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ACCELERATED COMMUNICATION

A novel method of affinity-purifying proteins using a bis-arsenical fluorescein

KURT S. THORN,[1] NARIMAN NABER,[2] MARIJA MATUSKA,[2] RONALD D. VALE,[1,2,3] AND ROGER COOKE[2]

[1]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143
[2]Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143
[3]The Howard Hughes Medical Institute, University of California, San Francisco, California 94143

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Abstract

Genetically-encoded affinity tags constitute an important strategy for purifying proteins. Here, we have designed a novel affinity matrix based on the bis-arsenical fluorescein dye FlAsH, which specifically recognizes short α-helical peptides containing the sequence CCXXCC (Griffin BA, Adams SR, Tsien RY, 1998, *Science 281*:269–272). We find that kinesin tagged with this cysteine-containing helix binds specifically to FlAsH resin and can be eluted in a fully active form. This affinity tag has several advantages over polyhistidine, the only small affinity tag in common use. The protein obtained with this single chromatographic step from crude *Escherichia coli* lysates is purer than that obtained with nickel affinity chromatography of 6xHis tagged kinesin. Moreover, unlike nickel affinity chromatography, which requires high concentrations of imidazole or pH changes for elution, protein bound to the FlAsH column can be completely eluted by dithiothreitol. Because of these mild elution conditions, FlAsH affinity chromatography is ideal for recovering fully active protein and for the purification of intact protein complexes.

Keywords: affinity tag; FlAsH; protein purification

The use of genetically-encoded affinity tags is now a standard method of purifying proteins (reviewed in Uhlén & Moks, 1990; LaVallie & McCoy, 1995; Makrides, 1996; Nilsson et al., 1997; Hannig & Makrides, 1998). This technique allows for simple purification of a protein of interest by fusing to it a tag with affinity for a stationary phase. Most affinity tags are small-molecule binding proteins (e.g., maltose binding protein, glutathione S-transferase). However, the size of these proteins can potentially interfere with the protein to which they are fused. A few short peptides, which are potentially less perturbing, have been used as affinity tags. The most common ones are the 6xhistidine tag and the FLAG tag (a six amino acid antibody epitope). However, both these affinity tags have disadvantages. The FLAG tag requires the use of an expensive antibody affinity matrix and as a result has not received widespread use. The polyhistidine tag, which binds to metal ions, is very widely used, but requires somewhat harsh conditions (either high concentrations of imidazole or low pH) for elution, which can disrupt macromolecular complexes. In addition, small amounts of metal ions that elute with the protein can inactivate many enzymes. The purity of the eluted protein can be low because many histidine-rich proteins can bind to and elute from metal affinity resins, contaminating the purified protein.

Recently, a fluorescent dye has been developed that specifically interacts with tetracysteine containing helices (Griffin et al., 1998). This compound, known as FlAsH (Fluorescein Arsenical Helix binder), has been shown to specifically interact with proteins tagged with a CCXXCC containing helix. The interaction is readily reversed by incubation with small dithiols such as ethanedithiol. The specificity and reversibility were shown by fluorescent enhancement of the FlAsH compound on binding to tagged proteins in vivo. In the present study, we have modified this compound for use as a stationary phase for purifying tagged proteins.

Results and discussion

Development of the FlAsH affinity matrix

To attach the FlAsH compound to a column media, it was modified to contain a primary amine at the 5 position of the fluorescein (Fig. 1). This was accomplished by acylating amino-fluorescein with β-alanine. Following cleavage of the protecting group, the β-alanyl fluorescein was then converted to the mercuric acetate derivative (Karush et al., 1964; Shipchandler & Fino, 1986) and then to the bis-arsenical derivative by transmetallation as described

Reprint requests to: Roger Cooke, University of California, San Francisco, Department of Biochemistry, Box 0448, 513 Parnassus Avenue, San Francisco, California 94143; e-mail: cooke@cgl.ucsf.edu.

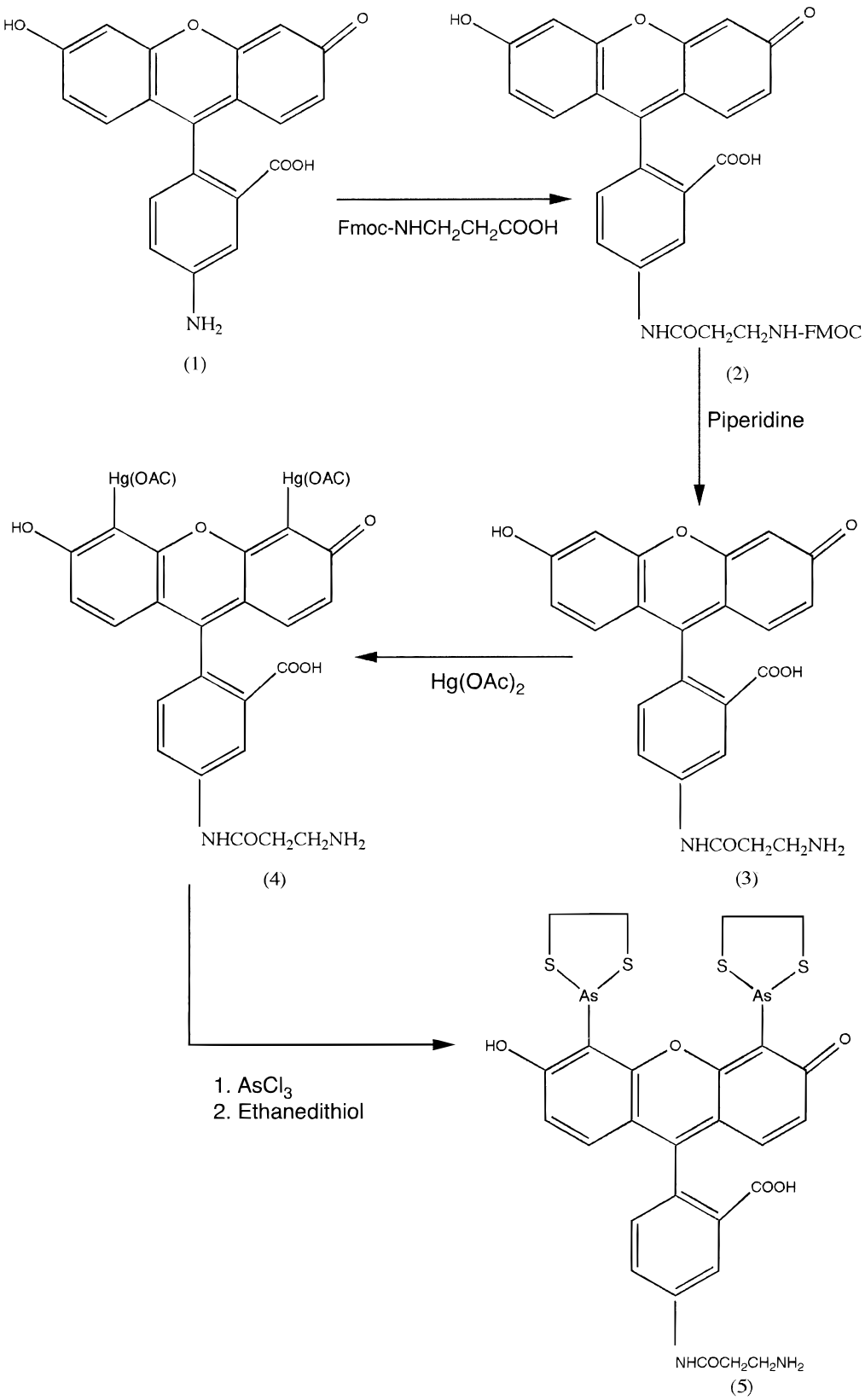

Fig. 1. Synthesis of 4′,5′-bis(1,2,3-dithioarsolan-2yl)5-(3-aminopropanamide)fluorescein (β-alanyl FlAsH). Aminofluorescein was modified to contain a primary amine in the 5 position to allow attachment to an NHS-functionalized stationary phase. This was done by acylating aminofluorescein (1) with β-alanine. The β-alanyl fluorescein (2) was then converted to the bis-arsenical derivative (5). This compound was then used for protein purification.

for synthesis of the original FlAsH compound (Griffin et al., 1998). The active compound, β-alanyl FlAsH, has very similar spectroscopic properties to the previously described FlAsH compound. The primary amine on the β-alanyl FlAsH readily reacts with N-hydroxysuccinamide (NHS) functionalized agarose beads to give a stable covalent linkage.

Purification of kinesin by FlAsH affinity chromatography

To test the FlAsH affinity purification, we prepared kinesin constructs C-terminally tagged with the peptide WEAAAREACCREC-CARA. This peptide specifically chelates FlAsH via the four cysteines. We chose kinesin as our test protein because it is easily tested for activity by microtubule gliding. We prepared both a monomeric construct, encoding the first 339 residues of human conventional kinesin (K339FL), and a dimeric construct encoding the first 560 residues (K560FL). The introduction of this peptide tag on the C-terminus of K560 did not change either the expression level or the solubility of the protein when expressed in *Escherichia coli* as compared to an identical construct with a C-terminal 6xHis tag (data not shown). Thus, the CCXXCC tag does not impair protein solubility or expression.

Incubation of high-speed supernatant from *E. coli* expressing K339FL followed by batch elution with 1,2-ethanedithiol (EDT) resulted in a major product (90% pure) that was the tagged kinesin (Fig. 2A). The single band that contaminates this protein is not a kinesin degradation product, as determined by immunoblotting, but was not consistently found in our FlAsH purifications. Despite the presence of this single contaminant, the protein is much purer than a similar polyhistidine tagged protein (K339GFP-6xHis) purified by NiNTA affinity chromatography (Fig. 2C). Experiments performed with a FlAsH column (rather than in batch) with more extensive washing gave improved protein purity (not shown). The resin capacity was determined by coupling known amounts of purified FlAsH compound to the affinity resin and determining the amount of protein that could be purified. The yield of tagged protein was 10% of the bound FlAsH compound.

Mass spectrometry of the purified K339FL showed a single peak of molecular weight 39,765 (expected: 39,770) and no higher molecular weight peaks, indicating that the K339FL is not forming covalent complexes. Purified K339FL eluted on a gel filtration column as a single peak at the same volume as comparable monomeric kinesins. This demonstrates that the addition of the tag does not cause aggregation of K339FL in solution.

We also tested kinesin eluted from the FlAsH column for activity, without dialysis or buffer exchange. K560FL purified by FlAsH-affinity chromatography (not shown) was fully active in microtubule gliding assays (29.8 $\pm$ 6.3 μm/min vs. 24.3 $\pm$ 9.4 μm/min for K560GFP), consistent with previously observed values (Case et al., 1997; Woehlke et al., 1997). In contrast, small amounts of Ni^{2+} displaced from metal affinity columns have been found to inhibit kinesin activity. FlAsH should remain on the column matrix, as it is covalently bound.

In initial purification experiments, 1,2-ethanedithiol (EDT) was used to elute the specifically bound protein. However, EDT has an extremely unpleasant smell, and it oxidizes and aggregates with the tagged protein after an overnight incubation in aqueous solution, leading to a loss of protein. To avoid these problems, we tested two other dithiol elution agents: dithiothreitol (DTT) and 2,3-dimercaptopropanesulfonate (DMPS). Although not as efficient as EDT at low concentrations, 50 mM DTT completely eluted the bound protein (Fig. 2). With DTT elution, the protein showed no oxidation or aggregation problems. Similarly, DMPS, which eluted protein from the FlAsH column at the same concentrations as EDT (not shown), did not cause precipitation on prolonged incubation and is odor-free.

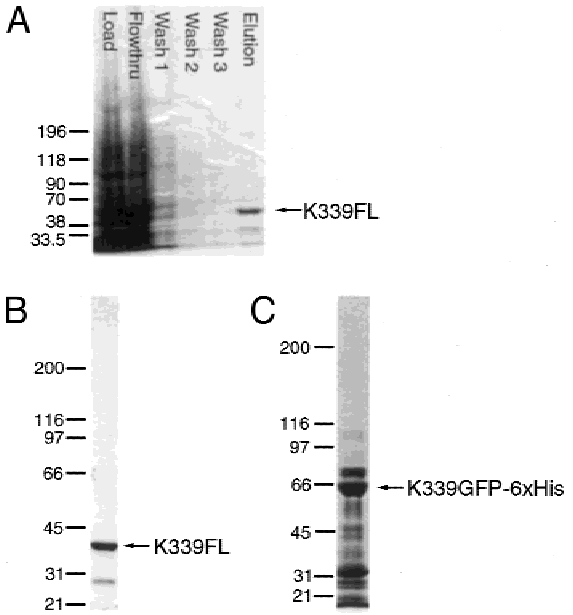

Fig. 2. A: The initial K339FL purification by FlAsH affinity column. One milliliter of *E. coli* high-speed supernatant was bound to 0.1 mL of FlAsH beads and incubated for 1 h. After three 1 mL batch washes, the tagged protein was eluted by incubation with 12 mM EDT. **B:** Purification of k339FL using DTT as the eluant. Beads were incubated as in **A**, then washed five times with 1 mL of buffer containing 0.1 mM DTT. Protein was then eluted by five 0.2 mL batch washes with buffer plus 50 mM DTT. The protein is much purer than a control 6xHis tagged protein shown in **C**. **C:** A similar protein (K339GFP-6xHis) purified by metal affinity chromatography. This protein was expressed and lysed under the same conditions as K339FL, and bound to 1 mL NiNTA resin (Qiagen, Hilden, Germany). The resin was washed with 20 mL wash buffer (pH 6.0 phosphate, 250 mM NaCl), and then eluted with pH 8.0 phosphate, 500 mM imidazole.

Advantages of FlAsH affinity chromatography

Our purification of tagged kinesins by FlAsH affinity chromatography is fast and yields very pure protein. This protein has a higher purity than that obtained by nickel affinity chromatography of 6xHis tagged kinesins, demonstrating one of the major advantages of this method.

FlAsH affinity chromatography is a highly specific protein purification method. It is based on the regiospecific interaction of two arsenics in FlAsH with two pairs of cysteines in a target alpha helix. The only requirement for binding is that the protein of interest contain the motif CCXXCC within an alpha helix. This motif is rarely found in proteins, and labeling tagged proteins in vivo (Griffin et al., 1998) indicate that there are very few eukaryotic proteins that bind to FlAsH. In contrast, many organisms contain histidine-rich proteins, and binding of these proteins to metal ion resins is a major source of contamination. FlAsH affinity chromatography has a number of very desirable features. The affinity tag (CCXXCC motif) can be attached at either the N- or C-terminus

of the protein or can be incorporated into an existing helix within the protein. The ability to incorporate the tag into an existing structural element within the protein is not possible with other affinity tags. The affinity tag does not interfere with kinesin function and is not expected to perturb the function of other proteins when added at either terminus.

The FlAsH resin is compatible with many commonly used buffer components. Buffers containing primary amines (e.g., Tris) and divalent metal chelators, both of which interfere with Ni-resin purifications, are fully compatible with FlAsH. The FlAsH-peptide interaction is also stable to 1 M NaCl. Reducing agents containing a single thiol, such as β-mercaptoethanol, can be used in concentrations up to at least 5 mM, and DTT can be included at concentrations up to 1 mM without interfering with protein binding to the FlAsH resin. The elution of tagged proteins from FlAsH columns occurs under gentle conditions. Bound proteins are eluted by millimolar concentrations of a dithiol (DMPS or DTT), which is unlikely to perturb protein structure or protein–protein interactions. In contrast, many other purification methods require large changes in ionic strength or pH for elution. This gentle elution of bound protein makes FlAsH affinity chromatography ideal for purification of macromolecular complexes.

The high affinity and regiospecificity of the FlAsH-CCXXCC interaction raises the potential for the use of this technique for attaching tagged proteins onto the surface of a bead or coverslip in a site-specific and oriented manner. This could be useful for functional assays of protein arrays on surfaces.

Materials and methods

Synthesis of 4′,5′-bis(1,2,3-dithioarsolan-2yl) 5-(3-aminopropanamide) fluorescein

Fmoc-β-alanine was purchased from Novabiochem. All other starting materials were purchased from the Aldrich Chemical Company (Milwaukee, Wisconsin). Thin layer chromatography (TLC) was carried out using Baker-flex silica gel plates with fluorescent indicator (245 nm). FlAsH chromatography was performed on Beakers Silica gel for flash chromatography purchased from VWR. MALDI-MS analysis was performed using a Voyager-DE (PerSeptive Biosystems, Framingham, Massachusetts) with a gentistic acid matrix (Sigma, St. Louis, Missouri). Elemental analysis was performed by S.F Analytical Laboratories.

5-(3-FMOC-aminopropanamide)fluorescein (2)

Dicyclohexylcarbodiimide (4 mmol, 827 mg) was added to Fmoc-β-alanine (3.5 mmol, 1.1 g) dissolved in 0.4 mL of DMF. 4-Amino fluorescein (0.5 mmol, 174 mg) dissolved in 0.7 mL of DMF was added to the mixture. The mixture was stirred overnight at room temperature. The next day the mixture was centrifuged and the precipitate was separated. The supernatant was evaporated to dryness using a rotovap, and the residue was dissolved in hexane. The product was purified twice by flash chromatography using 3:7 cyclohexane:ethylacetate (R_f = 0.64). Yield: 0.135 mmol (26%), λ_{max} = 499 nm, m/z = 420 (theoretical m+H$^+$ = 419).

5-(3-aminopropanamide)fluorescein (3)

Dry piperidine (0.65 mmol) was added to 0.135 mmol of 5-((5-Fmoc-aminoethyl)aminocarbonyl)fluorescein dissolved in 2.6 mL of DMF. The mixture turned red immediately. The progress of the reaction was monitored by TLC in cyclohexane:ethylacetate 3:7. The reaction was stirred for 2 h at room temperature, evaporated to dryness, and the product precipitated by addition of ether. The red precipitate was washed with ether several times and dried. Yield: 0.09 mmol (13.8%).

4′,5′-Bis(acetoxymercuri)-5-(3-aminopropanamide) fluorescein (4)

5-((5-Aminoethyl)aminocarbonyl)fluorescein (0.09 mmol) was dissolved in 15 mL of 2% acetic acid and 6.5 mL of ethanol and warmed to 50 °C. Mercuric acetate (0.2 mmol, 51.8 mg) dissolved in 0.65 mL of acetic acid was added dropwise to the fluorescein solution. The reaction mixture was left at 50 °C for 1 h and then at room temperature overnight. The light orange solution turned pale yellow after 10 min and red after 2 h. The next day a red precipitate was obtained, and the mixture was evaporated to dryness. The precipitate was washed with a total of 35 mL of water in five portions to remove excess mercuric acetate. Yield: 0.08 mmol, λ_{max} = 505 nm, Hg = 42.4% by weight (theoretical value 42.7%).

4′,5′-Bis(1,2,3-dithioarsolan-2yl)5-(3-aminopropanamide) fluorescein (5)

The above mercuric derivative (0.043 mmol, 40 mg) was placed in a 25 mL two neck flask equipped with a cooling condenser. N-methylpyrrolidinone (0.6 mL), diisopropylethylamine (0.032 mL, 0.346 mmol), $AsCl_3$ (0.075 mL, 0.87 mmol), and a catalytic amount of palladium acetate were added. After 1 h the mixture turned to a clear orange-red solution. The mixture was left overnight and then quenched by the addition of 0.09 mL ethanedithiol and 15 mL of 2 M MOPS at pH 7. The resulting yellow precipitate was removed by centrifugation, and the solution that contains the product was used in this form.

This product was also purified using a spehasil peptide C85um ST 4.6/100 high-performance liquid chromatography column (Pharmacia, Uppsala, Sweden) using a 20–90% DMF/1 mM phosphate (pH 7.0) gradient. The pink product (β-alanyl FlAsH) eluted at 95% DMF. The product has an absorption maxima of 508 nm and gives a 20-fold fluorescence enhancement on incubation with the FlAsH-tag peptide (WEAAAREACCRECCARA; synthesized by the HHMI peptide synthesis facility and used without purification). For some experiments, the product was purified in a similar fashion using low pressure chromatography on hydrophobic substituted sepharose. Matrix-assisted laser desorption ionization mass spectrometry gave a mass of 753 (theoretical m+H$^+$ 752).

The final compound was coupled to Affigel-10 or −15 (Biorad) or to HiTrap-NHS columns (Amersham-Pharmacia) at 3 μmol/mL resin. Binding was done in an isopropanol/DMF/water mixture for 1–2 h at room temperature. The resin was then washed with several volumes of isopropanol and unreacted NHS groups were quenched by incubation with 1 M ethanolamine in isopropanol for 30 min. Coupling was followed by absorbance; typically greater than 90% coupling was achieved. The resulting resin was then washed with several volumes of buffer and stored in 1 mM DTT in isopropanol until use.

Proteins and assays

Constructs encoding K339FlAsH and K560FlAsH were prepared by using polymerase chain reaction to replace the GFP sequence in

constructs K560GFP (Case et al., 1997) and K339GFP with the FlAsH-tag sequence (WEAAAREACCRECCARA). Bacterial high speed supernatants were prepared essentially as described in Case et al. (1997), except that cell lysis was performed with a Microfluidizer 110S (Microfluidics Corp.). The supernatants were frozen in liquid nitrogen and stored at −80 °C until needed. Microtubule gliding assays were performed as described for untagged kinesins (Case et al., 1997). Protein quantitation was performed by densitometry of Coomassie-stained SDS-PAGE gels using BSA as a standard. Gel filtration was performed on a Superose 6 PC 3.2/30 column in 25 mM PIPES pH 6.8, 200 mM NaCl, 2 mM $MgCl_2$, and 1 mM EGTA. MALDI-MS analysis was performed using a Voyager-DE (PerSeptive Biosystems) with a sinapinic acid matrix (Sigma)

Purification

Batch purification was performed by incubating 100 μL of FlAsH resin with 1 mL of bacterial high speed supernatant for 1 h at 4 °C with end-over-end rotation. The FlAsH resin was then pelleted and washed 4 × 1 mL with wash buffer (80 mM PIPES pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM DTT). The specifically bound protein was then eluted 5 × 200 μL with 50 mM DTT in wash buffer. Each elution was incubated for 5 min to allow equilibration.

Column purification was performed by loading 20 mL of bacterial high speed supernatant onto a 1 mL FlAsH column at 0.5 mL/min. The column was washed with buffer A (25 mM PIPES, 2 mM $MgCl_2$, 1 mM EGTA, 0.1 mM ATP, 5 mM βME) until the A_{280} returned to baseline. The column was then washed 3 × 2 mL with 1 mM DTT in buffer A with 5 min pauses between each wash to allow equilibration. Protein was then eluted with 50 mM DTT in buffer A 5 × 2 mL with 5 min pauses. Elution with 2,3-dimercaptopropanesulfonate was performed in the same manner except that 10-fold lower concentrations of DMPS were used.

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