The Aspartyl Replacement of the Active Site Histidine in Histidine-containing Protein, HPr, of the Escherichia coli Phosphoenolpyruvate:Sugar Phosphotransferase System Can Accept and Donate a Phosphoryl Group

SPONTANEOUS DEPHOSPHORYLATION OF ACYL-PHOSPHATE AUTOCATALYZES AN INTERNAL CYCLIZATION

(Received for publication, March 19, 1999, and in revised form, May 17, 1999)

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The active site residue, His₁⁵, in histidine-containing protein, HPr, can be replaced by aspartate and still act as a phosphoacceptor and phosphodonor with enzyme I and enzyme II, respectively. Other substitutions, including cysteine, glutamate, serine, threonine, and tyrosine, failed to show any activity. Enzyme I ¹₅ → Asp HPr is increased 10-fold and ¾max is decreased 1000-fold compared with wild type HPr. The phosphorylation of Asp₁⁵ led to a spontaneous internal rearrangement involving the loss of the phosphoryl group and a water molecule, which was confirmed by mass spectrometry. The protein species formed had a higher pI than His₁⁵ → Asp HPr, which could arise from the formation of a succinimide or an isoimide. Hydrolysis of the isolated high pI form gave only aspartic acid at residue 15, and no isoaspartic acid was detected. This indicates that an isoimide rather than a succinimide is formed. In the absence of phosphorylation, no formation of the high pI form could be found, indicating that phosphorylation catalyzed the formation of the cyclization. The possible involvement of Asn₁² in an internal cyclization with Asp₁⁵ was eliminated by the Asn₁² → Ala mutation in His₁⁵ → AspHPr. Asn₁² substitutions of alanine, aspartate, serine, and threonine in wild type HPr indicated a general requirement for residues capable of forming a hydrogen bond with the Nε atom of His₁⁵, but elimination of the hydrogen bond has only a 4-fold decrease in ¼cat/¾m. The function of accepting and donating a phosphoryl group from enzyme I to the sugar-specific enzymes II was established for Escherichia coli. These events result in phosphoprotein formation with a Nε²-P-histidine in both enzyme I (6) and the enzyme IIₐₐ₇₉₉₇ domains (7) and a more unstable Nε³-P-histidine in HPr (8, 9). The structure of HPr from a number of species is now well established from both x-ray diffraction and NMR spectrometry approaches. The overall structure of the HPrs is described as an open-faced β-sandwich with a βαβαβαβ fold (see reviews in Refs. 10–12). In order to help specify phosphoryl transfer to the Nε³ atom of the His₁⁵ imidazole ring, various residues have been proposed to form hydrogen bonds with the Nε² atom of the His₁⁵ imidazole. In E. coli, the involvement of a glutamate residue was suggested (9) and was identified as Glu₆₅ by the first ¹H NMR structure (13) and the 2.0 Å resolution x-ray structure (14), the latter showing that the interaction was with the C-terminal α-carboxylate. Replacement or deletion of Glu₆₅ did not indicate a significant role for this residue (15). NMR spectral properties of His₁⁵ in E. coli HPr were consistent with hydrogen bonding to the Nε² atom (16), and van Nuland et al. (17) suggested Asn¹² as the most likely residue. Subsequently, in the 2.5 Å resolution structure of the complex of the JeI₄ monoclonal antibody Fab fragment with HPr, the Asn₁² side chain was found hydrogen-bonded to the Nε² atom of His₁⁵ (18).

Asn₁² in E. coli HPr has been investigated because it is a site of deamidation, which occurs through the formation of a succinimide to form aspartate and isoaspartate at residue 12 (19). The succinimide formation (Fig. 1A), especially at Asn-Gly pairs in a sequence leads to about 70% isoaspartic acid formation (20–23). This unusual amino acid can be repaired to aspartic acid by protein carboxylmethyltransferase (L-isoaspartate-(D-aspartate)-methyltransferase) in peptides (24), and the effective repair of residue 12 isoaspartic acid in HPr has been described (25). The substitution of aspartate or isoaspartate at residue 12 has modest effects on the phosphoacceptor role of HPr (19).

The formation of succinimides can occur at aspartate residues but usually under different conditions than those that prevail for asparagine residues. A stable succinimide has been characterized in somatotropin where cyclization of an aspartyl residue under acidic conditions allows the isolation of the succinimide that is labile at alkaline pH (26). A structure of a succinimide formed by an aspartyl residue at acidic pH has recently been described in lysozyme (27). In both cases, the hydrolysis of the succinimide yielded both isoaspartic and aspartic acid. The mechanism of succinimide formation is not the only route by which deamidation can occur (20–23), and among
the possibilities is the formation of an imide shown in Fig. 1B.

In this paper, we describe the effects of other substitutions at residue 12 and the lack of an absolute requirement for a histidine at residue 15 of HPr. Aspartate substitutes for histidine at residue 15 in phosphoryl acceptance and transfer, albeit inefficiently. In addition, the P-aspartyl residue leads to a spontaneous chemical rearrangement with the characteristics of catalyzed imide formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzyme I, the enzymes II*-*sor, and DNA oligonucleotides were obtained as described previously (28). Ampholytes were from Amersham Pharmacia Biotech. DEAE paper (D81) was from Whatman. The Quik-Change site-directed mutagenesis kit was obtained from Stratagene. [32P]Phosphoenolpyruvate (PEP) was produced as described previously (29).

**Mutagenesis**—His15 → Asp and His15 → Glu HPr mutants were produced by Dr. J. W. Anderson. Asn12 → Asp HPr has been described (19), and all other mutations were produced by the Quik-Change site-directed mutagenesis kit according to the manufacturer’s instructions. All mutations were in the ptsH gene incorporated into pUC19 (15). His15 → Ala HPr was obtained from M Scholtz (Texas A & M).

**Protein Expression and Purification**—HPr and mutant HPrs were expressed in E. coli strain ESK108, which is ptsH (28), using the pUC(ptsH) plasmids with HPr expression under the control of its own promoter. Homogeneous protein was produced as described previously (15). Yields were 50–500 mg of protein/30 g of wet weight of cells.

**Isolation of His15 → Asp HPr Derivatives**—His15 → Asp HPr (3 mg) was phosphorylated at 37 °C for 10 min in 10 mM potassium phosphate buffer, pH 7.0, with 5 mM PEP and 0.1 mg enzyme I. The three forms of His15 → Asp HPr, phosphorylated (lower pI), unphosphorylated, and cyclized (higher pI) were separated by anion exchange chromatography, Mini-Q-Sepharose column, and an Amersham Pharmacia Biotech Gra-difrac system at 4 °C. The reaction mixture was loaded with 10 mM citrate-phosphate buffer, pH 4.6, and the column was 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.

**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. Data were acquired and analyzed using an Applied Biosystems Inc. model 601A data system (30). The sequencing was carried out by Dr. S. Mackenzie (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada).

**Mass Spectrometer Analysis**—Mass spectrometry was performed using a Perseptive Biosystems Voyager ELITE matrix-assisted laser di-
ode ionization-time of flight spectrometer at the Plant Biotechnology Institute. The samples were run in linear mode.

Crystalization of HPr Mutants and Determination of the Tertiary Structures of Mutant HPrs—Crystals of His$^{15} \rightarrow$ Asp HPr were grown by the hanging drop vapor diffusion method at 14 °C. Washing and seeding of microcrystals was used (31). Crystals formed in 0.1% citrate phosphate buffer, pH 4.4, and 20–25% saturated ammonium sulfate. Crystals of the high pl form of His$^{15} \rightarrow$ Asp HPr, containing a putative cyclized Asp$^{15}$ were grown similarly. Synchrotron diffraction data for His$^{15} \rightarrow$ Asp HPr were collected with a Brandeis CCD detector at the Brookhaven National Laboratory (Upton, NY). For the high pl form, data were collected at the Photon Factory (Tsukuba, Japan) using a wavelength of 1.0 Å and a screenless Weissenberg camera. The data were processed using DENZO and SCALEPACK (32). The structures were solved as has previously been described for Ser$^{65} \rightarrow$ Asp HPr (33) using the Amore suite of programs (34) with molecular replacement with wild type HPr (11). Refinement was performed using the X-PLOR 3.1 package (35).

Construction of ptsH Strains and Enzyme IIA$^{\text{gc}}$ Production—The gene for enzyme IIA$^{\text{gc}}$, cer, was isolated by polymerase chain reaction from pTHSIC9 (36) and introduced into pT7-7 (37) using the NdeI and BamHI restriction endonuclease sites. Enzyme I$^{\text{IEF}}$ was expressed in E. coli strain ESK262, which is Kan$^{\text{R}}$:ptsH, following mid-log phase induction by 0.5 mM isopropylthiogalactoside. This strain was constructed by ligating the Kan$^{\text{R}}$ gene from pUC4 into the PstI restriction endonuclease site in ptsH in pGAB65 (38). The linearized plasmid was used to transform E. coli strain DPB271 (39), which is recD, and a ptsH gene replacement derivative was selected by kanamycin resistance. This E. coli strain ESK150 had no HPr detectable by assay, phosphorylation, or immunoreactivity as determined by standard methods (40). E. coli strain was a derivative of strain BL21 pLysS (37), which was transduced with P1-plage grown on strain ESK150 to produce a Kan$^{\text{R}}$:ptsH strain ESK262. Enzyme I was also overproduced in this strain using a similar plasmid.$^2$

Protein Methylation—His$^{15} \rightarrow$ Asp HPr and derivatives were assayed for methyl accepting ability by incubation with S-adenosyl-l-methionine-$^{14}$C)methionine and l-isooaspartate-(o-aspartate) O-methyltransferase. Wild type HPr was used as a control. The assays were performed by J. D. Lowenson and S. Clarke (UCLA) as described previously (25).

Other Methods—Standard methods have been described for characterization of HPr:protein determinations (15, 28), isoelectric focusing, (19), SDS-polyacrylamide gel electrophoresis and autoradiography (42), rates of phosphohydrolisis (9), and enzyme I and enzyme IIA$^{\text{inc}}$ assays (15, 28, 43).

RESULTS

His$^{15} \rightarrow$ Asp HPr Can Act as a Phosphoacceptor—The following substitutions for His$^{15}$ in E. coli HPr were made: alanine, asparagine, aspartate, cysteine, glutamate, glutamine, serine, threonine, and tyrosine. Except for His$^{15} \rightarrow$ Asp HPr, none of these mutations showed any detectable activity when tested for activity with enzyme I by: (a) a spectrophotometric assay for enzyme I activity (43); (b) $^{32}$P-protein labeling by PEP detected by SDS-polyacrylamide gel electrophoresis and autoradiography (42); (c) a gel shift of a band on an isoelectric focusing (IEF) gel because of the introduction of the phosphoryl group (9); and (d) in vivo complementation of the fermentation negative phenotype of the ptsH strain, E. coli ESK108.

His$^{15} \rightarrow$ Asp HPr, when incubated with PEP, enzyme I, and Mg$^{2+}$, revealed two new species, one with a lower pl and another with a higher pl (Fig. 2, A and B). The formation of the high pl form was not efficient at room temperature (22 °C) but was readily detected at 37 °C (Fig. 2C). When $^{32}$PPEP was used, the lower pl band was shown to contain $^{32}$Pphosphate by autoradiography; the higher pl band did not have a phosphoryl group (Fig. 2, D and E). Enzyme I phosphotransfer activity was measured using His$^{15} \rightarrow$ Asp HPr and was shown to have a $K_m$ of 66 μM for His$^{15} \rightarrow$ Asp HPr (wild type HPr $K_m$ 6 μM) and a $V_{\text{max}}$ that was 0.1% of that obtained with wild type HPr.

$^2$S. Brokx, J. Taylor, F. Georges, and E. B. Waygood, submitted for publication.

P-Asp HPr Can Act as a Phosphodonor—The impairment of the enzyme I reaction was large, and thus much higher amounts of enzyme I were used, greater than 100-fold compared with equivalent experiments with wild type HPr. Assays of the enzymes I$^{\text{E116S}}$ and His$^{15} \rightarrow$ Asp HPr would require impractical amounts of enzyme I to meet the requirements of independence of the enzyme II reaction from P-HPPr generation (42). For this reason, sugar phosphorylation was not measured. In the experiment described below, which showed enzyme IIA$^{\text{gc}}$ phosphorylation using $^{32}$PPEP, the protein preparations required the purification of all the PTS proteins from strains of E. coli that did not produce HPr. When this was done, phosphorylation of enzyme IIA$^{\text{gc}}$ that was dependent upon the presence of His$^{15} \rightarrow$ Asp HPr could be shown (Fig. 3). To assess...
reactions with other enzymes IIAsugar, an in vivo approach was used. When His\(^{15}\)\(^{3}\) Asp HPr was overproduced in vivo, it would not complement sugar fermentation in the ptsH strain, E. coli ESK108. His\(^{15}\)\(^{3}\) Asp HPr has a 10,000-fold impairment; Ser\(^{46}\)\(^{3}\) Asp HPr is the next most impaired HPr described, 1000-fold, and its overproduction in vivo results in delayed fermentation (33). His\(^{15}\) \(\rightarrow\) Asp HPr is not effective in the overall function of the PTS.

Characterization of the High pI Species of His\(^{15}\) \(\rightarrow\) Asp HPr—
The pH stability of the different pI species was found by carrying out the phosphorylation reaction and then putting samples at different pHs and following the progress of the loss of species by IEF. These results showed that the high pI form was more stable at acidic pH and that the P-Asp15 HPr was more stable at alkaline pH.

The deamidation events at Asn\(^{38}\) and Asn\(^{12}\) in HPr (19, 25) suggested that the higher pI form might be a succinimide ring (Fig. 1). This form results in the loss of a water molecule from the protein, a net loss of 18 mass units. Moreover, when the succinimide hydrolyzes, the normal distribution of products is about 70% isoaspartyl and 30% aspartyl. The resulting HPr species have very similar pIs and have been distinguished by the detection of doublet bands on IEF gels (19). In gels such as shown in Fig. 2, there was no indication of doublet bands for any of the pI species. No increase in protein carboxymethyl transferase methylating activity could be detected with His\(^{15}\) \(\rightarrow\) Asp HPr or the derivatives following phosphorylation. The methylation reaction requires isoaspartyl residues. In addition, N-terminal sequencing of His\(^{15}\) \(\rightarrow\) Asp HPr preparations and the derivatives obtained after dephosphorylation gave normal recoveries of an aspartyl residue at position 15. If an isoaspartyl residue forms, the sequencing reactions do not proceed through the isoaspartyl residue (22).

The high pI form of His\(^{15}\) \(\rightarrow\) Asp HPr was purified as described under “Experimental Procedures.” Mass spectroscopy showed a molecular species with 18 mass units less than His\(^{15}\) \(\rightarrow\) Asp HPr (Fig. 4). The isolated high pI form was sequenced from the N terminus, and the sequencing did not proceed beyond residue 14. Incubation of the isolated high pI form at pH 9 led to reversion to the normal His\(^{15}\) \(\rightarrow\) Asp HPr, and N-terminal sequencing identified only an aspartyl residue at position 15 with normal recoveries. When phosphorylation of the reverted form was carried out as described in Fig. 2, the appearance of the phosphorylated and higher pI species was the same (results not shown). These results confirm an unusual structure at residue 15 and the reversibility of the whole process. These findings are consistent with either a succinimide ring formation followed by a very constrained hydrolysis reaction to yield only aspartate or the formation of an isomide from which hydrolysis would always yield an aspartyl residue (Fig. 1).

Stability of P-Asp HPr—Phosphohydrolysis of P-Asp HPr
was investigated at several pHs. The comparisons with P-His HPr are given in Fig. 5.

**Tertiary Structure of His$^{15}$ → Asp HPr**—The structure of His$^{15}$ → Asp HPr was determined as described under “Experimental Procedures.” Crystallographic parameters are shown in Table I. The 1.5 Å resolution structure of His$^{15}$ → Asp HPr is essentially the same as the 2.0 Å resolution structure of wild type HPr (14). However, His$^{15}$ → Asp HPr had the two differences found in both the 1.6 Å resolution structure of Ser$^{36}$ → Asp HPr (33) and the 2.5 Å resolution structure of wild type HPr bound to the Fab fragment of the HPr-specific monoclonal antibody Je142 (18); neither the tight β-turn involving Asn$^{12}$ nor any torsion angle strain at residue 16 was found. The Asp$^{15}$ residue was well defined (Fig. 6A). One of the oxygen atoms of the Asp$^{15}$ carboxyl group is in essentially the same position as the N$^{11}$ atom in the His$^{15}$ imidazole ring of wild type; the relative distances between the position of the His$^{15}$ N$^{12}$ atom in wild type and the positions of the two Asp$^{15}$ carboxyl oxygen atoms in His$^{15}$ → Asp HPr are 1.0 and 2.0 Å (Fig. 6B). The C-terminal carboxyl group of Glu$^{85}$, which has been found hydrogen-bonded with the N$^{2}$ atom of His$^{15}$, is found in the same position in His$^{15}$ → Asp HPr (Fig. 6B) as described in wild type and Ser$^{46}$ → Asp HPrs (14, 33), but no hydrogen bond is formed. The side chain of Asp$^{15}$ is involved in no hydrogen bonds.

Formation of a succinimide or an isoidime have optimal main chain ϕ angles and side chain χ$^{1}$ dihedral angles: ϕ = −120°, χ$^{1}$ = +60°, and ϕ = +60°, χ$^{1}$ = +120°, respectively (20). For the residue 15 in His$^{15}$ → Asp HPr, these angles are ϕ = −170°, χ$^{1}$ = +61°, which are considerably closer to the ideal values for succinimide formation.

In addition to this structure, the high pI form, which contained the putative cyclized form, was crystallized, and diffraction data were collected within 10 days. The unit cell and refinement parameters are very similar to the normal His$^{15}$ → Asp HPr (Table II). Only a well defined aspartyl residue was found at residue 15 (Fig. 6A) and in essentially the same position as in the normal His$^{15}$ → Asp HPr (Fig. 6C).

**The Double Mutant of HPr: His$^{15}$ → Asp and Asn$^{12}$ → Ala**—A novel cyclic compound involving Asn$^{12}$ and Asp$^{15}$ was possible. To eliminate the possible involvement of Asn$^{12}$ in the formation of the high pI form, the double mutant of HPr, His$^{15}$ → Asp and Asn$^{12}$ → Ala was made and purified. In wild type HPr, the Asn$^{12}$ → Ala mutation causes a small impairment in the enzyme I reaction (Table II). In His$^{15}$ → Asp HPr, the Asn$^{12}$ → Ala mutation leads to no detectable change in the formation of P-Asp HPr or the high pI species, and the IEF gel is essentially the same as presented in Fig. 2.

**Deamidation and Cyclization at Residue 15**—In order to follow more closely the events at position 15, the complications with respect to deamidation events at Asn$^{12}$ and Asn$^{38}$ were eliminated by creating the following two triple mutants Asn$^{12}$ → Ala, His$^{15}$ → Asp, and Asn$^{38}$ → Ala, and Asn$^{12}$ → Ala, His$^{15}$ → Asn, and Asn$^{38}$ → Ala. The events of either cyclization and/or deamidation were carried out at 60 °C and at pH 5.0, 7.0, 8.0, and 10.0 followed by separation of products on IEF gels as shown in Fig. 7. These gels show that there was no indication of either cyclization or deamidation even after 90 min at either pH 5.0 or pH 10.0, suggesting that the location at residue 15 has no unusual propensity to either cyclize or produce a succinimide specifically. Deamidation of Asn$^{15}$ would have caused a band shift on the IEF gels, and none was observed even though the main chain ϕ angle and side chain χ$^{1}$ dihedral angle of Asp$^{15}$ (and presumably Asn$^{15}$) were near optimal for succinimide formation.

**Properties of Asn$^{12}$ Substitutions**—The following mutants were made in wild type HPr: Asn$^{12}$ → Ala, Asn$^{12}$ → Ser, and Asn$^{12}$ → Thr. Each was expressed and purified, and kinetic parameters for enzyme I were determined. The results are presented in Table II.

**DISCUSSION**

The active site of HPr has two conserved residues, His$^{15}$ and Arg$^{17}$, and various investigations of the structure of HPr indicate that a residue with hydrogen bonding potential should be found at residue 12 in HPrs from several species (18, 19, 44, 45). Substitution of Asn$^{12}$ in *E. coli* HPr by either serine or threonine has little effect on the phosphorylation of HPr by enzyme I. Serine is found in *Bacillus subtilis* HPr and threonine in *Staphylococcus aureus* and *Streptococcus faecalis* HPrs. The removal of the hydrogen bonding potential by replacement with alanine results in only modest changes (Table II), very similar to that previously reported for Asn$^{12}$ → Asp HPr. The approximately 4-fold change in $k_{cat}/K_{m}$ will presumably affect the physiological efficiency of HPr, but the hydrogen bonding potential of residue 12 does not appear to be a major requirement for the mechanism of phosphoryl transfer. These modest changes in activity concur with the lack of direct evidence in NMR spectra for a hydrogen bond between His$^{15}$ and residue 12 (45–48, 50, 51).

In contrast to the flexibility of requirement at residue 12, it was expected that histidine would be an absolute requirement at residue 15. However, His$^{15}$ → Asp can be phosphorylated, and donate phosphate to at least IIA$^{36}$ but with much reduced efficiency. The aspartyl residue is a partial structural analogue of histidine as shown in Fig. 6; one of the carboxyl oxygen atoms is structurally equivalent to the N$^{2}$ atom in histidine. Phosphoryl transfer between P-histidines and acyl phosphates is well established. Acetate kinase, in which a γ-glutamyl phosphate is formed (52), interacts with enzyme I to form a N$^{2}$-P-histidine (53, 54). Reactions in chemotaxis involve transfers of phosphoryl groups between N$^{2}$-P-histidine in CheA (41) and aspartyl phosphate in CheY (49), which is an example of two component sensor systems. An interesting aspect of the aspartyl substitution of His$^{15}$ is that phosphorylation catalyzes formation of a cyclized compound. Cyclization reactions to form succinimides are established for both asparagine and aspartate, and the production of isoaspartyl from the hydrolysis of succinimides is established (20–23). Although isoaspartyl formation is favored, constraints in a protein structure might cause the formation of only aspartyl or only isoaspartyl. It has been proposed (20, 23) that a second form of cyclization can occur to yield an isoidime (Fig. 1),
His$^{15}$ → Asp HPr Cyclization

The accession numbers are: His$^{15}$ → Asp HPr, 1cm3, and the high pl species, 1cm2.

| TABLE I | Refinement parameters |
|------------------|-----------------------|
| His$^{15}$ → Asp HPr | High pl species |
| Unit cell | $a = 25.37 \text{ Å}$, $b = 45.34 \text{ Å}$, $c = 27.62 \text{ Å}$, $\beta = 104.0^\circ$ | $a = 25.92 \text{ Å}$, $b = 45.97 \text{ Å}$, $c = 27.22 \text{ Å}$, $\beta = 104.2^\circ$ |
| Space group | $P_{2_1}$ | $P_2_1$ |
| Resolution | 1.5 Å | 1.8 Å |
| Water molecules | 72 | 43 |
| Final R | 19.1% | 20.0% |
| Number of reflections | 7761 | 5670 |
| Completeness | 96.5% | 97.5% |

The complete lack of detection of significant amounts of isoaspartic acid and the ability to isolate a cyclic intermediate with 18 mass units less than His$^{15}$ → Asp HPr are difficult to reconcile with the formation of a succinimide. It is suggested that the structure formed is an isoisomide, which has not been found before in proteins.

Acknowledgments—The following are thanked for contributions to this work: George Wong for help with protein purifications; Katherine Dixon, who made the first observation of phosphorylated His$^{15}$ → Asp HPr phosphorylation during an undergraduate research project; Kim Napper and Joan Smallshaw produced E. coli strain ESK150 by gene replacement; James Talbot for the construction of E. coli strain ESK258; Jon Lowensen and Steve Clarke (UCLA) for the protein methylation assays; Sam Mackenzie (Plant Biotechnology Institute, National Research Council of Canada) for amino acid sequencing; Dr. M. Suzuki, The Photon Factory for help with synchrotron data collection; and Jeremy Lee for discussions on the formation of isoisomides.

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