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Dynamics of energy charge and adenine nucleotides during uncoupling of catabolism and anabolism in *Penicillium ochrochloron*

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ABSTRACT

Filamentous fungi are able to spill energy when exposed to energy excess by uncoupling catabolism from anabolism, e.g. via overflow metabolism. In current study we tested the hypothesis that overflow metabolism is regulated via the energetic status of the hyphae (i.e. energy charge, ATP concentration). This hypothesis was studied in *Penicillium ochrochloron* during the steady state of glucose- or ammonium-limited chemostat cultures as well as during three transient states (i) glucose pulse to a glucose-limited chemostat, (ii) shift from glucose-limited to ammonium-limited conditions in a chemostat, and (iii) ammonium exhaustion in batch culture). Organic acids were excreted under all conditions, even during exponential growth in batch culture as well as under glucose-limited conditions in a chemostat. Partial uncoupling of catabolism and anabolism via overflow metabolism was thus constitutively present. Under all tested conditions, overflow metabolism was independent of the energy charge or the ATP concentration of the hyphae. There was a reciprocal correlation between glucose uptake rate and intracellular adenine nucleotide content. During all transients states a rapid decrease in energy charge and the concentrations of nucleotides was observed shortly after a change in glycolytic flux ("ATP paradoxon"). A possible connection between the change in adenine nucleotide concentrations and the purine salvage pathway is discussed.

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Introduction

When filamentous fungi are exposed to energy excess, they adjust their metabolism like other microorganisms in order to spill energy and to avoid an energetic collapse, i.e. they uncouple catabolism from anabolism (Larsson *et al.* 1995; Teixeira De Mattos & Neijssel 1997; Gallmetzer & Burgstaller

2002). The degree of uncoupling between catabolism and anabolism is an important determinant for the efficiency of energy conversion, as it influences the amount of energy available for a cell. The more catabolism and anabolism are uncoupled, the more the efficiency of energy conversion is lowered: energy, which is theoretically made available by one metabolic process, is not completely used by another

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metabolic process (Larsson *et al.* 1995; Teixeira De Mattos & Neijssel 1997).

The degree of uncoupling has severe consequences on the yield of biomass and biotechnological products. From a biotechnological point of view uncoupling may therefore be either highly desired or exceedingly undesired. Typical biotechnological processes, where microorganisms are forced to a high degree of uncoupling, are for example citric acid production with *Aspergillus niger* or ethanol production with *Saccharomyces cerevisiae*. This is in contrast to biotechnological processes like heterologous protein production or biomass production, where as much as possible of the energy contained in the energy source should be converted into protein or biomass, respectively. In any case, knowledge about the mechanism and control of uncoupling is crucial, as it allows a specific manipulation and optimisation of cultivation conditions or strains.

Filamentous fungi are able to uncouple catabolism from anabolism at different cellular levels:

At the level of substrate-level phosphorylation via futile cycles (also called: substrate cycles). A futile cycle is defined as a cyclic set of enzymatic reactions, which only results in the consumption of ATP, NADH or NADPH (Hue 1982; Jennings & Burke 1990). In filamentous fungi there is evidence that synthesis and catabolism of so-called “physiological buffering agents” like glycerol involve phosphorylation and dephosphorylation (Jennings & Burke 1990).

At the level of oxidative phosphorylation via an “uncoupling” protein in the respiratory chain or via “alternative” respiration. Both processes change the P/O ratio. The presence of “alternative” respiration in filamentous fungi has been frequently reported, e.g. in *A. niger* (Kirimura *et al.* 1987, 1996, 2000; Prömper *et al.* 1993; Zehentgruber *et al.* 1980), in *Aspergillus fumigatus* (Tudella *et al.* 2003), in *Neurospora crassa* (Edwards *et al.* 1974), in *Penicillium ochrochloron* (Gallmetzer & Burgstaller 2002) and in *Ustilago maydis* (Juarez *et al.* 2004).

At the level of metabolism, for example via overflow metabolism (synonyms: incomplete oxidation, aerobic fermentation). Overflow metabolism is the excretion of either intermediary metabolites from primary metabolism or compounds derived from these metabolites. It is commonly suggested, that the main prerequisite for overflow metabolism is (i) a restriction in growth by a nutrient other than the carbon source and (ii) an excess of the carbon source (Foster 1949; Tempest & Neijssel 1984; Gallmetzer & Burgstaller 2002).

The present work focuses on the third mechanism of energy spilling. Uncoupling of catabolism and anabolism in filamentous fungi has been most intensively studied with regard to overflow metabolism, especially citric acid excretion by *A. niger* (e.g. Kubicek 1988a, b; Steinböck *et al.* 1991; Matthey 1992; Kubicek *et al.* 1994; Röhr *et al.* 1996; Karaffa & Kubicek 2003; Legisa & Matthey 2007; Papagianni 2007). Although this topic has been a focus of interest for almost a century, there is still no general and conclusive explanation for the biochemical mechanisms leading to citric acid excretion in filamentous fungi (Sauer *et al.* 2008). This is partly due to the scarce biochemical knowledge compared to well investigated organisms like *S. cerevisiae*

or *Escherichia coli*. Indeed, data derived from these organisms cannot be uncritically transferred to filamentous fungi. Furthermore, most data on overflow metabolism in filamentous fungi are derived from high-yielding citric acid producing strains of *A. niger* and again, the obtained picture cannot always be generalised. Additionally, the factors reported to influence overflow metabolism vary considerably between different strains and cultivation conditions (Kristiansen *et al.* 1982), which is possibly due to differences in cellular organisation and metabolic regulation (Anastassiadis & Rehm 2005).

The intracellular signal, which triggers or impedes overflow metabolism, is still unknown. As overflow metabolism is regarded to be an energy spilling process (Gallmetzer & Burgstaller 2002), it is obvious to suppose a connection between organic acid excretion and the energetic state of the hyphae. Interestingly, in filamentous fungi this relationship has rarely been addressed (Kubicek *et al.* 1980; Leangon *et al.* 2000). Commonly the energy state of a cell is characterized by the energy charge (EC), which was also used for filamentous fungi (Slayman 1973; Kubicek *et al.* 1980; Pitt & Bull 1982; Wynn *et al.* 2001; Nasution *et al.* 2006b). In at least one case it was proven that the activity of a fungal enzyme of the primary metabolism changed as predicted by the energy charge concept (pyruvate carboxylase of *Penicillium camembertii*; Stan 1972).

Therefore the main hypothesis tested in this study was as follows: *Overflow metabolism is an energy spilling process and is thus regulated via the energy status (i.e. EC or ATP concentration). Changes in these parameters lead to respective changes in overflow metabolism as predicted by the energy charge concept, i.e. an increasing energetic state causes an increase in organic acid excretion (in order to spill energy) and vice versa.* We investigated this hypothesis with several transient states in chemostat and bioreactor batch cultures of *P. ochrochloron*, leading to a different degree of uncoupling between catabolism and anabolism and thus to a different degree of overflow metabolism. *P. ochrochloron* excretes organic acids and is resistant to high concentrations of heavy metals (Tuthill *et al.* 2001). It has been used for bioleaching of heavy metals from industrial wastes (Schinner & Burgstaller 1989; Burgstaller *et al.* 1992).

Materials and methods

Organism. *Penicillium ochrochloron* (CBS 123823) was isolated in 1986 from the soil of the copper works Montanwerke Brixlegg (Tyrol, Austria) and in 1989 incorrectly identified as *Penicillium simplicissimum* (Franz *et al.* 1991). A recent reidentification by three independent institutions revealed that this strain is the closely related species *P. ochrochloron* (Vrabl *et al.* 2008).

Preculture. A filamentous growing preculture was produced as described in Gallmetzer *et al.* (1998) using a high buffering capacity and osmolarity in the medium (1 M HEPES) as well as an alkaline pH of 7.3.

Batch and continuous cultivation were performed either in a Biostat M bioreactor (Sartorius/Braun, Melsungen, Germany) or in a KLF 2000 bioreactor (Bioengineering, Wald, Switzerland), both with a working volume of 1.7 L. Equipment, culture conditions and media were as described in Vrabl *et al.* (2008). A steady state was assumed when at least four residence times had passed after the start of continuous cultivation. The

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