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The respiratory NADH dehydrogenase of the cyanobacterium *Anabaena variabilis*: purification and characterization

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An NADH dehydrogenase, tightly bound to the thylakoid membrane, was purified and characterized. The enzyme was solubilized with deoxycholate and purified by ammonium sulfate precipitation, gel filtration and repeated FPLC anion-exchange chromatography. A purification factor of 870 was achieved. SDS-polyacrylamide gel electrophoresis of the purified enzyme showed one major component of 17 kDa and a minor one at 52 kDa. Activity staining after isoelectric focusing and subsequent SDS gel electrophoresis corroborated the determination of the apparent molecular weight. The enzyme exhibited a K_m for NADH of 22 μM and a V_{max} of 17.4 μmol NADH oxidized per mg protein per min. Various quinones including phylloquinone but not ferricyanide and cytochrome *c*-550 are electron acceptors in vitro. Compared to membrane-bound pyridine-nucleotide dehydrogenases of mammalian mitochondria the *Anabaena* enzyme was poorly inhibited by rotenone. Reconstitution experiments revealed FAD, not FMN as a covalently bound prosthetic group of the NADH dehydrogenase. It is suggested that the enzyme is operating in the respiratory chain.

Introduction

NADPH and NADH are donors for respiratory electron transport in *Anabaena variabilis* [1–3] and other cyanobacterial species [4–6]. In *A. variabilis* NADPH oxidation coupled to respiratory electron transport is performed by a thylakoid-associated ferredoxin–$NADP^+$ oxidoreductase (EC 1.18.1.2) [7,8]. Apparently, NADH oxidation is catalyzed by a separate membrane-bound dehydrogenase [7].

In mammalian mitochondria the corresponding enzyme at the beginning of the respiratory electron transport is NADH–quinone oxidoreductase (EC 1.6.99.3) composed by 26 protein subunits [9]. The bacterial counterpart has 1–3, in most cases only one subunit [10–12]. Pyridine nucleotide oxidation in plant mitochondria is performed by four different dehydrogenases with various K_m values and specificities for NADH and NADPH [13].

The isolation of a membrane (thylakoid)-bound enzyme from a cyanobacterium encounters several difficulties as compared to mitochondria (or facultative) heterotrophic bacteria without photosynthetic pigments present in the respiratory membranes. A close interaction of respiratory and photosynthetic electron transport is evident [14] with respiration being localized mainly on the thylakoid membrane. Thus, large amounts of lipophilic chlorophyll and carotenoid have to be removed during preparation. Furthermore, respiration in cyanobacteria has very low rates of about 5–15% of photosynthetic electron transport [15,16] with a seemingly low content of respiratory enzymes. In this paper, the isolation and partial characterization of a cyanobacterial, thylakoid-bound NADH dehydrogenase is reported for the first time.

Materials and Methods

Culture conditions. Anabaena variabilis (ATCC 29413) was grown autotrophically under nitrogen-fixing conditions in batch cultures (10 litre) in a mineral medium according to Ref. 17. The culture was bubbled with air enriched by 1.6% CO_2 (v/v) and illuminated with 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent light, growth temperature was

Abbreviations: DCPIP, dichlorophenolindophenol; FAD, flavin adenine dinucleotide; FNR, ferredoxin–$NADP^+$ oxidoreductase; FMN, flavin adenine mononucleotide; FPLC, fast performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; *p*-CMPS, *p*-chloromercuriphenylsulfonic acid; TTFA, thenoyltrifluoroacetone.

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35°C. Chlorophyll determinations were performed according to Ref. 18.

Photometric assays. Dehydrogenase activity was determined by absorbance decrease at 340 nm in a reaction medium containing 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1% (w/v) Genapol X-080 (or Triton X-100), 0.1 mM NADH, 0.1 mM menadione (or acceptor as indicated) in a total volume of 1 ml. 10 μM FAD was added routinely to assay purified fractions after the first Mono-Q purification.

Protein determination. The protein assay was performed according to Bradford [19] with the dye concentrate of Bio-Rad and bovine serum albumin as a standard.

Gel electrophoresis. SDS-PAGE was accomplished according to Ref. 20 with an acrylamide content of 15% followed by staining with Coomassie R-250 and silver stain according to Ref. 21. Non-denaturing PAGE was performed in a gel with 10% acrylamide containing 0.1% Triton X-100 instead of SDS. Running buffer was 25 mM Tris base and 192 mM glycine. Electrophoresis was operating with 10 mA constant current for 2 h under cooling to 10°C. Immediately after electrophoresis activity staining was performed using the formazan-dye reaction [22]. The gel was incubated in a medium containing 50 mM Tris-HCl (pH 8.7), EDTA 0.5 mM, 0.1% Triton X-100 and 0.5 mg/ml nitroblue tetrazolium (Fluka). Formazan was formed after addition of NADH (0.5 mM final concentration). Isoelectric focusing was performed in a gel containing Pharmalyte D2 (Pharmacia) to generate a pH gradient from pH 3 ot pH 10 according to the procedure described in the corresponding Pharmacia manual. Activity staining was accomplished as described above.

Column chromatography. A preparative gel filtration column Sephacryl S-300 (Pharmacia) was used (60 cm length, 2.8 cm diameter). Mono Q 5/5 anion-exchange column chromatography was performed by the FPLC system of Pharmacia (Uppsala, Sweden).

Chemicals. NADH, FAD were purchased by Boehringer (Mannheim, F.R.G.), the sodium deoxycholate, menadione and FMN were from Sigma (Deisenhofen, F.R.G.). Phylloquinone and ubiquinone-1 were kindly provided by Hoffmann La Roche (Basel, Switzerland). Genapol X-080 was purchased from Calbiochem (Frankfurt, F.R.G.).

Results

Purification of NADH dehydrogenase

Anabaena variabilis (filaments, 50 g fresh weight) were suspended in washing buffer (50 mM Tris-HCl, 0.25 mM EDTA (pH 7.5)) to a chlorophyll content of 0.5 to 1 mg/ml and passed through a French-pressure cell at 6895 kPa. The homogenate was washed three times with washing buffer and centrifuged at 100 000 × g for 60 min which resulted in a crude fraction of membranes. After resuspending the membranes in washing buffer to a chlorophyll content of 0.5 mg/ml, deoxycholate (10% stock solution) was added dropwise to a final concentration of 0.1%. The suspension was stirred for 20 min on ice and centrifuged 60 min at 100 000 × g. Under these conditions 10–15% of chlorophyll-containing material and 30–40% of the NADH dehydrogenase are solubilized without stimulating activity. Up to 80% of the dehydrogenase could be solubilized, when increasing the detergent concentration (e.g. to 0.5%). The specific activity, however, was lowered. Ammonium sulfate was added (35%) to precipitate residual membrane material and chlorophyll, leaving about 70% of NADH dehydrogenase in the supernatant, which was concentrated by precipitation with 80% ammonium sulfate. The resulting precipitate was resuspended in washing buffer including 0.05% Genapol X-080 and 20% glycerol. The suspension was desalted on a Sephadex G-25 column (PD 10, Pharmacia) equilibrated with the same buffer and yielded the 'prepurified fractions'. In subsequent fractionation steps 10% glycerol was included in all buffers. Isolated heterocystous thylakoids of *A. variabilis* (prepared according to Ref. 23), exhibited NADH oxidation with a specific activity 2–3-times higher than thylakoids obtained with vegetative cells.

The prepurified fractions were applied to gel filtration on a preparative Sephacryl S-300 column (Fig. 1). The fractions containing NADH dehydrogenase were completely separated from NADPH-oxidizing activity. The inset of Fig. 1 shows the activity staining of the prepurified NADH-oxidizing enzyme after non-denaturing gel electrophoresis. A single band of NADH-induced formazan formation appeared. The same band was obtained when the detergent concentration was increased or the detergent changed (not documented).

NADH-oxidizing fractions collected from gel filtration were pooled and applied to a Mono Q anion-exchange chromatography (FPLC technique). NADH dehydrogenase was eluted by a NaCl gradient at 250 mM NaCl (Fig. 2A), yielding a purification factor of 8.5. However, a major part of the fraction was contaminated by a protein with an apparent molecular weight of 55 kDa. NADH dehydrogenase was further purified by re-chromatography on Mono Q with elution buffer containing a reduced concentration of detergent (0.01% Genapol X-080) as shown in Fig. 2B. About 50% of the NADH-oxidizing activity was eluted at 350 mM NaCl. Table I summarizes the data of a typical purification procedure, the deviations of activity and yield from different preparations are given in parentheses.

A 870-fold purification was achieved based on the specific activity of NADH oxidation. The V_{max} is 17.4 μmol NADH oxidized per mg protein per min. The yield of active protein was low due to incomplete elu-

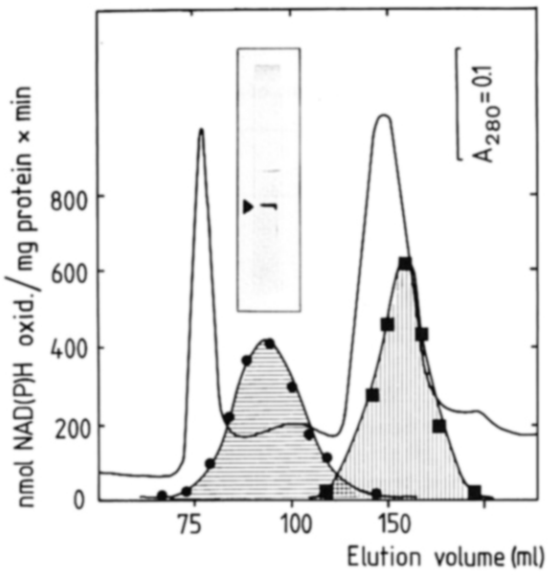

Fig. 1. Fractionation of the prepurified enzyme on a preparative Sephacryl S-300 column. Elution medium contained 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 10% glycerol, 0.05% genapol X-080. Flow rate was 0.6 ml/min. Activities of NADH oxidation are shaded by horizontal lines, NADPH oxidation by vertical lines. Inset: Activity staining of the prepurified NADH-oxidizing fraction after non-denaturing gel electrophoresis. The band indicated by the arrow was formed after addition of NADH (see Materials and Methods), the upper band being due to phycobiliproteins.

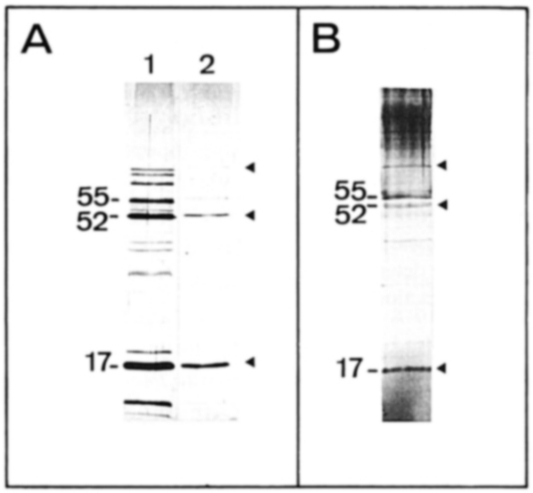

Fig. 3. (A) SDS-PAGE of NADH dehydrogenase of *A. variabilis* (silver stain). Lane 1, First Mono Q purification; lane 2, 870-fold purified fraction according to Table I. (B) The band of NADH-induced formazan formation after isoelectric focusing (1st dimension) was excised and layered onto a SDS gel to run the second dimension. The protein bands are clearly visible, the dark zones originate from free formazan dye.

tion of the anion-exchange column with decreased detergent concentration and to loss of flavin. The procedure yielded highly purified protein in microgram quantities. Elution with higher detergent concentrations yielded more enzyme activity present in less purified fractions.

Characterization

In Fig. 3A SDS-polyacrylamide gel electrophoresis patterns of the NADH-oxidizing fractions are shown. A major component of 17 kDa and a less intensive band at 52 kDa were retained after the last purification step, lane 2. The major contaminating protein with 55 kDa (see lane 1) was removed by Mono Q anion-exchange chromatography with 0.01% Genapol X-080. The data were confirmed by a different experimental approach as shown in Fig. 3B. Isoelectric focusing of solubilized thylakoids and activity staining resulted in a single NADH-induced formazan band (compare, Fig. 1, inset), which was excised and layered onto a SDS electrophoresis gel for a run in the second dimension. The protein pattern was similar to that obtained after column chromatography purification steps. A major 17-

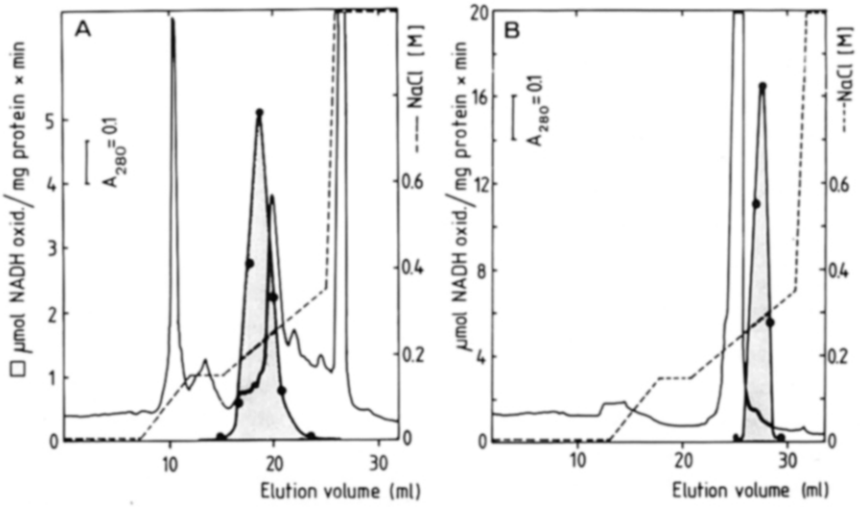

Fig. 2. Elution of NADH dehydrogenase from the anion-exchange column Mono Q 5/5 (FPLC technique). (A) Active fractions from Sephacryl S-300 purification according to Fig. 1 were run with buffer containing 0.05% genapol x-080. (B) Active fractions of (A) (first Mono Q fractionation) were separated in buffer containing 0.01% Genapol X-080. Flow rate was 0.8 ml/min.

TABLE I

Purification of NADH dehydrogenase from A. variabilis

The data are from a typical preparation. The yield is based on activity. Values in parenthesis show deviations of five comparable preparations.

	Specific activity (μmol NADH oxidized per mg protein per min)	Yield (%)	Purification factor
Homogenate	0.02	100	1
Membranes after washing	0.032 (0.032–0.062)	70 (40–70)	1.6
Solubilization by detergent, $(NH_4)_2SO_4$ fractionation	0.075 (0.075–0.115)	40 (20–50)	3.7
Sephacryl S-300	0.6 (0.4–0.6)	38	30
Mono Q, 0.05% Genapol	5.1 (3.5–5.1)	38	255
Mono Q, 0.01% Genapol	17.4	6	870

kDa component and minor 52-kDa and 70-kDa bands were found. The 70-kDa component, however, was absent in some preparations without loss of activity.

Table II shows activities with various acceptors for the in vitro test of NADH dehydrogenase. A preference of the dehydrogenase for quinones is obvious compared to one-electron acceptors like ferricyanide and cytochrome *c*-550. The same acceptor properties were obtained with crude membrane fractions before solubilization (data not shown). For rates with phylloquinone the low solubility in water has to be considered when comparing the oxidation rates with those obtained with soluble quinones like menadione, ubiquinone-1 and 2,5-dimethylbenzoquinone.

With the prepurified enzyme a K_m value for NADH of 22 μM was determined in our assay system. Evaluation of the data by a double-reciprocal plot (Fig. 4) resulted in a straight line indicative of a single enzyme being responsible for NADH oxidation. Further purification did not change the K_m value significantly. The purified enzyme did not oxidize NADPH in the assay system described in Materials and Methods.

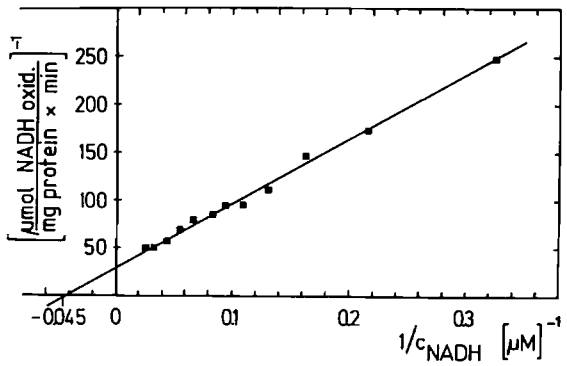

Fig. 4. Determination of K_m. Double-reciprocal plot of prepurified NADH dehydrogenase (assay system NADH → menadione).

Various inhibitors of respiratory dehydrogenase activities were applied to the NADH dehydrogenase of *A. variabilis* as shown in Fig. 5. Relatively low inhibitor activities were observed with the partially purified fraction (after gel filtration). An I_{50} value of 40 μM was measured for rotenone, a potent inhibitor of mammalian mitochondrial NADH–quinone oxidoreductase. TTFA and the mercuric compound p-CMPS poorly

TABLE II

Various acceptors for NADH dehydrogenase of A. variabilis

Figures are given in μmol NADH oxidized per mg protein per min. Activities were measured before column chromatography (prepurified fraction) and after the first Mono Q fractionation. The assay contained 10 μM FAD. n.d., not determined.

Electron acceptor	NADH oxidation	
	prepurified fraction	After 1st Mono Q step
None	0	0
0.1 mM menadione	0.077	4.1
0.1 mM 2,5-dimethyl-benzoquinone	0.079	4.0
0.5 mM ubiquinone-1	0.085	4.0
0.1 mM phylloquinone	0.027	1.0
0.1 mM DCPIP	0.018	n.d.
0.02 mM cytochrome *c*-550	0	0
0.1 mM ferricyanide	0	0

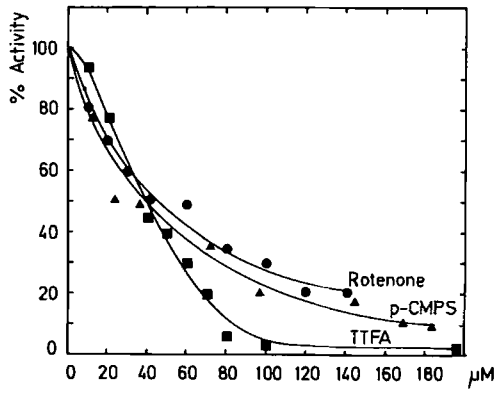

Fig. 5. Inhibition of NADH dehydrogenase of *A. variabilis* by rotenone (●——●), TTFA (thenoyltrifluoroacetone) (■——■) and *p*-CMPS. (*p*-chloromercuriphenylsulfonic acid) (▲——▲). 100% activity corresponds to 950 nmol NADH oxidized per mg protein per min.

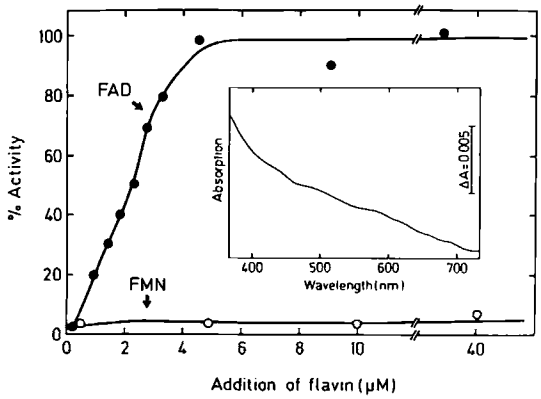

Fig. 6. Reconstitution of NADH dehydrogenase activity by addition of FAD and FMN to the assay. The fraction eluting after the Mono Q purification step (compare, Table I) was assayed after storage. Inset: Absorption spectrum of an 870-fold purified fraction of NADH dehydrogenase (see Table I and Fig. 3, lane 2).

affected *Anabaena* dehydrogenase. The sensitivity to inhibitors did not change after further purification steps.

The first purification steps including gel filtration (Fig. 1) did not inactivate NADH dehydrogenase. After repeated anion-exchange chromatography (Fig. 2A, B) however, and especially after storage at −20 °C 50% of activity or less were recovered. This was due to loss of the cofactor FAD as indicated by the reconstitution experiment in Fig. 6. Micromolar concentrations of FAD added to the assay system completely reconstituted the original activity, which had been lost during storage or after anion-exchange chromatography. The reaction was specific for FAD, FMN had no effect. Although participation of FAD in the redox reaction could be shown, the absorption spectrum of the purified enzyme did not provide clear evidence for the existence of a flavin (Fig. 6B). Apparently, flavin is lost during purification. In early purification steps the spectrum is overlapped by chlorophyll and carotenoid absorption.

Discussion

The purification of NADH dehydrogenase from thylakoids of *A. variabilis* was complicated by the low enzyme content, by denaturation and loss of the cofactor FAD. The dehydrogenase characterized in this study is highly specific for NADH and not responsible for NADPH-oxidizing activity. Separation by gel filtration (Fig. 1) leads to the conclusion that NADH dehydrogenase and NADPH dehydrogenase are different enzymes, confirming our earlier conclusions [7]. Contradictory results as reported for other cyanobacterial preparations [24,25] may be explained by assuming NADH-oxidizing fractions still contaminated with NADPH-oxidizing activity, probably FNR (compare, Ref. 8).

Apparently, the dehydrogenase characterized in this study is the only thylakoid-bound NADH dehydrogenase in *A. variabilis* as is evidenced by different approaches. Firstly, gel filtration of the prepurified enzyme resulted in a single symmetrical elution of NADH-oxidizing activity (Fig. 1). Activity staining of non-denaturing PAGE exhibited a single band of NADH-induced dye formation (Fig. 1, inset). Thirdly, an identical K_m was found by double-reciprocal plots using either the prepurified (Fig. 4) or a highly purified preparation, indicating that we were dealing with one dehydrogenase only during the purification procedure. Different K_m values for NADH found previously by oxygen-uptake measurements with isolated thylakoids from *A. variabilis* [2] may be explained by different affinities of the same enzyme to NADH in dark or light when the enzyme is still integrated in the intact membrane.

Acceptor studies revealed a preference of the enzyme for quinones in the prepurified as well as in a highly purified state (Table II). One-electron acceptors like potassium ferricyanide and cytochrome *c* are no good acceptors, which was also found with a partially purified NADH–plastoquinone oxidoreductase from *Nostoc muscorum* [26]. Inhibitor characteristics of the *Anabaena* enzyme point to a protein different from complex I from mammalian mitochondria since the latter is completely inhibited by nanomolar concentrations of rotenone [27]. Plant mitochondria exhibit both rotenone-sensitive and rotenone-resistant NADH dehydrogenase activities [28,29]. The *Escherichia coli* enzyme is inhibited by comparatively high rotenone concentrations [30], *p*-CMPS inhibits NADH dehydrogenases from plant mitochondria [31] more effectively than the *Anabaena* enzyme. The marginal effect of TTFA was also observed with *Anacystis* [5].

Participation of FAD in NADH-oxidizing activity indicates a similarity to bacterial membrane-bound dehydrogenases, since FAD was found in dehydrogenase fractions of bacterial origin [10,11,32], while FMN is the prosthetic group in the dehydrogenase of mammalian mitochondria [33]. Bacterial enzymes have one subunit in most cases, for example a 65 kDa unit from an alkalophilic *Bacillus* [11], 63 kDa from *Bacillus subtilis* [10], 44 kDa from *Bacillus caldotenax* [34] and 43 kDa from *E. coli* [35]. A two-subunit dehydrogenase was reported for *Paracoccus denitrificans* [36] and a three-subunit dehydrogenase for *Vibrio alginolyticus* [37]. A dehydrogenase of *A. variabilis* with a 17-kDa peptide as the major component appears to be comparatively small, a possible participation of a 52-kDa component, however, cannot be excluded as yet by our data.

Summarizing, we suggest the protein described is a genuine respiratory enzyme of *Anabaena* (NADH–quinone oxidoreductase). NADH supports oxidative phosphorylation with isolated *Anabaena* thylakoids [3].

Furthermore, the presence of higher NADH-oxidizing activity in heterocyst preparations (not documented) parallels the observation that respiratory components, especially cytochrome oxidase, are found in higher quantities in heterocysts [38,39].

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