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Review

Fungal respiration: a fusion of standard and alternative components

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Abstract

In animals, electron transfer from NADH to molecular oxygen proceeds via large respiratory complexes in a linear respiratory chain. In contrast, most fungi utilise branched respiratory chains. These consist of alternative NADH dehydrogenases, which catalyse rotenone insensitive oxidation of matrix NADH or enable cytoplasmic NADH to be used directly. Many also contain an alternative oxidase that probably accepts electrons directly from ubiquinol. A few fungi lack Complex I. Although the alternative components are non-energy conserving, their organisation within the fungal electron transfer chain ensures that the transfer of electrons from NADH to molecular oxygen is generally coupled to proton translocation through at least one site. The alternative oxidase enables respiration to continue in the presence of inhibitors for ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase. This may be particularly important for fungal pathogens, since host defence mechanisms often involve nitric oxide, which, whilst being a potent inhibitor of cytochrome *c* oxidase, has no inhibitory effect on alternative oxidase. Alternative NADH dehydrogenases may avoid the active oxygen production associated with Complex I. The expression and activity regulation of alternative components responds to factors ranging from oxidative stress to the stage of fungal development.

Keywords: Mitochondria; Fungus; Mitochondrial electron transport; NAD(P)H:ubiquinone oxidoreductase; Alternative oxidase; Protonmotive force

1. Introduction

The animal paradigm for respiration involves interaction of three large protein complexes: NADH:ubiquinone oxidoreductase (Complex I), ubiquinol:cytochrome *c* oxidoreductase (Complex III) and cytochrome *c* oxidase (Complex IV). In each complex electron transport is coupled to proton translocation, with the resultant protonmotive force (pmf) being used for ATP synthesis and transport of metabolites. Each complex has characteristic inhibitors, e.g. rotenone and piericidin (Complex I), antimycin and myxothiazol (Complex III) and cyanide (Complex IV). Electrons from succinate reach ubiquinone without coupled proton transfer (Complex II).

This pathway provides a fixed route for passage of electrons from NADH to molecular oxygen. In plants it has been known for many years that there are alternative NADH dehydrogenases and an alternative oxidase, each associated with lower efficiency

Abbreviations: AOX, alternative oxidase; COX, cytochrome *c* oxidase; $\Delta\Psi$, membrane potential; EPR, electron paramagnetic resonance; NO, nitric oxide; pmf, protonmotive force; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid

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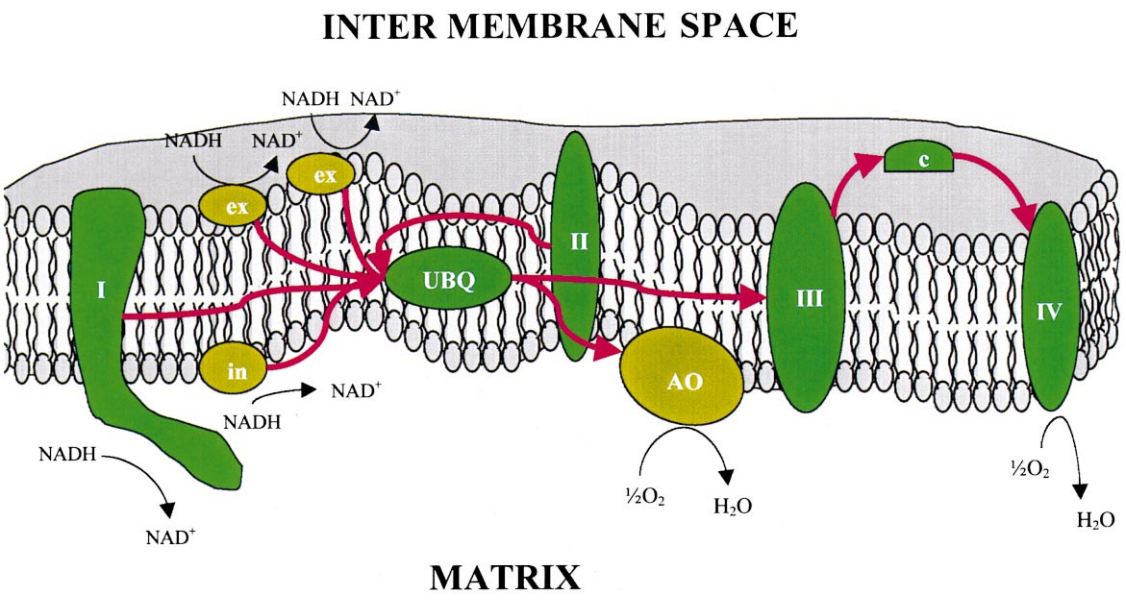

Fig. 1. Composite scheme of fungal mitochondrial electron transport. I, II, III, IV, core electron transport complexes; ex, external NADH:ubiquinone oxidoreductase; in, internal NADH:ubiquinone oxidoreductase; UBQ, ubiquinone:ubiquinol pool; c, peripheral cytochrome *c*. Arrows indicate direction of electron flow.

for proton pumping. These respectively bypass Complex I, and Complexes III plus IV, but ubiquinone is always involved. However, until recently there were few comparable studies with fungi. New technologies enable researchers to explore biochemical, bioenergetic and molecular mechanisms of this fascinating cellular process. The advances have been particularly marked for filamentous plant pathogenic fungi, which have historically proved difficult to study.

The primary aim of this review is to focus on the non-standard aspects of electron transport in fungi, although attention will be given to recent significant advances in understanding of 'core' respiratory components. This will enable comparisons to be made both with other forms of life and within the fungal kingdom. The integration of standard and alternative components is shown in Fig. 1.

2. The standard respiratory complexes in fungi

2.1. Complex I

Complex I couples the transfer of electrons from NADH to ubiquinone with translocation of protons across the inner mitochondrial membrane, for which a stoichiometry of 4 H^+ per 2 e^- is now generally accepted (for example, [1–3]). In fungi, Complex I has been studied in most depth in *Neurospora crassa*, including determination of its structure, while much is also known for *Aspergillus niger*. It seems that Complex I in fungi is very similar to its counterpart in other forms of life. The number of subunits is at least 35, of which seven are encoded by the mitochondrial genome [4]. As in other eucaryotes, it forms an L-shaped structure with the redox groups associated with NADH oxidation (FMN, FeS centres) located within the peripheral arm [5,6], while subunits associated with proton translocation and ubiquinone are within the hydrophobic membrane region (Fig. 2) [7,8]. By pulse labelling *N. crassa* with radiolabelled amino acids, evidence has been obtained for separate assembly of the peripheral and hydrophobic arms, prior to the in situ formation of the complete complex [9].

The mechanism of coupling to proton translocation deserves brief comment, because the semiquinone state of ubiquinone is implicated in active oxygen production (see below). Two or three independent ubiquinone reduction sites (Q-sites) have been identified by inhibitor studies, while two different ubisemiquinones, identified by electron paramagnetic resonance (EPR) spectroscopy, are formed during steady-state turnover. Perhaps the strongest

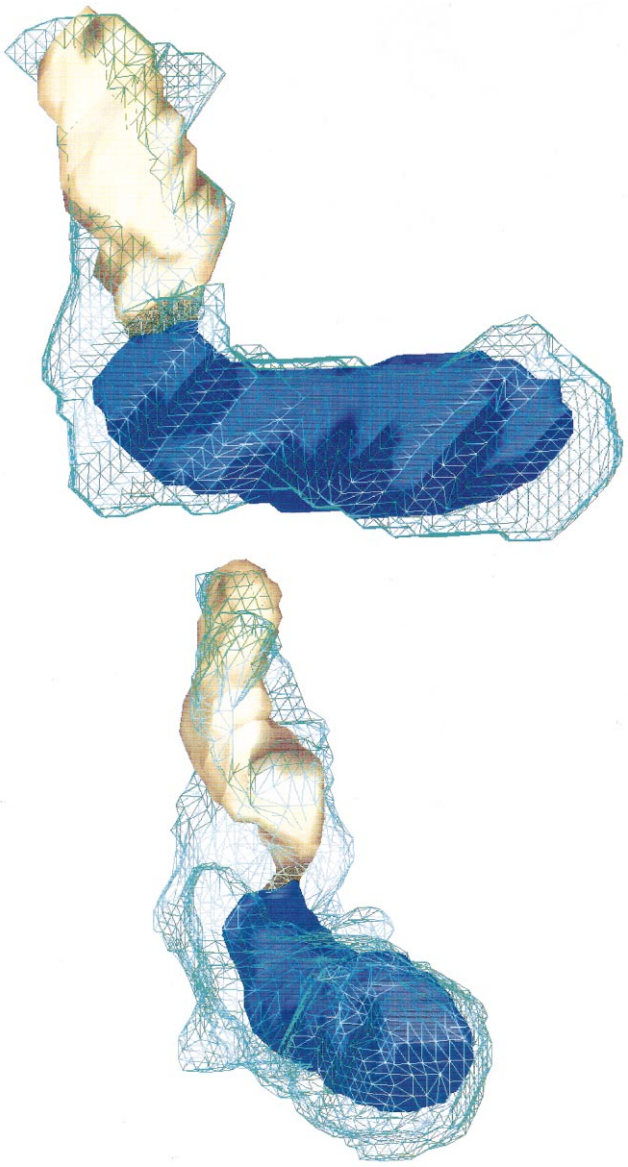

Fig. 2. Three-dimensional models of complex I from *N. crassa* and *Escherichia coli*. Grey wire model, *N. crassa*; solid model, *E. coli*. Lengths of peripheral and membrane arms are approx. 25 nm. Figure reproduced courtesy of Dr T. Friedrich [11].

mechanistic candidate for proton translocation is a bifurcated reaction, similar to that observed within Complex III [5,10]. However, this has yet to be experimentally validated.

In mitochondria from *N. crassa*, a recent spectrophotometric study detected a novel redox group, probably in the membrane arm of Complex I [11]. The experimental sample was prepared by adding NADH in excess over dioxygen. After reduction was complete, the remaining NADH was oxidised by addition of lactate dehydrogenase (LDH) and pyruvate. Relative to an untreated reference, the spectrum showed an absorbance peak at 295 nm and broad trough centred at 425 nm. EPR analysis of the LDH/pyruvate reoxidised sample established that the N2 FeS cluster was about 50% reduced, while the other FeS clusters were all fully oxidised. The chromophore was apparently unique to Complex I of *N. crassa* [11].

Complex I has been found in nearly all of the fungi tested. However, it is notably absent from *Saccharomyces cerevisiae* (baker's yeast) [12]. Two other fungi without Complex I are *Saccharomyces carlsbergii* and *Kluyveromyces lactis*. The implications of this unexpected absence are discussed below.

2.2. Complex II

The oxidation of succinate to fumarate has a higher redox potential than most other couples of standard metabolism, and has its own mechanism of coupling to ubiquinone reduction via Complex II. Although Complex II spans the membrane, it does not translocate protons. The prosthetic groups are flavin (FAD), three FeS clusters (2Fe-2S, 3Fe-4S and 4Fe-4S) and *b*-type haem. The role of cytochrome *b* is poorly characterised, since it is not readily reducible by succinate in bovine mitochondria and is not required for reduction of artificial electron acceptors. Complex II comprises four polypeptides, which in yeast are encoded by the nuclear genes *SDH1*, *SDH2*, *SDH3* and *SDH4* [13–16]. The main catalytic groups (FAD and FeS) are located in Sdh1p and Sdh2p [15,17]. The other two peptides provide membrane anchoring and include sites for quinone binding, as well as ligands for the *b*-type haem [17]. Interestingly, whilst the catalytic peptides retain high homology at the primary amino acid level throughout mitochondria and bacteria, considerable variation is seen within the smaller membrane anchoring peptides. Until recently, it was proposed that SDH of *S. cerevisiae* contained no *b*-type cytochrome; however, a recent study by Oyedotun and Lemire demonstrated the presence of an amount of cytochrome b_{562} stoichiometric to covalent FAD (0.92 ± 0.11 mol/mol) [18]. Detection was achieved by difference spectroscopy of fumarate-oxidised, dithionite reduced mi-

tochondria. Additional support for the presence of this *b*-type cytochrome was obtained from SDH3 and SDH4 deletion mutants, which lacked the corresponding absorbance peak at 562 nm [18].

2.3. Complex III

Complex III (bc_1 complex; ubiquinol:cytochrome *c* oxidoreductase) is the best characterised of the major respiratory components and is found in mitochondria from a wide range of sources. The overall mechanism shows remarkable conservation, not only in mitochondria but also in procaryotes, enabling functional comparisons to be made (for example, [19,20]). For fungi, the first high-resolution structure was recently obtained for Complex III co-crystallised with a bound antibody Fv fragment (Fig. 3; *S. cerevisiae*) [19], although low resolution 2D crystals were obtained earlier for *N. crassa* [21]. The *S. cerevisiae* bc_1 complex comprises ten subunits, of which only the cytochrome *b* peptide is mitochondrially encoded [22–24]. Import into the mitochondria is directed by targeting sequences, usually within the first 30 amino acids of the N-terminus. Although no consensus sequences have been identified, each targeting sequence is composed predominately of hydrophobic residues with between one and three charged residues able to form an α-helix, which is generally cleaved upon import [25].

In yeast, as with all other bc_1 complexes examined to date, four redox centres are involved in energy conservation. These are located within three subunits: cytochrome *b*, a membrane anchored FeS protein (ISP) and cytochrome c_1 (also membrane anchored). The cytochrome *b* peptide comprises eight transmembrane helices with the two haem groups, b_H and b_L, located between helices B and D. Surrounding the metalloprotein nucleus of the complex are the remainder of the subunits, which include two core proteins which face the matrix, COR1 and QCR2 (Fig. 3). These two subunits show homology with

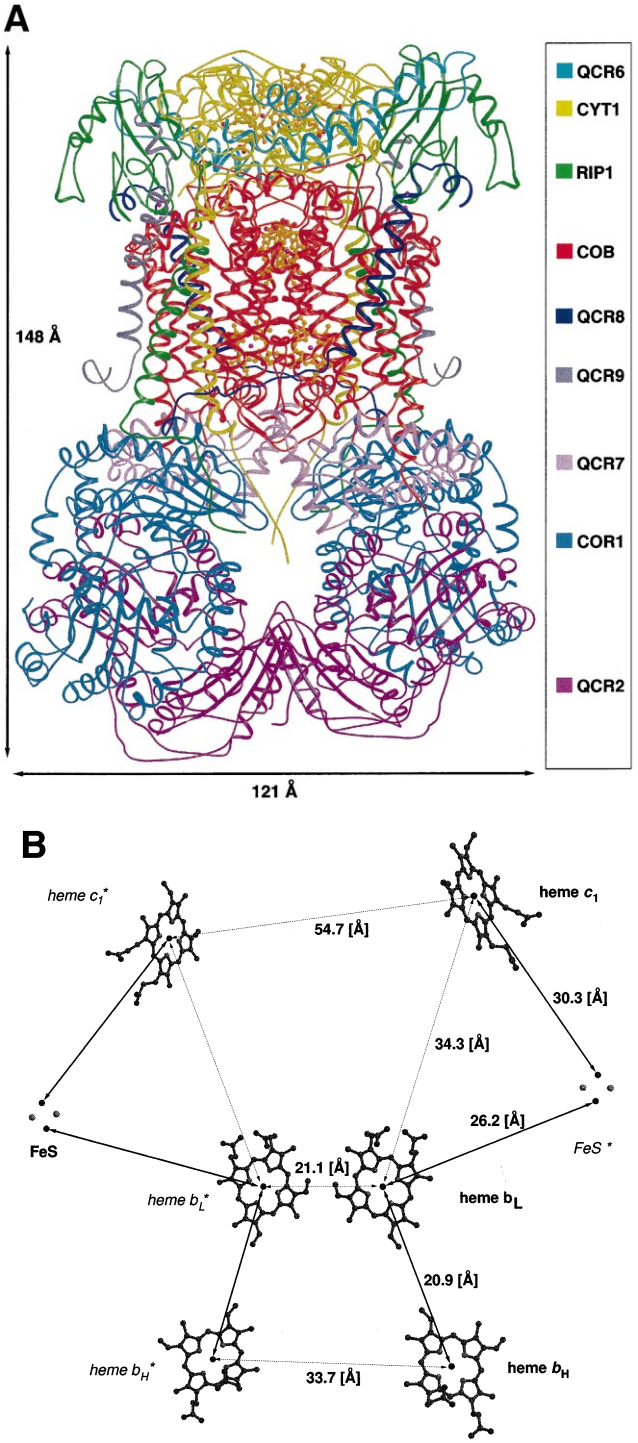

Fig. 3. Structural model of the dimeric bc_1 complex from *S. cerevisiae*. (A) The dimer is viewed parallel to the membrane, with the matrix at the bottom. Peptides are depicted as ribbons with each of the nine subunits colour coded as indicated. Relative positions of the redox centres and co-factors are depicted in the same orientation as for the ribbon model. (B) Solid lines indicate distances required for electron transfer within each monomer, with dotted lines indicating distances between redox centres from either monomer. Images courtesy of Dr Carola Hunte [19].

mitochondrial peptidases involved in processing newly imported proteins. However, unlike their equivalents in bovine mitochondria, the yeast subunits are proteolytically inactive and are not involved in cytochrome *c* reductase activity [26–28]. Nevertheless, defects in either of these core proteins prevents correct assembly of the yeast bc_1 complex [26,27].

Redox reactions of ubiquinol are coupled to a complex series of protonation and deprotonation events which occur at two distinct sites: Q_o and Q_i [29–31] as described by Mitchell's Q-cycle hypothesis [32]. However, the precise mechanisms have yet to be fully understood [33].

The Q_o site is located on the positive side (p-side) of the inner mitochondrial membrane and formed from the C-terminal of helix *c*, helix cd_1 and loop *ef* together with the presenting tip of the extrinsic domain of FeS protein [19]. Oxidation of quinol occurs via a bifurcated reaction, initiated at this site with the loss of one electron which is transferred along a high potential chain to the Rieske FeS centre. The resultant ubisemiquinone is stabilised within the Q_o site, facilitating transfer of the second electron to haem b_L. This second transfer reaction destabilises bound quinone in the Q_o site promoting movement of the reduced FeS protein (see below) and loss of quinone from this site. Current opinion is that rotation of the FeS protein provides two specific functions: to prevent the second electron associated with ubisemiquinone from being transferred directly to cytochrome c_1, and to ensure better contact with cytochrome c_1 for direct electron transfer [34,35]. The precise mechanisms of proton transfer and release into the intermembrane space remain unclear. However, based on recent crystal data and mutagenesis studies, His181 and Glu272 (yeast numbering) are involved, probably as primary proton acceptors, although other residues are likely to be involved directly or indirectly in proton transfer and release [19,36].

Electron transfer from the bound and stabilised semiquinone at the Q_o site to the Q_i site, located at the negative side (n-side) of the membrane, occurs through the low potential b_L haem and the high potential b_H haem, and is electrogenic. Interestingly, as two electrons are required to be transferred along this potential chain this may imply that two semiquinone molecules are required to occupy the Q_o site [37,38]. From analysis of current crystal data, the pocket forming the Q_o site would appear large enough to accommodate two such molecules, possibly in a stacked conformation.

The reduction of ubiquinone to ubiquinol at the Q_i site is coupled to proton uptake from the matrix compartment. As with proton release from the Q_o site, the precise mechanisms remain unresolved. Analysis of the yeast structure implicates His202 and Asp229 as potential proton donors to the bound quinone/semiquinone molecule at the Q_i site [19]. Yet from their orientations within the yeast crystal, reprotonation would be hindered unless residue side chain movement occurs, either in response to proton transfer or in conjunction with the loss of quininol from the pocket. Interestingly, in the bovine crystal structure, the corresponding histidine side chain was in a different orientation [39], which may indicate that such movement is possible. Alternatively, the orientation of the histidine may differ in yeast and animals, and therefore the mechanisms of protonation and deprotonation may be similarly species specific.

Other fungal research has used site directed mutagenesis to study the FeS protein. Its location within different crystal structures indicates that the head domain is mobile (57° rotation upon reduction) but the membrane spanning region remains essentially fixed [20,40,41]. Such an observation may indicate that mobility in vivo is essential for the electrons from ubiquinol to follow a bifurcated pathway. In *S. cerevisiae*, the highly conserved tether region has been the focus of mutagenesis studies (85-TADVLAMA-92) [42]. A series of point mutations for Ala86, Ala90, Ala92 and Glu95 led to catalytic activities of 22–56% of the wild type value, as did deletion of VLA (88–90) or AMA (90–92). In addition, insertion of a triple alanine motif between Asp87 and Val88 decreased activity by 90%. There was no sign of any alteration in the assembly of Complex III, or its concentration within the inner mitochondrial membrane.

Other apparent differences between fungal and mammalian bc_1 complex concern the processing and association of the FeS protein. During processing and assembly, the targeting peptide is cleaved in yeast during a two step processing reaction by a soluble matrix processing peptidase and an inter-

mediate peptidase [43]. This is unlike the bovine targeting peptide which is retained in the mature protein [39]. Interestingly, the yeast subunit is more tightly bound, preventing its ready dissociation [44,45].

The precise mechanisms for reduction and oxidation of cytochrome c_1 have yet to be fully determined. However, analysis of yeast, chicken and beef heart bc_1 complexes has identified that movement of the Rieske protein away from the Q_o site ensures electron density contact between Cys160 (chicken numbering) and a Gly residue (G107) located between helix $\alpha 2'$ and the haem bracing Pro111. As the calculated distance over which electron transfer must occur in this conformation is 8.2 Å, the estimated rate of transfer is $4.8–80 \times 10^6$ s^{-1}, and well within the predicted range for the reaction rate. Interestingly, electron transfer from bovine c_1 to c is dependent upon a complex formed between these two proteins and the 'hinge' subunit (subunit QCR6 in yeast) [46], whereas formation of such a complex is not essential in yeast as shown by deletion mutants [47]. Rather, formation of the complex appears to enhance binding of cytochrome c [47].

Recently, fungal bc_1 complex has acquired its own importance as a fungicide target for phytopathogens. Two classes of commercial inhibitors have been developed based on the methoxyacrylate and oxazolidinone chemistries, such as azoxystrobin and famoxadone [48,49]. Both compounds block electron transfer at the Q_o site. Interestingly, the methoxyacrylate class was developed from strobilurin A, which is produced by the fungus *Strobilurus tenacellus*. A study of several inhibitor producing species found that each had immunity to its own inhibitor. In *S. tenacellus*, the bc_1 complex was highly similar to its counterpart in *S. cerevisiae* with respect to subunit composition, spectral characteristics and midpoint potentials of the haem centres [50]. The different inhibitor profile was attributed to replacement of Pro254 (or Ser254, found in many fungi) by Gln and replacement of Asn261 by Asp. Similar amino acid exchanges were found in *Schizosaccharomyces pombe*, which was also naturally resistant to methoxylacrylates. Resistant populations of certain pathogens, which have developed since such inhibitors became in widespread agrochemical use, have been correlated with a single point mutation, Gly143Ala (yeast numbering) [51].

2.4. Complex IV

Complex IV (cytochrome c oxidase; COX) is the terminal oxidase of the core respiratory chain. It belongs to a haem-copper oxidase family in which the catalytic cycle is coupled to proton translocation, with an accepted stoichiometry of two protons pumped per two electrons. Crystal structures have recently been obtained at 2.8 Å resolution for both beef heart mitochondria [52] and a procaryote, *Paracoccus denitrificans* [53]. In *S. cerevisiae*, the predicted amino acid sequences for each subunit show high similarity to their bovine equivalents, enabling the bovine crystal structures to be used as a template for the fungal enzyme. In agreement with previously available biochemical data, crystallographic data have confirmed that the active site is associated with subunit I, whereas subunit II forms the dinuclear, mixed valence copper centre (Cu_A) [54]. Histidine residues have been identified as forming ligands to the two haems and the Cu_B centre, with His240 (bovine numbering) forming both a ligand to Cu_B and covalently bonding to Tyr244 [55]. It has been proposed that this histidine-tyrosine adduct may form a radical essential for reduction of oxygen.

S. cerevisiae has proved useful for site directed mutagenesis of Complex IV, the aim being to elucidate critical stages in the coupling to proton translocation. Whilst not fully understood, the current mechanism of proton translocation involves two hydrophilic channels, in which the critical amino acids are Asp and Lys [54]. Protons translocated from the matrix to the intermembrane space, and those consumed during oxygen reduction enter through these channels. For full catalytic activity, mutagenesis studies have shown that both channels must be functional [56–58]. However, the precise mechanism of proton translocation remains unclear. Several hypotheses have been put forward, with the glutamate trap system currently favoured [56]. This mechanism does not require a proton carrier to transfer protons across a hydrophobic barrier located within the enzyme, which separates the matrix and cytoplasmic sides ('histidine cycle' hypothesis) [58]. Rather, proton translocation occurs via a conserved glutamate (Glu243, yeast numbering) and propionates of the two haems [59]. Considerable experimental evidence in support of this hypothesis, and the role of the

residue in proton translocation, has been presented. For example, single mutations close to a conserved glutamate (Glu243, yeast numbering) have shown that perturbation of its p*K* disrupts proton pumping without affecting the redox properties of the adjacent haem a_3 [57]. The results supported a 'glutamate trap' mechanism, also involving the propionate groups of haem a_3, as opposed to an alternative 'histidine cycle' hypothesis. However, a recent report concerning the effect of amino acid replacement in the *P. denitrificans* COX questions the essential requirement of this Glu in proton translocation and activity. Whilst both these parameters are eliminated following mutation of Glu278 (*P. denitrificans* numbering), both can, at least in part, be restored providing a second mutation at Gly275 (*P. denitrificans* numbering) is introduced in addition to a third mutation of a Phe→Try which is in close proximity to Glu278 in the mature protein [60].

As with many respiratory components, little information is available for fungi other than *S. cerevisiae*. Cyanide generally causes at least a partial inhibition of mitochondrial oxygen consumption (for example [61–64]). For *Gaeumannomyces graminis* var. *tritici*, cyanide binding spectra have been obtained with isolated mitochondria, while difference spectra identifying both haem groups (*a* and a_3) have been reported for several other species [61–65].

3. Alternative NADH:ubiquinone oxidoreductases in fungi

3.1. Introduction

In most animals Complex I provides the sole mechanism for entry of electrons from NADH into the respiratory chain. As a consequence, NADH oxidation is totally inhibited by rotenone and piericidin. In addition, external (cytoplasmic) NADH can only be used by an indirect process such as the malate-aspartate shuttle. This involves cytoplasmic and mitochondrial malate dehydrogenase and transaminases, and antiporters with a net inward transfer of 1 H^+ per NADH. By contrast, in fungi as in plants it seems that alternatives to Complex I are widely distributed [66–74]. These either enable direct oxidation of external NADH, or act in parallel with Complex I to give rotenone insensitive oxidation of internal NADH.

Three classes of NADH:ubiquinone (UQ) oxidoreductase are now recognised. Complex I is assigned to class 1, while the alternative dehydrogenases of plant and fungal mitochondria belong in class 2 (for example, [75,76]). In class 3, electron transfer is coupled to Na^+ pumping, but this is restricted to bacteria [77,78]. The dehydrogenases in class 2 are encoded by a single nuclear gene and have a mature peptide molecular mass of 50–60 kDa. The only prosthetic group is FAD, by contrast with the FMN and multiple FeS centres of Complex I. The greatest functional difference from Complex I is that electron transport to UQ is not coupled to proton translocation.

3.2. Internal and external NADH dehydrogenases: similar proteins, different targeting

Before turning to fungi in detail, it is useful to consider a recent study for potato mitochondria. Like many plants, potatoes have both internal and external alternative NADH dehydrogenases (NDA and NDB respectively) [79]. Gene cloning has established that NDA and NDB are markedly similar to the yeast NDI1, with sequence identity of about 30–40% [79]. Both NDA and NDB have NADH and FAD binding motifs, while neither has any indication of membrane spanning α-helices. Peptide uptake studies showed that the NDA precursor was readily imported into mitochondria, with removal of a short targeting peptide after transit. By contrast, the NDB precursor did not reach the mitochondrial matrix, despite an N-terminal targeting sequence. Instead, it only crossed the outer mitochondrial membrane. Both proteins became bound to the inner mitochondrial membrane, but their different targeting led to locations on opposite sides. Relative to the centre of the lipid bilayer, they would be almost mirror images of each other, as shown in Fig. 4. In this respect they seem to be unique among mitochondrial proteins. There is evidence for distinct external and internal NADH dehydrogenases in many species, but this study provides the clearest evidence for their topology and interrelation.

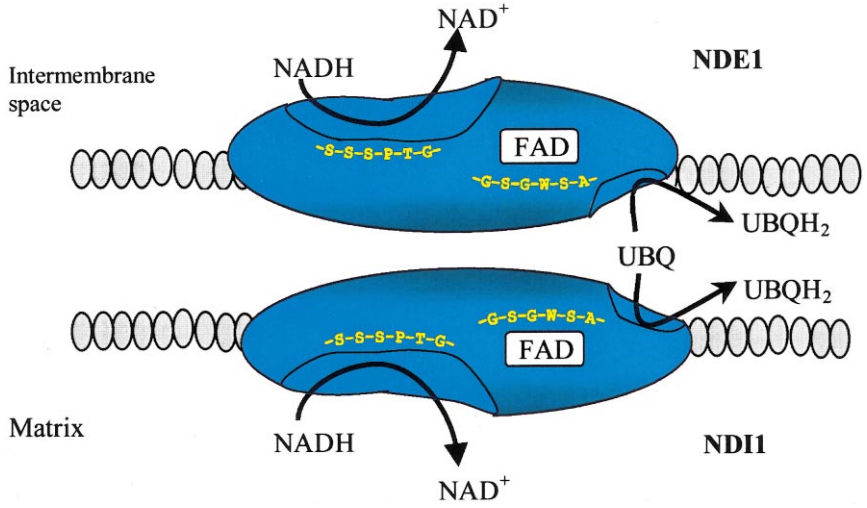

Fig. 4. Organisation of alternative internal and external NADH dehydrogenases. The internal and external NADH dehydrogenases of yeast (NDI1 and NDE1 respectively) are depicted as similar proteins with opposite topologies in the inner mitochondrial membrane. The experimental evidence relates to potato mitochondria, as discussed in the text.

3.3. *S. cerevisiae*

As mentioned above, *S. cerevisiae* lacks Complex I. Instead, reducing equivalents are delivered to the respiratory chain via three NADH dehydrogenases: NDE1, NDE2 (both external) and NDI1 (internal) [66,72,73,80]. All are insensitive to rotenone or piericidin and lack FeS redox centres, while all are nuclear encoded with mitochondrial targeting sequences near the N-terminus. It would appear that their interrelation is very similar to that shown in Fig. 4. The characterisation is most complete for NDI1 [66]. The mature protein is a single subunit with molecular mass 53 kDa and non-covalently bound FAD [80]. A protonmotive force was essential for import of its precursor, while a matrix peptidase was required for cleavage of the targeting sequence [66]. In null [81] and knockout mutants, growth was inhibited when turnover of pyruvate dehydrogenase and the TCA cycle was required, but not with fermentable carbon sources [82]. These findings are consistent with matrix NADH as the substrate.

The interrelation of NDI1 with a proton pumping Complex I has been studied by transfection of human embryonic kidney cells [83]. In these cells Complex I was still present, but NDI1 transfection led to a decreased efficiency for coupling of NADH oxidation to ATP synthesis (P/O ratio down from 2.4 to 1.8), while P/O ratios for succinate oxidation were unchanged. These results are consistent with an absence of proton pumping in NDI1. An additional feature of the study was the use of flavone to inhibit NDI1; further research is needed to determine whether this is a useful general tool for inhibition of class 2 NADH dehydrogenases.

Although biochemical and NDI1 null analysis of *S. cerevisiae* had indicated the presence of external NADH dehydrogenase(s) [80–82,84], molecular confirmation has only recently been obtained [72]. A key observation was the presence of two open reading frames (YMR145c and YGL085w), with predicted sequence identity to the NDI1 protein of 48% and 46% respectively, and a predicted mutual identity of 63% [72]. Both putative proteins (NDE1 and NDE2 respectively) contained extensions of 30–45 amino acids, proposed to be involved in subcellular localisation. The disruption of NDE1 led to reduced NADH dehydrogenase activity for cells cultivated in glucose, and reduced NADH to O_2 electron transfer for isolated mitochondria. However, disruption of NDE2 had no effect. A second study noted that commercial NADH often contains ethanol as an impurity, which could lead to misleading results in O_2 uptake studies [73]. Although the absence of NDE2 did not produce a clear phenotype, complete abolition of NADH oxidation was only observed with both NDE1 and NDE2 deleted. In shake flasks the double mutant had a reduced growth rate with ethanol or

galactose as substrate, but growth with glucose was unchanged. With glucose, there are evidently alternative mechanisms for reoxidation of cytosolic NADH, such as glycerol 3-phosphate dehydrogenase and an ethanol/acetaldehyde shunt [72].

3.4. N. crassa

The other fungus in which alternative NADH dehydrogenases have been extensively studied is *N. crassa* [70,74]. There is evidence for rotenone insensitive oxidation of both matrix and cytoplasmic NADH, implying the presence of both internal and external class 2 dehydrogenases. Unlike *S. cerevisiae*, Complex I is also present.

A cDNA clone encoding a dehydrogenase with strong amino acid homology with NDI1, NDE1 and NDE2 of *S. cerevisiae* has recently been obtained [85]. Heterologous expression confirmed that it encoded a single peptide with an apparent molecular mass of 64 kDa. Whilst the predicted sequence contained NADH and FAD binding motifs, a predicted Ca^{2+} binding domain was also identified which is absent from the yeast sequences [85]. A similar Ca^{2+} binding domain was observed in the external dehydrogenase from potato (NDB; see above), suggesting that Ca^{2+} may have a regulatory role for both proteins [86,87]. A location in the inner mitochondrial membrane was confirmed by increased protease sensitivity after outer membrane disruption by hypotonic swelling. The authors favoured a location facing the matrix, but this was less certain.

4. The alternative oxidase in fungi

4.1. Introduction and detection

The alternative oxidase (AOX) acts in parallel with the bc_1 complex for oxidation of ubiquinol and also catalyses the four electron reduction of dioxygen to water (Fig. 1) [88]. In comparison with other components of fungal respiration, understanding of its function, regulation and molecular organisation is relatively backward. However, in the past 5 years agrochemical interest in the respiratory chain as a fungicide target has provided a strong impetus for its study in plant pathogens [48,49]. This research has been influenced by what is already known about the AOX in plants.

The AOX is nuclear encoded and has been found in most fungal genomes tested, with *S. cerevisiae* and *Sch. pombe* as two exceptions [89,90]. The protein has been detected by immunoblot analysis of isolated mitochondria [62,64,91]. Such experiments have relied on cross-reaction with monoclonal antibodies raised against plant AOX's (from *Sauromatum guttatum* [62,91] and maize [64]) which recognise the sequence 300-RADEAHHRDVNH-311 [92].

To test for AOX activity one must measure O_2 consumption, typically with a Clark-type oxygen electrode. Such analysis has identified the fungal AOX as sensitive to hydroxamic acids (e.g. salicylhydroxamic acid (SHAM)) and alkyl gallates (e.g. *n*-propyl gallate) [93]. Conversely, AOX is not affected by cyanide, azide, carbon monoxide and nitric oxide, all of which inhibit cytochrome *c* oxidase [93,94]. These properties are exactly as for AOX in plants. One of the key questions for fungal AOX is whether it is constitutively expressed, and if so, is it an active component of the electron transport chain. The time frame for AOX nuclear induction to insertion and activation requires between 3 and 5 h [95,96]. Therefore, if inhibition of oxygen consumption by AOX inhibitors either alone or in combination with core electron transport inhibitors occurs within the 10 to 20 min it takes to routinely perform these assays, then AOX must be already present within the mitochondria, and hence is not induced.

4.2. Expression characteristics

Unlike the core components of fungal respiration, the fungal AOX appears to have complex mechanisms for regulation of expression. Early studies concluded that it was only expressed when the core pathway for ubiquinol oxidation was inhibited, for example by antimycin or cyanide [97–99]. This pattern of induction is also described in more recent references [100–103]. However, myxothiazol had little effect on AOX induction in *Hansenula anomala* [104]. These workers found that superoxide induced AOX, regardless of the functional state of the respiratory chain. The lack of response to myxothiazol was attributed to an absence in any rise in superoxide production, on addition of this Q_o site inhibitor.

However, regulation need not be solely at the level of transcription. A recent study of *Magnaporthe grisea* concluded that transcription was constitutive, but translation and activation only occurred under stress conditions [105]. The stress could be a partial or complete cessation of electron flux through the core pathway, or elevated levels of H_2O_2. It was found that cycloheximide induced accumulation of AOX transcript, but not its activation, indicating that degradation of transcript occurs via factors sensitive to this compound.

There is also evidence for developmental regulation. In *Botrytis cinerea*, respiration in 24 h cultures was sensitive to inhibitors of the core pathway [64]. However, within 48 h of germination in liquid culture, respiration in *B. cinerea* becomes sensitive to AOX inhibitors but insensitive to inhibition of Complex III (Joseph-Horne and Ishii, unpublished observations, 1999). It is not clear why this shift towards AOX utilisation should occur during a rapid and energetically demanding phase of growth. An earlier study with *G. graminis* had concluded that expression was constitutive [62]. However, a similar developmental regulation might have gone unnoticed, since spores were not tested.

4.3. Sequence data and molecular modelling

Molecular analysis has only been possible since the generation of antibodies against the AOX from *S. guttatum* (see above). These enabled the isolation of cDNA encoding a plant AOX and subsequently aided in isolation of genes from *H. anomala* and *N. crassa* [106–109]. The only other fungal species for which the full sequence is known are *A. niger* [110], *M. grisea* [105,111] and *Candida albicans* [112]. In addition, there are partial sequence data for *B. cinerea*, *G. graminis*, *Rhizoctonia solani*, *Stagonospora*

Fig. 5. Comparison of fungal and plant alternative oxidase primary amino acid sequences indicating helical organisation. Sequence keys are: magph, *M. grisea*; canal, *C. albicans*; neucr, *N. crassa*; hanan, *H. anomala*; aspng, *A. niger*; arath, *A. thaliana*; saugu, *S. guttatum*. The boxed Cys is that associated with dimer formation and pyruvate regulation in the plant AOX. Proposed metal ligands are similarly boxed.

nodorum and *Tapesia acuformis* [111]. A pileup for AOX sequences from fungi is shown in Fig. 5. It is noteworthy that sequence identity between plant and fungal AOX is relatively poor [110–113]; for example, identity between *M. grisea* and *S. guttatum* is 23.9%. This can be contrasted with a high amino acid identity between AOX from different plants; for example, identity between *S. guttatum* and *Arabidopsis thaliana* is 54.4%. Clearly, low identity at the amino acid level may explain some of the differences in function and regulation between plant and fungal AOX.

Indirect evidence has built up for iron at the active site of the plant AOX, despite the absence of any EPR signal or absorbance above 350 nm [114]. The inhibitor profile of the fungal AOX is identical (see Section 4.1), implying that its active site is essentially the same. Sequence and biochemical analysis has indicated similarities between AOX and members of the 'RNR R2-like protein' subgroup of the diiron carboxylate family [115,116]. The name is derived from the R2 domain of ribonucleotide reductase (RNR), while other members include methane monooxygenase and stearoyl acyl carrier protein Δ^9-desaturase. These proteins all contain a binuclear Fe centre coordinated by His and carboxylate residues. The active site is defined by two pairs of helices forming a four helix bundle. Two antiparallel helices, one in each pair, contain a critical EXXH motif. These motifs supply the residues for iron coordination [117,118].

In the absence of crystallographic data, any model for the AOX can only be regarded as provisional. The first model was derived from amino acid sequence data for a relatively small number of plants [116]. Three conserved EXXH motifs were identified, of which two were predicted to be in non-membrane spanning domains. Since the third was in a predicted transmembrane region, it was assumed not to provide ligands to the diiron centre. However, further sequence data revealed that one of the EXXH motifs chosen by Siedow et al. [116] to form ligands to the diiron centre was not always present whereas the EXXH motif previously thought to be within the transmembrane region was in fact highly conserved. This, together with discrepancies for ligand spacing, order and helical orientation compared with the structures of all other RNR R2-like family proteins led Andersson and Nordlund to propose a revised model for the plant AOX (Fig. 6A) [119].

Fig. 6B depicts the first molecular model for fungal AOX [111]. At an early stage in its preparation, examination of ligand spacing within the diiron centre revealed closest similarity between *M. grisea* AOX and Δ^9-desaturase, for which the structure is known. By employing Δ^9-desaturase as a template, a structure was developed which shows strong similarity to all members of this subfamily. The orientation of the four helices is consistent with Andersson and Nordlund's model for the plant AOX [119]. In both, a hydrophobic connecting sequence between the two helical pairs is proposed to act as a membrane anchoring region, since it includes several conserved positively charged residues. Significantly, the prediction is that this structural feature is interfacial at the membrane aqueous interface, rather than transmembrane. A channel to a less hydrophobic region close to the active site was identified as a possible site for quinol binding. Indeed, in AOX from *A. thaliana*, mutation of two residues close to this region has been shown to affect quinol binding [120]. For other diiron carboxylases this hydrophobic region has been implicated in protein-protein interactions. Given that both fungal and plant AOX are believed to exist in a dimeric form, such a role is also conceivable.

4.4. Comparison with AOX modulation in plants

Whilst inhibition profiles and model building shows plant and fungal AOX to be very similar, regulation and function of AOX appear less conserved between the two kingdoms. In particular, plant AOX has positive regulation by pyruvate, while pyruvate has no known effect on the fungal AOX [91,111]. It is known that pyruvate binds to plant AOX with formation of a thiohemiacetal [121]. Mutagenesis of Cys78 in *A. thaliana* abolished pyruvate regulation [122]. Significantly, this cysteine is conserved throughout all known plant AOX sequences, and is the likely site of pyruvate binding. However, the pileup of fungal AOX sequences in Fig. 5 shows that only *H. anomala* possesses a cysteine close to Cys78 of *Arabidopsis*. Thus an absence of pyruvate regulation in fungal AOX is not surprising.

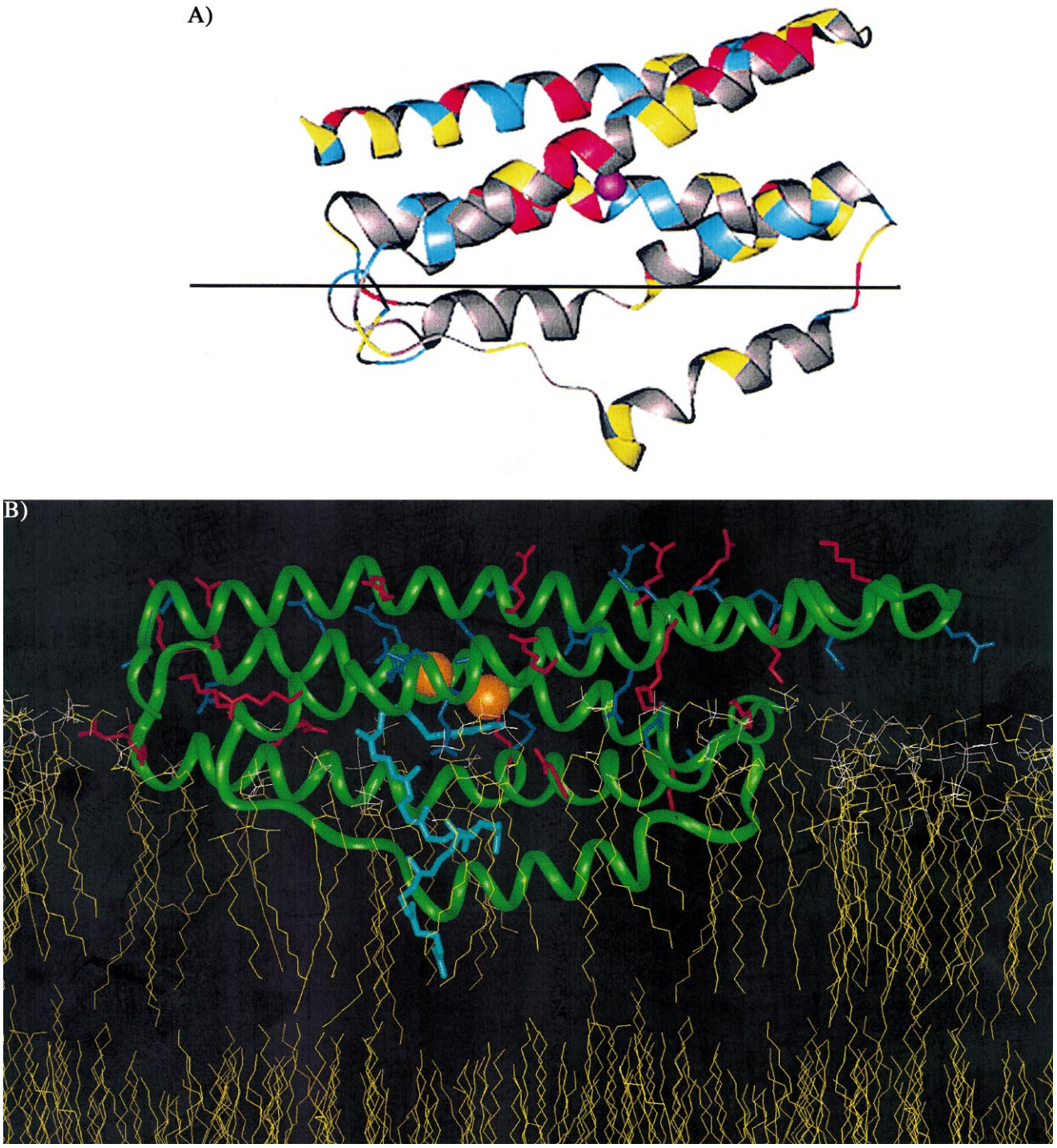

Fig. 6. Predicted molecular models of (A) plant and (B) fungal alternative oxidase. (A) Ribbon model for *S. guttatum*, adapted from Andersson and Nordlund [119]. Grey residues are hydrophobic, yellow are hydrophilic uncharged, blue are positive, red are negative and purple sphere represents the diiron centre. (B) Fungal AOX model, including the predicted orientation in the membrane. The model was developed from *M. grisea* AOX and adapted from Joseph-Horne et al. [111]. Side chains of charged groups are shown (Asp, Glu, Lys and Arg). Diiron centre is shown as orange spheres. Ubiquinol is represented as magenta sticks.

Regulation of plant AOX has additional complexities which include the existence of monomeric (high activity) and dimeric (low activity) forms. The dimer is stabilised by formation of an S-S bond between the two Cys78 [122,123]. However, other protein-protein interactions are evidently also important, since covalent binding of pyruvate does not prevent dimer formation. In most fungal AOX there is no comparable cysteine to that identified in plant AOX to form the disulphide bond (as just explained), but there could still be a dimeric state [62]. Probing SDS-PAGE gels of mitochondrial proteins from *G. graminis* with an antibody against AOX from *S. guttatum* gave two signals, attributed at the time to the monomeric and dimeric forms of AOX [62]. However, when mitochondria from *N. crassa* and *Pichia stipitis* were probed with the same antibody, the dimeric form could not be detected, even after pretreatment with the Cys-Cys cross-linker, bis-maleimidohexane [91]. Thus the importance and presence of a dimeric form of the fungal AOX is still questionable.

5. Rationale for alternative respiratory pathways in fungi

5.1. The problem of oxidative damage

During normal respiration in animals, it is estimated that superoxide is generated from 1–2% of all electrons transported [124,125]. Complex I is strongly implicated in this generation, due to the low redox potential required for one electron reduction of dioxygen to superoxide. The cytoplasm and mitochondrial matrix of all cells contain mechanisms to protect against oxidative damage (e.g. SOD, catalase, peroxidases), but such mechanisms can only work at finite rates. There will always be a background rate of oxidative damage [126]. The level that can be tolerated without significant inhibition of growth or viability will depend on the cell's metabolic state.

Extensive studies with other eucaryotic cells show that oxidative damage is increased by disruption of the respiratory chain at any of the four complexes [127–129]. Reactive oxygen species (ROS) generation appears particularly rapid following inhibition at sites of semiquinone oxidation or reduction, which would inevitably increase the steady-state concentration of the reactive semiquinone state. For fungal pathogens, inhibition of COX by nitric oxide (NO) is a particular threat, as NO is generated both enzymatically and non-enzymatically during the plant hypersensitivity response to infection [130–133]. Whereas the standard COX is sensitive to NO in only nanomolar concentrations [134,135], AOX is unaffected [94,136]. Interestingly, high levels of NO (micromolar) have been reported to inhibit Complex I within the murine macrophage cell line, J774, in a redox dependent, progressive and selective manner [137]. Whilst a similar inhibitory effect of NO has not been identified against fungal Complex I, the presence of AOX in combination with internal and/or external NADH dehydrogenases should enable NADH oxidation and electron flux rates to be maintained in the presence of NO, thereby preventing accumulation of semiquinone and decreasing ROS generation.

5.2. Alternative pathways are consistent with protonmotive force generation

In plants, photorespiration leads to formation of glycine, which must be oxidised even when the cell has no respiratory ATP requirement. In addition, plants in the Araceae (e.g. *S. guttatum*) use uncoupled respiration for thermogenesis during flowering [138,139]. The existence of these processes has led to the frequent assumption that alternative pathways are invariably uncoupled, in plants and (by extension) in fungi [140,141].

Electron flow through Complex I plus the AOX leads to a predicted P/O ratio of 1.0 (for external ATP synthesis, if one assumes the standard stoichiometry of 4 H^+ per ATP and 4 H^+ pumped by Complex I). The P/O ratio for the full respiratory chain is 2.5 (10 H^+ pumped). At 40% efficiency, the alternative oxidase need not be negligible as a source of ATP. Indeed there is evidence from fungi that the Complex I/AOX pathway can drive ATP synthesis, without any observable loss of cell viability [64]. The inhibition of AOX has been shown to cause a rapid collapse of mitochondrial $\Delta\Psi$ in isolated mitochondria from *G. graminis* and *Fusarium oxysporum*, and in whole mycelia of *B. cinerea*, *Sclerotinia homeocarpa* and *G. graminis* ([61]; Joseph-Horne, unpublished

data, 1999). In *G. graminis*, inhibition of AOX decreased the rate of ATP synthesis, regardless of whether electron flow could still occur through the bc_1 complex [62].

Further support in favour of a role of fungal AOX in mitochondrial energy production comes from its regulation by nucleotides [142,143]. Unlike the plant AOX, which is upregulated by ATP [138,144], no increased activity has been observed for fungal AOX by raising the ATP concentration. By contrast, a strong stimulatory effect for AMP, ADP, dAMP and GMP has been reported for AOX in *H. anomala* [143], *N. crassa* [91,140] and *P. stipitis* [91]. This stimulation was not an unspecific property of nucleotides, since ATP, cAMP, CMP and UMP were all ineffective. Such regulation lends support to a role for the fungal AOX in mitochondrial ATP production.

In contrast to data supporting a role for fungal AOX in pmf generation, the heterologous expression of a plant AOX in mitochondria of *Sch. pombe* led to decreased cellular growth rates and yields, implying that electrons were partitioned away from the core pathway [145]. However, this yeast does not possess an AOX gene, so it is likely to lack the regulatory mechanisms needed for successful AOX utilisation. The AOX was also from a plant, as opposed to a fungus. It may be more revealing to study the controlled overexpression of a fungal AOX in fungi that normally possess this protein.

6. Conclusions

The apparent ecological success of *S. cerevisiae* and the few other fungi without Complex I focusses attention on the rationale for multiple forms of NADH dehydrogenase. Compared with most fungi, yeast is unusually adapted towards anaerobic growth and, by implication, cytoplasmic (i.e. external) production of NADH. The external NADH dehydrogenase in conjunction with COX will give a P/O ratio of 1.5, while for external NADH the full chain would lead to P/O = 2.25 (since 1 H^+ is used in conversion to internal NADH).

Two connections can be drawn with the problem of oxidative damage (see above). In the first place, there seem to be no reports linking the alternative NADH dehydrogenases with ROS formation. This could partly be lack of data, but the alternatives may be 'safer' when a high efficiency for carbon utilisation is not the prime consideration. Second, when the conventional pathway is inhibited by NO (or any other inhibitor of Complex III or IV), diversion of electrons to the AOX may enable ATP synthesis to continue, and decrease the overreduction that leads to oxidative damage. This could be particularly relevant for plant and animal pathogens.

Unless the aim is uncoupled respiration, a fungus must avoid pairing the alternative NADH dehydrogenases and AOX together, since that would lead to zero proton pumping. It may be significant that no fungi are known in which Complex I is absent and AOX is present – a combination that would make any electron flow through AOX totally uncoupled.

In summary, fungi utilise extensive degeneracy within their respiratory chains, yet the requirement for such organisation remains largely unclear. In addition, the complex regulation for each branch point is also poorly understood. Whilst the fungal mitochondrial electron transport chain may appear similar to that of plants, it is clear that caution must be applied when assigning function and regulation for the fungal components based on their homologues in plants. Therefore it remains essential to search for the answers to these questions with continued diligence and an open mind.

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