Identification of a mitochondrial alcohol dehydrogenase in *Schizosaccharomyces pombe*: new insights into energy metabolism

Paul G. CRICHTON¹, Charles AFFOURTIT¹ and Anthony L. MOORE²

Department of Biochemistry, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, U.K.

In the present study we have shown that mitochondria isolated from *Schizosaccharomyces pombe* exhibit antimycin A-sensitive oxygen uptake activity that is exclusively dependent on ethanol and is inhibited by trifluoroethanol, a potent inhibitor of ADH (alcohol dehydrogenase). Ethanol-dependent respiratory activity has, to our knowledge, not been reported in *S. pombe* mitochondria to date, which is surprising as it has been concluded previously that only one ADH gene, encoding a cytosolic enzyme, occurs in this yeast. Spectrophotometric enzyme assays reveal that ADH activity in isolated mitochondria is increased ~16-fold by Triton X-100, which demonstrates that the enzyme is located in the matrix. Using genetic knockouts, we show conclusively that the novel mitochondrial ADH is encoded by *adh4* and, as

such, is unrelated to ADH isoenzymes found in mitochondria of other yeasts. By performing a modular-kinetic analysis of mitochondrial electron transfer, we furthermore show how ethanol-dependent respiratory activity (which involves oxidation of matrix-located NADH) compares with that observed when succinate or externally added NADH are used as substrates. This analysis reveals distinct kinetic differences between substrates which fully explain the lack of respiratory control generally observed during ethanol oxidation in yeast mitochondria.

Key words: alcohol dehydrogenase, mitochondrion, modular kinetics, NADH dehydrogenase, respiration, *Schizosaccharomyces pombe*, ubiquinone pool.

INTRODUCTION

Over the last two decades, *Schizosaccharomyces pombe* has proved an invaluable model organism to study biological processes that are fundamental to higher eukaryotes. This has greatly improved the understanding of, most notably, mechanisms by which the cell cycle is controlled and damaged DNA is repaired (reviewed in [1]). In our laboratory, *S. pombe* has been used for a different purpose, namely to functionally express the plant alternative oxidase [2]. This cyanide-insensitive mitochondrial respiratory enzyme is wasteful in terms of energy conservation, which is why considerable attention has been paid to the clarification of its physiological function [3]. Functional expression of the protein in *S. pombe* has provided valuable information on structure–function relationships of the oxidase [4–6] and, moreover, has resulted in this yeast becoming a useful model of plant mitochondrial electron transfer [7].

Although *S. pombe* is widely used as a model system, relatively little fundamental information is available on the mitochondrial energy metabolism of this yeast [8,9]. From the few studies published to date, it is clear that the respiratory chain of *S. pombe* contains at least two substrate dehydrogenases (oxidizing succinate and external NADH respectively) that both reduce ubiquinone, which is subsequently oxidized by the cytochrome pathway [8,9].

In addition to NADH and succinate, mitochondria from other yeasts (*Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*, for example) are able to use ethanol as a respiratory substrate [10–13]. Ethanol-dependent respiratory activity is generally attributed to the presence of mitochondrial ADH (alcohol dehydrogenase) isoenzymes [8–13]. In *S. pombe* only one ADH gene has been reported that relates to the mitochondrial ADH genes identified in other yeasts [14]. As this gene is believed

to encode a cytosolic enzyme (see, e.g., [15]), it has been widely assumed that *S. pombe* does not contain a mitochondrial ADH isoenzyme and, as a result, differs from other yeasts in how it metabolizes ethanol [16].

In the present study, we show unequivocally that *S. pombe* mitochondria exhibit ADH activity. Using genetic knockout strains, we confirm that the enzyme responsible is ADH4, the first protein of its type to be shown to play a role in mitochondrial function.

EXPERIMENTAL

Sample preparation

S. pombe cells were batch-cultured in glucose minimal medium [17] supplemented with 0.4 mM adenine, 1.1 mM leucine and 0.7 mM uracil. Mitochondria were isolated as described previously [6] from the following two strains: Sp.011 (*h*[−], *ade6-704*, *leu1-32*, *ura4-D18*) and JY743 carrying gene disruptions (see [18] for details) at either *adh1* (*h*[−], *leu1-32*, *ura4-D18*, *adh1*Δ::*ura4*⁺) or *adh4* (*h*[−], *leu1-32*, *ura4-D18*, *adh4*Δ::*leu1*⁺). Mitochondrial protein amounts were estimated using bicinchoninic acid [19] with BSA as a standard.

Respiratory analysis

Mitochondrial oxygen consumption was measured at room temperature (24–25 °C) using a Clark-type electrode (Rank Brothers). Mitochondria (0.4–1.2 mg) were suspended in 2.4 ml of reaction medium containing 0.65 M mannitol, 20 mM Mops (pH adjusted to 6.8 with NaOH), 1 mM MgCl₂, 5 mM Na₂HPO₄ and 10 mM NaCl (medium A). For modular-kinetic experiments, the reduction level of the Q (ubiquinone) pool was measured

Abbreviations used: ADH, alcohol dehydrogenase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Q(H₂), (reduced) ubiquinone.

¹ Present address: MRC Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2XY, U.K.

² To whom correspondence should be addressed (email a.l.moore@sussex.ac.uk).

Table 1 Oxygen consumption (a) and ADH activity (b) in isolated *S. pombe* mitochondria

(a) Concentration of substrates and effectors were 1.8 mM NADH, 1 μ M CCCP (stock dissolved in propan-2-ol), 0.15 M ethanol, 0.5 mM NAD⁺, 1.7 μ M antimycin A (in propan-2-ol) and 95 mM 2,2,2-trifluoroethanol. (b) ADH activity was measured in the absence or presence of 0.1% (v/v) Triton X-100. All experiments were performed at pH 6.8 and results are means $\pm$ S.D. for three to nine separate mitochondrial preparations.

(a) Oxygen consumption

Substrate	Respiratory rate (nmol of O ₂ · min ⁻¹ · mg ⁻¹)
NADH	67 $\pm$ 13
NADH + CCCP	332 $\pm$ 54
Ethanol	53 $\pm$ 7
Ethanol + NAD ⁺	65 $\pm$ 12
Ethanol + NAD ⁺ + CCCP	88 $\pm$ 8
Ethanol + NAD ⁺ + CCCP + antimycin A	3 $\pm$ 0
Ethanol + NAD ⁺ + 2,2,2-trifluoroethanol	17 $\pm$ 6

(b) ADH activity

Triton X-100	ADH activity (nmol of NADH · min ⁻¹ · mg ⁻¹)
Absent	44 $\pm$ 5
Present	716 $\pm$ 159

simultaneously with respiratory activity using a vessel adapted to accommodate glassy carbon, platinum and Ag/AgCl reference electrodes as described in [20]. For these experiments, 1 μ M Q₁ was included in the reaction medium. Where necessary, Q-reduction measurements were corrected for NADH- or ethanol-induced artefacts by subtracting electrode responses observed in the absence of Q₁.

ADH activity

ADH activity was followed spectrophotometrically as the production of NADH ($\epsilon_{\text{NADH}, 340} = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) using a PerkinElmer Lambda 16 spectrophotometer. Assays were performed with 17–24 μ g of mitochondrial protein at 24–25 °C in medium A (pH 6.8) that included 1.7 μ M antimycin A and 2 mM NAD⁺. Reactions were initiated by adding 100 mM ethanol.

RESULTS**Identification of a mitochondrial ADH**

S. pombe exhibits mitochondrial ADH activity

Mitochondria isolated from *S. pombe* contain an active external NADH dehydrogenase [8,9] that rapidly oxidizes exogenous NADH (Table 1a). This respiratory activity ($\sim 67 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) was stimulated 5-fold by the addition of the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazine), indicating that electron transfer is tightly coupled to proton translocation and, hence, that the mitochondrial inner membranes were relatively intact. Interestingly, when ethanol was added to *S. pombe* mitochondria, electron-transfer rates were achieved that are comparable with those observed with NADH (Table 1a). This ethanol-dependent oxygen uptake activity was marginally increased by the addition of NAD⁺ (Table 1a) and appears to depend on the integrity of the mitochondrial inner membrane, since it was significantly reduced in osmotically shocked mitochondria (results not shown). NAD⁺-stimulated ethanol-dependent respiration rates, however, were similar in intact and disrupted mitochondria, implying that ethanol-dependent respiratory activity requires matrix NAD⁺. NAD⁺-stimulated

ethanol-dependent oxygen uptake was stimulated ~ 1.4 fold by CCCP (Table 1a).

Ethanol-dependent respiratory activity is sensitive to antimycin A ($\sim 95\%$ inhibition) and 2,2,2-trifluoroethanol ($\sim 74\%$ inhibition) (Table 1a), specific inhibitors of mitochondrial complex III [21] and ADH [22] respectively. These observations strongly suggest that ethanol-dependent oxygen consumption in *S. pombe* mitochondria, similar to other yeasts (see, e.g., [10–13]), is accounted for by a matrix-located ADH that oxidizes ethanol resulting in reduction of endogenous NAD⁺, which is subsequently re-oxidized by the respiratory chain. In *Saccharomyces cerevisiae*, the acetaldehyde produced during this reaction can also be oxidized, which results in additional formation of NADH [23]. This appears not to occur in *S. pombe* mitochondria, as acetaldehyde inhibits ethanol-dependent oxygen consumption ($\sim 98\%$ at 8 mM) and does not sustain a respiratory activity when added separately (results not shown).

The presence of a matrix-located ADH in *S. pombe* is confirmed unambiguously by the spectrophotometric data presented in Table 1(b), which show that oxidation of ethanol can be increased approx. 16-fold by the addition of the detergent Triton X-100. This result clearly demonstrates that the major part of ADH activity in our mitochondrial sample is latent and becomes apparent only when membranes are disrupted by detergent. Such latency has been observed in other yeasts (see, e.g., [10,24]) and implies that nearly all NADH formed in the assay lacking Triton X-100 is shielded from detection by a membrane. This membrane is undoubtedly mitochondrial, since ADH activity is linked to antimycin A-sensitive oxygen consumption (Table 1a). Given that only the mitochondrial inner membrane and not the outer membrane is impermeable to NADH [13], it can be concluded that the observed ethanol-dependent respiratory activity in *S. pombe* mitochondria involves a matrix-located ADH.

The molecular nature of mitochondrial ADH in *S. pombe*

The presence of a mitochondrial ADH in *S. pombe* is somewhat surprising given that only one ADH gene (*adh1*), encoding a cytoplasmic protein, has been reported to date [14]. Using *adh1* as a probe, Southern blot analysis did not reveal any homologous sequences in *S. pombe* [14], which is in contrast with other yeasts where multiple *adh1*-like genes are usually present [15]. Recently, however, a protein has been identified in *S. pombe* [18] that is unrelated to ADH1, but similar to ADH isoenzymes from the fermentative bacterium *Zymomonas mobilis* (ADH2) and *Saccharomyces cerevisiae* (ADH4) [25,26]. Using algorithms described in [27–29], we predict that, unlike ADH1, this novel ADH protein (now termed ADH4) is targeted to mitochondria, and may therefore account for the mitochondrial ADH activity reported in the present paper. To test this notion, we measured respiratory activity in mitochondria isolated from *S. pombe* cell lines in which either ADH1 or ADH4 was ablated ($\Delta adh1$ and $\Delta adh4$ respectively).

From the data presented in Table 2, it is clear that mitochondria isolated from $\Delta adh1$ cells exhibit ethanol oxidation rates ($60 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) that are comparable with wild-type activities (cf. Table 1). In striking contrast, no ethanol-dependent activity was detectable in mitochondria isolated from the $\Delta adh4$ strain. This clear difference between $\Delta adh1$ and $\Delta adh4$ mitochondria confirms that the observed mitochondrial ADH activity in *S. pombe* can be attributed entirely to ADH4. Although quantitatively somewhat different from Sp.011, it is clear that mitochondria from JY743 cell lines oxidize NADH at appreciable rates that are stimulated significantly by CCCP (Table 2), confirming the respiratory competence of both the $\Delta adh1$ and $\Delta adh4$ strains.

Table 2 Ethanol-dependent respiratory activity in mitochondria from $\Delta adh1$ and $\Delta adh4$ mutant strains of *S. pombe*

Concentration of substrates and effectors were 0.15 M ethanol, 1 μ M CCCP (stock dissolved in propan-2-ol) and 1.8 mM NADH. All experiments were performed at pH 6.8 and results are means $\pm$ S.D. for three separate mitochondrial preparations.

Substrate	Respiratory rate (nmol of O ₂ · min ⁻¹ · mg ⁻¹)	
	$\Delta adh1$	$\Delta adh4$
Ethanol	60 $\pm$ 12	0 $\pm$ 0
Ethanol + CCCP	59 $\pm$ 16	—
NADH	94 $\pm$ 24	45 $\pm$ 12
NADH + CCCP	221 $\pm$ 31	135 $\pm$ 48

Modular kinetic analysis of *S. pombe* respiration

Respiratory control

The data shown in Table 1(a) reveal that mitochondrial ethanol-dependent oxygen consumption in *S. pombe* is not increased significantly by CCCP. This relative lack of respiratory control during ethanol oxidation has been observed previously in other yeast mitochondria, and has been attributed to a relatively high degree of proton leak in the presence of this respiratory substrate [30]. However, the coupled rates of ethanol oxidation are comparable with the equivalent rates observed with NADH, which would suggest that the proton conductivity of the mitochondrial inner membrane in *S. pombe* is not affected significantly by 1% (v/v) ethanol.

From studies with plant and yeast mitochondria, it has become clear that respiratory control ratios are substrate-dependent, since they reflect the kinetic interaction between several components of the respiratory system, including substrate dehydrogenases [31,32]. For example, oxygen consumption by *S. pombe* mitochondria is stimulated by CCCP more strongly when NADH is used as a substrate than when succinate is employed [7]. To assess whether the lack of respiratory control during ethanol oxidation could be explained in a similar manner, we performed a modular kinetic analysis to gain more detailed insights in the kinetics of the respiratory system in *S. pombe* mitochondria oxidizing succinate, ethanol or external NADH. In this analysis, we simplified the respiratory system conceptually to comprise modules that either reduce or oxidize a common Q pool (see [31] for details).

Oxidation of succinate

From the simultaneous oxygen uptake and Q-reduction measurements shown in Figure 1, it can be seen that succinate is oxidized in *S. pombe* mitochondria at ~ 28 nmol · min⁻¹ · mg⁻¹, with a concomitant Q-reduction level of $\sim 34\%$ (Figure 1A). Subsequent sequential addition of ATP and glutamate leads to increases in both respiratory activity and Q reduction, since both compounds activate succinate dehydrogenase [7,9]. Valuable information may be gained when the respective steady-state respiratory rates are plotted as a function of Q reduction (Figure 1B). When interpreting such a Q-kinetic plot, it is important to realize that respiratory steady states result from the interplay between Q-reducing (in this case succinate dehydrogenase with the dicarboxylate carrier) and QH₂ (reduced ubiquinone)-oxidizing (cytochrome pathway) modules. Since ATP and glutamate specifically affect Q reduction, a single model representing coupled QH₂-oxidation kinetics should suffice to describe the steady states shown. Indeed, the data in Figure 1(B) are fitted

Figure 1 The kinetic dependency of succinate-dependent oxygen consumption activity on the reduction level of the Q pool

(A) Typical oxygen consumption (solid line) and Q-reduction (broken line) traces illustrate the stimulatory effect of ATP (0.2 mM) and glutamate (9 mM) on the oxidation of succinate (9 mM). The numbers adjacent to the traces represent specific O₂-uptake rates (nmol · min⁻¹ · mg⁻¹) and Q-reduction levels (fraction QH₂ in total Q pool). (B) Steady-state respiratory rates obtained with succinate alone (○) or in the cumulative presence of ATP (□) and glutamate (Δ) are shown as a function of the Q-redox poise. Results are means $\pm$ S.D. for three independent mitochondrial preparations and were modelled with hyperbolic equations as described in [33].

readily to a single hyperbolic equation (cf. [33]) that accounts for the theoretically inevitable fact that cytochrome pathway activity is zero when the Q pool is fully oxidized.

Oxidation of ethanol

Ethanol-dependent respiratory rates are plotted as a function of Q reduction in Figure 2(A), both in the absence and the cumulative presence of ADP and CCCP. Under coupled conditions, oxygen is consumed at ~ 52 nmol · min⁻¹ · mg⁻¹ at a Q-redox poise of $\sim 78\%$. Importantly, this steady state is modelled well by the curve that describes cytochrome pathway activity (Figure 2A, curve 1). This implies that ethanol does not alter the kinetics of QH₂ oxidation under coupled conditions, indicating that it affects neither the components of the cytochrome pathway nor the integrity of the mitochondrial inner membrane. The rate of ethanol oxidation is increased only marginally by ADP and CCCP (to ~ 58 and 61 nmol of O₂ · min⁻¹ · mg⁻¹ respectively), whereas sequential addition of these compounds decreases the Q-redox poise to 71 and 43%. Obviously, the resulting respiratory steady states do not fall on the curve describing coupled QH₂-oxidation kinetics

Figure 2 Kinetic interplay between QH_2 -oxidizing and Q-reducing pathways in *S. pombe* mitochondria

Respiratory steady states were achieved upon the oxidation of ethanol (0.15 M, closed symbols) or NADH (1.8 mM, open symbols) in the absence (diamonds) or the cumulative presence of ADP (0.1 mM, circles) and CCCP (1 μM , triangles). Results are means $\pm$ S.D. for two to three separate mitochondrial preparations and were modelled with hyperbolic equations as described in [33]. Curves representing QH_2 -oxidizing kinetics under coupled conditions (labelled '1.' in **A** and **C**) are identical with the one presented in Figure 1(B). Descriptions of these kinetics in the presence of ADP and CCCP (labelled '2.' and '3.' respectively in **C**) are based on the data shown in **(B)** (circles and triangles respectively) and include the origin, since cytochrome pathway kinetics with respect to the Q-redox poise should be substrate-independent and zero when the Q pool is fully oxidized. The intersects of curves reflecting the kinetics of Q-reducing (labelled NADH and ethanol) and QH_2 -oxidizing modules (**C**) indicate steady states attained during the oxidation of ethanol (lower case letters) or NADH (upper case letters) in the absence (a and A) or the cumulative presence of ADP (b and B) and CCCP (c and C).

(Figure 2A), since control by the protonmotive force is relieved by ADP and CCCP. During ethanol-dependent respiration, the Q-reducing module comprises mitochondrial ADH and a substrate

dehydrogenase that oxidizes matrix NADH. Since *S. pombe* does not contain a protonmotive complex I [34], it is unlikely that activity of this internal NADH dehydrogenase is affected by ADP or CCCP. It is therefore reasonable to expect that all steady states shown in Figure 2(A) are describable by a single Q-reduction model, which indeed appears to be the case (Figure 2A, ethanol curve). Note that this model accounts for the theoretical inevitability that Q-reducing activity is zero when the Q pool is fully reduced.

Oxidation of external NADH

In Figure 2(B) Q-reduction kinetics during ethanol oxidation are compared directly with those observed during oxidation of externally added NADH. In the latter case, a Q-reduction level of $\sim 95\%$ is achieved, allowing a respiratory activity of $\sim 65 \text{ nmol of O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (see also Table 1a). This activity is stimulated significantly by ADP (1.5-fold) and CCCP (5-fold), whereas Q reduction is hardly affected by either compound (Figure 2B). This shows that the external NADH dehydrogenase exhibits a rather high elasticity towards the reduction level of the Q pool. In contrast, Q-reduction kinetics during ethanol oxidation are such that activity ($\sim 50\text{--}60 \text{ nmol of O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) is almost fully independent of the Q-redox poise.

Apparent lack of respiratory control during ethanol oxidation is due to kinetic characteristics of the Q-reduction module

Differences in the behaviour of Q-reducing modules cause clear system-kinetic differences between *S. pombe* mitochondria oxidizing either NADH or ethanol. In Figure 2(C), modelled data are shown that reflect both Q-reduction kinetics and cytochrome pathway behaviour under various energetic conditions. It is evident that lowering the protonmotive force with ADP or CCCP predominantly affects the oxygen-consumption rate during NADH oxidation, whereas it has an exclusive effect on the Q-redox poise during ethanol oxidation (Figure 2C, upper and lower case letters respectively). These results explain clearly why NADH-dependent respiratory activity is stimulated considerably by CCCP, whereas ethanol-dependent activity is not (Table 1a).

DISCUSSION

In the present study, we have demonstrated that the fission yeast *S. pombe* expresses a functional ADH isoenzyme that is specifically located in the mitochondrial matrix (Table 1), and, using genetic knockout strains, we have identified the gene responsible (Table 2). This gene (*adh4*) encodes a novel ADH protein that is not homologous with any of the mitochondrial (ADH1-like) isoenzymes found in other yeasts, which would explain why it has not been discovered before. In this respect, our results are of significant interest as they convincingly challenge the apparent general acceptance in the literature that *S. pombe* does not contain a mitochondrial ADH. An exception to this general notion is a study by Tsai et al. [35], who reported gel-electrophoretic data that would indeed suggest the occurrence of a mitochondrial ADH in *S. pombe*. Although the results of these authors have often been overlooked, probably because confirmation of the subcellular location of the various isoenzymes is lacking [35], they are clearly in agreement with our observations.

As is the case in other yeasts [10–13], mitochondrial ADH activity sustained considerable oxygen consumption when *S. pombe* mitochondria were incubated in the exclusive presence of ethanol (Table 1). This indicates that ADH reduces matrix

NAD⁺, which is subsequently re-oxidized by the respiratory chain. Although perhaps not surprising, our data therefore confirm that the mitochondrial electron-transfer chain of *S. pombe* contains an active internal NADH dehydrogenase that couples oxidation of matrix NADH to reduction of ubiquinone. Importantly, however, it turns out that electron transfer is controlled differently during NADH- and ethanol-dependent respiration, such that control is exerted predominantly by QH₂ oxidation when NADH is used as substrate, and by Q reduction when ethanol is employed (Figure 2C). The apparent lack of respiratory control (Tables 1 and 2), often attributed to detrimental effects of ethanol on the integrity of mitochondrial membranes [30], can be explained fully by the particular distribution of control during ethanol oxidation.

In general, the physiological function of yeast mitochondrial ADH enzymes is unclear. It has been proposed, however, that they form an ethanol–acetaldehyde shuttle with their respective cytosolic counterparts, which facilitates equilibration of cytoplasmic and mitochondrial NAD⁺/NADH ratios [13]. In *Saccharomyces cerevisiae*, for example, this is thought to be of particular relevance during anaerobic growth, when the NADH produced in the matrix by assimilatory reactions cannot be oxidized by the respiratory chain. *Saccharomyces cerevisiae* strains lacking mitochondrial ADH3 display impaired anaerobic growth [10], which would agree with this notion. As *S. pombe* cells lacking ADH4 also display reduced rates of anaerobic growth [18], it is conceivable that ADH4 fulfils a function in this system similar to that of ADH3 in *Saccharomyces cerevisiae*.

S. pombe ADH4 does not show significant amino acid sequence homology with ADH1 or any other member of the ‘group I-type’ NAD(P)⁺-dependent ADH enzyme family (as classified in [15]). Instead, it shares considerable sequence identity with ADH4 from *Saccharomyces cerevisiae* (56%) and ADH2 from *Zymomonas mobilis* (52%), both of which belong to the ‘group III-type’ iron-activated, NAD(P)⁺-dependent ADH enzyme family [25,26]. To our knowledge, *S. pombe* ADH4 is the first group III-type protein that has been shown to play a role in mitochondrial metabolism. This raises the possibility that these enzymes also function in mitochondria of other species. Interestingly, in this respect, an *S. pombe* ADH4 homologue has recently also been discovered in humans, where it is expressed during foetal development as well as in adult liver [36]. Inspection of the amino acid sequence of this enzyme reveals the presence of an ~40-amino-acid N-terminal extension that we predict, using the algorithms published in [27–29], to target the protein to mitochondria.

To summarize, we have identified a novel mitochondrial ADH enzyme in *S. pombe* and have demonstrated in detail how the activity of this enzyme is linked to *in vitro* respiration. With this information, we believe that we have provided valuable new insights into the energy metabolism of this widely used model organism.

We express our gratitude to Professor Yuko Giga-hama (Asahi Glass Co., Tokyo, Japan) for providing the $\Delta adh1$ and $\Delta adh4$ strains of *S. pombe*. P.G.C. thanks the School of Life Sciences, University of Sussex, for postgraduate funding.

REFERENCES

- Carr, A. M. (2002) DNA structure dependent checkpoints as regulators of DNA repair. *DNA Repair* **1**, 983–994
- Albury, M. S., Dudley, P., Watts, F. Z. and Moore, A. L. (1996) Targeting the plant alternative oxidase protein to *Schizosaccharomyces pombe* mitochondria confers cyanide-insensitive respiration. *J. Biol. Chem.* **271**, 17062–17066
- Moore, A. L., Albury, M. S., Crichton, P. G. and Affourtit, C. (2002) Function of the alternative oxidase: is it still a scavenger? *Trends Plant Sci.* **7**, 478–481
- Albury, M. S., Affourtit, C., Crichton, P. G. and Moore, A. L. (2002) Structure of the plant alternative oxidase: site-directed mutagenesis provides new information on the active site and membrane topology. *J. Biol. Chem.* **277**, 1190–1194
- Albury, M. S., Affourtit, C. and Moore, A. L. (1998) A highly conserved glutamate residue (Glu-270) is essential for alternative oxidase activity. *J. Biol. Chem.* **273**, 30301–30305
- Crichton, P. G., Affourtit, C., Albury, M. S., Carré, J. E. and Moore, A. L. (2004) Constitutive activity of *Sauromatum guttatum* alternative oxidase in *Schizosaccharomyces pombe* implicates residues other than conserved cysteines in α -keto acid activation. *FEBS Lett.* **579**, 331–336
- Affourtit, C., Albury, M. S., Krab, K. and Moore, A. L. (1999) Functional expression of the plant alternative oxidase affects growth of the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **274**, 6212–6218
- Jault, J. M., Comte, J., Gautheron, D. C. and Di Pietro, A. (1994) Preparation of highly phosphorylating mitochondria from the yeast *Schizosaccharomyces pombe*. *J. Bioenerg. Biomembr.* **26**, 447–456
- Moore, A. L., Walters, A. J., Thorpe, J., Fricaud, A. C. and Watts, F. Z. (1992) *Schizosaccharomyces pombe* mitochondria: morphological, respiratory and protein import characteristics. *Yeast* **8**, 923–933
- Bakker, B. M., Bro, C., Kötter, P., Luttk, M. A. H., van Dijken, J. P. and Pronk, J. T. (2000) The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*. *J. Bacteriol.* **182**, 4730–4737
- Luttk, M. A. H., Overkamp, K. M., Kötter, P., de Vries, S., van Dijken, J. P. and Pronk, J. T. (1998) The *Saccharomyces cerevisiae* NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. *J. Biol. Chem.* **273**, 24529–24534
- Overkamp, K. M., Bakker, B. M., Kötter, P., van Tuijl, A., de Vries, S., van Dijken, J. P. and Pronk, J. T. (2000) *In vivo* analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria. *J. Bacteriol.* **182**, 2823–2830
- von Jagow, G. and Klingenberg, M. (1970) Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. *Eur. J. Biochem.* **12**, 583–592
- Russell, P. R. and Hall, B. D. (1983) The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **258**, 143–149
- Reid, M. F. and Fewson, C. A. (1994) Molecular characterization of microbial alcohol dehydrogenases. *Crit. Rev. Microbiol.* **20**, 13–56
- Shain, D. H., Salvatore, C. and Denis, C. L. (1992) Evolution of the alcohol dehydrogenase (*ADH*) genes in yeast: characterization of a 4th *ADH* in *Kluyveromyces fragilis*. *Mol. Gen. Genet.* **232**, 479–488
- Murray, J. M., Carr, A. M., Lehmann, A. R. and Watts, F. Z. (1991) Cloning and characterization of the *rad9* DNA-repair gene from *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **19**, 3525–3531
- Sakurai, M., Tohda, H., Kumagai, H. and Giga-Hama, Y. (2004) A distinct type of alcohol dehydrogenase, *adh4⁺*, complements ethanol fermentation in an *adh1*-deficient strain of *Schizosaccharomyces pombe*. *FEMS Yeast Res.* **4**, 649–654
- 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) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85
- Moore, A. L., Dry, I. B. and Wiskich, J. T. (1988) Measurement of the redox state of the ubiquinone pool in plant mitochondria. *FEBS Lett.* **235**, 76–80
- Nicholls, D. G. and Ferguson, S. J. (2002) Respiratory chains. In *Bioenergetics* 3, p. 118, Academic Press, London
- Dickenson, C. J. and Dickinson, F. M. (1978) Inhibition by ethanol, acetaldehyde and trifluoroethanol of reactions catalysed by yeast and horse liver alcohol dehydrogenases. *Biochem. J.* **171**, 613–627
- Boubekur, S., Camougrand, N., Bunoust, O., Rigoulet, M. and Guerin, B. (2001) Participation of acetaldehyde dehydrogenases in ethanol and pyruvate metabolism of the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **268**, 5057–5065
- Young, E. T., Sloan, J., Miller, B., Li, N., van Riper, K. and Dombek, K. M. (2000) Evolution of a glucose-regulated ADH gene in the genus *Saccharomyces*. *Gene* **245**, 299–309
- Conway, T., Sewell, G. W., Osman, Y. A. and Ingram, L. O. (1987) Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. *J. Bacteriol.* **169**, 2591–2597
- Williamson, V. M. and Paquin, C. E. (1987) Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. *Mol. Gen. Genet.* **209**, 374–381
- Bannai, H., Tamada, Y., Maruyama, O., Nakai, K. and Miyano, S. (2002) Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* **18**, 298–305

- 28 Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localisation of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005–1016
- 29 Claros, M. G. and Vincens, P. (1996) Computational methods to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.* **241**, 779–786
- 30 Bakker, B. M., Overkamp, K. M., van Maris, A. J. A., Kötter, P., Luttik, M. A. H., van Dijken, J. P. and Pronk, J. T. (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **25**, 15–37
- 31 Affourtit, C., Krab, K. and Moore, A. L. (2001) Control of plant mitochondrial respiration. *Biochim. Biophys. Acta* **1504**, 59–70
- 32 Krab, K. (1995) Kinetic and regulatory aspects of the function of the alternative oxidase in plant respiration. *J. Bioenerg. Biomembr.* **27**, 387–396
- 33 Van den Bergen, C. W. M., Wagner, A. M., Krab, K. and Moore, A. L. (1994) The relationship between electron flux and the redox poise of the quinone pool in plant mitochondria: interplay between quinol-oxidizing and quinone-reducing pathways. *Eur. J. Biochem.* **226**, 1071–1078
- 34 Wood, V., Gwilliam, R., Rajandream, M. A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S. et al. (2002) The genome sequence of *Schizosaccharomyces pombe*. *Nature* **415**, 871–880
- 35 Tsai, C. S., Shi, J. L., Beehler, B. W. and Beck, B. (1992) Enzyme-activities of D-glucose metabolism in the fission yeast *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **38**, 1313–1319
- 36 Deng, Y., Wang, Z., Gu, S., Ji, C., Ying, K., Xie, Y. and Mao, Y. (2002) Cloning and characterization of a novel human alcohol dehydrogenase gene (ADHFe1). *DNA Seq.* **13**, 301–306

Received 2 August 2006/13 September 2006; accepted 25 September 2006

Published as BJ Immediate Publication 25 September 2006, doi:10.1042/BJ20061181