Biochemistry (Moscow), Vol. 69, No. 5, 2004, pp. 580-584. Translated from Biokhimiya, Vol. 69, No. 5, 2004, pp. 712-718.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-265, February 29, 2004.

NADH Oxidation by Mitochondria from the Thermogenic Plant *Arum orientale*

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Received November 24, 2003
Revision received December 18, 2003

Abstract—The enzyme content of the mitochondrial respiratory chain was investigated in the heat-producing plant *Arum orientale*. It is shown that mitochondria isolated from thermogenic tissues of this plant (with respect to non-thermogenic tissues of *A. orientale* or to *Zea mays*) demonstrate significantly elevated levels of activities of two non-coupled NADH dehydrogenases oxidizing intramitochondrial and cytoplasmic NADH pools. It is postulated that operation of a completely non-coupled respiratory chain consisting of non-coupled NADH:quinone oxidoreductases and cyanide-resistant alternative quinol-oxidase is the main mechanism of heat production in thermogenic plants.

Key words: thermogenic plants, *Arum orientale*, NADH dehydrogenase, alternative oxidase, Complex I

Some plants can maintain a significant temperature gradient between their flower (inflorescence) body and the environment. This gradient can be quite high and even reach 35°C. Significant self-heating of flowers, inflorescences, or cones is widely distributed between different families of primitive seed plants, this phenomenon being described for some species of aroids (Araceae), lotus (Nelumbonaceae), water lilies (Nymphaeaceae), Dutchman's pipes (Aristolochiaceae), palms (Arecaceae and Cyclanthaceae), custard apples (Annonaceae), magnolias (Magnoliales), and cycads (Cycadaceae) [1]. The reason for such heat production possibly involves enhancement of scent production to attract pollinators and/or protection from freezing. Inflorescence of heat-producing plants is accompanied by an extremely powerful increase in respiration, so powerful that it is often called a "metabolic explosion". This leads to significant heat production, up to 1 W/g of the flower. Due to these intriguing properties, the mitochondria from thermogenic tissues of plants have been intensively investigated. It has been shown that their respiratory activity (in contrast to respiration of mitochondria from non-thermogenic tissues) is almost completely cyanide-resistant [2]. This fact is connected with increased content of very active, cyanide-resistant alternative oxidase (AltOx) (for review see [3, 4]). The activity of this enzyme is not coupled with energy conservation; thus, the entire free energy of the ubiquinol-oxidase reaction in thermogenic plant mitochondria is directly converted into heat. The activity of AltOx is now believed to be the main mechanism of heat production in plants. However, for fast ubiquinol-oxidase activity of AltOx a very fast enzyme(s) must also operate in the initial part of the respiratory chain of the thermogenic plant mitochondria in order to reduce ubiquinone rapidly as well. This hypothetical enzyme(s) should oxidize NADH (because it is the main respiratory substrate for the mitochondrial respiratory chain) and should be non-coupled (or uncoupled), with energy conservation not being limited by generated proton motive force ($\Delta\mu_{H^+}$).

NAD(P)H can be oxidized by plant mitochondria via at least four different enzymes. Intramitochondrial NADH is oxidized by Complex I, the activity of which is coupled with $\Delta\mu_{H^+}$ generation, and by a non-coupled enzyme (NDin) similar to bacterial NDH-2 type enzymes [5, 6]. Plant mitochondria can also directly oxi-

Abbreviations: $\Delta\mu_{H^+}$) transmembrane H^+ electrochemical potential difference; AltOx) alternative cyanide-resistant oxidase; BSA) bovine serum albumin; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; dNADH) reduced nicotinamide hypoxanthine dinucleotide; DTT) 1,4-dithio-DL-threitol; NDex) external NADH:quinone oxidoreductase; NDin) internal non-coupled NADH:quinone oxidoreductase; NDPex) external NADPH:quinone oxidoreductase; SHAM) salicylhydroxamic acid.

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0006-2979/04/6905-0580

dize external (cytoplasmic) NAD(P)H via at least two non-coupled enzymes—external NADH:quinone oxidoreductase (NDex) and external NADPH:quinone oxidoreductase (NDPex) also localized in the inner mitochondrial membrane [5-7]. This oxidation of cytoplasmic NAD(P)H is fully dependent upon Ca^{2+}, which stimulates activities of NDex and NDPex [8].

The levels of all these abovementioned NADH-consuming enzymes can vary significantly depending on plant species, kind of tissue, and plant growth conditions [8-10]. It is well known that mitochondria from the thermogenic plant *Arum maculatum* possess very high NADH-oxidase activity [7, 11], but the contribution of all these mentioned above NAD(P)H-consuming enzymes in plant respiration during thermogenesis is still not known. This work was devoted to answering this question. We show that mitochondria isolated from thermogenic tissues of *Arum orientale* demonstrate extraordinarily high levels of activities of two non-coupled NADH dehydrogenases—NDin and NDex—oxidizing, respectively, intramitochondrial and cytoplasmic NADH pools.

MATERIALS AND METHODS

Plant materials. Inflorescences of *Arum orientale* in the late γ- and early δ-stage (as defined in [2]) and corms of this plant were collected in the N. V. Tsitsin Main Botanical Garden of the Russian Academy of Sciences (Moscow). Flowering female inflorescences of *Zea mays* were obtained from a farm in the Voronezh district, Russia.

Isolation of mitochondria. Mitochondria were isolated using a Percoll self-generated gradient method [12, 13] with some modifications. An *A. orientale* spadix (~0.8 g), *A. orientale* corms (~30 g), or *Z. mays* spadices (~30 g) were disintegrated using a mortar and pestle and homogenized in a Potter homogenizer with 5-10 volume/weight excess of medium A containing 350 mM sucrose, 1 mM EDTA, 20 mM Hepes-Tris (pH 7.4), 2 mM 1,4-dithio-DL-threitol (DTT), and 0.2 mg/ml bovine serum albumin (BSA). Unbroken tissue, cell debris, nuclei, and etioplasts were separated by successive centrifugations at 1000*g* for 5 min and 4000*g* for 10 min. The mitochondrial pellet was obtained from the supernatant after the third centrifugation (14,000*g*, 10 min). It was further purified on a Percoll self-generating gradient. Mitochondrial suspension in medium A (~0.5 ml) was placed on the top of 8.5 ml of 21% Percoll solution in medium A and centrifuged for 30 min at 48,000*g*. The mitochondrial fraction located close to the gradient bottom was collected, diluted with medium A, and precipitated with centrifugation at 25,000*g* for 5 min. The pellet was washed with medium B containing 350 mM sucrose, 20 mM Hepes-Tris (pH 7.4), 2.5 mM $MgSO_4$, 0.2 mM EGTA, 2 mM DTT, and 1 mg/ml BSA, resuspended in ~100 μl of the same medium, and immediately used for measurements of activities.

Measurement of activities. NADH, NADPH, and dNADH oxidation by mitochondria were measured at 30°C with a Hitachi 557 spectrophotometer (Japan) at 340 nm. The extinction coefficient ε_{340} taken for NADH, NADPH, and dNADH was 6.22 $mM^{-1} \cdot cm^{-1}$.

External NAD(P)H oxidation was measured in medium B (see above) supplemented with 0.5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 0.12 mM NADH or NADPH. The assay was started by addition of 1 mM $CaCl_2$ to activate the NAD(P)H dehydrogenases [9].

Internal (d)NADH oxidation was measured in medium B containing 0.2 mM EGTA to inhibit external NAD(P)H-dehydrogenases at NADH or dNADH concentration of 0.12 mM. The reaction was initiated by adding alamethicin (40 μg/ml) to permeabilize the mitochondrial membranes as described in [14]. Alamethicin is an antibiotic that can form in membranes channels of rather large size, providing its permeability for NADH [14, 15], thus alamethicin can be used for *in situ* catalytic assay of intramitochondrially located enzymes [16]. In control experiments, it was demonstrated that the effect of alamethicin on plant mitochondria is the same as in the case of animal mitochondria (data not shown).

The contribution of cytochrome *c* oxidase to total mitochondrial respiration was estimated for non-thermogenic tissues by inhibition of NADH oxidase caused by 1 mM KCN. In the case of *A. orientale* spadix mitochondria, inhibition by cyanide was investigated in the presence of 1 mM salicylhydroxamic acid (SHAM). The total inhibition of respiration in the presence of both 1 mM SHAM and 1 mM cyanide in all cases exceeded 95% of the initial activity.

Mitochondrial protein was determined by the bicinchoninic acid method [17] using BSA as the standard.

RESULTS

Arum maculatum is a typical species used to study plant heat production. In this work, we used *Arum orientale*, which is a species closely related to *A. maculatum* growing wild in Russia (Northern Caucasus).

Mitochondria from thermogenic (spadix) or non-thermogenic (corm) tissues of *A. orientale* were isolated and various NADH-oxidase activities of these organelles were measured. As seen from the table, *Arum* spadix mitochondria oxidize intra- and extramitochondrial NADH as well as intramitochondrial dNADH. It is noteworthy that the NADH-oxidase activities of *Arum* spadix mitochondria were several-fold stimulated in the presence of 1 mM pyruvate (table), which can be caused by activation of alternative cyanide-resistant oxidase (AltOx) by this α-keto acid [3, 18]. Thus, the NADH-oxidase activities

measured in the absence of pyruvate may be underestimated, especially on *Arum* spadix mitochondria due to elevated level of this oxidase [18]. In this work, for further calculation only the activities measured in the presence of pyruvate were used.

Using the activities measured it is possible to calculate the specific activities of different mitochondrial NADH-dehydrogenases (Fig. 1). The activities of external NAD(P)H:quinone oxidoreductases—NADH-dehydrogenase (NDex) and NADPH-dehydrogenase (NDPex)—were determined using activation of NADH- or NADPH-oxidation by intact mitochondria in the presence of calcium ions, respectively (as described in [9]).

The activities of internal NADH:quinone oxidoreductases, i.e., Complex I and internal non-coupled NADH-dehydrogenase (NDin), were determined using their different substrate specificities. It is well known that Complex I can oxidize either NADH or its analog dNADH (reduced nicotinamide hypoxanthine dinucleotide), while non-coupled NADH dehydrogenases utilize only NADH, but not dNADH [19]. In our assays, the rate of dNADH oxidation was almost completely inhibited by 15 μM rotenone, while the NADH oxidation was only partially sensitive to this inhibitor. In various preparations, the rotenone-sensitive part of NADH oxidase activity was equal to dNADH-oxidase activity (data not shown). This indicates that Complex I from plant mitochondria (as well as Complex I from animal mitochondria and bacterial NDH-1) oxidizes NADH and dNADH with equal rates. Hence, as described previously [19], we regarded the level of the dNADH oxidase activity as Complex I activity and the difference between the NADH- and dNADH-oxidase levels as NDin activity. This method let us determine precisely the Complex I activity against the very high NDin activity background, which is impossible to measure using the conventional rotenone-inhibition technique.

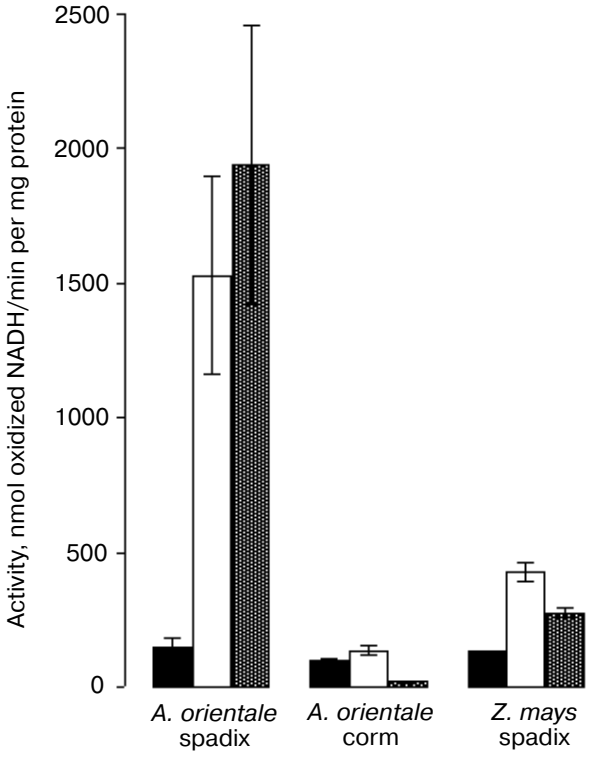

Fig. 1. NADH oxidation activities of different NADH-dehydrogenases in mitochondria purified from thermogenic (*A. orientale* spadix) or non-thermogenic (*A. orientale* corm and *Z. mays* spadix) tissues: Complex I (black rectangles), NDin (white rectangles), NDex (gray rectangles).

As seen in the table, mitochondria isolated from different parts of *A. orientale* (in contrast to *A. maculatum* mitochondria [7, 20]) demonstrated a very low level of external NADPH oxidation. Thus, it can be concluded that in *A. orientale* spadix mitochondria the NDPex activity is negligible, and it seems that this enzyme is not absolutely necessary for thermogenesis of at least this studied *Arum* species.

However, mitochondria isolated from *A. orientale* spadix demonstrated significantly elevated level of two other non-coupled NADH dehydrogenases—NDex and NDin (Fig. 1). Activities of these two enzymes in *Arum* spadix mitochondria are several times higher than the previously reported NADH-dehydrogenase activities of mitochondria from other plant species [6, 9]. As seen in Fig. 1, the significant enhancement of NDex and NDin activities in *A. orientale* spadix mitochondria is a characteristic property of mitochondria isolated only from *spadix* of this plant. The NDex and NDin activities of *Arum* corm mitochondria were, respectively, ~100- and ~10-times lower than in *Arum* spadix mitochondria. Thus, higher NDex and NDin activities were seen only in thermogenic tissues of *A. orientale*. At the same time, the significant enhancement of NDex and NDin activities is a characteristic property of mitochondria isolated only from spadix of a *heat-producing plant*. As seen in Fig. 1, mitochondria isolated from *Z. mays* spadix (female inflorescence from non-thermogenic plant that is closely related morphologically to *Arum* spadix) did not demonstrate this unique pattern. This means that the elevated level of NDex and NDin activities in plant seems to be not organ- or species-specific and apparently correlates only with heat production.

It is noteworthy that the Complex I activity was about the same in all the cases tested (table and Fig. 1). Thus, it is the *specific* induction of activities of non-coupled NADH-dehydrogenases (NDex and NDin) in thermogenic tissues, not a general increase in total NADH oxidation via all the mitochondrial enzymes. In the *Arum* spadix, the increase in NDex and NDin activities against the constant Complex I activity background should result in extremely low participation of Complex I in total NADH oxidation by these mitochondria.

Rate of oxidation of different reduced pyridine dinucleotides by mitochondria isolated from different plant tissues

Activity	*A. orientale* spadix*		*A. orientale* corm**		*Zea mays* spadix**	
	– pyruvate	+ pyruvate	– pyruvate	+ pyruvate	– pyruvate	+ pyruvate
Ca^{2+}-dependent NADH oxidation	520 ± 85	1940 ± 520	19 ± 3	19.5 ± 2.5	242 ± 15	276 ± 17
Ca^{2+}-dependent NADPH oxidation	20 ± 4	22 ± 4	0	0	23 ± 3	24 ± 4
Alamethicin-dependent dNADH oxidation	100 ± 20	150 ± 35	92 ± 10	95 ± 12	123 ± 7	130 ± 6
Alamethicin-dependent NADH oxidation	474 ± 93 (20%)***	1680 ± 410 (5%)***	206 ± 24 (97%)***	230 ± 30 (88%)***	490 ± 30 (95%)***	556 ± 38 (87%)***

Note: Activities were measured as described in "Materials and Methods" and are given in nmol of reduced pyridine dinucleotide consumed per 1 min per 1 mg protein. Where indicated, the pyruvate concentration was 1 mM.

* Average from four independent mitochondrial preparations.

** Average from two independent mitochondrial preparations.

*** Degree of NADH-oxidase activity inhibition by cyanide (see "Materials and Methods" for details).

It is also noteworthy that induction of NDex and NDin activities in thermogenic tissues was accompanied by the well-known induction of cyanide-resistant AltOx activity (table), which is also non-coupled to $\Delta\mu_{H^+}$ generation. As a result of this, the portion of energy-conserving enzymes in total respiration of *Arum* spadix was not higher than 5%, and in this case free energy of oxidation of respiratory substrates should be directly and almost entirely converted into heat.

DISCUSSION

Various living organisms are able to maintain a temperature gradient between their bodies and the environment. It is obvious that respiration is the main energy source for such heat production in any heterotrophic tissue because only this reaction is characterized by significant excess of free energy. However, the mechanisms of transformation of this energy into heat can differ in different organisms or different tissues of a single organism. In general, heat can be formed by three independent mechanisms: 1) by hydrolysis of ATP formed by oxidative phosphorylation (respiration $\rightarrow \Delta\mu_{H^+} \rightarrow$ ATP $\rightarrow$ heat); 2) by dissipation of proton motive force generated by proton-translocating enzymes of the respiratory chain (respiration $\rightarrow \Delta\mu_{H^+} \rightarrow$ heat); 3) by substrate oxidation via non-coupled enzymes of the respiratory chain (respiration $\rightarrow$ heat) [21].

Thermoregulation is generally assumed to be a specific property of higher animals—mammals and birds. The mitochondrial respiratory chain of these organisms contains only enzymes coupled with energy conservation. Thus, thermogenesis of these animals can be achieved only using the first and the second mechanisms, i.e., ATP hydrolysis (by trembling of muscles, for example) or $\Delta\mu_{H^+}$ dissipation (by the activity of thermogenin in brown adipose tissue, for example) [21].

However, some seed plants can also demonstrate significant heat production [1]. It is not yet known what kind of mechanism(s) is preferentially used in this case. There are several controversial reports in this field.

On one hand, uncoupling proteins have been shown to operate in plant mitochondria [13, 22, 23]. Moreover, in inflorescence of the skunk cabbage *Symplocarpus foetidus* the temperature-sensitive and thermogenic tissue-specific induction of uncoupling protein SfUCPb was demonstrated [24]. This can indicate that in this case $\Delta\mu_{H^+}$ dissipation is used as a heat-production mechanism.

On the other hand, it is well known that the respiratory chain of plant mitochondria (with respect to the respiratory chain of animal mitochondria) can contain several enzymes of an alternative branch of substrate oxidation, non-coupled with energy conservation [25]. This makes it possible for a plant to use the direct mechanism of respiratory energy transformation into heat, omitting intermediate stages of energy conservation in the form of $\Delta\mu_{H^+}$ or ATP. Indeed, mitochondria from thermogenic organs of plants contain very high level of AltOx, the activity of which is not coupled with energy conservation [1, 3, 4]. Thus, in these mitochondria $\Delta\mu_{H^+}$ generation at Complexes III and IV is almost completely bypassed. In this work it has been shown that NADH oxidation by mitochondria from thermogenic tissues of *A. orientale* also occurs via non-coupled enzymes (NDin and NDex), and the energy conservation by Complex I is bypassed too. This means that the activity of the entire respiratory chain of mitochondria from *A. orientale* thermogenic tissues is almost completely non-coupled to $\Delta\mu_{H^+}$ generation (see Fig. 2), and the free energy of respiration is directly and entirely transformed into heat. We would like

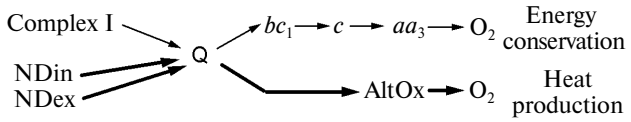

Fig. 2. A putative scheme of the mitochondrial respiratory chain of *A. orientale*. The electron transport pathway dominating in heat-production stages is shown by heavy arrows.

to point out that the portion of the energy-conserving branch of the respiratory chain in *Arum* spadix mitochondria is less than 5% of the total respiratory activity. Thus, at least in the case of *A. orientale* it is difficult to assume any significant participation of uncoupling proteins (UCPs) in the heat production. This statement is in accordance with the recent observation that there is no thermogenic tissue-specific induction of putative uncoupling protein HmUCP in the heat-producing arum lily *Helicodiceros muscivorus* [26].

It is noteworthy that simultaneous induction of non-coupled NADH:quinone oxidoreductase(s) and non-coupled (or "partially" coupled) terminal quinol-oxidase has been also reported for some specific growth conditions of bacteria (respiratory protection of nitrogenase complex in *Azotobacter vinelandii* [19, 27]) and fungi (the xylose-metabolizing yeast *Pichia stipitis* [28]). It seems that the induction of the alternative, non-coupled branch of the respiratory chain is a universal mechanism of living organisms for achieving extremely rapid respiration used for heat production, lowering of intracellular oxygen concentration, regulation of metabolic fluxes, or other functions. Only Metazoa are an exception from this rule. Apparently, the enzymes of this non-coupled branch were lost in the early stages of animal evolution. Thus, in the subsequent origin of thermoregulation, heat-producing animals had to utilize such complex mechanisms of thermogenesis as dissipation of energy that has been conserved in the form of $\Delta\mu_{H^+}$ or ATP. Alternatively, these complicated mechanisms of heat production in thermogenesis of higher animals can allow mammals and birds to more intensively regulate this process and as a result to maintain an exactly constant body temperature.

We are grateful to Prof. V. P. Skulachev for many suggestions. We thank Dr. T. Yu. Konovalova for help with collecting *A. orientale*.

This work was supported in part by the Russian Foundation for Basic Research (grants 04-04-48101 and 04-04-48344). Y. V. B. is indebted to the Russian Foundation for Basic Research for special assistance for young scientists (grant 03-04-06016), V. N. P. is grateful to CRDF for financial support (grant VZ-010-02 (Annex 03)).

REFERENCES

1. Seymour, R. S. (2001) *Biosci. Rep.*, **21**, 223-236.
2. James, W. O., and Beevers, H. (1950) *New Phytol.*, **49**, 353-374.
3. Affourtit, C., Albury, M. S., Crichton, P. G., and Moore, A. L. (2002) *FEBS Lett.*, **510**, 121-126.
4. McIntosh, L. (1994) *Plant Physiol.*, **105**, 781-786.
5. Möller, I. M. (2002) *Trends Plant Sci.*, **7**, 235-237.
6. Rasmusson, A. G., Svensson, A. S., Knoop, V., Grohmann, L., and Brennicke, A. (1999) *Plant J.*, **20**, 79-87.
7. Roberts, T. H., Fredlund, K. M., and Möller, I. M. (1995) *FEBS Lett.*, **373**, 307-309.
8. Möller, I. M. (1997) *Physiol. Plant.*, **100**, 85-90.
9. Svensson, A. S., and Rasmusson, A. G. (2001) *Plant J.*, **28**, 73-82.
10. Svensson, A. S., Johansson, F. I., Möller, I. M., and Rasmusson, A. G. (2002) *FEBS Lett.*, **517**, 79-82.
11. Cook, N. D., and Cammack, R. (1985) *Biochim. Biophys. Acta*, **827**, 30-35.
12. Wagner, A. M., and Moore, A. L. (1997) *Biosci. Rep.*, **17**, 319-333.
13. Popov, V. N., Markova, O. V., Mokhova, E. N., and Skulachev, V. P. (2002) *Biochim. Biophys. Acta*, **1553**, 232-237.
14. Grivennikova, V. G., Kapustin, A. N., and Vinogradov, A. D. (2001) *J. Biol. Chem.*, **276**, 9038-9044.
15. Bertsova, Y. V., and Bogachev, A. V. (2002) *Biochemistry (Moscow)*, **67**, 622-626.
16. Gostimskaya, I. S., Grivennikova, V. G., Zharova, T. V., Bakeeva, L. E., and Vinogradov, A. D. (2003) *Analyt. Biochem.*, **313**, 46-52.
17. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Analyt. Biochem.*, **150**, 76-85.
18. Hoefnagel, M. H. N., Rich, P. R., Zhang, Q., and Wiskich, J. T. (1997) *Plant Physiol.*, **115**, 1145-1153.
19. Bertsova, Y. V., Bogachev, A. V., and Skulachev, V. P. (1998) *Biochim. Biophys. Acta*, **1363**, 125-133.
20. Cottingham, I. R., and Moore, A. L. (1984) *Biochem. J.*, **224**, 171-179.
21. Skulachev, V. P. (1988) *Membrane Bioenergetics*, Springer, Berlin.
22. Vercesi, A. E., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cuccovia, I. M., and Chaimovich, H. (1995) *Nature*, **375**, 24.
23. Laloi, M., Klein, M., Riesmeier, J. W., Müller-Röber, B., Fleury, C., Bouillaud, F., and Ricquier, D. (1997) *Nature*, **389**, 135-136.
24. Ito, K. (1999) *Plant Sci.*, **149**, 167-173.
25. Siedow, J. N., and Umbach, A. L. (1995) *Plant Cell*, **7**, 821-831.
26. Ito, K., Abe, Y., Johnston, S. D., and Seymour, R. S. (2003) *J. Exp. Bot.*, **54**, 1113-1114.
27. Bertsova, Y. V., Bogachev, A. V., and Skulachev, V. P. (2001) *J. Bacteriol.*, **183**, 6869-6874.
28. Shi, N. Q., Cruz, J., Sherman, F., and Jeffries, T. W. (2002) *Yeast*, **19**, 1203-1220.