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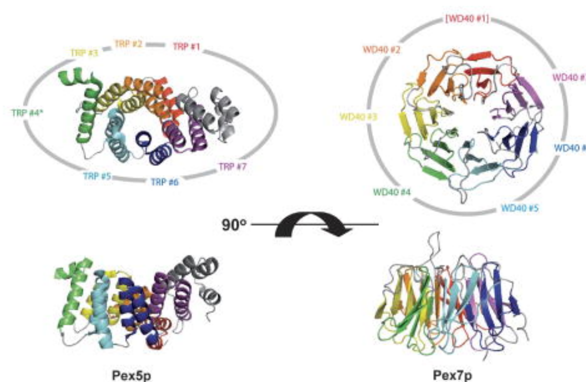

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Intradomain LexA rotation is a prerequisite for DNA binding specificity

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Abstract In the absence of DNA damage the LexA protein represses the bacterial SOS system. We performed molecular dynamic simulations of two LexA dimers bound to operators. Our model predicted that rotation of the LexA DNA binding domain, with respect to the dimerised C-terminal domain, is required for selective DNA binding. To confirm the model, double and quadruple cysteine LexA mutants were engineered. Electrophoretic mobility-shift assay and surface plasmon resonance showed that disulfide bond formation between the introduced cysteine residues precluded LexA specific DNA binding due to blocked domain reorientation. Our model could provide the basis for novel drug design.
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Keywords: LexA repressor; DNA binding; Domain rotation

1. Introduction

The transcriptional repressor LexA is a key regulator of the SOS system of *Enterobacteriaceae*. It binds as a dimer to operator sequences of approximately 40 genes involved in DNA repair and survival and sterically occludes RNA polymerase [1]. In addition to the SOS genes, LexA regulates synthesis of protein bacteriocins, including colicins produced by *E. coli* [2], as well as the dissemination, synthesis and secretion of virulence factors of some well characterized pathogens [3,4].

The LexA repressor has two structurally distinct domains, the N-terminal DNA binding domain (NTD) and the C-terminal dimerization domain (CTD) [5–7]. The two domains are separated by a highly hydrophilic and solvent exposed hinge region composed of residues Gln-70 to Glu-74 [7]. The NTD contains three α helices followed by two antiparallel β strands extending from Met-1 to Leu-69 [8]. The CTD exhibits a β-stranded structure, harboring a serine-protease cleavage-site region and the catalytic core [7]. LexA monomers interact entirely by the CTD [7,9]. The C-domain dimerization observed in the solved crystal structures of several mutant LexA proteins persists also in the operator-bound form [10]. Four mutant crystal structures revealed two distinct conformations of the LexA cleavage site region: (i) cleavage compatible and (ii) non-cleavable conformation [7].

LexA interacts with specific DNA sequences via a variant of the classical helix-turn-helix motif [8]. Helix III contains residues responsible for specific recognition of DNA [11]. Based on a number of studies employing biochemical and biophysical methods, hydrogen bonds and hydrophobic interactions between the LexA DNA binding domain and bases of the DNA sequence were previously determined by docking the LexA NMR solution structure onto DNA [12].

The goal of our study was to resolve whether a conformational change is required for LexA to discern specific DNA sequences. In the present study, an in silico model of the interaction of two LexA dimers with two overlapping operators of the colicin K activity gene, *cka*, was constructed using the Chemistry at HARvard Macromolecular Mechanics (CHARMM) program [13]. The model showed that re-orientation of the DNA binding domain with respect to the C-terminal dimerization domain enables specific LexA-DNA recognition. To confirm our model, we engineered double and quadruple cysteine mutants and employing electrophoretic mobility-shift assay (EMSA) and surface plasmon resonance (SPR) experiments, we were able to distinguish between an unbound and operator-bound LexA conformation.

2. Materials and methods

2.1. Computer modeling of the LexA dimer in solution and in the operator-docked form

All calculations were carried out on the GNU/Linux-based clusters of personal computers CROW at the National Institute of Chemistry in Ljubljana [14] and performed using the CHARMM biomolecular simulation program [13]. Structural and dynamical aspects of the systems were visualized via VMD software [15].

The LexA monomer model in the operator-docked form was built encompassing (i) LexA N-domain residues A1–A76 of the modeled LexA dimer-*recA* operator complex pdb ID: 1MVD [10]; (ii) C-terminal domain residues A77–A198 of the crystal structure pdb ID: 1JHH [7] with modified Ala119 to Ser as in the wild-type (WT); (iii) residues A199–A202 were added as in the 1MVD modeled structure. Subsequently, the complete structure of the dimer in LexA docked form was modeled according to 1JHH CTD interactions.

The model of the LexA dimer free in solution was modeled as described above except the model obtained NTD and hinge residues (2–76) from the pdb ID: 1JHH.

Missing hydrogen atoms were added with the HBUILD tool from CHARMM [13]. The ABNR energy minimization was used to optimize the LexA dimer coordinates.

2.2. Molecular dynamic DNA binding simulations

Straight B-DNA of the *cka* operator site [16] was built by the Model.it Server [17]. Two LexA dimers in the DNA docked form were positioned ~15 Å above the target sites. To assemble the two LexA dimers bound to the *cka* DNA, distance restrains using NOE module of 1.5–2.5 Å and 3.5–5.0 Å, respectively for the favorable hydrogen bonds and the hydrophobic interactions were performed [12]. Trajectories of the two LexA dimers bound to the *cka* operator were generated in abundance of constrains at 22 and 37 °C and covered 1.3 ns of

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molecular dynamics. The CHARMM potential energy function was taken in the form as described [13]. For the implicit solvent, we chose GBSW (version c30a1) [18]. The all-atom parameter set PARAM ALL27 library for proteins and nucleic acids was used [19,20]. From the simulation the structural model of a LexA bound form was obtained.

2.3. Construction of expression plasmids, protein expression and purification

The *lexA* gene was cloned into the pT7-7 expression vector [21] to construct plasmid pMB1. Plasmid variants of the LexA repressor (Table 1) were constructed according to the QuickChange® Site-directed Mutagenesis Kit manual (Stratagene). Construction details are presented in Supplementary data S1. Large-scale expression and isolation of proteins by Ni-NTA chromatography was performed as previously described [22]. Protein purification is presented in Supplementary data S2.

2.4. Oligomeric state of LexA and LexA-C13-C91, fluorescence measurements and circular dichroism (CD) spectroscopy

These experiments were performed as described in Supplementary data S3, S4 and S5.

2.5. Agarose gel mobility shift assays

The mobility shift assays were performed essentially as previously described [23]. Primers K2 and K6 were used to amplify the 270 bp fragment harboring the two *cka* LexA boxes from plasmid pKCT1 [24]. Details are provided in Supplementary data S6.

2.6. Surface plasmon resonance (SPR)

SPR measurements were performed on a Biacore X (Biacore, Sweden) apparatus at 25 °C. Approximately 30 µg of the amplified DNA fragment with the two *cka* LexA boxes described above was digested by XbaI restriction endonuclease. Biotin-11-dUTP (20 µM) and dATP, dCTP, dGTP (200 µM, Fermentas) were added enzymatically to the 3′ terminus using 5 U of Klenow fragment (Fermentas) in 50 mM Tris–HCl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM DTT reaction buffer. Reaction volume was 100 µl. The biotinylated DNA was precipitated with sodium acetate (0.3 M, pH 7.0) and ethanol and subsequently gel-purified (QIAquick kit, Quiagen). The streptavidin (SA) sensor chip surface was washed three times in 50 mM NaOH, 0.5 M NaCl and 0.15 M SDS. 10 ng/ml of the biotinylated DNA fragment in PBS buffer (0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.2) was passed for 15 min at 2 µl/min across the flow cell 1 to immobilize 120 response units (RU). The SA chip surface was washed as stated above to remove non-specifically adsorbed DNA. The interaction between LexA or LexA-C13-C91 and the chip-immobilized DNA was studied by injecting solutions of the desired concentration of proteins in oxidised and reduced form in 20 mM NaH_2PO_4, 200 mM NaCl, pH 7.4 at 40 µl/min for 1 min. Dissociation was followed for 5 min. The sensor chip was regenerated by injection of 0.15 M SDS (8 s at 40 µl/min) in between consecutive injections of two FPLC buffer injections (1 min at 40 µl/min).

2.7. Complementation of temperature sensitive lexA 41 mutant by cysteine lexA mutants

E. coli strain DM936 [25] was transformed with plasmid pMB1, pIVB, pIVB13/91, pIVB20/87 to complement the temperature sensitive *lexA*41 mutation or as a control DM936 with the pT7-7 vector without *lexA*. Transformants were grown in LB medium containing ampicillin (100 µg/ml) at 28.0 °C and at 42.5 °C until they reached the stationary phase when the cells were examined by bright field microscopy (Eclipse TE300 Nikon microscope) and cell counts were determined.

3. Results and discussion

3.1. Structural model of the two LexA dimers interacting with the cka operators

A model of the LexA dimer free in solution was constructed on the basis of the previously published LexA crystal structure of pdb ID: 1JHH [7], and as described in Section 2. For specific interaction of the LexA NTD with major groove residues [12], we observed that a change in the relative orientation between the CTD and NTD must occur with respect to the dimerised CTD [7,9]. A previous study also indicated that a conformational change could be necessary to dock the LexA dimer to the *recA* operator model [10]. Therefore, LexA in the docked form was modeled and used in molecular dynamic DNA binding simulations.

Using the CHARMM program [13] 1.3 ns of molecular dynamics of the two LexA dimers interacting with the two *cka* operators were simulated (Fig. 1A). The operators overlap by one base pair [16]. The relative position between the two LexA dimers interacting with the *cka* operators shows the net angle of approximately 180° about axis passing through the DNA backbone which is in agreement with the proposed interaction between LexA dimers binding to the colicin A activity gene *caa* operator DNA [26]. As predicted previously [12], our model also confirms that the LexA DNA binding domains share the central overlapping bases of the two operators in depletion of sterical clashes and add complex additional protein–protein contacts. Our model revealed that the centrally bound LexA monomers adopt the same binding geometry as the two external monomers.

We observed that LexA induced DNA bending and the presence of an intrinsic bend of DNA centered on the operator region.

When compared to the crystal structure of a previously described LexA mutant [7], predictably a rotation of the NTD by approximately 180° relative to the CTD was observed in the operator bound form (Fig. 1B). Both DNA binding domains most probably exhibit counter clockwise rotation. At the end of the simulation, the structures of the NTD and CTD remained essentially unaltered. Both domains could be easily superimposed with a backbone root mean square deviation of 2.3 for residues 1–65 of NTD and 2.5 for residues 75–202 of CTD (Fig. 1B and C). Our data indicate that the LexA

Table 1
Analysis of in vitro LexA binding and in vivo complementation of WT and LexA mutants

Repressor variant	Amino acid change	Expression plasmid	EMSA binding efficiency[a]		DM936 cell count[b]	
			50%	100%	28.0 °C	42.5 °C
LexA	WT	pMB1	0.51	0.76	3×10^9	8×10^8
LexA-C13-C91	D13C, Q91C	pIVB13/91	0.63	1.10	3×10^9	6×10^8
LexA-C20-C87	S20C, P87C	pIVB20/87	0.60	1.10	4×10^9	5×10^8
LexA-C13-C20-C87-C91	D13C, S20C, P87C, Q91C	pIVB	1.09	1.53	4×10^9	1×10^8
Without LexA	–	pT7-7	–	–	3×10^8	1×10^5

[a]nM concentration of added reduced protein.
[b]CFU/ml in stationary phase.

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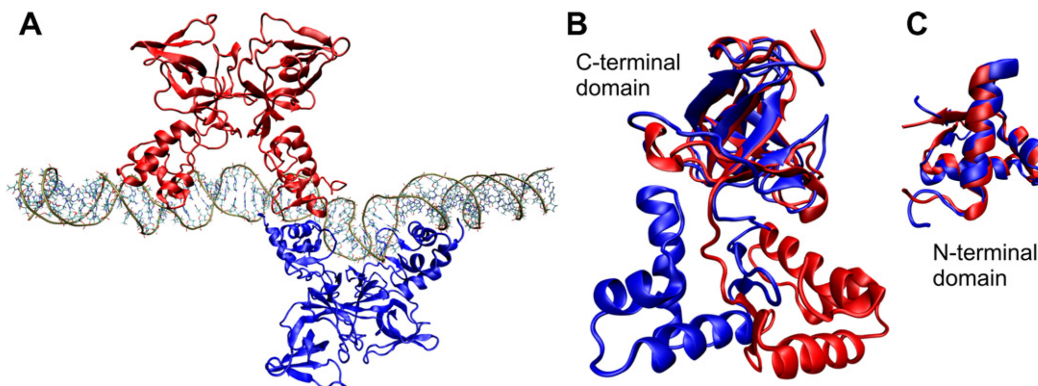

Fig. 1. Proposed model of the fully occupied LexA-*cka* complex and comparison of LexA crystal structure model versus LexA operator-bound model. (A) Two LexA dimers interacting with the regulatory region of the colicin K activity gene *cka*, are presented in red and blue, respectively. Molecular dynamics of 1.3 ns were simulated at 22 °C in the implicit solvent model. DNA sequence of the SOS-regulated gene contained two *cka* operators (marked are the SOS boxes) overlapping by 1 bp (underlined): CTGTACATAAAACCAGTGGTTATATGTACAG-3` centered on the 59 bp *cka* sequence. (B) Model of the LexA monomer derived from the crystal structure (pdb ID: 1JHH; red) superimposed on the CTD of the predicted LexA operator-bound form after 1.3 ns of molecular dynamics simulation (blue). (C) the superposition of NTDs.

hinge region can, besides physically separating the two domains [27], also assume different conformations.

3.2. Structural properties of LexA and mutants

To confirm the significance of a conformational change in the LexA protein upon specific DNA binding, we constructed LexA double and quadruple cysteine mutants from the naturally cysteineless LexA. On the basis of the LexA crystal structure (pdb ID: 1JHH) amino acid residues at the interface between the NTD and CTD were modified (Fig. 2A). Hence in the oxidised form intramolecular disulfide bond formation trapped the LexA conformation observed in solution [7].

Three LexA cysteine mutants, LexA-C13-C91, LexA-C20-C87 and LexA-C13-C20-C87-C91 were constructed, expressed in *E. coli* and purified to homogeneity. The purity of the proteins was analysed by SDS–PAGE electrophoresis. A small portion of covalently bound dimers were present in the LexA-C20-C87 and quadruple cysteine mutants, as indicated by SDS–PAGE with and without DTT (not shown). As no dimers were detected in the LexA-C13-C91 protein isolate (Fig. 2B) it was further structurally characterised. The mobility of the oxidized form of LexA-C13-C91 differed from that of the reduced and WT LexA protein, suggesting that disulfide formation was spontaneous (Fig. 2B).

Introduction of modifications into the LexA protein could result in secondary and tertiary structure perturbations as well as dimer formation. Nonetheless, the far-UV CD spectra of the WT LexA and oxidized LexA-C13-C91 mutant revealed comparable secondary and tertiary structure (see Supplementary Fig. S7A) implying that the introduced cysteine residues could pair only when the CTD is in the non-cleavable conformation (11). Our results thus confirm the existence of a noncleavable LexA confirmation in solution. The determined CD spectra were comparable to that described previously for LexA [28], exhibiting an α-helix minima peak at 208 nm and inflection point at 222 nm.

The 23 kDa LexA protein forms stable dimers at nanomolar concentrations under different conditions [29]. Employing gel filtration chromatography of the purified LexA and LexA-C13-C91 we found that both protein samples exhibited a molecular weight of approximately 50 kDa (Fig. 2C), indicating dimerisation. In addition, LexA contains a single tryptophan residue at position 201 which is partially buried within the dimer interface surface [28,29]. At 25 °C the intrinsic tryptophan fluorescence emission spectra were comparable for both proteins (see Supplementary Fig. S7B). Disruption of dimers due to denaturation by heating resulted in identical red-shift in emission spectra of both proteins, confirming that they exhibit the same dimerization mode.

In summary, the performed structural characterisation showed that the mutant protein LexA-C13-C91 has no large perturbations in its secondary structure, is dimeric in solution and that the disulfide is spontaneously formed in solution.

3.3. Functional properties of LexA and LexA mutants

The in vivo ability of the LexA cysteine mutants to regulate the SOS system was verified. For this purpose we followed growth of the *E. coli* strain DM936, expressing a temperature sensitive LexA protein and harbouring either a plasmid with the WT *lexA*, one of its cysteine variants or the control plasmid pT7-7. Determination of colony forming units (CFU) and bright field microscopy revealed at 42.5 °C, cell division for all strains harbouring the *lexA* encoding plasmids while almost no growth and cell division was detected for the control culture DM936 pT7-7 (Table 1). Our results thus showed that under reduced in vivo conditions, activity of the cysteine LexA repressor derivates was comparable to that of the WT protein.

In vitro evidence that a conformational change is required for specific DNA binding was provided by EMSA and SPR which showed that upon disulfide formation between the modified Cys residues, the required conformational change was blocked and specific DNA binding abolished. EMSA was performed with the 270 bp PCR-amplified fragment harboring the *cka* operator sequences used for molecular modeling. Specific binding of the oxidised LexA-C20-C87, LexA-C13-C20-C87-C91 or LexA-C13-C91 mutant proteins to the *cka* operator was not observed even at 2.40 nM of protein (data not shown). On the other hand, in the reduced form all mutant repressor proteins bound specifically to DNA. At concentrations of approximately 0.60 nM, 50%

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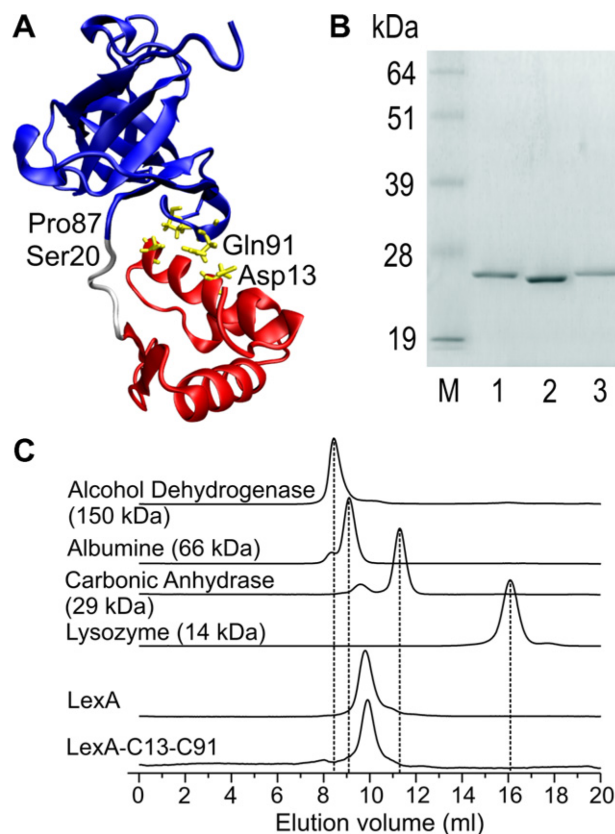

Fig. 2. Structural properties of purified LexA and LexA-C13-C91. (A) A ribbon representation indicating positions of modified residues in LexA mutants. The LexA CTD and NTD are presented in blue and red, respectively and hinge region in white. (B) 10% SDS–PAGE of LexA and LexA-C13-C91. M, molecular weight marker; 1, reduced LexA-C13-C91; 2, oxidised LexA-C13-C91; 3, the WT LexA. Mutant was reduced prior to the experiment by 30 min of incubation in 20 mM dithiothreitol. (C) Gel filtration chromatography to determine LexA and LexA-C13-C91 elution time in relation to molecular mass standards. Gel chromatography was performed on a Superdex™ –75 column (Amersham Biosciences) equilibrated in 50 mM NaH_2PO_4, 500 mM NaCl, pH 7.4, at a flow rate of 0.3 ml/min.

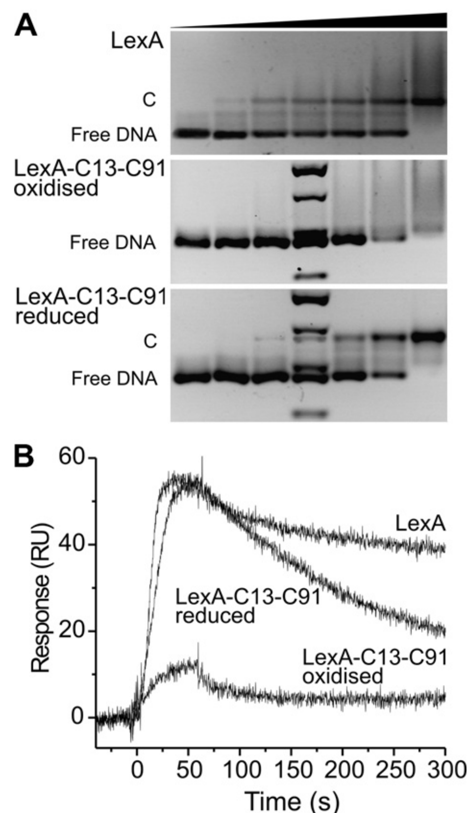

Fig. 3. In vitro DNA binding properties of LexA and LexA-C13-C91. (A) Electrophoretic mobility shift assay in 20 mM Tris, 200 mM NaCl, pH 8.0. Complex formation between the native LexA or mutant LexA-C13-C91 derivate in oxidised or reduced form and a 270 bp DNA fragment (50 nM) harboring the two *cka* operators. The following concentrations were used (from left to right): 0.02, 0.06, 0.15, 0.19, 0.23, 0.51, 0.76 nM for LexA; 0.02, 0.06, 0.10, 0.21, 0.34, 0.63, 1.10 nM for the reduced or oxidised LexA-C13-C91. Lower bands correspond to unbound DNA and the upper more slowly migrating bands are composed of protein–DNA complexes (A). For LexA-C13-C91 a 100 bp DNA ladder was included in the middle lane to show that no non-specific binding occured. (B) SPR sensograms of the 14 nM WT LexA and LexA-C13-C91 binding to the immobilized 120 response units of the DNA fragment at 25 °C. Flow rate was 40 μl/min.

and at 1.10 nM all of the *cka* operator sites were involved in complex formation with the double LexA-C20-C87 (EMSA not shown) or LexA-C13-C91 cysteine mutant (Fig. 3A). Lower affinity of binding was observed for the quadruple mutant (Table 1).

SPR analysis was employed to elucidate LexA and LexA-C13-C91 double mutant binding to the 270 bp *cka* fragment harboring the two operators in real time. Sensograms showed stable binding of LexA to the *cka* operator region (Fig. 3B). Comparable association was observed for the reduced LexA-C13-C91, while dissociation was more rapid, in agreement with lower affinity observed in the EMSA. This implies that the residues that were replaced by cysteines have some functional role in DNA binding. On the other hand, decreased association and reversible, non-stable binding was observed for the oxidised LexA-C13-C91.

In summary, two independent functional assays showed that a LexA conformational change is required for stable and specific DNA operator sequence binding.

In conclusion, our results demonstrate that a large conformational change in the LexA protein, predictably via rotation of the NTD with regard to the CTD, is required for binding to specific DNA sequences. As in addition to regulating genes involved in DNA repair and survival the LexA protein has been shown to be involved in dissemination of antibiotic resistances in *Vibrio cholerae* [30], dissemination of virulence factor genes in *V. cholerae* [3] and *Staphylococcus aureus* [31], induction of chromosomal virulence gene expression in *S. aureus* [4], as well as regulation of type III secretion system in enteropathogenic *E. coli* (EPEC) [32] and virulence factor synthesis of *S. aureus* [4] the presented binding model could provide the basis for drug intervention. We propose the cleft bordered by the CTD and NTD in operator-bound or LexA free form in solution, as a possible novel drug target.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.09.006.

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