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The functional catalytic unit involved in proton pumping by rat liver cytochrome-*c* reductase and by cytochrome-*c* oxidase

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The effect of partial inhibiton on the protonmotive stoichiometry of cytochrome-*c* reductase and cytochrome-*c* oxidase in intact rat liver mitochondria was examined using myxothiazol and cyanide as inhibitors, respectively. No decrease in the stoichiometry of either enzyme was found. It is shown that this result is consistent with the individual electron transfer units in each case being fully coupled to proton translocation but not with pairs of electron transfer units working in concert in dimers.

Introduction

The mammalian mitochondrial osmoenzymes cytochrome-*c* reductase (cytochrome bc_1 complex; EC 1.10.2.2) and cytochrome-*c* oxidase (EC 1.9.3.1) are protein complexes. The 'basic enzyme unit' in each case appears to consist of single copies of each of the constituent polypeptide subunits (11 and 13, respectively [1,2]). Both enzymes can be dimeric [3,4], but it seems well established that the basic enzyme unit contains the minimal catalytic unit for their electron transfer function. This conclusion is derived principally from three types of experiment. First, an interconversion between monomeric and dimeric forms can be achieved for both enzymes by changing the detergent and/or the ionic strength, the monomeric form in each case retaining most of the electron transfer activity of the dimeric form [5–9]. Second, titration curves of electron transfer activity versus inhibitor concentration are generally consistent with maximal inhibition occurring at one inhibitor molecule per basic enzyme unit. Such evidence is especially strong for cytochrome-*c* reductase because a wide range of inhibitors, many tight-binding ($K_d <$ 10^{-10} M, see Ref. 10 for a review), are available [11–15], but the same conclusion has been reached for cytochrome-*c* oxidase by titration of its activity with cyanide (either directly [16], or indirectly, by monitoring the steady-state reduction level of cytochrome *c* in submitochondrial particles [17]). Third, radiation-inactivation analysis indicates that the target size for the electron transfer activity of cytochrome oxidase is smaller than the basic enzyme unit [18–21]–in fact, except for the slightly larger values obtained by Suarez et al. [19], the reported target sizes correspond well with the combined molecular weights of the oxidase subunits I and II which are known to contain the four redox centres [22]. Recently, Nugent and Bendall [23] reached a similar conclusion for the chloroplast enzyme, the cytochrome *bf* complex, which is closely related to the mitochondrial cytochrome-*c* reductase. They found a target size consistent with an electron transfer catalytic unit composed of only one copy each of cytochromes *b* and *f* together with one copy of the Rieske iron-sulphur polypeptide.

In this paper we are concerned with whether the proton-pumping activities of cytochrome-*c* reductase and cytochrome-*c* oxidase are associated with individual electron transfer units (which, in both cases, we assume are contained within the basic enzyme units), or whether pairs of electron transfer units acting in concert within dimers are required for this activity. To distinguish between these possibilities, we have examined the effect of partial inhibition on the protonmotive stoichiometry of the two enzymes in intact rat liver mitochondria, using myxothiazol to inhibit the reductase and cyanide to inhibit the oxidase. We find no decrease in the stoichiometry of either enzyme, a result consistent with individual electron transfer units being fully coupled to proton translocation.

Abbreviations: $\Delta H_o^+/2e^-$, the change in the quantity of H^+ in the suspension medium per $2e^-$ accepted by oxidant; $\leftarrow H_o^+/2e^-$, the protonmotive stoichiometry, i.e. the number of protons output into the suspension medium per $2e^-$ accepted by oxidant (see, e.g., Ref. 26 for the relationship between $\Delta H_o^+/2e^-$ and $\leftarrow H_o^+/2e^-$); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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Materials and Methods

Rat liver mitochondria were isolated and protein estimations were carried out as described before [24]. All mitochondrial suspensions were kept on ice for use on the same day as preparation.

The apparatus and procedure for the oxidant pulse and rate/pulse experiments were as described in Mitchell et al. [25] and Moody et al. [26], respectively. For the high sensitivity detection of oxygen, current flow across a Clark-type oxygen electrode (fitted with a 13 μm thick Teflon membrane) was measured as the voltage change across a 1 MΩ resistor in series with the electrode. This arrangement was only used at oxygen tensions below about 10% of ambient so that the polarising voltage was always between -0.6 V and -0.8 V. Changes in the polarising voltage over this range had no significant effect on the sensitivity of the electrode. The response of the electrode under the conditions of the experiments described in this paper was slow; a 90% response time of about 15 s was found.

Other specific experimental details are given in the figure legends.

Reagents were supplied either by the Sigma Chemical Co. (Dorset, U.K.) or by BDH (Dorset, U.K.) except for choline base, obtained from Fluka AG (Buchs, Switzerland), and myxothiazol, obtained from Boehringer Mannheim (East Sussex, U.K.). Before use the choline base was purified using activated charcoal, and the rotenone was recrystallized twice in ethanol.

Rationale

Note that: (1) it is assumed that the individual electron transfer units are contained within the basic enzyme units; (2) no assumption is made as to the state of dimerisation of rat liver mitochondrial cytochrome-*c* reductase and cytochrome-*c* oxidase in situ; and (3) non-cooperative inhibitor binding is assumed, as is known to be the case for myxothiazol and cyanide [11,16,17].

If x is the fraction of basic enzyme units with an inhibitor bound, then the fraction of dimers that have 0, 1 and 2 sites inhibited are, respectively, $(1-x)^2$, $2x\cdot(1-x)$ and x^2.

If we assume that uninhibited dimers pump protons with an $\leftarrow H_o^+/2e^-$ of A, the half-inhibited dimers pump protons with an $\leftarrow H_o^+/2e^-$ of B, and that all uninhibited basic enzyme units have the same electron transfer activity, we find that

$$\leftarrow H_o^+/2e^- = \frac{2\cdot(1-x)^2\cdot A + 2x\cdot(1-x)\cdot B}{2\cdot(1-x)^2 + 2x\cdot(1-x)}$$

$$= (1-x)\cdot A + x\cdot B \qquad (1)$$

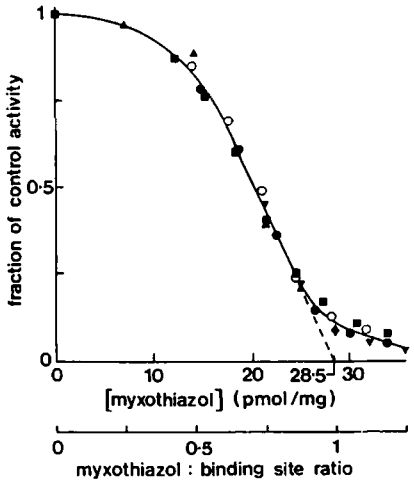

Fig. 1. Titration of rat liver mitochondrial succinate oxidase activity with myxothiazol. The rate of oxygen uptake by a suspension of rat liver mitochondria (1.2 mg of mitochondrial protein/ml) was measured in 0.1 M potassium phosphate buffer (pH 7.2), containing 200 mM sucrose, 3 mM potassium succinate, 240 nM FCCP, 15 ng valinomycin/mg of mitochondrial protein and myxothiazol at the concentrations shown. The concentration of the myxothiazol stock solution was determined spectroscopically using ε ($mM^{-1}\cdot cm^{-1}$) = 10.5 at 313 nm [10]. The results of five titrations using four mitochondrial preparations (▲ and ▼ are for the same preparation) are shown. Control activity was 314 ± 47 (S.D.) nmol of oxygen per min per mg of mitochondrial protein.

If the functional unit of proton pumping is associated with a pair of electron transfer units within a dimer then $B=0$ so that $\leftarrow H_o^+/2e^- = A\cdot(1-x)$, i.e., the stoichiometry decreases in direct relationship to the fraction of basic enzyme units that are inhibited. If the functional unit of proton pumping is associated with an individual electron transfer unit within a monomer then $B=A$ so that $\leftarrow H_o^+/2e^- = A$, i.e., the protonmotive stoichiometry is independent of the fraction of basic enzyme units that are inhibited.

Results

The protonmotive stoichiometry of cytochrome-c reductase on binding of substoichiometric levels of myxothiazol

Myxothiazol is a specific tight-binding inhibitor of mammalian cytochrome-*c* reductase ($K_d < 10^{-10}$ M, [10]). Fig. 1 shows sample titrations of the succinate oxidase activity of suspensions of rat liver mitochondria with myxothiazol. The concentrations of myxothiazol binding sites in the mitochondrial suspensions used in this work were estimated from such curves by extrapolation as shown (see Ref. 13) and found to be in the range 30 ± 2 (S.E., $n=6$) pmol/mg of mitochondrial protein. This range is lower than that found by Estabrook [[27] (70–80 pmol/mg) for the antimycin binding sites in rat liver mitochondria but is consistent with measurements from difference spectra ([succinate reduced + antimycin] − oxidised) of the cytochrome *b* content of simi-

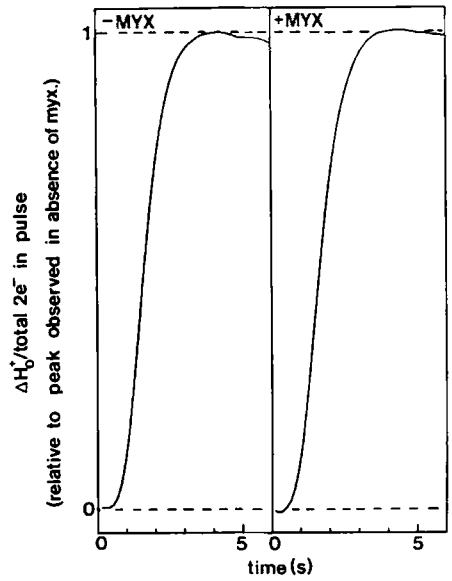

Fig. 2. The effect of myxothiazol on proton pumping by mitochondrial cytochrome-*c* reductase. Mitochondria (6 mg of mitochondrial protein/ml) were incubated anaerobically at 25 °C in 150 mM KCl containing 1 mM EGTA, 1 mM glycylglycine, 2 mM choline chloride and 20 μg/ml carbonic anhydrase (medium pH, 7.0–7.15). Valinomycin (0.1 μg/mg of mitochondrial protein), rotenone (0.6 μM) and myxothiazol (added as indicated at 0.45 mol/mol of binding sites) were added as aerobic solutions in ethanol at 1, 1.5 and 2 min, respectively, after the start of the incubation. A pre-pulse of potassium ferricyanide (40 nmol) (see Ref. 26), and *N*-ethylmaleimide (33 nmol/mg of mitochondrial protein) were added anaerobically at 10 min and 14 min, respectively. Electron transfer through cytochrome-*c* reductase was initiated after about 20 min pre-incubation with a pulse of anaerobic 10 mM potassium ferricyanide added using a remote-controlled syringe. In both cases, a total of about 40 nmol ferricyanide was added at about 65 nmol/s; the average time-courses of $\Delta H_O^+/2e^-$ relative to the peak value reached in the absence of myxothiazol for seven pulses are shown; and the average age of the mitochondrial suspensions used was the same. The average concentration of myxothiazol binding sites was 32 pmol/mg of mitochondrial protein.

lar mitochondrial suspensions, assuming one binding site per two oxidant-inducible haems *b* as the present text is ambiguous.

The effect of myxothiazol on the protonmotive stoichiometry of cytochrome-*c* reductase was examined by the oxidant-pulse method [25]. It was found that the time taken for the pulse of oxidant (ferricyanide) to be consumed was insensitive to myxothiazol at myxothiazol : binding site ratios up to at least 0.65, the rate of electron transfer from cytochrome *c* to ferricyanide being rate-limiting. At a myxothiazol : binding site ratio of 1 the time taken for the pulse to be consumed was almost doubled, corresponding to an average electron transfer rate of 15 nmol · min^{-1} · (mg of mitochondrial protein)$^{-1}$. This rate is consistent with the uncoupled rate of oxygen uptake observed at the same concentration of myxothiazol with succinate as substrate (see, e.g., Fig. 1). The results in Fig. 2 show that at a myxothiazol : binding site ratio of 0.45 there is no significant effect on the protonmotive stoichiometry of cytochrome-*c* reductase. Note that at this concentration myxothiazol has no effect on the kinetics of ΔH_O^+ so that the time-courses with and without myxothiazol can be directly compared without need for a correction for H^+ back flow. If the functional unit for proton pumping were associated with pairs of electron transfer units operating in concert within dimers we would have expected a 45% decrease in apparent protonmotive stoichiometry. Since this was not observed we conclude that the functional unit for proton pumping by cytochrome-*c* reductase is associated with individual electron transfer units.

The protonmotive stoichiometry of cytochrome-c oxidase on binding of substoichiometric levels of cyanide

Cyanide has low affinity for fully-reduced cytochrome-*c* oxidase, but under turnover conditions it behaves as a moderately tight-binding inhibitor (K_d = 10^{-3}–10^{-4} M and 10^{-7}–10^{-8} M, respectively [28]). Hence, during the anaerobic pre-incubation required for oxygen-pulse experiments the oxidase can be essentially uninhibited, but once turnover is started by the addition of oxygen the level of inhibition increases as the cyanide binds. When the oxygen is consumed the enzyme returns to the fully-reduced state and the cyanide slowly dissociates ($t_{1/2}$ for bovine heart enzyme reported to be about 10 s [29] or about 80 s [28]). These effects are illustrated by the experiments shown in Fig. 3 where anaerobic mitochondria in the presence of ascorbate/TMPD were pulsed with oxygen. In the absence of cyanide the oxygen pulses were consumed too fast to be detected with the Clark-type electrode used (Fig. 3B). In the presence of cyanide the first oxygen pulse of each series was almost indetectable (Fig. 3A, pulses 1 and 5), but during subsequent pulses the amount of oxygen detected increased considerably reflecting a progressive decrease in the rate of oxygen consumption. When the time between each pulse was increased (Fig. 3A, compare pulses 6–8 with pulses 2–4) the increase in oxygen

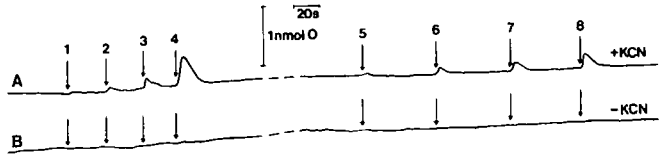

Fig. 3. The effect of cyanide on the oxygen detected on pulsing anaerobic mitochondria with oxygen. Mitochondria (6 mg of mitochondrial protein/ml) were incubated anaerobically at 25 °C in 150 mM KCl containing 1 mM EGTA, 1 mM glycylglycine, 5 mM ascorbate, 200 μM TMPD and 20 μg/ml carbonic anhydrase (medium pH, 7.0–7.15). Valinomycin (0.1 μg/mg of mitochondrial protein) was added 1 min after the start of the incubation. Cyanide was present as indicated at 120 μM. After 20 min pre-incubation a series of four pulses of air-saturated 150 mM KCl (about 20 nmol oxygen each) were made at 30-s intervals (A, 1–4 and B, 1–4). After 25 min pre-incubation a further series of four pulses were made at 1-min intervals (A, 5–8 and B, 5–8). All pulses were the same size.

detected was smaller reflecting the increased dissociation of the cyanide between pulses.

The effect of cyanide on the protonmotive stoichiometry of cytochrome-*c* oxidase was examined by the oxygen-rate/pulse method in the presence of antimycin and myxothiazol, using hexammineruthenium(II) ($[Ru(NH_3)_6]^{2+}$) as reductant [26]. The rate/pulse method is a modification of the conventional oxygen-pulse method [24,25] where electron flow through the oxidase is initiated and maintained by addition of oxygen at a constant rate to suspensions of initially anaerobic mitochondria. The rate of oxygen consumption during the rate-pulse is determined by the rate of oxygen addition, the oxidase being far from rate-limiting. This method has the advantage over the conventional pulse method in this context in that cytochrome-*c* oxidase can be substantially inhibited (the results of a simulation study in Ref. 26 would suggest at least 90%) before ΔH_O^+ generation during the pulse is significantly slowed. Therefore, the time-courses of ΔH_O^+ with and without cyanide can again be directly compared without need for a correction for H^+ back flow. A decrease in the protonmotive stoichiometry concomitant with cyanide binding would be easily seen provided the oxidase became sufficiently inhibited. The results in

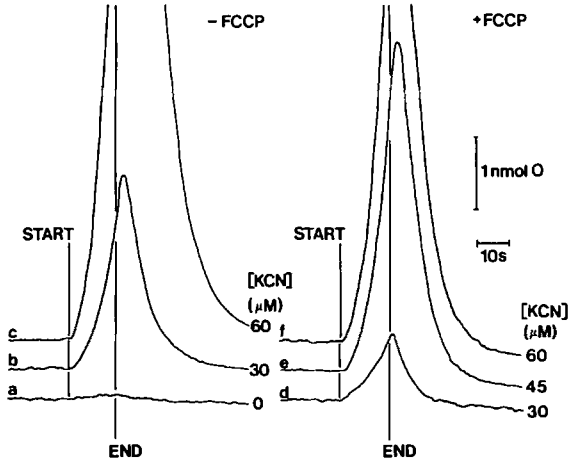

Fig. 4. The effect of cyanide on the oxygen detected during oxygen-rate/pulses. Experimental details were as described in the legend to Fig. 2 with the following differences/additions. Choline chloride was omitted from the medium. Since hexammineruthenium(II) is autoxidisable it was more convenient to add hexammineruthenium(III) to the medium (120 μM). Most of this was reduced by endogenous reductants during the anaerobic preincubation before the addition of antimycin (0.1 μg/mg of mitochondrial protein) and myxothiazol (0.15 μg/mg of mitochondrial protein) as anaerobic solutions in ethanol at 14.5 min and 15 min, respectively. Cyanide was present at the concentrations indicated. FCCP, where indicated, was present at 1 μM. Electron transfer through cytochrome-*c* oxidase was initiated after about 20 min pre-incubation by adding air-saturated 150 mM KCl (preincubated at 25 °C) at a constant rate using a remote-controlled syringe. In all cases the rate-pulses were the same size consisting of a total of about 50 nmol oxygen added at about 3.2 nmol/s.

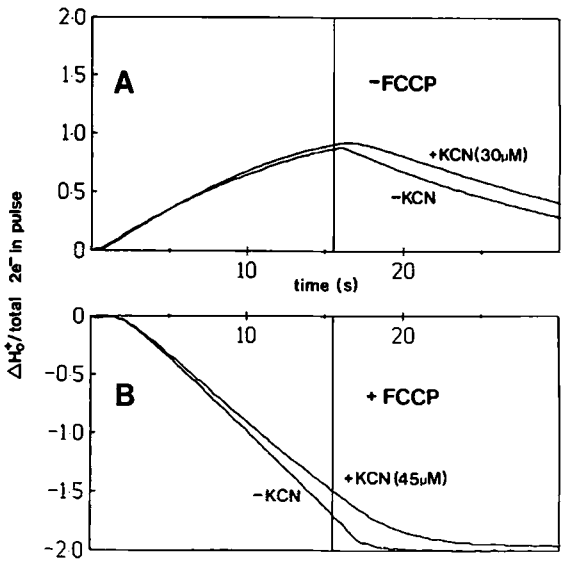

Fig. 5. The effect of cyanide on proton pumping by mitochondrial cytochrome-*c* oxidase. Experimental details were as described in the legends to Figs. 4 and 2. Cyanide was present at the concentrations indicated. FCCP, where indicated, was present at 1 μM. Electron transfer through cytochrome-*c*-oxidase was initiated after about 20 min pre-incubation by adding air-saturated 150 mM KCl (preincubated at 25 °C) at a constant rate using a remote-controlled syringe. The figure shows the average time-courses of $\Delta H_O^+/2e^-$: time-courses without FCCP are the averages from 12 rate-pulses; those with FCCP are the averages from eight rate-pulses. The rate-pulses were all of the same size, i.e. a total of about 50 nmol oxygen added at about 3.2 nmol/s. The vertical lines mark the end of the oxygen addition. The average age of the mitochondrial suspensions used was the same in each case.

Fig. 4 show that this condition was more than satisfied at the concentration of cyanide used here (30 μM) since the level of oxygen observed shortly after rate-pulses with cyanide was always at least 10-times greater than that observed without cyanide (compare Fig. 4, a and b). We conclude from this that the level of inhibition by the end of the rate-pulses was greater than 90%.

The average time-courses for a number of rate-pulses with and without cyanide are shown in Fig. 5A and it can be seen that cyanide binding causes no decrease (in fact, there is a slight increase) in the apparent protonmotive stoichiometry of cytochrome-*c* oxidase. From this we conclude that, like cytochrome-*c* reductase, the functional unit for proton pumping by cytochrome-*c* oxidase is associated with individual electron transfer units.

The final $\Delta H_O^+/2e^-$ value reached in the presence of the uncoupler FCCP, i.e., the apparent stoichiometry for the net scalar reaction of cytochrome-*c* oxidase, was somewhat less than the theoretical value of −2 when cyanide was present (Fig. 5B). Possible explanations for this and the slight increase in the protonmotive stoichiometry will be discussed later. Note that cyanide was found to be less effective in the presence of FCCP (Fig. 4, compare d with b) and that the concentrations

used in the presence and absence of FCCP were 45 μM and 30 μM, respectively, so that the time-courses with cyanide in Fig. 5, A and B should not be compared directly.

Discussion

We have shown in this paper that partial inhibition of the osmoenzymes cytochrome-*c* reductase and cytochrome-*c* oxidase in rat liver mitochondria, using myxothiazol and cyanide, respectively, does not decrease the protonmotive stoichiometry of either enzyme. This result allows us, using the reasoning laid out in the Rationale, to eliminate the possibility that the proton pumping activities of these enzymes are associated with pairs of electron transfer units operating in concert in dimers, and is consistent with the individual electron transfer units in each case being fully coupled to proton translocation. It should be noted that this reasoning is based on the assumption that the electron transfer unit of both enzymes is contained within the basic enzyme unit (defined as consisting of one copy of each constituent polypeptide [1,2]). While there seems to be no question that this is true for cytochrome-*c* oxidase, it is not universally accepted for cytochrome-*c* reductase, and models where the dimer is necessary for the electron transfer function of this enzyme are still current (e.g., the 'double Q-cycle' model introduced by De Vries et al. [30]). Weiss and co-workers favour a 'dimeric' electron transfer model for the enzyme isolated from *Neurospora crassa* [31] and have found a titre of one inhibitor molecule per dimer for this enzyme using antimycin [32] (as did Graan and Ort [33] for the cytochrome *bf* complex with DBMIB). Also, De Vries [34] has failed to observe a monomeric form of reductase under ostensibly the same conditions as those used successfully in Ref. 5. However, we feel that the weight of evidence (see the Introduction), especially that from inhibitor titrations [11–15], favours a 'monomeric' rather than a 'dimeric' electron transfer unit for cytochrome-*c* reductase.

Finel and Wikström have reported results that suggest that for cytochrome oxidase proton pumping is only observed when the dimeric form is reconstituted into phospholipid vesicles [35]. It should be pointed out that the present results are compatible with this since we have only demonstrated that there is no mechanistic requirement for the dimer with respect to proton pumping. A structural requirement where the conformation of the basic enzyme unit that is appropriate for proton pumping can only occur in dimers is still possible. Our results show that this conformation would also have to be present in a basic enzyme unit whose counterpart in a dimer had been inhibited.

A slight increase in protonmotive stoichiometry in the presence of cyanide was consistently observed. One possible explanation for this increase is that cyanide binds preferentially to any uncoupled mitochondria present in the mitochondrial suspension (see Ref. 26 for a discussion of the effect of mitochondrial damage on protonmotive stoichiometry). However, the observation that cyanide was less effective when the uncoupler FCCP was present (Fig. 4, compare d with b) seems to eliminate this possibility. Another possibility is that a proton is released to the medium on cyanide binding. This would cause an apparent increase in $\Delta H_o^+/2e^-$ of between 0.02 and 0.04, taking the concentration of oxidase in our mitochondrial suspensions to be between 50 and 100 pmol/mg (i.e., twice the concentration of reductase) and is supported by the observation of a difference of this size between the final $\Delta H_o^+/2e^-$ values observed with and without cyanide in the presence of the uncoupler FCCP (Fig. 5B). However, a difficulty with this explanation is that Konstantinov et al. [36] have shown that the cyanide binding site in oxidase is in protonic contact with the mitochondrial matrix and not with the medium.

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