Intramolecular rearrangements as a consequence of the dephosphorylation of phosphoaspartate residues in proteins

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Abstract **Aspartate phosphorylation induces changes in protein conformation that are used to regulate processes ranging from gene expression and cell differentiation to cell motility and the generation of electrochemical gradients across membranes. We show here that dephosphorylation of the phosphoaspartate in the chemotaxis response regulator CheY can result in the loss of a water molecule that may be due to formation of a succinimide intermediate.**

Key words: Response regulator; Phosphoaspartate; Succinimide; CheY; OmpR

1. Introduction

Signal transduction in microorganisms and plants frequently involves His-Asp phosphorelay networks of sensor histidine protein kinases and response regulator proteins [1–3]. The histidine kinases catalyze the transfer of phosphoryl groups from ATP to histidine imidazole side chains, which serve as donors for phosphotransfer to a highly conserved aspartate residue in response regulator proteins. Phosphorylation induces changes in response regulator conformation that activate responses ranging from changes in gene expression to alterations in cell motility [4–6]. The phosphorylated response regulators have an intrinsic autophosphatase activity that acts to limit the duration of the phosphorylated state.

The intrinsic phosphatase activities of different response regulators vary by four orders of magnitude depending on their physiological function. Thus, the half-life for the phosphorylated response regulator that mediates second-to-second changes in cell motility, phospho-CheY, is about 10 s [7], whereas phospho-Spo0F, a response regulator that controls the initiation of endospore production in *Bacillus subtilis*, has a half-life of 3 h [8]. Intrinsic phosphatase activities of response regulators are generally modulated in vivo through interactions with their cognate histidine kinase or another protein specific to a given regulatory system [9].

The mechanism of response regulator autophosphatase activity has not been defined. Although it has been demonstrated that residues adjacent to the phosphoaspartate side chain influence the kinetics of the reaction [8], the magnitude of these effects is small compared to the diverse range of phosphorylation half-lives of different response regulators. It has generally been assumed that the phosphatase reaction proceeds essentially as a reversal of the phosphohistidine phosphotransfer reaction with water or hydroxide taking the place of the histidine imidazole side chain. The recent co-crystal structure of CheY and CheZ supports this as the mechanism of CheZ-mediated dephosphorylation [10].

There is an alternative possibility, however; the phosphoryl group could be displaced by intramolecular nucleophilic attack at the aspartate β-carboxyl group. That this sort of alternative mechanism might be present in response regulators is suggested by two previous results. The first comes from an investigation using mass spectrometry of the phosphorylation of OmpR, the response regulator that mediates osmotic responses in *Escherichia coli*. In addition to the unphosphorylated and phosphorylated OmpR proteins, a third species was detected with a mass approximately 18 Da less than that of the unphosphorylated protein, corresponding to the loss of a water molecule [11]. This form was not seen in the absence of phosphorylation, so it presumably arose as a consequence of dephosphorylation. The second comes from studies of a protein–protein phosphotransfer system, termed the PTS, that functions in many bacteria to control sugar uptake and metabolism [12]. The PTS is distinct from His-Asp phosphorelay signal transduction systems in that phosphoryl groups are passed directly from one histidine to another rather than from histidine to aspartate. The phosphoaccepting histidine of a PTS phosphotransfer protein, the *E. coli* HPr protein, was mutated to aspartate. The phosphotransfer activity of this His15Asp HPr was retained through formation of a phosphoaspartate in place of the normal phosphohistidine [13]. Interestingly, the phosphoaspartate form of HPr exhibited an autophosphatase activity, and an investigation of the mechanism indicated that the reaction involved displacement of the phosphoryl group by nucleophilic attack at the aspartate β-carboxyl with concomitant formation of a cyclic succinimide intermediate. The succinimide form of a protein has a mass one water molecule less (−18 Da) than the original.

In this paper we show that phosphorylation of CheY using any of several small-molecule phospho-donors results in the formation of a dehydrated species detected using mass spec-

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trometry. Our result suggests that this intramolecular dephosphorylation results from succinimide formation.

2. Materials and methods

Except as noted, all chemicals and buffers were of reagent grade and purchased from standard suppliers. Trypsin was type XI, DPCC treated from Sigma.

Salmonella typhimurium CheY was purified from an overproduction plasmid in *E. coli* as described previously [14]. Protein concentration was determined using absorbance at 280 nm.

Mass spectra measured using a high-performance liquid chromatography system inline with an electrospray mass spectrometer (HPLC/MS) were acquired in the mass spectrometry facility of the Princeton University Department of Chemistry. The HPLC (LC 1100, Hewlett Packard) had a Luna C-8 reverse-phase column (Phenomenex). The electrospray mass spectrometer (MSD, Hewlett Packard) is coupled to a PC for data collection and analysis. CheY was mixed with other reagents to a final concentration of 100 μM CheY, 20 mM $MgCl_2$, 50 mM Tris pH 7.5. For phosphorylated samples, acetyl phosphate (or phosphoramidate or carbamoyl phosphate) was added to a final concentration of 25 mM and the mixture incubated for 10 min at 37°C. Typically, 10–40 μl of CheY sample was injected with a flow rate of 0.35 ml/min. A gradient of 10–80% acetonitrile plus 0.1% trifluoroacetic acid in H_2O was used to elute the CheY from the column. A UV absorbance detector set to 280 nm was used to determine the position of the elution peak of CheY.

Protein isoAsp content was assayed using the ISOQUANT Isoaspartate Detection Kit (Promega), using the radioactive detection protocol. In one set of experiments, duplicate samples with 200 μM CheY were incubated for 5 min at 37°C with 20 mM $MgCl_2$, 40 mM HEPES pH 7.5 in a volume of 25 μl. Samples also contained either 20 mM acetyl-phosphate (phosphorylated) or 20 mM acetic acid and 20 mM KH_2PO_4 (control). Samples were denatured with either four volumes 100 mM Tris, 10 M urea pH 9.2 (8 M urea final) or four volumes of 95°C 100 mM pH 8.7 Tris. After incubating 50 min at room temperature, all samples were diluted with 375 μl 100 mM HEPES pH 7.5 such that the final CheY concentration was 10 μM. 25 μl of these final solutions was used in each 50 μl ISOQUANT assay. A second set of experiments was done with the additional step of a 2 h trypsin digestion at 30°C before the ISOQUANT assay. The trypsin was inhibited by the addition of phenylmethylsulfonyl fluoride (PMSF) before the assay. A third set of experiments used duplicate 50 μl samples of 20 μM CheY incubated for 5 h at 37°C with 20 mM $MgCl_2$, and 100 mM HEPES pH 7. Phosphorylated samples also contained 20 mM acetylphosphate. Samples were digested for 2 h at 30°C with trypsin, PMSF was added, and then 25 μl was used in each 50 μl ISOQUANT assay.

3. Results and discussion

To investigate the possibility of succinimide formation as an intermediate in phospho-CheY dephosphorylation, we performed mass spectrometric analyses. Using HPLC/MS the mass of the unmodified CheY protein was verified by a single peak at 13 993 or 13 994 Da, in agreement with the mass (13 994 Da) calculated from the amino acid sequence (Fig. 1A). When CheY was phosphorylated using acetyl-phosphate, phosphoramidate, or carbamoyl phosphate, an additional major species was detected with a mass 18 Da less than that of unmodified CheY. This form of CheY represented one third or more of the total detected in the reaction mixture (Fig. 1B,C; data not shown for carbamoyl phosphate). A control reaction with acetyl-phosphate, but no Mg^{2+}, was run in which CheY should not be phosphorylated, and as expected, the mass spectrum shows only a single peak of unmodified CheY. Phospho-CheY is relatively unstable, and only a trace was detectable as a small peak with a mass of 14 073 in our experiments. Presumably, during the electrospray MS procedure, phospho-CheY was hydrolyzed via an intramolecular attack, leading to the large peak 18 Da below native CheY. Thus, the result previously obtained with the response regulator OmpR seems to be a general feature of response regulator biochemistry [11].

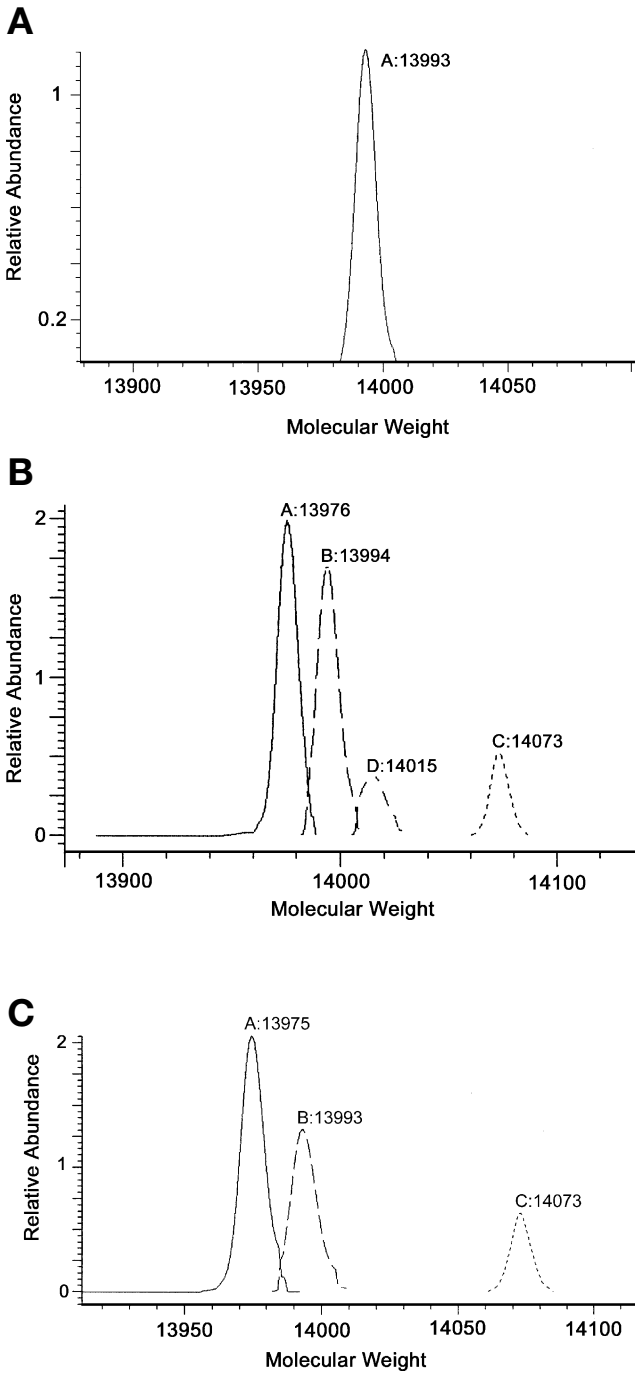

Fig. 1. Mass spectrum of CheY. A: Control without phosphorylation. Native CheY has a predicted molecular mass of 13 994 amu. B: After incubation with 25 mM acetyl-phosphate for 10 min at 37°C and then desalting by reverse-phase HPLC, additional peaks arise. The species with mass 13 976 amu (−18) corresponds to a dehydrated form of CheY, which is attributed to the succinimide form. The peak at 14 073 amu (+79) is attributed to phosphorylated CheY (a small peak at 14 015 probably represents a sodium adduct of CheY). C: A similar result is obtained after incubation of CheY with phosphoramidate.

The chemistry of succinimide formation in proteins has

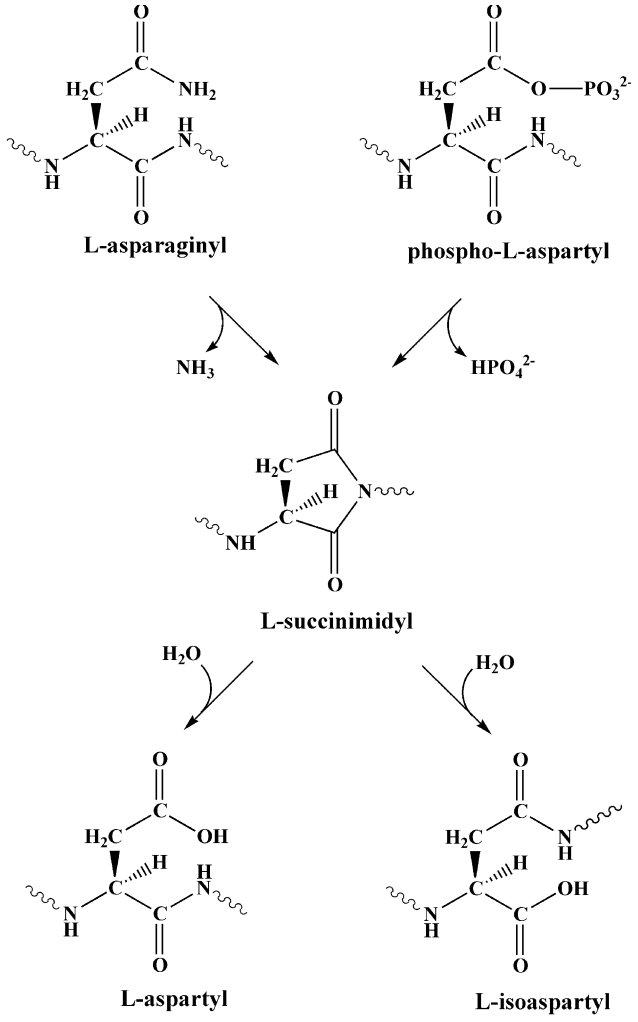

Fig. 2. Formation and breakdown of a succinimide. Succinimide residues have been shown to form from aspartyl and asparaginyl residues. They may also be able to form as an intermediate in phosphoaspartate hydrolysis.

been thoroughly investigated through studies of the spontaneous hydrolysis of asparagine side chain amides to aspartate and ammonia that is a hallmark of protein ageing [15,16]. The mechanism involves nucleophilic attack by the main chain amide nitrogen of the amino acid following the asparagine with concomitant displacement of the side chain amido group (Fig. 2). The cyclic succinimide that results from this reaction tends to be short-lived. Hydrolysis can proceed by water or hydroxide attack at either of the imide linkages. Whereas attack at the β-carboxyl leaves a normal polypeptide chain with aspartate in place of asparagine, attack at the α-carboxyl routes the polypeptide chain through the aspartate side chain producing an isoaspartyl linkage. The free isoaspartyl α-carboxyl group produced by this linkage is a substrate for a specific protein carboxyl methyltransferase that functions to repair isoaspartyl lesions in ageing proteins by methylating the isoaspartate α-carboxyl side chain [16]. The α-carboxyl methyl ester produced by this enzyme leads to reformation of the cyclic succinimide allowing possible ring opening through attack at the β-carboxyl with consequent formation of a normal aspartate residue [17].

One would expect that a phosphoryl group would also function as a good leaving group in much the same way as a methyl ester. Although rates of succinimide formation are primarily determined by the presence of a good leaving group at the β-carboxyl, they are also highly dependent on the primary and tertiary structure of the protein. Cyclic succinimide formation requires a main chain aspartate or asparagine ψ angle of −120°. This is rare in proteins [16] and as a consequence only a small subset of asparagines undergo spontaneous deamidation in native structures. Table 1 shows a compilation of the ψ angles for the active site Asp in response regulator structures. In CheY the ψ angle of the phosphoaccepting Asp57 is between +143.9° and +176.1° in X-ray crystal structures, which suggests that cyclization would not be favored [18]. However, evidence for a ψ angle much more appropriate for succinimide formation is provided by the nuclear magnetic resonance (NMR) solution structure of activated CheY. Complexation with the phosphate analog beryllofluoride (BeF_3^{2-}) causes a shift in the main chain conformation of CheY, bringing the Asp57 ψ angle to −158.2°, a value that is much closer to the ideal for succinimide formation. This rotation is accompanied by a shift in distance from the main chain amide nitrogen to the Asp57 β-carboxyl carbon of 3.49 Å to 2.96 Å [19]. Of the other response regulators in Table 1, NtrC is the only other protein whose conformation seems compatible with succinimide formation, but clearly crystallization can alter the conformation relative to the solution structure.

To look for the formation of isoAsp residues as a consequence of phosphorylation, we assayed CheY under two different types of conditions. In the first, CheY was incubated with acetyl-phosphate for 5 min at 37°C and then denatured using either urea or 95°C buffer. The goal of this was to attempt to disrupt secondary structure elements that might be directing the hydrolysis of succinimides back to Asp rather than isoAsp. In this experiment for the control sample we found 0.007 ± 0.003 mol isoAsp/mol CheY compared to 0.004 ± 0.003 for CheY phosphorylated with acetyl-phosphate. For CheY denatured with urea we found 0.005 ± 0.003 mol isoAsp/mol CheY compared to 0.006 ± 0.003 for phosphorylated CheY. Similarly, CheY denatured with 95°C buffer gave

Table 1
Structural parameters for the phosphoaccepting Asp residues from response regulator structures

Protein	PDB entry	ψ (°)	N to β-COOH distance (Å)
CheY	2CHE	143.9	3.49
CheY/CheA	1AO0	161.1	2.91
CheY ~ BeF_3^{2-} (NMR)	1DJM	−158.3	2.96
CheY ~ BeF_3^{2-} (X-ray)	1FQW	176.1	2.97
FixJ	1DBW	162.8	3.37
FixJ ~ P	1D5W	156.86	2.95
NarL	1AO4	117.3	3.75
NtrC	1NTR	−172.3	4.22
NtrC	1DC7	−148.8	4.18
NtrC ~ P	1DC8	111.3	3.68
PhoB	1BOO	122.1	3.45
Spo0A	1DZ3	127.3	3.74
Spo0A ~ P	1QMD	156.2	3.22
Spo0F	1NAT	125.4	3.66

The ψ angle of the phosphoaccepting Asp residue is indicated as well as the distance from the main chain amide nitrogen to the Asp side chain β-carboxyl carbon.

0.006 ± 0.003 mol isoAsp/mol CheY in both the absence and presence of acetyl-phosphate. This set of assays was conducted without trypsin digestion of CheY, but similar results were obtained using trypsin. In the second set of experiments CheY was allowed to incubate with an excess of acetyl-phosphate for 5 h to ensure that a large number of rounds of phosphorylation occurred. In this experiment as well the number of isoAsp residues detected was not significantly different in the presence or absence of acetyl-phosphate. Thus, we cannot detect any phosphorylation-dependent isoAsp formation. Since formation of an isoAsp from a succinimide requires the attack of a water molecule on the main chain α-carboxyl carbon, features of protein secondary structure can prevent access of solvent water molecules to this position, thus influencing the ratio of Asp to isoAsp produced on ring opening (Fig. 2) [16,17]. 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 [18].

The physiological importance of intramolecular dephosphorylation mechanisms in CheY is still unclear. It is conceivable that succinimde formation could be of physiological importance under some conditions as a regulatory mechanism. CheY has been identified as a member of the haloacid dehalogenase (HAD) superfamily based on its active site geometry [20]. This superfamily includes both the P-type ATPases and the dehalogenases. The reaction mechanism of most dehalogenase enzymes includes a covalent intermediate formed with an active site Asp residue. This intermediate is hydrolyzed via attack on the Asp as demonstrated by ^{18}O incorporation experiments [21,22], consistent with the formation of a succinimide during the hydrolysis of the covalent intermediate. Perhaps the use of covalently modified aspartates in the response regulators and other members of the HAD superfamily relates in some way to the possibility of succinimide formation from aspartate.

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