The *Saccharomyces cerevisiae* succinate-ubiquinone reductase contains a stoichiometric amount of cytochrome b_{562}

Kayode S. Oyedotun, Bernard D. Lemire*

The Medical Research Council of Canada Group in the Molecular Biology of Membranes, Department of Biochemistry, University of Alberta, Edmonton, Alba T6G 2H7, Canada

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Abstract The *Saccharomyces cerevisiae* succinate-ubiquinone reductase or succinate dehydrogenase (SDH) is a tetramer of non-equivalent subunits encoded by the *SDH1*, *SDH2*, *SDH3*, and *SDH4* genes. In most organisms, SDH contains one or two endogenous *b*-type hemes. However, it is widely believed that the yeast SDH does not contain heme. In this report, we demonstrate the presence of a stoichiometric amount of cytochrome b_{562} in the yeast SDH. The cytochrome is detected as a peak present in fumarate-oxidized, dithionite-reduced mitochondria. The peak is centered at 562 nm and is present at a heme:covalent FAD molar ratio of 0.92 ± 0.11. The cytochrome is not detectable in mitochondria isolated from *SDH3* and *SDH4* deletion strains. These observations strongly support our conclusion that cytochrome b_{562} is a component of the yeast SDH.

Key words: Succinate dehydrogenase; Cytochrome b_{562}; Dithionite; Fumarate-oxidizable; Covalent flavin; Mitochondrion; Yeast; Heme

1. Introduction

Succinate-ubiquinone reductase, also called succinate dehydrogenase (SDH), is a membrane bound enzyme of the Krebs cycle and the mitochondrial respiratory chain. It catalyzes the oxidation of succinate to fumarate coupled to the reduction of quinone. A related enzyme, fumarate reductase (FRD), which catalyzes the reduction of fumarate to succinate coupled to quinone oxidation, is present in anaerobic cells respiring with fumarate as the terminal electron acceptor. This enzyme is functionally and structurally similar to SDH [1–3].

Generally, SDH is a tetramer of non-equivalent subunits: a flavoprotein subunit (about 70 kDa), an iron-sulfur protein subunit (about 27 kDa) and two smaller hydrophobic polypeptides of about 17 and 13 kDa [1,2,4,5]. The flavoprotein and the iron-sulfur protein subunits comprise the catalytic domain. The dicarboxylate active site resides in the flavoprotein subunit, near a covalently attached FAD cofactor. The iron-sulfur protein subunit contains three iron-sulfur clusters that are involved in electron transfer from FAD to quinone. This catalytic domain can transfer electrons from succinate to artificial electron acceptors such as phenazine methosulfate (PMS) or potassium ferricyanide. However, the reduction of natural quinones or quinone analogs requires the presence of the membrane-anchoring domain, which is typically comprised of the two smaller polypeptides [1,6–8]. The membrane domain attaches the catalytic subunits to the inner membrane of mitochondria or to the cytoplasmic membrane of bacteria. In addition, it contains the binding site(s) for quinone and may also ligand one or two *b*-type hemes.

The flavoprotein and iron-sulfur subunits are well conserved across species at the primary amino acid level. In contrast, there is considerable variability in subunit composition and primary amino acid sequence of the membrane domain [9–14]. Some SDHs have a single anchor subunit while others have two; some have two hemes in their anchor subunits, while others have one or none. The variability has made it difficult to identify residues that may be involved in cofactor binding or in subunit-subunit interactions through sequence comparisons. In the absence of high-resolution structural information, the yeast SDH promises to be a good model system for studying structural requirements for cofactor interactions through genetic approaches. *Saccharomyces cerevisiae* is able to grow either by fermentation, where functional SDH is not required, or by respiration, where its presence is essential [7,8,15,16].

In *S. cerevisiae*, SDH is composed of four subunits encoded by the nuclear *SDH1*, *SDH2*, *SDH3*, and *SDH4* genes [7,8,17–20]. The hydrophilic subunits, Sdh1p and Sdh2p, which comprise the catalytic domain and are involved in succinate oxidation, are anchored to the inner mitochondrial membrane by the small, hydrophobic subunits, Sdh3p and Sdh4p. These are believed to contain two quinone binding sites by analogy to the bovine SDH, the *Bacillus subtilis* SDH, and the *Escherichia coli* FRD [1,2,5,21–25]. Each subunit is predicted to each contain three membrane-spanning domains [7,8,26].

Based on heme content and the number of anchor polypeptides, SDH and FRD membrane anchor domains have been classified into four types, A–D [6]. The yeast SDH and *E. coli* FRD fall into the D structural subtypes, containing two anchor polypeptides without heme. The supposed absence of heme is based on a published abstract [27]. In this earlier report, the authors isolated SDH by a procedure involving cholate solubilization, ammonium sulfate fractionation, and density gradient centrifugation. This yielded a preparation containing a substoichiometric molar ratio of heme to flavin (less than 0.2). The final purification step involved immunoaffinity chromatography, which further reduced the heme to flavin molar ratio to 0.06. The influence of this abstract has persisted in the literature [2,6,28]. Recently, investigators working with the yeast complex III demonstrated the presence of a fumarate-oxidizable heme in dithionite-reduced mitochondria corresponding to cytochrome *b* in SDH [29,30].

In this report, we demonstrate the presence of a stoichio-

*Corresponding author. Fax: (1) (403) 492-0886.
E-mail: bernard.lemire@ualberta.ca

Abbreviations: SDH, succinate dehydrogenase; FRD, fumarate reductase; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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metric amount of heme in the yeast SDH by monitoring the fumarate-oxidizable spectrum in dithionite-reduced mitochondria. Fumarate oxidation of dithionite-reduced mitochondria produces a peak centered at 562 nm, hence we designate the heme as cytochrome b_{562} (previous reports have referred to the putative heme of yeast SDH as cytochrome b_{560}, by analogy to cytochrome b_{560} of the bovine SDH [8,31]). The peak is absent in mitochondria isolated from *SDH3* and *SDH4* knock-out strains. These observations strongly favor the conclusion that this cytochrome is a component of the yeast SDH.

2. Materials and methods

2.1. Media, strains and yeast culture conditions

The yeast media used are SD (0.67% yeast nitrogen base, 2% glucose), SG (0.67% yeast nitrogen base, 3% glycerol), and semisynthetic media (0.3% yeast extract, 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 0.06% $MgSO_4.7H_2O$, 0.05% $CaCl_2$, 0.003% $FeCl_3$, 2% galactose or 2% lactate). Yeast strains were initially cultivated on SD for 2 days to select for plasmid retention. Single colonies were picked to inoculate 100 ml of semisynthetic galactose or lactate medium and grown aerobically at 30°C for 24 h. The precultures were diluted 100-fold into semisynthetic galactose or lactate medium and grown aerobically at 30°C to an OD_{600} of about 3, yielding approximately 3 g (wet weight) of cell paste per liter of culture.

The yeast strains, MH125 and sdh4W2, and the *E. coli* strain, DH5α, have been described [26,32]. sdh3W3 was constructed by targeted gene disruption [33]. Briefly, a 0.13 kb *Kpn*I fragment was removed from *SDH3* gene and replaced with a 0.83 kb *Kpn*I fragment containing the *TRP1* gene to yield the plasmid pSDH3-TRP1. A 1.7 kb fragment (containing the disrupted *SDH3* gene) was isolated and transformed into the yeast strain, MH125. Tryptophan prototrophs were selected on SD medium supplemented with 20 μg/ml each of histidine, uracil, adenine sulfate, leucine and lysine. At least five stably transformed tryptophan prototrophs had lost the ability to grow on SG and were further examined.

2.2. Cytochrome content determination

Total absorption spectra of yeast mitochondria were measured as the dithionite-reduced minus ferricyanide-oxidized spectra. The heme content of SDH was determined spectrally as the dithionite-reduced minus the dithionite-reduced/fumarate-oxidized difference spectrum [29,34]. Mitochondria isolated from strains grown on semisynthetic lactate or galactose medium [26] were suspended to 185 pmol of covalently attached FAD/ml (2.5–3.2 mg protein/ml) in 0.65 M sorbitol, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 0.3% fatty acid-free bovine serum albumin, pH 6.5. Lauryl maltoside (Boehringer Mannheim) was added to a final concentration of 0.1% to reduce spectral noise. Spectra were recorded at 22°C on a Hewlett Packard 8453 diode array spectrophotometer with a 1 cm path length. The molar absorption coefficients used were: 21.4 mM^{-1} cm^{-1} for cytochrome aa_3, 18 mM^{-1} cm^{-1} for cytochrome cc_1, and 24 mM^{-1} cm^{-1} for cytochrome b_{562}+b_{565} [35]. This latter value was also used for fumarate-oxidizable cytochrome b_{562} [29].

2.3. Electrophoresis and Western blot analyses

Mitochondrial proteins (10 μg) were separated using a 13% Tricine gel system [36] and transferred electrophoretically to a polyvinylidene difluoride membrane as described [37]. The blot was treated with a rabbit polyclonal antiserum raised against a peptide consisting of the amino-terminal 12 residues of the mature Sdh3p. Detection of antigens was with a peroxidase-labeled goat anti-rabbit secondary antibody and the enhanced chemiluminescence detection system (Amersham).

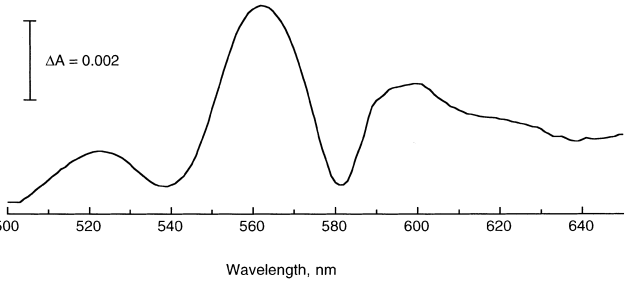

Fig. 1. Difference absorption spectrum of mitochondria isolated from lactate-grown MH125 cells. Mitochondria were suspended to a covalent flavin level of 185 pmol/ml and the dithionite-reduced minus dithionite-reduced/fumarate-oxidized spectrum determined. Mitochondria were reduced by adding a few grains of dithionite and oxidized with 40 mM fumarate (final concentration).

2.4. Polymerase chain reaction

Genomic DNA was isolated from the *SDH3* knock-out strain, sdh3W3, and the parental strain, MH125, and used as templates in the polymerase chain reactions. The following primers were used: 5′-GTTAAAGGATGTTCTAGTGG-3′ (upstream primer) and 5′-ATAGGTTTGTGTTAATCGGC-3′ (downstream primer), corresponding to nucleotides −307 to −326 and +719 to +738 of the *SDH3* gene, respectively. PCR conditions were as previously described [16].

2.5. Miscellaneous methods

Covalently attached flavin was measured on a Perkin-Elmer luminescence spectrometer model LS50 using a riboflavin standard as described [16]. Values are averages of triplicate determinations after subtracting background levels. Protein determination was carried out as described [16].

3. Results and discussion

3.1. Fumarate-oxidizable spectrum of yeast mitochondrial membranes

Since cytochrome b_{562} of complex III is considerably more abundant than that of SDH in *S. cerevisiae*, the spectrum of the latter is not readily observable in the classical difference absorption spectrum of yeast mitochondria. In the bovine SDH, excess fumarate rapidly oxidizes dithionite-reduced cytochrome b_{560}. The other chromophores reduced by dithionite can be subsequently reoxidized by ferricyanide [38], suggesting a unique property of cytochrome b_{560} (the ability to be selectively oxidized by fumarate after dithionite reduction). In vitro, SDH can catalyze the reduction of fumarate to succinate,

Table 1
Concentrations of cytochromes in yeast mitochondrial membranes

Cytochrome	Wavelength pairs (nm)	Concentration (mol/mol of covalent FAD)
aa_3	605–630	6.5 ± 0.1
c+c_1	550–540	12.9 ± 1.1
b_{562}+b_{565} (complex III)	562–590	8.1 ± 1.1
b_{562} (complex II)	562–578	0.92 ± 0.11

Concentrations of the cytochromes aa_3, c+c_1 and b_{562}+b_{565} were calculated, using the indicated wavelength pairs, from the dithionite-reduced minus ferricyanide-oxidized difference spectrum of MH125 mitochondria (not shown) while that of cytochrome b_{562} of complex II was calculated from the spectrum in Fig. 1. Values represent means of triplicate determinations ± S.E.M.

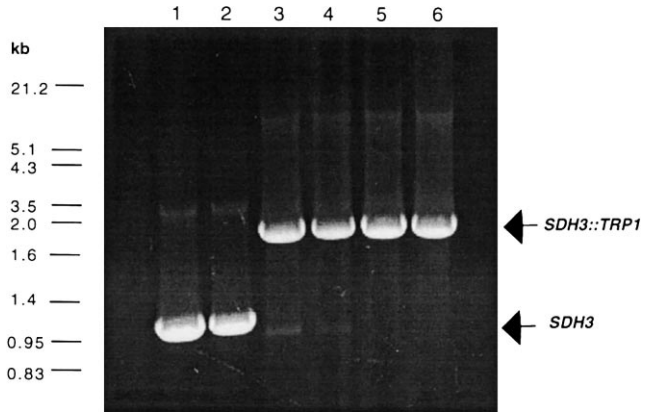

Fig. 2. Agarose gel analysis of PCR products obtained using the upstream and downstream primers (see Section 2) with MH125 genomic DNA (lanes 1 and 2) or genomic DNA from tryptophan-prototrophic, respiration-deficient transformants carrying the *SDH3* disruption mutations (lanes 3–6). The deletion of 0.13 kb of SDH3 coding sequence and the insertion of the 0.83 kb TRP1 DNA is expected to result in the production of a 1.76 kb band in the mutant as opposed to a 1.06 kb band in the wild type.

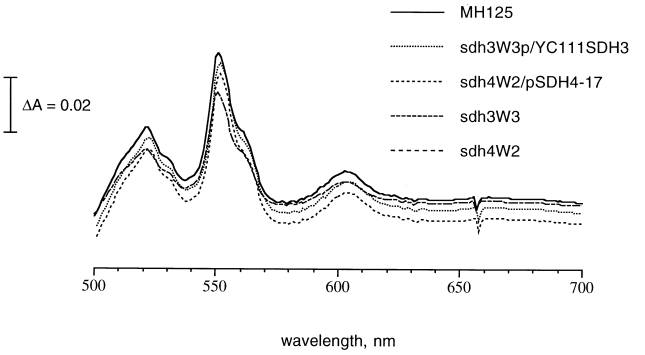

Fig. 4. Difference absorption spectra of mitochondria isolated from wild type and mutant *S. cerevisiae* strains. Mitochondria were isolated from galactose-grown cells, suspended to a covalent flavin level of 185 pmol/ml and the dithionite-reduced minus ferricyanide-oxidized spectrum determined. Mitochondria were reduced by adding a few grains of dithionite and oxidized with 10 μM potassium ferricyanide (final concentration). The SDH activities (measured as the succinate-dependent, malonate-sensitive, phenazine methosulphate-mediated reduction of dichlorophenol indophenol, DCPIP) of wild type mitochondria is 130 nmol DCPIP/min/mg protein, while sdh3W3 and sdh4W2 mutant mitochondria have less than 4% of the wild type activity.

the reverse of the physiologically important reaction. By exploiting this property, investigators working on yeast complex III have demonstrated the presence of a *b*-type cytochrome in SDH [29,34]. We used a similar approach to determine the cytochrome *b* content of the yeast SDH. Shown in Fig. 1 is the fumarate-oxidizable spectrum of mitochondria isolated from lactate-grown MH125 cells. The major peak, which appears within 1 min after the addition of fumarate, is symmetrical and is centered at 562 nm. There is only a small peak at 605 nm, indicating that cytochrome aa_3 remains reduced. Addition of ferricyanide leads to the reoxidation of cytochromes cc_1, complex III-associated cytochrome *b* and cytochrome aa_3 (data not shown). The amount of cytochrome b_{562} calculated from the spectrum is 0.92 ± 0.11 mol/mol of covalent FAD (Table 1), suggesting the presence of a single heme per SDH holoenzyme. Also shown in Table 1 are the mitochondrial contents of cytochromes aa_3, $c+c_1$ and $b_{562}+b_{565}$ calculated from dithionite-reduced minus ferricyanide-oxidized difference spectra (data not shown).

3.2. Construction of an SDH3 knock-out mutant

Residues likely to be involved in heme coordination are usually contributed by the SDH anchor polypeptides [2,6]. We investigated the effects of *SDH3* and *SDH4* knock-out mutations on the mitochondrial contents of cytochrome b_{562}. To do this, we constructed an *SDH3* knock-out strain, sdh3W3, by targeted gene disruption. sdh3W3 has lost the ability to grow on minimal medium containing non-fermentable carbon sources; this ability could be restored with the plasmid YC111SDH3, encoding the wild type *SDH3* gene. The disruption was confirmed by PCR (Fig. 2) and Western blotting (Fig. 3). Interestingly, Sdh3p is not detectable in mitochondria isolated from sdh4W2 (Fig. 3, lane 3). This suggests that in the absence of Sdh4p, sdh3p is not stable.

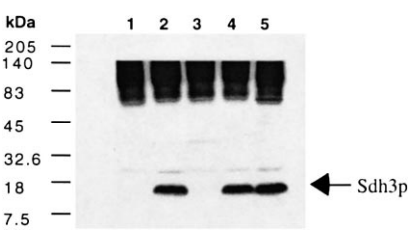

Fig. 3. Western blot analysis of mutant and wild type mitochondria. Mitochondria were isolated from galactose-grown MH125, sdh3W3 and sdh4W2 knock-out strains and 10 μg electrophoresed on 13% Tricine SDS-PAGE, and immunoblotted using anti-Sdh3p antibody. Lanes 1–5: sdh3w3, sdh3W3/pYC111SDH3, sdh4W2, sdh4W2/pSDH4-17, and MH125 respectively.

3.3. Difference absorption spectra of mutant and wild type mitochondrial membranes

Depicted in Fig. 4 are the absorption spectra of mitochondrial membranes isolated from galactose-grown mutant and wild type yeast strains. The total cytochrome spectra of mitochondria isolated from both wild type and mutant strains are comparable. Similarly, the fumarate-oxidizable cytochrome *b* peak, centered at 562 nm, is present in mitochondria from the wild type strain, MH125 or the plasmid-complemented strains, sdh3W3/pYC111SDH3 and sdh4W2/pSDH4-17 (Fig. 5). In contrast, the fumarate-oxidizable cytochrome b_{562} spectrum is not detectable in sdh3W3 and sdh4W2 mitochondria (Fig. 5), strongly suggesting that it is a component of the yeast SDH.

To further demonstrate the reliability of the fumarate-oxidizable spectra in detecting a heme associated with SDH, we used the SDH-specific inhibitor, malonate. Malonate competes with succinate and fumarate for the SDH dicarboxylate binding site. Thus, as expected, malonate prevents the oxidation of cytochrome b_{652} by preventing the binding of fumarate to SDH holoenzyme present in MH125 mitochondria (Fig. 5).

In summary, we demonstrate here that: (1) the *S. cerevisiae* SDH contains a stoichiometric amount of cytochrome b_{562}, (2) the fumarate-oxidizable heme is not detectable in the absence of either of the SDH anchor polypeptides, Sdh3p and Sdh4p or in the presence of malonate. The present study is the first to demonstrate the presence of a stoichiometric amount

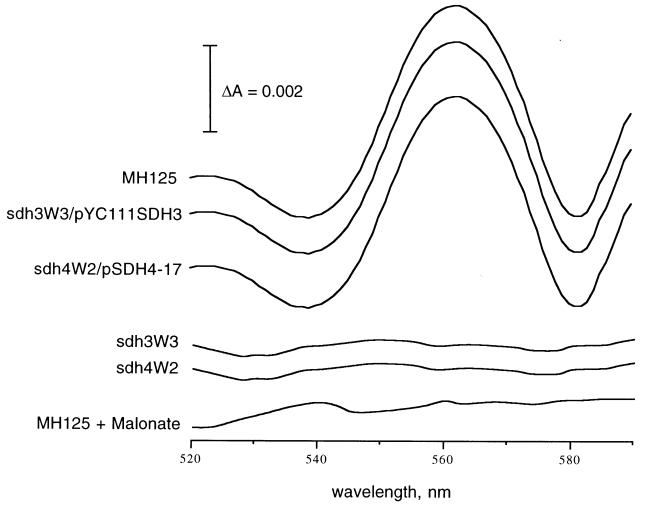

Fig. 5. Fumarate-oxidizable spectra of mutant and wild type *S. cerevisiae* strains. Mitochondria were isolated from galactose-grown cells, suspended to a covalent flavin level of 185 pmol/ml and the dithionite-reduced minus dithionite-reduced/fumarate-oxidized spectrum determined. Mitochondria were reduced by adding a few grains of dithionite and oxidized with 40 mM fumarate (final concentration). Malonate was added to a final concentration of 40 mM prior to the addition of fumarate.

of heme in yeast SDH. Our data contradict an earlier study reporting a substoichiometric molar ratio of heme to covalent FAD in an immuno-affinity purified SDH [27]. We believe that the purification procedure employed in the earlier study leads to the loss of heme. The use of whole mitochondria in this study may have prevented the loss of heme. Even mammalian SDH, which is uniformly acknowledged to contain heme, is usually purified with substoichiometric amount of heme, suggesting the chromophore is easily lost [1].

The results presented in Table 1 suggest a ratio of SDH to complex III of 1:4 since complex III contains two *b*-type cytochromes. SDH to complex III ratios ranging from 1:2 to 1:10 have been reported for different respiratory chain sources [39]. Our determination of a cytochrome b_{562} to covalent FAD ratio of 0.92 in SDH is not influenced by the stoichiometry of the individual respiratory chain complexes. The yeast respiratory chain may function in a kinetically different manner than mammalian respiratory chains [40].

Is it possible that the fumarate-oxidizable cytochrome b_{562} does not reside in SDH, but is linked to it by an electron transport pathway? We consider this possibility unlikely for two reasons. First, a fumarate-oxidizable cytochrome residing out of SDH has not been reported in any system. Second, we have constructed a mutant in Sdh4p that renders the enzyme incapable of quinone reduction. Yet, this mutant still displays a fumarate-oxidizable cytochrome (data not shown). Thus, electron transport beyond the quinone is not necessary to oxidize cytochrome b_{562}, placing it within SDH in an electron transport pathway between fumarate and ubiquinone.

Is there a role for cytochrome b_{562} in electron transfer from succinate to ubiquinone? Electron transfer in SDH from donor to acceptor occurs via multiple domains. The pathway of electron transfer in SDH is believed to be: succinate → FAD- → iron-sulfur → quinone [1,5]. For the reverse reaction, in which fumarate is reduced to succinate, the pathway of electron flow is in the opposite direction [41]. The role of heme in this scenario remains obscure. Many SDHs contain *b*-type hemes but the content and redox properties of these hemes vary considerably. The *B. subtilis* SDH contains two *b*-type hemes [9,11], while the *E. coli* and *Ascaris suum* SDHs have one [10,12,13]. In *B. subtilis*, the heme with the higher potential ($E'_m = +65$ mV) is reduced by succinate while the heme with the lower potential ($E'_m = -95$ mV) is not [9,11]. The heme in *E. coli* SDH is fully reducible [10] while that of *A. suum* SDH is only partially reduced by succinate [12,13]. Bovine SDH cytochrome b_{560} is not readily reducible by succinate [14]. In *B. subtilis* SDH, the cytochrome plays a structural role [42], while loss of heme does not impair the assembly of the *E. coli* enzyme [28]. The role of heme in the succinate-mediated reduction of ubiquinone will be addressed in further studies.

All cytochromes in SDHs so far are known to be coordinated by two histidine residues [2,6]. In the yeast Sdh3p, His-156 is positioned in the putative transmembrane helix 2 and may be such a ligand. Preliminary results show that replacing His-156 to Tyr impairs growth on non-fermentable carbon sources (K.S. Oyedotun, unpublished observation). However, a second conserved His that serves as a heme ligand in other SDHs is replaced by Cys-109 in the yeast Sdh4p. Therefore, determining the heme ligands in the yeast SDH may reveal unusual coordination. Further studies will be required to define the redox properties of SDH cytochrome b_{562}. To do this, it will be necessary to overexpress or purify SDH, due to the paucity of this cytochrome in yeast mitochondria. These studies are currently in progress.

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