Targeting the Plant Alternative Oxidase Protein to Schizosaccharomyces pombe Mitochondria Confers Cyanide-insensitive Respiration*

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The Sauromatum guttatum alternative oxidase has been expressed in Schizosaccharomyces pombe under the control of the thiamine-repressible nmt1 promoter. Alternative oxidase protein and activity were detected both in spheroplasts and isolated mitochondria, indicating that the enzyme is expressed in a functional form and confers cyanide-resistant respiration to S. pombe, which is sensitive to inhibition by octyl-gallate. Protein import studies revealed that the precursor form of the alternative oxidase protein is efficiently imported into isolated mitochondria and processed to its mature form comparable to that observed with potato mitochondria. Western blot analysis and respiratory studies revealed that the alternative oxidase protein is expressed in the inner mitochondrial membrane in its reduced (active) form. Treatment of mitochondria with diamide and dithiothreitol resulted in interconversion of the reduced and oxidized species and modulation of respiratory activity. The addition of pyruvate did not effect either the respiratory rate or expression of the reduced species of the protein. To our knowledge this is the first time that the alternative oxidase has been effectively targeted to and integrated into the inner mitochondrial membrane of S. pombe, and we conclude that the expression of a single polypeptide is sufficient for alternative oxidase activity.

A major feature of the plant mitochondrial respiratory chain is the presence of a second terminal oxidase, in addition to cytochrome c oxidase, namely the cyanide- and antimycin-resistant alternative oxidase (1–5). The alternative oxidase is an integral inner membrane protein, which branches from the main respiratory chain at the level of the ubiquinone pool. Electron flow through this oxidase is not coupled to ATP synthesis, and the enzyme catalyzes the reduction of oxygen to water and not hydrogen peroxide (1–5). In recent years most attention has been focused upon the regulation and nature of the alternative oxidase.

Extensive kinetic analyses of the regulation of alternative pathway activity in a variety of plant tissues suggest that activity is determined by the redox poise of the ubiquinone pool (6–8), the amount of alternative oxidase protein (8, 9), the mitochondrial concentration of α-keto acids (particularly pyruvate) (10), the redox status of the alternative oxidase intermolecular sulfhydryl/disulfide system (11), the total amount of ubiquinone (12), and the activity of the quinone-reducing enzymes (9). The mechanism of interplay between all of these various regulatory systems that ultimately defines the total capacity and engagement of the alternative oxidase is, however, still not fully understood.

Following the isolation of a cDNA encoding the alternative oxidase protein, initially from Sauromatum guttatum (13) and later from the yeast Hansenula anomala (14) and other plant tissues (see Refs. 3 and 4), details are emerging as to the structural organization and possible nature of the active site of this interesting yet enigmatic terminal oxidase. For instance, hydropathy analyses of the plant alternative oxidase sequences have revealed the presence of two transmembrane α-helices that are located centrally in the body of the protein, being flanked by two hydrophilic domains that extend into the mitochondrial matrix (2, 3, 15). Further amino acid sequence comparisons have revealed that all of the plant alternative oxidases contain, in the carboxyl-terminal hydrophilic domain of the protein, two copies of the iron-binding motifs that are found in the class I group of di-iron-carboxylate proteins such as ribonucleotide reductase R2 and methane monoxygenase (16, 17). Based upon the presence of these motifs and using the highly conserved residues located in the carboxyl-terminal hydrophilic domain of the protein, a hypothetical structure of the catalytic site has been proposed (16, 18). In this hypothetical structure, the active site contains a coupled binuclear iron center analogous to that found in methane monoxygenase and ribonucleotide reductase (17).

Although such sequence information is of importance in the elucidation of the structure of the alternative oxidase, physical characterization of the properties of this enzyme has been hampered by the lack of suitably purified preparations. Plant tissues have proved to be a difficult source from which to purify the enzyme, and its counterpart in H. anomala has yet to be demonstrated as being kinetically equivalent to that of the plant enzyme (1, 3). A cDNA from Arabidopsis thaliana (19) has been successfully used to complement an Escherichia coli mutation that appears unable to grow aerobically, suggesting that the expression of a single protein species is sufficient to obtain a functional enzyme. To date, however, the alternative oxidase has not been expressed in eukaryotic cells such as yeast and, in particular, has not been targeted to mitochondria of non-plant origin.

In this paper we have investigated the possible use of Schizosaccharomyces pombe as a suitable system for genetic analysis of the plant alternative oxidase. S. pombe has proved to be an excellent model for the study of higher eukaryotes (20, 21) and has been recently proved useful in the functional expression of
the chloroplast 2-oxoglutarate/malate translocator (22). To our knowledge this is the first report on the functional expression of the plant alternative oxidase in yeast mitochondria. The alternative oxidase is expressed in its active form (the reduced species) in S. pombe spheroplasts and mitochondria resulting in cyanide- and antimycin-resistant respiratory activity. As in plants, modulation of the redox status of the sulfhydryl/disulfide linkage results in alterations in mitochondrial electron flux via this pathway. Such results indicate that the alternative oxidase protein is correctly targeted to the inner mitochondrial membrane, imported, processed, and correctly folded and iron inserted into the active site to form an active enzyme.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The S. pombe strain was sp. 011 (ade6–704, leu1–2, ura4-D18, h+.). For amplification of plasmids and preparation of pAOSG81 (13), the E. coli strain M110 was used. Media and growth conditions were as described by Murray et al. (23) with the additional inclusion of 75 mM iron and lack of Mn2+ and the presence or absence of 0.1 mM thiamine.

**Plasmid Construction**—The wild-type alternative oxidase on a 1.33-kilobase pair BspH/EcoR V fragment from pAOSG81 was ligated to pREP1 (24) containing an Nco I site in place of the published Nde I site, which had been replaced with Nco I and Smal I. This created pREP1/AOX, which contained the alternative oxidase coding sequence under the control of the nmt1 promoter.

**General Molecular Biology Methods**—S. pombe cells were transformed using a modified lithium acetate method (25). Other methods were as described by Sambrook et al. (26).

**Isolation of Mitochondria and Respiratory Measurements**—S. pombe mitochondria were isolated from 1.5-liter cultures grown overnight in minimal medium as described previously (27) but without additional purification on Percoll gradients. Mitochondria from fresh potato tubers were isolated and purified as described by Whitehouse et al. (28). Respiratory activity was measured polarographically using a Rank oxygen electrode in 2 ml of reaction medium (mitochondrial samples) containing 0.75 M sorbitol, 0.4 M mannitol, 10 mM MOPS (pH 6.8), 10 mM MgCl2, 5 mM K2HPO4, and 0.3% BSA as described previously (27).

Oxygen consumption by spheroplasts was determined in a medium containing 0.75 M sorbitol, 0.4 M mannitol, 10 mM MOPS (pH 6.8), and 0.1% (w/v) BSA.

**Gel Electrophoresis and Western Blotting**—Equal amounts of protein (approximately 15 μg) were separated by gel electrophoresis on 10% SDS-polyacrylamide gels without mercaptoethanol essentially according to Laemmli (29). The separated proteins were transferred to nitrocellulose membranes (0.45-mm pore size) according to the method of Towbin et al. (30). The transfer buffer contained 200 mM glycine, 12.5 mM Tris, and 10% methanol (pH 7.2), and proteins were transferred at 100 V (constant voltage) for 1 h. The filters were initially incubated with 0.1% Tween 20, 0.35 mM E64, 10 mM EDTA (pH 6.8), 10 mM MgCl2, 5 mM KH2PO4, and 0.3% BSA as described previously (27).

Oxygen consumption by spheroplasts was determined in a medium containing 0.75 M sorbitol, 0.4 M mannitol, 10 mM MOPS (pH 6.8), and 0.1% (w/v) BSA.

**ATP, 9 μM creatine phosphate, 120 μM creatine kinase.** 440 μM of purified mitochondrial protein were used per import reaction, which were performed in siliconized scintillation vials at 25 °C for 30 min in a shaking water bath. Import was stopped by addition of valinomycin to a final concentration of 0.1 mg/ml. Proteinase K was added to 110 μM of import buffer at a final concentration of 2 μg/ml, and incubation was continued for 30 min on ice. Phenylmethylsulfonyl fluoride was then added to both proteinase K-treated and untreated samples to a final concentration of 1 mM. Mitochondria were resolated and resuspended in SDS sample buffer, and proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% gels as described above. Unless otherwise stated all chemicals were obtained from Sigma (Pode, Dorset, United Kingdom).

**RESULTS**

The plant alternative oxidase was introduced into yeast by transformation of S. pombe with pREP1-AOX and selection for leucine prototrophy. The resulting plasmid contained the S. guttatum alternative oxidase expressed under control of the nmt1 promoter, which is repressed by thiamine. Fig. 1 shows respiratory activity and Western blot analysis of S. pombe spheroplasts prepared from cells grown overnight in minimal media in the presence and absence of thiamine. Oxygen uptake measurements indicate that spheroplasts prepared from both types of these cells exhibit rapid rates of oxygen consumption, which were stimulated by FCCP. Importantly the data summarized in Fig. 1A show that the functional expression of the alternative oxidase in S. pombe grown in the absence of thiamine results in cyanide-resistant respiration as indicated by the relative insensitivity of the respiratory activity to KCN (or myxothiazol). Furthermore Fig. 1A indicates that the respiratory activity in spheroplasts expressing the alternative oxidase protein is sensitive to octyl-gallate, a potent inhibitor of alternative pathway activity (32). The respiratory activity of spheroplasts prepared from cells grown in the presence of thiamine and in the presence of FCCP (Fig. 1B) was comparable to that observed in Fig. 1A, except that it was very sensitive to the subsequent addition of 1 mM KCN. Western blot analysis, using antibodies specific for the alternative oxidase (AOA antibodies; Ref. 33), reveals that the protein is only expressed in spheroplasts obtained from cells grown in the absence of thiamine (Fig. 1C) and that, in comparison with Arum maculatum mitochondria, the alternative oxidase protein (when immunoblotted in the absence of reductant) is expressed mainly in its reduced

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1 The abbreviations used are: MOPS, 4-morpholinoethanesulfonic acid; mHSP70, heat shock protein 70 located in the mitochondrial matrix; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; AOA, alternative oxidase, all antibodies; DTT, dithiothreitol; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

**FIG. 1.** Expression and activity of the alternative oxidase in S. pombe spheroplasts. Spheroplasts were prepared from cells grown either in the absence or presence of thiamine. Respiratory activity (A and B) was determined as indicated under "Experimental Procedures" in the presence of 10 mM glucose, 1 μM FCCP, and 1 mM KCN. Oxyd gallate was added to a final concentration of 2 μM. Rates on the respiratory traces are in nmol of O2.
In an attempt to determine whether the cyanide-resistant respiratory activity observed in Fig. 1 was mitochondrial in origin, S. pombe mitochondria isolated from both types of cells were probed with mthHSP70 and AOA antibodies. The mthHSP70 antibodies only recognize an HSP70 protein, which is specifically localized in the mitochondrial matrix (34). In this experiment purified A. maculatum mitochondria were used as a positive control (Fig. 2, A and B, lane 3). It is clear from the immunoblots shown in Fig. 2 that the AOA antibodies only recognize a protein in lanes 1 and 3 (Fig. 2A), whereas the HSP70 antibodies cross-react with a protein in lanes 1-3 (Fig. 2B), confirming the presence of mitochondria from both types of cell. Such results indicate that the alternative oxidase protein is expressed and targeted only to mitochondria isolated from cells grown in the absence of thiamine.

When the alternative oxidase protein was transcribed and translated in vitro, import experiments revealed (Fig. 3) that the protein was efficiently imported into the matrix of wild-type S. pombe and potato mitochondria. The precursor form of the produced alternative oxidase protein from the S. guttatum cDNA clone had an apparent molecular mass of 42 kDa, and following incubation the apparent molecular mass was approximately 32 kDa, suggesting that the presequence had been removed following import to produce the mature form of the protein. Of particular importance was the finding that following proteinase K treatment the precursor form of the protein was processed to the same size as observed in potato mitochondria (compare position of mature protein in lanes 2 and 4). Close inspection of Fig. 3 also reveals that there is more of the proteinase K-insensitive precursor form of the alternative oxidase protein when import experiments were performed with S. pombe mitochondria than with potato mitochondria. Although the reason for the presence of these intermediates is at present unclear, it may be due to the formation of translocation-arrested precursor proteins. Such intermediate forms of the imported protein have previously been observed with some plant mitochondria, and we have suggested that this may be due to limiting amounts of the mthHSP70 severely restricting the import process (35). Nevertheless the results expressed in Fig. 3 indicate that the plant alternative oxidase precursor protein can be cleaved by S. pombe mitochondria at the same site as by the plant processing enzymes.

Fig. 4 shows a typical series of respiratory traces using mitochondria isolated from cells grown in the presence (Fig. 4A) or absence (Fig. 4B) of thiamine. Both sets of mitochondria actively oxidize NADH as a respiratory substrate with comparable rates of respiration, both of which were stimulated by FCCP. The respiratory activity of mitochondria isolated from cells grown in the presence of thiamine, however, was potently inhibited by antimycin (Fig. 4A), whereas mitochondrial respiration in cells expressing the alternative oxidase protein was approximately 30% resistant to the inhibitor. Subsequent addition of octyl-glutamate to these mitochondria resulted in total respiratory inhibition (Fig. 4B), confirming that the antimycin-resistant respiration was due to engagement of the alternative oxidase. Interestingly the addition of 5 mM pyruvate or \( \alpha \)-ketoglutarate (not shown), both of which have previously been demonstrated to activate the plant alternative oxidase (4, 36), had no effect upon alternative pathway activity in S. pombe mitochondria, perhaps suggesting that the protein is expressed in its fully activated form.

Umbach and Siedow (11) have previously shown that the plant alternative oxidase exists as a covalently (oxidized) or non-covalently linked (reduced) dimer within the mitochondrial inner membrane. Although Fig. 2 indicated that the recombinant alternative oxidase exists primarily, in transformed S. pombe mitochondria, in its reduced form, it was of interest to determine if the protein species could be interconverted to its oxidized form in a manner analogous to that observed with plant mitochondria (3). The immunoblot shown in Fig. 5 indicates that in isolated S. pombe mitochondria the alternative oxidase protein is expressed mainly in its reduced form. Interestingly the AOA antibodies recognize not only the oxidized and reduced forms of the enzyme, but also a protein species with a molecular mass of approximately 42 kDa. It is tempting to speculate that this protein species represents the precursor form of the alternative oxidase (2). When S. pombe mitochondria were incubated with 20 mM DTT on ice for 30 min, the oxidized species were largely converted to the reduced species with a concomitant increase in respiratory activity. Similarly, treatment of mitochondria with diamide resulted in a greater proportion of the protein existing in its oxidized form and a decrease in respiratory activity. However, in contrast to the results obtained with plant mitochondria, it was only possible to oxidize a small proportion of the reduced form of the protein, suggesting that the enzyme exists mainly as a non-covalently-linked dimer when expressed in S. pombe.

**DISCUSSION**

The results described in this paper are, to our knowledge, the first to report on the functional expression of the plant alternative oxidase in yeast mitochondria. Although the alternative oxidase has previously been expressed in *E. coli* (19), detailed analysis of its properties in this system have yet to emerge. Unlike *H. anomala* (37), a cyanide-resistant alternative ox-
dase cannot be induced in S. pombe by growing the cells on antimycin, and previous characterization of these mitochondria indicates that S. pombe mitochondria possess only cytochrome c oxidase as a terminal oxidase (27). S. pombe is therefore a useful system to study the expression of foreign gene products such as the chloroplast 2-oxoglutarate/malate translocator (22) and the alternative oxidase. Although considerable information is available on the molecular biology and kinetic regulation of the alternative oxidase in plants, a convenient and rapid system to study structure-function relationships of this protein has not previously been available. It is readily apparent from the results depicted in Figs. 1 and 2 that in the absence of thiamine the plant alternative oxidase is expressed in S. pombe and furthermore that expression of a single gene confers cyanide-resistant respiration to these cells, in contrast to that proposed to occur in soybean (38). The protein import experiments illustrated in Fig. 3 also demonstrate that a plant mitochondrial leader sequence effectively targets the protein to the inner mitochondrial membrane of S. pombe and additionally that, once the protein has been imported into the matrix, the mature form of the protein has the same molecular mass as that observed with potato mitochondria. The oxygen uptake traces depicted in Fig. 4 indicate that the alternative oxidase is expressed in a functional form, indicating that following import and processing the protein is correctly folded and importantly, the necessary enzymes for the incorporation of iron are present in S. pombe. Furthermore such observations indicate the expression of a single polypeptide is sufficient for enzyme activity, which is in contrast to that observed in thermogenic tissues (39, 40) and soybean (4, 41).

Immunoblots using the AOA antibodies (Figs. 1C and 5) revealed that the oxidase protein is present predominantly in its reduced (most active) form both in spheroplasts and isolated mitochondria. Regulation of enzyme activity in S. pombe does, however, bear some resemblance to the plant enzyme inasmuch as treatment of mitochondria with diamide to oxidize the sulf-hydryl bond results in some loss of respiratory activity (Fig. 5), which can be partially restored by subsequent re-reduction of this bond by DTT (11). The immunoblots also revealed a protein band of an apparent molecular mass of 42 kDa. Although the identity of this band is uncertain, it is interesting to speculate that it represents the precursor form of the alternative oxidase (2), suggesting that expression of the alternative oxidase in the inner mitochondrial membrane is maximal and may well be limited by availability of mtHSP70. mtHSP70 is required for efficient protein import and has previously been demonstrated by our laboratory to be developmentally regulated in plant systems (35). We are currently co-expressing the mtHSP70 protein along with the alternative oxidase in attempts to ascertain whether this idea is correct and to optimize protein expression.
The results described in this paper have considerable implications with respect to future structure-function studies of the alternative oxidase protein. We have recently postulated that the active site of the alternative oxidase contains a binuclear iron center comparable to that observed in methane monooxygenase and ribonucleotide reductase (16, 18). Within this center, the two iron atoms are coordinated by two histidines, two glutamates (one of which is bridging), one aspartate, and two water molecules. A bridging μ-hydroxo atom has also been included to account for the lack of absorbance above 350 nm.

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