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FEBS Letters 340 (1994) 109–113

FEBS 13707

FEBS LETTERS

The Cu_A site of the caa_3-type oxidase of *Bacillus subtilis* is a mixed-valence binuclear copper centre

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Received 22 December 1993

Abstract

A copper-containing domain of the caa_3-type oxidase from *Bacillus subtilis* has been expressed as a water-soluble protein in the cytoplasm of *Escherichia coli*. Electron paramagnetic resonance (EPR) spectra of this purple domain show well-resolved lines in the g_z resonance, both at X-band and S-band frequencies. Interpretation of EPR spectra and analytical data indicate a binuclear copper site consisting of one Cu^{2+} and one Cu^{1+}. This copper site closely resembles Cu_A in subunit II of cytochrome *c* oxidase and is shown here to be a mixed-valence [Cu^{2+}–Cu^{1+}] binuclear centre.

Key words: Cytochrome *c* oxidase; Cu_A; Binuclear copper centre; Nitrous oxide reductase

1. Introduction

Cytochrome *c* oxidase is a haem/copper-containing enzyme that catalyzes the last step of the respiratory pathway in mitochondria and many aerobic bacteria. It has been proposed that electrons from ferrocytochrome *c* first reduce the copper centre, called Cu_A [1]. Subsequently an internal electron flow from Cu_A and/or the low-spin haem results in reduction of oxygen at the binuclear catalytic site of the high-spin haem and a copper ion, Cu_B. The coupling of the oxygen reduction to a translocation of protons accross the membrane results in conservation of the free redox energy [2].

Despite extensive spectroscopic investigation of the Cu_A center, both its structure and function are still a matter of dispute, as reviewed by [3]. A milestone has been the spectroscopic evidence that the Cu_A site of cytochrome *c* oxidase is closely related to the centre A of the nitrous oxide (N_2O) reductase [4–7]. Moreover, alignment of protein sequences of cytochrome oxidase subunits II and N_2O reductases from several sources indicates significant homology [4,7,8].

Based on a seven-line hyperfine splitting observed in multifrequency electron paramagnetic resonance (EPR) experiments, Kroneck et al. [5,9] proposed that the Cu_A-like site of N_2O reductase can be described as a binuclear mixed-valence copper centre. It is tempting to assume that the Cu_A site in cytochrome oxidase has a similar configuration, however, EPR analysis of the latter enzyme is complicated by the interference of haems as well as by the poor resolution of hyperfine structure. Nevertheless, several EPR studies favour the binuclear Cu_A model [5,6,10]. Recently, a Cu_A site has been constructed into a domain of a quinol oxidase from *Escherichia coli*, by introducing the copper ligands into subunit II of cytochrome *bo* [7]. Quantitative analyses of this engineered copper site support the binuclear nature of Cu_A [7,11]. By site-directed mutagenesis five copper ligands have been identified: two cysteines, two histidines and a methionine [11].

Subunit II of the *Bacillus subtilis* caa_3-type cytochrome *c* oxidase (CtaC) is most likely composed of an amino-terminal integral membrane anchor domain, an extramembranous Cu_A-domain and a carboxy-terminal cytochrome *c* domain (Fig. 1) [12]. Here we report the cloning and expression of the Cu_A domain of *B. subtilis* CtaC in *E. coli*. By means of electrospray mass spectroscopy, EPR at X-band and S-band frequencies, and chemical analysis of oxidized and reduced copper, evidence is presented that Cu_A is a mixed-valence binuclear copper centre.

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Abbreviations: DMSO, dimethyl sulfoxide; ES-MS, electrospray mass spectrometry; EPR, electron paramagnetic resonance; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strain BL21(DE3), a λ lysogen containing the T7 RNA polymerase gene under lacUV5 promoter control [13], and derivatives were

SSDI 0014-5793(94)00091-9

grown in rich medium (YT), with addition of ampicillin (100 μg/ml) where appropriate.

2.2. Recombinant DNA techniques

General DNA techniques were carried out essentially as described by [14]. Sequencing of DNA fragments cloned in derivatives of bacteriophage M13 [15] was performed using an Applied Biosystems 370A DNA Sequencer.

2.3. SDS-PAGE and amino-terminal sequence analysis

SDS-PAGE was carried out according to Laemmli [16]. For sequence analysis proteins were blotted to polyvinylidine difluoride membranes, Coomassie-stained protein bands were cut out and the amino-terminal sequence was directly analyzed on an Applied Biosystems 473A Peptide Sequencer.

2.4. Construction and expression

A water soluble Cu_A domain was generated using the polymerase chain reaction (PCR), with as a template the genomic *B. subtilis* clone λ10, carrying the *cta* gene cluster [12]. In this construct the ATG start codon has been introduced as a *Nco*I site (underlined) in the amino-terminal primer (no. 114, 5′-GCGCGCCATGGAGCTAGCGGACACATCACC; encoding MELADTSP); no signal sequence was included. The *Hin*dIII site (underlined) in the carboxy-terminal primer (no. 117, 3′-TATAAGTCTACAGCAGAGAGCCATTAAAAGCTTGCGCG; encoding YKSTAESH*) is adjacent to a stop codon that was introduced in the putative linker region between the Cu_A-domain and the cytochrome *c*-domain (Fig. 1). The PCR fragment was cloned into a pET3d-derived expression vector [13], called pET.B2, and transformed to *E. coli* BL21(DE3), as described before [7].

2.5. Expression and purification

Cultivation of *E. coli* BL21(DE3)/pET.B2, in YT medium supplemented with 1 mM $CuCl_2$, and subsequent preparation of cell free extract was performed essentially as described before [7]. From 3 liter culture, the cell free extract (70 ml) was applied to a Q-Sepharose fast flow column (Pharmacia, 2.6 × 4 cm) that was equilibrated with 20 mM Tris-HCl pH 8.0, 0.1 mM $CuCl_2$, 2% (v/v) DMSO, 0.2 mM PMSF. Proteins were eluted when the NaCl concentration was elevated in a step-wise manner. A purple form of protein B2 eluted at 50 mM NaCl, a colourless form at 100 mM. The purple peak fractions were collected (9 ml) and concentrated using Filtron Microsep microconcentrators to 2 ml. Of the concentrated fraction 1.0 ml was loaded on a PD-10 column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 8.0) and 2% (v/v) DMSO. The purple fractions were pooled, and concentrated to 0.4 ml, resulting in a protein concentration of approx. 250 μM, as determined with the BCA reagent (Pierce) using BSA as a standard. This sample was stored in liquid nitrogen.

2.6. Spectroscopy

Optical spectra were recorded on an Aminco DW2 UV/Vis spectrophotometer at room temperature, in the dual wavelength mode, with the reference set at 578 nm. A filter was used in the near-infrared wavelength region. EPR spectroscopy was performed with a Varian E-9 spectrometer operating at X-band with a home-made He-flow cryostat. S-band measurements were made with a Bruker microwave unit and a rectangular cavity. Settings were as described in the legend of Fig. 3. The molecular weight of the purified B2-domain has been determined by electrospray mass spectrometry (ES-MS). The purified B2-domain was 10 times diluted in HPLC-grade distilled water, and loaded on a small Sephadex G-25 column, equilibrated with distilled water. The coloured fractions were pooled, the concentration was approx. 10 μM. A Sciex API III electrospray mass spectrometer was calibrated with myoglobin; the settings of the spectrometer were as described before [11]. Spectra were deconvoluted as described by [17].

2.7. Chemical determination of copper

Copper was determined with 2,2′-biquinoline-4,4′-dicarboxylic acid (BCA) in alkaline solution as supplied by Pierce. An extinction coefficient at 540 nm for reduced copper complex with BCA was determined (ε_{540} = 6.7 $mM^{-1} \cdot cm^{-1}$). Determination of the oxidation/reduction state of copper (Cu^{2+}/Cu^{1+}) was based on the fact that BCA only complexes Cu^{1+} [18]. Artefactual reduction of copper by cysteine residues and via the 'Biuret' reaction were effectively blocked by addition of 20 mM EDTA, which preferentially complexes Cu^{2+}. First after full colour development due to originally reduced copper, the amount of oxidized copper was determined by measuring the increase in absorbance at 540 nm after reducing Cu^{2+} (bound to EDTA) to Cu^{1+} with Na-dithionite.

3. Results and discussion

3.1. Expression and purification of CuA domain

In order to investigate the nature of the Cu_A-centre of cytochrome *c* oxidase, we used a combined molecular genetic and spectroscopic approach. In spectroscopic investigations of cytochrome *c* oxidase the detection of Cu_A has always been difficult due to interference of haems. In the present study the Cu_A domain from the caa_3-type oxidase of *B. subtilis* has been overexpressed in the cytoplasm of *E. coli* in a water soluble form: protein B2. Densitometric scanning of SDS-PAGE gels indicates that protein B2 comprises approx. 30% of the total cell protein; it migrates with an apparent mass of about 17 kDa (not shown). From the elution profile on a Superdex 75 gel filtration column the molecular mass of protein B2 was estimated to be between 15–20 kDa (not shown). Both observations fits well with the calculated molecular mass of the B2 monomer: 16.9 kDa.

A purple form of the B2 protein was purified to near homogeneity by anion-exchange chromatography. These coloured fractions of protein B2 were collected and further characterized spectroscopically (see below). Typically, however, about half of the B2 protein eluted as a colourless form at a higher salt concentration. The latter fraction could not be reconstituted with copper. A similar phenomenon has been reported previously for two

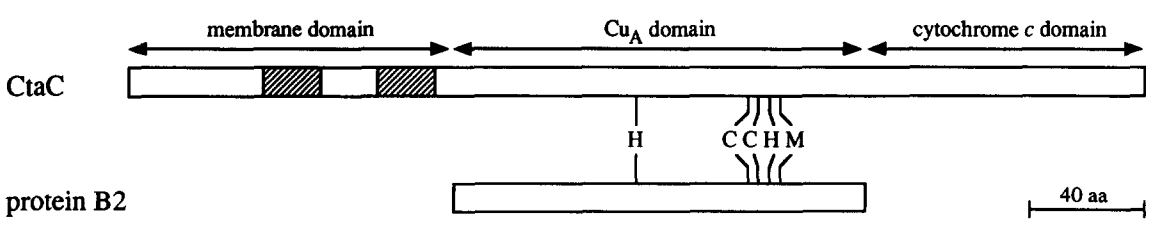

Fig. 1. Subunit II of the caa_3-type oxidase of *B. subtilis* (CtaC) may be composed of three domains as indicated. In the designed water-soluble Cu_A domain (protein B2) a methionine has been introduced at the amino-terminal site and a stop-codon at the carboxy-terminal site of the putative Cu_A domain. The proposed copper ligands [11] are indicated.

other copper proteins expressed in *E. coli*: the engineered Cu_A domain (purple CyoA) [7,11] and azurin [19].

3.2. Optical properties

In intact cytochrome *c* oxidase the broad absorbance peak in the near-infrared (800–830 nm) is the only part of the Cu_A spectrum that is not covered by haem absorbance. The optical spectrum of the *Bacillus* Cu_A domain, however, shows absorbance maxima at 365 nm, 480 nm, 530 nm in addition to the broad band between 775–800 nm, with a possible shoulder around 830 nm (Fig. 2). The apparent blue-shift of the latter band is also reported for the caa_3-type oxidase of *Bacillus* PS3, where the corresponding band is located at 780 nm [20]. It is obvious that the optical spectrum of protein B2 is very similar to that of the purple CyoA domain [7], and to the absorbance peaks ascribed to centre A of N_2O reductase from *P. stutzeri* (cf. [4,20]). Addition of reductants Na-dithionite and Na-ascorbate led to bleaching of the visible absorbance.

3.3. EPR spectroscopy

In the EPR spectrum of the N_2O reductase a seven-line hyperfine splitting is observed, which has been interpreted to originate from a binuclear Cu_A-like centre (centre A) [5,9]. On basis of the aforementioned similarities, it is not unlikely that Cu_A in cytochrome *c* oxidase is a binuclear center as well [4–8]. This model would be in agreement with accurate metal analysis of cytochrome oxidase that shows a stoichiometry of three coppers per two heme irons [22,23]. However, the EPR spectrum of Cu_A in cytochrome *c* oxidase recorded at Q-band, X-band, C-band and S-band frequencies does not show a clear (seven-line) hyperfine splitting in any of the three resonances. This is in part due to spectral overlap of the g_y resonance of haem *a* with the g_z resonance of Cu_A.

EPR analysis of the *Bacillus* domain, however, shows a (partly) uncovered Cu_A spectrum (Fig. 3). The lineshape of the X-band EPR spectrum of protein B2 (Fig. 3A) is typical for Cu_A, with $g_z = 2.178$, $A_z = 3.82$ mT and $g_{x,y} = 1.99$–2.03 (cf. [6,10]). In addition, the Cu_A-like signal of this soluble domain rapidly relaxes because even at temperatures between 20–40 K it is difficult to saturate, and it has broadened beyond detection at 135 K (not shown). All these features are very similar to those of Cu_A in intact cytochrome *c* oxidase (cf. [10]) and centre A in N_2O reductase [6].

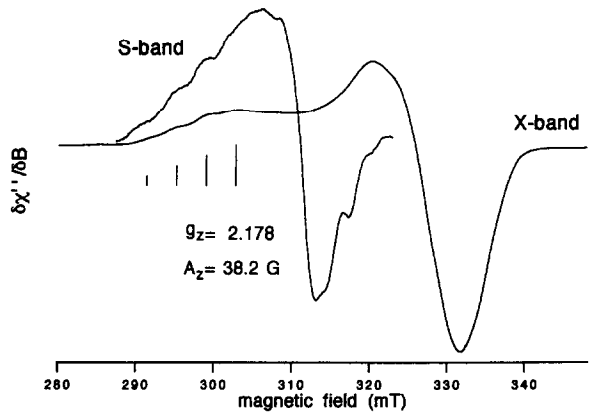

Fig. 3. X-band and S-band EPR spectra of protein B2. Spectra recorded at both X-band and S-band frequencies are plotted on the same magnetic-field scale, aligned at $g_z = 2.178$. The S-band spectrum is an average of 40 spectra. Vertical lines mark the position and relative intensities of the first four lines of a seven-line hyperfine pattern in the g_z resonance. Experimental: X-band, S-band: modulation amplitude 1.0, 1.25 mT; microwave power 20, 8 mW; temperature 30, 12 K; frequency: 9.232, 3.959 GHz.

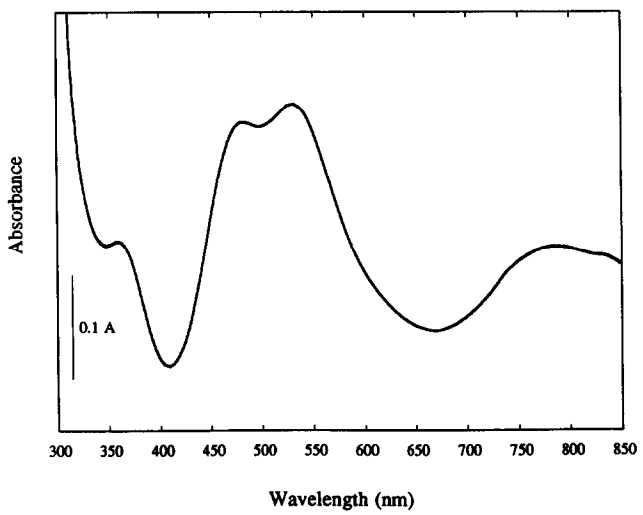

Fig. 2. Optical spectrum of the purified protein B2.

Apart from the Cu_A signal, a type 2 copper signal is observed, see e.g. the hyperfineline at 285 mT in the X-band spectrum. Under these experimental conditions this signal, which is ascribed to adventitious copper, is saturated and therefore hardly visible in the spectra shown in Fig. 3, even though it is present in an amount equivalent to that of Cu_A (see Table 1). Attempts have been made to remove this type 2 copper. Gel filtration at different pH values in the presence of either EDTA, CDTA, or cyanide were ineffective; addition of specific copper-complexing compounds such as BCA or diethyldithiocarbamate destroyed the Cu_A site. Only dilution followed by exchange of the Tris buffer with distilled water did remove some of the type 2 copper (see below).

The type 2 copper signal disturbs the fine details of the spectrum, in particular in the *g*-perpendicular region, and thus prevents an accurate analysis of the EPR signal by simulation. An alternative approach to discriminate between a mononuclear and a binuclear copper site is comparison of EPR spectra recorded at X-band and S-band frequency (Fig. 3). The g_z-resonance of Cu_A of protein B2 shows 4 (X-band) and 3–4 (S-band) equidistant lines with $A_z = 3.82$ mT. This clear hyperfine splitting could not be observed in intact cytochrome *c* oxi-

dase because of spectral interference by the g_y resonance of heme *a* (see e.g. [6]). The relative intensity (i.e. amplitude × linewidth) of the hyperfine lines observed is close to the 1 : 2 : 3 : 4(: 3 : 2 : 1) ratio expected for a binuclear copper center consisting of two equivalent copper ions. A mononuclear copper center would show four equidistant hyperfine lines with equal intensities.

By plotting the spectra obtained at the two microwave frequencies on the same magnetic field scale, the magnitude and position of the hyperfine splittings in the g_z line can be directly compared, provided that the two spectra are properly aligned. The spectra in Fig. 3 have been aligned at $g_z = 2.178$. It is evident that the position of the g_z-hyperfine lines coincide (the position of hyperfine lines is frequency independent, provided the correct value of g_z is used as the reference point). This coincidence implies that the g_z-hyperfine lines observed in the S-band and X-band spectra are part of a seven-line pattern originating from two interacting copper nuclei. Analysis in terms of a mononuclear copper site would imply an alignment of the S- and X-band spectra with $g = 2.220$ as the reference point (i.e. between the second and third hyperfine line) in which case the two hyperfine patterns do not match at all (not shown).

3.4. *Electrospray mass spectrometry*

Additional evidence in favour of a binuclear Cu_A model is provided by electrospray mass analysis of protein B2. The calculated molecular mass of apo-protein B2 as deduced from the DNA sequence is 16,785 Da; this includes the amino-terminal methionine residue which was detected in the amino-terminal amino acid sequence (MELADT). The molecular mass of the apo- and holoform of protein B2 has been determined by electrospray-mass spectrometry in three experiments in which the amount of formic acid was varied (Fig. 4). The rational of this titration is that the copper ligands are protonated at lower pH values, thereby removing the copper from the Cu_A site. At the highest concentration of formic acid (Fig. 4C) only apo-protein is present: the molecular mass is determined at 16,784 Da, which is in excellent agreement with that calculated from the DNA sequence. The molecular mass of holo-B2 (Fig. 4A,B) is about 126–128 Da larger than that of the apoprotein, consistent with the presence of two copper atoms in the holoprotein.

Table 1.
Determination of the copper concentration of protein B2 by EPR and chemically with BCA in the presence of 20 mM EDTA.

	Cu_A	Extraneous Cu	Cu^{1+}	Total Cu
EPR	271 μM (34%)	255 μM (32%)	–	797 μM*
BCA	–	–	273 μM (34%)	804 μM

The concentration of B2 protein was determined to be 250 μM. The total copper content deduced from EPR measurements (*) was calculated by assuming that Cu_A represents two Cu/spin = 1/2 system.

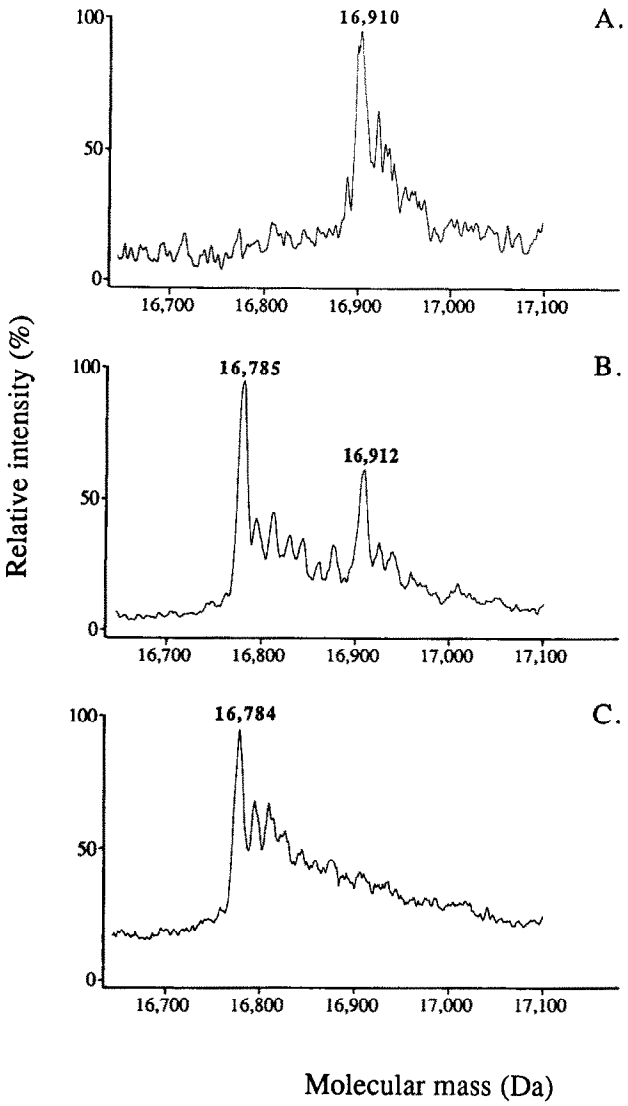

Fig. 4. Electrospray mass spectra of protein B2. (A) protein B2 in water (holo-form); (B) protein B2 in 0.05% formic acid; (C) protein B2 in 0.3% formic acid (apo-form). The calculated mass of the B2 domain is 16,785 Da.

3.5. *Quantitation of Cu^{1+} and Cu^{2+}*

The X-band EPR spectra of Cu_A and of adventitious copper have been quantitated. Both signals represent approx. the same amount of $S = 1/2$ system (Table 1). Furthermore, the EPR detectable copper comprises about two-third of total amount of copper, as determined by chemical analysis with BCA in alcaline solution. Analysis of the redox state of copper in the B2 protein preparation with BCA, indicates that about one third of the total amount of copper is Cu^{1+} (Table 1). The electrospray mass data indicate that the preparation is homogeneous, i.e. it does not contain apo-protein B2 (see Fig. 4A); this observation is important for the proper interpretation of the quantitative copper analysis.

3.6. Cu_A is a binuclear, mixed-valence copper centre

The chemical copper analysis and the determined redox state are consistent with the quantitation of the EPR signals: about one-third of copper is present as Cu^{1+} (Table 1), a state which is, in general, EPR silent. The presence of three copper atoms per monomeric B2, as determined by chemical analysis (Table 1), is not necessarily inconsistent with the ES-MS data. To prepare the sample for ES-MS analysis, protein B2 is first diluted and subsequently transferred from 20 mM Tris to distilled water. This procedure results in a 40–70% loss of type 2 copper, as analyzed chemically (not shown). A similar removal of adventitious copper during preparation for ES-MS has been demonstrated for the purple *E. coli* CyoA domain [7,11]. Unfortunately, the B2 protein in distilled water could not be concentrated to the level required for EPR studies.

Interpretation of the analytical data and the EPR spectra at X-band and S-band frequencies indicate that the B2 protein contains one adventitious Cu^{2+} site and one binuclear copper site consisting of one Cu^{2+} and one Cu^{1+}. The latter site closely resembles Cu_A in subunit II of cytochrome *c* oxidase and is shown here to be a mixed-valence [Cu^{2+}–Cu^{1+}] binuclear center.

Acknowledgements: Dr. Matti Saraste (EMBL, Heidelberg) is gratefully acknowledged for stimulation and collaboration at early stages of this project and for valuable discussions, Willem Reijnders (VU, Amsterdam) assisted with the DNA sequencing, Roel van der Schors (VU, Amsterdam) determined the N-terminal amino acid sequence, Dr. Kenneth Morand (EMBL, Heidelberg) performed the ES-MS measurements. We also acknowledge Dr. Simon Albracht and H.J. Gerritsen for sharing thier expertise to construct an interface for our EPR spectrometer and Dr. Simon Albracht for his help with the S-band measurements. C.v.W. had a fellowship from the Netherlands Organization for the Advancement of Research, J.v.d.O. has a fellowship from Royal Dutch Academy of Science.

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