A HighlyConserved Glutamate Residue (Glu-270) Is Essential for Plant Alternative Oxidase Activity*

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We have previously demonstrated that expression of a Sauromatumguttatumalternative oxidase in Schizosaccharomycesspombeconfers cyanide-resistant respiratory activity on these cells (Albury, M. S., Dudley, P., Watts, F. Z., and Moore, A. L. (1996) J. Biol. Chem. 271, 17062–17066). Using this functional expression system we have investigated the active site of the plant alternative oxidase, which has been postulated to comprise a non-heme binuclear iron center. Mutation of a conserved glutamate (Glu-270), previously postulated to be a bridging ligand within the active site, to asparagine abolishes catalytic activity because mitochondria containing the E270N mutant protein do not exhibit antimycin A-resistant respiration. Western blot analysis, using antibodies specific for the alternative oxidase, revealed that the E270N mutant protein was targeted to and processed by S. pombe mitochondria in a manner similar to that of the wild-type protein. It is possible that lack of antimycin A-insensitive respiration observed in mitochondria containing the E270N mutant protein is due to incorrect insertion of the mutant alternative oxidase into the inner mitochondrial membrane. However, Western blot analysis of subfractionated mitochondria shows that both wild-type and E270N alternative oxidase are specifically located in the inner mitochondrial membrane, suggesting that misfolding or lack of insertion is unlikely. These results provide the first experimental evidence to support the structural model in which the active site of the alternative oxidase contains a coupled binuclear iron center.

A distinctive feature of the plant mitochondrial respiratory chain is the presence of two respiratory pathways for the reduction of oxygen (1–4). In addition to a cytochrome c oxidase, higher plants contain an alternative oxidase, an integral membrane protein, which branches from the cytochrome pathway at the level of the ubiquinone pool (1). The alternative pathway catalyzes the four-electron reduction of oxygen to water (1). Unlike cytochrome c oxidase, electron flow through the alternative oxidase is not coupled with proton translocation, and the resultant free energy is lost as heat (1). The alternative oxidase is insensitive to inhibitors of the cytochrome pathway such as antimycin A, cyanide, and myxothiazol but is inhibited by octyl- and propyl-gallates and SHAM (3).1 In non-thermogenic plants expression of the alternative oxidase is induced by a number of stresses including chilling and wounding (see Refs. 2 and 4) and appears to be developmentally regulated (5) in a tissue-specific manner (6). Activity of the alternative oxidase appears to be regulated by a number of systems including the redox state of a sulfhydryl/disulfide linkage (7) and by α-keto acids, in particular pyruvate (8). The role of the alternative oxidase in thermogenesis is well known (4), and in non-thermogenic plants the enzyme is thought to provide an overflow pathway involved in the turnover of trichloroacetic acid cycle intermediates (9) and has also recently been proposed to play a role in the defense mechanism against oxidative stress (10, 11).

cDNA sequences encoding the alternative oxidase have been isolated from a variety of plant species, as well as from fungi and trypanosomes (4). Amino acid comparison shows a high degree of homology especially between the plant sequences. Hydropathy analysis reveals the presence of two hydrophobic regions, thought to be transmembrane α-helices, with hydrophilic regions flanking each side and extending into the mitochondrial matrix (1, 12). Sequence analysis shows the presence of three copies of an iron-binding motif (Glu-X-X-His), two of which are located in the carboxyl-terminal hydrophilic domain of the protein at the base of the second transmembrane helix (13, 14). These two motifs correspond to those found in the class I group of di-iron-carboxylate proteins such as R2 (R2 subunit of ribonucleotide reductase) and MMO. 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 of the alternative oxidase has been proposed (13, 14). In this hypothetical structure (Fig. 1) the active site contains a coupled binuclear iron center.

We have previously reported on the first successful functional expression of the Sauromatumguttatum alternative oxidase in Schizosaccharomycesspombe mitochondria (15). Expression of the alternative oxidase in S. pombe confers cyanide-insensitive respiration upon these cells with characteristics similar to those observed in plants. Furthermore, kinetic analysis of S. pombe mitochondria reveals that the alternative

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1 The abbreviations used are: SHAM, salicylhydroxamic acid; PCR, polymerase chain reaction; MOPS, 3-(N-morpholino)propanesulfonic acid; AOA, alternative oxidase all - antibodies; MMO, methane monoxygenase.
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FIG. 1. Diagrammatic representation of the liganding pattern for the proposed di-iron center of the alternative oxidase. The residue numbers refer to the amino acid sequence for the unprocessed polypeptide from S. guttatum (17), and the structure is based upon the model previously outlined (13, 14).

oxidase contributes up to 20% toward the total mitochondrial respiratory activity and that its expression decreases the respiratory activity and that its expression decreases 

Experimenal Procedures

Strains and Growth Conditions—The S. pombe strain used was sp011 (ade6–704, leu1–2, ura4–D18, h+). Yeast media and growth conditions were as described previously (15). The Escherichia coli strains JM101 and JM110 were used for amplification of plasmids.

Site-directed Mutagenesis—Overlap extension PCR (16) was used to convert glutamate (GAG) to asparagine (AAT), which also introduced a recognition site for the restriction enzyme BsmI. PCR reactions were carried out in 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 200 ng of each primer, 10 ng of template DNA, and 2 units of Taq DNA polymerase (Boehringer Mannheim) made up to 100 μL and overlaid with mineral oil. Samples were subjected to an initial denaturation period (94 °C, 5 min) and then 30 cycles of denaturation (94 °C, 1 min), annealing (67 °C, 1 min), and extension (72 °C, 1 min) followed by a final extension (72 °C, 3 min) using a TRIO thermoster (Biomera, Maidstone, UK). Primers Sg265 (5’-ACCGCATCTTCTTCCAGCGCGGTTACG-3’) and Sg265 (5’-TAGGAAGGTGATCATTCTCCTCCAGTATG-3’) (alteration underlined) were used to generate a 234-base fragment from plasmid pAO6G81 (17). Primers Sg267 (5’-GGAGGAAATGTTGCACTCCTACACGGAG-3’) (alteration underlined) and T7-plus (5’-CGACTCATAAGGCGGAATTTGGTACC-3’) were used to generate a 595-base fragment from the same plasmid. An overlap extension reaction containing 100–200 ng of purified PCR-generated fragments and primers Sg162 and T7-plus was performed using the conditions described above. The resultant fragment was treated with polynucleotide kinase and ligated to Smal-digested, phosphatase-treated pUC18. The mutation was initially identified by restriction analysis. The mutant fragment was excised on a Sphl-HindIII fragment and ligated to pAO6G81 digested with Spfl and HindIII. The entire cloned fragment was sequenced using the Sequenase kit, version 2.0 (Amersham Pharmacia Biotech). The orientation of the mutant cDNA in the Bluescript vector wasverified by digestion with EcoRI followed by ligase of the resultant fragments. The mutant alternative oxidase on a BspHI-BamHI fragment was ligated to pREP1 (a modified version of pREP1 (18) in which the NdeI site was replaced with NcoI) that had been digested with NcoI and BamHI. The resultant plasmid pREP1-E270N contained the mutant alternative oxidase under control of the nmt1 promoter.

General Molecular Biology Procedures—S. pombe cells were transformed using lithium acetate (19). Other methods were as described by Sambrook et al. (20).

Isolation and Subfractionation of Mitochondria—S. pombe mitochondria were prepared from logarithmic phase 2-liter cultures grown overnight in minimal medium in the presence or absence of thiamine. Mitochondria were isolated as described previously (21) but with the following modifications. After centrifugation at 12,000 × g for 10 min in 4 °C, the mitoplast fraction was resuspended in 10 volumes of shock medium (5 mM EGTA, 1 mM ATP, 10 mM MgCl₂, 5 mM sodium succinate, 10 mM MOPS (pH 6.8), and 0.1% bovine serum albumin) with 0.5% bovine serum albumin. After centrifugation at 12,000 × g for 10 min at 4 °C, the mitoplast fraction was resuspended in 10 volumes of shock medium and passed through a 20 K French Pressure Cell (SLM Instruments, Urbana, IL) at 8,000 kg cm⁻². The mitoplast fraction was removed by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatant fraction, containing inner membrane fragments and matrix proteins, was centrifuged at 150,000 × g for 1 h at 4 °C. The pellet containing inner membrane fragments was resuspended in a minimal amount of shock medium. The supernatant containing the matrix fraction was dried by rotary evaporation and then resuspended in water.

Respiratory Measurements—Respiratory activity was measured polarographically using a Rank oxygen electrode (Rank Brothers, Cambridge, UK) in 2 ml of reaction medium (0.65 mM mannitol, 0.35 mM EGTA, 10 mM MOPS (pH 6.8), 10 mM MgCl₂, 5 mM K₂HPO₄, and 0.3% bovine serum albumin) as described previously (21).

Gel Electrophoresis and Western Blotting—Separation of proteins on SDS-polyacrylamide gels, transfer to nitrocellulose membranes, and probing with antibodies were carried out as described previously (15). Antibodies against the alternative oxidase were raised in mice (23). Antibodies against nHSP70 (heat shock protein of 70 kDa located in the mitochondrial matrix) were raised in rabbit against a synthetic peptide representing ten amino acids from the Pisum sativum mitochondrial protein (24).

Until otherwise stated, all chemicals were obtained from Sigma (Poole, UK).

RESULTS

Site-directed mutagenesis was carried out on the S. gutta- tum alternative oxidase to mutate Glu-270 to asparagine. The presence of the E270N mutation was initially identified by restriction analysis and confirmed by sequencing. Both the wild-type and mutant alternative oxidase were introduced into S. pombe by transformation with pREP1-AOX (15) and

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pREP1-E270N, respectively, with selection for leucine prototrophy.

Fig. 2 shows the results of typical respiratory and Western blot analyses performed with S. pombe mitochondria containing the wild-type or E270N alternative oxidase prepared from cells grown in the presence or absence of thiamine. Respiratory activity measurements (Fig. 2A) indicate that mitochondria from the three types of cells readily oxidized NADH as a respiratory substrate and that all were coupled to oxidative phosphorylation because oxygen uptake rates could be stimulated by 1 mM carbonyl cyanide m-chlorophenylhydrazone. Close scrutiny of the state 4 rates suggests that the NADH-dependent rate was the highest in mitochondria isolated from cells expressing the alternative oxidase protein (Fig. 2A, panel 1). At first glance this may be in accordance with the idea that expression of a heterologous integral membrane protein, such as the alternative oxidase, may result in non-specific effects on membrane proton permeability (25), thereby increasing the state 4 rate in comparison with the control (Fig. 2A, panel 2). Analysis of a number of mitochondrial preparations (n = 21) in which the alternative oxidase was either present (∼thiamine) or absent (+thiamine), however, revealed that this difference in state 4 rates was not significant. Instead it was found that the highest state 4 rate was observed in mitochondria in which the alternative oxidase had not been expressed (Fig. 2A, panel 2).

Fig. 2A (panel 1) also reveals that the respiratory activity of mitochondria isolated from cells containing wild-type oxidase, grown in the absence of thiamine, was partially resistant to the addition of 3.5 μM antimycin A. Subsequent addition of 2.5 μM octyl-gallate totally inhibited this activity, demonstrating that the antimycin-resistant respiration was due to alternative oxidase activity. Respiratory activity of mitochondria isolated from cells grown in the presence of thiamine (Fig. 2A, panel 2) was completely inhibited by antimycin A. Western blot analysis, using AOAbodies, reveals that the alternative oxidase protein was only expressed in mitochondria isolated from cells grown in the absence of thiamine (Fig. 2B).

In contrast to the wild-type alternative oxidase, mitochondria isolated from cells transformed with pREP1-E270N grown in the absence of thiamine did not exhibit any antimycin A-insensitive respiration (Fig. 2A, panel 3). However, it can be seen from Fig. 2B that the alternative oxidase antibodies do recognize a protein (lane 3) that has the same molecular mass as both the wild-type alternative oxidase (lane 1) and a control from Arum maculatum. It is also apparent from Fig. 2B that identical unprocessed forms (15) of both the wild-type and mutant oxidase can be detected (lanes 1 and 3, respectively). It is conceivable that lack of respiratory activity observed with the E270N form of the alternative oxidase (Fig. 2A, panel 3) is not a result of changes in the active site but is merely due to incorrect folding or lack of insertion of the protein into the inner mitochondrial membrane. To establish that the mutant alternative oxidase was correctly targeted to the inner membrane, mitochondria from cells expressing the wild-type or mutated form of the alternative oxidase were subfractionated into inner membrane and matrix fractions. When these fractions were probed with AOAbodies (Fig. 3A) a protein was recognized in the inner membrane fraction from cells expressing the wild-type (lane 2) and the E270N mutant protein (lane 5). However, the AOAbodies did not identify any protein within the matrix fraction from either type of cells (lanes 3 and 6). Probing with mHSP70 antibodies (Fig. 3B), which recognize an HSP70 protein located in the mitochondrial matrix or loosely associated with the inner surface of the inner membrane (24), confirms that matrix fraction did indeed contain protein. It can therefore be concluded that the immunoblots shown in Fig. 3 are consistent with the idea that the E270N mutant protein is targeted to and integrated into the inner mitochondrial membrane in a manner identical to that of wild-type protein. Such results make it highly unlikely that lack of antimycin A-insensitive respiration of the mutant protein (Fig. 2A, panel 3) is due to incorrect insertion of this protein into the inner mitochondrial membrane. Successful incorporation into the inner membrane, in turn, suggests that the E270N mutant protein is correctly folded and that lack of activity is not due to misfolding of the protein.

Fig. 2. Respiratory activity and expression of the wild-type and E270N alternative oxidase in S. pombe mitochondria. Mitochondria were prepared from cells containing the wild-type alternative oxidase (panels 1 and 2) or alternative oxidase with the E270N mutation (panel 3) grown in the absence (−) or presence (+) of thiamine. A, respiratory activity was determined as described under “Experimental Procedures” with additions as indicated: 2 mM NADH, 1 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP), 3.5 μM antimycin A (AA), and 2.5 μM octyl-gallate (OG). Numbers on the trace refer to specific oxygen consumption rates in nmol of O2 min−1 mg protein−1. B, immunoblot of mitochondria from the same samples as in A probed with AOAbodies. The A. maculatum lane refers to mitochondria isolated from spadices of this organism. The position of the molecular mass standard (kDa) is shown on the left.
DISCUSSION

In the present work we have probed the active site of the plant alternative oxidase by site-directed mutagenesis. Although a number of other groups have employed mutagenesis techniques to investigate the plant alternative oxidase, specifically residues involved in inhibitor binding (26) and the role of the conserved cysteines in the regulation of oxidase activity (27), to our knowledge none have used such techniques to investigate the active site of this enzyme.

Although the nature of the active site of the alternative oxidase has remained elusive for a considerable number of years, analysis of a number of cDNAs from a variety of organisms has revealed that the carboxyl-terminal portion of all of the alternative oxidase sequences contains two copies of a Glu-X-X-His iron binding motif (see Refs. 1–4 and 11). Identification of these sequence motifs led to a structural model of the alternative oxidase being proposed in which the catalytic site comprises a binuclear iron center comparable with that found in MMO and R2 (Refs. 13 and 14 and Fig. 1). One of the key residues within the binuclear iron site in both MMO and R2 is a glutamate residue that serves as a bidentate ligand bridging the two iron atoms (28). In the model of the catalytic site of the plant alternative oxidase the bridging carboxylic acid residue is Glu-270 (Fig. 1), which is analogous to Glu-144 of MMO and Glu-115 of R2. In R2 this glutamate residue has previously been demonstrated to be essential for enzyme activity because the mutation severely inhibits iron binding (reducing the amount of bound iron to less than half of that observed with wild-type protein) by eliminating a bridging ligand, thereby resulting in loss of activity (29). The close similarity of the proposed active site of the alternative oxidase to that of R2 would suggest that mutation of the corresponding bridging ligand in the plant oxidase should equally destabilize iron binding and hence inhibit enzyme activity.

To test the model of the active site and the pivotal importance of the bridging carboxylate, we engineered Glu-270 of the S. guttatum alternative oxidase expressed in S. pombe to an asparagine residue. Immunoblots (Figs. 2 and 3) revealed that the E270N mutated form of the alternative oxidase is still effectively targeted to S. pombe mitochondria. As alternative oxidase protein was imported and processed to the same size as wild-type oxidase, such results suggest that the lack of antibiotic-resistant respiration observed with the E270N mutant is not due to the absence of oxidase protein. Because the single difference between the wild-type and mutated form of the alternative oxidase is the conversion of Glu-270 to an asparagine residue, it can be concluded that mutation of this residue has profound inhibitory effects upon the activity of the alternative oxidase. Although we cannot be certain at present, it is highly likely that such inhibitory effects upon enzyme activity are due to the loss of bound iron within the di-iron center, suggesting that this residue is both structurally and functionally important.

It is possible that mutation of a glutamate to an asparagine residue, which results in a loss of a negative charge, could have resulted in, following mitochondrial import and processing, a destabilization of the three-dimensional structure of the protein such that it either did not form an integral membrane protein or interfered with the protein fold of the active site. The subfractionation experiments summarized in Fig. 3 support the idea that the E270N mutant protein is specifically localized within the inner mitochondrial membrane in a manner similar to that observed with wild-type protein. Hence it can be concluded that loss of enzymic activity of the mutated protein is unlikely to be due to lack of integration of the protein within the inner membrane. Furthermore published results suggest that one of the remarkable features of di-iron proteins, such as R2, is the stability of its three-dimensional fold. For instance, crystallography studies have shown that the oxidized and reduced forms of R2 are identical (30). Similarity in fold not only extends to a binuclear manganese form of R2 (31) but also to all mutant structures solved to date (see Ref. 29). Persson et al. (29) concluded from such results that metals contribute very little to the protein fold in R2 and furthermore that tolerance of the protein structure to charge differences and mobility of side chains within the vicinity of the di-iron site should be very high. It is therefore unlikely that lack of enzymic activity of the E270N alternative oxidase reported in this paper is due to disruption of protein fold, particularly because the mutation is located within the four-helix bundle that acts as the scaffold to bind the iron atoms. A more plausible explanation is, as indicated above, that such a mutation eliminates the bridging ligand and thus severely inhibits iron binding.

Berthold (26) has used random mutagenesis to identify residues in the Arabidopsis thaliana alternative oxidase expressed in E. coli, which may be involved in inhibitor binding. Interestingly, two of these mutations are located in helix 1 (see Refs. 13 and 14) of the four-helix bundle and therefore place the binding site for the inhibitor SHAM close to the proposed active site. Because SHAM is thought to interfere with binding of reduced ubiquinone (26), such studies are compelling evidence in favor of the active site being located in the carboxyl-terminal region. Additional studies into the active site are focusing on the role of other carboxylates and the possible role of tyrosine, located in the putative hydrophobic pocket, in oxygen activation.

The yeast heterologous expression system, which we are currently using to answer questions about the structure and function of the plant alternative oxidase can also be extended to allow analysis of the oxidase from other systems. For instance, in view of the close structural similarity and antibody cross-reactivity of the alternative oxidase in trypanosomes (32, 33), the yeast expression system that we have developed may prove extremely useful in screening for inhibitors of trypanosomal alternative oxidase and hence in future pharmaceutical development of anti-parasitic agents.

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