Topological organization of the Rieske iron-sulphur protein and subunit IV in the cytochrome *bc₁* complex of *Rhodobacter sphaeroides*

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The ubiquinol-cytochrome *c₂* oxidoreductases (cytochrome *bc₁* complex) of *Rhodobacter sphaeroides* contains highly conserved cytochrome *b*, cytochrome *c₁* and Rieske FeS subunits, as well as a unique 14 kDa polypeptide, designated as subunit IV, thought to function as a ubiquinol-binding protein [Yu and Yu (1991) *Biochemistry* 30, 4934–4939]. As the topology of subunit IV is unknown and that of the FeS subunit remains a matter of debate, both the inner (cytoplasmic) and outer (periplasmic) surfaces of the intracytoplasmic membrane (ICM) were digested with proteinase K, and cleavage products were identified by immunoblotting. In uniformly oriented chromatophore vesicles (inner ICM surface exposed), fragments of approx. 4 and 1 kDa were removed from subunit IV and the FeS protein respectively.

Neither subunit IV nor the FeS protein was cleaved from the outer ICM surface as exposed in osmotically protected spheroplasts or as presented to proteinase K after microencapsulation of the protease in unilamellar liposomes and fusion of these structures to chromatophore vesicles. Studies with the isolated *bc₁* complex, however, suggested that the C-terminal domain of the Rieske FeS, thought to reside on the periplasmic side of the ICM, was resistant to proteinase K. Overall, these results suggest a single N-terminal transmembrane helix for the FeS protein, with exposure of the N-terminus to the cytoplasm and an orientation in which a major, N-terminal portion of subunit IV is located in the cytoplasm with the predicted C-terminal transmembrane domain anchoring this polypeptide to the membrane.

INTRODUCTION

The ubiquinol-cytochrome *c₂* oxidoreductase (cytochrome *bc₁* complex) occupies a central position in the mitochondrial respiratory chain and in cyclic electron flow of photosynthetic bacteria [1]. In most photosynthetic bacteria, the *bc₁* complex consists of three catalytic subunits: cytochrome *b*, cytochrome *c₁*, and the Rieske FeS protein [2–5]. An additional polypeptide (subunit IV) is tightly associated with the *bc₁* complex isolated from *Rhodobacter sphaeroides* [6], and immunoinhibition studies suggest that this 14 kDa protein is essential for oxidoreductase activity [7]. Moreover, both subunit IV and cytochrome *b* have been implicated in ubiquinone binding by photoaffinity labelling [8].

Definitive structural and functional studies of the *R. sphaeroides* cytochrome *bc₁* complex depend upon a detailed knowledge of the topological arrangement of each subunit within the membrane. It is now well established that cytochrome *c₁* is located mainly in the periplasm and contains a single, C-terminal membrane-spanning anchor [9,10], whereas cytochrome *b* consists of eight transmembrane helices with both the N- and C-termini located at the cytoplasmic surface of the intracytoplasmic membrane (ICM) [11,12]. In contrast, the topology of the Rieske FeS protein remains a matter of debate in both mitochondrial [13–15] and bacterial [10,16] *bc₁* complexes, as well as in the homologous cytochrome *b₆f* complex of chloroplast thylakoids [17,18]; models in which this subunit either contains a single N-terminal membrane span [13,16,17] or lacks transmembrane helices [15,18] have been proposed. Identification of a C-terminal stretch of predominantly non-polar residues in subunit IV by amino acid sequence analysis [6], together with the hydropathy profile derived from the deduced primary structure [19], have suggested that this polypeptide contains a C-terminal membrane anchor. Clearly, biochemical methods are essential to establish the membrane topology of this unique polypeptide as well as that of the FeS protein.

In the present study, sealed, uniformly oriented chromatophore vesicles (with the cytoplasmic surface of ICM exposed) and osmotically protected spheroplasts (with much of periplasmic surface of ICM available) were prepared from *R. sphaeroides* and digested with proteinase K to further assess the membrane topology of the Rieske FeS protein and of subunit IV. The inner (periplasmic) surface of chromatophores was also digested by a recently developed procedure in which chromatophore vesicles are fused to unilamellar liposomes [20] in which proteinase K is encapsulated [10]. Although no digestion of either of these subunits could be demonstrated on the periplasmic side of the membrane, immunoblotting analyses of cleavage products obtained with chromatophores suggested that the bulk of subunit IV is located on the cytoplasmic side of the ICM, and confirmed the possibility that the *R. sphaeroides* Rieske FeS protein contains a single N-terminal transmembrane helix.

MATERIALS AND METHODS

Preparation of chromatophores

R. sphaeroides (wild-type strain NCIB 8253) was grown photoheterotrophically at 1800 lux (1 lux $\approx$ 10.76 foot candles) as described previously [6], and chromatophores were isolated from French-press extracts by rate-zone sedimentation in sucrose density gradients [20]. The chromatophore fraction was pelleted by ultracentrifugation, resuspended in a minimal volume of 1 mM Tris/HCl, pH 7.5, containing 25% (v/v) glycerol and stored at -80°C . Uniformly oriented chromatophores for topology studies were obtained by chromatography on a Sepharose 2B-CL column [20], and stored at 4°C in the dark. Assays of ferrocyclochrome *c* photo-oxidation in the presence and absence of octyl- β -D-glucopyranoside suggested that these preparations consist of $\sim 90\%$ 'inside-out' ICM vesicles with the cytoplasmic aspect of the ICM exposed [20].

Preparation of spheroplasts

Spheroplasts were prepared essentially as described previously [21]. Photoheterotrophically grown cells were pelleted and washed twice with 1 mM Tris/HCl, pH 7.5, resuspended to an A_{680} of 6.5 in 50 mM Tris/HCl, pH 7.5, containing 25% (w/v) sucrose, and stirred at room temperature for 10 min, followed by addition of 0.1 vol. of lysozyme (6.4 mg/ml) and EDTA solutions (20 mg/ml) respectively. After incubation, the spheroplasts were pelleted and suspended to the same volume in 50 mM Tris/HCl, pH 7.5, containing 25% sucrose, 200 mM NaCl and 1 mM CaCl_2 .

Digestion of chromatophores and spheroplasts with proteinase K

Osmotically protected spheroplasts and column-purified chromatophores were digested with proteinase K, a broad-specificity endoprotease, by previously described procedures [10]. The digested spheroplasts were centrifuged at 12 100 *g* in a Sorvall SS-34 rotor and osmotically lysed in 10 mM Tris/HCl, pH 7.5, 2 mM phenylmethanesulphonyl fluoride (PMSF), sonicated and recentrifuged at 12 100 *g* to remove unlysed spheroplasts. The supernatant was further centrifuged at 48 200 *g* for 30 min. The pelleted spheroplast-derived membrane fraction was sonicated again in the same PMSF-containing buffer.

Isolation of cytochrome bc_1 complex

The bc_1 complex was extracted from chromatophores with dodecyl β -D-maltoside (Sigma) and purified by a procedure similar to that of Andrews et al. [22], except that the final Sepharose CL-4B chromatography step was omitted. The purified bc_1 complex was stored in 50% (v/v) glycerol at -80°C .

Polyacrylamide gel electrophoresis

Chromatophores and the sonicated spheroplast-derived membranes were precipitated with an equal volume of 12% (w/v) trichloroacetic acid. Pellets were exposed to NH_3 vapour for ~ 30 min on ice, the NH_3 was evaporated, and the residues were dissolved in sample buffer [10] and denatured by boiling for 1 min. SDS/PAGE was performed in a Mini Protean II apparatus (Bio-Rad) using the buffer system of Laemmli [23]; the separating gel was prepared with 14% (w/v) acrylamide. The protein standards (Bio-Rad) used to calibrate the gels had the following molecular masses (Da): lysozyme, 14 400; soybean trypsin inhibitor, 21 500; carbonic anhydrase, 31 000; ovalbumin, 45 000; BSA, 66 200; and phosphorylase *b*, 97 400. The procedure for quantitative detection of *c*-type cytochromes in SDS/PAGE has been described [10], and the values obtained were shown by densitometric scans of identical samples to vary by $\pm 5\%$ [10]. Protein content was determined using the method of Lowry et al. [24].

Immunological procedures

Antisera against reaction centre subunit H and the Rieske FeS protein were raised in rabbits by C. N. Hunter and D. J. Purvis respectively as described by Purvis et al. [6] and were purified further by DEAE-Affi-Gel Blue column chromatography as described by Bio-Rad, followed by ultrafiltration using a YM-30 membrane in a Centricon-30 microconcentrator (Amicon). Antibodies to subunit IV were raised at Pocono Rabbit Farm and Laboratory (Canadensis, PA, U.S.A.), by injection of 2 mg of subunit IV protein isolated from the purified bc_1 complex by preparative SDS/PAGE. IgGs were further purified from the

antisera by ammonium sulphate precipitation and DEAE-Affi-Gel Blue chromatography and stored at -20°C in the presence of 0.1% (w/v) sodium azide. Immunoblots with the purified bc_1 complex showed that the subunit IV antisera also contained antibody to the more highly immunogenic Rieske FeS protein; these antibodies were removed partially by addition of isolated FeS protein to the purified IgG during the blotting procedure. Immunoblotting was performed as described by Towbin et al. [25] using peroxidase-labelled goat anti-(rabbit IgG) (Bio-Rad) as the second antibody and 4-chloro-1-naphthol for staining.

Assay of ubiquinol-cytochrome *c* oxidoreductase activity

The reaction mixture (1 ml) contained 50 mM sodium phosphate buffer, pH 7.0, 50 μM cytochrome *c*, 1 mM EDTA and trypsin-treated bc_1 complex (3 μg of protein). The reaction was initiated with 25 μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decylubiquinone). The specific enzyme activity of the untreated bc_1 preparation was $\sim 52 \mu\text{mol}$ cytochrome *c* reduced $\cdot \text{min}^{-1} \cdot \text{mg}$ protein $^{-1}$.

RESULTS

Approaches to proteolytic digestion at the periplasmic ICM surface

Proteinase K was microencapsulated in phosphatidylglycerol liposomes and rapidly fused to chromatophores in the presence of Ca^{2+} [20] to facilitate exclusive and controlled digestion at the inside (periplasmic) surface of the membrane [10]. Immunoblot

Figure 1 Effect of proteinase K on cytochrome c_1 in chromatophore and spheroplast preparations

Membrane preparations were digested with 5.0×10^{-3} p.k.u. $\cdot \text{ml}^{-1}$ at 35°C for the indicated times and subjected to SDS/PAGE (a p.k.u. represents one unit of proteinase K activity under the assay conditions defined by Theiler and Niederman [10]). 60-PK indicates chromatophore and spheroplast preparations incubated at 35°C for 60 min without proteinase K. (a) Cytochrome c_1 detected by haem staining. (b) Densitometric scans of photographic negatives obtained using a Joyce-Loebl Chromoscan 3 densitometer; integration of the cytochrome c_1 peak showed that, in spheroplasts, $\sim 55\%$ was degraded after 60 min; further digestion was not observed thereafter (results not shown). The lack of complete cytochrome c_1 digestion apparently resulted in part from inaccessibility of sequestered ICM regions to macromolecular probes, which may represent as much as 25% of the inner ICM surface [33]; this would suggest that $\sim 70\%$ of the available cytochrome c_1 was degraded after 60 min.

Figure 2 Effect of proteinase K on reaction-centre subunit H in chromatophore and spheroplast preparations

The conditions of digestion were the same as in Figure 1. (a) Subunit H and its cleavage product (H*) were detected by immunoblotting. Whereas no cleavage of subunit H was observed in spheroplasts, a 1–2 kDa fragment was removed from this protein in chromatophores, as reported previously [10,30]. The remainder of the cytoplasmically localized globular domain of subunit H [31] has been shown to be refractory to protease digestion. (b) Densitometric scans of photographic negatives obtained using a Joyce–Loebl Chromoscan 3 densitometer.

analysis indicated that both subunit IV and the Rieske protein remained intact during a 60 min incubation (results not shown). This is consistent with a limited exposure of subunit IV at the periplasmic aspect of the ICM and confirms the lack of Rieske protein cleavage observed in [10], despite evidence that the bulk of this subunit is located in the periplasm [16,26–29]. It was previously shown [10] that 70–75% of the cytochrome c_1 , a marker of the periplasmic ICM surface [9,10], was degraded following liposome–chromatophore fusion, whereas reaction-centre subunit H [30,31], a marker for the cytoplasmic surface, remained intact.

The possibility that the fusion conditions induced structural changes affecting the proteolytic susceptibility of the FeS protein and subunit IV was examined in osmotically protected spheroplasts, shown previously to be largely intact with a ‘right-side out’ orientation of over 90% [32] and to be useful for topological analyses of the *R. sphaeroides* ICM [21,30,32]. The disappearance of ~70% of the available cytochrome c_1 (Figure 1) after 60 min of proteinase K digestion and a failure to degrade reaction-centre subunit H (Figure 2) showed that these preparations were suitable for the present study.

Cleavage of subunit IV and the Rieske iron-sulphur protein in spheroplasts and chromatophores

Immunoblot analysis (Figure 3) also showed that subunit IV was not cleaved from the periplasmic surface of the ICM as exposed in the spheroplast preparations. In chromatophores, however, ~4 kDa of subunit IV was partially removed after incubation at 35 °C, whereas after 60 min at 50 °C, the subunit IV band had disappeared and only the 10 kDa IV* (see Figure 3) band was

Figure 3 Effect of proteinase K on subunit IV in chromatophore and spheroplast preparations

The conditions of digestion were the same as in Figure 1. Subunit IV and its cleavage product (IV*) were detected by immunoblotting with subunit IV antibody (a) and with IgG that was enriched for subunit IV antibody as described in the Materials and methods section (b). The lack of cleavage of subunit IV in the spheroplast preparations was suggested by the unchanged intensity of subunit IV in immunoblots, as an apparent artifactual band was found near the bottom of the gel (results not shown).

Figure 4 Effect of proteinase K on the Rieske FeS protein in chromatophore and spheroplast preparations

The conditions of digestion were the same as in Figure 1. The Rieske protein (FeS) and its cleavage product (p1) were detected in chromatophores by immunoblotting with IgG obtained from the antisera raised against subunit IV which also contained Rieske FeS antibodies. Antibodies raised against the Rieske FeS protein by Purvis et al. [6] were used for immunoblotting of the spheroplast preparation. The slightly reduced staining intensity in the Rieske protein after 60 min of spheroplast digestion is evidently an artifact of the immunoblotting procedure because no cleavage products were observed.

observed. The mass of the Rieske FeS protein was also reduced by proteinase K in the chromatophore preparations (Figure 4); at 50 °C, this subunit was completely converted to a 19 kDa p1 cleavage product. A second ~16 kDa degradation product reported previously [10] was not observed here at either 35 or 50 °C. No cleavage of the Rieske protein was apparent in the digested spheroplasts, confirming the results obtained with the liposome delivery system.

Cleavage of the Rieske iron-sulphur protein in the isolated cytochrome bc_1 complex

The formation of an ~16 kDa cleavage product (p2) from the Rieske subunit when the isolated *R. sphaeroides* bc_1 complex was digested with proteinase K (Figure 5) supports the possibility that this protein exists in the periplasm in a compact, protease-resistant conformation [34]. The electrophoretic mobility of this p2 cleavage product was identical to that obtained when the bc_1

Figure 5 Comparison of digestion of isolated cytochrome bc_1 complex by proteinase K and chymotrypsin

The bc_1 complex (100 μ g), isolated as described by Andrews et al. [22], was digested with 1 μ g of chymotrypsin (CH) or proteinase K (PK) for 1 h at 35 °C. The Rieske protein (FeS) and its cleavage product (p2) were detected by immunoblotting with antibodies raised against the Rieske FeS protein by Purvis et al. [6].

Figure 6 Effect of trypsin on subunit IV in the isolated cytochrome bc_1 complex

The bc_1 complex (1 mg of protein $\cdot$ ml $^{-1}$) in 50 mM Tris/HCl buffer, pH 8.0, containing 10% (v/v) glycerol and 0.01% (w/v) dodecyl maltoside was digested with trypsin (2.5 μ g $\cdot$ mg of protein $^{-1}$) in the presence of 0.5% (w/v) sodium cholate. Cholate has been shown to specifically expose subunits of the cytochrome b_6f complex to proteolytic digestion [35]. Aliquots were removed at the indicated times and the reaction was terminated with 5 mM PMSF. The cleavage pattern was analysed by SDS/PAGE after solubilization at 37 °C for 2 h, and was stained with Coomassie Brilliant Blue. The subunits and apparent molecular masses observed were: cytochrome b (cyt b), 43 kDa; cytochrome c_1 (cyt c_1), 33 kDa; Rieske FeS protein (FeS), 20 kDa; subunit IV, 14 kDa. IV' and IV'' are the major and minor subunit IV cleavage products respectively.

complex was digested with chymotrypsin, which was shown in detergent-treated spheroplast-derived vesicles to cleave the Rieske subunit directly downstream from the putative N-terminal membrane anchor and to release a water-soluble form of the protein containing the FeS cluster [16].

Limited proteolytic digestion of subunit IV in the isolated cytochrome bc_1 complex

Figure 6 shows that when the isolated bc_1 complex was subjected to limited proteolytic digestion with trypsin, subunit IV was specifically cleaved while the other subunits appeared to remain intact. This procedure, which has resulted in selective degradation of polypeptides of the mitochondrial cytochrome bc_1 [34] and chloroplast cytochrome b_6f complexes [35] and has aided in delineating their possible functions, gradually gave rise to a subunit-IV-derived fragment of 9–10 kDa. This product may have arisen from cleavage at trypsin-sensitive sites identified in the isolated protein by Purvis et al. [6], suggesting that the N-terminal portion was removed. Although a smaller fragment was seen near the gel front, the larger one was the main cleavage

product, as demonstrated by immunoblotting with subunit IV antibodies (results not shown). The portion of the subunit IV protein removed with trypsin was not involved in catalytic reactions of the bc_1 complex, as no significant change in ubiquinol-cytochrome c reductase activity was observed even after cleavage had reached completion (results not shown).

DISCUSSION

In contrast to the simpler prokaryotic cytochrome bc_1 complexes [1–6], preparations of the mitochondrial ubiquinol-cytochrome c oxidoreductase contain as many as eight additional polypeptides, designated as supernumerary subunits [1], that lack prosthetic groups and have no apparent direct catalytic role in energy transduction [36–44]. Subunits in the molecular-mass range 14–17 kDa have been implicated as Q-binding proteins by photoaffinity labelling [36,37], and several other supernumerary polypeptides have been shown to be required both for the regulation of electron transport [38–41] and for assembly and processing of the redox-active subunits [39,40,42–44]. The single non-catalytic polypeptide (subunit IV) of the *R. sphaeroides* cytochrome bc_1 complex therefore provides a unique opportunity to examine the role of a supernumerary subunit without the complexities of multiple constituents.

Immuno-inhibition studies [7] have suggested that subunit IV is essential for the activity of the *R. sphaeroides* bc_1 complex. All four subunits were retained from detergent-solubilized chromatophores by an anti-subunit IV immunoaffinity column, confirming that subunit IV is an integral component of the overall complex [6]. Anti-(subunit IV) IgG also inhibited the ubiquinol-cytochrome c reductase activity of the isolated complex, but this may have resulted from antibody-induced conformational changes, rather than direct interference with the catalytic activity of redox centres. A more detailed understanding of any functional roles of subunit IV requires elucidation of the topological organization of this polypeptide within the ICM.

The lack of subunit IV cleavage both in spheroplast preparations and liposome-chromatophore fusion products, together with removal of a mass of $\sim$ 4 kDa from this polypeptide at the surface of chromatophores, is consistent with a model in which subunit IV is anchored to the ICM through a transmembrane α -helix near the C-terminus [19]. This would leave $\sim$ 80 amino acid residues, comprising the hydrophilic N-terminal domain, exposed to the cytoplasm. The 15 C-terminal residues thought to face the periplasmic side were not apparently attacked by the protease, presumably because of a compact conformation or a close association with the membrane.

This overall topological arrangement is also supported by the preponderance of positively charged amino acids immediately upstream from the putative C-terminal transmembrane segment, which are typically found on the cytoplasmic side of the membrane ('positive inside rule' of von Heijne [45]) and are important determinants of correct insertion of integral membrane proteins [46]. Subunit IV lacks a cleavable N-terminal signal sequence [19], and the C-terminal membrane anchor [6,19] apparently functions as a stop-transfer sequence [46]. The lack of a cleavable transit sequence may assure that the bulk of this protein remains in the cytoplasm. Whereas cytochrome c_1 also contains a C-terminal membrane anchor [9,10], the apocytochrome is apparently synthesized with a cleavable signal sequence at the N-terminus that targets this protein to the periplasm [22,47].

This proposed topology for subunit IV has possible functional implications for the cytochrome bc_1 complex, which catalyses electron transfer from ubiquinol to cytochrome c_2 and contains

a periplasmic quinol oxidation (Q_o) site and a cytoplasmic quinone reduction (Q_i) site, thought to function in a Q-cycle mechanism [48]. Because subunit IV is a possible Q-binding protein [8,19], and the Q-binding domain is believed to be localized within amino acid residues 81–90, the topological results presented here suggest that this region is located on the cytoplasmic side of the ICM. Thus, subunit IV may be involved in quinone reduction, and in conjunction with cytochrome *b*, may form part of the ubiquinone-binding pocket within the Q_i site. It is noteworthy, however, that anti-(subunit IV) IgG did not affect the e.p.r. signal characteristic of the ubisemiquinone radical generated at the Q_i site [7], and that no role for subunit IV could be established here by limited trypsin digestion of the isolated bc_1 complex; this resulted in selective removal of a 4–5 kDa subunit IV fragment, but no reduction in ubiquinol-cytochrome *c* reductase activity.

Although a mass of ~1 kDa was removed from the Rieske FeS protein when chromatophores were digested with proteinase K, no cleavage could be observed at the periplasmic surface of the ICM as exposed either in spheroplasts or in chromatophore-liposome fusion products. The failure to cleave the FeS protein on the periplasmic side of the membrane is in contrast to the considerable biophysical and genetic evidence for the localization of the functional domain, including the 2Fe-2S cluster, at or near this surface [26–29]. Proteolytic digestion studies of the isolated bc_1 complex presented here suggest that the inability of proteinase K to cleave the FeS protein on the periplasmic side of the intact ICM is attributable to the protease resistance of the C-terminal domain of this subunit. In isolated bc_1 preparations, both proteinase K and chymotrypsin gave rise to 16 kDa fragments; the latter may have cleaved the FeS subunit at the same site as in detergent-treated spheroplast-derived vesicles [16], which resulted in release of a water-soluble product of 152 residues with an intact 2Fe-2S cluster. Moreover, it is possible that in the isolated complex, proteinase K cleaved the FeS protein at a similar site which requires detergent treatment for exposure and is unavailable in either spheroplasts or chromatophore-liposome fusion products.

Overall, these results support a topology for the Rieske FeS subunit in *R. sphaeroides* [16] which predicts a single N-terminal transmembrane helix; this leaves 13 N-terminal residues that are apparently exposed sufficiently to the cytoplasm to permit their proteolytic cleavage. A stretch of mainly hydrophobic residues (Lys33–Lys52 in mammalian mitochondria and Asp13–Pro33 in *R. sphaeroides*) is generally agreed to be required for FeS protein association with the bc_1 complex [13–17]. González-Halphen et al. [15] have shown that a peptide with this mitochondrial sequence competes with the intact protein for reconstitution, suggesting that this domain represents a site of protein–protein interactions with the oxidoreductase, rather than a transmembrane anchor. The Rieske FeS protein of the *Chlamydomonas reinhardtii* b_f complex has also been shown recently to contain a hydrophobic segment near the N-terminus [49] which is thought to be the site of association of the FeS subunit with other proteins of the complex [18], based upon extraction of this subunit with chaotropic agents and the limited ability of the isolated protein to bind detergents [18]. In contrast, the demonstration by Van Doren et al. [16] that a small amount of the Rieske subunit containing an intact FeS cluster was assembled in the cytoplasmic membrane of *R. sphaeroides* in the absence of cytochromes *b* and c_1 suggests that the bacterial protein interacts directly with the lipid bilayer interior. Although the N-terminal hydrophobic sequence of the chloroplast FeS subunit is also thought to function as an uncleaved lumen targeting sequence [49], proteins with long hydrophobic stretches are usually not

secreted from bacterial cytoplasmic membranes [46,50]. Further examination of the topology of the *R. sphaeroides* Rieske FeS and subunit IV proteins will involve N-terminal sequence analysis of appropriate proteolytic fragments, and strategies for their isolation are currently being developed in this laboratory.

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