FEBS Letters 348 (1994) 181–184

FEBS 14214

Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria

Ann L. Umbach[a], Joseph T. Wiskich[b], James N. Siedow[a,*]

[a]*Department of Botany, Duke University, Box 90338, Durham, NC 27708-0338, USA*
[b]*Botany Department, University of Adelaide, North Terrace, Adelaide SA, Australia*

Received 18 May 1994

Abstract

Two factors known to regulate plant mitochondrial cyanide-resistant alternative oxidase activity, pyruvate and the redox status of the enzyme's intermolecular disulfide bond, were shown to differently affect activity in isolated soybean seedling mitochondria. Pyruvate stimulated alternative oxidase activity at low levels of reduced ubiquinone, shifting the threshold level of ubiquinone reduction for enzyme activity to a lower value. The disulfide bond redox status determined the maximum enzyme activity obtainable in the presence of pyruvate, with the highest rates occurring when the bond was reduced. With variations in cellular pyruvate levels and in the proportion of reduced alternative oxidase protein, a wide range of enzyme activity is possible in vivo.

Key words: Cyanide-resistant oxidase; Plant mitochondrion; Pyruvate activation; Dimeric enzyme; Regulatory disulfide bond

1. Introduction

The cyanide-resistant alternative oxidase of plant mitochondria accepts electrons from the ubiquinone pool of the mitochondrial electron transport chain and catalyzes the subsequent reduction of oxygen to water. Unlike the electron transfer pathway from the ubiquinone pool to cytochrome *c* oxidase, energy is not conserved in this reaction so the alternative pathway has been considered 'wasteful' from a bioenergetic perspective [1]. Circumstantial evidence, however, indicates that this pathway is of critical importance to plant metabolism under certain physiological and environmental conditions [2–4]. Nevertheless, how alternative oxidase activity is regulated and integrated with general plant metabolism is not well understood.

Two means of regulation of alternative oxidase activity are well-established. One involves variation in the amount of alternative oxidase protein present in the membrane. Changes in protein amount, as determined by immunoblotting, correlate positively with changes in alternative oxidase activity under some circumstances. Examples include development of the *Sauromatum guttatum* Schott thermogenic floral appendix [5,6] and induction by chilling [7] or salicylic acid [8] of alternative oxidase activity in tobacco cell suspension cultures. The second regulatory feature occurs at the level of the electron transport chain where the extent of ubiquinone pool reduction determines the degree of engagement of the alternative pathway [9,10]. The oxidase shows significant activity only after a threshold level of ubiquinone pool reduction has been achieved, beyond which activity increases as a non-linear function of reduced ubiquinone.

Recently, additional potential regulatory mechanisms for the alternative oxidase have been reported. Millar et al. [11] demonstrated that pyruvate stimulates alternative oxidase activity, the activation being especially dramatic for mitochondria with inherently low alternative oxidase rates. Succinate and malate were also shown to activate the alternative oxidase by a mechanism clearly distinct from quinone pool reduction [12]. Additionally, Umbach and Siedow [13] found that the alternative oxidase protein exists in mitochondria as a dimer. The dimer can occur in a disulfide-linked, less active state or in a more active state when the disulfide bond has been reduced.

Here we further characterize the effects of pyruvate and the redox state of the dimer disulfide bond system on alternative oxidase activity and begin to address how these two regulatory mechanisms might interact. In soybean seedling mitochondria, pyruvate reduces the threshold level of quinone pool reduction at which the alternative oxidase becomes engaged, while the redox state of the disulfide bond affects maximum enzyme activity.

*Corresponding author. Fax: (1) (919) 684 5412;
email: jsiedow@acpub.duke.edu

Abbreviations: Diamide, azodicarboxylic acid; DTT, dithiothreitol; Q_r, reduced mitochondrial ubiquinone; Q_t, total mitochondrial ubiquinone pool; Q_r/Q_t, proportion of the ubiquinone pool in the reduced state; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHAM, salicylhydroxamic acid.

2. Materials and methods

Soybean seedlings (*Glycine max* [L.] Merr. cv Ransom) were grown in a greenhouse [13] and cotyledons were harvested at 7 to 12 days. Preparation of Percoll gradient-purified mitochondria, treatment of

SSDI 0014-5793(94)00600-Z

isolated mitochondria with 20 mM DTT and 3 mM diamide, SDS-PAGE, immunoblotting and densitometry were as described previously [13]. Oxygen uptake and the reduction state of the mitochondrial ubiquinone pool were measured simultaneously with a combined oxygen electrode and voltametric apparatus [9]. Reaction conditions were modified as in [13] and included 0.15 mM ATP to activate succinate dehydrogenase. Respiration was initiated by addition of 4.5 to 5 mM succinate and, after one or two state 3/state 4 transitions driven by ADP [13], 5 to 6 μM myxothiazol was added to inhibit the main respiratory pathway, followed by addition of pyruvate (4.3 to 5 mM). The level of reduced ubiquinone (Q_r) was varied by sequential additions of malonate, up to a final concentration of 4 mM, to inhibit succinate dehydrogenase. The total ubiquinone pool (Q_t) was considered to be equivalent to the reduced quinone measured when the reaction medium was allowed to become anaerobic [9]. Respiration rates were evaluated relative to the proportion of the ubiquinone pool in the reduced state (Q_r/Q_t). The oxidation of ubiquinol-1, a direct electron donor to the alternative oxidase [14], was monitored using the same experimental set-up and reaction medium. After addition of mitochondria to the reaction chamber, myxothiazol and 2 μM ubiquinol-1 were added sequentially. Oxidation was initiated by subsequent addition of 5 mM pyruvate. At 2 μM ubiquinol-1, oxygen uptake by the alternative oxidase was undetectable, but the change in amount of reduced quinone was readily measured.

3. Results

Activity of the alternative oxidase of isolated soybean cotyledon mitochondria was measured over a range of Q_r/Q_t as described in section 2. In the presence of 4.3 mM pyruvate, the alternative oxidase response to Q_r/Q_t was modified so that substantial activity was achieved at low levels of Q_r/Q_t, where little activity appeared in the absence of added pyruvate (Fig. 1). Glyoxylate, at the same concentration, had an identical effect on alternative oxidase kinetics (Fig. 1). Similar results were obtained with soybean leaf mitochondria (data not shown). These α-keto acids shifted the value of the Q_r/Q_t threshold where the alternative oxidase becomes active to a lower state of ubiquinone pool reduction. Immunoblots of cotyledon mitochondria sampled during the activity determinations plus and minus pyruvate showed no effect of pyruvate on the redox status of the alternative oxidase protein disulfide bond (data not shown).

The effect of pyruvate was evident as well in direct measurements of oxidation of ubiquinol-1 by the alternative oxidase. When 2 μM ubiquinol-1 was supplied to cotyledon mitochondria without pyruvate, almost no oxidation was detected beyond that which could be attributed to the equilibration of ubiquinol-1 with the endogenous mitochondrial ubiquinone pool (data not shown). However, in the presence of 5 mM pyruvate, a pronounced rate of ubiquinol-1 oxidation was achieved (Fig. 2, control). Glyoxylate was also found to stimulate ubiquinol-1 oxidation (data not shown). These results are consistent with α-keto acid enhancement of alternative oxidase reactivity at low concentrations of reduced quinone, exogenous reduced substrate in this case. In addition, the rate of the pyruvate-stimulated ubiquinol-1 oxidation was affected by the redox state of the alternative oxidase intermolecular disulfide bond. Treatment of

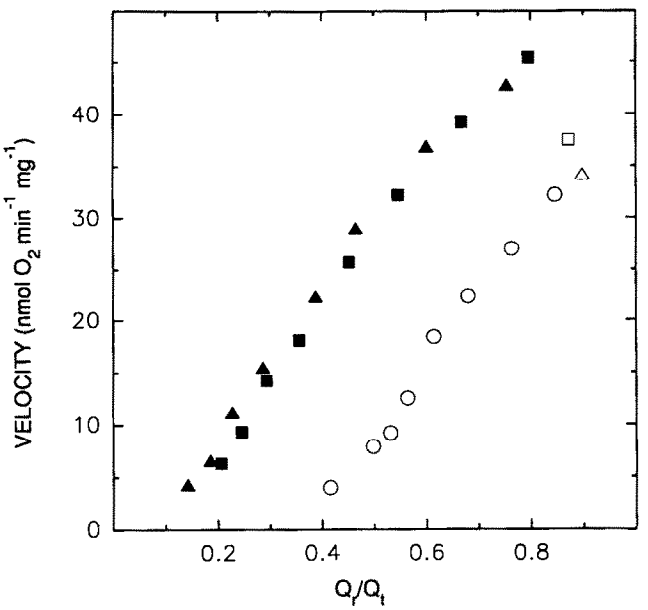

Fig. 1. Relationship of isolated soybean cotyledon mitochondria alternative oxidase activity (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein) and the level of mitochondrial ubiquinone reduction (Q_r/Q_t) in the presence and absence of pyruvate and glyoxylate. A representative experiment is shown. Succinate-supported respiration and ubiquinone reduction were monitored as described in section 2. For the pyruvate and glyoxylate treatments, 4.3 mM pyruvate or glyoxylate was added after myxothiazol. The Q_r/Q_t level was subsequently varied by incremental additions of malonate; ○, no pyruvate; △, before pyruvate addition; ▲, plus 4.3 mM pyruvate; □, before glyoxylate addition; ■, plus 4.3 mM glyoxylate.

cotyledon mitochondria with 20 mM DTT, which fully reduces the disulfide bond [13], stimulated the endogenous rate of ubiquinol-1 oxidation (Fig. 2, DTT), while 3 mM diamide treatment, which leads to quantitative formation of the disulfide through sulfhydryl oxidation [13], decreased the rate of ubiquinol-1 oxidation to near the background rate seen in the absence of pyruvate (Fig. 2, diamide). For 8-day-old soybean root mitochondria, reduction and oxidation of the disulfide bond had the same effects on alternative oxidase rates as seen with cotyledon mitochondria (data not shown).

To determine how the pyruvate effect and disulfide bond redox status might interact, alternative oxidase activity was measured over a range of Q_r/Q_t in the presence or absence of 5 mM pyruvate, after the cotyledon mitochondria were pretreated with diamide or DTT to fully oxidize or reduce, respectively, the alternative oxidase protein disulfide bond. Immunoblots showed that the alternative oxidase was initially present as a 60%/40% mixture of oxidized and reduced dimers in control mitochondria and confirmed the efficacy of the DTT and diamide treatments ([13], data not shown). The separate effects of the two regulatory mechanisms were clearly evident. At high Q_r/Q_t (e.g., before addition of malonate to inhibit succinate oxidation), the redox state of the disulfide bond had an overriding effect on alternative

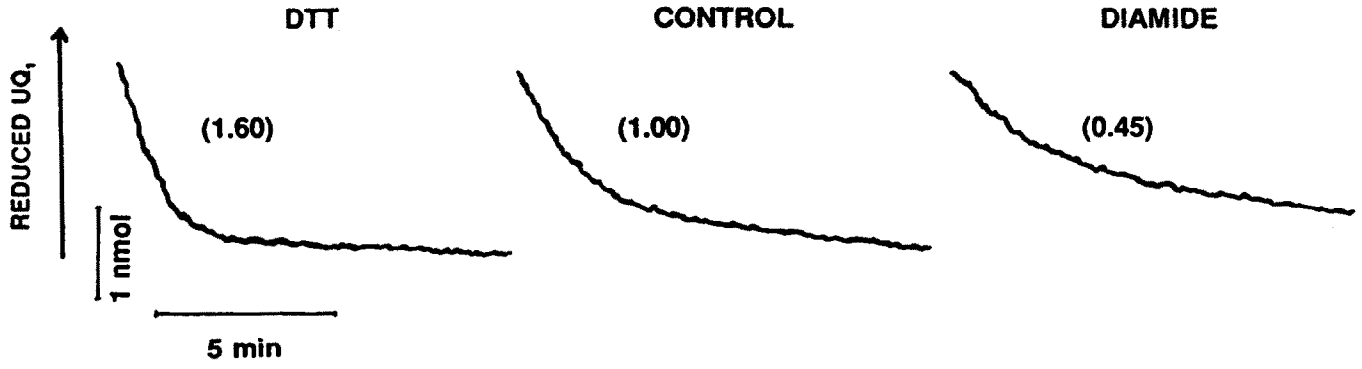

Fig. 2. Effect of alternative oxidase disulfide bond redox state on the rate of ubiquinol-1 (UQ_1) oxidation. A representative experiment with 7-day-old soybean cotyledon mitochondria is shown. The disulfide bond redox state was changed by pretreatment of the isolated mitochondria with either 20 mM DTT or 3 mM diamide [13]. Ubiquinol-1 oxidation rates through the alternative oxidase were subsequently determined as described in section 2, with 2 μM ubiquinol-1 and 5 mM pyruvate in the presence of 6 μM myxothiazol. Numbers in parenthesis indicate the magnitude of the initial slopes, relative to the control.

oxidase activity (Table 1). Pyruvate addition stimulated the control, DTT-, and diamide-treated mitochondria alternative oxidase rates about 10%. In contrast, DTT treatment alone stimulated alternative oxidase activity 54% and oxidation by diamide inhibited activity 72%. Eight-day-old soybean root mitochondria displayed a qualitatively similar pattern. While percent stimulation of activity by pyruvate was much larger for root than for cotyledon mitochondria, the maximum alternative oxidase activity was again achieved with DTT-treated mitochondria in the presence of pyruvate (data not shown). For cotyledon mitochondria, reduction of the disulfide bond had only a small effect on the Q_r/Q_t range over which the oxidase was active; enzyme activity declined precipitously in either control or DTT-treated mitochondria as Q_r/Q_t decreased (Fig. 3A). In contrast, in the presence of 5 mM pyruvate, for both DTT-treated and control mitochondria, the alternative oxidase was active at markedly lower levels of Q_r/Q_t, with reduced-enzyme rates being higher throughout (Fig. 3B). For diamide-treated mitochondria, alternative oxidase activity remained low, regardless of Q_r/Q_t or pyruvate treatment (Table 1; Fig. 3B).

Table 1
Effect of pyruvate on alternative oxidase activity of control, DTT-, and diamide-treated soybean cotyledon mitochondria

Treatment[b]	Alternative oxidase activity (nmol $O_2 \cdot min^{-1} \cdot mg^{-1} \cdot$ protein)[a]	
	−Pyr	+Pyr
Control	93	100
DTT	143	159
Diamide	26	29

[a] Oxygen uptake was measured as described in section 2 with 5 mM succinate as respiratory substrate. '−Pyr' rates were measured in the presence of 6 μM myxothiazol and '+Pyr' rates were measured subsequently, after addition of 5 mM pyruvate.
[b] Isolated 7-day-old soybean cotyledon mitochondria were treated with 20 mM DTT to reduce the alternative oxidase disulfide bond or with 3 mM diamide to oxidize the bond [13].

4. Discussion

Two recently-reported regulatory mechanisms of alternative oxidase activity, pyruvate stimulation [11] and redox status of the alternative oxidase dimer intermolecular disulfide bond [13], are shown here to affect activity differently. Pyruvate markedly broadens the range of Q_r/Q_t over which the enzyme is active, essentially lowering the 'effective' K_m of the alternative oxidase for reduced ubiquinone. Glyoxylate has the same effect on activity (Fig. 1) which is consistent with the stimulation of alternative oxidase by hydroxypyruvate, another α-keto acid, previously observed with soybean mitochondria [11]. Thus, the alternative oxidase is found to have substantial activity at relatively low Q_r/Q_t levels in the presence of pyruvate and related metabolites. The stimulation of alternative oxidase by succinate and malate [12], possibly a related phenomenon, is another instance where metabolite levels can affect alternative oxidase activity, although whether the effect is mediated through response to Q_r/Q_t or the formation of pyruvate is not yet known. In light of these findings, the paradigm of a constant threshold Q_r/Q_t level below which the alternative oxidase is inactive [9,10] may need to be modified. Depending on the endogenous pyruvate, or other metabolite, levels, 'threshold' Q_r/Q_t could vary considerably such that, under the appropriate metabolic conditions, the alternative pathway could achieve substantial rates without saturation of the cytochrome pathway. If so, previous interpretations of some experimental results may need re-evaluation. Alternative pathway engagement has been operationally derived from the change in respiratory rate upon addition of saturating amounts of the inhibitor SHAM [15]. Yet under the circumstances outlined above, SHAM addition could result in little or even no net change in rate, because electrons could be shunted to the unsaturated cytochrome *c* oxidase pathway. No change in rate would be incorrectly interpreted as a lack of engagement of the alternative pathway.

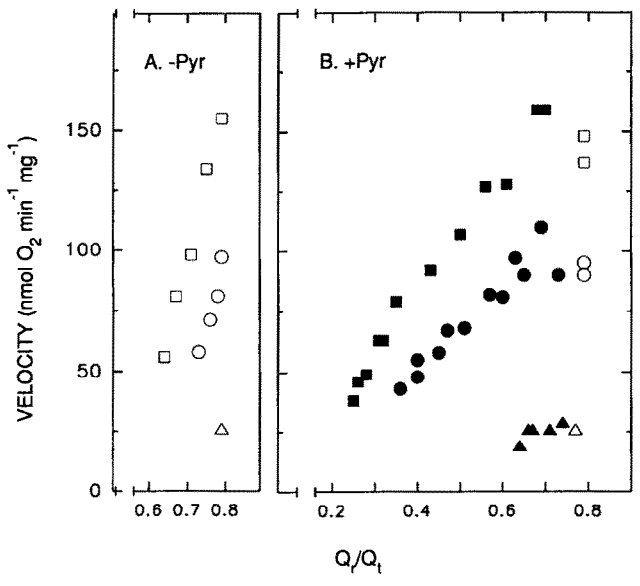

Fig. 3. Combined effect of pyruvate and disulfide bond redox state on the alternative oxidase response to the level of ubiquinone reduction. The disulfide bond redox state of 7-day-old soybean cotyledon mitochondria was changed as described for Fig. 2. Measurements were conducted as for Fig. 1. but with 5 mM succinate, 6 μM myxothiazol and 5 mM pyruvate. (A) In the absence of pyruvate; (B) in the presence of 5 mM pyruvate. For both A and B, open symbols are rates either without or before the addition of pyruvate; closed symbols are rates in the presence of pyruvate. ●, untreated (control); ■, DTT-treated; ▲, diamide-treated.

The redox state of the intramolecular disulfide bond apparently can affect alternative oxidase capacity by acting as a governor on the enzyme's activity. While pyruvate facilitated ubiquinol-1 oxidation, the rate achieved was dependent on whether the disulfide bond was in the oxidized or the reduced state. Factors such as oxidant/reductant effects on other mitochondrial electron transport chain complexes can be ruled out as affecting alternative oxidase activity in this assay since ubiquinol-1 acts as a direct donor to the alternative oxidase [14]. Also, with succinate as substrate and for a given range of Q_r/Q_t, maximum activity (with or without pyruvate) was dependent on whether the disulfide bond was oxidized or reduced (Fig. 3). Thus, pyruvate can stimulate the alternative oxidase, but the maximum rate achieved will be also dependent on the degree of oxidation or reduction of the disulfide bond in situ.

That pyruvate and the disulfide redox state have different effects is consistent with different sites of action for these factors on the alternative oxidase protein. Based on predictions of alternative oxidase protein topology and on sequence similarities, the relevant sulfhydryl group is located on the matrix side of the inner mitochondrial membrane, most probably near the beginning of the first of the two putative membrane-spanning helices of the protein [13], while pyruvate is postulated to act at the region between the two membrane-spanning helices which is localized toward the inter-membrane space [11]. The observation that pyruvate is an allosteric effector of alternative oxidase activity [11] is consistent with the recent characterization of the alternative oxidase as a dimeric enzyme [13], allostery being uncommon with monomeric enzymes [16].

Generalizations concerning the newly recognized complexity of regulation of alternative oxidase activity must be made cautiously. The nature of the pyruvate effect is not yet thoroughly characterized. The number of different tissues and plant species in which either the disulfide bond redox state or the pyruvate effect has been investigated is limited, and an in vivo role for either regulatory mechanism has yet to be clearly established. However, for isolated soybean seedling mitochondria, pyruvate and disulfide bond redox state, individually and together, can have large effects on alternative oxidase kinetics and activity.

Acknowledgements: This work was supported by a grant from the National Science Foundation (DCB 90–19735) to J.N.S. and an Australian Research Council grant to J.T.W.

References

[1] Moore, A.L. and Siedow, J.N. (1991) Biochim. Biophys. Acta 1059, 121–140.
[2] Lambers, H. (1982) Physiol. Plant. 55, 478–485.
[3] Purvis, A.C. and Shewfelt, R.L. (1993) Physiol. Plant. 88, 712–718.
[4] Theodorou, M.E. and Plaxton, W.C. (1993) Plant Physiol. 101, 339–344.
[5] Elthon, T.E. and McIntosh, L. (1987) Proc. Natl. Acad. Sci. USA 84, 8399–8403.
[6] Rhoads, D.M. and McIntosh, L. (1992) Plant Cell 4, 1131–1139.
[7] Vanlerberghe, G.C. and McIntosh, L. (1992) Plant Physiol. 100, 115–119.
[8] Rhoads, D.M. and McIntosh, L. (1993) Plant Physiol. 103, 877–883.
[9] Dry, I.B., Moore, A.L., Day, D.A. and Wiskich, J.T. (1989) Arch. Biochem. Biophys. 273, 148–157.
[10] Siedow, J.N. and Moore, A.L. (1993) Biochim. Biophys. Acta 1142, 165–174.
[11] Millar, A.H., Wiskich, J.T., Whelan, J. and Day, D.A. (1993) FEBS Lett. 329, 259–262.
[12] Lidén, A.C. and Åkerlund, H.-E. (1993) Physiol. Plant. 87: 134–141.
[13] Umbach, A.L. and Siedow, J.N. (1993) Plant Physiol. 103, 845–854.
[14] Rich, P.R. (1978) FEBS Lett. 96, 252–256.
[15] Møller, I.M., Bérczi, A., van der Plas, L.H.W. and Lambers, H. (1988) Physiol. Plant. 72, 642–649.
[16] Lipscomb, W.N. (1983) Annu. Rev. Biochem. 52, 17–34.

Note added in proof

After acceptance of this manuscript, H.Y. Steiner informed us about the identification of a peptide transporter from *Arabidopsis* that is not identical to NTR1 but also belongs to the same family of related proteins (Steiner, H.Y. et al, Plant Cell, in press).