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Structural Investigation of a Phosphorylation-Catalyzed, Isoaspartate-Free, Protein Succinimide: Crystallographic Structure of Post-Succinimide His15Asp Histidine-Containing Protein[†,‡]

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ABSTRACT: Aspartates and asparagines can spontaneously cyclize with neighboring main-chain amides to form succinimides. These succinimides hydrolyze to a mixture of isoaspartate and aspartate products. Phosphorylation of aspartates is a common mechanism of protein regulation and increases the propensity for succinimide formation. Although typically regarded as a form of protein damage, we hypothesize succinimides could represent an effective mechanism of phosphoaspartate autophosphatase activity, provided hydrolysis is limited to aspartate products. We previously reported the serendipitous creation of a protein, His15Asp histidine-containing protein (HPr), which undergoes phosphorylation-catalyzed formation of a succinimide whose hydrolysis is seemingly exclusive for aspartate formation. Here, through the high-resolution structure of postsuccinimide His15Asp HPr, we confirm the absence of isoaspartate residues and propose mechanisms for phosphorylation-catalyzed succinimide formation and its directed hydrolysis to aspartate. His15Asp HPr represents the first characterized protein example of an isoaspartate-free succinimide and lends credence to the hypothesis that intramolecular cyclization could represent a physiological mechanism of autophosphatase activity. Furthermore, this indicates that current strategies for succinimide evaluation, based on isoaspartate detection, underestimate the frequencies of these reactions. This is considerably significant for evaluation of protein stability and integrity.

Post-translation alterations of protein structure, whether through deliberate enzyme-mediated modification or spontaneous covalent damage, have the ability to influence structure and activity. Analogous to how different strategies of enzymatic modification are associated with specific types of amino acids, particular residues also have unique propensities for spontaneous alteration based on their chemical characteristics. For example, aspartates and asparagines can undergo intramolecular cyclization to form a five-member succinimide ring through the nucleophilic attack of the adjacent peptide bond amide nitrogen on the side-chain carbonyl of either residue (*1−3*). Succinimides are unstable and undergo rapid hydrolysis to a mixture of aspartate and isoaspartate products (Figure 1A). Whether the succinimide was derived from aspartate or asparagine, the hydrolysis products are the same such that the reaction represents deamidation of asparagines and isomerization of aspartates.

Formation of isoaspartates has significant potential for disruption of protein structure and function through both the introduction of an additional methylene unit (CH_2) into the main chain and the reduction of the length of the side chain, which can influence its ability to participate in stabilizing hydrogen bonds and salt bridges. Furthermore, deamidation of asparagine changes the net charge at the reacting position which can further compromise protein stability. The physiological significance of isoaspartate formation is supported by the presence of the highly conserved enzyme protein isoaspartate methyltransferase (PIMT),[1] which methylates isoaspartates in both peptide and protein contexts (*4, 5*). Seemingly consistent with a general repair function, PIMT is present in a broad range of prokaryotic and eukaryotic organisms (*6, 7*) and recognizes isoaspartyl residues in most amino acid sequence contexts (*8*). PIMT catalyzes transfer of a methyl group from *S*-adenosylmethionine to the α-carboxyl group of an isoaspartate to stimulate re-formation of the succinimide. Subsequent hydrolysis of this ring results in a fraction of the original isoaspartates being converted to aspartates.

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[1] Abbreviations: HPr, histidine-containing protein; PIMT, protein isoaspartyl methyltransferase; PTS, phosphoenolpyruvate:sugar phosphotransferase system; PS-His15Asp HPr, post-succinimide His15Asp HPr; pI, isoelectric point; rmsd, root-mean-square deviation; DSIP, Iso-Delta Sleep-Inducing Peptide; NMR, nuclear magnetic resonance; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; NCS, noncrystallographic symmetry.

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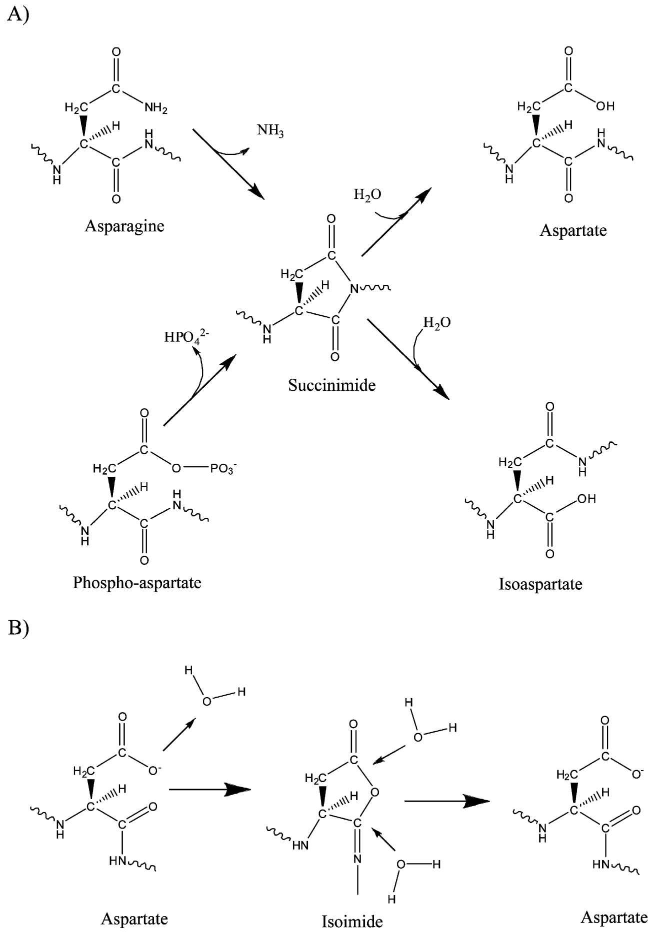

FIGURE 1: Mechanism of succinimide and isoimide formation and hydrolysis. (A) Succinimide formation and hydrolysis. Mechanism of succinimide formation and hydrolysis from an asparagine and a phosphoaspartate. A similar mechanism occurs from aspartate, with the distinction being release of a water molecule rather than a phosphoryl group upon ring formation. Also shown are the products which emerge from succinimide hydrolysis with aspartates being produced from hydrolysis at the side-chain carbonyl and isoaspartates from hydrolysis at the main-chain carbonyl. (B) Isoimide formation and hydrolysis. Hydrolysis of an isoimide ring results in aspartyl products independent of the point of attack of the incoming water molecule.

However, as isoaspartates remain the predominant product of succinimide hydrolysis, multiple rounds of repair are required for complete conversion of an isoaspartate to aspartate. Furthermore, this represents true repair only when the initiating residue was an aspartate as asparagines are not regenerated. The consequences of PIMT deficiency have been reported for a number of species and include fatal seizures in mice (*9*), poor dauer stage survival for *Caenorhabditis elegans* (*10*), and increased sensitivity to environmental stressors in *Escherichia coli* (*11*). All of these phenotypes are an apparent consequence of accumulation of isoaspartyl-containing proteins.

Succinimide formation is the rate-limiting step in the transformation of self-reactive asparagines and aspartates with half-lives of days to weeks (*12*). These rates are influenced by the structural and sequence context of the reacting residue and in particular the identity of the residue in the immediate C-terminal position (*12*). The greatest rates of cyclization are observed when the reacting residue is followed by a glycine, which likely reflects the structural flexibility imparted by this residue on the main chain. Main-chain flexibility is believed to be a key structural determinant for succinimide propensity with cyclization most frequently observed in regions of undefined secondary structure (*1*). Specifically, succinimide formation is favored when the main chain of the reacting residues is able to adopt a conformation of approximately −120° (*1*). This is a rare conformation for native structures and likely limits the self-reactivity of most asparagines and aspartates.

As a dual consequence of their slow rates of formation and chemical instability, succinimides are difficult to directly observe. As such, most strategies for quantification of these reactions focus on characterization of the isoaspartate product rather the succinimide itself. For example, a number of techniques employing electron capture dissociation mass spectrometry have been described for detection and quantification of isoaspartates (*13, 14*). These approaches target either diagnostic fragment ions (*14*) or the incorporation of $H_2{}^{18}O$ (*15−17*). Alternatively, and more commonly, enzymatic assays, based on the PIMT enzyme, for quantification of isoaspartate content in peptides and proteins have been established (*18*). In this capacity, PIMT has found application as a bioreagent for quantification of the transformation of aspartates and asparagines through succinimide-based mechanisms, most notably as a commercially available kit (ISOQUANT Promega).

On the basis of their stability and relative ease of detection, isoaspartates are a logical biomarker for succinimide events. However, this approach is likely to underestimate the frequency of ring formation as isoaspartyl residues do not represent an exclusive or consistent product of succinimide breakdown. Isoaspartates typically account for 65−85% of the residues emerging from succinimide hydrolysis, and this ratio can vary in different protein contexts (*2*). The variability of the ratio of products which emerge from succinimide hydrolysis likely reflects structural limitations imposed by the protein that influence patterns of succinimide hydrolysis.

Patterns of succinimide hydrolysis are sensitive to the structural context in which they are contained as parameters that limit access of an incoming water molecule to either of the succinimide carbonyl groups influence the ratio of products formed. Hydrolysis at the side-chain succinimide carbonyl produces an aspartate, while hydrolysis at the main-chain carbonyl results in an isoaspartate (Figure 1A). It is therefore theoretically possible for protein structure to restrict isoaspartate formation by blocking access of incoming water molecules to the main-chain succinimide carbonyl group. As such, while an isoaspartate offers strong evidence of succinimide occurrence, it is presumptuous to interpret the absence of isoaspartates as an absolute indicator of the absence of succinimide formation. The demonstration of a protein which undergoes isoaspartate-free succinimide hydrolysis would offer reason to conceptually separate succinimides from isoaspartates. Our fortuitous creation of a protein in which succinimide formation is catalyzed by phosphorylation, and appears to undergo exclusive hydrolysis to an aspartate, seems to provide such an example and offers an ideal system for investigating such particulars of succinimide behavior.

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) functions through a series of phosphotransfer events from enzyme I to histidine-containing protein (HPr) to a sugar-species IIA protein (*19*). HPr is a single-domain, nonglycosylated protein with no disulfide bonds. Depending upon the bacterial strain of origin, HPr varies in size from 6.7 to 15 kDa and adopts a highly conserved open-faced β-sandwich composed of four antiparallel β-strands and three α-helicies (*20*). HPr undergoes phosphorylation at the $N^{\delta 1}$ position of His15 (*21*), but this active center histidine can be mutated to aspartate, His15Asp HPr, while the ability to phosphotransfer from enzyme I and to enzyme $IIA^{glucose}$ via a phosphoaspartyl intermediate is retained (*22*). Other PTS phosphotransfer proteins have shown similar potential for substitution of active site histidines with the general trend of histidines which are phosphorylated at the $N^{\delta 1}$ position being functionally replaced with aspartate and histidines which are phosphorylated at the $N^{\varepsilon 2}$ position being functionally replaced with glutamate (*23*). This likely reflects the importance of conserved positioning of the phosphoacceptor atom.

Phosphorylation of the active site aspartate of His15Asp HPr leads to a spontaneous internal rearrangement with the formation of an unphosphorylated protein species with a pI higher than that of the original unmodified protein. This species can be isolated by ion exchange chromatography for detailed investigation. Our group has demonstrated that the high-pI species exhibits (a) changes in mass consistent with loss of a water molecule, (b) a +1 charge relative to the unmodified protein, (c) sensitivity to basic conditions, and (d) the presence of a nonstandard peptide bond at the phosphoacceptor position (*22*). All of these observations are consistent with succinimide formation. In the absence of phosphorylation, formation of the high-pI species is not observed, indicating that the rearrangement is phosphorylation-catalyzed. The ability of phosphorylation of an aspartate to catalyze succinimide formation was not unanticipated as covalent modification of aspartyl side chains is known to increase reactivity. For example, the β-methyl ester derivatives, as formed by the PIMT enzyme, have been shown to form succinimides 24000 times faster than the corresponding unmodified aspartate residues (*2*). Modification of the carboxyl group of aspartic acid through phosphorylation would also be expected to increase the rate of ring formation by providing a better leaving group.

While succinimide theory would predict the rapid accumulation of isoaspartate residues as a consequence of dephosphorylation via a succinimide intermediate, we instead observed within PS-His15Asp HPr (a) retention of activity of the protein after multiple rounds of phosphorylation, (b) retention of a standard peptide bond at the reacting position, and (c) the fact that the post-succinimide His15Asp HPr does not function as a PIMT substrate (*22*). These observations are consistent with retention of aspartate at position 15 and are therefore inconsistent with typical succinimide hydrolysis.

In the original work, we proposed two alternate explanations for the apparent absence of isoaspartates following resolution of the high-pI form of His15Asp HPr. First, the absence of isoaspartates could result from the formation of a novel five-member isoimide ring in which aspartates emerge as the exclusive product of ring hydrolysis (Figure 1B). Alternatively, steric restraints imposed by the protein of succinimide hydrolysis could exclude the isoaspartyl product.

From crystallographic determinations of wild-type and His15Asp *E. coli* HPr, Athmer et al. (*24*) observed that the C-terminal residue, Glu85, is positioned to limit access of an incoming water molecule to the main-chain carbonyl of position 15. As the required point of hydrolysis for formation of isoaspartate, this offers a structural rationalization for the breakdown of the high-pI species of His15Asp HPr exclusively to aspartate. This was confirmed through mutational analysis in which deletion of the C-terminal residue (His15Asp Δ85 HPr) did not influence the ability of the protein to undergo phosphorylation or formation of the high-pI species but did permit rapid accumulation of isoaspartates.

An attempt to determine the crystallographic structure of the succinimide-containing intermediate of His15Asp HPr proved unsuccessful as the succinimide was hydrolyzed during crystallization. The structure revealed an aspartate at position 15; however, due to the nonphysiological pH (pH 4.5) and high salt concentrations of the crystallization conditions, it was not possible to conclusively say whether this would also reflect the products which would form under physiological conditions (*22*).

The example of phosphorylation-catalyzed succinimide formation in His15Asp HPr, with subsequent hydrolysis exclusively to aspartate, is sufficiently extraordinary to warrant further structural investigation to verify the absence of isoaspartates as well as to offer structural explanation for catalyzed ring formation and directed hydrolysis to aspartate. With these objectives in mind, we present the high-resolution structure of post-succinimide His15Asp HPr (PS-His15Asp HPr). This protein was generated from hydrolysis of the isolated high-pI species at physiological pH and salt concentrations. The unambiguous electron density and absence of alternate positions of the active site main-chain atoms verify the exclusive production of aspartate residues following succinimide hydrolysis. These findings are further confirmed through N-terminal sequencing and PIMT assays. This definitively confirms the ability of the protein structure to influence, and even eliminate, isoaspartate formation during succinimide hydrolysis. From this high-resolution structure, we offer structural rationalizations for both catalyzed succinimide formation and exclusive hydrolysis to aspartate through limitation of attack at the main-chain succinimide carbonyl group.

These findings are biologically significant, in general, as they offer strong evidence to support the hypothesis that succinimide formation may serve as an effective mechanism for removal of modifying groups from aspartates. Furthermore, that succinimide formation can occur in a manner independent of isoaspartate formation suggests the estimated frequency of succinimide events is likely underestimated both *in vivo* and *in vitro*. This is significant both in evaluation of protein and peptide integrity and in assigning potential biological roles to these rearrangements.

MATERIALS AND METHODS

Protein Expression and Purification. His15Asp HPr was overexpressed in *E. coli* strain ESK108, F′ trp thi rpsL ptsH465 recA56, using the puc19(ptsH) plasmid under the control of the ptsH promoter (*25*). Homogeneous protein was produced as previously described (*25*). Enzyme I was purified by methods previously described (*26*).

Phosphorylation of His15Asp HPr. His15Asp HPr (3 mg) was phosphorylated at 37 °C for 60 min in 10 mM potassium phosphate buffer (pH 7.0) with 5 mM phosphoenolpyruvate, 5 mM MgCl$_2$, and 0.3 mg of enzyme I.

Isolation of the Succinimide-Containing His15Asp HPr Intermediate. The phosphorylation reaction mixture was separated by anion exchange chromatography using a Mini-Q-Sepharose column and an Amersham Pharmacia Biotech Gradifrac system at 4 °C. The reaction mixture was loaded with 10 mM citrate-phosphate buffer (pH 4.6) and the column eluted at 2 mL/min with a 20 mL gradient to 0.07 M NaCl in the same buffer. Fractions (0.5 mL) were collected, and protein elution was monitored at 214 nm. Isoelectric focusing confirmed the ability of the ion exchange protocol to separate the three forms of the protein, phosphorylated (lower pI), unphosphorylated, and cyclized (higher pI). The isolated high-pI species was dialyzed overnight against phosphate-buffered saline (pH 7.4). Following dialysis, this form of the protein migrated the same as unmodified His15Asp HPr, indicating hydrolysis of the succinimide. This species was used for structural investigations.

Isoelectric Focusing. Isoelectric focusing gels were constructed using a Bio-Rad capillary tray. Gels were created by dissolving 1.1 g of sucrose in 10.3 mL of 5% acrylamide with subsequent addition of ampholytes (600 μL of 20% pH 3.0−10.0, 40% pH 3.5−5.0, and 40% pH 5.0−7.0). Gels were set by the addition of 50 μL of 5% ammonium persulfate, 25 μL of riboflavin 5-phosphate (2.5 mg/mL), and 5.0 μL of TEMED. The mixture was poured under the glass plate and allowed to set under a light source for 30 min. Polymerized gels were removed from the tray, and electrode wicks soaked in 1 M NaOH (cathode) and 1 M phosphoric acid (anode) were positioned at either side of the gel. The gel was placed, glass side down, on a precooled (4 °C) flat bed Pharmacia IEF apparatus, and samples (20 μL) were loaded and run for 2 h at 8 W with a 1000 V upper limit.

Crystallization and Data Collection. PS-His15Asp HPr was crystallized by the hanging drop vapor diffusion method at 14 °C. Crystals formed in 0.1 M citrate-phosphate buffer (pH 4.4) and 20−25% saturated ammonium sulfate. X-ray diffraction data were collected using a flash-cooled crystal on the 14-BMC beamline at BioCARS, Advanced Photon Source (Argonne National Laboratory, Chicago, IL). The total oscillation range was 180°, using radiation with a wavelength of 0.9984 Å. The crystal−detector distance was 75 mm. The crystal diffracted to 1.0 Å resolution with an overall completeness of 71%. The data were indexed and scaled with DENZO and SCALEPACK (*27*). The crystal space group was identified as $P2_1$ on the basis of symmetry and systematically absent reflections.

Solution and Refinement of the Crystallographic Structure. The structure of the His15Asp mutant in space group $P2_1$ was determined by molecular replacement using PHASER (*28*) with the Ser46Asp mutant of HPr (PDB entry 1OPD) as the starting model. The structure was refined using Refmac5 in the CCP4 package. Between refinements, the model was visually adjusted and water molecules were added using the molecular graphics program COOT (*29*). Non-crystallographic symmetry (NCS) restraints were invoked between molecules A and B in the asymmetric unit during the initial stages of refinement, and the NCS restraints were relaxed in the later stages of refinement. All molecular figures were generated with Pymol (*30*). This structure has been submitted to the RCSB Protein Data Bank as RCSB entry rcsb046605 and PDB entry 3CCD.

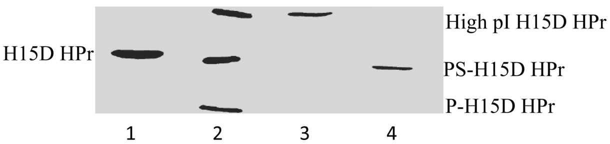

FIGURE 2: Phosphorylation of His15Asp generates three species of the protein. His15Asp HPr (3 mg) was phosphorylated at 37 °C for 60 min in 10 mM potassium phosphate buffer (pH 7.0) with 5 mM phosphoenolpyruvate, 5 mM MgCl$_2$, and 0.3 mg of enzyme I. Protein species were separated by isoelectric focusing: lane 1, unphosphorylated His15Asp HPr; lane 2, His15Asp HPr under phosphorylating conditions with the generation of both the phosphorylated and high-pI species; lane 3, isolated high-pI His15Asp HPr; and lane 4, isolated high-pI His15Asp HPr dialyzed overnight against PBS.

Molecular Modeling. The phosphorylated Asp15 and the succinimide ring at position 15 were initially modeled visually, and the modeled protein(s) was subjected to energy minimization using MacroModel (*31*). The Merck molecular force field was used with water as the solvent medium, and the minimization was carried out by the molecular dynamics simulated annealing method.

Methyltransferase Assays. Methyltransferase assays were performed using the ISOQUANT isoaspartate detection kit (Promega) according to the manufacturer's specifications for the radioactive protocol. For each experiment, 50 pmol of the Iso-Delta Sleep-Inducing Peptide (DSIP) reference standard and either 200 pmol of His15Asp HPr and PS-His15Asp HPr or 100 pmol of wild-type HPr were analyzed. Percentage isoaspartate contents were calculated as (sample counts per minute − blank counts per minute) × (picomoles of reference standard)/(reference standard counts per minute − blank counts per minute) × (picomoles of protein). PIMT assays were performed on both the intact proteins and the proteolytic fragments generated by treating the samples with a 1:50 (w/w) ratio of trypsin to substrate for 4 h at 30 °C. Digestions were halted with the addition of phenylmethanesulfonyl fluoride to a final concentration of 100 mM.

N-Terminal Sequencing. Protein sequencing was performed using an Applied Biosystems Inc. model 471A sequencer equipped with a model MG5 microgradient pump and a blot cartridge for polyvinylidene difluoride-type membranes, and data were analyzed using an Applied Biosystems Inc. 601A data system. Sequencing was carried out by Dr. S. MacKenzie (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK).

RESULTS

Phosphorylation of His15Asp Generates Three Protein Species. Incubation of His15Asp HPr under phosphorylation conditions generates three species of the protein, one with a pI higher than that of the unmodified protein and the other with a pI lower than that of the unmodified protein (Figure 2). On the basis of previous experiments, we have demonstrated that the lower-pI species corresponds to the phosphorylated form of the protein (*22*).

Crystallographic Structure. PS-His15Asp HPr crystallizes in space group $P2_1$ with two molecules in the asymmetric unit and the following unit cell dimensions: $a = 33.65$ Å, $b = 42.30$ Å, $c = 48.30$ Å, and $\beta = 89.95°$. The two molecules

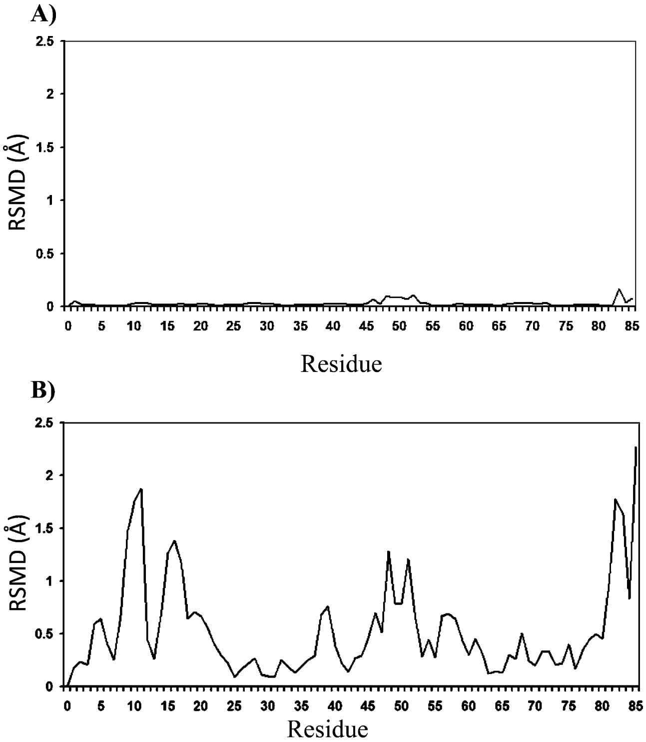

FIGURE 3: Regions of structural deviation between His15Asp HPr and PS-His15Asp HPr. Root-mean-square deviations were calculated for the α-carbons using the LSQKAB program from CCP4: (A) rmsds of the α-carbons of the two molecules within the asymmetric unit of PS-His15Asp HPr and (B) rmsds of the α-carbons of pre- and post-succinimide PS-His15Asp HPr.

(A and B) in the asymmetric unit are identical, the root-mean-square deviation (rmsd) between the two molecules being 0.04 Å (Figure 3A). All further discussions refer to molecule A only. Notably, this is distinct from the unit cell adopted by the unmodified His15Asp HPr which, under identical crystallization conditions, crystallized in space group $P2_1$ with one molecule in the asymmetric unit and the following unit cell dimensions: $a = 25.37$ Å, $b = 45.34$ Å, $c = 27.62$ Å, and $\beta = 104.0°$.

The tertiary structure of PS-His15Asp HPr was determined as described in Materials and Methods. Data collection and refinement parameters are listed in Table 1. Due to its small size and relative ease of crystallization, HPr has been extensively characterized structurally and employed as a model for various structure−activity investigations. The structure we report here is the highest-resolution determination performed to date. The overall structure of PS-His15Asp HPr is consistent with previous crystallographic investigations of *E. coli* HPr, including the wild type (*32*), His15Asp HPr (*22*), and Ser46Asp HPr (*33*). Overall, the structure forms an open-faced *β*-sandwich composed of four antiparallel *β*-strands and three helicies. The four strands form a single, pleated *β*-sheet on one face of the protein, and the three α-helicies lie on one side of the sheet. The active center is situated in a relatively unstructured turn between *β*-strand 1 and α-helix 1.

Overall Conformation. The overall structure of PS-His15Asp HPr is in close agreement with our previous structural determination of the unmodified form of His15Asp HPr. As a relative measure of structural deviations between the structures, it should be noted that the rmsd between the two molecules of PS-His15Asp HPr in the asymmetric unit was only 0.04 Å (Figure 3A) whereas comparison of the main-chain atoms in the pre- and post-succinimide structures reveals a rmsd of 0.7 Å (Figure 3B). In particular, there are three noted regions of structural deviation between the pre- and post-succinimide forms of His15Asp HPr: the minor helix B, the C-terminal residue Glu85, and the active center.

Helix B is a small, irregular helix composed of residues 46−53, where residues 46 and 47 conform to an α-helical conformation, residue 48 is a transition point, and residues 49−53 are in a 3_{10}-helical conformation. Apart from the active site, the α-carbon atoms of these residues have the greatest variability in HPr structural determinations and the flexibility of this region is believed to be important to the physiological functions of HPr. A highly conserved Ser46 N-caps this helix and is phosphorylated in Gram-positive bacteria to regulate HPr activity (*34*). While HPrs from Gram-negative bacteria are not modified at this site, the functional consequences can be mimicked with a Ser46Asp mutation that results in activation of the protein (*33*). One of the most significant structural alterations of phosphorylation of Ser46 or its mutation to aspartate is the promotion of formation of a more idealized α-helix as a result of the stabilizing interaction of these negatively charged groups with the helix dipole (*33*).

In the post-succinimide His15Asp structure, a more idealized α-helix is observed in terms of the hydrogen bonds that are formed and their geometry throughout the helix. Furthermore, in the post-succinimide structure, the main-chain carbonyl group of Phe48 is able to form a hydrogen bond with the main-chain amide of Gln51, whereas in the pre-succinimide, the carbonyl group of Phe48 hydrogen bonds with water. The stabilization of this helix in the post-succinimide structure is believed to be a consequence of a sulfate anion from the crystallization solution that is in the proximity of Ser46 which causes the differential positioning within helix B of the pre- and post-succinimide forms of His15Asp HPr and illustrates the sensitivity of this region of the protein to electrostatic influence (Figure 4A).

The second region of structural deviation is at the C-terminus. The positioning and involvement of the C-terminus in the active site of HPr have been a point of disagreement between structural and functional investigations. While the involvement of the C-terminus in the active center is supported by NMR investigations of HPr (*35*) as well as most, but not all (*36*), crystallographic investigations, mutation or deletion of the C-terminal residues does not have a significant impact on HPr phosphotransfer function (*25*). It does, however, have a significant impact on the production of isoaspartyl residues during phosphorylation-catalyzed succinimide formation and hydrolysis in His15Asp HPr. We have previously demonstrated that this residue functions as a steric restraint to limit the attack of a water molecule on the main-chain carbonyl at position 15 and thereby prevent isoaspartate formation (*24*). In spite of the difference in the positions of the α-carbons of the C-terminal region, the α-carboxyl is similarly positioned in each of the structures to limit access to the main-chain carbonyl of residue 15 (Figure 4B). The similar positioning of this region in two different crystallographic investigations of His15Asp HPr, which crystallized in the same space group, but with different unit cell dimensions, to minimize the potential influence of

Table 1: Data Collection and Refinement Statistics of Post-Succinimide His15Asp HPr

Data Collection Statistics

unit cell dimensions		resolution range (Å)	48.3−1.0 (1.06−1.0)
a (Å)	33.65	no. of reflections measured	331691
b (Å)	42.30	no. of unique reflections	73053 (944)
c (Å)	48.30	$R_{\text{sym}}{}^{a}$	0.021 (0.15)
β (deg)	89.95	completeness (%)	71 (23)
no. of molecules in asymmetric unit	2	redundancy	2.6
space group	$P2_1$	mean $I/\sigma(I)$	16.0 (4.0)

Refinement Statistics[b]

resolution limits (Å)	26.3−1.0 (1.03−1.0)	rmsd from ideal geometry	
R_{free}	0.20 (0.26)	bond distances (Å)	0.01
R_{work}	0.19 (0.26)	bond angles (deg)	1.2
R_{cryst}	0.19	dihedral angles (deg)	5.9
total no. of reflections	57397	improper angles (deg)	0.08
no. of non-H atoms	1318	Ramachandran statistics	
no. of water molecules	198	most favored (%)	90.7
mean B factor for protein atoms (Å^2)	9.71	favored (%)	9.3
		additional allowed (%)	0
		disallowed (%)	0

[a] $R_{\text{sym}} = \sum|\langle I_{hkl}\rangle - I_{hkl}|/|I_{hkl}|$, where $\langle I_{hkl}\rangle$ is the average intensity over symmetry-related reflections and I_{hkl} is the observed intensity. [b] $R_{\text{value}} = |F_o| - |F_c|/|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. For R_{free}, the sum is done on the test set reflections (5% of the total reflections). For R_{work}, the sum is done on the remaining reflections. For R_{cryst}, the sum is done on all reflections included in the resolution range.

packing interactions, further supports the proposed role of this region of the protein in preventing isoaspartyl formation.

The final region of structural deviation between the pre- and post-succinimide structures of His15Asp HPr is in the stretch of amino acids linking β-strand 1 to α-helix 1, which corresponds to the active site region. While the positioning of this region is generally well conserved in the various crystallographic determinations of *E. coli* HPr, even for different space groups, NMR investigations indicate the potential for alternate positioning of this region (*35, 37*). The shift corresponds to a general repositioning of the main chain to bring it closer to the core of the protein as well as a tightening of the cleft that contains the active site region. The cusps of this cleft are defined by Pro11 and Pro18; in the pre-succinimide structure, the distance between the α-carbons of these residues is 11.85 Å, whereas in the post-succinimide structure, this closes to 9.97 Å (Figure 4C).

Structure of the Active Site. Changes in conformation at the active site region are obviously of interest as they may indicate the presence of isoaspartyl residues. In spite of the moderate repositioning within the active center region, the electron density of the main-chain atoms is very well defined with no indication of alternate placements, in particular those that would be required for the inclusion of an additional methylene group as necessitated by an isoaspartate (Figure 5). Both the side-chain and main-chain atoms of residues 15 and 16 were maximally refined at 100% occupancy with mean B-factors of 8.01 and 8.46 Å^2, respectively, for each molecule in the asymmetric unit. The average mean B-factor value of 8.23 Å^2 for this region is lower than the average mean B-factor for the protein of 9.71 Å^2. Collectively, this high-resolution structure shows no evidence for the presence of an isoaspartyl group in post-succinimide His15Asp HPr.

N-Terminal Sequencing. N-Terminal sequencing depends on the presence of standard peptide bonds and is unable to proceed through succinimide or isoaspartyl residues. The modification of His15Asp HPr occurs sufficiently close to the N-terminus to permit N-terminal sequence analysis to provide a qualitative measure of nonstandard residues at the phosphoacceptor site. For His15Asp HPr, the anticipated sequence was obtained through the active center; however, for high-pI His15Asp HPr, the sequencing reaction failed after the fourteenth round, indicating the presence of a nonstandard peptide bond. This is consistent with the presence of a succinimide. Hydrolysis of this species to generate PS-His15Asp HPr, under the same conditions used the protein for crystallographic investigations, re-established the ability to sequence past the reacting residue. This is supportive of the presence of an aspartate rather than an isoaspartate (Figure 6A).

Protein Isoaspartate Methyltransferase Assays. PIMT assays were performed to provide quantitative analysis of isoaspartate content in the wild type, His15Asp HPr, and PS-His15Asp HPr. For these three proteins, an identical baseline content of isoaspartyl residues is observed, with fewer than five isoaspartates per 100 protein molecules (Figure 6B). A low frequency of isoaspartate generation is anticipated for HPr as the protein has been shown to deamidate at Asn12 and Asn38 (*38*). That wild-type HPr, which has a hisitidine at the phosphoacceptor position which is unable to undergo succinimide or isoaspartate formation, has isoaspartate content identical to that of His15Asp and PS-His15Asp HPr indicates these isoaspartates are independent of the phosphoacceptor site.

On the basis of the hypothesis that structural restraints of the active site may impede isoaspartate formation, similar steric interference may limit the ability for PIMT to recognize isoaspartates in this region. However, proteolytic digestion of these proteins did not increase the number of isoaspartates detected, indicating that protein structure is not interfering with its ability to function as a PIMT substrate (Figure 6B).

Conserved Positioning of the Phosphoacceptor Site. The ability of the aspartate to act as a functional substitution for the His15 Nδ1 phosphorylation event is rationalized on the basis of the conserved geometric positioning of phosphoacceptor atoms of aspartate and histidine. Consistent with this

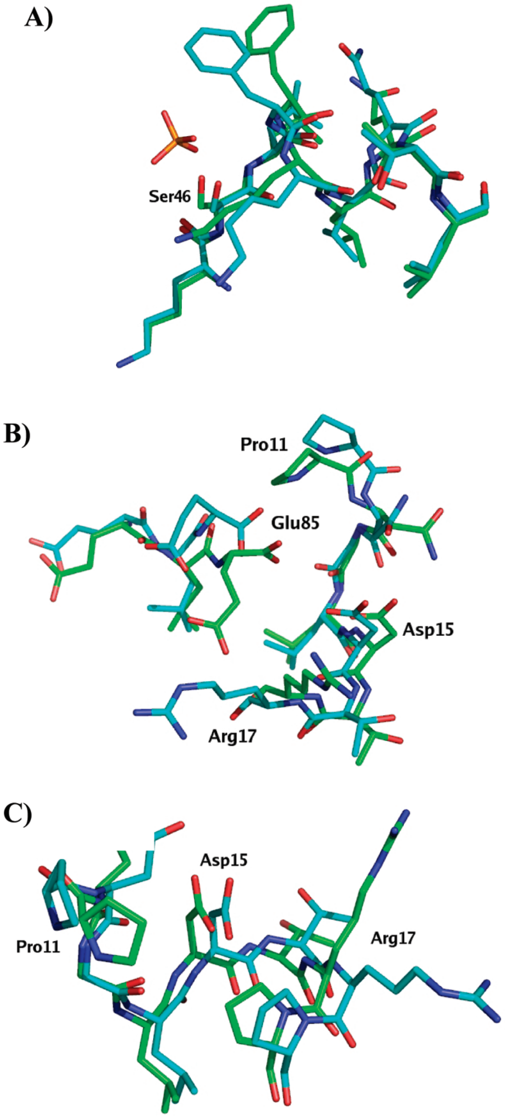

FIGURE 4: Structural deviation between His15Asp and PS-His15Asp HPr. These structures were created with Pymol (*30*), and in each case, molecules with green carbon−carbon bonds represent the PS-His15Asp HPr whereas molecules with blue carbon−carbon bonds represent His15Asp HPr. Structural comparisons are presented for the following. (A) Minor helix B. Formation of a more idealized helix in His15Asp Hpr is believed to be a consequence of a sulfate anion from the crystallization solution. (B) C-Terminus. The similar positioning of this region in two different crystallographic investigations of His15Asp HPr supports the proposed role in preventing isoaspartyl formation. (C) Active center. This involves a general repositioning of the main chain to bring it closer to the core of the protein as well as a tightening of the cleft that contains the active site region.

hypothesis, one of the side-chain carboxyl group oxygens of His15Asp occupies essentially the same position as the N$^{\delta 1}$ atom of the imidazole ring of the wild-type structure for both the pre- and post-succinimide forms of His15Asp (Figure 7). The electron density of position 15 clearly corresponds to that of an aspartate group with all of the non-hydrogen atoms of the side chain clearly defined. There is no additional electron density for which we cannot account near this position to suggest the presence of an isoaspartyl side chain.

The Conformation of the Active Site Is Consistent with Succinimide Formation. The formation of a succinimide within a protein is generally limited by the unique conformations required for side-chain reactivity with the main chain. Specifically, succinimide formation is favored when the main-chain ψ angles and side-chain χ_1 dihedral angles are approximately −120° and 60°, respectively (*1*). For the PS-His15Asp HPr, these angles are −169° and 60°, respectively. The amide nitrogen of residue 16 is positioned only 3.5 Å from the carboxyl oxygen of the phosphoacceptor Asp15. This positioning is predictive of succinimide formation and offers a partial structural rationalization of the ability of the protein to undergo rapid succinimide formation. However, that the high-pI intermediate is not observed in the absence of phosphorylation indicates that the favorable conformation, in the absence phosphorylation, is insufficient to initiate succinimide formation.

Phosphorylation-Catalyzed Succinimide Formation. The ability of phosphorylation to catalyze succinimide formation in His15Asp HPr likely occurs as a dual function of the phosphoryl group providing a better leaving group as well as the ability of phosphorylation to induce conformations which favor succinimide formation. The NMR structure of phosphorylated wild-type HPr demonstrates that the phosphoryl group forms hydrogen bonds with the main-chain amides of residues 16 and 17 (*37*). On the basis of the conserved positioning of the carboxyl oxygens of Asp15 with the phosphoryl-accepting site, it is reasonable to assume similar interactions occur with the phosphoacyl group of phosphorylated His15Asp. On the basis of conservation of conformation in phosphorylated His15Asp, the ψ and χ_1 angles are −169° and 60°, respectively, which are highly conducive to succinimide formation. Furthermore, the side-chain carbonyl group is brought within 2.4 Å of the amide nitrogen of residue 16 which is again highly favorable for succinimide formation (Figure 8A).

In addition to offering a more favorable conformation of the reactive side chain for succinimide formation, the anticipated interaction between the phosphoacyl group and the main chain is also expected to initiate a concerted chemical reaction in which extraction of protons from the main-chain amides by the phosphoryl group renders the phosphoryl group an even better leaving group, further increasing rates of succinimide formation (Figure 8B).

DISCUSSION

Traditionally, protein succinimides are regarded as a form of nonspecific damage affecting asparagines and aspartates which are vulnerable on the basis of their sequence and structural contexts. This vantage point is supported by numerous examples of proteins which have been shown to undergo spontaneous and physiological formation of isoaspartates, including calbindin (*39*), calmodulin (*40*), somatotropin (*41*), and collagen (*42*), as well as the detrimental effect these nonstandard residues have on protein function. The presence of an apparent repair enzyme, PIMT, which is widely expressed in most organisms supports the physiological occurrence and importance of isoaspartyl formation through succinimide intermediates.

It has alternatively been suggested that succinimide formation may serve as a molecular, biological clock (*43*−*45*). This could serve to indicate protein age for regulating longevity, not unlike PEST sequences (*46*) or the N-end rule (*47*), or could serve to trigger biological events by providing

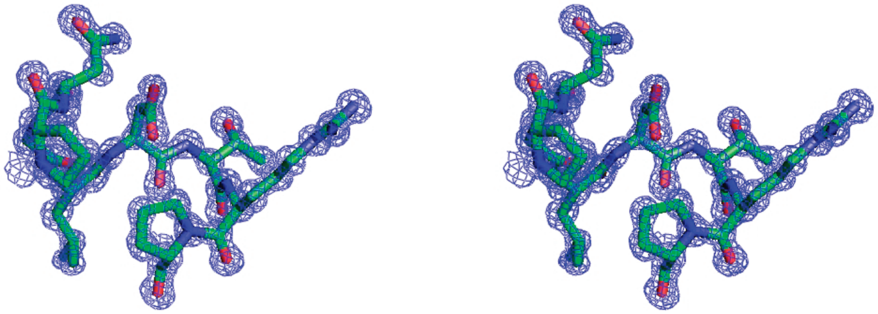

FIGURE 5: Stereographic representation of electron density of th active site region. Electron density of the active site region of post-succinimide His15Asp HPr at 1 Å resolution. The clear electron density for Asp15 as well as the absence of additional electron density that is unaccounted for in the active center region argues against the possibility of an isoaspartyl residue at position 15.

A)

	Residue						
	10	**11**	**12**	**13**	**14**	**15**	**16**
His15Asp	A	P	N	G	L	D	T
High pI His15Asp	A	P	N	G	L	NS	NS
PS-His15Asp	A	P	N	G	L	D	T

No detectable signal (NS).

B)

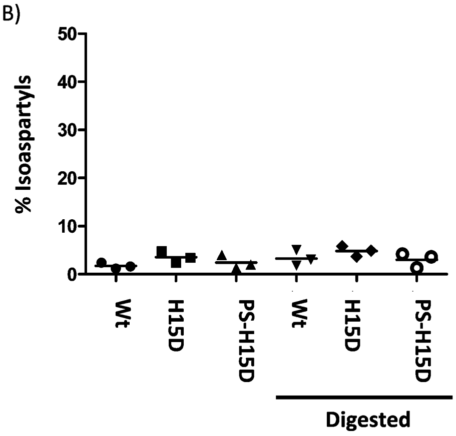

FIGURE 6: Amino acid analysis and methyltransferase assays for detection of isoaspartyls in PS-His15Asp HPr. (A) Amino acid sequencing. The inability for N-terminal sequencing to proceed past nonstandard peptide bonds, such as succinimides or isoaspartyls, provides a qualitative assessment for the presence of these residues. The inability to sequence past the 14th residue of high-pI His15Asp HPr indicates the presence of a succinimide, while detection of correct sequences in positions 15 and 16 of PS-His15Asp HPr indicates breakdown to an aspartyl product. Not shown are the first 10 residues of each protein. (B) Methyltransferase assays were performed using the ISOQUANT isoaspartate kit (Promega) according to the manufacturer's instructions. Isoaspartate content is expressed as a percentage of modified residues per 100 molecules of protein. Isoaspartate contents were determined for the wild type, His15Asp HPr, and PS-His15Asp HPr for both intact and proteolytic fragments, to eliminate any influence of protein structure on the ability to act as a PIMT substrate. Each data point represents a separate PIMT assay.

alternative protein isoforms of distinct functions. From this perspective, the deliberate placement of aspartates or asparagines within susceptible regions of the polypeptide would cause predictable rates of isoaspartate formation to predetermine protein lifespan or initiate changes in protein function. Importantly, however, the considerations of succinimide formation as a form of protein damage, or as a molecular clock, are both influenced by the dogma that succinimide formation and isoaspartate production are inseparably linked. Our demonstration of isoaspartate-free succinimide cycling expands and complicates the potential physiological roles for succinimide formation as well as their detection for protein quality assurance purposes.

First, the example of His15Asp HPr demonstrates that succinimide formation represents an effective strategy for removal of phosphoryl groups from modified aspartic acid residues and could represent a mechanism of autophosphatase activity for the numerous proteins which are regulated through phosphorylation of aspartyl residues. Specifically, signal transduction in microorganisms and plants frequently occurs through two-component systems which involve His-Asp phosphorelay networks of sensory histidine protein kinases and response regulator proteins (*48, 49*). The phosphorylated response regulators have intrinsic autophosphatase activities to limit the duration of the phosphorylated state. These autophosphatase activities vary by 4 orders of magnitude for different response regulators depending on their physiological function (*50, 51*).

The mechanism of response regulator autophophatase activity has generally been assumed to proceed as a reversal of the phosphohistidine reaction with water replacing the imidazole side chain. Alternately, through succinimide formation, the phosphoryl group could be displaced by the intramolecular nucleophilic attack at the β-carboxyl group. There are two notable examples to support this model. First, a mass spectrometric investigation of the phosphorylation of OmpR, the response regulator that mediates osmotic responses in *E. coli*, relieved a third species, in addition to the unphosphorylated and phosphorylated OmpR proteins, with a mass approximately 18 Da lower than that of the unmodified protein (*52*). This dehydrated form of the protein was dependent upon phosphorylation, so it presumably arose as a consequence of dephosphorylation. An identical dehydrated species was observed as a consequence of phosphorylation of the chemotaxis response regulator CheY (*53*), although succinimide formation does not appear to be a principle source of autophosphatase activity for this particular response regulator (*54*).

Phosphorylated aspartate residues are also observed outside of the response regulators, specifically, within the haloacid dehydrogenase superfamily, which includes the P-type ATPases and the dehalogenases. For most dehalogenase enzymes, the reaction mechanism involves 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 (*55, 56*) and is

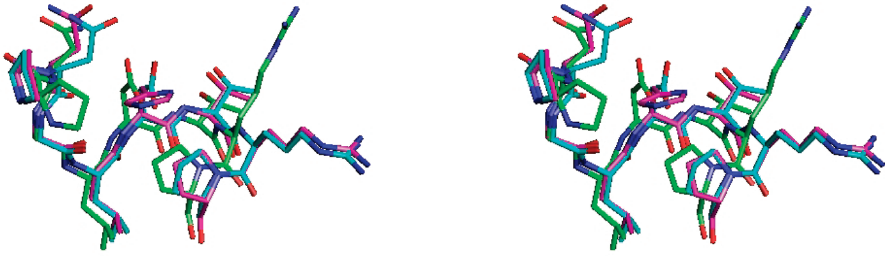

FIGURE 7: Stereographic comparison of the phosphoacceptor site of the wild type, His15Asp HPr, and PS-His15Asp HPr. This figure was created with Pymol (*30*). Molecules with green carbon−carbon bonds represent the post-succinimide structure, molecules with blue carbon−carbon bonds the pre-succinimide structure, and molecules with magenta carbon−carbon bonds wild-type HPr. For both the pre- and post-succinimide His15Asp structures, the carboxyl group oxygen occupies a position similar to that of the $N^{\delta 1}$ atom of the phosphoacceptor His15 residue of wild-type HPr.

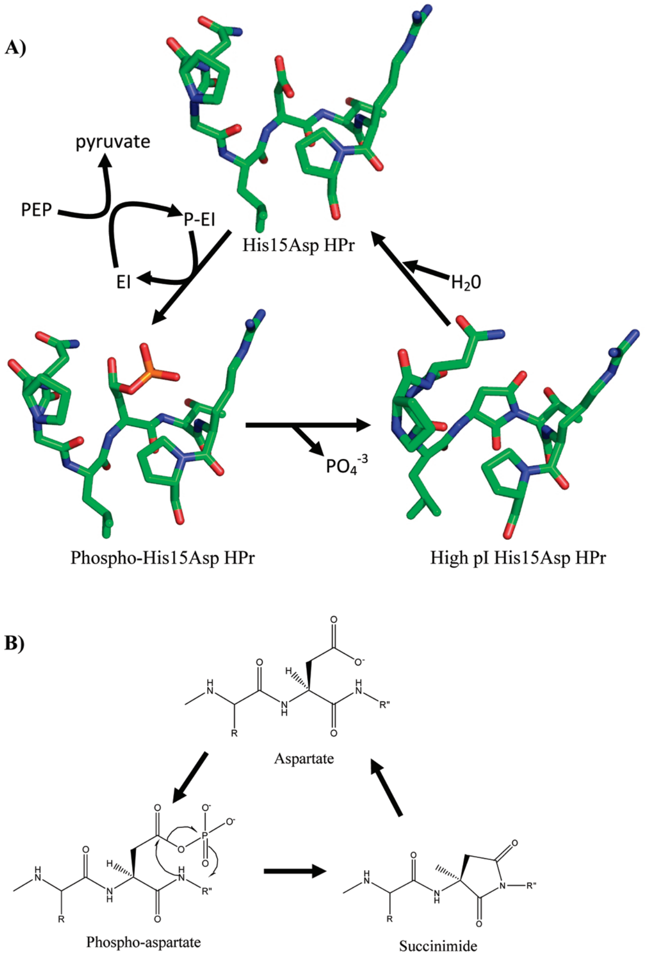

FIGURE 8: Structural mechanism for succinimide-based autophosphatase activity. (A) Structural isomers of phosphorylation and succinimide-based dephosphorylation of His15Asp are modeled on the basis of the crystallographic structure. (B) General mechanism for catalyzed succinimide formation from a phosphoaspartate with subsequent hydrolysis to aspartate.

consistent with succinimide formation during hydrolysis of the covalent intermediate.

In addition to a potential role in serving as a source of autophosphatase activity of phosphoaspartyl residues, the demonstration of succinimide hydrolysis exclusively to an aspartate also is significant for the consideration of these modifications as a measure of protein damage. Experimentally, it is easier to quantify isoaspartates than succinimides in proteins. As a result, and as a consequence of the assumption that succinimide formation is inseparable from isoaspartate formation, strategies for quantification of these reactions are based on the occurrence of isoaspartate. Our demonstration of isoaspartate-independent succinimide hydrolysis indicates these approaches likely underestimate the frequency of this modification. This could be significant in the evaluation of the structural integrity of peptides and proteins. This is particularly important as it has been shown that deamidation can significantly influence the quality of vaccines (*57, 58*) and other peptide and protein pharmaceuticals (*59, 60*).

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