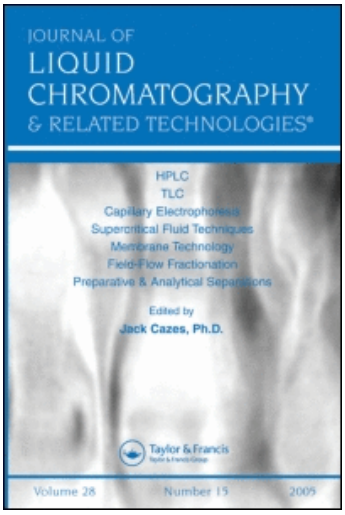

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Berger, G. , Girault, G. , Andre, F. and Galmiche, J. -M.(1987) 'Application of HPLC to the Purification of Coupling Factor CF_1 From Spinach Chloroplasts and of Some of its Subunits', Journal of Liquid Chromatography & Related Technologies, 10: 7, 1507 — 1517
To link to this Article: DOI: 10.1080/01483918708066783
URL: http://dx.doi.org/10.1080/01483918708066783

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(7), 1507-1517 (1987)

APPLICATION OF HPLC TO THE PURIFICATION OF COUPLING FACTOR CF_1 FROM SPINACH CHLOROPLASTS AND OF SOME OF ITS SUBUNITS

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ABSTRACT

A simple and rapid method of purification of coupling factor CF_1, from spinach chloroplast crude extracts, is described, involving anion exchange HPLC on DEAE Pak. Separation of β , $CF_1-\delta$, $CF_1-\varepsilon$ subunits is performed on the same column, after dissociation of the bound enzyme by extensive washing at low ionic strength. Purity of the fractions is checked by urea-SDS polyacrylamide electrophoresis and by fluorescence spectrometry.

INTRODUCTION

In chloroplasts, ATP synthesis is catalyzed by the hydrosoluble portion CF_1 of the membrane bound proton translocating CF_0-CF_1 ATPase. CF_1 has a Mr of 416,000 and a five subunit structure with a stoichiometry $\alpha_3\ \beta_3\ \gamma\ \delta\ \varepsilon$ (1). The different subunits have respective Mr of 59,000, 56,000, 34,000, 21,000 and 16,000.

Coupling factor CF_1 from spinach chloroplasts is usually prepared by EDTA treatment with or without chloroform, followed by chromatography on DEAE Sephadex with ammonium sulfate gradient or by sucrose gradient centrifugation (2-8).

In order to determine the role and the characteristics of subunits (α, β γ, δ , ε) or groups of subunits of CF_1 (CF_1- ε , CF_1- δ , CF_1- δ - ε), different separations have been performed.

By preparative SDS polyacrylamide gel electrophoresis (SDS PAGE), small quantities of the different subunits can be isolated (7,9). The CF_1- δ complex is preferentially obtained when the DEAE Sephadex chromatography is performed at a neutral pH (2,5,8). It is also isolated on hydroxylapatite with detergents and phosphate gradient (7,10), or on Mono Q with lithium borate buffer, ethylene glycol and sodium chloride gradient (8). The CF_1- ε complex is separated in the same conditions as the CF_1- δ complex (8) or on DEAE cellulose with a glycerol-ethanol mixture (11), or on hydroxylapatite after action of Triton X 100 (10).

Pyridine treatment of CF_1 and DEAE cellulose chromatography in urea gives δ (12, 13) or δ and ε (2, 5). The small subunit ε is also prepared on DEAE cellulose (11) by washing the CF_1 bound to the column with a glycerol- ethanol mixture. δ subunit can be purified on hydroxylapatite column in presence of octylglucoside and phosphate (10). Methods for purification of the other subunits are less numerous ; they are sometimes incomplete and require the combination of two types of chromatography. Fractions containing ε , α+δ , α+ δ + γ , β + γ , β , are obtained by chromatography on hydroxylapatite in presence of 0,1 % SDS (4). These mixtures are then resolved by chromatography on Biogel P 300 in presence of 0,1 % SDS (4) or 0,1 % LDS and 6 M urea (6). Partial separation is also achieved on DEAE Sephadex in 7 M urea, with chloride gradient. γ , β , β + α and α are eluted successively (14). Finally, β subunit is purified by HPLC on Mono Q, by elution with lithium borate buffer, 20 % ethylene glycol and NaCl gradient (8).

We describe here a simple method of purification of CF_1 and of some of its subunits, using a preparative HPLC column, allowing to prepare sufficient quantities for biophysical and biochemical studies.

MATERIALS AND METHODS

Spinach chloroplasts are prepared as in (3) and CF_1 is extracted by EDTA, sucrose and chloroform treatment as in (2). The extract is then passed on a DEAE cellulose column equilibrated with 50 mM Tris-HCl, 2 mM EDTA, pH 7.8 and CF_1 is eluted with the same medium supplemented with NaCl 0.4 M, precipitated with ammonium sulfate (50 % saturation) and eventually stored at 5°C.

Several preparations are pooled, dialysed against 20 mM Tris HCl, 0.5 mM EDTA pH 8, and centrifuged half an hour at 40,000 g just before HPLC purification.

HPLC is performed on a Waters apparatus, consisting in two pumps M 510, a solvent programmer M 660, an injector U $_6$ K and a spectrophotometer M 481. Crude extracts are chromatographed on Protein Pak DEAE 5 PW Waters Column, 21.5 mm x 150 mm. Flow rate is set at 4 ml/min. The column is initially equilibrated at pH 8 (20 mM Tris HCl), about 30-50 mg protein (in 1,5 ml dialysed against 20 mM Tris-HCl, 0.5 mM EDTA pH 8) are injected and a linear ammonium sulfate gradient (0 to 1 M) is applied during 30 min. The column is then reequilibrated with initial buffer and the ionic strength is controlled by saline concentration detector (IBF).

Fractions corresponding to purified coupling factor are pooled, precipitated by ammonium sulfate (50 % saturation) for concentration and dialysed during the night against 20 mM Tris-HCl, 0.5 mM EDTA pH 8. The solution is submitted to 3 cycles of freezing-thawing, centrifuged at 40.000 g during 15 min and supernatant is injected on the DEAE Pak column. Extensive washing of the column by 20 mM Tris HCl pH 8 (4 ml/min during one to two hours) leads to partial fractionation of CF_1 : β subunit, CF_1- δ and CF_1-ε complexes are eluted from the column by an ammonium sulfate gradient (0 to 0.8 M during 30 or 40 min).

Protein chromatographic elution is followed at 280 nm, protein dosages are performed with Bio Rad protein assay (15), using bovine serum albumin as a standard or by U.V. spectrum, assuming for CF_1 and its subunits an $E^{1\%}_{1cm}$ of 2.1 at 280 nm (16).

Fluorescence spectra (excitation at 280 nm, emission between 280 nm and 340 nm) of the different fractions are performed with spectrofluorimeter JY 3D Jobin et Yvon.

SDS-Polyacrylamide gel electrophoresis in urea are performed as in (17).

RESULTS

In the conditions described above, CF_1 is eluted in a rather sharp peak (Fig. 1) and separated from other proteins present in the crude extract, especially the ribulose bisphosphate carboxylase which elutes at a lower ionic strength (6).

The fluorescence spectrum is chosen as a test of purity. The ratio Emission at 308 nm/Emission at 340 nm depends on the tryptophan content of the protein : when the latter is very low, as in CF_1 (3,6 mol tryptophane/mol of enzyme (9)), the fluorescence spectrum is typically that of tyrosine, which has a maximum at 308 nm. In other proteins, the maximum is shifted towards 320-340 nm (Fig. 2).

By successive injections of crude extracts, about 250 mg of CF_1 can be purified in one day. The purest fractions, according to fluorescence ratio

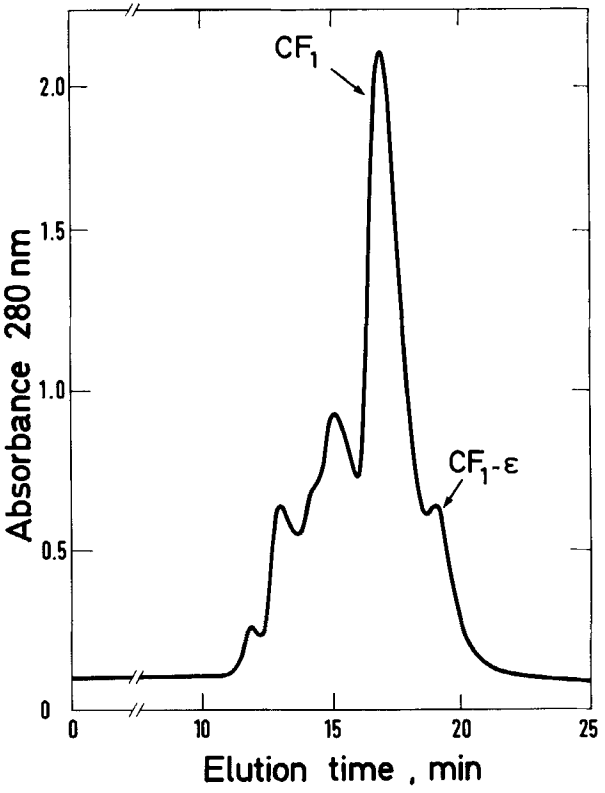

Fig. 1 : HPLC of CF_1 crude extract on Protein Pak DEAE 5 PW 21.5 mm x 150 mm. Flow rate 4 ml/mn. Initial buffer 20 mM Tris-HCl pH 8. Linear ammonium sulfate gradient 0 to 1 M in 30 min. The major peak is CF_1, identified by its fluorescence spectrum (Fig. 2) and by SDS urea PAGE (Fig. 3).

($>1,7$), which represent from 2/3 to 3/4 of total quantity, are used for fractionation into subunits.

The urea-SDS polyacrylamide electrophoresis pattern shows the five subunits (Fig. 3). δ subunit is not lost during this purification, the alkaline pH preventing its detachment as Finell and coll. (8) have mentionned. No band corresponding to ribulose bisphosphate carboxylase (especially the small subunit S, which migrates under the ε subunit (9)) is visible on the gels.

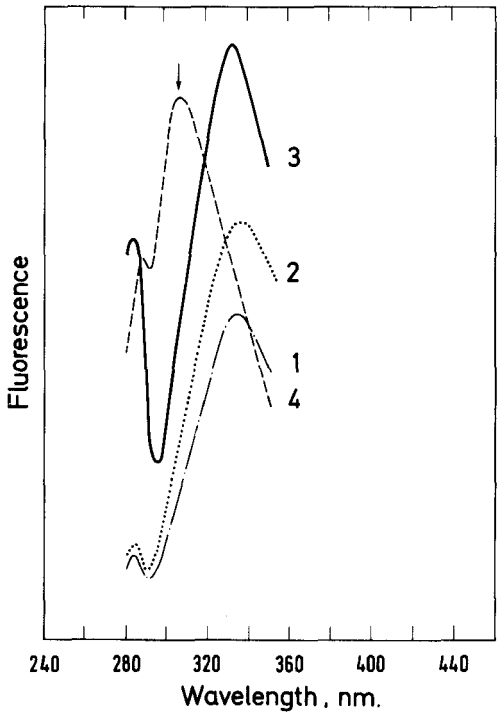

Fig. 2 : Fluorescence spectra of different chromatographic fractions (cf. Fig. 1). Excitation wavelength 280 nm. Spectra n°1, 2, 3, correspond to contaminants. The arrow indicates the CF_1 fraction (n°4)

The chromatogram Fig. 1 shows the presence of a small peak after that of CF_1. This peak becomes more important when, after injection of crude extract, ammonium sulfate gradient is not established immediately and the column is washed with initial buffer. SDS-PAGE shows that this peak corresponds to CF_1-ε complex. During the washing of the column, ε subunit is detached from the fixed CF_1.

The extensive washing of the column (1h30) with the initial buffer, after the injection of purified CF_1, leads to a new chromatogram (Fig. 4), with three major peaks. They are identified by electrophoresis as β subunit, CF_1- δ and CF_1- ε complexes (Fig. 5). CF_1 is no more visible and CF_1-δ replaces it in the elution profile.

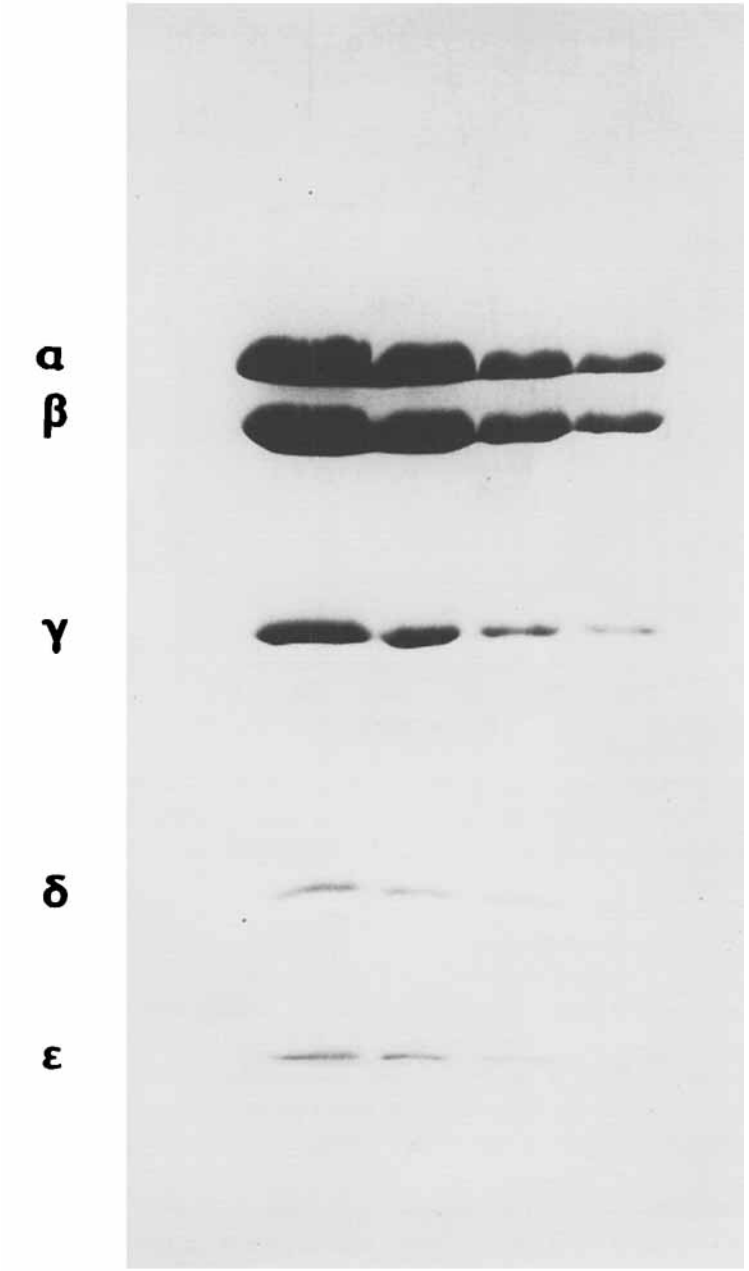

Fig. 3 : SDS-urea PAGE (8 M urea, 12 % polyacrylamide) of HPLC purified CF_1, at four different concentrations.

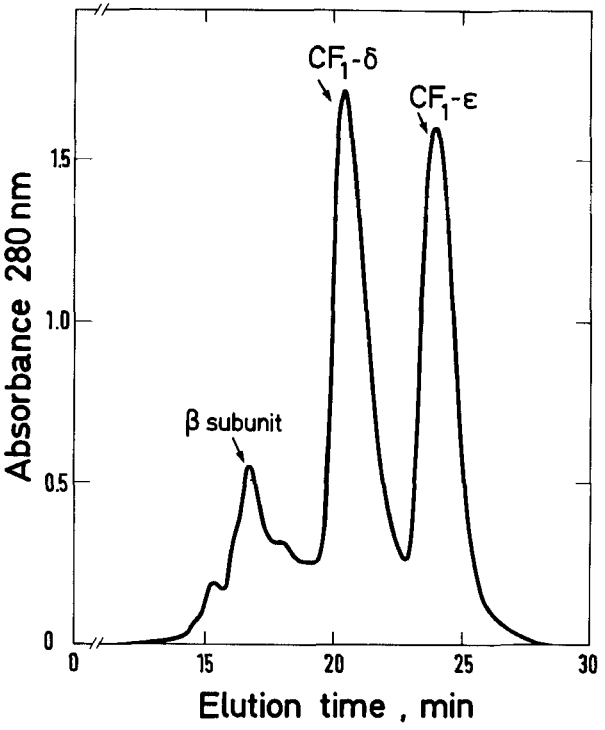

Fig. 4 : Separation of CF_1 subunits by HPLC. Injection of about 100 mg of HPLC purified CF_1. Column washing 1 h 30 min with 20 mM Tris-HCl pH 8, flow rate 4ml/min. Linear ammonium sulfate gradient 0 to 0.8 M in 40 min. Peaks eluting at 17 min, 21 min and 24 min are identified respectively as β subunit, CF_1-δ and CF_1- ε , by SDS urea PAGE (Fig. 5).

The purity of these fractions is quite good according to the SDS-PAGE and the fluorescence ratio (Emission at 308 nm/Emission at 340 nm) of the β subunit and of CF_1- ε complex are higher than those of CF_1- δ or CF_1, which contain ε subunit, the only one subunit to include tryptophan in its composition (9).

The relative quantities of the different peaks are quite reproducible in these conditions. It seems that well-defined fractions of δ and ε subunits are eluted, giving CF_1- δ and CF_1- ε . Some molecules of CF_1 are completely dissociated, giving β subunits. The corresponding small subunits δ , ε and

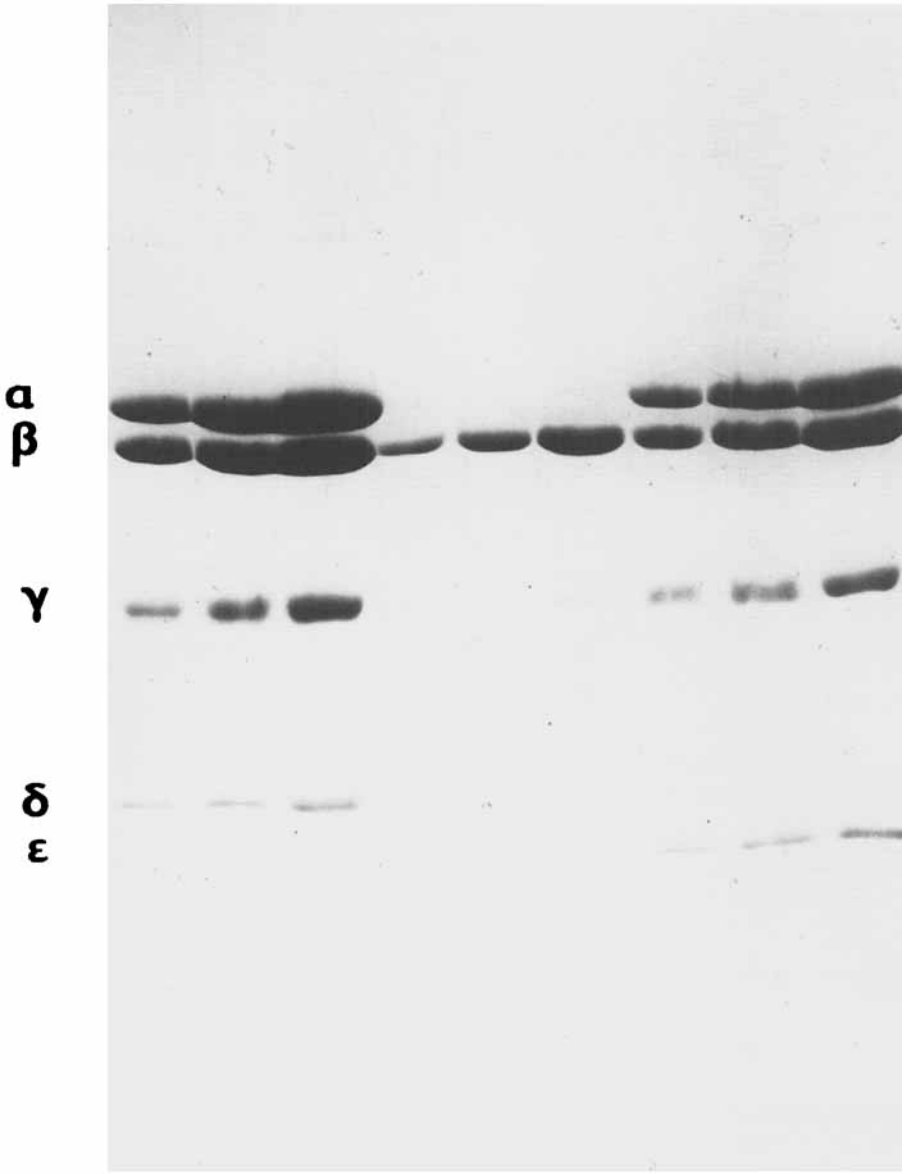

Fig. 5 : SDS-urea PAGE (8 M urea, 12 % polyacrylamide) of HPLC separated CF_1 subunits.
From left to right, CF_1-ε , β subunit, CF_1- δ , each one at three different concentrations.

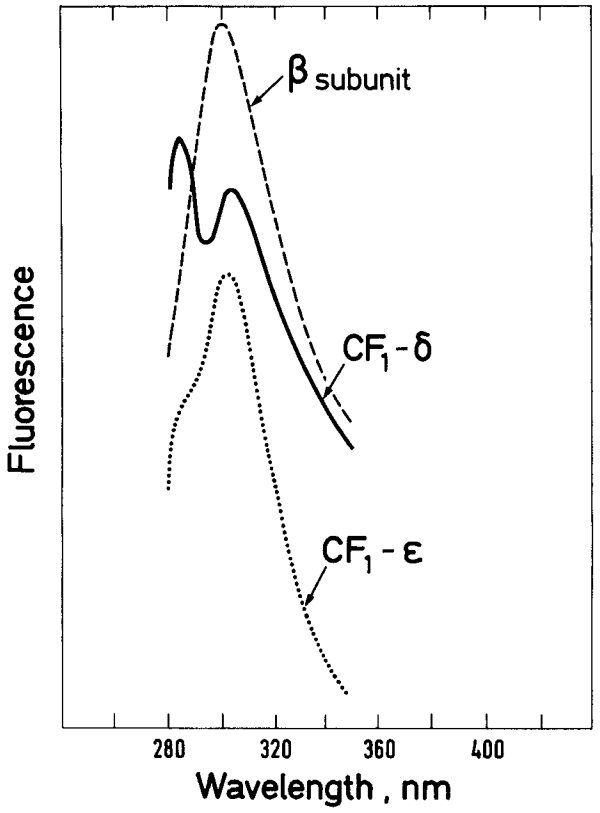

Fig. 6 : Fluorescence spectra of different CF_1 subunits identified by SDS urea PAGE (Fig. 5). Excitation wavelength 280 nm.

most likely γ are eluted during the washing of the column. However, α subunit is not eluted, even with 1 M ammonium sulfate. Further studies are thus necessary to obtain these other subunits.

The overall yield of the recovered β and subunit complexes is around 40 to 50 % (Bio Rad assay).

CF_1- δ and CF_1- ε are water soluble and stable and are stored at 5°C after ammonium sulfate precipitation. Quantities between 20 and 40 mg are prepared per run.

β subunit precipitates within half an hour at ambient temperature after chromatography. It is stored at 5°C in solution in 2M urea or precipitated by

ammonium sulfate for storage and redissolved in 4 M urea before use. Quantities between 2,5 and 5 mg are prepared per run.

CONCLUSION

In conclusion, spinach chloroplast coupling factor CF_1 can be purified from crude extracts in a simple and rapid way by HPLC. A second chromatography involving an extensive washing separates small subunits and gives $CF_1-\delta$, $CF_1-\varepsilon$ complexes and a pure subunit β.

The advantages brought by HPLC over the precedent methods consist in rapidity, quantity and purity of the materials. These properties are essential for enzymatic studies carried with $CF_1-\varepsilon$ (18) or for structural studies and ATP fixation carried with β subunit (19).

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Mmes Jeanne Corvoisier and Jeanine Le Cruer for their skillfull work of preparation of CF_1 crude extracts and to Mlle Patricia Belle for typing the manuscript.

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