20 mM EDTA or 20 mM Mg^{2+} (Table 2). Denatured phospho-DrrA was prepared by the addition of 1% SDS, and the unfolded state of the protein under this condition was confirmed by CD analysis at 25 °C (data not shown). Denatured phospho-DrrA and acetyl phosphate exhibited decreasing stability at increasing temperatures, and throughout the temperature range, the denatured protein and small molecule acyl phosphates had comparable half-lives. These half-lives were similar to those observed in native phospho-DrrA in the presence of Mg^{2+}, but significantly shorter than those found in native phospho-DrrA in the absence of divalent cations. In contrast, only minor differences in the stability of acetyl phosphate were observed in the presence of 20 mM Mg^{2+} or 20 mM EDTA. This is consistent with the previously reported observation that Mg^{2+}-catalyzed hydrolysis of acetyl phosphate proceeds independently of the uncatalyzed reaction and is first-order in Mg^{2+} (*28*); thus low concentrations of Mg^{2+} have minimal effects on the nonenzymatic hydrolysis of acetyl phosphate.

Effect of HpkA on Phospho-DrrA Dephosphorylation. Dephosphorylation of response regulators can be mediated by their intrinsic phosphatase activity or by the phosphatase activity of their corresponding histidine protein kinase or other auxiliary proteins. To examine the phosphatase activity of the histidine protein kinase, HpkA57 was added to purified phospho-DrrA at 55 °C under nondenaturing conditions. When substoichiometric amounts of HpkA57 were added to phospho-DrrA a small increase in the rate of dephosphorylation of the response regulator was observed (Figure 6). The half-life of phospho-DrrA decreased approximately 1.5-fold in the presence of HpkA57. The magnitude of the increase in the rate of dephosphorylation observed with HpkA57 is much less than that reported for other histidine kinases containing well-defined phosphatase activities, for which dephosphorylation rate enhancements of >10-fold are typically observed (*30*−*33*). To examine whether reversal of the phosphotransfer reaction was contributing to the slight acceleration of phospho-DrrA dephosphorylation in the presence of HpkA57, the effects of nucleotides were examined. Whereas no significant changes in the dephosphorylation rates of phospho-DrrA occurred upon addition of either ATP or ADP in the absence of HpkA57, addition of ADP in the presence of HpkA57 resulted in an increased rate of phospho-DrrA dephosphorylation (Figure 6). Since the complete reverse reaction requires ADP to dephosphorylate phospho-HpkA57 by regeneration of ATP, these data suggest that reversal of the phosphotransfer reaction contributes to the enhanced rate of phospho-DrrA dephosphorylation observed in the presence of HpkA57. Further evidence for this mechanism comes from direct observation of transfer of radiolabeled phosphoryl groups from phospho-DrrA to HpkA57 when stoichiometric amounts of the two proteins are incubated in the absence of ADP (data not shown).

DISCUSSION

Thermostability of HpkA57 and DrrA Proteins. Purification procedures for DrrA and the cytoplasmic region of HpkA were developed exploiting the thermostability of these proteins relative to the heat denaturable mesophilic host proteins. Migration of the proteins during gel-filtration chromatography at the end of the purification procedures was consistent with the expected monomeric state of DrrA and a dimeric state for HpkA57. In cell lysates and at early steps in purification, HpkA57 migrated aberrantly in SDS−

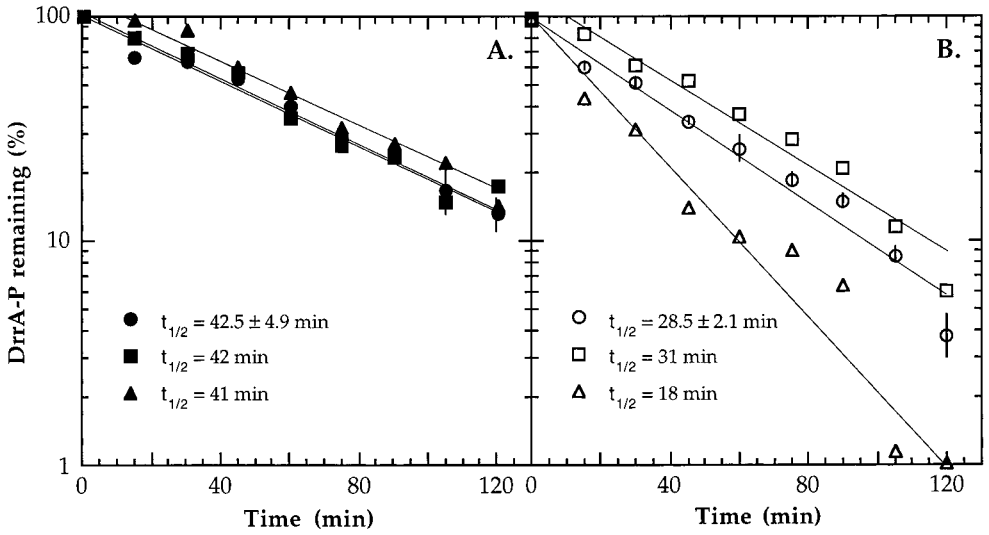

FIGURE 6: Effect of HpkA57 and nucleotides on the dephosphorylation rate of phospho-DrrA. [^{32}P]Phospho-DrrA (10 μM) was incubated in a final volume of 100 μL of 25 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, pH 8.0, at 55 °C containing 1 μM HpkA57, 1 mM ATP, or 1 mM ADP as indicated. Reactions were initiated by the addition of HpkA57 in the case of those reactions in which the influence of the histidine kinase was assayed, and either ATP, ADP, or buffer. At the indicated times, 10-μL aliquots were removed from the reactions and quenched by the addition of 10 μL of 2X SDS Laemmli sample buffer. Samples were analyzed as described in Experimental Procedures. (A) Phospho-DrrA stability in the absence of HpkA57: no nucleotide (●), ATP (■), and ADP (▲). (B) Phospho-DrrA stability in the presence of HpkA57: no nucleotide (○), ATP (□), and ADP (△). For assays in the absence of nucleotide, vertical bars show the range of values obtained in two independent experiments.

polyacrylamide gels. Native polyacrylamide gels were successfully used to monitor HpkA57 until purified to homogeneity, at which point it exhibited normal mobility during SDS gel electrophoresis.

A central issue in the adaptation of proteins to extreme environmental conditions is the conservation of a functional state optimized with respect to enzyme activity and conformational flexibility. For both DrrA and HpkA57, unfolding occurs at relatively high temperatures. The thermal denaturation of DrrA was observed to be irreversible, perhaps attributable to the multidomain structure of the protein. The apparent T_m of 95 °C is in the range expected for proteins from *T. maritima*, which grows at 50−90 °C. HpkA57 exhibited a reversible thermally induced transition at 75 °C. The lower temperature may reflect a destabilization of the protein caused by removal of the 57 N-terminal amino acid residues containing the transmembrane domain and flanking sequence. In both proteins, the temperatures of the transitions corresponded closely with the temperatures at which maximum rates of enzymatic activities, autophosphorylation of HpkA57 and autodephosphorylation of DrrA, were observed.

Smaller structural transitions were also observed for both proteins at approximately 35 °C, presumably representing a reorganization within the folded protein. These structural perturbations apparently influence function as evidenced by breaks at similar temperatures in the Arrhenius plots of enzymatic rate constants. Significantly higher activation energies are associated with the phosphorylation/dephosphorylation reactions at ambient temperatures as compared to reactions at elevated temperatures. Similar structural and functional transitions at ambient temperatures have previously been observed for other enzymes from thermophiles (*34*, *35*).

Chemistries of Autophosphorylation and Phosphotransfer. The primary sequences of HpkA and DrrA contain all of the conserved residues, including the phosphorylation sites, characteristic of histidine protein kinases and response regulator proteins (*8*). Thus it was expected that phosphorylation would occur at a histidine residue in HpkA57 and at an aspartic acid residue in DrrA. The rates of phosphate hydrolysis determined for denatured phospho-HpkA57 and phospho-DrrA under different chemical conditions were indeed consistent with this assumption. Phospho-HpkA57 was unstable in acidic conditions but stable in alkaline solutions similar to the stabilities reported for many phosphorylated mesophilic histidine protein kinases (*4*, *18*, *19*, *36*−*38*) and for phospho-Enzyme I of the bacterial phosphotransferase system (*20*). Phospho-DrrA was unstable in both acidic and alkaline conditions, resembling the stability observed for acyl phosphates (*22*, *28*, *39*, *40*). Mutation of Asp54 to Cys resulted in a DrrA protein incapable of transferring phosphate from HpkA57. Furthermore, HpkA57 was able to serve as a phosphodonor for the *E. coli* response regulator PhoB, and DrrA could transfer phosphate from the *E. coli* histidine protein kinase PhoR. This exhibition of cross-talk between homologous proteins isolated from dissimilar organisms suggests that very similar chemistries are used in both the mesophilic and thermophilic two-component phosphotransfer systems.

Dephosphorylation of Phospho-DrrA. The lifetimes of the phosphorylated states of previously characterized mesophilic response regulators vary significantly, with half-lives ranging from seconds to hours (*2*−*5*). In these proteins, intrinsic autophosphatase activities together with dephosphorylating activities of auxiliary proteins determine the lifetimes of the phosphorylated response regulators in vivo. At 80 °C, phospho-DrrA has a half-life of a few minutes, similar to that of many mesophilic response regulators. Like other two-component systems that function to regulate gene expression, and in which the phosphorylated response regulators have half-lives in the range of minutes, the cognate kinase,

HpkA57, has no significant phosphatase activity against the response regulator, DrrA (*41*).

In thermophilic response regulators, the stability of the relatively labile acyl phosphate might be expected to pose special problems. The stability of a representative acyl phosphate, acetyl phosphate, was found to decrease substantially with increasing temperature, with acetyl phosphate having a half-life of only a couple of minutes at 80 °C. Throughout the temperature range from 4 to 80 °C, native phospho-DrrA, in the presence of Mg^{2+}, has half-lives just slightly greater than those of acetyl phosphate or SDS-denatured phospho-DrrA. Thus it appears that the overall effect of the folded protein is to stabilize the phospho-aspartate.

This slight stabilization actually reflects a balance between significant opposing influences. Like most previously characterized mesophilic response regulator proteins, DrrA exhibits autophosphatase activity that can be affected by the removal of divalent metal ions with EDTA. Under such conditions, the half-life of phospho-DrrA was found to be approximately 12-fold greater than the half-lives of acetyl phosphate or SDS-denatured phospho-DrrA. DrrA itself seems to be acting to protect the labile acyl phosphate from hydrolysis. The 1.5-fold stabilization of native phospho-DrrA relative to an isolated acyl phosphate can thus be considered to result from a 12-fold stabilization by the folded protein coupled with an 8-fold destabilization resulting from the divalent cation-dependent autophosphatase activity. Why relatively large opposing effects are used to achieve a minor alteration in stability is an intriguing question. The most obvious explanation is that such a strategy provides greater potential for regulation. It is also conceivable that the intrinsic destabilization may be an obligatory consequence of the presence of a divalent cation, perhaps required for other purposes such as phosphotransfer or restructuring of the active site in the presence of the phosphoryl group. Alternatively, the mechanism may simply reflect a logical evolutionary path from other response regulators.

The mechanism of protection of the phospho-aspartate from hydrolysis can be readily envisioned as a shielding of the phosphorylated residue from water, the proposed nucleophile of the hydrolytic reaction. Homology modeling of DrrA based on the known three-dimensional structures of other regulatory domains failed to reveal any unusual features surrounding the active site region. Sequestering of the phospho-aspartate most likely results from specific positioning of residues in the active site region to allow limited solvent accessibility to the labile phospho-anhydride bond. The enhanced stability of the protein and the long lifetime of the phosphorylated state at low temperatures raise the possibility that this issue as well as others regarding the phosphorylated state may eventually be addressed by biophysical and structural characterization of the phosphorylated response regulator protein.

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