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Characterization of the alternative oxidase protein in the yeast *Hansenula anomala*

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The cyanide-resistant respiratory pathway is induced by respiratory inhibitors in the yeast *Hansenula anomala*. A monoclonal antibody against the alternative oxidase in the higher plant *Sauromatum guttatum* cross-reacted with a 36-kDa mitochondrial protein induced by antimycin A in *H. anomala* and with a protein encoded by a cDNA which was previously cloned for an antimycin A-inducible mRNA in the yeast. There was a similarity in the amino acid sequence between the cDNA-encoded protein and the plant alternative oxidase protein. We propose that the 36-kDa mitochondrial protein encoded by the cDNA is a component of alternative oxidase in *H. anomala*.

Cyanide-resistant respiration; Alternative oxidase; Amino acid sequence similarity; Mitochondria; *Hansenula anomala*

1. INTRODUCTION

Mitochondria from most higher plants, some fungi and trypanosomes contain the cyanide-resistant respiratory pathway that transports electrons directly from ubiquinol to oxygen. Although a cyanide-resistant terminal oxidase called 'alternative oxidase' is thought to be involved in the pathway, little is understood about its exact biochemical nature (for review see [1]).

The cyanide-resistant respiratory activity is induced in the fungus *Neurospora crassa* [2] and the yeast *Hansenula anomala* [3] by agents which inhibit mitochondrial functions. For example, the pathway is induced by treatment with inhibitors of the normal cyanide-sensitive respiratory pathway such as antimycin A in *H. anomala* [3]. This induction of the cyanide-resistant respiratory activity is accompanied by induction of a 36-kDa mitochondrial protein [4–6]. Recently, we cloned a cDNA for an antimycin A-inducible mRNA from the yeast [7]. The mRNA is expressed under conditions that induce the cyanide-resistant respiration, and encodes a 36-kDa mitochondrial protein. Therefore, we have proposed that the 36-kDa protein is involved in the cyanide-resistant respiratory pathway in *H. anomala*.

Duroquinol oxidase showing inhibition characteristics similar to the alternative pathway (cyanide-resistant and SHAM-sensitive) has been purified from the thermogenic plant, *Sauromatum guttatum*, spadix mitochondria [8]. The purified preparation contained three immunologically related proteins of 35, 36 and 37 kDa as major protein species. The amount of these proteins clearly correlates with the alternative oxidase activity in mitochondria, indicating that these proteins are responsible for the alternative pathway [8]. Further, one of monoclonal antibodies against these polypeptides (AOA antibody) reacted with the three proteins and inhibited the duroquinol oxidase activity [9]. It has, therefore, been strongly suggested that these proteins constitute alternative oxidase in *S. guttatum*.

The AOA antibody is very useful in characterization of the alternative oxidase protein, because it reacts with proteins from many higher plant species [9–11] and *N. crassa* [2]. In the present work, we have characterized the alternative oxidase protein in *H. anomala* using the monoclonal antibody against the plant alternative oxidase.

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2. MATERIALS AND METHODS

2.1. *Materials*

A monoclonal antibody against the alternative oxidase from *S. guttatum*, designated AOA [9], was a kind gift from Dr. L. McIntosh, Michigan State University. A monoclonal antibody against *Escherichia coli* β-galactosidase and a horseradish peroxidase-conjugated anti- mouse IgG antibody were products of Promega. ECL Western blotting detection system, *E. coli* β-galactosidase, antimycin A and Immobilon-P membrane were purchased from Amersham, BRL, Sigma and Millipore, respectively.

2.2. *Purification of mitochondria*

Hansenula anomala strain LKBY-1 grown aerobically was treated with antimycin A to induce the cyanide-resistant respiration as described previously [3]. Spheroplasts were prepared, and mitochondria were isolated, as described previously [5].

2.3. *Synthesis of the fusion protein*

Part of the protein encoded by the cloned cDNA for an antimycin

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A-inducible mRNA was expressed in *E. coli* as a hybrid protein fused to β-galactosidase using the λgt11 phage vector. Construction of the recombinant phage and purification of the fusion protein were described previously [7].

2.4. *Western blotting*

Western blotting was performed essentially as described by Hiser and McIntosh [10] except that PVDF membranes and the ECL Western blotting detection system were used.

3. RESULTS

Proteins in mitochondria from *H. anomala* cells, in which the cyanide-resistant respiration was induced by antimycin A, were analyzed by Western blotting with the monoclonal antibody (AOA antibody) against *S. guttatum* alternative oxidase. A polypeptide with a molecular mass of about 36 kDa reacted with the AOA antibody (Fig. 1, lane 2). On the other hand, no immunoreactive protein was detected when mitochondria from the cells exhibiting little cyanide-resistant respiration activity were used (Fig. 1, lane 1).

We previously cloned a cDNA for an mRNA induced by antimycin A in *H. anomala* cells, and prepared a polyclonal antibody against the cDNA-encoded protein synthesized in *E. coli* [7]. It seems that the cDNA encodes a protein involved in the cyanide-resistant respiratory pathway [7]. In addition, Western blotting with our polyclonal antibody and the AOA antibody revealed that a mature form of the protein encoded by our cDNA in mitochondria was the same mobility on an SDS-polyacrylamide gel as the 36-kDa protein reactive to the AOA antibody (data not shown). We therefore

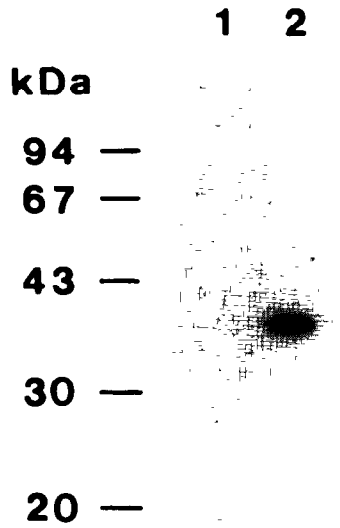

Fig. 1. Western blot analysis of *H. anomala* mitochondrial protein with the AOA monoclonal antibody against the alternative oxidase in *S. guttatum*. Proteins (10 μg) in mitochondria purified from control cells (lane 1) and antimycin A-treated cells (lane 2) were electrophoresed on an 11% polyacrylamide gel containing SDS.

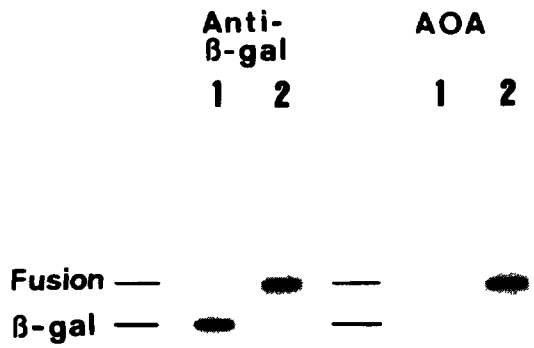

Fig. 2. Western blot analysis of the protein encoded by the cDNA for antimycin A-inducible mRNA with the AOA antibody (right) and the antibody against β-galactosidase (left). Proteins (0.5 μg) were electrophoresed on a 7% polyacrylamide gel containing SDS. Lane 1, *E coli* β-galactosidase; lane 2, the fusion protein prepared as described in Section 2.

tested whether the protein encoded by the cDNA reacts with the AOA antibody; namely, part of the protein, a polypeptide from Asn-12 to Ala-298 in Fig. 3, was expressed in *E. coli* as a fusion protein with β-galactosidase, and the fusion protein was subjected to Western blotting. As shown in Fig. 2, the fused protein reacted with the AOA antibody (lane 2 in the right side panel). Since *E. coli* β-galactosidase did not react with the antibody (Fig. 2, lane 1 in the right side panel), the AOA antibody must have recognized a region encoded by the cDNA.

We further compared the amino acid sequence deduced from the nucleotide sequence of our cDNA [7] with that of the cDNA for a precursor protein of plant alternative oxidase [12]. When the two sequences were aligned as shown in Fig. 3, 130 amino acid residues were identical (38% identity). In particular, a close similarity between the two sequences was observed in the C-terminal half; namely, residues 137–295 in the yeast sequence. In addition, two putative membrane-bound regions (residues 144–155 and 200–219) corresponded to hydrophobic regions of the plant protein (Fig. 3).

4. DISCUSSION

In the present work, we have shown that a monoclonal antibody against a plant alternative oxidase reacts with a 36-kDa protein in the mitochondria in *H. anomala* cells treated with antimycin A and that the protein encoded by a cDNA for an antimycin A-inducible mRNA is reactive with this antibody. Further, there

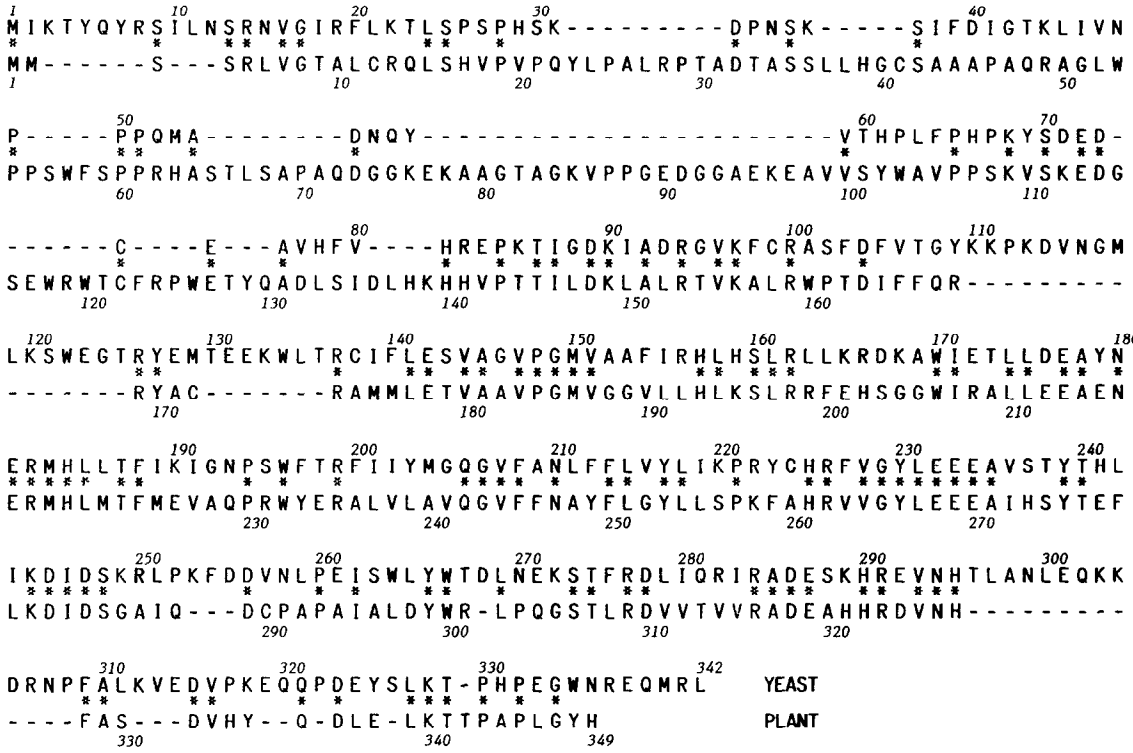

Fig. 3. Comparison between amino acid sequences of the protein encoded by the cDNA and the precursor protein of plant alternative oxidase. Gaps (–) were inserted to make a maximum alignment. Asterisks indicate positions of the same amino acid residues.

is a similarity between the amino acid sequences deduced from the nucleotide sequences of the cDNA for the *H. anomala* protein and the plant alternative oxidase. These results suggest that the 36-kDa mitochondrial protein encoded by the cDNA for antimycin A-inducible mRNA is a component of the alternative oxidase in the yeast.

The role of the three polypeptides in functional alternative oxidase is still remained uncertain in *S. guttatum* [1]. In most cases of higher plants, multiple protein bands in a region of 35–40 kDa have been reported to react with the AOA antibody in Western blotting [9–11]. In *N. crassa*, the AOA antibody reacts with two mitochondrial proteins of 36.5 and 37 kDa, which appeared in cells treated with chloramphenicol to induce the alternative pathway and seem to be derived from a single structural gene by modification [2]. In contrast, our work shows that only a single protein of 36 kDa reacts with the antibody in the case of *H. anomala*. We propose that only one of the immunoreactive proteins in higher plants and *N. crassa*, and not at all at the same time, may be required for the function of the alternative oxidase.

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