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# Photolabelling of mutant forms of the S1 subunit of pertussis toxin with NAD+

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The S1 subunit of pertussis toxin catalyses the hydrolysis of $NAD^+$ ($NAD^+$ glycohydrolysis) and the $NAD^+$-dependent ADP-ribosylation of guanine-nucleotide-binding proteins. Recently, the S1 subunit of pertussis toxin was shown to be photolabelled by using radiolabelled $NAD^+$ and u.v.; the primary labelled residue was Glu-129, thereby implicating this residue in the binding of $NAD^+$. Studies from various laboratories have shown that the *N*-terminal portion of the S1 subunit, which shows sequence similarity to cholera toxin and *Escherichia coli* heat-labile toxin, is important to the maintenance of both glycohydrolase and transferase activity. In the present study the photolabelling technique was applied to the analysis of a series of recombinant-derived S1 molecules that possessed deletions or substitutions near the *N*-terminus of the S1 molecule. The results revealed a positive correlation between the extent of photolabelling with $NAD^+$ and the magnitude of specific $NAD^+$ glycohydrolase activity exhibited by the mutants. Enzyme kinetic analyses of the *N*-terminal mutants also identified a mutant with substantially reduced activity, a depressed photolabelling efficiency and a markedly increased $K_m$ for $NAD^+$. The results support a direct role for the *N*-terminal region of the S1 subunit in the binding of $NAD^+$, thereby providing a rationale for the effect of mutations in this region on enzymic activity.

## INTRODUCTION

Pertussis toxin (PT) is a hexameric protein toxin produced by *Bordetella pertussis* that plays a central role in the pathogenesis of pertussis or whooping cough [1]. PT is composed of five different subunits, termed S1 ($M_r$ 28000), S2 ($M_r$ 23000), S3 ($M_r$ 22000), S4 ($M_r$ 11000), and S4 ($M_r$ 9000) [2]. Most of the pathobiological effects of PT appear to be a consequence of the ability of the S1 subunit to catalyse the $NAD^+$-dependent ADP-ribosylation of guanine-nucleotide-binding regulatory proteins of the adenylate cyclase complex in eukaryotic cells [3,4]. PT also appears to be an important component in acellular vaccines against pertussis [5–7].

The operon encoding the PT subunits has been cloned and sequenced [8,9], and the individual subunit genes have been expressed in *Escherichia coli* [10–13]. The isolation and expression of the S1 subunit gene has permitted studies designed to identify those regions of the S1 subunit that are important to enzymic activity and that contribute to the formation of potentially important antigenic determinants. Such information would facilitate the production of a non-toxic form of PT that retains protective immunogenicity for use as a potential vaccine constituent. We and others have recently identified a small region of the S1 subunit located near the *N*-terminus (residues 8–14 in the mature protein) that is required for the expression of detectable enzyme activity [14–17]. This region also exhibits a high degree of amino acid sequence similarity (identity in seven of eight residues) to sequences found in the *N*-terminal regions of two other bacterial ADP-ribosylating toxins, cholera toxin (CT) and *E. coli* heat-labile toxin I (HLT) [8,9,18]. Analyses of this region using single-amino-acid substitutions showed that certain replacements could suppress both $NAD^+$ glycohydrolase and ADP-ribosyltransferase activity without appreciably affecting the reactivity of the S1 subunit with a protective anti-S1 monoclonal antibody [15]. Importantly, this antibody has been shown to be directed to a conformational epitope [19], thus suggesting that the single-amino-acid substitutions did not affect enzyme activity through gross conformational changes. Although these studies suggested that the region delineated by amino acids 8–14 is essential to expression of the enzymic activities of the S1 subunit, they did not address the specific function that this region has in the enzymic mechanism.

To help define the specific role of the *N*-terminal similarity region in the enzymic mechanism, we undertook experiments which were designed to examine the interaction of S1 with $NAD^+$ by using photoaffinity-labelling techniques. The experiments were initially prompted by the studies of Collier and co-workers that showed that other ADP-ribosylating toxins, such as diphtheria toxin (DT) and *Pseudomonas aeruginosa* exotoxin A (ETA), could be specifically labelled with radioactive $NAD^+$ and u.v. at glutamic acid residues which appeared to be in the vicinity of the active site [20–22]. The labelling did not require the addition of a photoactivatable group to the $NAD^+$, and was postulated to occur through the photoexcitation of one or both of the two nitrogenous bases in the $NAD^+$ molecule. The studies also identified the labelled amino acids in DT and ETA as Glu-148 and Glu-553 respectively [20,21]. Substitution of these glutamic acid residues resulted in abrogation of enzyme activity and strongly supported the proposition that they represented active-site residues that were directly involved in $NAD^+$ binding [23,24]. Recently, both Cockle [25] and Barbieri *et al.* [26] showed that the purified S1 subunit of PT could also be photolabelled by $NAD^+$ using u.v. irradiation and that the labelling was accompanied by a loss of enzyme activity. The label was largely found at a residue corresponding to glutamic acid-129 and suggested that this residue was at or near the $NAD^+$-binding site. In the present study we investigated the potential role of the *N*-terminal similarity region in $NAD^+$ binding and catalysis by using both u.v.-induced photolabelling by $NAD^+$ and enzyme-kinetic analyses of recombinant forms of the S1 subunit that contain mutations in this region.

Abbreviations used: PT, pertussis toxin; CT, cholera toxin; HLT, *Escherichia coli* heat-labile toxin; DT, diphtheria toxin; ETA, *Pseudomonas* exotoxin A; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; DTT, dithiothreitol.

## MATERIALS AND METHODS

### Materials

PT was purified from *B. pertussis* strain 3779 using the method of Sekura *et al.* [27]. The S1 subunit was purified from PT by the method of Burns *et al.* [28]. [*carbonyl*-$^{14}$C]NAD$^+$ (44 mCi/mmol) and [U-*adenine*-$^{14}$C]NAD$^+$ (285 mCi/mmol) were obtained from Amersham International. All other reagents were obtained from Sigma Chemical Co.

### Preparation of recombinant S1 subunits

The construction of expression vectors containing gene sequences encoding mutant forms of S1 subunit with *N*-terminal deletions and mutations within the region spanning amino acids 8–15 has been described previously [11,15,16]. The recombinant proteins were recovered from *E. coli* cells as inclusion bodies and were extracted with 8 M-urea and dialysed exhaustively against 50 mM-Tris/HCl, pH 8.0 [15]. The amount of recombinant S1 protein in each preparation was determined by scanning laser densitometry of proteins separated by SDS/PAGE and stained with Coomassie Brilliant Blue R-250. The preparation and partial purification of a recombinant S1 analogue lacking the 47 *C*-terminal amino acids (rS1d) have been described elsewhere [12,29]. Assays of total protein were performed by using the bicinchoninic acid method and a reagent kit supplied by Pierce Chemical Co. The recombinant S1 molecules constituted 20–40% of the total protein in the preparations.

### NAD$^+$ glycohydrolase assay

NAD$^+$ glycohydrolase activity was measured as the ability to catalyse the release of nicotinamide from [*carbonyl*-$^{14}$C]NAD$^+$ as described previously [12,16]. Briefly, reaction mixtures (0.05 ml) containing 100 $\mu$M-ATP, 20 mM-dithiothreitol, 1.0% (w/v) CHAPS, 30 $\mu$M-[*carbonyl*-$^{14}$C]NAD$^+$, 50 mM-Tris/HCl, pH 8.0, and 0.5–1.0 $\mu$g of recombinant S1 were incubated for 1–3 h at 30 °C. The amount of labelled nicotinamide released was determined using Dowex AG1 X-2 ion-exchange resin [20]. For determination of $K_m$ and $k_{cat.}$ values, the wild-type and mutant subunits were assayed at final concentrations ranging from 10 to 40 $\mu$g/ml using final NAD$^+$ concentrations of 5, 7.5, 10, 15, 20, 25, 30, 50, 75, and 100 $\mu$M. The data were analysed by double-reciprocal plots and the $K_m$ and $V_{max.}$ values were obtained by use of least-squares linear-regression analysis.

### Photolabelling reaction

The procedure used was similar to that described by Carroll & Collier [20]. Reaction mixtures containing 1.0% CHAPS, 20 mM-DTT, 50–100 $\mu$M [*carbonyl*-$^{14}$C]NAD$^+$ or [U-*adenine*-$^{14}$C]NAD$^+$, 50 mM-Tris/HCl, pH 8.0, and 1–2 $\mu$M-PT (or 1–2 $\mu$M-S1 subunit) were chilled on ice and placed as 0.1 ml drops on inverted lids of microwell plates (Linbro; no. 76-032-05) that had been pre-chilled and kept cold in an ice/water bath. The droplets were then irradiated at a distance of 5 cm with a 15 W germicidal lamp (GE G15T8) for various times. Control reactions contained buffer instead of enzyme or consisted of mixtures that were incubated for an equivalent period of time in the dark. After irradiation, aliquots (0.05 ml) of the reaction mixtures were mixed with 0.01 ml of 6 M-guanidinium chloride containing 5 mg of ovalbumin/ml and applied to filter-paper squares impregnated with 10% (w/v) trichloroacetic acid and 0.1% (w/v) $H_3PO_4$. The filters were washed, air-dried, and the amount of radioactivity on each filter was determined by liquid-scintillation spectrometry. For determination of the stoichiometry of incorporation into the recombinant proteins the specific radioactivity of the labelled NAD$^+$ as provided by the manufacturer was employed, and a theoretical $M_r$ of 26005 was used for the recombinant or wild-type S1 subunit. The data (c.p.m.) obtained were corrected for counting efficiency before calculation. For some experiments, aliquots of the reaction mixtures were analysed by SDS/PAGE and fluorography after dilution with an equal amount of double-strength electrophoresis sample buffer [30] and heating to 95 °C for 5 min.

## RESULTS AND DISCUSSION

When whole PT was incubated with radioactive NAD$^+$ labelled in the carbonyl position and exposed to u.v. irradiation, the S1 subunit of the toxin was found to contain covalently incorporated label (Fig. 1). None of the other subunits (S2, S3, S4, or S5), or the carrier protein (ovalbumin; $M_r$ 45000) that was included in the reaction mixture to prevent non-specific adsorption, was labelled. Labelling of the holotoxin was found to proceed to a level of approx. 0.3–0.4 mol of ligand/mol of toxin after 1 h, and no significant incorporation (less than 1% of control) was observed when the reaction mixtures were incubated in the dark (results not shown). The photolabelling reaction was decreased to near-background levels (i.e. no u.v.) when a 100-fold molar excess of unlabelled NAD$^+$ was included in the reaction mixture; however, the reaction was not inhibited by a similar molar excess of unlabelled nicotinamide. Collectively these findings indicated that, under the conditions employed, the photolabelling was specific for the S1 subunit and for the site of NAD$^+$ binding.

Photolabelling of purified S1 subunit exhibited apparent saturation and proceeded to a level of approx. 0.6 mol of

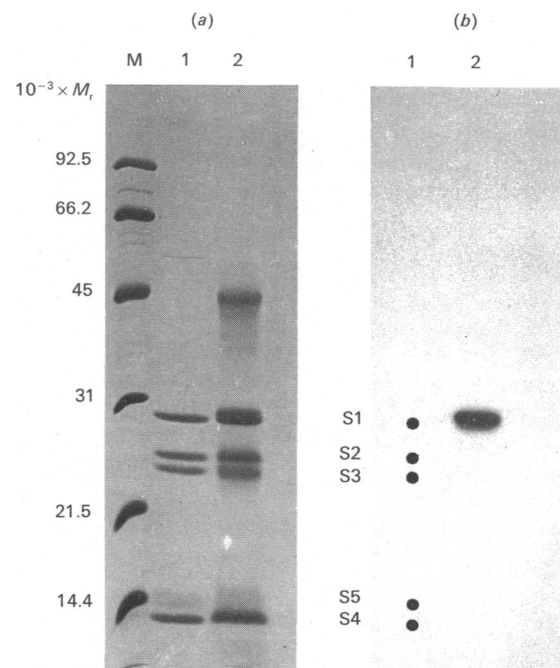


**Fig. 1. Photolabelling of the S1 subunit of PT with NAD$^+$**

PT (3.7 $\mu$M) was photolabelled with [*carbonyl*-$^{14}$C]NAD$^+$ (50 $\mu$M) for 1 h as described in the Materials and methods section. The reaction mixture also contained ovalbumin (1 mg/ml) to prevent non-specific adsorption. The reaction products were analysed by SDS/PAGE in a 12% gel. (*a*) Gel stained with Coomassie Brilliant Blue R-250. The positions of the various subunits of PT are indicated. Lane 1, untreated PT; lane 2, PT photolabelled with NAD$^+$. The stained band in lane 2 at a position corresponding to $M_r$ 45000 represents the ovalbumin added to the reaction mixture. Lane M contained $M_r$ markers. (*b*) Fluorogram of the gel shown in panel (*a*). The relative positions of the stained untreated subunits are indicated in lane 1.

ligand/mol of S1 subunit (Fig. 2). The maximum amount of incorporation observed after more prolonged labelling (greater than 2 h) was approx. 0.7 mol of ligand/mol of S1 subunit; failure to achieve stoichiometric levels of incorporation may reflect the sensitivity of either the S1 subunit or $NAD^+$, or both, to destruction by u.v. light under the conditions employed. Fig. 2 also shows that label was not effectively transferred to the S1 subunit from $NAD^+$ containing $^{14}C$ in the adenine moiety, indicating that only the nicotinamide moiety of $NAD^+$ is covalently linked during the reaction. These results using the purified S1 subunit are similar to those reported by Cockle [25].

In previous work, we described a series of mutant forms of the S1 subunit genes that were expressed at high levels in *E. coli*

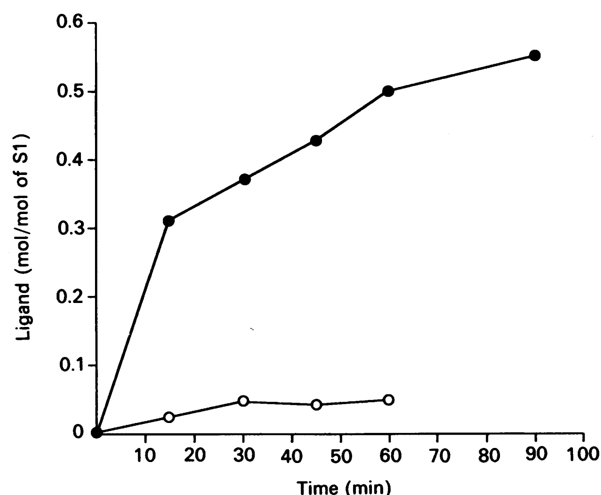


**Fig. 2. Photolabelling of purified S1 with [*carbonyl*-$^{14}$C]$NAD^+$ and [U-*adenine*-$^{14}$C]$NAD^+$**

Purified S1 (1.7 $\mu$M) was photolabelled using either [*carbonyl*-$^{14}$C]$NAD^+$ (50 $\mu$M) (●) or [U-*adenine*-$^{14}$C]$NAD^+$ (50 $\mu$M) (○). At the times indicated, samples were removed from each reaction mixture and the amount of trichloroacetic acid-precipitable radioactivity was determined. The values represent the means of duplicate determinations from which the background values were subtracted. Background incorporation was determined from equivalent reaction mixtures incubated in the dark.

[15,16]. These constructs were designed to investigate the potential role of a small *N*-terminal region of the S1 subunit in the expression of enzyme activity. This region, which we previously termed the 'homology box' [16], spans residues 8–15 in the mature polypeptide and bears considerable sequence similarity (identity in seven of eight residues) to regions located near the *N*-termini of CT and HLT. We found that those molecules truncated beyond amino acid-15, as well as a mutant in which arginine-9 was changed to lysine, lacked detectable $NAD^+$ glycohydrolase activity [15,16].

To examine further the role of the 'homology box' in enzymic activities, we assayed the enzyme kinetic parameters of mutant S1 subunits and their ability to be photolabelled with $NAD^+$. When the series of *N*-terminal deletion and substitufion mutants were evaluated in parallel for photolabelling and $NAD^+$ glycohydrolase activity, a general correspondence between the amount of labelling observed after 1 h and relative $NAD^+$ glycohydrolase activity was observed (Table 1). It should be noted that the recombinant WT gene encodes an S1 molecule that lacks any vector-derived fusion peptides, but lacks the first two aspartic acid residues of authentic S1 [16] and that all of the mutant subunits, except for rS1d, were derived from this construct. In each case where $NAD^+$ glycohydrolase activity was reduced below reliably detectable limits (less than 5% of control), the extent of photolabelling was uniformly decreased to less than 10% of that exhibited by the wild-type subunit. Analysis of the reaction products by SDS/PAGE and fluorography revealed that the labelling was confined to the recombinant S1 molecules and was not associated with other contaminating *E. coli* proteins (results not shown).

The deletion mutant lacking the first six amino acids of the authentic S1 subunit (del6) was photolabelled with efficiency similar to that of the wild-type enzyme, despite a reduction in specific $NAD^+$ glycohydrolase activity by a factor of approx. 3. Kinetic measurements using this mutant revealed a 4-fold decrease in the value of the $K_m$ for $NAD^+$ (Table 1); however, the value for $k_{cat.}$ was also decreased by 6-fold. The simultaneous decreases in $K_m$ and $k_{cat.}$ could account for the disparity between the specific enzyme activity and the degree of labelling, and suggests that the removal of the six amino acids immediately preceding the 'homology box' may have the effect of relieving steric or other constraints on $NAD^+$ binding and at the same time decreasing the catalytic efficiency. Replacement of serine-12

**Table 1. Photolabelling and $NAD^+$ glycohydrolase kinetic constants for S1 *N*-terminal mutants**

Photolabelling and measurement of $NAD^+$ glycohydrolase activity were determined as described in the Materials and methods section. Photolabelling of the S1 analogues with $NAD^+$ was determined after 1 h and the values given represent the means of triplicate determinations. $NAD^+$ glycohydrolase activity was determined using 1 $\mu$g of each S1 analogue. The values for $K_m$ and $k_{cat.}$ were derived from at least two experiments. The values for $k_{cat.}$ were determined from the $V_{max.}$ intersects of double-reciprocal plots. Abbreviation: ND, not determined.

| S1 analogue | Photolabelling [mol of $NAD^+$/mol of S1 (% of control ± S.D.)] | $NAD^+$ glycohydrolase [pmol of nicotinamide released/min per $\mu$g (% of control ± S.D.)] | $K_{mNAD}$ [$\mu$M (relative $K_m$)] | $k_{cat.}$ [$min^{-1}$ (relative $k_{cat.}$)] |
|---|---|---|---|---|
| WT | 0.33 (100) | 4.02 (100) | 21.6 (1.00) | 0.12 (1.00) |
| del6 | 0.29 (87.5±5.0) | 1.42 (35.3±2.3) | 5.5 (0.25) | 0.02 (0.16) |
| del14 | 0.01 (2.1±1.6) | 0.15 (3.8±0.8) | ND | ND |
| del16 | 0.01 (4.5±2.5) | 0.06 (1.6±1.5) | ND | ND |
| Y8F | 0.26 (78.8±1.7) | 5.3 (132.1±7.4) | ND | ND |
| R9K | 0.01 (3.6±1.3) | 0.09 (2.2±0.4) | ND | ND |
| D11E | 0.05 (16.1±1.9) | 0.36 (9.1±2.0) | 359.5 (16.7) | 0.09 (0.75) |
| S12G | 0.15 (44.3±3.0) | 1.9 (47.2±3.1) | 12.4 (0.57) | 0.01 (0.08) |
| R13K | 0.03 (9.4±2.9) | 0.24 (6.1±1.0) | ND | ND |
| R9N/S12G | 0.01 (3.9±2.7) | 0.09 (2.2±0.6) | ND | ND |

with glycine (S12G) resulted in a decrease in specific $NAD^+$ glycohydrolase activity of approx. 50% relative to that of wild-type S1 and a similar decrease in photolabelling (Table 1). The $K_m$ for $NAD^+$ of S12G was reduced by a factor of approx. 2, and the $k_{cat.}$ value associated with this construct was reduced by a factor of approx. 10. In contrast, when aspartic acid-11 was replaced with glutamic acid (D11E) the specific $NAD^+$ glycohydrolase activity and photolabelling were co-ordinately reduced to levels of approx. 9 and 16% respectively, and the $K_m$ for $NAD^+$ was increased by a factor of approx. 17. The $k_{cat.}$ value of D11E was similar to that of the wild-type construct.

We have also found that a separately derived mutant form of S1 (rS1d) that lacks the *C*-terminal 47 amino acids can be photolabelled in partially purified form (80%) with $NAD^+$ with an efficiency similar to that of authentic S1 (0.43 mol of ligand/mol of rS1d after 40 min). A similar finding has been made by Barbieri *et al.* [26], employing a mutant S1 that lacks the *C*-terminal 54 amino acids. The mutant rS1d exhibits a specific $NAD^+$ glycohydrolase activity that is almost identical with that of authentic S1 and has a similar $K_m$ for $NAD^+$, but has significantly reduced (by a factor of 15–20) ADP-ribosyltransferase activity [29]. These results suggest that photolabelling is related to the ability of the S1 subunit to interact with $NAD^+$ and is not dependent upon the presence of a catalytically optimal interaction with the acceptor substrate.

The results of the experiments using the *N*-terminal mutants indicate that perturbations within the 'homology box', especially in proximity to Arg-9, have significant effects on the binding of $NAD^+$, as judged by photolabelling and alterations of the Michaelis constant. Although nominal effects on catalysis were observed in the case of mutants del6 and S12G, the dramatic increase in $K_m$ exhibited by D11E, coupled with its diminished photolabelling capacity, suggests that this region is directly involved in the interaction with $NAD^+$. In the case of mutants del6 and S12G, the changes might impart small structural deviations that do not permit the alignment of the nicotinamide moiety of $NAD^+$ with Glu-129 in a fashion that is favourable to photoactivatable cross-linking.

Studies using deletions and site-directed mutagenesis have implicated several regions of the S1 subunit of PT in the enzymic mechanism of ADP-ribosylation. Several studies have now shown that deletion or alteration of the *N*-terminal region that shows identity with that of CT and HLT can significantly diminish the ADP-ribosyltransferase activity of the S1 subunit [14–17]. Chemical modification or mutagenic replacement of Trp-26, the only tryptophan residue in the catalytic domain of the S1 subunit, also results in drastic reductions in enzyme activity [14,31]. Deletion of Glu-129 or substitution of Glu-129 in the S1 subunit with glycine or aspartic acid results in loss of detectable enzyme activity and has led to the proposal that this residue represents the functional equivalent of Glu-148 of DT and Glu-553 of ETA [17,31]. A specific role for Glu-129 is also supported by the results of Cockle [25], who initially showed that this residue was the primary site of photolabelling with radioactive $NAD^+$, and by Barbieri *et al.* [26], who demonstrated that substitution of this residue with either glycine or aspartic acid abolished the photolabelling of the S1 subunit. We favour the interpretation that residues within the *N*-terminal region of sequence similarity in S1 are also involved directly in $NAD^+$ binding. On the basis of our previous observation of the profound effect exerted by the single substitution mutant R9K [15] on both glycohydrolase and transferase activity, we propose that arginine-9 might also be directly involved in $NAD^+$ binding. This could occur, for example, through ion-pairing between the guanidino group of arginine and the pyrophosphate group of $NAD^+$. Other recent findings also support an important role for this residue in the enzymic mechanism. Pizza *et al.* [32] have recently described mutant versions of intact PT that incorporate single-amino-acid substitutions at several positions in the S1 subunit. The substitutions included lysine for arginine-9, leucine for arginine-13 and glycine for glutamic acid-129. Among these mutations, the arginine-9-to-lysine substitution yielded a specific ADP-ribosyltransferase activity that was less than either of the two other mutants by a factor of 5. This finding suggests that arginine-9 is equally or more important than glutamic acid-129. It is also noteworthy that we have determined that substitution of arginine-9 with a variety of amino acids results in similar decreases in enzymic activity, whereas substitution of arginine-58, which is located in a second region of sequence similarity, or tryptophan-26, results in a significantly lesser decrease [33; Y. Lobet & W. Cieplak, Jr., unpublished work].

The results described herein provide a basis for the mechanistic interpretation of the effects associated with mutagenic alterations in the *N*-terminal region on the enzymic activities of the S1 subunit of PT, and support the proposal that mutagenic alteration of this region will be useful in the construction of a non-toxic, but immunogenic, recombinant holotoxin, like those described recently by Pizza *et al.* [32] for vaccine use.

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