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PURIFICATION AND CRYSTALLIZATION OF ZITB, A ZINC TRANSPORTER FROM *ESCHERICHIA COLI*

Katherine Kao, DaXiong Fu

ABSTRACT

Cellular zinc homeostasis is essential to human health. Zinc transporters transport zinc ions into and out of cells to maintain cellular zinc concentrations in a narrow range. Several membrane proteins have been shown to facilitate transmembrane fluxes of zinc ions, however, structures of these zinc transporters are unknown. The purpose of this work is to express, purify and crystallize a Zinc transporter, ZitB for crystallographic studies. ZitB was over-expressed as a His-tagged membrane protein using a pET15b expression vector hosted in *E. coli* BL21 cells. Purification of ZitB was achieved by preparation of ZitB-containing membrane vesicles, followed by detergent extraction, and completed with Ni-NTA metal affinity and size exclusion chromatography. The molecular identity of the purified ZitB was confirmed by mass spectrometry, which showed the expected molecular weight of 35.2kDa. Crystallization trials of ZitB were conducted at 20 °C, using a series of low molecular weight PEGs as precipitants. Micro-crystals were grown in 25% PEG 1K, whereas only amorphous precipitations were observed in PEG 400 and 600. In conclusion, this work yielded highly purified ZitB protein and defined an initial crystallization condition for ZitB.

INTRODUCTION

Zinc is important in all living organisms; however, when present in excess, Zinc can be toxic. Zinc ion is hydrophilic, therefore transport across the hydrophobic membrane bilayer of all cells requires a special membrane transport system. To balance the intracellular and extracellular levels of Zinc in a cell requires careful regulation of membrane transport. It is advantageous to study further the mechanism of Zinc transport, e.g. binding site chemistry and transport mechanism, as well as the structural conformation of the Zinc transporter membrane proteins because such studies will yield useful and important information in the future relevant to drug design and diagnosis of medical problems and diseases associated with Zinc.

ZitB, a transmembrane protein from *E. coli*, is a member of the Cation Diffusion Facilitator (CDF) family found in many species, ranging from prokaryotes to eukaryotes. This family is involved specifically with the transport of transition metal ions, such as Co, Zn, and Cd, and was first described by Nies and Silver in 1995 ([4,5]). The proteins in the CDF family share some common characteristics, including the presence of six transmembrane domains with strong conservation in the four N-terminal spanners [5]. Members of the CDF family vary in size (from 276-549 amino acyl residues), location, polarity, and sequence. Proteins of the CDF family in eukaryotes also differ from those in prokaryotes in the possession of histidine-rich cytoplasmic loops between transmembrane spanner 4 and 5. [5]

ZitB has been found to be a zinc membrane transporter, and was first associated with zinc transport by Grass et. al [1] through ZitB expression and analysis in *E. coli*. The second major advance to studying and characterizing ZitB was through functional analysis of ZitB. Site-directed mutagenesis further characterized the function of essential amino acid residues located in hypothetical transmembrane domains with Zinc resistance abolished in five mutants. H159R and D163E substitutions in the putative transmembrane helix 5, D186E substitutions in transmembrane helix 6 resulted in complete loss of resistance and H53R mutation in putative transmembrane helix 2 was unable to confer zinc resistance, E214A did not show zinc sensitivity in *E. coli*. The conservation of E214 in the carboxy-terminal domain in bacterial zinc transporters also appears to be involved in zinc function [2]. In addition to presenting evidence that the transmembrane protein was involved in Zinc transport, researchers have also postulated ZitB to be an antiporter, a type of transporter which couples the electrochemical gradient of ions or organic solutes to drive transport reactions [3]. ZitB in particular, exchanges Zn^{2+} for K^+ [2]. However, all of the above studies on ZitB were carried out in whole cells of *E. coli*, therefore, experimental results may be affected by the presence of other membrane transporters in the intact cell.

Membrane protein isolation and purification is a key issue for effective functional and structural determination. To precisely characterize the transport function of a membrane transporter requires pure membrane protein sample reconstituted with the lipid bilayers. Pure membrane protein samples are also needed to study the biological properties of the membrane proteins, such as protein-ligand binding properties or protein-protein interactions. Up to date, the best way to determine the three-dimensional structure of membrane proteins is through X-ray crystallography. To get the crystals for crystallographic studies, a pure and high quality sample of a membrane protein is a must. Membrane protein purification remains a difficult task, hampered by the hydrophobicity and instability of many membrane proteins when removed from the lipid bilayer. In this study, we have developed an effective approach to purify ZitB from overexpressed *E. coli* cells, and have defined an initial condition for ZitB crystallization. These results provide a basis for future structural and functional study of ZitB

METHODS/MATERIALS

Materials

All DNAse, protease inhibitor cocktail, and thrombin were purchased from Sigma (St. Louis, MO). Lysozyme was from Amresco (Solon, OH). n-dodecyl-β-D-Maltopyranoside (DDM) was from Anatrace (Maumee, OH) and all Polyethyleneglycol (PEG) was purchased from Fluka (Milwaukee, WI) as was the glycerol used in crystallization. All other chemicals were purchased commercially with the highest available quality.

ZitB Expression and Membrane Preparation

A suspension of BL21pLys competent cells (Novagen, Madison, WI) with ZitB-N-His plasmid DNA was inoculated into a 6 liter auto-induction culture after a 6-hour incubation at 37 °C, 300RPM in a C25K incubator shaker (New Brunswick Scientific, Edison, NJ) and the culture was left to incubate at 37°C, 300RPM overnight. [7] The cells were harvested the next day by centrifugation (Beckman Coulter, Avanti J-20 XP, Fullerton, CA) for 15 min at 7000 rpm and the pellet was resuspended in 20mM Tris HCl, 100mM NaCl, 2mM β-Mercaptoethanol (β-Me), 1mg/ml lysozyme, 0.1 mg/ml DNAse, 0.1% saturated Phenylmethanesulfonyl fluoride (PMSF), pH 8.5. The suspension was incubated and allowed to shake for 30 min at 37 °C, 300RPM. Saturated PMSF in 100% ethanol was added at 15, and 30 min. After 30 min, the cell membrane was ruptured using a microfluidizer (Microfluidics Corporation, model 110S, Newton, MA) at 110-120 psi, with 40µL of the protease inhibitor cocktail (Sigma, St. Louis, MO) added after each pass. After 4 passes, the suspension was centrifuged at 7000 rpm (DuPont Sorvall RC-5B Refrigerated Superspeed Centrifuge, Chadds Ford, PA) for 15 minutes to remove the cell walls, and the supernatant was then ultracentrifuged (Beckman Ultracentrifuge XL-100K, Fullerton, CA) at 50K for 45 min. The pellets, which contain membrane vesicles, were resuspended in 20 mM HEPES, 300mM NaCl, 2mM β-Me, pH 7.5; the resuspension was followed by ultra centrifugation (Beckman Coulter Optima XL-100K ultracentrifuge Fullerton, CA) at 45K for 45 min. The resulting pellet was resuspended in 40 ml buffer which contains 20 mM HEPES, 100mM NaCl, 2mM β-Me, 20% Glycerol, pH 7.5, and then was quick frozen in liquid N_2 and stored at -80 °C.

Isolation of ZitB by Metal Affinity Chromatography

All of the following was performed at 4 °C. Three buffers were prepared; equilibrating/desalting buffer (containing 20 mM HEPES pH 7.5, 100 mM NaCl, 20% glycerol, 0.05% DDM, 2mM β-Me), wash buffer (containing 20 mM HEPES pH 7.5, 300 mM NaCl, 30mM Imidazole, 20% glycerol, 0.05% DDM, 2mM β-Me), elution buffer (containing 20 mM HEPES pH 7.5, 100 mM NaCl, 500mM Imidazole 20% glycerol, 0.05% DDM, 2mM β-Me). Membrane vesicles were thawed at room temperature and added to 7% DDM. The mixture was stirred and 0.1% PMSF and 2mM β-ME were added at 1 and 15 min. At 30 min, the mixture was centrifuged at 50K for 45 min. The supernatant was passed through a DEAE Sepharose Fast Flow column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) that had been prewashed with double deionized H_2O, charged with 2M NaCl and equilibrated with equilibrating buffer. The flow through from the DEAE column was then passed through a Ni-NTA column (Qiagen, Valencia, CA) that had also been prewashed and equilibrated with equilibrating buffer. After loading the sample, the Ni-NTA column was washed with 80 ml wash buffer, and the wash was then followed by application of elution buffer at a flow rate of 2ml/min. The flow through gathered from the elution buffer was then passed through a gel filtration column (Pharmacia Biotech, Piscataway, New Jersey and Amersham Biosciences AB, Uppsala, Sweden) to remove imidazole. Overnight thrombin digestion and concentration was performed in 10K Dialysis cassettes (Pierce, Rockford, IL) that were placed in Dialysis buffer (same as desalting/equilibrating buffer with the exception of pH adjustment to 8.0 for thrombin digestion) and 8% PEG 35K for overnight concentration or 20% PEG 35K for 3-4 hours concentration to 20-30 mg/ml. Protein concentration

PEG 400	PEG 600	PEG 1k
12.5% PEG, 15% glycerol	12.5% PEG, 15% glycerol	12.5% PEG, 15% glycerol
17.5% PEG, 15% glycerol	17.5% PEG, 15% glycerol	17.5% PEG, 15% glycerol
20% PEG, 15% glycerol	20% PEG, 15% glycerol	20% PEG, 15% glycerol

Table 1: PEG solutions/crystallization

HEPES pH 6.6	HEPES pH 7.0	HEPES pH 7.25	HEPES pH 7.5	HEPES pH 7.77	HEPES pH 8.0
NaCitrate pH 4.5	NaCitrate pH 5.22	NaCitrate pH 5.5	NaCitrate pH 5.75	NaCitrate pH 6.0	DDH_20

Table 2: Buffer solutions/crystallization

was determined by UV absorption at 280 nm. (Perkin-Elmer Lambda UV/Vis Spectrometer EZ 201, Norwalk, CT).

High Performance Liquid Chromatography Purification (HPLC)

HPLC purification was performed using the concentrated His-tag cut ZitB. HPLC buffer was prepared (12.5% glycerol, 100mM NaCl, 20mM HEPES, 0.2mM β-Me, 0.05% DDM), filtered, and degassed. The protein at 20-30mg/ml was ultracentrifuged at 50K for 20 min (Beckman coulter Optima TLX ultracentrifuge, Fullerton, CA). 500µl of concentrated protein was injected into the column and the sample was collected by its UV absorption at 280 nm. The protein was concentrated using Amicon Centrifugal filter devices (Bedford, MA) to 14 mg/ml in preparation for crystallization studies. The concentrated protein was then stored at 4°C.

SDS Page

10 µl of the following samples were loaded into a SDS gel (Biorad, Hercules, CA): SDS molecular weight standard (Biorad, Hercules, CA), DEAE column flow through, wash column flow through, Ni-NTA flow through, ZitB with His-tag, ZitB without His-tag.

Mass Spectrometry

Mass Spectrometry was performed on the protein with and without His-tag (Perseptive Biosystems, Voyager–DE Biospectrometry workstation, Framingham, MA) in Matrix containing saturated 4HCAA in 1:2 (v/v) mixture of water and acetonitrile.

Crystallization of ZitB

Crystallization of ZitB was done through vapor diffusion method by hanging drop using 24 well cell culture plates (Hampton, Laguna Niguel, CA). Solutions of PEG and buffer were prepared as summarized in table 1&2. All solutions were filtered using Nalgene 0.2µm syringe filters. 800 µl of the appropriate PEG/glycerol, 100 µl 0.05% DDM, 100 µl buffer was added to each well. The well solutions in the plates were mixed on a shaker at about 20°C. 1.5µl each of well solution and protein were placed onto each siliconized cover slip (Hampton, Laguna Niguel, CA) and inverted over a greased well (Dow Corning High Vacuum Grease, Hampton, Laguna Niguel, CA).

RESULTS

Protein Purification

His–tagged ZitB was purified to homogeneity by a single step Ni chelate affinity chromatography. This is supported by a single band of His-tagged ZitB after it was eluted from Ni-NTA column (Figure 1a). After overnight thrombin digestion, the 6-Histine tag was completely removed, as shown by mass spectrometry (Figure 2a and b). However, the molecular weight difference of His-tagged ZitB and wild type ZitB (~1.7 kDa) is not large enough to be detected by SDS PAGE. The corresponding ZitB band did not show up in the lanes of DDM solubilized membrane vesicles from the DEAE column flow-through. This result indicates that over-expressed ZitB only contributes a small amount of total membrane proteins and thus could be not detected in SDS PAGE. From six liters of cell culture, the yield of ZitB is only about 50 mg. The His-tag removed ZitB was further purified by size exclusion HPLC. Analytical size exclusion HPLC analysis showed a major mono-disperse species of ZitB with minor aggregation (less than 10%) (Figure 1b). Besides DDM, we also tested two other non-ionic detergents, β-OG and DM, to solubilize ZitB.

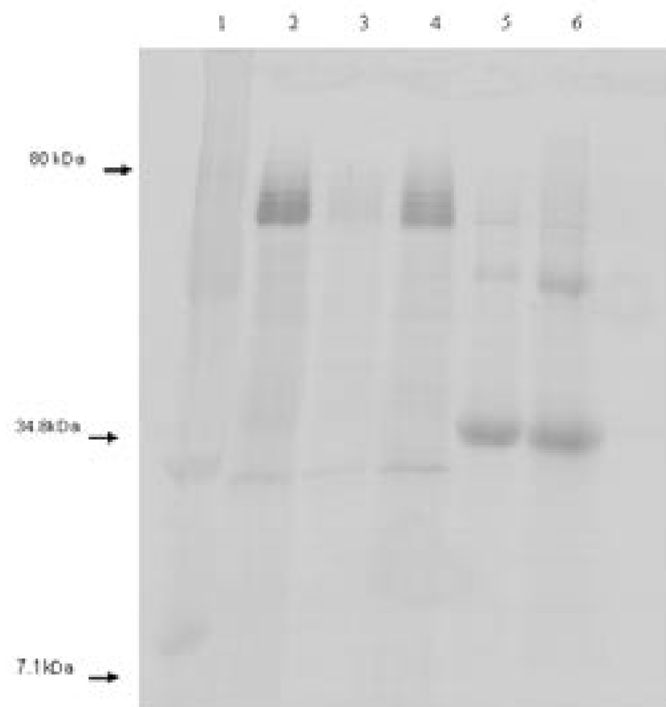

***Figure 1a*: SDS PAGE.** Lane 1: molecular weight standard, lane 2: flow through from the DEAE column, lane 3: flow through from the wash procedure, lane 4: flow through from the Ni-NTA, lane 5: ZitB with His tag, lane 6: ZitB without His tag.

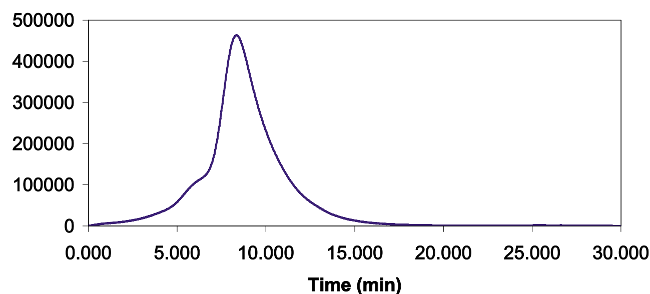

Figure 1b: HPLC profile. This profile is a rerun of the sample after HPLC purification.

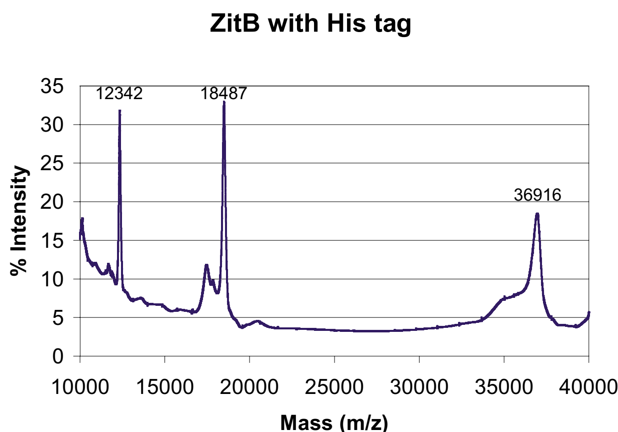

Figure 2a: Mass Spectrum of ZitB with His-tag. The three peaks correspond to +1, +2, and +3 charged peaks of ZitB.

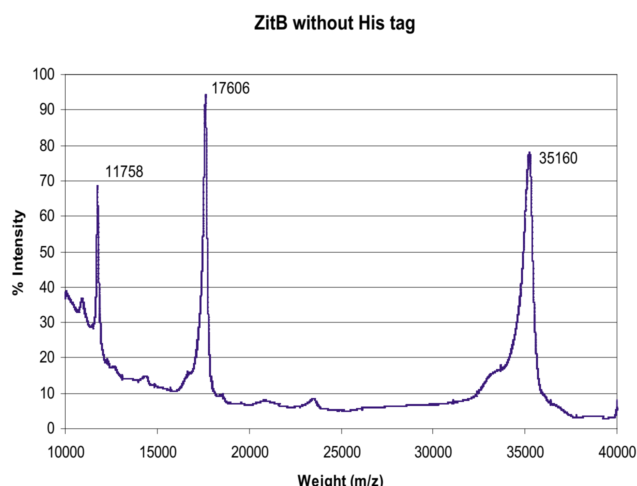

Figure 2b: Mass Spectrum of ZitB without His-tag. The three peaks correspond to +1, +2, and +3 charged peaks of ZitB.

β-OG caused ZitB denaturation and precipitation, while DM solubilized ZitB aggregated and came out in the void peak of HPLC (data not shown). Therefore, DDM is the best detergent for ZitB purification among these non-ionic detergents

The average molecular weight of +1, +2, and +3 charged peaks of ZitB-His is 36.97kDa while the average molecular weight of ZitB without His-tag is 35.21 kDa by mass spectrometry. The expected molecular weight of ZitB-His is 36.94kDa while the expected molecular weight of ZitB without His-tag is 35.20kDa. The mass spectrum of ZitB with His-tag shows a small additional peak with a mass of about 35.06 kDa (figure 2a). This indicates that a small portion of the protein sample was degraded during the purification and isolation process. The difference in molecular weight between the cut and uncut samples is 1.76kDa and is in good agreement with the calculated molecular weight of the His-tag, 1.74 kDa. The mass spectrum of ZitB without His-tag (figure 2b) shows peaks that are definitive, clear and well defined. Mass spectrometry results thus confirm the molecular identity and purity of ZitB. Mass Spectrometry also indicate the successful completion of the thrombin digestion process.

B. Crystallization

A two dimensional crystallization screen of ZitB (Table 1&2) with conditions ranging from 12.5%-25% PEG 400, 600, 1K and pH 4.5-8.0 (in citrate or HEPES buffer) resulted in heavy to light precipitation in all trials. Micro crystals formed in 25% PEG 1K, pH 4.5-6.0 Na Citrate accompanied by light precipitate. A picture of ZitB micro crystals is given in figure 3a,b.

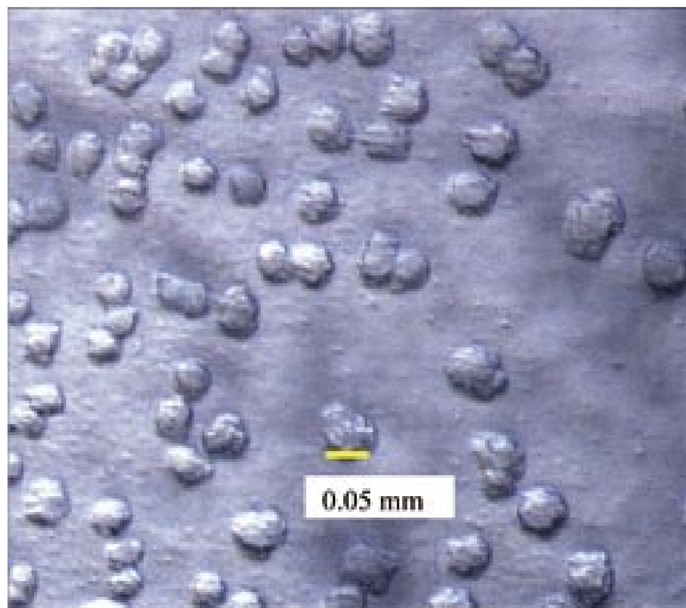

Figure 3a: Microcrystals of ZitB. Microcrystals of ZitB grown in 25%PEG 1K, pH 5.75 (Na Citrate buffer). The size of the crystals is about 0.05 mm.

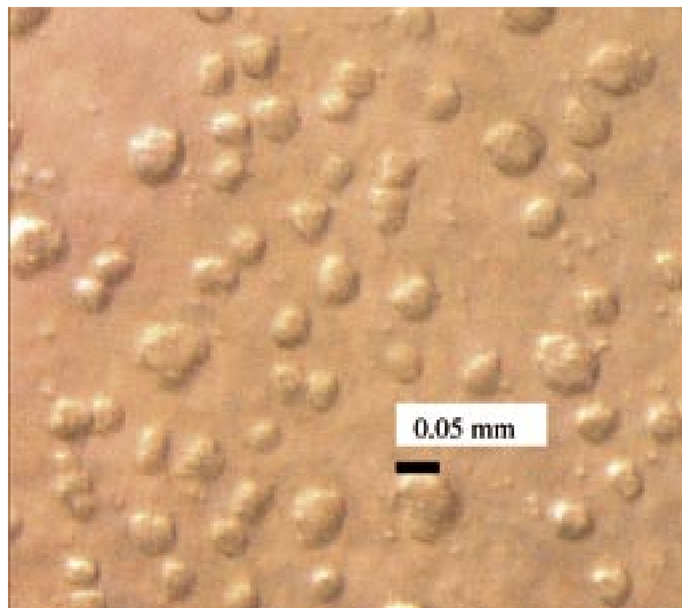

Figure 3b: Microcrystals of ZitB. Microcrystals of ZitB grown in 25% PEG 1K, pH 6.0 (Na Citrate buffer). The size of the crystals is about 0.05 mm.

DISCUSSION AND CONCLUSION

Membrane proteins are essential for biological systems. In order to study the structure and function of membrane proteins, obtaining a high yield and purity of the target protein is the first and most important step. However, the high hydrophobicity and poor stability of membrane proteins make it a difficult task to obtain high purity membrane proteins. In this study, we have successfully developed a series of efficient approaches to obtain highly pure ZitB from overexpressed *E. coli* cells. The effectiveness of each purification step was confirmed by SDS PAGE (Figure 1a), and the molecular identity and purity of ZitB were also confirmed by SDS PAGE (Fig. 1a), size exclusion HPLC (Fig.1b), and mass spectrometry (Fig. 2a, 2b). The percentage of over-expressed ZitB in total *E. coli* membrane proteins was small and could hardly be detected by SDS PAGE. However, we were able to efficiently purify His-tagged ZitB from solubilized membrane vesicles by one single step of Ni affinity chromatography. The histidine tag was successfully removed by thrombin digestion and ZitB was further purified to high quality by size exclusion HPLC. The small peaks at 17.45kDa and 34.93 kDa in the mass spectrum of His-tagged ZitB (Fig 2a) indicates a small portion of ZitB may have been degraded during purification. This suggests that in future purification procedures, we may need to increase the amount of protease inhibitors. Detergents have played a significant role in membrane protein purification because they keep the membrane protein soluble in aqueous solution, however, too much detergent in solution may destabilize the protein. After testing several nonionic detergents at different concentrations, we found that 0.05% DDM is the optimal condition for ZitB in aqueous solution. As DDM has a lower critical micellar concentration (CMC) compared to β–OG and DM, this result indicates that low CMC non-ionic detergents are favorable for ZitB solubilization. The total amount of ZitB purified from 6-liter cell culture was about 50 mg, measured by protein OD at 280 nm. This large quantity makes it possible to carry on crystallization studies as well as other biochemical studies

The conditions that were tested for the crystallization of ZitB caused the formation of light to heavy precipitate in all drops. Microcrystals formed after 7 days and were accompanied by light precipitate that did not clear up. Microcrystals appeared in PEG 1K solutions in the pH range 6-7.3. For future studies, it may be useful to experiment with different buffers within this pH range, as well as with higher molecular weight PEG such as PEG 1.5K and 2K, given that low molecular weight PEGs (400, 600) were unsuccessful and yielded precipitate only. It can also be concluded that ZitB is very sensitive to the concentration of 25% PEG 1K at high pH as no micro crystals formed at pH higher than 6.5. The crystallization screening study provided an initial condition for future crystallization refinement.

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