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Studies on the proton-translocating NADH:ubiquinone oxidoreductases of mitochondria and *Escherichia coli* using the inhibitor 1,10-phenanthroline

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Abstract

Mitochondrial NADH:ubiquinone oxidoreductase (complex I) is uncompetitively inhibited by 1,10-phenanthroline (OP). EPR spectroscopy of submitochondrial particles indicates that OP, similarly to rotenone, inhibits electron transfer between the Fe–S clusters of complex I and the ubiquinone pool. The proton-translocating NADH dehydrogenase (NDH1) of *E. coli* is more sensitive to OP than is NDH1 of *Paracoccus*. EPR spectroscopy of membranous *E coli* NDH1 shows that two slow- and one fast-relaxing Fe–S clusters become detectable upon reduction by NADH in the presence of OP. However, none of them resembles the mitochondrial cluster 2

Key words: Orthophenantroline; Iron–sulfur cluster; NADH dehydrogenase; Bacterial NDH1; Mitochondrial complex I

1. Introduction

The mitochondrial NADH:ubiquinone oxidoreductase (complex I) from bovine heart is a very large membrane enzyme which is composed of more than 40 protein subunits, and several different redox centres. The latter are one FMN molecule and four to six EPR-detectable Fe–S clusters [1]. Bacterial counterparts of complex I are composed of fewer subunits, but they are also large enzymes which contain FMN and several Fe–S clusters [2]. The bacterial enzymes were generally named NDH1, to distinguish them from an alternative membranous NADH:quinone oxidoreductase which is called NDH2. The latter does not contain Fe–S clusters and, in contrast to NDH1, does not couple the redox reaction to proton translocation across the membrane [2]. Recent cloning and sequencing works revealed that there is a high degree of homology between the *Paracoccus* and *E. coli* genes that encode the respective NDH1 subunits, and between them to complex I [3,4].

The *Paracoccus* NDH1 is largely similar to the mitochondrial complex I in the EPR characteristics of its Fe–S clusters and in its inhibitors sensitivity [3]. On the other hand, the enzyme from *E. coli* is much less similar to complex I in such properties [2,5,6]. In contrast to the large difference in their rotenone sensitivity, the inhibition of both *Paracoccus* and *E. coli* NDH1 by capsaicin is nearly identical [7]. However, the inhibition sites of capsaicin in the respiratory chain of mitochondria and bacteria have not been fully characterised, and it appears to inhibit at several different sites. This is indicated by the partial inhibition (25%) by capsaicin of the ferricyanide reduction activity in bovine submitochondrial particles (SMP), and its partial inhibition of the alternative NADH:quinone reductases of *S. cerevisiae* and *E. coli* [7].

The iron chelator, 1,10-phenanthroline (OP), was previously shown to inhibit NADH oxidation by SMP when the acceptor was oxygen, but not when it was ferricyanide or juglone [8,9]. OP is a known inhibitor of photosystem II, where it blocks the oxidation of photo-reduced primary acceptor by the secondary acceptor [10]. In the photosynthetic bacterial reaction centre the binding site of OP was shown, by X-ray crystallography, to be at the Q_B pocket near a histidine side chain that is also ligating the non-haem iron [11].

In this study we have examined the type and site of inhibition by OP in both mitochondrial complex I and *E. coli* NDH1. It is demonstrated that OP inhibits electron transfer between the Fe–S clusters and the ubiquinone pool in both systems.

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Abbreviations DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; EPR, electron paramagnetic resonance; OP, 1,10-phenanthroline (orthophenanthroline); PMSF, phenylmethylsulfonyl fluoride; SMP, submitochondrial particles.

2. Materials and Methods

2.1 Chemicals

1,10-Phenanthroline was purchased from Sigma. 2,3-Dimethoxy-5-

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methyl-6-*n*-decyl-1,4-benzoquinone (DB) was synthesised according to [12] by Dr. Hase in the Department of Organic Chemistry, University of Helsinki.

2.2. Bacterial strains

E. coli strains GO103 [13] and NKSO2 [14] were gifts from Prof. R.B. Gennis, University of Illinois. The *Paracoccus* strains used were either the 'wild type' Pd1222, or a strain lacking subunit I of cytochrome aa_3 (MR13; Raitio and Wikström, submitted for publication).

2.3. Bacterial growth and membranes preparation

Cells of both *Paracoccus* and *E. coli* were grown under high aeration as previously described [15], except that the fermentor was 23 l (B. Braun/Diessel Biotech GmbH ES 15). In addition, the bacteria were grown in media in which succinate was replaced by DL-malate (15 g/l). Log-phase cells were collected by centrifugation and suspended in a cold buffer containing 500 mM sucrose, 5 mM EDTA and 40 mM Tris-Cl, pH 8.0, to about 100 g wet cells/l. The protease inhibitors PMSF and benzamidine were then added (0.5 mM), followed by the addition of lysozyme to final concentration of 0.2 mg/ml. The suspension was mixed for 30 min and centrifuged for 20 min at 10,000 × *g*. The pellets were suspended in 2 mM EDTA, 10 mM potassium phosphate, pH 8.0, to the same volume as the cell suspension. Protease inhibitors were added as above and the suspension mixed vigorously. After 5 min, $MgSO_4$ was added to a concentration of 10 mM, followed by few grains of solid DNase I. The suspension was mixed for 25 min, and centrifuged for 30 min at 40,000 × *g*. The upper, coloured layer of the pellet was suspended in a small volume of buffer containing 2 mM EDTA, 0.5 mM PMSF and 10 mM potassium phosphate, pH 8.0. It was homogenised and used within 30 min to prepare the EPR samples, or for the activity and inhibition assays.

2.4. Submitochondrial particles and complex I preparations

Submitochondrial particles were prepared from rat liver according to [16]. Complex I was purified from bovine heart mitochondria essentially according to [17].

2.5. Enzymatic activity assays

NADH oxidase activity in the membrane samples, either bacterial or SMP, was assayed polarographically using a Clark-type oxygen electrode. The buffer for all the samples was 100 mM potassium phosphate, pH 7.5, and the assays were carried out at 25°C. The NADH:DB oxidoreductase activity of isolated complex I was assayed spectrophotometrically at 340 nm, in a buffer containing 1 mM EDTA, 0.5 mg/ml sonicated soybean phospholipid and 20 mM Tris-HCl, pH 7.4. The NADH concentration was 0.1 mM and the DB concentration was either 0.05, 0.1 or 0.2 mM (Table 1). The assay was carried out at 30°C, and started by the addition of enzyme to a final concentration of 15 nM, as determined from its FMN content.

2.6. EPR spectroscopy and sample preparation

EPR spectroscopy was carried out using a Bruker ESP-300 spectrometer equipped with an ESR-900 liquid helium cryostat (Oxford Instruments). Spectra were acquired and handled using Bruker ESP 1600 software, version 2.2. The EPR parameters were: microwave frequency 9.44 GHz, modulation frequency 100 kHz, modulation amplitude 0.2 mT, time constant 41 ms and sweep speed 0.48 mT/s. The spectra shown are each an average of 4 scans.

The SMP samples were prepared from frozen particles (48.8 mg protein/ml) that were stored at − 70°C. The inhibitors, 10 mM OP or 50 μM rotenone were added first, followed by 5 mM NADH. The samples were mixed vigorously and kept on ice in an open tube for 1 min before transfer into EPR tubes and freezing in liquid N_2.

The *E. coli* samples for EPR spectroscopy were prepared from fresh membranes of strain NKSO2 (41.7 mg protein/ml). OP was added first to a final concentration of 10 mM, followed by NADH (5 mM), or potassium succinate (10 mM), or both. The samples were then treated as described above.

Protein concentrations were determined according to [18].

3. Results and discussion

The sensitivity of NADH:DB oxidoreductase activity of isolated complex I from bovine heart mitochondria to OP is very similar to that of the NADH:O_2 oxidoreductase activity of rat liver SMP (Fig. 1). The observed I_{50} were close to 0.3 mM in both systems, regardless of differences in the source of material and the assay method. These result are in good agreement with previous reports [8,9], and they indicate that complex I is the main site of inhibition by OP in the inner mitochondrial membrane.

In order to test whether OP is a competitive inhibitor of isolated complex I, its inhibition was studied in the presence of different concentrations of ubiquinone analogue DB. The results show that while the rate of NADH oxidation is highly dependent on the DB concentration, the I_{50} is not (Table 1). This suggests that the inhibition by OP is non-competitive with respect to DB, and hence with respect to ubiquinone (Q_{10}) in the mitochondrial membrane.

The binding site of OP in the bacterial photosynthetic reaction centre was precisely located by crystallising the protein in the presence of the inhibitor [11]. Such a tool is not yet available in the study of complex I, but the inhibition site with respect to the different Fe–S clusters can be detected by EPR spectroscopy. EPR spectra of rat liver SMP that were incubated in the presence of NADH and either OP or rotenone are shown in Fig. 2. A spec-

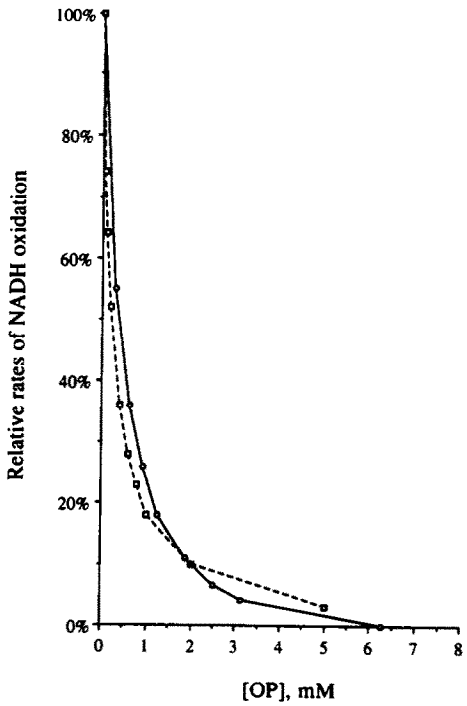

Fig. 1. Inhibition of the electron transfer activities of membranous and isolated complex I by OP. NADH:O_2 oxidoreductase activity was assayed in rat liver SMP (solid line and circles), and NADH:DB oxidoreduction in isolated complex I (dashed line and squares). The DB concentration was 0.10 mM.

trum of oxidised SMP from the same batch is also shown for comparison. The relatively isotropic and large signal of cluster S3 of succinate dehydrogenase ($g = 2.01$) is similar in all the spectra. This is a trinuclear Fe–S cluster which is EPR detectable, below 20 K, only in the oxidised state ($S = 1/2$), but not in the reduced state ($S = 2$) [19]. Due to this property the signal of S3 in the EPR spectra of Fig. 2 indicates that both inhibitors prevented anaerobiosis of the SMP suspension, in spite of the high concentration of both NADH and membranes. The spectra in the presence of OP and rotenone are similar, and in all of them reduced cluster 2 is easily seen at $g = 1.92$ and 2.05. In Fig. 2A it can also be seen that cluster 1b is reduced ($g = 1.94$), while reduced cluster 3 (N4) at $g = 1.88$ and 2.10, and cluster 4 (N3) at $g = 1.86$ and 2.04 [20], can be detected more easily in Fig. 2B under very high radiation power (Albracht's nomenclature is used [20], while Ohnishi's nomenclature is given in brackets). It can thus be concluded that the inhibition site of OP in mitochondria, similarly to that of rotenone, is between all the Fe–S clusters of complex I and the ubiquinone pool.

Several bacteria contain a proton-translocating NADH:ubiquinone oxidoreductase (NDH1). We have studied the inhibition of such enzymes by OP in freshly prepared membranes of *Paracoccus* and *E. coli*. The results were surprising since in contrast to rotenone or even capsaicin, *E. coli* NDH1 is somewhat more sensitive to OP than the *Paracoccus* enzyme (Fig. 3). Moreover, *E. coli* NDH1 could be nearly completely inhibited by raising the OP concentration to 10 mM, but this had only a minor effect on the activity of the *Paracoccus* NDH1 (not shown). The possibility that the OP-insensitive activity in *Paracoccus* was catalysed by an alternative NADH dehydrogenase was rejected since it was fully sensitive to rotenone.

NDH1 of *E. coli* was mainly studied using strain NKSO2 which lacks NDH2 [14]. The latter enzyme, NDH2 of *E. coli* was also tested, but in strain GO103 in which the NADH dehydrogenases were native [13]. This was done by comparing the inhibitions of NADH oxidase and deamino-NADH oxidase activities, and it was found that NDH2 is not significantly inhibited by OP.

In light of the results from the mitochondrial complex I (Fig. 2), and the photosynthetic reaction centre [11], it seems likely that OP also inhibits *E. coli* NDH1 near the ubiquinone binding site. If so, the Fe–S clusters of this enzyme should be reduced and EPR-detectable after short incubation in the presence of OP and NADH.

In a previous study of the Fe–S clusters of membranous NDH1 from *E. coli*, one binuclear cluster was detected and designated $[N\text{-}1]_E$ [5]. However, in a recent study on the isolated enzyme, three different binuclear clusters were reported [6]. One of them, N1a, has a very low midpoint potential and is thus expected to be EPR-invisible under the conditions used here. The second one was named N1b ($g_{x,y,z} = 1.94, 1.94, 2.03$), due to its similarity to the mitochondrial cluster 1b. The third binuclear cluster, named N1c ($g_{x,y,z} = 1.92, 1.95, 1.99$), is unique to *E. coli* NDH1 and no homologous cluster is currently known in any other enzyme of this family [6].

The *E. coli* NDH1 is a labile enzyme even in the native membrane, and its activity as well as EPR spectrum may

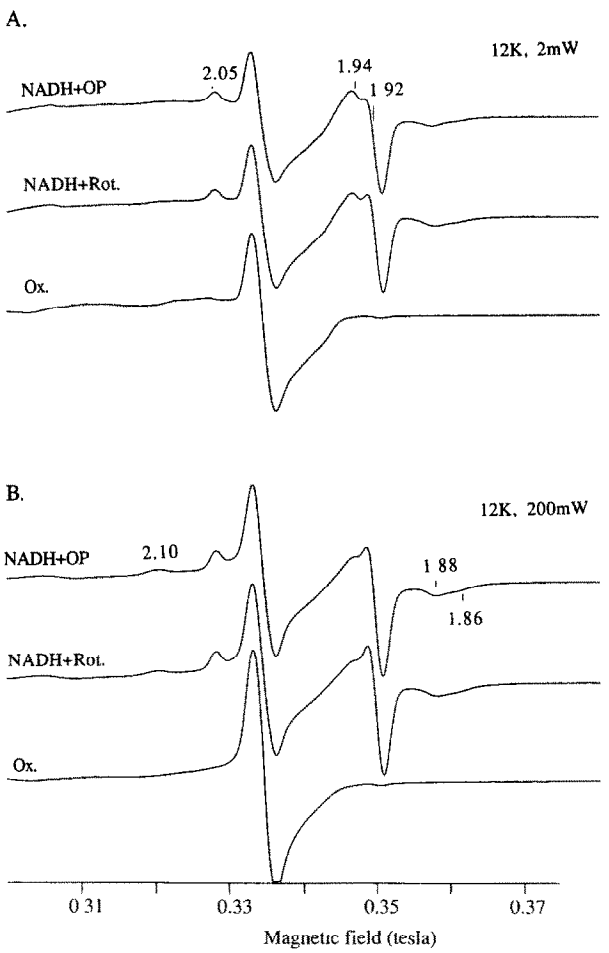

Fig. 2. Determination of the inhibition site of OP in rat liver SMP by EPR spectroscopy. Spectra were collected at 12 K, the radiation frequency was 9.44 GHz, and the radiation power was either 2 mW (A), or 200 mW (B). The samples were incubated in the presence of NADH and either OP or rotenone, or without any additions (Ox.). Selected g values are marked on the traces. For other details see section 2.

Table 1
The dependence of the NADH:DB oxidoreductase activity of isolated complex I, and its inhibition by OP, on the concentration of ubiquinone analogue, DB

[DB](mM)	NADH oxidation rate (μmol/nmol FMN · min)	I_{50} for OP (mM)
0.20	0.51	0.25
0.10	0.42	0.25
0.05	0.10	0.25

The NADH oxidation rates (steady state) are those in the absence of OP. For other details see section 2.

change during long incubation [5]. In order to minimise this we have only used, for both activity and EPR experiments, freshly prepared membranes from cells that were harvested on the same day (see section 2). The EPR spectra (Fig. 4) were recorded from such membranes incubated in the presence of OP together with either NADH, or succinate, or both. Spectroscopy was carried out under different conditions of temperature and radiation power, in order to detect both slow and fast relaxing clusters, as well as to allow direct comparison to the spectra in [6]. The EPR spectrum of NADH-reduced membranes at 40 K indicates the presence of at least one slow-relaxing Fe–S cluster (Fig. 4A). This spectrum contains the transitions previously assigned to cluster $[N\text{-}1]_E$ [5], but in addition, another signal at $g = 1.96$ is clearly detectable. The same signal is also present in the sample that was reduced with NADH plus succinate, but it is absent from the succinate-reduced sample (Fig. 4A). In Fig. 2B it can be seen that the spectrum of the NADH-reduced sample at 16 K is very similar to the 40 K spectrum (Fig. 4A), except for the appearance of a large signal from cluster S3 of succinate dehydrogenase at the lower temperature. This signal indicates that succinate dehydrogenase remains oxidised in the presence of both NADH and OP [19], and hence that OP prevented anaerobiosis. The similarity between the spectra of NADH-reduced samples at 40 K and 16 K includes the '$g = 1.96$' signal. This signal was not previously discussed by Meinhardt et al. [5], but it might be detectable in their NADH-reduced and piericidin-inhibited sample (Fig. 5B of [5]).

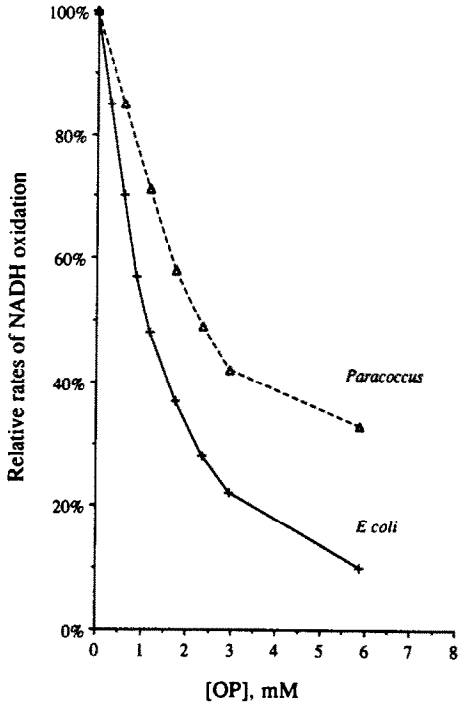

Fig. 3. Inhibition of the NADH:O_2 oxidoreductase activity of NDH1 from both *E. coli* and *Paracoccus* by OP. Freshly prepared membranes of the *E. coli* strain NKSO2, and the *Paracoccus* strain MR13 were used in this experiment.

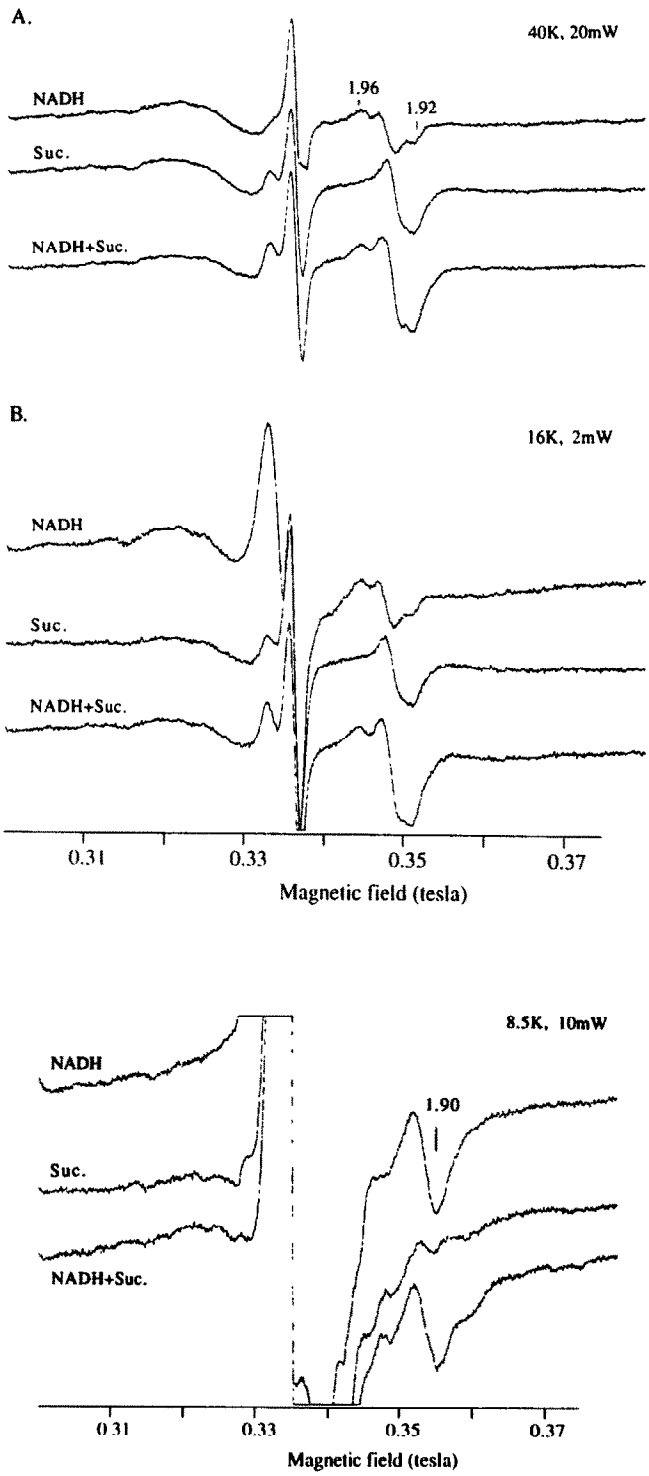

Fig. 4. EPR spectra of *E. coli* membranes after short incubation with OP and different reductants. Freshly prepared *E. coli* membranes (strain NKSO2) were incubated with OP, followed by the addition of either NADH or succinate or both. The EPR conditions were: (A) 40 K and radiation power of 20 mW; (B) 16 K and 2 mW; (C) 8.5 K and 10 mW. For other details see legend to Fig. 2 and section 2.

The '$g = 1.96$' signal probably arises from the g_y transition (at $g = 1.95$) of the unique cluster N1c [6]. It is thus suggested that two binuclear clusters, N1b and N1c, are

detectable in the OP-inhibited and NADH-reduced membranes of *E. coli* (Fig. 4A and B).

At 8.5 K and radiation power of 10mW the slow-relaxing clusters are saturated, and new signals appear (Fig. 4C). The clearest one is at $g = 1.90$, which may arise from the tetranuclear cluster N4 [6]. This signal was detected in a previous study upon incubation of the membranes in the presence of piericidin A and NADH [5]. Hence, its appearance in the OP-inhibited and NADH-reduced samples supports the suggestion that the inhibition site of OP in *E. coli* NDH1 is near the piericidin A site, as does the appearance of the '$g = 1.96$' signal. In addition, and independently of the previous results, the simultaneous presence of reduced NADH dehydrogenase and oxidised succinate dehydrogenase (Fig. 4B) strongly suggests that OP inhibits electron transfer between the Fe–S clusters of *E. coli* NDH1 and the ubiquinone pool.

An interesting but yet unanswered question is whether or not the *E. coli* NDH1 contains a high potential tetranuclear cluster, homologous to the mitochondrial cluster 2 [6]. Such a cluster was not detected in the EPR spectra shown in this work (Fig. 4), nor was it clearly seen in previous studies [5,6]. However, at present we cannot exclude its presence in *E. coli* NDH1, and more experiments have to be done under different redox and EPR conditions.

The results of this work show that OP, like rotenone and piericidin A, inhibits ubiquinone reduction by the proton-translocating NADH:ubiquinone oxidoreductase. However, the differences in sensitivity to these inhibitors between *Paracoccus* and *E. coli* suggest that the inhibition site of OP is not identical to those of either rotenone or piericidin A. This could be employed for detailed studies on the ubiquinone binding site(s) in complex I and in bacterial NDH1.

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