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Possible involvement of superoxide anion in the induction of cyanide-resistant respiration in *Hansenula anomala*

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A chemiluminescence study showed that Q_i site inhibitors such as antimycin A induce O_2^- generation in respiring cyanide-sensitive mitochondria from the yeast, *Hansenula anomala*. The O_2^- generation was suppressed by radical scavengers such as flavone, butylated hydroxyanisole, and Co_0. Induction of cyanide-resistant respiration in *H. anomala* cells by Q_i site inhibitors was also inhibited by these radical scavengers. Furthermore, antimycin A-induced synthesis of the mitochondrial 36-kDa protein, which is thought to be the alternative oxidase functional in the cyanide-resistant respiratory pathway, was abolished by the addition of flavone. These observations suggest that O_2^- is somehow involved in the induction of cyanide-resistant respiration.

Mitochondria; Superoxide anion; Chemiluminescence; Cyanide-resistant respiration

1. INTRODUCTION

The electron transfer mechanism of the ubiquinol:cytochrome *c* reductase in mitochondria is described more or less consistently by a Q-cycle scheme [1]. Ubiquinone is re-reduced by a two step mechanism at the Q_i site, where the electron transfer is blocked by antimycin A [1,2]. Antimycin A is known to induce cyanide-resistant respiration in fungi, yeast and *Euglena* [3]. It has been established that the cyanide-resistant respiratory pathway branches off from the normal cyanide-sensitive mitochondrial respiratory chain at the CoQ (ubiquinone) level and involves an alternative oxidase [3,4]. We have reported that the induction of cyanide-resistant respiration by Q_i site inhibitors [5] and sulfur compounds [6] in the yeast *Hansenula anomala* is accompanied by the appearance of a 36-kDa protein in mitochondria, suggesting strongly that this protein is the alternative oxidase [7]. However, virtually nothing is known of the mechanism by which synthesis of the 36-kDa protein is induced by the inducers. The 36-kDa protein is encoded by a nuclear gene [3,8] and is synthesized as a 39-kDa precursor in the cytosol and undergoes processing to the mature form [5]. How is the signal generated in the mitochondrion by the inducers transduced to the nucleus to express the specific gene? A clue to this problem is the fact that the induction requires molecular oxygen [8], and we have suggested the involvement of a certain active oxygen species in the induction [6]. Since it has been reported that the addition of Q_i site inhibitors to respiring mitochondria induces the generation of superoxide anion (O_2^-) [9–11], we decided to study the possible role of O_2^- in the inductions of cyanide-resistant respiration. The results thus obtained suggest that O_2^- is actually involved in the induction.

Abbreviations: MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one; SOD, superoxide dismutase; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

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2. MATERIALS AND METHODS

2.1. *Materials*

SOD from bovine erythrocyte and antimycin A were purchased from Sigma, and MCLA from Tokyo Kasei Co., Tokyo, Japan.

2.2. *Preparation of mitochondria*

Cyanide-sensitive mitochondria were prepared from freshly harvested cells of *H. anomala*, as previously described [5]. These mitochondria retained clear respiratory control response to added ADP. For the chemiluminescence study, the mitochondria fraction was suspended in 0.6 M sorbitol, 5 mM EDTA, and 0.2 mM phenylmethanesulfonylfluoride (adjusted to pH 7.4 with Tris, 22.4 mg protein/ml), and added to the assay mixture described below.

2.3. *Analytical methods*

Cyanide-resistant respiration activity of the cells was determined as previously described [8]. Sodium dodecylsulfate (SDS)-polyacrylamide slab gel electrophoresis, determination of apparent molecular mass, and autoradiography were carried out as described [5].

2.4. *Estimation of O_2^- generation*

Since microsomal and mitochondrial fractions contain cytochrome *c*-reducing systems, the conventional cytochrome *c* method for the detection of superoxide anion cannot be used. The sensitivity of the chemiluminescence method [10,12] used in this study is about 10 times

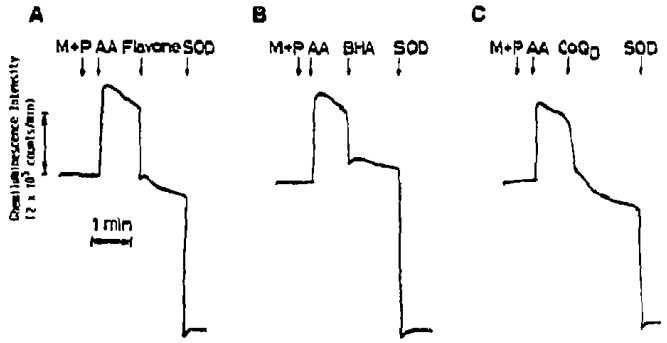

Fig. 1. Effects of antioxidants on the antimycin A-dependent O_2^- generation in the mitochondria. The cyanide-sensitive mitochondria (0.112 mg) were used. M + P, 7.5 mM malate plus 7.5 mM pyruvate; AA, 20 μM antimycin A; SOD, 0.5 μM; flavone, 0.1 mM; BHT, 0.1 mM; CoQ_0, 0.1 mM.

higher than that of the adrenochrome method, which is widely used for the detection of O_2^- in mitochondrial systems [9]. Differing from ESR [11], the detection can be carried out under just similar conditions to those for the assay of respiration activity.

The mitochondrial fraction (around 0.1 mg protein) suspended in 1 ml of 0.3 M sucrose, 10 mM Tris, 10 mM potassium phosphate, 10 mM potassium chloride, 0.05 mM $MgCl_2$, and 4 μM MCLA (pH 7.0 with Tris) was incubated at 30°C in a chemiluminescence reader (Aloka, BLR-102). Substrates and reagents were added as indicated in the figure legend.

3. RESULTS AND DISCUSSION

As shown in Fig. 1, MCLA-dependent chemiluminescence in the cyanide-sensitive mitochondria system was increased by the addition of antimycin A in the presence of malate plus pyruvate as respiratory substrate. In all cases shown in Fig. 1, the chemiluminescence was significantly suppressed by SOD, indicating that the luminescence is originated from an O_2^--mediated reaction. Other Q_i site inhibitors, such as funiculosin and heptylhydroquinoline-*N*-oxide, also showed similar effects (data no shown). The generation of O_2^- in mitochondria has so far been detected only with submitochondrial particles [9,10] or in the presence of an uncoupler [11]. This is the first report to identify the O_2^- generation in the almost intact mitochondria retaining respiratory control. Subsequent addition of flavone, BHA, and CoQ_0 (0.1 mM) significantly repressed the antimycin A-dependent O_2^- generation in mitochondria. Similar results were obtained using flavanone, BHT, and CoQ_{10} (data not shown).

Table I

Effects of radical scavengers on the antimycin A-dependent induction of cyanide-resistant respiration

Additions	CN^--resistant respiration (nmol O_2/min/mg)
None	0.04
10 μM antimycin A	4.16
10 μM antimycin A + 1 mM flavone	0.32
10 μM antimycin A + 1 mM flavanone	0.76
10 μM antimycin A + 1 mM BHA	1.45
10 μM antimycin A + 1 mM BHT	3.39
10 μM antimycin A + 1 mM CoQ_0	2.83
10 μM antimycin A + 1 mM CoQ_{10}	2.95

Five ml of freshly harvested cell suspension (52.4 mg wet cells/ml) in 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose with or without the additions as indicated was shaken under air at 30°C for 60 min. Fifty-μl samples were withdrawn and assayed for cyanide-resistant respiration activity in a 1-ml glass chamber.

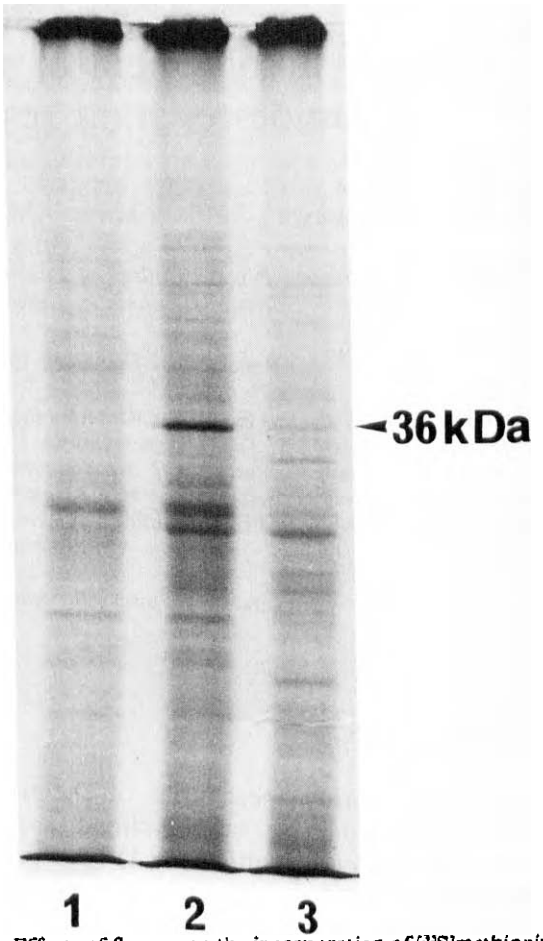

Fig. 2. Effects of flavone on the incorporation of [^{35}S]methionine into mitochondrial proteins in the presence of antimycin A. To 20 ml of a cell suspension (A_{600} = 25) in 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose was added 10 μM antimycin A in the presence and absence of 1 mM flavone, and the mixture was aerobically shaken at 30°C. After 35 min, the cells were pulse-labeled with [^{35}S]methionine (9.25 MBq), and 20 min later chased with cold methionine (1 mM). The mitochondrial sample isolated as described [5] (50 μg protein) was subjected to SDS-polyacrylamide slab gel electrophoresis (11% gel), followed by autoradiography for 2 days. Lane 1, no addition; lane 2, antimycin A; lane 3, antimycin A and flavone.

As previously reported [6], radical scavengers such as flavone inhibited the induction of cyanide-resistant respiration. As shown in Table I, both flavone and flavanone (1 mM) strongly inhibited the antimycin A-dependent induction of cyanide-resistant respiration. An-

tioxidants such as BHA, BHT, CoQ_0, and CoQ_{10} also inhibited the induction to a lesser extent. The inhibitory effect of flavone on the induction is not due to the blockade of the electron transport to ubiquinone, since 1 mM flavone had no effect on the cyanide-sensitive O_2 uptake in the mitochondria using malate plus pyruvate as a substrate, unlike the situation in higher plant mitochondria [13].

Then, the effect of flavone on the biosynthesis of the 36-kDa protein was examined (Fig. 2). In the presence of antimycin A, the nuclear gene was expressed followed by the de novo synthesis of the 39-kDa precursor protein, which is converted into the 36-kDa mature form in the mitochondria [5]. The cells were pulse-labeled for 20 min with [^{35}S]methionine and the mitochondria isolated from those cells were analyzed by SDS-polyacrylamide slab gel electrophoresis and subsequent autoradiography. When the cells were incubated with antimycin A, the 36-kDa protein appeared in the mitochondria (Fig. 2, lane 2), whereas simultaneous addition of flavone caused a significant decrease of this protein (lane 3), indicating that flavone specifically inhibits the biosynthesis of the 36-kDa protein. Accordingly, flavone is deduced to block a certain step of the signal transduction before the expression of the gene by radical scavenging action [14].

From the above results, O_2^- generated in the mitochondria in the presence of Q_i site inhibitors is suggested to be involved in the induction mechanism of cyanide-resistant respiration. Therefore, O_2^- might play a role in the signal formation toward nuclei to express the alternative oxidase gene. The report on a possible involvement of active oxygen species in the signal transduction mechanism from mitochondria toward nuclei is without precedent. However, recently a role of reactive oxygen radicals as widely used messengers has been postulated [15] and several lines of evidence support this fact. Further investigation is in progress to elucidate the detailed O_2^--involved signal transduction mechanism to express the alternative oxidase gene.

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