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Effect of His192 Mutation on the Activity of Alginate Lyase A1-III from *Sphingomonas* Species A1

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Abstract The alginate lyase A1-III gene of *Sphingomonas* species A1 is composed of 1,077 nucleotides, encoding a protein (359 amino acids) with a molecular mass of 40,322 Da. Recombinant A1-III expressed in *Escherichia coli* exhibited the same full enzymatic activity as native A1-III. In order to identify the critical residue for activity, a site-directed mutation was introduced into the A1-III gene (H192A, His192→Ala). Recombinant A1-III (H192A) exhibited a significant decrease in enzyme activity (one-thirty thousandth of that of A1-III), without any conformational change, as detected by the CD spectra in the far UV region. Also, the chemical modification of wild-type A1-III with methyl 4-nitro benzene sulfonate resulted in a 40% decrease from the initial activity, whereas the same modification of A1-III (H192A) produced no change in the activity. The role of His192 on the catalytic process was also explored based on a model of A1-III docked with mannuronic acid into the active site.

Key words: Alginate lyase, mutation, site-directed mutagenesis, active site, chemical modification

Sphingomonas species A1 intracellularly produces three kinds of alginate lyase, A1-I, -II and -III. It would appear that alginate lyase A1-I can be autocatalytically processed to form A1-II and -III [5]. However, the substrate specificities of the latter two lyases differ; that is, A1-II preferentially acts on non-acetylated alginates produced by brown seaweed, whereas A1-III efficiently liquefies acetylated alginates produced by bacteria [10].

The three-dimensional structure of an A1-III crystal at a resolution of 1.78 Å has already determined [16] it to be abundant in helices with a deep tunnel-like cleft in a novel (α_6/α_5)-barrel structure, similar to the (α_6/α_6)-barrel found in glucoamylase and cellulase. This structure presents the possibility that alginate molecules may penetrate into the cleft to interact with the catalytic site of A1-III. Several residues important for the interaction with the substrate, including His 192, were identified in the active site of the enzyme.

It has been previously reported that the action of alginate lyase proceeds by the extraction of a hydrogen from a C-5 atom followed by the donation of a proton to the oxygen of the glycosidic bond to be cleaved, thereby resulting in the formation of a product with a double bond between C-4 and C-5 [2, 3, 8]. Although several authors assume that the histidine residue is a proton acceptor [3, 8], there is no direct evidence that the residue is involved in a catalytic step.

In order to understand the catalytic mechanism of A1-III, it is necessary to investigate the catalytic site of this enzyme. This paper describes the characterization of an A1-III mutant, A1-III (H192A), in which His 192 was replaced with Ala by site-directed mutagenesis.

Materials and Methods

Materials

In order to substitute an alanyl residue for a histidinyl residue at the 192th position, two oligonucleotides were synthesized by a DNA synthesizer. The synthetic oligonucleotide primers used for constructing A1-III (H192A) were as follows: primer 1 (sense): 5'-CG AGT TGC TGC AAC AAT GCC TCG TAT TGG CGC GG-3'; primer 2 (antisense): 5'-CC GCG CCA ATA CGA GGC ATT GTT GCA GCA ACT CG-3'. To overexpress A1-III (H192A), an expression vector (pET14b) and *E. coli* host strain [HMS174(DE3)pLysS] were purchased from Novagen, Inc. (Madison, WI, U.S.A.). The vector pET14b includes a gene coding for a His-tag sequence.

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Site-Directed Mutagenesis

To replace His 192 with Ala, site-directed mutagenesis was performed using plasmid pALY1-3 [15] as the template and two synthetic oligonucleotides, described above, as primers according to the manufacturer's directions in a Quick Change Site-Directed Mutagenesis kit (Stratagene, CA, U.S.A.). The resultant plasmid containing the mutation was designated as pALY1-3 (H192A).

Sequence and Manipulations of DNA

The nucleotide sequence of the A1-III (H192A) gene was determined by the dideoxy-chain termination method using an automated DNA sequencer model 377 (Applied Biosystems Division of Perkin-Elmer, Foster City, CA, U.S.A.) [13]. The subcloning, transformation, and gel electrophoresis were performed as described previously [12].

Construction of the Overexpression System of A1-III (H192A)

To subclone the A1-III (H192A) gene into the expression vector (pET14b), a PCR was performed by using pALY1-3 (H192A) as the template and two synthetic oligonucleotides as primers [17]. The A1-III (H192A) gene amplified by the PCR was digested with *Nde*I and *Bam*HI, and ligated with a *Nde*I- and *Bam*HI-digested expression vector (pET14b). The resultant plasmid was designated as pET14b-A1-III (H192A). The *E. coli* strain of HMS174(DE3)pLysS was transformed with pET14b-A1-III (H192A).

Purification of A1-III (H192A)

E. coli cells overproducing the A1-III (H192A) mutant were grown in 15 l of an LB medium [12] at 28°C for 5 h, and then IPTG was added to the cultures at a concentration of 1 mM, followed by a continuous culture at 16°C for 24 h. The cells were harvested by centrifugation at 7,000 ×g at 4°C for 10 min, washed in a 5 mM Tris-HCl buffer, at pH 7.2, and then resuspended in the same buffer. The cells were ultrasonically disrupted (Insonator, Kubota Model 201 M, Tokyo, Japan) at 9 kHz at 0°C for 40 min, then the clear solution (250 ml) obtained after centrifugation at 13,000 ×g at 4°C for 20 min was used as the cell-free extract. This extract (15 ml, 31.7 mg in 20 mM Tris-HCl, pH 7.2) was applied to a column of Ni^{+}-chelating Sepharose (1.6×10 cm), and the adsorbed proteins in the column were eluted with a 300 mM imidazole buffer after washing with a 20 mM imidazole buffer. The eluent was then applied to a SP-Sephadex column (1.6×10 cm) equilibrated with a 20 mM Tris-HCl buffer, pH 7.5. The enzyme was eluted with a linear gradient of NaCl (from 0 to 0.4 M) in a 20 mM Tris-HCl buffer, pH 7.5 (21 ml) and 1.5 ml-fractions were collected every 0.5 min. The active fractions, which were eluted with about 0.24 M NaCl, were dialyzed against a 50 mM Tris-HCl buffer, pH 7.5, containing 30% saturated ammonium sulfate. The dialysate was then applied to a Phenyl-Superose (hydrophobic) column (1.6×10 cm) equilibrated with 30% saturated ammonium sulfate in 50 mM Tris-HCl buffer, pH 7.5. The enzyme was eluted with a linear gradient of ammonium sulfate (from 30 to 0%) in 50 mM Tris-HCl buffer, pH 7.5 (34 ml), and 1.5 ml-fractions were collected every 0.5 min.

The active fractions, which were eluted with 17–15% saturated ammonium sulfate, were used as purified A1-III (H192A). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm the homogeneity of A1-III (H192A) [7].

Spectral Measurement

The absorption spectra were measured with a Hitachi U-2001 recording spectrophotometer at 25°C. The circular dichroism (CD) in the far ultraviolet region was measured using a JASCO J-720C spectropolarimeter at 25°C with a 0.2-mm-pathlength cell. The ionic strength of the buffer (20 mM Tris-HCl, pH 7.5) was adjusted to 0.08 by 0.2 M NaCl.

Alginate Lyase Assay

The A1-III (H192A) was assayed in a mixture (2.0 ml) containing 0.1% alginate, a 50 mM Tris-HCl (pH 7.0) buffer, and 0.5 μg/ml enzyme. The reaction was monitored at 25°C for 12 or 24 h, depending on the increase in absorbance at 235 nm in comparison with A1-III (wild-type) [14], because the activity exhibited by A1-III (H192A) was too low. One unit (U) of the enzyme activity was defined as the amount of enzyme required to increase the absorbance by 1.0 at 235 nm per min. The specific activity was expressed as ΔE_{235} per mg of protein. The protein content was determined by the method of Bradford [1] with bovine serum albumin as the standard, and also by measuring the absorbance at 280 nm using $E_{280,\ 1\%}$= 32.8 [5].

Chemical Modification of His Residues by MNBS

The chemical modification of the His residues in A1-III (wild-type) or A1-III (H192A) was carried out using methyl-4-nitrobenzene sulfonate (MNBS) at 25°C, as previously described by Marcus and Dekker [9]. The enzyme was incubated with 0.1 M MNBS, then the reaction was terminated by 1 M imidazole after an appropriate time interval. The enzyme was assayed at 25°C in a 1-ml reaction mixture containing 0.1% alginate and a 50 mM Tris-HCl buffer, pH 7.2, by monitoring the increase in the absorbance at 235 nm resulting from the double bonds formed in the reaction products.

Modeling of a Mannuronic Acid in the Active Site of A1-III

One mannuronic acid was manually located in the active site of A1-III based on an A1-III coordinate (PDB, 1AQZ)

using a program package of Turbo-Frodo (Bio-Graphics Co.). The position of the sugar was confirmed by the following criteria: (i) the direction of the sugar chain was parallel to the direction of the active cleft; (ii) The carboxyl group of the sugar interacted with the side-chain of Arg239; (iii) There was no steric interaction between the sugar and protein residues. There were two possible conformations of the sugar which had reversed direction sugar chains.

RESULTS AND DISCUSSION

Mutagenesis of Alginate Lyase A1-III

Based on the native A1-III structure [16], the amino acid residues involved in alginate degradation have already been clearly established. Accordingly, to elucidate the role of His 192 in the active site of A1-III, His 192 was substituted with Ala by site-directed mutagenesis. The mutation was confirmed by an analysis of the DNA sequence of the A1-III (H192A) gene. The overexpression system for A1-III (H192A) was constructed by inserting the A1-III (H192A) gene downstream of the His-Tag gene in pET14b. The inserted gene in the constructed plasmid [pET14b-A1-III (H192A)] was controlled by a T7 promoter and expressed in the form of a fusion protein with a His-tagged sequence (MGSSHHHHHHSSGLVPRGSH) in the N-terminal. The transformant *E. coli* strain [HMS174(DE3)pLysS] with pET14b-A1-III (H192A) produced a substantial amount of the mutant enzyme in soluble form.

Purification and Characterization of A1-III (H192A) Expressed in *E. coli* Cells

The transformant was grown at 16°C in an LB broth in the presence of 1 mM IPTG to induce gene expression. A1-III (H192A) was purified 13.2-fold from *E. coli* cells with an activity yield of 10.9% using Ni^{2+}-chelating Sepharose, SP-Sephadex, and Phenyl-Superose columns. The purified A1-III (H192A) with a 42,000 molecular mass was homogeneous according to SDS-PAGE (Fig. 1).

The results of the purification are summarized in Table 1.

The CD spectra of the wild-type and the H192A mutant are shown in Fig. 2. There was no significant difference found in the CD spectra between A1-III (H192A) and A1-III (wild-type), thus indicating that the substitution of H192 with Ala did not affect the secondary structure of the enzyme. Both spectra gave estimated secondary structural contents of 45.3% α-helices, 11.5% β-strands, and 15.7% turns using the method of Hennessey and Johnson [4]. These values are in good agreement with those previously determined by X-ray crystallography (44.3% α-helices, 0% β-strands, and 14.1% turns) [16].

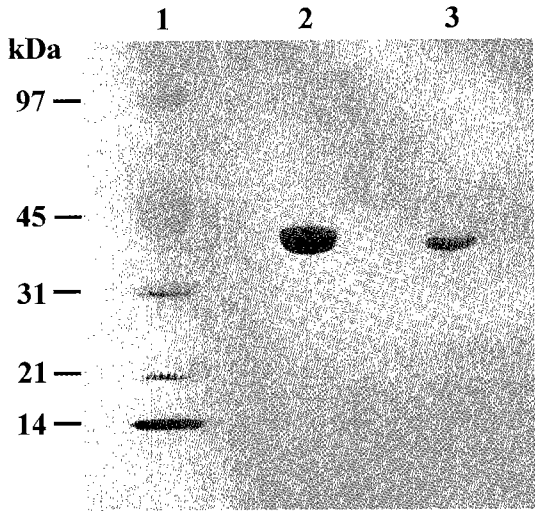

Fig. 1. Electrophoretic profile of A1-III (wild-type) and A1-III (H192A).
The purified A1-III and H192A mutant from *E. coli* cells were subjected to SDS-PAGE. Lane 1, molecular weight standards (from top): phosphorylase b (97,400); ovalbumin (45,000); carbonic anhydrase (31,000); trypsin inhibitor (21,500); and lysozyme (14,400); lane 2, A1-III (H192A); lane 3, A1-III (wild-type).

A1-III (H192A) activity was measured and the V_{max} and K_m values were compared with those of A1-III (wild-type), as shown in Table 2. The V_{max} of A1-III (H192A) was only one-thirty-thousandth of that of A1-III (wild-type). In contrast, the K_m value of the mutant was reduced to about one tenth of that of the wild-type enzyme. This result suggested that His 192 was an essential residue for the activity of A1-III and that the mutant had a stronger affinity to the substrate than the wild-type.

The optimal pH of A1-III (wild-type) and A1-III (H192A) was compared by examining the enzyme activities using different pH buffers (0.1 M Tris-HCl buffer, from pH 7.1 to pH 9.6, at 25°C). The optimal pH of A1-III (wild-type) was 8.2, while that of A1-III (H192A) was 8.0. These results suggest that the substitution of one amino acid, that is, Ala from His, had no influence on the optimum pH of A1-III (Fig. 3).

Table 1. Purification of the A1-III H192A mutant.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	4,608	0.5253	0.000114	100.0	1.0
Ni^{2+}-chelating Sepharose	500	0.4402	0.00088	83.8	7.7
SP-Sepharose	150	0.2137	0.00142	40.7	12.5
Phenyl-Superose	38	0.0572	0.00151	10.9	13.2

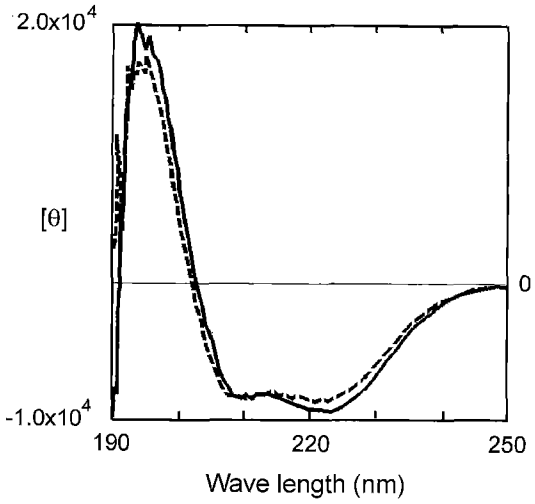

Fig. 2. CD spectral curves of A1-III (wild-type) and A1-III (H192A). The spectra were measured in a 20 mM Tris-HCl buffer at pH 7.5 and 20°C. The ionic strength was adjusted to 0.2. -----, A1-III (wild-type); ——, A1-III (H192A).

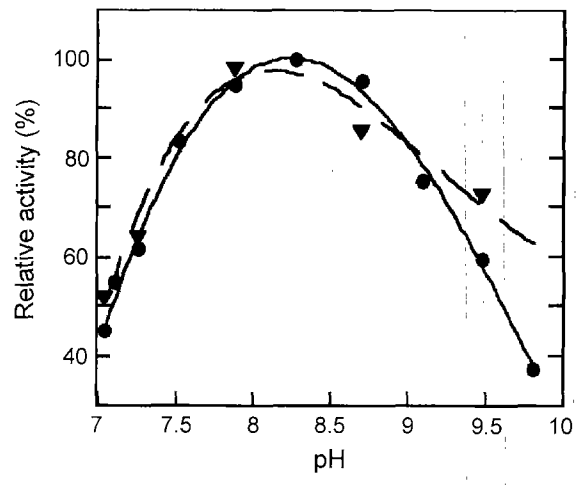

Fig. 3. Optimal pH of A1-III (wild-type) and A1-III(H192A). The enzyme activity was measured at 25°C for 1 min for A1-III and 20 h for A1-III (H192A). The activity of 1 ml of a reaction mixture containing 0.1% alginate solutions dissolved in 0.1 M NaCl, the pHs of which were adjusted to the desired values using HCl or NaOH, was determined by monitoring the increase in the absorbance at 235 nm resulting from the double bonds formed in the reaction products. ●, A1-III (wild-type); ▼, A1-III (H192A).

Chemical Modification by MNBS

The methylation of the His residues of A1-III (wild-type) by MNBS reduced the original activity by 40%, as shown in Fig. 4. This incomplete inactivation suggests an instability of MNBS during the incubation time. In contrast to A1-III (wild-type), there was little change in the remaining activity of A1-III (H192A) when using the reagent (Fig. 4). This result shows that His 192 in A1-III (wild-type) can be responsible for an inactivation of the enzyme by chemical modification, and suggests that His192 is an essential residue for the catalytic reaction of this enzyme. On the basis of the results of the remaining activity, CD spectrum, and chemical modification, it appears that, although His192 is a critical residue for degrading the substrate, it has no effect on the binding of the substrate to the enzyme.

Location and Possible Role of His192 in the Active Site of A1-III

As shown in Fig. 5a, His 192 is located in the seventh helix of A1-III and its side-chain is extended to the center of the active cleft [16]. This residue is also conserved in all mannuronate specific bacterial alginate lyases [16]. The replacement of His 192 with Ala 192 decreased the V_{max} value thirty-thousand-fold compared to that of the wild-type enzyme without significantly affecting the K_m value or secondary structure content. The result of the chemical modification of the histidine residues also supported the importance of His 192 in the activity of the enzyme.

In order to further explore the role of His192 in the active site of A1-III, a mannuronic acid model was located in the presumed active site of the enzyme [16]. There were

Table 2. Kinetic parameters of A1-III wild-type and H192A mutant enzymes.

	A1-III (Wild-type)	A1-III (H192A)
V_{max} (ΔE_{235}/min/mg protein)	72.7±3.07	0.00244±0.000078
K_m (%)	0.028±0.0062	0.0027±0.00052

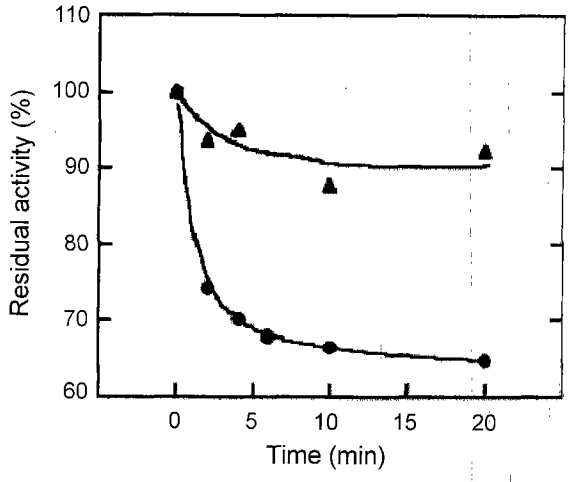

Fig. 4. Chemical modification of histidine residues with MNBS of A1-III (wild-type) and A1-III (H192A) at 25°C. The enzyme was incubated with 0.1 M MNBS, and the reaction was terminated by 1 M imidazole after an appropriate time interval. The enzyme was assayed at 25°C in 1 ml of a reaction mixture containing 0.1% alginate and a 50 mM Tris-HCl buffer, pH 7.2, and the activity was determined by monitoring the increase in the absorbance at 235 nm resulting from the double bonds formed in the reaction products. ●, A1-III (wild-type); ▼, A1-III (H192A).

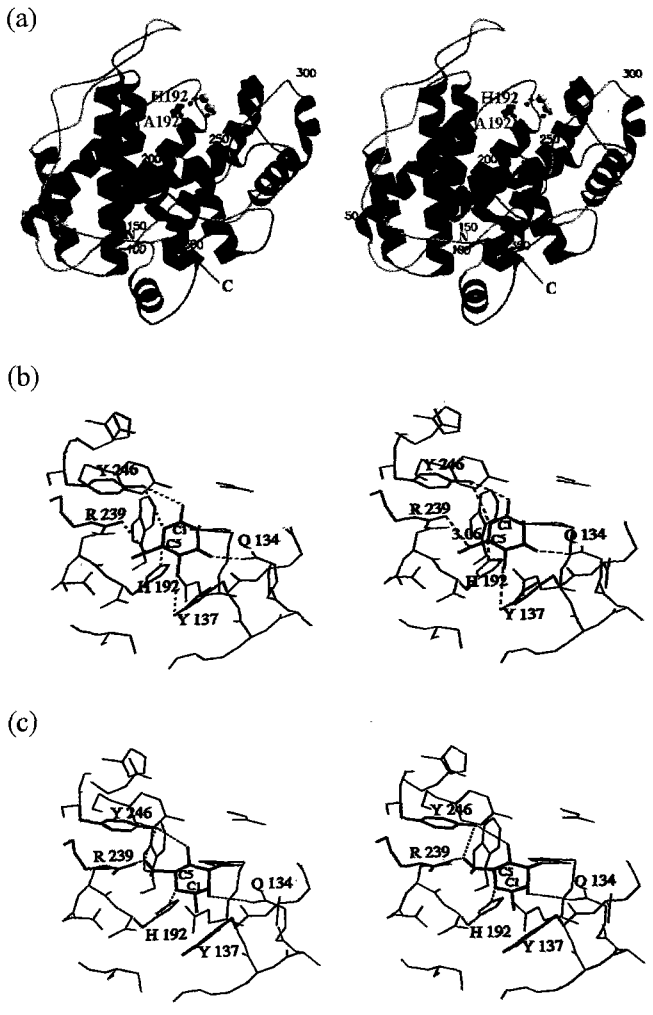

Fig. 5. The position of His 192 and two possible models of β-mannuronic acid bound in the active site of A1-III (stereo view). (a) The position of His 192 (red) and Ala 192 (green) are represented as ball-and-stick models in a ribbon diagram of A1-III. The S-S bridges are shown as yellow spheres. The figure was drawn by MOLSCRIPT [6]. (b) C-5 hydrogen located near the His 192 side-chain, (c) C-5 hydrogen located on the other side of the His 192 side-chain. Both models of mannuronic acid (red colored) have $^4C_1$-pyranosid forms yet reversed directions along the active site cleft. Tyr137, Arg239, and Tyr246 which interact with the mannuronic acid, are colored green. His 192 is colored orange. The possible hydrogen bonds are shown as dotted lines.

two possible orientations included in the docked model of the sugar residue (Fig. 5). Although the sugar models could make favorite interactions with protein residues, they also had an inverted direction sugar ring along the active cleft. In both models, the carboxyl groups of mannuronic acid interacted with the side-chain of Arg239. In the direction as shown in Fig. 5b, the mannuronic acid interacts with the side-chains of Gln 134, Tyr 137, His 192, Arg 239, and Tyr 246. The hydrogen of C-5 faced the side-chain of His192, thereby indicating that the side-chain of His192 could act as a proton acceptor. In contrast, in the reverse direction sugar as shown in Fig. 5c, the mannnuronic acid could also interact with the side-chains of His 192, Arg239, and Tyr246. In this case, however, the hydrogen of C-5 faced the opposite side of the His192 side-chain, and was near to the Tyr 246 side-chain. Here, the His 192 side-chain interacted with O-5 of the sugar ring.

The catalytic mechanism of polysaccharide lyases is believed to proceed by an initial extraction of a C-5 proton followed by the donation of hydrogen to the oxygen of the glycosidic linkage to be cleaved, resulting in the formation of a double bond between C-4 and C-5 atoms and the cleavage of the glycosidic bond [3]. It has also been suggested that a His residue is responsible for the extraction of hydogen from a C-5 atom [3, 8]. Based on a manually reoriented substrate model of a hyaluronate lyase/product complex determined by an X-ray crystallographic analysis, Ponnuraj *et al.* [11] proposed a mechanism involving three residues, His399, Tyr408, and Asn 349, in which His399 and Tyr408 act as a proton acceptor and proton donor, respectively. This seems to agree with the case of alginate lyaseA1-III in Fig. 5b, in which His192 and Tyr137 seem to act as a proton acceptor and proton donor, respectively. In the case of Fig. 5c, Tyr 246 seems to act as both a proton acceptor and donor. The latter case cannot be eliminated because His192 is still important in maintaining the productive binding of the substrate.

In order to clarify the fine enzymatic mechanism of A1-III, further studies are currently underway to determine the structure of the H192A mutant in a complex with substrates.

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