Microbiology, Vol. 72, No. 4, 2003, pp. 403–407. Translated from Mikrobiologiya, Vol. 72, No. 4, 2003, pp. 453–458.

EXPERIMENTAL ARTICLES

The Alternative Oxidase of *Yarrowia lipolytica* Mitochondria Is Unable To Compete with the Cytochrome Pathway for Electrons

V. K. Akimenko[1], A. Yu. Arinbasarova, N. M. Smirnova, and A. G. Medentsev

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

[1]E-mail: akimenko@ibpm.serpukhov.su

Received January 3, 2002

Abstract—The activity of the cyanide-resistant alternative oxidase (pathway) of *Yarrowia lipolytica* mitochondria was studied as a function of the activity of the major, cyanide-sensitive, cytochrome pathway. The contribution of the alternative oxidase to the total respiration of mitochondria was evaluated by measuring the rate of oxygen consumption in the presence of cyanide (an inhibitor of the cytochrome pathway). The potential activity of the cytochrome pathway was evaluated spectrophotometrically, by measuring the oxidation rate of cytochrome *c* by ferricyanide, which accepts electrons from complex III (cytochrome *c*) of this pathway. The oxidation of succinate by mitochondria in the presence of ferricyanide and cyanide was accompanied by oxygen consumption due to the transfer of electrons through the alternative pathway. The subsequent addition of ADP or FCCP (an uncoupler of oxidative phosphorylation in the cytochrome pathway) completely inhibited the consumption of oxygen by the mitochondria. Under these conditions, the inhibition of the alternative pathway by benzohydroxamic acid failed to affect the transfer of electrons from cytochrome *c* to ferricyanide. Benzohydroxamic acid did not influence the rate of ferricyanide reduction by the cytochrome pathway occurring in controlled state 4, nor could it change the phosphorylation quotient ATP/O upon the oxidation of various substrates. These findings indicate that the alternative pathway is unable to compete with the cytochrome respiratory chain for electrons. The alternative pathway transfers only electrons that are superfluous for the cytochrome chain.

Key words: yeasts, cyanide-resistant respiration, alternative oxidase (pathway), regulation, physiological role.

Cyanide-resistant respiration is common to many higher plants, fungi, yeasts, and protozoa [1–3]. This respiration is due to the functioning of cyanide-resistant oxidase (also called alternative oxidase, or alternative pathway), which transfers electrons from reduced ubiquinone (coenzyme Q) to oxygen independently of the main cytochrome respiratory chain [1].

Alternative oxidase is located in the inner mitochondrial membrane [4, 5] and is insensitive not only to cyanide but also to azide, CO, antimycin A, and myxothiazol [1–3]. On the other hand, this pathway is specifically inhibited by benzohydroxamic acid (BHA) and its derivatives [6]. The alternative pathway branches from the cytochrome respiratory chain at the level of ubiquinone [7]. The alternative oxidase–mediated oxidation of the substrates that donate their electrons to ubiquinone (α-glycerophosphate, succinate, and exogenous NADH) was shown not to be coupled to phosphorylation [8, 9]. However, the oxidation of NAD-linked substrates (like pyruvate + malate) is associated with the synthesis of ATP at the first coupling site. Moore and Siedow showed that the alternative oxidase reduces oxygen with the formation of water but not hydrogen peroxide or superoxide radicals [10]. The affinity of the cyanide-resistant alternative oxidase for oxygen is considerably lower than that of cytochrome oxidase, as is evident from a comparison of their K_m values with respect to oxygen ($K_m > 1$ μM and $K_m < 0.1$ μM, respectively) [10].

In spite of extensive investigations, the physiological role of the cyanide-resistant respiration of cells is far from well understood. One approach to this problem is to evaluate the relative contributions of the main cytochrome respiratory chain and the alternative pathway to the total respiration of mitochondria and whole cells. Early investigations along this line led to contradictory conclusions [11–16]. According to some authors, who employed inhibition analysis of plant mitochondrial respiration, the alternative oxidase transfers electrons only when the cytochrome pathway is inhibited or occurs in the controlled state 4 (in terms of Chance) [11–14]. At the same time, De Troostemberg and Nyns concluded that electrons are partitioned between the cytochrome respiratory chain and the alternative pathway merely in accordance with their electron acceptor activities [15]. Conversely, Hoefnagel *et al.* inferred from their experimental data that the two electron transfer pathways compete with one another

0026-2617/03/7204-0403$25.00

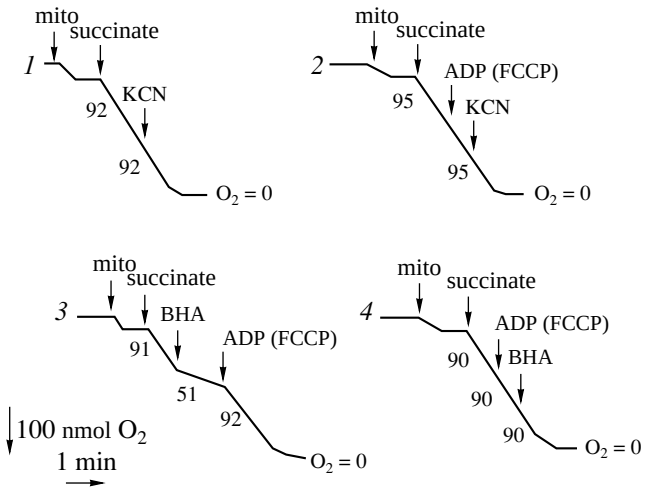

Fig. 1. The effect of cyanide and BHA on the oxidation of succinate by the cyanide-resistant *Y. lipolytica* mitochondria. Substances added: 1.1 mg/ml mitochondria (mito), 25 mM succinate, 0.5 mM ADP, 2 μM FCCP, 1 mM KCN, and 5 mM BHA. Figures alongside the curves indicate the rate of oxygen consumption in nmol/(min mg protein).

for electrons, at least during the oxidation of pyruvate [16].

The aim of the present work was to study the mechanism of electron partitioning between the alternative and the cytochrome pathways.

MATERIALS AND METHODS

The yeast *Yarrowia lipolytica* strain VKM Y-155 used in this study was obtained from the All-Russia Collection of Microorganisms (VKM). The strain was grown in a Reader medium containing 1% glucose as the sole source of carbon and energy. Mitochondria were isolated from stationary-phase yeast cells as described earlier [8].

The respiration of mitochondria was measured at 22–25°C using a Clark-type oxygen electrode. The respiration medium (2 ml) was 10 mM Tris–phosphate buffer (pH 7.0) containing 0.6 M mannitol, 0.5 mM EDTA, and 0.05% bovine serum albumin.

The reduction of ferricyanide by mitochondria was measured at the wavelength pair 470 and 420 nm using a C-356 Hitachi spectrophotometer (Japan).

The protein concentration was determined with the biuret reagent.

ATP in mitochondria was assayed as follows: Some time after the addition of a substrate and 0.5 mM ADP to mitochondria present in the measuring cell of the oxygen electrode, the mitochondria were inactivated by adding $HClO_4$ to a final concentration of 5%. The mixture was transferred into a test tube placed on ice and neutralized by adding the necessary amount of 2 N KOH. The precipitate was removed by centrifugation, and the concentration of ATP in the supernatant was determined in an enzyme-linked assay system containing 10 mM $MgCl_2$, 10 mM KCl, 5 mM EDTA, 1 mM $NADP^+$, 10 mM glucose, 1 U hexokinase, and 1 U glucose-6-phosphate dehydrogenase in 100 mM Tris–HCl buffer (pH 7.5). The NADPH formed in this reaction was quantified by measuring its fluorescence at 460 nm.

The phosphorylation quotient ATP/O was calculated as the ratio of the amount of ATP formed by mitochondria in a specific time to the amount of oxygen consumed in this time.

RESULTS AND DISCUSSION

The concurrent functioning of the phosphorylating respiratory chain and non-phosphorylating alternative pathway in cyanide-resistant mitochondria poses the problem of the relative contributions of these electron transport pathways to the total mitochondrial respiration. One of the approaches to this problem is the inhibition analysis of cyanide-resistant mitochondria. As can be seen from Fig. 1, the oxidation of succinate by cyanide-resistant mitochondria was not inhibited by cyanide (an inhibitor of the cytochrome *c* oxidase of the cytochrome pathway) either in the controlled (curve *1*) or in the uncontrolled (curve *2*) state. On the other hand, the oxidation of succinate was inhibited by benzohydroxamic acid in the mitochondria occurring in the controlled state (curve *3*) but not in the mitochondria occurring in the uncontrolled (active) state (curve *4*).

The absence of any inhibitory action of BHA on the respiration rate of actively respiring (i.e., occurring in the uncontrolled state) cyanide-resistant mitochondria can be explained in two ways. First, the respiration of such mitochondria may be due to the operation of the main electron transport chain alone, while the alternative pathway is not involved. Second, the alternative oxidase does function even in the actively respiring mitochondria, but the addition of BHA diverges electron flow from the alternative pathway to the cytochrome pathway.

It is difficult to decide which of these two explanations is valid, since it is difficult to experimentally estimate electron flows through the pathways when they operate concurrently. For instance, electron flows through the two pathways cannot be distinguished based on the formation rate of the end product, since both the cytochrome pathway (more specifically, its terminal cytochrome *c* oxidase) and the alternative oxidase transfer electrons to oxygen with the formation of the same end product, water [10].

To overcome this difficulty, we employed an approach in which the operation of cytochrome *c* oxidase was inhibited by cyanide, and electrons transferred by the main respiratory chain were accepted by ferricyanide. As this compound is unable to penetrate the inner mitochondrial membrane, it accepts electrons exclusively from the cytochrome *c* of the respiratory chain. As a result, the potential activity of this chain can

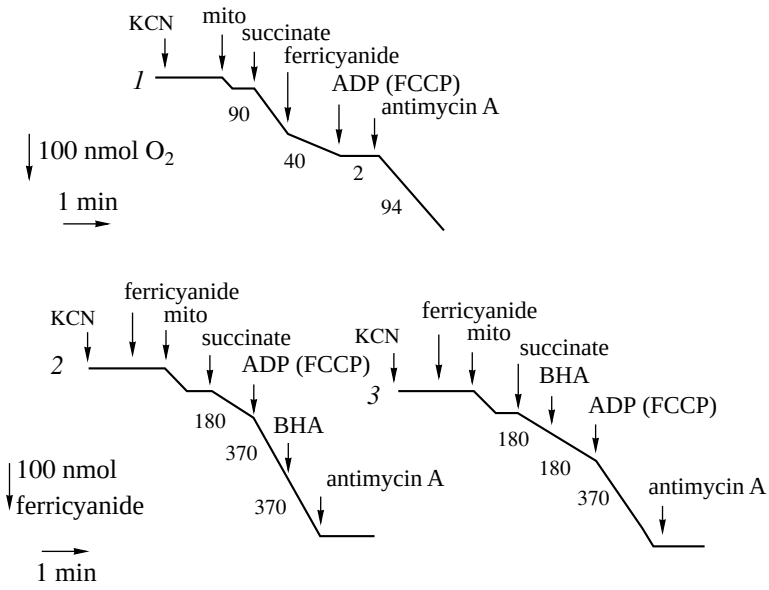

Fig. 2. The effect of respiratory inhibitors, ADP, and the uncoupler FCCP on the rates of (*1*) oxygen consumption and (*2*, *3*) ferricyanide reduction by the cyanide-resistant *Y. lipolytica* mitochondria. Substances added: 0.5 mg/ml mitochondria (mito), 25 mM succinate, 1 mM ADP, 1 μM FCCP, 1 mM KCN, 1 mM BHA, 1 mM ferricyanide, and 2 μM antimycin A. Figures alongside the curves indicate the rates of oxygen consumption and ferricyanide reduction in nmol/(min mg protein).

be estimated by measuring the rate of ferricyanide reduction in the presence of cyanide, whereas the activity of the concurrent alternative pathway in the same mitochondria can be estimated by measuring the rate of oxygen consumption. The addition of ferricyanide must evidently change the partition of electrons between the two electron transport pathways. If so, the relative contribution of alternative oxidase to the total respiration of mitochondria can be estimated from the change in the rate of oxygen consumption in response to the addition of ferricyanide. On the other hand, if the alternative pathway is able to compete for electrons with the main respiratory chain, this ability can be estimated from the change in the rate of ferricyanide reduction in response to the inhibition of the alternative pathway by BHA.

As can be seen from Fig. 2 (curve *1*), the addition of ferricyanide to the cyanide-resistant mitochondria oxidizing succinate in the presence of cyanide inhibited oxygen consumption by about 60%. The subsequent addition of ADP or the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) led to the complete inhibition of mitochondrial respiration. These data suggest that the respiration of cyanide-resistant mitochondria was about 40% due to the operation of the alternative pathway and about 60% due to the operation of the main respiratory chain. The activation of the main respiratory chain by ADP or FCCP diverged the electron flow from the alternative pathway to the main respiratory chain and thus blocked mitochondrial respiration, since the transfer of electrons to oxygen through the main respiratory chain was inhibited by cyanide. Paradoxically, the subsequent addition of antimycin A (an inhibitor of the main respiratory chain at the level of cytochrome *b*) restored the consumption of oxygen by the cyanide-resistant mitochondria (Fig. 2, curve *1*). This was obviously due to the triggering of the electron flow from the main respiratory chain to the alternative pathway, as is evident from the sensitivity of the antimycin A–activated mitochondrial respiration to BHA (data not presented).

The simultaneous recording of the rate of ferricyanide reduction (Fig. 2, curves *2*, *3*) showed that the addition of succinate to mitochondria in the presence of cyanide led to the reduction of ferricyanide at a certain rate (Fig. 2, curve *2*). The activation of the main respiratory chain by ADP or FCCP enhanced the rate of ferricyanide reduction. The addition of BHA did not influence the reduction of ferricyanide, whereas the subsequent addition of antimycin A completely inhibited it.

As is evident from Fig. 2, curve *3*, the inhibition of the alternative pathway by BHA under conditions when the main respiratory chain occurred in a controlled state (i.e., in the absence of ADP or FCCP) exerted no influence on the rate of ferricyanide reduction and hence on the rate of electron transfer via the main respiratory chain. In general, the data presented in Fig. 2 indicate that the inhibition of the alternative oxidase by BHA does not increase the electron flow through the main respiratory chain and, hence, the alternative pathway is unable to compete with the main respiratory chain for electrons.

To verify this inference, we studied the effect of cyanide and BHA on the efficiency of phosphorylation during the oxidation of various substrates by cyanide-resistant mitochondria. Since the transfer of electrons via the alternative pathway is not coupled to the phospho-

The effect of respiratory inhibitors on the efficiency of ATP synthesis by *Y. lipolytica* mitochondria

Substrate	Inhibitor	Oxygen consumed, nmol	ATP formed, nmol	ATP/O quotient
Pyruvate + malate	None	187 ± 18	492 ± 46	2.63 ± 0.07
	KCN	182 ± 16	188 ± 17	1.03 ± 0.12
	BHA	152 ± 15	402 ± 38	2.65 ± 0.07
Succinate	None	147 ± 14	235 ± 21	1.62 ± 0.12
	KCN	98 ± 9	3.92 ± 0.3	0.04 ± 0.006
	BHA	154 ± 15	250 ± 22	1.62 ± 0.11
α-Glycerophosphate	None	219 ± 22	370 ± 35	1.69 ± 0.06
	KCN	229 ± 23	11.5 ± 0.9	0.05 ± 0.008
	BHA	198 ± 18	310 ± 30	1.71 ± 0.12

Note: The concentration of KCN was 1 mM, the concentration of BHA, pyruvate, and malate was 5 mM, and that of succinate and α-glycerophosphate, 25 mM. The data are the means of triplicate measurements.

rylation of ADP [8, 9], the comparison of the phosphorylation efficiencies of cyanide-resistant mitochondria in the presence and absence of BHA may allow some inferences to be drawn as to the contribution of the alternative pathway to the total respiration of such mitochondria. As can be seen from the data presented in the table, the ATP/O quotient during the oxidation of succinate and α-glycerophosphate by cyanide-resistant mitochondria was, respectively, 1.64 and 1.71 in the presence of BHA and 1.62 and 1.69 in its absence. Therefore, BHA virtually did not influence the efficiency of mitochondrial phosphorylation upon the oxidation of these two substrates. Similarly, BHA influenced but little the ATP/O quotient during the oxidation of pyruvate + malate (ATP/O = 2.65 and 2.63 in the presence and absence of BHA). These data confirm the inference that almost all electrons derived from respiratory substrates in cyanide-resistant mitochondria are transferred to oxygen through the main respiratory chain. The fact that the oxidation of succinate and α-glycerophosphate by cyanide-resistant mitochondria in the presence of cyanide is characterized by an ATP/O ratio close to zero provides further indication that electron transfer through the alternative pathway is not coupled to phosphorylation. The oxidation of pyruvate + malate under these conditions yielded an ATP/O quotient close to unity, which can be explained by the fact that cyanide does not impair the normal functioning of the first phorphorylation site in the cyanide-resistant mitochondria.

Earlier investigations of the partition of electrons between the main and alternative respiratory chains of plant mitochondria gave contradictory results. For instance, Day [17] showed that the inhibition of the alternative oxidase of soybean mitochondria did not enhance the electron flow through the main respiratory chain. At the same time, Wilson [18] came to the conclusion that the alternative pathway of ming-bean mitochondria is able to compete with the main respiratory chain for electrons. This conclusion was based on the results of investigation of the effect of little oxygen pulses on the rate of ferricyanide reduction by the cyanide-resistant mitochondria under anaerobic conditions. Wilson believed that oxygen activated the alternative oxidase of ming-bean mitochondria and thus shifted the electron flow towards the alternative pathway, as a result of which the rate of ferricyanide reduction decreased. However, these experimental results can be interpreted in a different way. The absence of data on the degree of the cytochrome *c* oxidase inhibition by cyanide allows the suggestion to be made that reduced ferricyanide was oxidized by oxygen pulses via cytochrome *c* oxidase. Furthermore, Wilson did not investigate the effect of the alternative oxidase inhibitors on the reduction of ferricyanide by the cyanide-resistant ming-bean mitochondria, which casts further doubt on his interpretation of the experimental results.

After it was established that the alternative oxidase of plants is activated by keto acids (pyruvate etc.) [19], Hoefnagel *et al.* [16] showed that the addition of pyruvate to the cyanide-resistant soybean mitochondria occurring in the controlled state 4 slightly diminished (by 6%) the rate of ferricyanide reduction, whereas the inhibition of alternative oxidase by BHA augmented the rate of ferricyanide reduction by 7%. The addition of pyruvate to the mitochondria occurring in the uncontrolled state 3 (i.e., in the presence of ADP) did not influence the rate of ferricyanide reduction. At the same time, the activation of the main respiratory chain by ADP decreased the electron flux through the alternative pathway by 70% in the presence of pyruvate and by 63% in its absence. Based on these data, Hoefnagel *et al.* inferred that the alternative pathway activated by pyruvate is able to compete with the main respiratory chain for electrons and explained the disagreement between his data and those obtained by other investigators by differences in the type and age of plant tissues from which the mitochondria were isolated.

We believe that the data available in the literature [11–15, 17] and those presented in this paper strongly

suggest the validity of the Bahr and Bonner model [11, 12], according to which the alternative pathway fails to compete with the main respiratory chain for electrons and transfers superfluous electrons alone. The control of the main respiratory chain over the alternative pathway is beneficial for cells from the standpoint of the efficient utilization of respiratory substrates. When the level of ATP in cells is low and, hence, the level of ADP is high, the main respiratory chain is active and transfers almost all the available electrons through the phosphorylation sites. Conversely, when the ATP/ADP ratio in cells is high, the electron-transport activity of the main respiratory chain declines, and some electrons derived from respiratory substrates are transferred to oxygen through the non-phosphorylating alternative pathway. This allows cells to maintain their oxidative activity at a sufficiently high level without decreasing their ATP pool.

ACKNOWLEDGMENTS

This work was supported by grant no. 01-04-48680 from the Russian Foundation for Basic Research.

REFERENCES

1. Medentsev, A.G., Arinbasarova, A.Yu., and Akimenko, V.K., The Regulation and Physiological Role of Cyanide-Resistant Oxidase in Fungi and Plants, *Biokhimiya*, 1999, vol. 64, no. 11, pp. 457–1472.
2. Henry, M.F. and Nyns, E.J., Cyanide-Insensitive Respiration: An Alternative Mitochondrial Pathway Sub-Cell, *Biochimie*, 1975, vol. 4, no. 1, pp. 1–65.
3. Vanlenberghe, G.C. and McIntosh, L., Alternative Oxidase from Gene to Function, *Annu. Rev. Plant Physiol. Plant. Mol. Biol.*, 1997, vol. 48, pp. 703–734.
4. Rasmusson, A.G., Molle, I.M., and Palmer, J.M., Component of Alternative Oxidase Localized in the Matrix Surface of the Inner Membrane of Plant Mitochondria, *FEBS Lett.*, 1990, vol. 259, pp. 311–314.
5. Medentsev, A.G. and Akimenko, V.K., Localization of Cyanide-Resistant Oxidase in Mitochondria from the Yeast *Yarrowia lipolytica*, *Mikrobiologiya*, 1994, vol. 63, no. 3, pp. 417–423.
6. Schonbaum, G.S., Bonner, W.D., Storey, B.T., and Bahr, J.T., Specific Inhibition of the Cyanide-Insensitive Respiratory Pathway in Plant Mitochondria by Hydroxamic Acids, *Plant Physiol.*, 1971, vol. 47, no. 1, pp. 124–128.
7. Storey, B.T., Respiratory Chain of Plant Mitochondria: Point of Interaction of Alternate Oxidase with the Respiratory Chain, *Plant Physiol.*, 1976, vol. 58, pp. 521–525.
8. Akimenko, V.K., Golovchenko, N.P., and Medentsev, A.G., The Absence of Energy Conservation Coupled with Electron Transfer via the Alternative Pathway in Cyanide-Resistant Yeast Mitochondria, *Biochim. Biophys. Acta*, 1979, vol. 545, pp. 398–403.
9. Moore, A.L., Bonne, W.D., and Rich, P.R., The Determination of the Proton-Motive Force during Cyanide Insensitive Respiration in Plant Mitochondria, *Arch. Biochem. Biophys.*, 1978, vol. 186, pp. 298–306.
10. Moore, A.L. and Siedow, J.Ns., The Regulation and Nature of the Cyanide-Resistant Alternative Oxidase of Plant Mitochondria, *Biochim. Biophys. Acta*, 1991, vol. 1059, no. 1, pp. 121–140.
11. Bahr, J.T. and Bonner, W.D., Cyanide-Insensitive Respiration: I. The Steady States of Skunk Cabbage Spadix and Bean Hypocotyls Mitochondria, *J. Biol. Chem.*, 1973, vol. 248, pp. 3441–3445.
12. Bahr, J.T. and Bonner, W.D., Cyanide-Insensitive Respiration: II. Control of the Alternative Pathway, *J. Biol. Chem.*, 1973, vol. 248, pp. 3446–3450.
13. Lambers, H., The Physiological Significance of Cyanide-Resistant Respiration in Higher Plants, *Plant Cell Environ.*, 1980, vol. 3, pp. 293–302.
14. Laties, G.G., The Cyanide-Resistant, Alternative Pathway in Higher Plant Respiration, *Annu. Rev. Plant Physiol.*, 1982, vol. 33, pp. 519–555.
15. de Troostembergh, J.C. and Nyns, E.S., Kinetics of the Respiration of Cyanide-Insensitive Mitochondria from the Yeast *Saccharomycopsis lipolytica, Eur. J. Biochem.*, 1978, vol. 85, pp. 423–432.
16. Hoefnagel, M.H.N., Millar, A.H., Wiskich, J.T., and Day, D.A., The Cytochrome and Alternative Respiratory Pathways Compete for Electrons in the Presence of Pyruvate in Soybean Mitochondria, *Arch. Biochem. Biophys.*, 1995, vol. 318, pp. 394–400.
17. Day, D.A., *Molecular, Biochemical and Physiological Aspects in Plant Respiration*, Lambers, H. and van der Plas, L.H.W., Eds., SPB Academic, 1992, pp. 37–42.
18. Wilson, S.B., The Switching of the Electron Flux from the Cyanide-Insensitive Oxidase to the Cytochrome Pathway in Ming-Bean (*Phaseolus aureus* L.) Mitochondria, *Biochem. J.*, 1988, vol. 249, pp. 301–303.
19. Millar, A.H., Wiskish, J.T., Whelan, J., and Day, D.A., Organic Acid Activation of the Alternative Oxidase of Plant Mitochondria, *FEBS Lett.*, 1993, vol. 329, no. 3, pp. 259–262.