The Influence of Protein Structure on the Products Emerging from Succinimide Hydrolysis*

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Proteins are vulnerable to spontaneous, covalent modifications that may result in alterations to structure and function. Asparagines are particularly labile, able to undergo deamidation through the formation of a succinimide intermediate to produce either aspartate or isoaspartate residues. Although aspartates cannot undergo deamidation they can form a succinimide and result in the same products. Isoaspartyls are the principal product of succinimide-hydrolysis, accounting for 65–85% of the emerging residues. The variability in the ratio of products emerging from succinimide-hydrolysis suggests the ability of protein structure to influence succinimide outcome. In the H15D histidine-containing protein (HPr), phosphorylation of the active site aspartate catalyzes the formation of a cyclic intermediate. Resolution of this species is exclusively to aspartate residues, suggestive of either a succinimide with restrained hydrolysis, or an isoimide, from which aspartate residues are the only possible product. Deletion of the C-terminal residue of this protein does not influence the ability for phosphorylation or ring formation, but it does allow for isoaspartyl formation, verifying a succinimide as the cyclic intermediate in H15D HPr. Isoaspartyl formation in H15D Δ85 is rationalized to occur as a consequence of elimination of steric restrictions imposed by the C terminus on the main-chain carbonyl of the succinimide, the required point of nucleophilic attack of a water molecule for isoaspartyl formation. This is the first reported demonstration of the influence of protein structure on the products emerging from succinimide-hydrolysis.

Although numerous cellular mechanisms exist to ensure fidelity during protein synthesis, proteins are chemically unstable and may undergo spontaneous, post-translation alterations. Asparagine and aspartate residues are particularly reactive with the potential for the nucleophilic attack of the neighboring peptide bond nitrogen on the side-chain carbonyl resulting in the formation of a succinimide, an unstable five-member ring (1–3) (Fig. 1). Formation of a succinimide is the first and rate-limiting step in the metabolism of these residues.

The spontaneous hydrolysis of a succinimide yields either an aspartyl or isoaspartyl residue. Isoaspartyls, with the introduction of an extra carbon to the main-chain, have the greater potential for disruption of protein structure. Although there is variability of the specific distribution of products, isoaspartyls predominant, typically accounting for 65–85% of the emerging residues (1–2). The α-carbon of a succinimide is prone to racemization, resulting in the D-configurations of each of these products, although at a low yield (4).

The physiological importance of succinimide formation is indicated by the presence of an enzyme, 1-isoaspartyl methyltransferase, which specifically recognizes and methylates these non-standard amino acids (5–7). The enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine to the α-carboxyl group of 1-isoaspartyl residues and the β-carboxyl group of aspartyl residues. Methylation stimulates re-formation of the succinimide, and subsequent hydrolysis results in a fraction of the offending residues being converted to l-aspartate. The overall reaction therefore represents true repair when the initiating residue is an aspartate and partial repair from an asparagine. This enzyme is found in a broad range of both prokaryotic and eukaryotic organisms (8, 9) and can recognize isoaspartyl residues in most amino acid sequences (10). Mice lacking the enzyme show an accumulation of damaged proteins and suffer fatal seizures at an early age (11), Caenorhabditis elegans mutants having a disruption in the gene encoding this enzyme show poor dauer stage survival (12) and Escherichia coli mutants lacking the enzyme have increased sensitivity to stresses in stationary phase (13).

There have been numerous investigations of factors that influence rates of succinimide formation, including structural determinants at the level of both primary (14) and tertiary (15) structure, as well as investigations of environmental influences such as temperature (16), pH (17), and dielectric strength (18). In contrast, no investigations have been performed into the structural influences on patterns of succinimide hydrolysis. Such investigations are complicated by the slow rates of succinimide formation and the inability to directly quantify succinimide occurrence in proteins.

The fortuitous creation of a protein in which succinimide formation is autocatalyzed from a phosphoaspartyl residue has provided an ideal system in which to conduct such an investigation. As a consequence of the rapid cyclization, the succinimide-containing intermediate can be visualized and quantified by mass spectrometry and isoelectric focusing, allowing for discrimination of mutations that influence succinimide formation as opposed to hydrolysis.

The first two reactions of the phosphoenolpyruvate:sugar phosphotransferase system involve the soluble, non-sugar-specific, energy-coupling proteins, enzyme I and HPr. The active center His15 of HPr can be mutated to an aspartate, H15D HPr.
while retaining the ability to phosphotransfer from enzyme I to enzyme II\(\text{A}^{\text{Glc}}\) (EII\(\text{A}^{\text{Glc}}\)) although at reduced efficiency (19). The ability for aspartate to act as a functional substitution for the His15 \(\text{N}\text{\delta1}\) phosphorylation was rationalized on the basis of the similar geometric positioning of the aspartyl carboxyl oxygen atom with the \(\text{N}\text{\delta1}\) atom of histidine. Crystallographic determinations of both the wild type and H15D HPr verified the positioning of the aspartate carboxyl to within an angstrom of the relative position of the \(\text{N}\text{\delta1}\) atom of the His\(^{15}\) imidazole in the wild type structure (19).

It was observed on isoelectric focusing gels that phosphorylation of the H15D mutant of \(E.\text{coli}\) HPr resulted in the formation of a species of the protein that was more positively charged than the unmodified protein (19). This high pl species could be isolated by ion-exchange chromatography and displayed properties consistent with a succinimide ring: (a) changes in mass isolated by ion-exchange chromatography and displayed properties consistent with loss of a water molecule, (b) +1 in charge relative to the unmodified protein, (c) sensitive to basic conditions, and (d) the presence of a nonstandard peptide bond.

The ability of a phosphoacyl to undergo catalyzed ring formation was anticipated. Covalent modification of side-chains can increase reactivity; modification of the carboxyl group of aspartic acid influences the rate of ring formation by providing a better leaving group. \(\beta\)-Methyl ester derivatives have been shown to form succinimides 24,000 times faster than the corresponding unmodified aspartyl residues (2).

Following hydrolysis of the cyclized species of H15D HPr, neither amino acid analysis nor an isoaspartyl methyltransferase assay indicated the presence of isoaspartyl residues (19). N-terminal sequencing of the H15D HPr preparations and samples taken after dephosphorylation gave normal recoveries of an aspartyl residue at position 15. These sequencing reactions are dependent upon the presence of standard peptide bonds and do not proceed through isoaspartyl residues. These results are consistent with either the constrained hydrolysis of a succinimide or an isomide.

Isoimides have been suggested as alternate structures to succinimides through which deamidation may proceed (2). They have not, however, been verified experimentally in proteins. Hydrolysis of an isoimide yields only aspartyl residues regardless of the point of attack of the incoming water molecule (Fig. 2).

In \(E.\text{coli}\) HPr the active center region is complicated by the presence of the C terminus. The involvement of the Glu\(^{85}\) in the active center was initially suggested by two-dimensional NMR investigations of HPr (20). This was supported later by the 2.0 Å x-ray structure of wild type \(E.\text{coli}\) HPr in which there is an ion pair between the \(\alpha\)-carboxyl of Glu\(^{85}\) and the Ne2 atom of His\(^{15}\) (21). An identical positioning was demonstrated in the 1.5 Å x-ray structure of the S46D mutant of \(E.\text{coli}\) HPr (22). The two structures crystallized in different space groups, reducing the possibility that the positioning of these residues represents a crystallographic artifact. In the H15D \(E.\text{coli}\) x-ray structure at 1.5 Å, Glu\(^{85}\) is again positioned toward the active center region in a manner similar to that seen in the wild type structure (Fig. 3) (19).

In other species such as \(Bacillus\) \(subtilis\) (23) and \(Enterococcus\) \(faecalis\) the C terminus is extended by three residues and is positioned far from the active center (24).

In \(E.\text{coli}\) HPr mutations to the C-terminal residues, Glu\(^{85}\), Leu\(^{84}\), and Glu\(^{83}\) have been performed to assess the involvement of the C-terminal of HPr in phosphoryl group transfer (25). Contrary to structural information, mutagenesis does not support the role of the C terminus in the phosphotransfer function of \(E.\text{coli}\) HPr. The enzyme I kinetic parameters are unaffected by substitutions or deletion of the final three residues, as are the kinetic parameters with various enzyme II complexes (26).

Although the C terminus is not involved in the catalytic mechanism, it may represent the structural restraint preventing isoaspartyl formation from a succinimide in H15D HPr. Formation of an aspartyl from a succinimide requires the attack of a water molecule on the side-chain carboxyl-carbon, whereas isoaspartyl formation requires the attack to be on the main-chain carbonyl-carbon, a region sterically restricted by Glu\(^{85}\). In this work the influence of the C-terminal residue on isoaspartyl formation in H15D HPr is investigated.

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**FIG. 1.** Succinimide formation from an aspartate residue. Not shown is the formation of small amounts of \(n\)-aspartyl and \(n\)-isoaspartyl that also occurs.

![Succinimide formation from an aspartate residue](image_url)

L-Aspartic Acid

L-Succinimide

L-Isoaspartic Acid

**TABLE 1**

| Type of Residue | Formation Pathway |
|-----------------|-------------------|
| Aspartyl        | From succinimide  |
| Isoaspartyl     | From isoimide     |

*Note:* This table illustrates the pathways through which aspartyl and isoaspartyl residues can be formed.
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EXPERIMENTAL PROCEDURES

Mutagenesis

Mutants were prepared using a QuickChange Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions.

PROTEIN Expression and Purification

HPr and Mutant HPrs—HPr protein were over-expressed in E. coli strain ESK108, F’ trp thi rpsD. ptsH465 recA56, using pUC19( ptsH) plasmid under the control of the ptsH promoter (27). Homogeneous protein was produced as described previously (25).

Enzyme IIA^Glc—The gene for EIIA^Glc, crr, was isolated by polymerase chain reaction from pTSHIC9 (28) and introduced into pT7-7 (29), creating pT7-7.crr (19) following mid-log phase induction by 0.5 mM isopropylthiogalactoside. Purification of enzyme IIA^Glc and mutants was based on the methods described previously (30). Approximately 75 ml of the crude supernatant was applied to a 300-ml Q-Sepharose anion-exchange column (3.0 cm × 20 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, and 10 mM N-aminobenzamidine. The column was then eluted using a 2-liter salt gradient of 0–0.5 M KCl, and fractions of 10 ml were collected. Fractions containing EIIA^Glc were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). EIIA^Glc and mutants were estimated to be >90% pure judged by inspection of SDS-PAGE. Appropriate fractions were then pooled and dialyzed overnight against water. Samples were aliquoted and frozen at −20°C.

Mass spectrometry was performed using a PerSeptive Biosystems Voyager ELITE matrix-assisted laser diode ionization-time of flight spectrometer at the Plant Biotechnology Institute. Samples were run in linear mode.

Other Methods

Methods have been described for protein determinations (25, 27), SDS-PAGE (31), and enzyme I assays (25, 27, 32).

RESULTS

H15D Δ85 HPr Can Act as a Phosphoacceptor—The catalytic capabilities of the H15D Δ85 HPr mutant were demonstrated by (a) a spectrophotometric assay for enzyme I activity, (b) protein labeling by [32P]PEP detected by SDS-polyacrylamide gel electrophoresis and autoradiography, and (c) band-shift on an isoelectric focusing gel.

The H15D Δ85 HPr demonstrated very similar kinetics to that of wild type HPr, indicating that deletion of the C-terminal Glu85 residue from H15D HPr may help alleviate some of the additional negative charge introduced to the active center region by the H15D mutation, thus slightly decreasing the Km. Both the H15D HPr and H15D Δ85 have a Vmax of −0.1% of that of wild type HPr, indicating that deletion of the C-terminal

Methyltransferase Assay

Methyltransferase assays were performed using the ISOQUANT Isoaspartate Detection Kit (Promega) according to the manufacturer’s specifications for the radioactive protocol.

Production of [32P]PEP

[32P]PEP was made by incubating 0.2 mCi of [γ-32P]ATP (specific activity, 3000 Ci/mmol), 0.1 mg of homogeneous E. coli phosphoenolpyruvate carboxylase (provided by Dr. Hughes Goldie, University of Saskatchewan), 1 mM ATP, 12.5 mM KF, 5.0 mM MgCl2, and 1 mM oxaloacetate in 50 mM HEPES buffer (pH 7.5) in a final volume of 250 μl at 37°C for 10 min. The reaction was initiated with the addition of oxaloacetate. Reactions were stopped and stored with freezing at −20°C. The preparation was used without further purification.

Phosphorylation Conditions

Phosphorylation reactions consisted of 5 mM MgCl2, 25 μM [32P]PEP (specific activity, 800 Ci/mmol), and 50 mM HEPES buffer, pH 7.5, in a 20-μl volume. Protein concentrations of 5 μg of enzyme I, 10 μg of HPr, and 20 μg of enzyme IIA^Glc were used in all experiments, except where indicated otherwise. Mixtures were incubated at 37°C for 10 min. The reaction was halted with the addition of 2× SDS loading buffer and electrophoresed through a 15% SDS-PAGE. Following the run the gels were covered with commercial food wrap and exposed to x-ray film for 12 h at −80°C (31).

FIG. 3. The active center of H15D HPr.

FIG. 2. Isoimide formation from an aspartate residue.

L-Aspartic Acid

N16

OT2 85

L-Isoimide

L-Aspartic Acid

Mass Spectrometry

Mass spectrometry was performed using a PerSeptive Biosystems Voyager ELITE matrix-assisted laser diode ionization-time of flight spectrometer at the Plant Biotechnology Institute. Samples were run in linear mode.

Other Methods

Methods have been described for protein determinations (25, 27), SDS-PAGE (31), and enzyme I assays (25, 27, 32).
residue does not further compromise the catalytic activity of the mutant. This result is in agreement with the previously reported observation that deletion of the C-terminal residue of wild type E. coli HPr has little kinetic consequence in the ability of the protein to accept phosphotransfer from enzyme I (25).

H15D HPr, when incubated under phosphorylation conditions and examined by isoelectric focusing, reveals two new species, one with a lower pi and another with a higher pi. When \(^{32}\text{P}\) phosphatase was used, the lower pi band was shown to contain \(^{32}\text{P}\) phosphate (19). The higher pi species is the dehydrated, ring-containing species of the protein. Similar analysis of the H15D mutant of E. coli HPr resulted in an identical pattern of protein species arising from phosphorylation (Fig. 4).

**P-H15D Δ85 HPr Can Act as a Phosphodonor**—The ability of the P-H15D deletion mutants to phosphotransfer to an enzyme IIa protein was determined by "\(^{32}\text{P}\)" protein labeling by PEP detected by SDS-polyacrylamide gel electrophoresis and autoradiography. In the experiment described below, which shows enzyme IIa\(^{\text{Glc}}\) phosphorylation using "\(^{32}\text{P}\)" PEP, the protein preparations required the purification of all of the phosphoenolpyruvate:sugar phosphotransferase system proteins from strains of E. coli that do not produce HPr. When this was done, phosphorylation of enzyme IIa\(^{\text{Glc}}\) that was dependent upon the addition of H15D Δ85 HPr could be shown (Fig. 5).

As demonstrated in the lactate dehydrogenase assay, the impairment of the enzyme I reaction was large, requiring much higher amounts of enzyme I to be used, more than 100-fold compared with equivalent experiments with wild type HPr. Assays of the enzyme II\(^{\text{sug}}\) were unable to be performed for either the H15D or H15D Δ85 HPr because of the impractical amounts of enzyme I that would require to meet the criteria of independence of the enzyme II reaction from P-HPr generation (32). For this reason, sugar phosphorylation was not measured.

**Demonstration of Ring Formation in H15D Δ85 HPr**—Formation of either a succinimide or isomide involves the loss of a water molecule, this dehydrated species of protein can be identified by mass spectrometry. Mass spectrometric analysis of the unphosphorylated forms of H15D or H15D Δ85 HPr verified a single peak corresponding to the mass of all of the unmodified protein. Under phosphorylation conditions three species of each protein, corresponding to the unmodified, phosphorylated, and dehydrated forms of protein, were present.

Formation of either a succinimide or isomide from an aspartyl residue results in a loss of a negatively charged side-chain. Using isoelectric focusing it was possible to visualize this high pi species of H15D HPr (19). Isoelectric focusing of a phosphorylation mixture of the H15D Δ85 HPr demonstrates an identical pattern of protein species (Fig 4).

**Isoaspartyl Formation in H15D Δ85 HPr as a Consequence of Phosphorylation**—The distinguishing characteristics between a succinimide and an isomide are the products emerging from hydrolysis. Isoimidies are limited to aspartyl production, whereas succinimides produce a mixture of both aspartyl and isoaspartyl residues. Isoaspartyl formation, under both phosphorylation conditions, was measured for wild type, H15D, and H15D Δ85 HPr at various time points over a 5-h incubation period (Fig. 6a). For both wild type and H15D HPr the rate of isoaspartyl formation was \(\sim 0.2\) isoaspartyl residues/100 HPr molecules/h. For the H15D Δ85 HPr mutant the rate of isoaspartyl formation was \(5\) isoaspartyl residues/100 protein molecules/h.

Incubation of the wild type, H15D, and H15D Δ85 HPrs under phosphorylation conditions but without the inclusion of enzyme I did not affect the rates of isoaspartyl formation in either the wild type or H15D HPrs. The exclusion of enzyme I did, however, reduce the rate of isoaspartyl formation in H15D Δ85 to 0.2 isoaspartyl residues/100 protein molecules/h (Fig. 6b).

Isoaspartyl residues typically emerge as a consequence of deamidation of asparagine residues. Prolonged incubation of proteins under the temperatures used for phosphorylation is known to promote deamidation (1–3). In E. coli HPr there are 2 residues, Asn\(^{12}\) and Asn\(^{85}\), that are known to deamidate (33). As the background level of isoaspartyl formation, 0.2 isoaspartyls/100 protein molecules/h, is present in wild type HPr, these isoaspartyls can be attributed to deamidation and not to events associated with formation of the phosphaaspartyl residue.

**DISCUSSION**

Proteins are chemically unstable, susceptible to a variety of spontaneous covalent modifications that may result in alterations to both structure and function. Aspartate and asparagine residues are particularly labile with the potential to undergo cyclization between the side-chain carboxyl and the amide nitrogen of the n + 1 peptide bond to the formation of a succinimide. Succinimides are unstable intermediates that undergo spontaneous hydrolysis to a mixture of aspartyl and isoaspartyl residues. Although either of these products has the potential to be disruptive to protein structure, isoaspartyls are particularly problematic as they introduce an additional atom into the main-chain polypeptide.

Isoaspartyl formation has been proposed as a mechanism of aging in proteins, and the physiological significance of these reactions is supported by the presence of a proposed repair enzyme L-isoaspartyl methyltransferase (5–7). This enzyme shows unique specificity for methylation of the α-carboxyl
group of L-isoaspartic acid and the β-carboxyl group of D-aspartic acid. Methylation of these nonstandard amino acids stimulates reformation of the succinimide. Subsequent hydrolysis of the succinimide results in a fraction of the residues being converted to aspartates. For deamidation, the overall reaction represents partial repair of asparagine to an aspartyl residue. The repair is inefficient in that repeated cycles of methylation and succinimide formation are required for complete conversion of isoaspartate to aspartate.

Structural determinants of the protein may serve a protective function by influencing the extent of isoaspartyl formation from succinimide hydrolysis. Typically isoaspartyl residues emerge as the predominant product, accounting for 65–85% of emerging residues (1–3). The variability in the ratio of products suggests the influence of protein structure on hydrolysis, although no specific characterization of this influence has ever been performed. Formation of an isoaspartyl from a succinimide requires the nucleophilic attack of a water molecule on the main-chain carbonyl of the succinimide. Aspects of a protein that restrict access to this region are anticipated to favor formation of an aspartyl.

In H15D HPr phosphorylation of the aspartate residue initiates an intramolecular rearrangement to either a succinimide or isouimidic structure. Both isouimidides and succinimides involve cyclization of the side-chain carbonyl with main-chain atoms; isouimidides with the n-carboxyl group, succinimides with the n+1 amide group. Succinimide hydrolysis has potential for formation of aspartyl and isoaspartyl residues, whereas isouimide hydrolysis is limited to aspartyl production. Succinimides are accepted as intermediates of deamidation, isouimidides have never been conclusively demonstrated in a protein.

The exclusive production of aspartyl residues from the hydrolysis of the cyclized species of H15D HPr led to the conclusion that an isouimide was occurring (19). The possibility existed, however, that structural constraints imposed by the protein were preventing isoaspartyl formation from a succinimide. In E. coli HPr the C-terminal Glu85 residue is situated to prevent access to the main-chain carbonyl of residue 15 (Fig. 7),

FIG. 7. Model of the active center of a succinimide-containing H15D HPr.

FIG. 8. Model of the active center of a succinimide-containing H15D A85 HPr.
the required point of nucleophilic attack of an incoming water molecule to hydrolyze a succinimide to an isoaspartyl. One of the γ-carboxyl oxygens of Glu^{85} is situated 7.0 Å from the carbonyl carbon of a modeled succinimide.

Deletion of the C-terminal residue of E. coli HPr does not disrupt the kinetic or phosphotransfer abilities in either the wild type or H15D proteins. Mass spectrometry and isoelectric focusing both verify that phosphorylation of the H15D Δ85 mutant still initiates the formation of a ringed species of the protein. Modeling of the mutation (Fig. 8) illustrates how removal of the Glu^{85} residue opens access to the succinimide carbonyl-carbon group.

As predicted by modeling, the deletion of the C-terminal residue allows for the formation of isoaspartyl residues as a consequence of phosphorylation. This definitely demonstrates that a succinimide is produced in H15D Δ85 HPr and strongly suggests a succinimide in the dehydrated species of H15D HPr.

The formation of isoaspartyl residues in H15D Δ85 HPr are still lower than would be anticipated from the hydrolysis of an unrestrained succinimide, suggesting additional features of the active center region influence hydrolysis outcome. In the modeled structure the side-chain carbonyl of the succinimide has greater solvent exposure, whereas the main-chain carbonyl is directed more toward the protein, which would favor hydrolysis to an aspartate residue. Alternatively, we consider the possibility that a mixture of succinimides and isomides are being produced in the truncated form of the mutant protein. However, given that formation of each of these rings requires significantly different positions of the main-chain atoms it seems unlikely that this degree of conformational flexibility exists in the folded protein.

The demonstration of the influence of protein structure on the products emerging from succinimide hydrolysis has general importance for all proteins that undergo deamidation. Structural determinants that favor aspartyl production from deamidation may represent a mechanism to limit the deleterious consequences of deamidation of labile asparagine residues and limit dependence on the inefficient isoaspartyl methyltransferase repair system. The potential to undergo intramolecular rearrangements of asparagines without the production of isoaspartyl residues indicates that the identification and quantification of deamidation by isoaspartyl quantification may dramatically underestimate the extent of these reactions.

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