Stoichiometry of Proton Translocation Coupled to Substrate Oxidation in Plant Mitochondria

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ABSTRACT

The proton translocation coupled to the electron flux from succinate, exogenous NADH, and NAD⁺-linked substrates (malate and isocitrate) to cytochrome c and to oxygen was studied in purified potato (Solanum tuberosum) mitochondria using oxygen and ferricyanide pulse techniques. In the presence of valinomycin plus K⁺ (used as a charge compensating cation), optimum values of H⁺/2 e⁻ were obtained when low amounts of electron acceptors (oxygen or ferricyanide) were added to the mitochondria (1–2 nanogram [2 e⁻] equivalents per milligram protein). The stoichiometry of proton translocation to electron flux was unaffected in the presence of N-ethylmaleimide, an inhibitor of the P/H⁺ symport. With succinate as substrate, H⁺/2 e⁻ ratios were 4.0 ± 0.2 and 3.7 ± 0.3 with oxygen and ferricyanide as electron acceptors, respectively. With exogenous NADH, H⁺/2 e⁻ ratios were 4.1 ± 0.9 and 3.4 ± 0.2, respectively. The proton translocation coupled to the oxidation of NAD⁺-linked substrates (malate, isocitrate) was dependent upon the presence of adenylates (ADP, AMP, or ATP). For malate (+ glutamate) oxidation the observed H⁺/2 e⁻ ratios were increased from 3.6 ± 2.2 to 6.5 ± 0.5 in the presence of 20 micromolar ADP.

It is generally accepted that mitochondrial respiration results in the formation of a transmembrane electrochemical proton gradient generated by an electrogenic proton extrusion (15, 16). It is also widely accepted that ΔH⁺ is the intermediate between electron transport and ATP synthesis (3). However, despite considerable research on stoichiometry relationships, there is still uncertainty concerning the values of H⁺/2 e⁻ per site of phosphorylation (or H⁺/ATP ratios), and the ability of Cytc oxidase to extrude protons (3, 30).

Although considerable attention has been focused on these questions in mammalian systems, relatively little work has been performed on mitochondria isolated from microorganisms (9, 27, 29) or on plant mitochondria (17). The operation of an electronneutral K⁺/H⁺ antiporter has been suggested to explain the underestimation of H⁺/2 e⁻ ratios in both yeast and plant mitochondria (6, 17, 29).

In the studies reported here, the stoichiometry of the H⁺/2 e⁻ ratios associated with different spans of the respiratory chain has been reinvestigated using highly cyanide-sensitive plant mitochondria to avoid interference with the nonelectrogenic alternative pathway (19). Special attention has been given to the comparison between the proton translocation driven by exogenous NADH and that driven by succinate. These two substrates are coupled to phosphorylation sites 2 and 3 and are oxidized via two different dehydrogenases located on opposite sides of the inner mitochondrial membrane (for review, see 22). Using ferricyanide as an electron acceptor (acting at the Cytc level), it is possible, for these two substrates, to determine the efficiency of proton translocation at the level of complex III alone.

In plant mitochondria, the oxidation of NAD⁺-linked substrates, which involves the participation of complex I, is complicated by the presence of two different oxidation pathways for endogenously generated NADH (23). These pathways, which are respectively sensitive and insensitive to rotenone (5), exhibit different affinities for endogenously generated NADH (18) and may be regulated by adenine nucleotides (28). The H⁺/2 e⁻ stoichiometry for malate and isocitrate was investigated, keeping in mind the possibility of regulation of the mitochondrial electron flux at the level of complex I.

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria were prepared from potato tubers (Solanum tuberosum L.) as described previously (8). After purification on Percoll gradient, the mitochondria were resuspended in a reaction medium containing 0.3 M mannitol, 30 mM KCl, 7 mM MgCl₂, 1.5 mM MOPS (pH 7.0), and 1 mg ml⁻¹ BSA. The protein concentration was determined by Nesslerization.

Oxygen Uptake Measurements. Oxygen was measured polarographically with a Clark-type oxygen electrode (Hansatech Ltd, King’s Lynn, U.K.), coupled to a Servo chart recorder (Servotrace, model PED X 100). The chamber (2 ml) was enclosed in a thermostatically controlled water jacket at 25 ± 0.02°C.

pH Measurements. pH measurements were carried out simultaneously with oxygen determinations in the same chamber, using a prestandardized Beckman microcombination electrode coupled to a Beckman (Research) pH meter. The system was calibrated during each experiment by additions of known amounts of standard 0.015 N HCl. When HCl pulses (sufficient to change the pH by 0.1 unit) were injected into the cell containing the experimental medium, the electrode showed 80% of the final deflection in less than 2 s. Mitochondria (about 2.0–2.5 mg protein ml⁻¹) were incubated in 2 ml of reaction medium. The suspension was made anaerobic by flushing with N₂ after the addition of substrate. The electrode was sealed by a sleeve fitted tightly into the cell. Additions to the cell were made via a 1 mm gap in the sleeve. Catalase (Sigma) was added at a concentration of 1.25 mg ml⁻¹ in the medium before anaerobiosis and, afterwards, oxygenation of the medium was obtained by small additions of 0.5 to 4 μl of 16 to 80 mM H₂O₂, without any significant dilution of the mitochondrial suspension. Pulses of ferricyanide

Abbreviations: MOPS, 4-morpholinopropanesulfonic acid; CCCP, cyanide-m-chlorophenylhydrazone; NEM, N-ethylmaleimide; TPP⁺, tetraphenylphosphonium ion.
were made by addition of 1 to 20 µl of an O₂-free solution of 15 mM ferricyanide.

H⁺/O Ratios Determinations. According to Mitchell and Moyle (15), the H⁺/O ratios can be calculated in the experiments of oxygen pulse by extrapolating the H⁺ decay to the time when half of the added O₂ has been consumed, assuming a state 3 rate during the burst of respiration. In this work, due to the presence of valinomycin, the rate of oxidation corresponds to an uncoupled respiration which was calculated using large oxygen pulses (6). For both succinate and exogenous NADH, an extrapolation to zero time introduced negligible errors when low amounts of oxygen were added to anaerobic mitochondria.

Membrane Potential Determination. Membrane potentials were measured using the TPP⁺ electrode described by Kamo et al. (11). A thin and flexible electrode was constructed by use of a polyvinylchloride (PVC) tube and a PVC-based membrane containing tetraphenylboron as an ion exchanger. Ten mM TPP⁺ was placed inside the tube as an internal reference solution in which a silver wire, coated with AgCl, made the connection with a reference electrode (Ag/AgCl/KCl). For simultaneous measurements of membrane potential and pH, the reference electrode of the pH meter was taken as a common electrode. The signals from the pH meter and the TPP⁺ electrode were simultaneously fed to a dual-trace recorder (Servotrace, model PED X 100). The electrode was calibrated by successive additions of TPP⁺ up to 10 µM, each addition doubling the concentration of TPP⁺ in the mixture. Each doubling of the TPP⁺ concentration gave the same increase of the electrode potential: 17.8 mV (11). The absolute values of the membrane potential (Δψ) were calculated according to the Kamo et al. (11) equation:

\[ Δψ = 59 \log \left( \frac{V}{V'} \right) - 59 \log \left( \frac{10^{Δψ/19} - 1}{10} \right) \]

where \( V, V' \), and \( E \) stand for the mitochondrial volume, the volume of the incubation medium, and the deflection of the TPP⁺ electrode potential from the value prior to the injection of mitochondria, respectively. In these experiments, \( V \) was taken equal to a volume of 1 µl mg⁻¹ protein (14).

**RESULTS**

Effect of Valinomycin on Proton Translocation. Simultaneous changes in the external pH of the suspension medium and membrane potential in potato mitochondria oxidizing succinate during an oxygen pulse experiment are illustrated in Figure 1. For oxygen pulse equivalent to 1 ngatom O mg⁻¹ protein, a low proton ejection occurred and a rapid uptake of TPP⁺ indicated an important change in membrane potential which was estimated to be 140 mV. The subsequent anaerobic proton back-diffusion was quite slow, whereas the collapse of membrane potential occurred in approximately 20 s. For oxygen pulse equivalent to 10 ngatom O mg⁻¹ protein, about 8 nmol H⁺ mg⁻¹ protein were expelled and a larger membrane potential (170 mV) was generated. Anaerobic proton back-decay and collapse of membrane potential were reached after approximately 20 s. In the presence of valinomycin (20 ng mg⁻¹ protein), membrane potential collapsed and, for both small or large quantities of pulsed oxygen, proton extrusion was enhanced. Under conditions of small oxygen pulses due to the time response of the system, proton ejection lasted about 3 s but the burst of respiration was approximately 0.5 s (5) as calculated assuming an uncoupled rate of oxidation (6).

H⁺/O Stoichiometry with Succinate and Exogenous NADH. As shown in Figure 2 for both succinate and NADH oxidation, the values of the observed H⁺/O rates in the presence of valinomycin were constant for oxygen pulses ranging between 0.5 and 2 ngatom O mg⁻¹ protein; then they declined rapidly with increasing oxygen, especially with NADH. For succinate, the dependence of H⁺/O upon the amount of pulsed oxygen can be interpreted in terms of increased reentry of protons catalyzed by an H⁺/K⁺ antiport when the transmembrane ΔpH is enhanced in response to addition of large amounts of oxygen (6). With NADH, such an exchange likely occurs. According to the chemiosmotic theory, the presence of a protonophore (CCCP) collapses the proton movements driven by the redox pumps of the respiratory chain. Moreover, during succinate oxidation, the protons coming from the dehydrogenation of substrates are balanced by the uptake of protons used to form water at the level of Cyt oxidase (1). In potato mitochondria, in the presence of CCCP, no changes of pH occurred during succinate oxidation. By contrast, with NADH as substrate, a disequilibrium of one
proton per 2 $e^-$ occurred leading to a net alkalinization of the suspension medium (data not shown). In the experiments reported in Figure 2, this alkalinization was negligible for small oxygen pulses but was probably responsible for the fast decrease of the observed H$^+/O$ ratios with the amounts of added oxygen.

Based on determinations of proton ejection for small oxygen pulses, H$^+/O$ ratios averaged 4.0 ± 0.2 (10 independent experiments) and 4.1 ± 0.9 (five independent experiments) for succinate and NADH, respectively. These values are in good agreement with H$^+/O$ ratios reported for substrates involving the phosphorylation sites 2 and 3 in mammalian mitochondria (13, 15, 16, 24). However, stoichiometries higher than 4 have been observed for succinate in mammalian mitochondria, especially when N-ethylmaleimide was present to inhibit the H$^+/Pi$ symport (4, 7, 25).

**Effect of N-Ethylmaleimide.** It is well established that the H$^+/Pi$ symport is theoretically extremely active in most mitochondria and that Pi uptake due to the induced $\Delta \text{pH}$ results in a movement of protons into the matrix and hence in an underestimation of the H$^+/O$ stoichiometry (3). The effect of NEM, an inhibitor of the H$^+/Pi$ symport (3), on the H$^+/O$ stoichiometry in potato mitochondria is reported in Figure 3. Addition of only 1 mM phosphate caused 30% inhibition of the H$^+/O$ ratio, but an incubation with NEM abolished this effect at phosphate concentrations lower than 0.3 mM. Thus, the addition of NEM to mitochondria incubated in the presence of phosphate prevented the inhibition of proton extrusion caused by phosphate transport. However, in the absence of exogenous phosphate, addition of 60 ng NEM mg$^{-1}$ protein did not enhance proton extrusion and H$^+/O$ ratios. This experiment indicates that in plant mitochondria H$^+/O$ ratios determined by the oxygen pulse technique were not underestimated in proportion to phosphate transport. Similarly, the observed H$^+/O$ ratios with succinate were unaffected by the presence of rotenone, which suggests that no proton extrusion linked to malate oxidation or endogenous substrates occurred under these conditions (data not shown).

**H$^+/O$ Stoichiometry with NAD$^+$-Linked Substrates.** Proton translocation driven by malate (+ glutamate) was very low as long as valinomycin was not present to collapse the membrane potential. Moreover, the proton ejection taking place during the oxygen pulse was dependent upon the way of reaching anaerobiosis (Fig. 4). In the first case (A), the mitochondrial suspension was incubated for a few min in the presence of substrates and then made anaerobic by flushing with N$\_2$. Under these conditions, the subsequent oxygen pulse led to very little proton ejection. In the second case (B), anaerobiosis was rapidly ob-

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**Fig. 4.** Effects of ADP on proton translocation driven by malate (+ glutamate) oxidation in potato mitochondria. Oxygen pulses were given after anaerobiosis had been obtained by (A) oxidizing substrates and then flushing with N$\_2$ or (B) by flushing with N$\_2$ and then oxidizing substrates. Twenty mM malate; 10 mM glutamate; 20 ng valinomycin mg$^{-1}$ protein; 20 $\mu$M ADP when present; 2 to 2.5 mg ml$^{-1}$ mitochondrial protein. Oxygen pulse: 2 ng atom O mg$^{-1}$ protein.

**Fig. 5.** Dependence of observed H$^+/O$ ratios on the amount of oxygen added as a pulse to anaerobic potato mitochondria oxidizing malate (+ glutamate) in the absence or in the presence of adenylates. (A), 20 mM malate + 10 mM glutamate; (●), in the presence of 20 $\mu$M AMP; (○), in the presence of 20 $\mu$M ADP.

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When the proton translocation was low, addition of 20 $\mu$M ADP enhanced the rate of proton extrusion significantly and subsequent proton back diffusion occurred at a higher rate (Fig. 4A). Similar responses
were obtained with AMP or ATP. Determinations of H⁺/O ratios from malate (+ glutamate) provided values which were also dependent upon the amounts of pulsed oxygen (Fig. 5). For oxygen pulses ranging between 0.5 and 2 ng atom mg⁻¹ protein, H⁺/O ratios were maximum and rather constant, then they decreased for larger amounts of oxygen. The same dependence of H⁺/O ratios upon the amounts of oxygen was observed when adenylates were present.

Table I summarizes the mean values of H⁺/O ratios obtained with malate under typical experimental conditions. On the average, H⁺/O ratios were 3.6 ± 2.2 and 6.5 ± 0.5 in the absence or in the presence of ADP, respectively. Similar ratios were obtained when 20 μM AMP (6.3 ± 0.8) or 20 μM ATP (5.5 ± 0.5) was added instead of ADP. Addition of 10 μM rotenone significantly decreased H⁺/O ratios both in the absence or in the presence of ADP (Table I). Thus, when adenylates were present, this inhibitor of complex I dramatically reduced proton translocation. However, as illustrated in Table I, the addition of ADP to rotenone-treated mitochondria was still able to greatly enhance proton translocation and H⁺/O ratio.

Isocitrate as well as malate was poorly oxidized by potato mitochondria, and anaerobiosis was reached a long time after flushing N₂. Addition of small oxygen pulses to anaerobic mitochondria provided consistent proton translocation (H⁺/O = 3.5 ± 0.2). In the presence of 20 μM adenylates (ADP, AMP, or ATP), the stoichiometry of proton translocation was enhanced (H⁺/O = 5.3 ± 0.8).

H⁺/2 e⁻ Ratios with Ferricyanide as Electron Acceptor. The proton translocation driven by succinate and NADH oxidation was measured in potato mitochondria using ferricyanide as an electron acceptor instead of oxygen, since this artificial acceptor is known to interact mainly at the Cyt c level. With succinate as well as with NADH, the observed H⁺/2 e⁻ ratios were dramatically dependent upon the amounts of added ferricyanide (Fig. 6). When anaerobic mitochondria were pulsed with less than 2 ng (2 e⁻) equivalents mg⁻¹ protein, the H⁺/2 e⁻ ratios were 3.7 ± 0.3 and 3.4 ± 0.2 for succinate and NADH, respectively. For amounts of ferricyanide higher than 2 ng (2 e⁻) equivalents mg⁻¹ protein, proton translocation was dramatically diminished, especially with NADH as substrate. The lowering of the H⁺/2 e⁻ ratios is probably due to interactions of ferricyanide with mitochondrial electron carriers other than Cyt c, particularly when ferricyanide is added at high concentrations (1). For exogenous NADH, ferricyanide can also easily interact with the NADH dehydrogenase located on the outer mitochondrial membrane (20). This suggests that the values of H⁺/2 e⁻ for NADH are probably underestimated and that comparison between H⁺/O and H⁺/2 e⁻ (with ferricyanide) may be tentatively made from values obtained with succinate alone.

As clearly shown for succinate oxidation in yeast mitochondria (29) and illustrated in Figure 6, proton translocation with ferricyanide as electron acceptor had one component protonophore insensitive. Figure 6 shows that in potato mitochondria and in the presence of 4 μM CCP, approximately two H⁺ were produced per two ferricyanide ions reduced with succinate, whereas only

Table I. Effects of ADP and Rotenone on the H⁺/O Stoichiometry Linked to Malate Oxidation in Potato Mitochondria

<table>
<thead>
<tr>
<th>Conditions</th>
<th>H⁺/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate (+ glutamate)</td>
<td>3.6 ± 2.2</td>
</tr>
<tr>
<td>+ 20 μM ADP</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>+ 10 μM rotenone</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>+ 20 μM ADP + 10 μM rotenone</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>+ 10 μM rotenone + 20 μM ADP</td>
<td>3.9 ± 0.3</td>
</tr>
</tbody>
</table>

FIG. 6. Dependence of the observed H⁺/2 e ratios on the amounts of ferricyanide added to anaerobic mitochondria oxidizing succinate and exogenous NADH. Experimental conditions are described in "Materials and Methods." (●), 5 mM succinate; (O), 5 mM NADH. (-- --), in the absence of CCCP; (-----), in the presence of 3 μM CCCP.

1 H⁺ per 2 electron acceptor equivalents appeared with NADH. This suggests that the protons released in the presence of the protonophore correspond to the dehydrogenation of the substrate which is not compensated by H₂O formation, since ferricyanide is used as electron acceptor (1).

DISCUSSION

The experiments reported in this paper show that the H⁺/O stoichiometry can be estimated in plant mitochondria using the classical pulse oxygen technique described by Mitchell and Moyle (15, 16). In contrast to mammalian mitochondria (15) but as for yeast mitochondria (27), an electrical permeability must be created by valinomycin in order to obtain a maximal proton extrusion (Fig. 1; Ref. 6).

The results reported in this paper show that the H⁺/O ratio for exogenous NADH oxidation in plant mitochondria is close to 4 as for succinate when low amounts of oxygen (less than 2 ng atom O mg⁻¹ protein) are added to avoid the building up of a large ΔpH. At higher amounts of pulsed oxygen, the amounts of translocated protons are not proportional to the quantities of pulsed oxygen. For succinate, as previously suggested, this lack of linearity is probably due to the operation of an H⁺/K⁺ antiporter (6). With NADH as substrate, this lack of linearity is still more dramatic due to the large alkalinization which occurs during NADH oxidation.

In contrast to mammalian mitochondria (4, 25) NEM, which inhibits the H⁺/Pi symport, does not enhance proton extrusion in potato mitochondria. However, the effect of NEM on the proton extrusion and H⁺/O stoichiometry is not always observed in rat liver mitochondria (10). Moreover, the reasons put forward to explain the effect of NEM on the determinations of H⁺/O ratios are questionable since Moyle and Mitchell (21) have claimed that the H⁺/O ratios could be raised by NEM by favoring
the use of NADPH or isocitrate as endogenous reductants. Indeed, the absence of effect of NEM in potato mitochondria suggests that the preparations of isolated mitochondria were fully depleted of endogenous phosphate.

Interestingly, the values of the $H^+/2e^-$ ratio for the respiratory span succinate-Cyt c were close to the observed $H^+/O$ ratios (i.e. 4). This suggests that in plant mitochondria the Cyt oxidase complex (complex IV) does not contribute to the proton translocation linked to electron flow from succinate to oxygen. In both mammalian and yeast mitochondria, $H^+/O$ ratios of 4.0 have been observed for the succinate-O$_2$ respiratory span (13, 15, 16, 24, 27). However, higher values for $H^+/O$ ratios have been reported for the respiratory chain, two extra protons being supposed to be ejected at the level of Cyt oxidase (4, 30). According to Reynafarge et al. (25) and Di Virgilio et al. (7), the $H^+/2e^-$ per site would be 4, i.e. the $H^+/O$ ratio for succinate would be 8. In mung bean mitochondria Mitchell and Moore (17) have recently reported values of $H^+/O$ of 4.22 ± 2.16 for succinate oxidation. More accurately, using a spectrophotometric determination of the rate of respiration in mammalian mitochondrial, Papa et al. (24) have convincingly shown that the $H^+/O$ ratio for succinate oxidation is 4.0 instead of 6 or 8.

In the model assumed for $H^+$ ejection at site 2 in mammalian mitochondria (2), it is proposed that 2 $H^+$ are ejected by the protolytic oxidation of QH$_2$, the other 2 $H^+$ being ejected as the electron pair flows through the b-c complex. Consequently, substrates interacting with site 2, whether internally (like succinate) or externally (like glycerol phosphate) gave theoretical values of $H^+/2e^-$ ratios of 4. Based on this model, the theoretical $H^+/2e^-$ in plant mitochondria should be 4 for succinate and 3 for exogenous NADH, since the oxidation of NADH requires one external $H^+$.

Experimental values of $H^+/2e^-$ for the spans NADH-Cyt c and NADH-O$_2$ are 3.4 ± 0.2 and 4.1 ± 0.9, respectively. Determinations of $H^+/2e^-$ ratios with ferricyanide as an electron acceptor provide values which are likely to be underestimated due to the possible interaction of the electron acceptor with electron carriers other than Cyt c, such as the electron transport system of the outer membrane (20). Determinations of $H^+/O$ ratios are complicated, however, by the net uptake of protons during NADH oxidation, due to the disequilibrium between the net appearance of 1 $H^+$/mol of NADH coming from the substrate oxidation in the external medium, and the uptake of 2 $H^+$ e$^-$ transferred to oxygen at the level of Cyt oxidase. Indeed, the experimental data obtained with NADH and low amounts of oxygen provided values close to 4. This result suggests that, for the very low oxygen pulses (1 ngatom mg$^{-1}$ protein), the additional proton which is required for the oxidation of 1 mol of NADH was primary removed from the membranous vicinity of the dehydrogenase instead of the external medium.

The last observation reported in this paper concerns the special features of proton translocation linked to malate and isocitrate oxidation and the possible regulation of proton translocation by adenylates at the level of complex I. In potato mitochondria, the proton translocation driven by malate or isocitrate oxidation provides relatively low $H^+/O$ ratios whose mean value does not exceed 4. Moreover, with malate as substrate, even in the presence of glutamate, proton translocation appears to be dependent upon the experimental conditions used to reach the initial anaerobiosis necessary for oxygen pulses experiments (Fig. 4). Whatever the initial value of proton translocation driven by the NAD$^+\text{-}linked$ substrates (malate, isocitrate), addition of ADP brings the $H^+/O$ stoichiometry up to 6, a value consistent with the operation of three phosphorylating sites (15, 16). Similar values were obtained using AMP or ADP instead of ADP. Under the experimental conditions required to measure proton extrusion (no exogenous phosphate and high protein concentration), ATP can be slowly hydrolyzed to ADP (data not shown). The original observations by Rusness and Still (26) and additional experiments on potato mitochondria indicate that AMP can be rapidly converted to ADP via an adenylate kinase which is highly active in plant mitochondria. Consequently, because of the presence of adenylate kinase and ATP hydrolysis, under our conditions, the nucleotides AMP, ADP, or ATP coexist in underdetermined proportions. The observations reported in this paper do not indicate which species is more specifically active.

We have also observed that the addition of adenylates stimulate the rate of uncoupled malate oxidation (in the presence of valinomycin) to a maximal rate characteristic of the state 3 (data not shown). This suggests that adenylates permit the oxidation of endogenous substrate by generated NADH at full capacity which is not possible otherwise. Moreover, the effect of adenylates was not observed with succinate or exogenous NADH. This shows that these effectors could interact at the level of complex I. Besides, the adenylate-dependent proton extrusion driven by malate oxidation is highly sensitive to rotenone; but, in the presence of this inhibitor, a significant activation is still obtained (Table I). All these observations suggest that adenylates could interact more specifically with the NAD-linked enzymes themselves rather than with the respiratory chain, as suggested by Laties (12) and Sottibhandu and Palmer (28).

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