UDC 572.784:582.282
ISSN 1330-9862

review

(FTB-1190)

Regulation of the Cyanide-Resistant Alternative Respiratory Pathway in the Fungus *Acremonium chrysogenum*

*Erzsébet Sándor, Erzsébet Fekete and Levente Karaffa**

Department of Microbiology and Biotechnology, Faculty of Sciences, University of Debrecen, H-4010, P.O.Box 63, Debrecen, Hungary

Received: September 17, 2002
Accepted: January 24, 2003

Summary

This review summarises the current knowledge on the biochemical and physiological events that directly or indirectly alter the engagement of the cyanide-resistant alternative respiratory pathway in the cephalosporin C producer filamentous fungus *Acremonium chrysogenum*. Particular emphasis is placed on the role this activity plays in the overproduction of antibiotic, and also on the critical fermentation technology background that supports its operation.

Key words: Acremonium chrysogenum, alternative oxidase, respiration, oxygen, peroxides, cephalosporin C, soybean oil

Introduction

More than seven decades have passed since the observation of Genevois (*1*) on the inability of cytochrome c oxidase inhibitors like cyanide, azide or carbon-monoxide to completely suppress plant respiration. The phenomenon is accounted for by the presence of a single mitochondrial respiratory enzyme, the alternative oxidase (AOX). Plants (*2*), fungi (*3,4*) and protists (*5*) are known to possess this cyanide-resistant, salicylhydroxamic acid (SHAM) sensitive (*6*), non-phosphorylating and, therefore, energy-dissipating alternative respiratory route in their inner mitochondrial membrane in addition to the phosphorylating, cytochrome-dependent respiratory pathway (Fig. 1). Though resistance against cyanide was also observed in procaryotes, no matching sequences to the otherwise highly conserved fungal or plant AOX gene / protein are available in the databases, rendering the putative »bacterial AOX« nonexistent so far.

In thermogenic plants the heat that is dissipated during AOX activity is used to volatilise amines to attract pollinating insects (*7*). However, the widespread presence of the enzyme suggests that the AOX has a more general physiological role. It appears that the AOX is important under conditions where cytochrome pathway activity is somehow impaired. Reversely, the AOX allows Krebs-cycle turnover when the energy charge of the cell is high (*8*). Apart from thermogenesis, AOX is involved in the maintenance of the mitochondrial electron transport at low temperatures, and it may also take part in the stabilisation of the redox state of the ubiquinone pool in the inner mitochondrial membrane, thereby preventing the intracellular accumulation of harmful free oxygen radicals (*9–11*).

In plants, the intensity of the alternative route will depend on the amount of oxidase present and on the ratio of reduced ubiquinone to the total ubiquinone pool (*12*). Activity of the oxidase in plant mitochondria is increased upon reduction of an intersubunit disulphide bridge, yielding a non-covalently linked homodimeric protein. The reduced enzyme is further activated by pyruvate, which has been shown to react with a protein-

* Corresponding author; Phone: +36 52 316 666 ext. 2488; Fax: +36 52 533 677; E-mail: karaffal@tigris.klte.hu

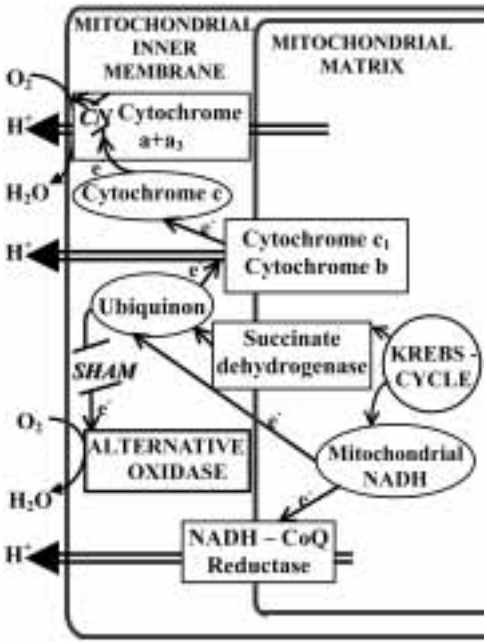

Fig 1. Cyamide resistant alternative respiratory route in the inner mitochondrial membrane

-derived sulphydryl moiety, most likely to form a thiohemiacetal (*13–16*). A conserved cysteine residue near the N-terminus of the protein is responsible for both disulphide bond formation and organic acid activation of the AOX. Engineering the AOX by replacing this cystein with a serine residue resulted in a permanently monomeric enzyme that was specifically stimulated by succinate but not by pyruvate (*17*).

Being an extremely conserved protein, it was a discovery of high significance that, in contrast to plant enzymes, fungal AOX displays the very features of the *in vitro* modified protein, *e.g.* it is permanently monomeric and is not activated directly by pyruvate. It was also revealed that a domain of about 40 amino acids responsible for the establishment of the disulphide bond in the plant enzyme is missing in fungal sequences. The fungal AOX is therefore stimulated by succinate rather than pyruvate, and also by purine nucleotides like ADP, AMP and GMP (*18*).

The possession of a functional AOX by the filamentous fungus *Acremonium chrysogenum*, producer of the commercially important cephalosporin C (CPC) antibiotic has been described independently by Telesnina *et al.* (*19*) and Kozma *et al.* (*20*). In this review, the factors regulating the activity of the alternative respiratory pathway in submerged *A. chrysogenum* cultures and the contribution of this route to CPC production will be summarised and discussed.

Time-Profile of the Alternative Respiratory Activity

Alternative respiration of *A. chrysogenum* displays a characteristic time-profile in submerged cultures. Cyanide-resistant oxygen uptake sharply increases during fast growth and gets lower as cultures reach stationary phase (*21*). Expression of these values as a percentage of the total uninhibited respiration, however, displays a different picture: overall respiratory activity (cytochrome-dependent and alternative combined) decreases much faster than that of the AOX alone (*22*). Consequently, the contribution of AOX to the total mycelial oxygen uptake significantly increases towards the end of cultivation.

Stationary phase cultures are largely carbon-depleted, with little potential left for further biomass production. In order to see whether the switch to the alternative respiratory route is due to a decrease in the specific growth rate or rather to the exhaustion of the carbon source, glucose-limited chemostat cultures were made, where dilution rate (specific growth rate under steady--state conditions) could be independently modified (*23*). Results revealed that *A. chrysogenum* AOX activity is in fact growth rate dependent, and high metabolic turnover can stimulate it even under glucose limitation. Conversely, most of the carbon sources with a slow uptake and/or catabolism (with the noteworthy exceptation of plant oils, see later) resulted in decreased AOX activity (*23*).

Alternative Respiration and Aeration

AOX activity of various organisms including plants and fungi have long been described to require »high« dissolved oxygen tensions, though very few quantitative data are available in this respect (*3,24,25*). Studies where oxygen input of submerged *A. chrysogenum* cultures was manipulated to yield dissolved oxygen concentrations in the range of 1–50 % of saturation (*26*) revealed that uninhibited respiratory activities above 5 % of saturation were independent of the actual oxygen tensions (*22*). To the contrary, AOX activity was found dependent on oxygen up to 30 % of saturation. Growth of the cultures with different oxygen supply were comparable, thus ruling out that the varying AOX activity could be the result of differences in the growth rate, but the carbon source (glucose) consumption was progressively and profoundly more rapid with increasing aeration (*22*). This could well be explained by the enhanced activity of the non-phosphorylating AOX, which is able to lower significantly the amounts of carbon without ATP production (*27*). As a consequence of its improved operation, more carbon had to be catabolised to restore the necessary ATP yield.

The activity of AOX depends on the availability of its main substrates, oxygen and NADH. Remarkably, time-course of both NADH and NAD^+-concentrations was largely independent of the aeration (*22*). In addition, highest AOX activity values coincided with a progressively decreasing NADH-concentration, indicating that the electron overflow hypothesis, *e.g.* that AOX acts to handle an excess of NADH which would exceed the capacity of the main cytochrome pathway, is not always valid, and that the alternative pathway, could also be active when the cytochrome route is not saturated (*22,23*).

Respiratory control, *e.g.* availability of ADP for oxidative phosphorylation did not influence AOX activity either, as proved by uncoupling substances that were otherwise taken up by *A. chrysogenum* mycelia (*22*). Inorganic phosphate (see later) was abundant in the me-

dium, thus modified dissolved oxygen tensions remained considered as the obvious regulator of AOX activity (*22*).

The production of reactive oxygen species (ROS), such as $O_2.^{(-)}$ and H_2O_2, is an unavoidable consequence of aerobic metabolism. Mitochondrial electron transport chain is a major site of ROS production. The capability of the alternative respiratory pathway to prevent the production of ROS has been proposed by several studies (*28–31*). Indeed, intracellular peroxide (IP) levels of mycelia proved to be aeration-dependent, while their increase caused by elevated oxygen input could be counteracted by the presence of the lipid peroxyl radical scavenger DL-α-tocopherol, and *vice versa*. Accordingly, the effect of oxygen on the cyanide-resistant alternative respiratory activity is mediated via changes in the size of the IP pool. Since a lipid peroxyl radical scavenger could hinder the IP production, an overwhelming majority of these molecules were suggested to be of lipophylic character. The primarily produced ROS are water-soluble, thus a secondary, tertiary, *etc*. production of peroxides due to the propagation of lipid peroxidation by peroxyl radicals was supposed to take place. These results were in good agreement with those obtained when peroxides were directly supplemented to *A. chrysogenum* cultures, or when catalase activities *in vivo* were inhibited by salicylic acid (*32*). Both treatments caused a substantial increase in the levels of IP and coincided with a stimulation of the AOX activity.

Alternative Respiration and CPC Production

Similarly to the activity of AOX, biosynthesis (and also the industrial production) of CPC by *A. chrysogenum* requires extensive aeration. In order to prove a causal relationship between AOX activity and CPC production, a multi-reactor system with gradually increasing oxygen tensions was used for the cultivation of the fungus (*26*). It was found that both AOX activity and CPC productivity increased progressively, yielding a correlation coefficient of over 0.9 and therefore suggesting a strong, although indirect evidence for the case. A direct evidence was served by the addition of SHAM into the culture medium. Specific inhibition of the alternative respiratory pathway reduced the CPC production rate practically to zero without inhibiting biomass production, indicating the crucial role of the AOX activity in CPC production (*22*).

In the course of CPC fermentation by *A. chrysogenum*, activities of the cytochrome-dependent respiratory enzymes decrease, whereas the activity of the AOX increases (*21*). The physiological function of this non-protonmotive respiration was suggested to be the removal of the reducing equivalents in excess. The synthesis of CPC requires a high rate of carbon catabolism, which will result in NADH production and subsequently, ATP generation. However, ATP consumption is limited by the absence of biosynthetic processes of equivalent capacity, resulting in a high energy charge and an overall decrease in catabolism. A switch from cytochrome-dependent respiration to the alternative route would enable the fungus to reoxidise its NADH without concomitant ATP production, allowing carbon catabolism to ensue.

The experiments described above were performed using a cultivation medium that supports CPC overproduction. Consequently, final CPC concentrations were comparable to those of the final biomass. When the experiments were repeated using a minimal medium with glucose and ammonium as a sole carbon and nitrogen source, respectively, final CPC concentrations became negligible to the biomass produced (*23*). Under such conditions, addition of SHAM into the culture medium (specific inhibition of the alternative respiratory pathway) exhibited no effect on the production of CPC. It was concluded, therefore, that CPC overproduction rather than CPC biosynthesis itself requires the operation of the alternative respiratory pathway, and that under non-producing conditions the relationship between AOX activity and CPC production is lost.

Alternative Respiration and Plant Oil Catabolism

To produce CPC by *A. chrysogenum* on an industrial scale, the fungus was cultivated in a complex medium with glucose (dextrane) and different plant oils as major carbon sources (*33,34*). Biochemical background of the beneficial effect of oils on CPC production is still not clear. It was therefore questioned whether the addition of soybean oil may also influence the activity of the alternative respiratory pathway.

The addition of soybean oil had no effect on the total respiratory rate. However, the respiratory activities of the soybean-oil-supplemented and non-supplemented cultures were differently affected by the addition of KCN, since soybean-oil-containing culture showed about twofold activity of the AOX when compared to the controls (*35*). In order to test whether there was a direct relationship between the presence of soybean oil and the formation of the AOX, its activity was also assayed in mycelia growing in the presence of different concentrations of soybean oil and glucose. Data showed that the activity of the SHAM-sensitive pathway indeed increases with the increase of soybean oil concentration (*35*).

Metabolism of soybean oil can involve the glyoxalate cycle. Growth in the presence of increasing concentrations of soybean oil indeed led to correspondingly increased activities of its key enzyme isocitrate lyase. In order to find out whether succinate, the product of the glyoxalate cycle, may stimulate the alternative respiratory pathway, thenoyltrifluoroacetone (TTFA) was used, which specifically inhibits succinate dehydrogenase (*36*). TTFA addition indeed had a more dramatic effect on respiration in mycelia growing in the presence of soybean oil. TTFA also appeared to specifically block the alternative respiratory activity, since further addition of KCN blocked respiration completely in both cultures (*35*).

As mentioned earlier, fungal AOX is stimulated by succinate at concentrations of 1–5 mM (*18*). These concentrations are likely to be physiologically relevant, since succinate, produced within the mitochondrial matrix via the glyoxalate cycle as described above, can reach concentrations as high as that. Data obtained using isolated

A. chrysogenum mitochondria confirmed that succinate is in fact a stimulator of the AOX activity.

Stimulation of the AOX by succinate may also proceed, similarly to the effect of oxygen, by increased IP production. The addition of glucose and soybean oil, respectively, to carbon-depleted *A. chrysogenum* cultures enhanced IP levels differently: glucose caused an approx. 6-fold, while soybean oil an almost 17-fold increase. On the other hand, both cyanide-resistant respiration and the IP levels were effectively suppressed by the lipid peroxyl radical scavenger DL-α-tocopherol. Importantly, CPC overproduction could also be hindered by DL-α-tocopherol. Inhibition of the oxidative metabolism of succinate by TTFA also resulted in a decrease of both the alternative respiration and the IP levels, but considerably more severely on soybean oil than on glucose. These results suggest that in addition to the stimulatory effect of succinate *per se*, succinate dehydrogenase and the cyanide-resistant alternative respiratory pathway may also be functionally connected via changes in the size of the IP pool.

Alternative Respiration and Phosphate Concentrations

The alternative respiratory pathway is uncoupled, *i.e.* electron flow via AOX is not hindered by the generation of a proton gradient, and thus it is not attached to the phosphorylation of ADP. In suboptimal concentrations, ADP and inorganic phosphate can both limit respiratory electron flow. It was hypothesized that under phosphate limiting conditions (which happen during such important commercial fermentations like citric acid or penicillin production), electron flow would switch from the cytochrome-dependent pathway to the alternative route. To test this hypothesis, *A. chrysogenum* mycelia were pregrown in a standard medium, and were then transferred into ones that contained phosphate in the concentration range of 0.1–5 g/L. Biomass production as well as overall respiratory activity started to decline below a phosphate concentration of 1 g/L. In contrast, alternative route has become progressively more engaged, and comprised almost 75 % of the total oxygen uptake at the lowest examined phosphate concentration.

Conclusions

Similarly to all other fungi studied to date, *A. chrysogenum* also possesses a cyanide-resistant alternative respiratory pathway in its mitochondria. It was shown that its activity can be stimulated by succinate rather than pyruvate, thereby indirectly confirming the established difference between plant and fungal AOX. *A. chrysogenum* AOX responds primarily to processes that involve the generation of ROS, including increased aeration, catabolism of certain carbon sources and ageing. It was proven that AOX activity plays a crucial role in CPC overproduction by removing the redundant reducing equivalents, and thus allowing the carbon catabolism to proceed. Under non-producing conditions, this relationship is lost. However, inhibition of the generation of ROS would inevitably result in decreased AOX activity and a subsequently reduced CPC production, irrespective of the technological background applied.

Acknowledgement

Research in our laboratory is grant-aided by the OTKA (Hungarian Scientific Research Fund; grant F 031985) and the FKFP (Fund for Research and Development in Higher Education; grant no. 0009/2001). E. S. is a recipient of an OTKA Postdoctoral Fellowship (grant no. D 37975).

References

1. M. L. Genevois, *Rev. Gén. Bot. 41* (1929) 252–271.
2. J. T. Bahr, W. D. Bonner Jr., *J. Biol. Chem. 248* (1973) 3441–3445.
3. C. P. Kubicek, O. Zehentgruber, H. El-Kalak, M. Röhr, *Eur. J. Appl. Microbiol. Biotechnol. 9* (1980) 101–115.
4. N. Minagawa, A. Yosimoto, *J. Biol. Chem. 101* (1987) 1141–1146.
5. W. Jarmuszkiewicz, C. M. Sluse-Goffart, A. E. Vercesi, F. E. Sluse, *Biosci. Rep. 2* (2001) 213–222.
6. G. R. Schonbaum, W. D. Bonner Jr., B. T. Storey, J. T. Bahr, *Plant Physiol. 47* (1971) 124–128.
7. B. J. D. Meeuse, *Ann. Rev. Plant. Physiol. 26* (1975) 117–126.
8. F. E. Sluse, W. Jarmuszkiewicz, *Braz. J. Med. Biol. Res. 31* (1998) 733–747.
9. A. M. Wagner, *FEBS Lett. 368* (1995) 339–342.
10. S. Chivasa, J. O. Berry, T. ap Rees, J. P. Carr, *Aust. J. Plant Physiol. 26* (1999) 391–399.
11. F. F. Millenaar, J. J. Benschop, A. M. Wagner, H. Lambers, *Plant Physiol. 118* (1998) 599–607.
12. M. H. N. Hoefnagel, J. T. Wiskich, *Arch. Biochem. Biophys. 355* (1998) 262–270.
13. A. L. Umbach, J. N. Siedow, *Plant Physiol. 103* (1993) 845–854.
14. A. L. Umbach, J. T. Wiskich, J. N. Siedow, *FEBS Lett. 348* (1994) 181–184.
15. A. H. Millar, J. T. Wiskich, J. Whelan, D. A. Day, *FEBS Lett. 329* (1993) 259–262.
16. A. L. Umbach, J. N. Siedow, *J. Biol. Chem. 271* (1996) 25019–25026.
17. I. Djajanegara, R. Holtzapffel, P. M. Finnegan, M. H. N. Hoefnagel, D. A. Berthold, J. T. Wiskich, D. A. Day, *FEBS Lett. 454* (1999) 220–224.
18. A. L. Umbach, J. N. Siedow, *Arch. Biochem. Biophys. 378* (2000) 234–245.
19. G. N. Telesnina, Yu. E. Bartoshevish, I. N. Krakhmaleva, R. M. Petyushenko, Yu. O. Sazykin, S. M. Navashin, *J. Basic Microbiol. 30* (1990) 273–278.
20. J. Kozma, L. Lucas, K. Schügerl, *Biotechnol. Lett. 13* (1991) 899–900.
21. J. Kozma, L. Lucas, K. Schügerl, *Appl. Microbiol. Biotechnol. 40* (1993) 463–465.
22. J. Kozma, L. Karaffa, *J. Biotechnol. 48* (1996) 59–66.
23. L. Karaffa, E. Sándor, J. Kozma, A. Szentirmai, *Biotechnol. Lett. 18* (1996) 701–706.
24. G. G. Laties, *Arch. Biochem. Biophys. 79* (1959) 364–77.
25. G. Larsson, S. O. Enfors, *Appl. Microbiol. Biotechnol. 21* (1985) 228–233.
26. J. Kozma, L. Karaffa, *Hung. J. Ind. Chem. 24* (1996) 225–227.
27. H. Lambers, *Plant Cell Environ. 3* (1980) 293–302.
28. A. C. Purvis, R. L. Shewfelt, *Physiol. Plant. 88* (1993) 712–718.

29. V. N. Popov, R. A. Simonian, V. P. Skulachev, A. A. Starkov, *FEBS Lett. 415* (1997) 87–90.

30. E. Braidot, E. Petrussa, A. Vianello, F. Macrí, *FEBS Lett. 451* (1999) 347–350.

31. D. P. Maxwell, Y. Wang, L. McIntosh, *Proc. Natl. Acad. Sci. USA, 96* (1999) 8271–8276.

32. L. Karaffa, K. Váczy, E. Sándor, S. Biró, A. Szentirmai, I. Pócsi, *Free Rad. Res. 34* (2001) 405–416.

33. C. H. Pan, E. Mckillip, C. H. Nash, S. V. Speth, *Develop. Ind. Microbiol. 23* (1982) 315–323.

34. V. V. Revin, S. A. Kasatkin, G. N. Cherkasova, V. T. Nikolaev, N. S. Iamashkina, M. N. Chabushkina, O. A. Popova, V. F. Belianina, *Antibiot. Khimioterap. 36* (1991) 5–8.

35. L. Karaffa, E. Sándor, J. Kozma, C. P. Kubicek, A. Szentirmai, *Appl. Microbiol. Biotechnol. 51* (1999) 633–638.

36. A. U. Igamberdiev, V. N. Popov, M. I. Falaeeva, *FEBS Lett. 367* (1995) 287–290.

Reguliranje alternativnog respiratornog puta otpornog na cianid u gljivi *Acremonium chrysogenum*

Sažetak

U radu su prikazane postojeće spoznaje o biokemijskim i fiziološkim procesima koji direktno ili indirektno mijenjaju djelovanje alternativnog respiratornog puta otpornog na cianid u filamentoznoj gljivi *Acremonium chrysogenum* proizvođaču cefalosporina C. Osobito je istaknuta uloga koju ova aktivnost ima pri prekomjernoj proizvodnji antibiotika, kao bitna osnova ovog fermentacijskog postupka.