Mechanism of Phosphatase Activity in the Chemotaxis Response Regulator CheY†

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ABSTRACT: Response regulator proteins are phosphorylated on a conserved aspartate to activate responses to environmental signals. An intrinsic autophosphatase activity limits the duration of the phosphorylated state. We have previously hypothesized that dephosphorylation might proceed through an intramolecular attack, leading to succinimide formation, and such an intramolecular dephosphorylation event is seen for CheY and OmpR during mass spectrometric analysis [Napper, S., Wolanin, P. M., Webre, D. J., Kindrachuk, J., Waygood, B., and Stock, J. B. (2003) *FEBS Lett 538*, 77−80]. Succinimide formation is usually associated with the spontaneous deamidation of Asn residues. We show here that an Asp57 to Asn mutant of the CheY chemotaxis response regulator undergoes an unusually rapid deamidation back to the wild-type Asp57, supporting the hypothesis that the active site of CheY is poised for succinimide formation. In contrast, we also show that the major route of phosphoaspartate hydrolysis in CheY occurs through water attack on the phosphorus both during autophosphatase activity and during CheZ-mediated dephosphorylation. Thus, CheY dephosphorylation does not usually proceed via a succinimide or any other intramolecular attack.

Response regulator proteins such as CheY are the downstream effectors of signal transduction in phosphorelay networks with sensor histidine protein kinases (*1*−*3*). Histidine kinases catalyze phosphotransfer from ATP to specific histidine imidazole side chains. The phosphate from the phospho-His is subsequently transferred to an Asp in a cognate response regulator protein. Phosphorylation induces changes in response regulator conformation that activate responses ranging from changes in gene expression to alterations in cell motility (*4*, *5*).

CheY is a doubly wound α/β protein composed of a central five-stranded parallel β-sheet flanked by five α-helices. The conserved aspartate is situated on the C-terminal edge of β-strand 3 in an acidic pocket containing two other highly conserved aspartate residues. The active site is also characterized by conserved lysine and threonine residues (*5*−*7*). The fold of the CheY monomer and the composition of the active site very closely resemble the features found in the receiver domain of other two-component response regulators. Through sequence alignment studies Ridder and Dijkstra (*8*) determined that the basic geometry of the active site including the identity of the conserved amino acids extends beyond response regulators and is more broadly characteristic of the larger HAD[1] superfamily of which dehalogenases and P-type ATPases are also members. This common geometry may imply mechanistic similarities among family members. Though overall structure and functions mediated by HAD proteins are diverse, all employ a covalent modification of an aspartate followed ultimately by hydrolysis to carry out nucleophilic displacement. In the case of response regulators and P-type ATPases, this nucleophilic attack is potentially enhanced by divalent cation binding at the active site.

Response regulators and other phosphorylated members of the HAD superfamily have an intrinsic autophosphatase activity. For response regulators, it has generally been assumed that the phosphatase reaction proceeds essentially as a reversal of the phospho-His phosphotransfer reaction with water or hydroxide taking the place of the histidine imidazole side chain (*9*). An alternative possibility, however, is that the phosphoryl group could be displaced by nucleophilic attack at the Asp β-carboxyl group. Figure 1 illustrates this reaction and compares it to the well-studied asparagine deamidation reaction, which is known to usually proceed through a succinimide intermediate (*10*−*12*). We recently hypothesized that the mechanism of phospho-Asp hydrolysis via a succinimide may play a role in the activation/inactivation mechanism of response regulator proteins such as CheY (*13*).

In vivo the CheY dephosporylation rate is increased by interaction with the CheZ phosphatase. The structure of the cocrystal between CheY and CheZ has led to the hypothesis that CheZ acts to accelerate hydrolysis by positioning a water molecule for inline attack on the CheY phospho-Asp (*14*). Hence, CheY dephosphorylation might proceed by one

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[1] Abbreviations: HAD, haloacid dehalogenase; D57N, Asp57 to Asn mutation; EDTA, ethylenediaminetetraacetic acid; H15D, His51 to Asp mutation; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPLC, high-performance liquid chromatography; HPLC/MS, an HPLC system inline with an electrospray mass spectrometer; IEF, isoelectric focusing; isoAsp, isoaspartyl residue; PEP, phosphoenol pyruvate; PMSF, phenylmethylsulfonal fluoride; RBIII, reaction buffer III; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEG buffer, Tris, EDTA, glycerol buffer; TFA, trifluoroacetic acid; WT, wild type.

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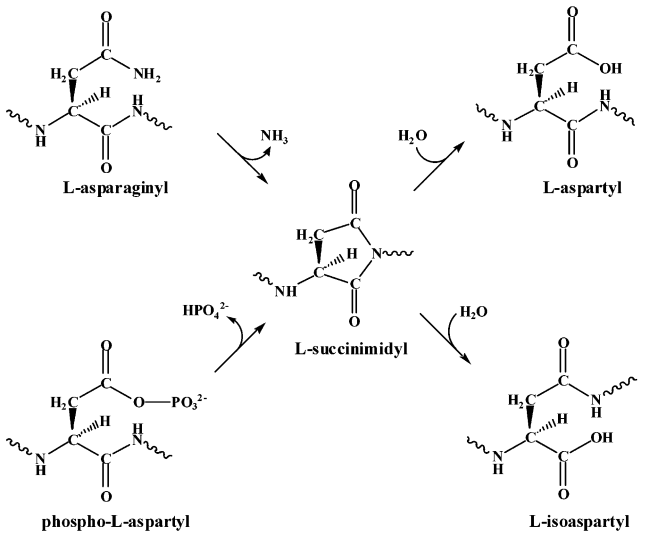

FIGURE 1: Formation and breakdown of a succinimide. Succinimide residues have been shown to form from Asp and Asn residues. They may also be able to form as an intermediate in phospho-Asp hydrolysis.

mechanism for spontaneous phospho-Asp hydrolysis, but a different mechanism when hydrolysis is mediated by CheZ.

We show here that the D57N mutant of CheY undergoes a metal-dependent deamidation back to Asp57, a result that supports the idea that the active site of CheY is poised for hydrolysis via attack at the β-carboxyl. The results of our studies of ^{18}O incorporation during dephosphorylation clearly show, however, that the major CheY dephosphorylation routes involve attack at the phosphorus.

MATERIALS AND METHODS

Buffers and Reagents. Except as noted, all chemicals and buffers were of reagent grade and purchased from standard suppliers. Water (95% ^{18}O) (normalized) was purchased from Icon Services, Inc. (Summit, NJ). Sequencing-grade modified trypsin was from Princeton Separations (Adelphia, NJ). HPLC-grade water, acetonitrile, acetic acid, and TFA were used for HPLC and HPLC/MS analysis.

Protein Purification. The WT and D57N variants of *Salmonella typhimurium* CheY were purified by ion-exchange and gel filtration chromatography as described previously (*15*), and concentrated to 1.18 and 0.47 mM, respectively. *S. typhimurium* CheA (*16*), *S. typhimurium* CheW (*17*), and all Q LZ-Tar_C (*18*) were purified as described previously. Protein concentration was determined using absorbance at 280 nm. *S. typhimurium* CheZ was purified from JM109/pME105 (*19*). Cells were washed into 0.1 M $NaPO_4$ (pH 7) plus protease inhibitors (Calbiochem) and lysed using a model M-110Y microfluidizer (Microfluidics Corp., Newton, MA), and the lysate was clarified by centrifugation at 100000*g* (24000 rpm in a Beckman SW-28 rotor). The lysate was precipitated twice with 43% $(NH_4)_2SO_4$, and dialyzed against TEG buffer consisting of 50 mM Tris, 1 mM EDTA, and 10% glycerol, pH 7.5. The protein was first separated using a 90 mL ion-exchange column with DEAE-650M resin (Tosoh Corp.) with a gradient of 0−500 mM NaCl in TEG buffer. The CheZ-containing fractions were pooled, precipitated in 50% $(NH_4)_2SO_4$, and resuspended in TEG buffer with 150 mM NaCl. This protein was applied to a Sephacryl S-200 column (Amersham Biosciences) equilibrated in the same buffer, and the CheZ-containing fractions were pooled, precipitated in 50% $(NH_4)_2SO_4$, and resuspended in 10 mM $NaPO_4$, 0.8 M $(NH_4)_2SO_4$, pH 7.0. The protein was applied to a phenyl-650M column (Tosoh Corp.) equilibrated in 10 mM $NaPO_4$, 0.8 M $(NH_4)_2SO_4$, pH 7.0, and eluted with a gradient of 0.8−0 M $(NH_4)_2SO_4$. CheZ-containing fractions were pooled, dialyzed back into TEG buffer, and concentrated to 3.6 mg/mL in an Ultrafree 30 concentrator (Millipopre). This preparation was approximately 70% pure as judged by a Coomassie R-250-stained SDS−PAGE gel scanned using a ScanMaker 4 (Microtek) with Photoshop software (Adobe) and with the intensity of the bands in the scanned image quantitated on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). The CheZ preparation was free of significant contaminating ATPase or nonspecific phosphatase activity. Protein concentration was determined by the Bradford-type Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard.

CheY Phosphatase Activity. The phosphatase activity of CheY was assayed using a coupled ATPase assay at 20 °C along with soluble complexes formed from CheA, CheW, and LZ-Tar_C (*20*). The complexes were formed from 10 μM CheA, 10 μM CheW, and 50 μM LZ-Tar_C in RBIII that consists of 25 mM Tris, 25 mM NaCl, 50 mM potassium glutamate, 5 mM $MgCl_2$, 5% DMSO, and 10% glycerol, pH 7.5. The coupled system used has final concentrations of 100 U/mL pyruvate kinase and lactate dehydrogenase, 10 mM PEP, 2 mM NADH, and 2 mM ATP in RBIII. In the assay, the coupled regeneration of ATP by consumption of NADH and the kinase activity of CheA (0.5 μM final) in the activated complexes were both much higher than the phosphatase activity of CheY (typically 3 μM final WT CheY and 3−10 μM final D57N CheY). Thus, the phosphatase activity of CheY was rate-limiting, and the measured ATPase activity (less background) was equal to the phosphatase activity of CheY. The ATPase rate was determined by monitoring the consumption of NADH at 340 nm in an 80 μL reaction volume in a 1 cm cuvette in a thermostated holder at 20 °C using a DU-530 spectrophotometer (Beckman-Coulter).

The effect of CheZ on the CheY phosphatase rate was also detemined using the coupled assay. CheY and CheZ were mixed together at a concentration of 0.1 μg/μL CheZ and 40 μM CheY. The ATPase rate of 2 μL of this CheY−CheZ mixture in an 80 μL reaction in RBIII was assayed and compared to the ATPase rate from CheY alone.

D57N CheY Deamidation. To make comparison with published deamidation rates for model peptides, samples of WT and D57N CheY were prepared in 150 mM pH 7.4 Tris (*21*). To each was also added 1 mM EDTA, 10 mM $MgCl_2$, or 10 mM $MnCl_2$ to determine the effect of having a metal ion present or absent in the active site. Samples were sterile filtered using 0.22 μM Spin-X filters (Corning) and kept at 36 °C in a warm room except when being assayed for CheY phosphatase activity. The increases in phosphatase activity were fit to exponential curves using KaleidaGraph software (Synergy Software).

Isoaspartate Detection. Protein isoAsp content was assayed using the ISOQUANT isoaspartate detection kit

(Promega), using the radioactive detection protocol. Samples of WT and D57N CheY were digested for 2 h at 30 °C with trypsin, 1 mM PMSF was added, and then 25 μL was used in each 50 μL ISOQUANT assay.

Isoelectric Focusing. To simulate the conditions where a dehydrated form of CheY is seen during HPLC/MS (*13*), CheY was phosphorylated, bound, and eluted from a reversed-phase column. A Waters model 510 HPLC with a Luna C-18 reversed-phase column (Phenomenex) equilibrated with 10% acetonitrile and 0.1% TFA was used. WT CheY was phosphorylated for 10 min at 37 °C in 50 mM Tris, pH 7.5, 20 mM acetyl phosphate, and 20 mM $MgCl_2$. This was injected onto the C-18 column at a flow rate of 1 mL/min and then eluted with a gradient of 10−80% acetonitrile. An ultraviolet absorbance detector set to 280 nm was used to monitor the elution of the CheY, and the peak fraction was collected for IEF analysis.

Alternately, CheY was simply phosphorylated and then diluted into acidic buffer. Phosphorylation conditions were the same as for the HPLC experiment, and then CheY was diluted 10-fold into 30% acetonitrile with 0.1% TFA in H_2O, or into 0.1% TFA in H_2O.

IEF gels were cast and run for a gel with a pH range of 4−6 following standard protocols (*22*). Bio-Lyte ampholytes in the pH 4−6 and 3−10 ranges (BioRad) were used, as well as ProtoGel premixed 30% acrylimide−0.8% bisacrylimide (National Diagnostics). The phosphoric acid analyte was put in the upper chamber where the samples were loaded. Loading the samples under acidic conditions should have stabilized any succinimide that was present (*23*). WT and D57N CheY were run as standards using a final buffer matched to the experimental samples.

^{18}O Incorporation Assays. For multiple-round dephosphorylation experiments, WT CheY, HEPES buffer, and $MgCl_2$ were diluted into 95% ^{18}O water to give a final concentration of 50 μM CheY, 140 mM HEPES, 15 mM $MgCl_2$, pH 7.6, in approximately 83% ^{18}O water. As a negative control, samples were prepared in ^{18}O water with 20 mM acetyl phosphate plus 5 mM EDTA, but no $MgCl_2$. These reaction mixtures were incubated overnight at 37 °C to ensure complete hydrolysis of the acetyl phosphate. The resulting production of acetic and phosphoric acid lowered the final pH to around 7.4.

To generate positive controls, the carboxy terminus of each peptide fragment was labeled with ^{18}O. CheY samples were digested with trypsin overnight at 30 °C in ∼81% ^{18}O water, and then formic acid was added to 5% to inactivate the trypsin (*24*). A portion of each experimental sample was exchanged back into natural-abundance water by using a CentriSpin-10 spin desalting column (Princeton Separations) equilibrated with 50 mM Tris, 5 mM EDTA, pH 8.0. After this buffer exchange, the resulting samples were digested overnight at 30 °C with trypsin. Control samples were prepared by diluting stock CheY protein into 50 mM Tris, 5 mM EDTA, pH 8.0, and digesting overnight at 30 °C with trypsin.

Additionally, Asp57-specific positive controls were generated essentially following the method described above where rapid D57N CheY deamidation is observed. D57N CheY was incubated in ∼83% ^{18}O water with $MnCl_2$ and 140 mM Tris until deamidation reached approximately 90% as determined by assaying for the appearance of ATPase activity as described above. Regardless of its mechanism, the deamidation event must lead to the incorporation of a single oxygen atom at Asp57. These samples were exchanged into Tris−EDTA buffer and processed with trypsin as described above for WT CheY.

The effect of CheZ on CheY was assayed by incubating 40 μM CheY and 10 μg of CheZ with 140 mM HEPES, 15 mM $MgCl_2$, and 20 mM acetyl phosphate, pH 7.6, in approximately 82% ^{18}O water ovenight at 37 °C. In control samples in normal-water buffer, the CheZ-mediated CheY phosphatase rate (measured as described above using the coupled ATPase assay) after the overnight incubation was still approximately 70% of the value at the beginning of the incubation, indicating that the majority of the CheZ was still active in the mixture. These samples were buffer exchanged and processed with trypsin as described above for WT CheY.

The mass spectra of the WT and deamidated D57N CheY tryptic peptides were measured by HPLC/MS. The HPLC/MS system uses an Ultimate Capillary HPLC system (Dionex), and the peptides were separated using a PicoTip column (New Objective) packed with Jupiter C4 resin (Phenomenex) which terminates in a 15 μm nanospray needle tip. A 1.35 μL sample of digested CheY was injected, and a gradient of 5−85% acetonitrile plus 0.1 M acetic acid at a flow of 0.170 μL/min was used to elute the peptides from the column. The nanospray needle introduces the sample into a LCQ DecaXP Plus ion trap mass spectrometer (Thermo Finnigan). The +2 charge species of selected peptide fragments were detected and high-resolution spectra obtained using two zoom scan windows from 900 to 910 amu and from 1372 to 1392 amu. The predicted peptide masses were calculated from the CHEY_SALTY entry in the SWISS-PROT database using the PeptideMass software tool (*25*).

Retention of Phosphate by CheY during HPLC. A Waters model 510 HPLC with a Luna C-18 reversed-phase column (Phenomenex) was used for all stand-alone HPLC experiments. CheA was phosphorylated using 0.2 mM [γ-^{32}P]ATP (5500 cpm/pmol) in 50 mM Tris, 50 mM KCl, 5 mM $MgCl_2$ pH 9 (*26*), and purified from free ATP using a CentriSpin-10 desalting column. About 40 μM ^{32}P-CheA (based on ^{32}P activity) and 10 μM CheY were mixed and immediately injected onto the C-18 column at a flow rate of 1 mL/min. The process of mixing and injection took about 40 s. A gradient of 10−80% acetonitrile plus 0.1% TFA in H_2O was used to elute the proteins. Fractions were collected for 30 s each. A 100 μL sample of each fraction was counted using a Beckman LS 6000SE scintillation counter. The remainder of each fraction was evaporated to dryness using a SpeedVac concentrator (Savant) and resuspended using SDS sample buffer with 90 mM NaOH. Half of each resuspended fraction was loaded and run on a 15% SDS−PAGE gel, and stained with Coomassie R-250. Coomassie-stained gels were scanned and the bands quantitated as described above.

RESULTS

Measurement of D57N CheY Deamidation. A significant deamidation of D57N CheY has previously been observed during in vitro assays of purified protein (*9*), and our recent observation of a possible succinimide intermediate during HPLC/MS (*13*) suggested to us that the active site of CheY might be poised for rapid deamidation. To quantitatively

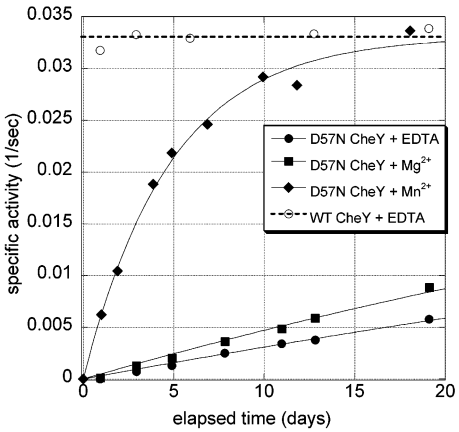

FIGURE 2: Recovery of D57N CheY phosphatase activity. D57N CheY and WT CheY were incubated at 36 °C in 140 mM Tris, pH 7.4, with $MnCl_2$, $MgCl_2$, or no divalent metal. Deamidation of D57N CheY back to functional CheY was measured using the coupled ATPase assay in the presence of activated CheA.

measure the deamidation rate of D57N CheY, both WT and D57N variants of CheY were incubated at 36 °C with the buffer and pH (150 mM Tris, pH 7.4) chosen to match that previously used for deamidation studies of model peptides (*27*). On the basis of peptide studies, predictions of deamidation time for Asn residues in proteins indicate that only 0.37% of Asn residues followed by Trp would have a deamidation half-time of less than 100 days, and none less than 25 days (*21*). For the peptide sequence Ser-Asn-Trp (the sequence around Asn57 in D57N CheY), the measured deamidation half-time was 76.8 days (*27*).

In contrast to the measured deamidation time for the Ser-Asn-Trp peptide, we found an unusually rapid spontaneous deamidation can occur in the D57N CheY protein (Figure 2). This rapid deamidation depends on the presence of divalent ions, with the increase being correlated to the relative binding affinity (*9*). With Mn^{2+}, $T_{1/2}$ was about 3.3 days, while with Mg^{2+} $T_{1/2}$ was estimated to be 45 days, and $T_{1/2}$ was about 70 days in the absence of metal. The value we estimate for the D57N CheY deamidation half-time in the absence of metal (70 days) is close to the value measured for the Ser-Asn-Trp peptide, suggesting that metal binding in the active site is required to accelerate deamidation. WT CheY showed essentially constant activity over the same time period (Figure 2), and the average measured hydrolysis rate of 0.0338 ± 0.0002 min^{-1} is very close to previously reported values of the spontaneous hydrolysis rate at 20 °C (*28*).

Since deamidation usually proceeds through a succinimide intermediate, the rapid deamidation of D57N lends support to our hypothesis that the active sites of response regulators such as CheY are poised for succinimide formation (*13*). This hypothesis arose as a result of our observation during HPLC/MS of a phosphorylation-dependent dehydrated (−18 Da) form of CheY, as well as from a similar observation previously reported for the response regulator OmpR (*29*). However, it is surprising that D57N CheY appears capable of complete deamidation back to the WT Asp57 form. During deamidation of Asn residues via a succinimide intermediate in model peptides (Figure 1), the majority of succinimides hydrolyze to isoAsp (*10*). In contrast, none of the D57N CheY protein was converted to isoAsp at position 57, on the basis of both the recovery of phosphotransfer activity and the fact that there was no substantial isoAsp formation for either the WT or D57N CheY protein incubated under equivalent conditions. After 20 days of incubation, for D57N CheY with Mn^{2+}, we measured 0.022 ± 0.002 mol of isoAsp/mol and 0.012 ± 0.003 mol of isoAsp/mol for WT CheY with Mn^{2+}.

IEF Assay for Succinimide Formation. On the basis of our previous observation of a dehydrated form of CheY during mass spectrometric analysis (*13*), and on the basis of the evidence above that D57N CheY is poised for deamidation, we performed IEF with WT CheY to look for a succinimide form directly. A succinimide intermediate as a consequence of aspartate phosphorylation has been observed by IEF for the H15D mutant of *E. coli* Hpr (*30, 31*). CheY was first phosphorylated with acetyl phosphate. The phosphorylated CheY protein was either injected onto the reversed-phase column, and the eluted protein collected, or simply diluted into an acidic buffer. In both cases the CheY was subjected to IEF to separate WT CheY from CheY with a +1 relative charge due to cyclization of Asp57 into a succinimide, but no +1 charge species of CheY was detected as a consequence of dephosphorylation (data not shown). D57N CheY was used as a standard to determine the effect of a +1 charge change on the position of CheY in the gel.

^{18}O Incorporation Assay To Determine the CheY Spontaneous Hydrolytic Mechanism. To further look for evidence of hydrolysis of phospho-CheY through water attack at the β-carboxyl, CheY was allowed to undergo dephosphorylation multiple times in ^{18}O-water-based buffer. CheY samples were exchanged back to natural-abundance-water-based buffer and then digested with trypsin. The tryptic fragments were analyzed by HPLC/MS to look for a mass shift due to incorporation of ^{18}O. We typically examined in detail both the Asp57-containing fragment and two additional fragments as an internal control. Using the zoom scan feature of the mass spectrometer, we obtained very accurate mass spectra. The average mass value was within about 0.1 amu of the predicted value for the +2 ion of each fragment. Table 1 shows the predicted masses of these CheY fragments, while Figure 3 shows typical mass spectra for tryptic fragments of CheY and tryptic fragments of CheY labeled uniformly at the carboxy-terminal oxygens with ^{18}O during the digest. As an Asp57-specific positive control we allowed D57N CheY to undergo deamidation in an ^{18}O-water-based buffer. Regardless of the deamidation mechanism, this must result in incorporation of one solvent oxygen at residue 57. For these samples we observe a net mass shift of 0.74 ± 0.05 mass unit in the +2 mass spectrum of the Asp57-containing fragment relative to the predicted *m*/*z* for this fragment (see Table 2 and Figure 4B). This indicates that about 74% of the fragments have ^{18}O incorporated, and is consistent with the approximately 70−80% labeling expected on the basis of the buffer content of ^{18}O and the degree of deamidation measured using the ATPase assay. This clearly confirms that we can observe ^{18}O incorporation at Asp57 and also demonstrates that ^{18}O incorporated at Asp57 is not lost during the sample handling procedures and HPLC/MS analysis.

If solvent oxygens are incorporated into CheY during the hydrolysis of phospho-CheY, we expect to see labeling at both oxygen atoms in Asp57 with ^{18}O. In this case, the mass spectrum of the +2 ion of the 2767 Da mass fragment would be expected to look like the rightmost panel in Figure 3B

Table 1: Tryptic Fragments of CheY Routinely Measured for This Study and Their Predicted Masses[a]

residues	sequence	predicted mass (Da)	predicted *m/z* of the +2 ion
45-69	LQAGGFGFIISDWNMPNMDGLELLK	2767.21	1384.61
92-118	ENIIAAAQAGASGYVVKPFTAATLEEK	2750.10	1376.06
73-90	ADSAMSALPVLMVTAEAK	1805.13	903.58

[a] The predicted peptide fragments and their masses were calculated from the sequence of *S. typhimurium* CheY from the CHEY_SALTY entry in the SWISS-PROT database using the PeptideMass software tool (*25*).

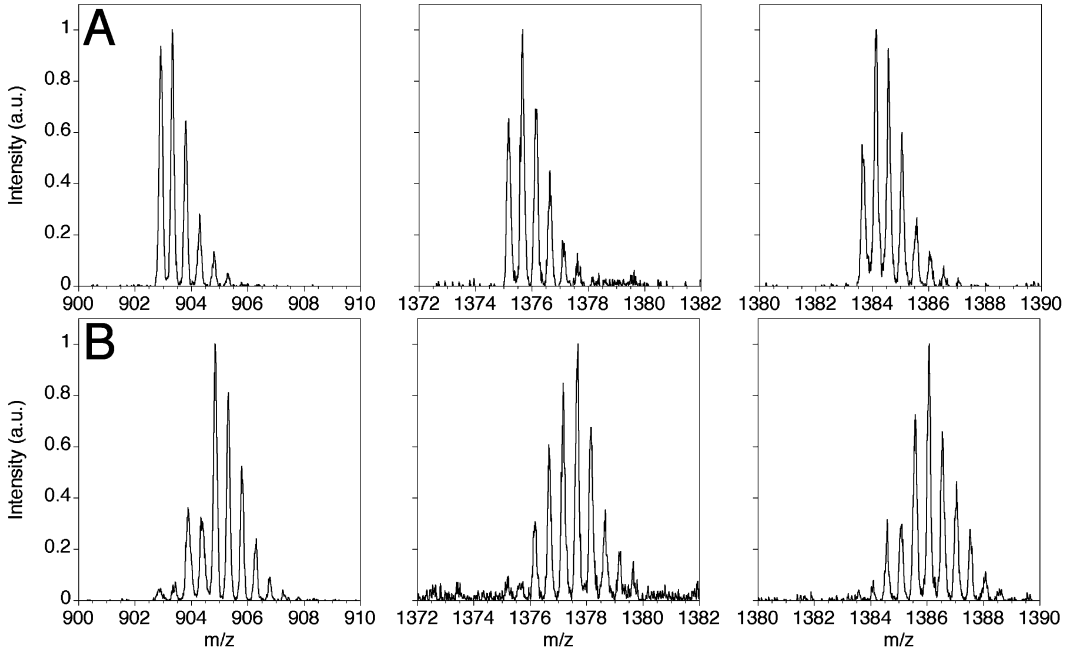

FIGURE 3: Mass spectra of three tryptic fragments of CheY. Representative mass spectra of the +2 ions of the fragments shown in Table 1. (A) Tryptic fragments generated by digestion of stock CheY protein. (B) Positive control generated by digesting CheY with trypsin in ^{18}O-water-based buffer. The mass spectrum of the +2 ion of the 2767.21 Da fragment generally had a lower signal-to-noise ratio than the other two fragments, perhaps due to less efficient ionization. The positive control fragments have a net mass shift of about 1.5 mass units, consistent with the expected 1.6 mass unit shift for 65.6% labeled with two ^{18}O atoms, and an additional 30.8% labeled with one ^{18}O atom. This expected labeling assumes 81% ^{18}O in the digest buffer and that the reaction with trypsin has come to its final equilibrium (*24*).

Table 2: Measured *m/z* Values of +2 Ions of CheY Tryptic Fragments Compared to Predicted Values[a]

sample	903.58	Δ	1376.06	Δ	1384.61	Δ
CheY control	903.47 ± 0.03	−0.11	1376.10 ± 0.05	+0.04	1384.56 ± 0.01	−0.05
D57N CheY deamidated in ^{18}O water	903.50 ± 0.04	−0.08	1376.13 ± 0.12	+0.07	1385.35 ± 0.05	+0.74
CheY reacted in ^{18}O water	903.48 ± 0.04	−0.10	1376.04 ± 0.06	−0.02	1384.54 ± 0.06	−0.07
CheY with CheZ reacted in ^{18}O water	903.53 ± 0.01	−0.05	1376.21 ± 0.07	+0.15	1384.54 ± 0.02	−0.07

[a] The measured *m/z* was determined by calculating the weighted average of the +2 charge spectrum. Values represent the average of measurements on three or four independent samples plus or minus the standard deviation. The predicted values of *m/z* are given in the column heads. The difference between the measured *m/z* and the predicted *m/z* is indicated in each column labeled "Δ". The CheY control samples were generated by digestion of stock CheY protein, D57N CheY was deamidated in ^{18}O-water-based buffer with $MnCl_2$, and CheY or CheY with CheZ were subjected to multiple rounds of phosphorylation/dephosphorylation by incubating in ^{18}O-water-based buffer with acetyl phosphate and $MgCl_2$.

and have a mass shift of around 1.6 mass units (based on the ^{18}O content of the buffer). In contrast, the results of multiple trials for experiments with CheY incubated with acetyl phosphate in ^{18}O-water-based buffer were consistent with zero or very low incorporation of ^{18}O into CheY at Asp57 during the dephosphorylation reaction (see Table 2 and Figure 4C).

^{18}O Incorporation Assay To Determine the CheZ-Mediated CheY Hydrolytic Mechanism. As expected, purified CheZ was found to substantially increase the CheY hydrolysis rate. Mixing CheY and CheZ at an approximately 10:1 molar ratio increased the CheY hydrolysis rate from 0.034 to 0.37 ± 0.05 min^{-1}. Thus, under these conditions, almost all the CheY dephosphorylation was mediated by CheZ. After incubation of CheY with CheZ and acetyl phosphate in ^{18}O-water-based buffer, the mass spectra of the CheY tryptic fragments were consistent with zero or very low incorporation of ^{18}O into CheY at Asp57 during the dephosphorylation reaction (see Table 2 and Figure 4D).

^{32}P Assay To Determine the Stability of Phospho-CheY during HPLC Analysis. We have previously reported that the HPLC/MS analysis of CheY phosphorylated with acetyl phosphate shows very little phosphoprotein, but instead a large fraction of the protein is observed to be in a dehydrated form (a mass of −18 Da relative to unmodified CheY) (*13*). Experiments with a stand-alone HPLC system were con-

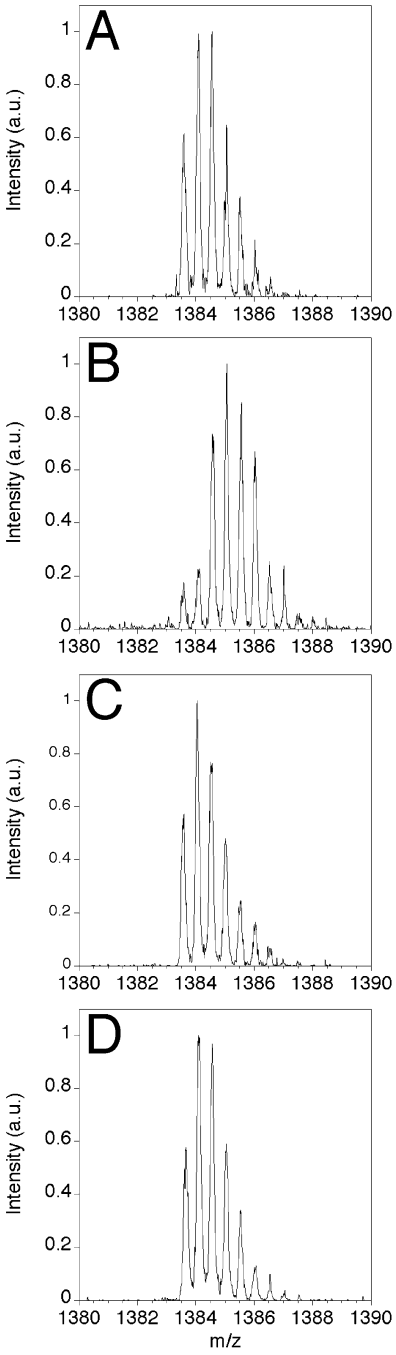

FIGURE 4: Mass spectra of the Asp57-containing CheY tryptic fragment. Representative mass spectra of the +2 ion of the 2767.21 Da tryptic fragment of CheY. (A) CheY negative control incubated in ^{18}O-water-based buffer with acetyl phosphate but no $MgCl_2$. (B) D57N CheY positive control generated by allowing D57N CheY to deamidate in ^{18}O-water-based buffer with $MnCl_2$. (C) CheY incubated with acetyl phosphate and $MgCl_2$ in ^{18}O-water-based buffer. (D) CheY incubated with CheZ, acetyl phosphate, and $MgCl_2$ in ^{18}O-water-based buffer.

ducted to determine whether the lack of phosphorylated CheY observed by HPLC/MS was due to hydrolysis that occurred during the HPLC separation, or during the subsequent mass spectrometric analysis.

To measure the fraction of phospho-CheY remaining after elution, CheY was labeled using ^{32}P-CheA. Since the phosphotransfer from CheA to CheY is very rapid (*26*, *32*), ^{32}P is transferred to CheY as soon as the proteins are mixed, while the excess CheA would be expected to maintain the

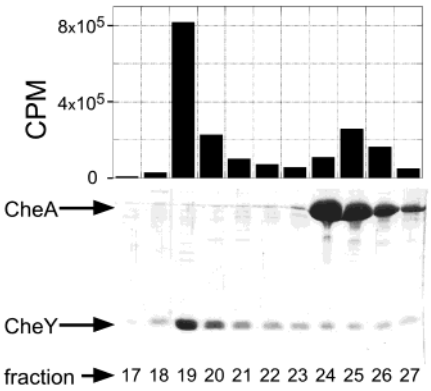

FIGURE 5: Retention of ^{32}P by CheY after reversed-phase HPLC. CheY was mixed with ^{32}P-CheA, injected onto a C-18 column, and eluted with a gradient of acetonitrile in water. Free ^{32}P passed through the column and appeared around fraction 6 (not shown). The fraction of phospho-CheY was determined for the fractions that elute before the CheA peak (fractions 17−23). CheY injected alone elutes as a tight peak, but some interaction between CheY and CheA must persist during the HPLC run as shown by the presence of a low level of CheY in fractions 24−27.

CheY at close to full phosphorylation during the time required to mix and inject the sample. Fractions from four separate HPLC injections were analyzed by SDS−PAGE and scintillation counting. Figure 5 shows a comparison for one injection of the radioactivity measured for fractions 17−27 as compared to a Coomassie-stained SDS−PAGE gel run using the same fractions. The results of these analyses indicated that from 48% to 73% (average 60%) of the CheY was still phosphorylated after HPLC separation. Thus, the majority of the dephosphorylation of CheY during HPLC/MS must occur during the mass spectrometric stage of the analysis. The results of the ^{18}O incorporation assays described above rule out attack at the phospho-Asp β-carboxyl as the normal route of phospho-CheY hydrolysis under the in vitro conditions tested. Together these results suggest that the dehydrated forms of CheY (*13*) and OmpR (*29*) seen during electrospray mass spectrometry probably result from an intramolecular dephosphorylation mechanism that is greatly enhanced by the in vacuo conditions of the mass spectrometric analysis. This dephosphorylation mechanism might involve the formation of a succinimide intermediate. Other mechanisms cannot be excluded, however. For instance, loss of water during electrospray mass spectrometric analysis of phosphoproteins by β-elimination is known to occur at phospho-Ser and phospho-Thr residues (*33*, *34*).

DISCUSSION

During the normal route for deamidation of Asn residues, a succinimide intermediate is formed and the majority of the product is usually converted to isoAsp (*10*). In the case of D57N CheY with Mn^{2+}, however, the product protein has an insignificant amount of isoAsp. One possibility suggested by the low isoAsp content is that some features of the active site geometry direct the hydrolysis of the succinimide. Formation of an isoAsp from a succinimide requires the attack of a water molecule on the main-chain α-carboxyl carbon, so if the protein secondary structure prevents access of solvent water molecules to this position, the ratio of Asp to isoAsp produced on ring opening will be altered (*11*, *12*). An examination of the crystal structure of CheY reveals that

the Asp57 main-chain α-carboxyl carbon is in fact packed against the hydrophobic core residues and sequestered from solvent (*13*, *35*). Another possibility that would explain the lack of isoAsp formation is that in D57N CheY with Mg^{2+} or Mn^{2+} the amide group is directly hydrolyzed by hydroxyl attack at the β-carboxyl. Catalyzed deamidation has been seen previously in dehalogenase enzymes (*36*, *37*), and both CheY and the dehalogenase enzymes are part of the HAD superfamily (*8*). Since enzymatic activity is restored in the deamidated dehalogenase enzymes, amide hydrolysis in these proteins must also proceed in a fashion that minimizes the production of isoAsp at the active site Asp residue.

We have previously hypothesized that dephosphorylation of Asp residues in CheY and other members of the HAD superfamily can proceed via a mechanism parallel to the normal mechanism for deamidation of Asn residues (Figure 1) (*13*). While CheY dephosphorylation may proceed via a succinimide intermediate under some conditions, this does not seem to be the preferred route. We have been unable to detect any succinimide form of CheY by IEF, and we could not detect any ^{18}O incorporation on the basis of the measured mass of the Asp57-containing fragment. Incorporation of solvent oxygen is clearly less than 10%, and our data are consistent with zero incorporation after multiple rounds of dephosphorylation. These results indicate that CheY dephosphorylation usually proceeds via water attack at the phosphorus during spontaneous hydrolysis as has been previously hypothesized (*9*). CheZ-mediated hydrolysis also proceeds via water attack at the phosphorus, confirming this hypothesis on the basis of the recently solved CheY−CheZ cocrystal (*14*). These results contrast with the findings of ^{18}O incorporation during the enzymatic reaction in dehalogenase members of the HAD superfamily (*38*, *39*). The mechanism of most dehalogenase enzymes involves the formation of a covalent adduct at the active site Asp residue. This intermediate must be hydrolyzed via a mechanism that involves water attack at the Asp β-carboxyl as demonstrated by ^{18}O incorporation in these dehalogenase enzymes.

There also remains an intriguing possibility that for CheY in vivo or for other members of the HAD superfamily dephosphorylation occurs at least in part via a succinimide intermediate. Perhaps such a side reaction could serve a regulatory function. It has been proposed that isoAsp formation may be a regulatory switch for some proteins (*40*), and HAD superfamily enzymes whose active site Asp is converted to isoAsp would presumably be nonfunctional until or unless converted back to Asp. The protein isoaspartate methyltransferase enzyme has been shown to recognize and repair isoAsp residues using an *S*-adenosylmethionine-dependent mechanism, and could be central to a regulatory pathway of this type (*40*−*44*).

It has also previously been speculated that there is an additional step in CheY activation when it binds to the flagellar motor (*45*). Perhaps this activated state is a succinimide or other covalent intramolecular intermediate. This would be consistent with our previous observation that, in the absence of the kinase CheA, overexpression of D57N CheY causes behavioral changes in vivo, while overexpression of WT CheY does not (*46*). One mechanism that must still be considered is that binding to the flagellar motor brings phospho-CheY into an active conformation where hydrolysis through the succinimide is favored.

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