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A 36-kDa mitochondrial protein is responsible for cyanide-resistant respiration in *Hansenula anomala*

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Antimycin A-dependent induction of cyanide-resistant respiration in *Hansenula anomala* was reversibly blocked by carbonylcyanide-*m*-chlorophenylhydrazone (CCCP). When the cells were pulse-labeled with [^{35}S]methionine in the presence of both antimycin A and CCCP, the radioactivity was incorporated into a 39 kDa mitochondrial protein. Upon removal of CCCP, this protein was processed into a 36 kDa form. The increase in the 36 kDa protein completely paralleled that in cyanide-resistant respiration activity, suggesting that the 39 kDa protein is the precursor of the 36 kDa protein, which is responsible for cyanide-resistant respiration.

Cyanide-resistant respiration; Alternative oxidase; Mitochondria; Precursor; Protein import

1. INTRODUCTION

Cyanide-resistant respiration has been widely found in higher plants, algae, fungi, some bacteria, and *Trypanosoma brucei brucei* [1,2]. This cyanide-resistant O_2 uptake constitutes a pathway 'alternative' to the main cyanide-sensitive cytochrome pathway. At present biochemical characterization of the cyanide-resistant oxidase ('alternative oxidase') remains incomplete [3–5] and there is still doubt as to the substantiality of the oxidase [6]. We reported the induction of cyanide-resistant respiration in the presence of respiratory inhibitors in the ascomycetous yeast, *Hansenula anomala* [7]. Cycloheximide completely blocked this induction, indicating the involvement of cytosolic de novo protein synthesis in the induction process. We have also shown that antimycin A-dependent induction of cyanide-resistant respiration in *H. anomala* is accompanied by the appearance of a 37-kDa protein in particulate fraction [8]. This protein has been demonstrated to be localized in mitochondria (Sakajo, S. et al., unpublished data).

In this study we report that a 39 kDa precursor of the 36-kDa protein accumulates in the presence of CCCP, which blocks the import of proteins into mitochondria [9,10], and that its processing to the 36 kDa form parallels the expression of cyanide-resistant respiration activity.

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; BSA, bovine serum albumin; PMSF, phenylmethane sulfonylfluoride

2. MATERIALS AND METHODS

2.1. *Materials*

Antimycin A and CCCP were purchased from Sigma, zymolyase 100T from Seikagaku Kogyo, and [^{35}S]methionine (37 TBq/mmol) from Amersham. *Hansenula anomala* LKBY-1 was grown and harvested as described [7].

2.2. *Preparation of spheroplasts and mitochondria*

Cells (ca. 18 g wet weight) were suspended in 80 ml of 20 mM potassium phosphate buffer (pH 7.4) containing 1.2 M sorbitol, 2 mM $MgCl_2$, 1 mM dithiothreitol and 0.15 mg/ml of zymolyase 100T, and shaken gently at 30°C for 30 min. The resultant spheroplasts were washed twice with 1.2 M sorbitol, collected by centrifugation, and suspended in 20 mM HEPES-KOH buffer (pH 7.5) containing 0.4 M mannitol, 5 mM EDTA, 0.1% BSA, and 0.2 mM PMSF. When necessary the suspension was homogenized, and the homogenate was centrifuged at $3000 \times g$ for 6 min to obtain a lysate. Crude mitochondria were precipitated from the lysate by centrifugation at $10000 \times g$ for 10 min.

2.3. *Analytical methods*

Cyanide-resistant respiration activity was determined as reported [7]. Sodium dodecyl sulfate slab gel electrophoresis, determination of apparent molecular mass, and autoradiography were carried out as described previously [8].

3. RESULTS AND DISCUSSION

At an uncoupling concentration (20 μM), CCCP inhibited the growth of *H. anomala* only by about 20% and had no effect on cyanide-sensitive and -resistant oxygen uptake. As shown in Fig. 1, the antimycin A-dependent induction of cyanide-resistant respiration was completely inhibited by simultaneous addition of CCCP. When added during the course of induction, CCCP caused immediate cessation of further induction of cyanide-resistant respiration. This action is thought to be reversible [11]. An interesting finding was that,

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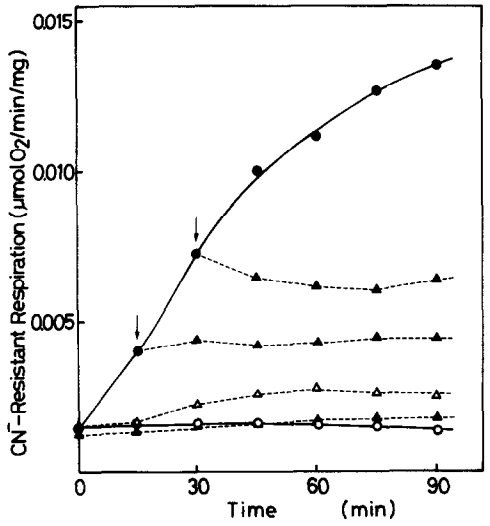

Fig. 1. Effect of CCCP on the induction of cyanide-resistant respiration. Ten ml of cell suspension (52.4 mg wet cell/ml) in 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose with or without 10 μM antimycin A was aerobically shaken at 30°C. At the indicated time point, an aliquot was withdrawn and assayed for cyanide-resistant respiration activity. CCCP (20 μM) was added at the time indicated by an arrow. ○, no addition; ●, antimycin A; △, CCCP; ▲, antimycin A and CCCP. Solid and dashed lines indicate the incubation in the absence and presence of CCCP, respectively.

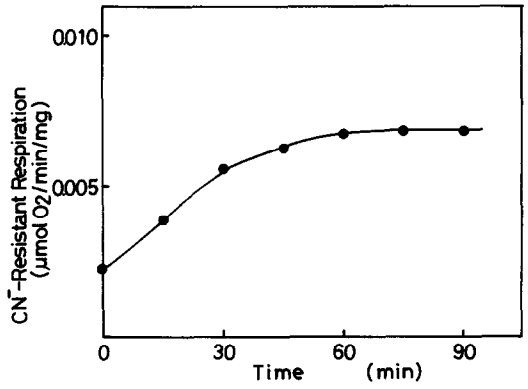

Fig. 2. Increase in cyanide-resistant respiration activity in the presence of cycloheximide after removal of CCCP. Cycloheximide (20 μM) was added to the cell suspension after the incubation for 45 min with antimycin A and CCCP as described in Fig. 1 followed by centrifugation. The cells thus sedimented were resuspended in 10 ml of 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose, 10 μM antimycin A, and 20 μM cycloheximide, and the suspension was shaken aerobically at 30°C. At indicated time point, an aliquot was withdrawn for determination of cyanide-resistant respiration activity.

upon removal of CCCP, cyanide-resistant respiration activity of the cells that had been incubated with both antimycin A and CCCP increased even in the presence of 20 μM cycloheximide, a potent inhibitor of the induction [7]. As shown in Fig. 2, this 'induction' in the presence of cycloheximide continued for about 60 min and then the activity reached a plateau, corresponding to 70% of the activity attained in the cells incubated for 45 min in the presence of antimycin A alone (see Fig. 1). These results suggested that an inactive component accumulating in the cells during the incubation with both antimycin A and CCCP is converted to an active one upon removal of CCCP, in the absence of de novo protein synthesis. It was tempting to assume that this active component is the 36 kDa mitochondrial protein, which has been implicated as a component of the cyanide-resistant respiratory pathway [8].

The antimycin A-dependent induction of cyanide-resistant respiration took place similarly in spheroplasts suspended in the induction buffer containing 1.2 M sorbitol and this induction was completely and reversibly inhibited by CCCP (data not shown). To make small-scale preparation of mitochondria simple and reproducible, we used spheroplasts in the following experiments. When spheroplasts were incubated with antimycin A and pulse-labeled with [^{35}S]methionine, the radioactive 36 kDa protein appeared in mitochondria (Fig. 3, lane 2) in confirmation of our previous finding [8], whereas simultaneous addition of CCCP resulted in the incorporation of radioactive methionine into a 39

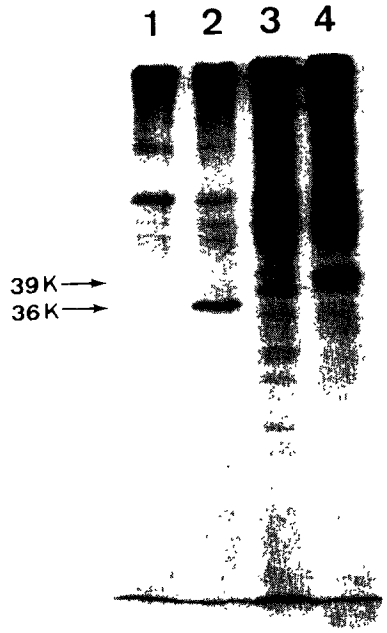

Fig. 3. Incorporation of [^{35}S]methionine into mitochondrial proteins in the presence of antimycin A and/or CCCP. To 20 ml of a spheroplast suspension (A_{600} = 12.5) in 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose and 1.2 M sorbitol was added either 10 μM antimycin A, 20 μM CCCP, or both, and the mixture was aerobically shaken at 30°C. After 35 min, the spheoplasts were pulse-labeled with [^{35}S]methionine (1.85 MBq), and 10 min later chased with cold methionine (1 mM). The mitochondrial sample isolated from the spheroplasts (60 μg protein) was subjected to SDS-PAGE (11% gel), followed by autoradiography for 6 days. Lane 1, no addition; 2, antimycin A; 3, CCCP; 4, antimycin A and CCCP.

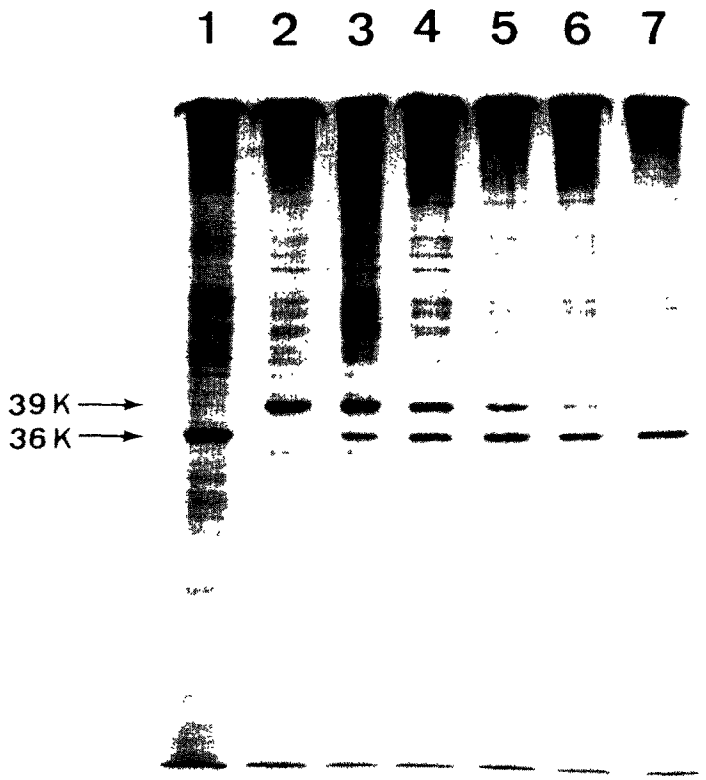

Fig. 4. Effect of the removal of CCCP on the pulse-labeled spheroplasts in the presence of antimycin A and CCCP. A spheroplast suspension (25 ml, $A_{600} = 12.5$) was incubated at 30°C with antimycin A and CCCP, labeled and chased as in Fig. 3. After the addition of 20 μM cycloheximide, the spheroplasts were collected, resuspended in 25 ml of 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose, 1.2 M sorbitol, and 20 μM cycloheximide, and then shaken at 30°C. At indicated time point 5 ml of the suspension was withdrawn to prepare mitochondria. Each 30 μg protein was applied to SDS-PAGE followed by autoradiography. Lanes 1 and 2 correspond to lanes 2 and 4 in Fig. 3, respectively. Lane 3, time 0; 4, 15 min; 5, 30 min; 6, 45 min; 7, 60 min.

kDa band (lane 4). Neither the radioactive 36 kDa nor the 39 kDa band was detected in control incubation without any addition (lane 1) and with CCCP alone (lane 3). In all cases the 39 kDa and 36 kDa bands could not be detected in the cytosolic fraction. The pulse-labeled spheroplasts that had been incubated with both antimycin A and CCCP were washed and resuspended in the CCCP-free buffer containing cycloheximide. As shown in Fig. 4, upon incubation of this suspension the accumulated 39 kDa protein was time-dependently converted to the 36 kDa protein.

CCCP dissipates mitochondrial membrane potential and thus blocks the import of precursor protein synthesized in the cytosol into mitochondria [9,10]. The accumulated precursors remain bound to proteinous receptors on the outer membrane [10]. Upon removal of CCCP the precursors are imported into mitochondria and converted to the mature form by the action of a processing peptidase [9,10]. From the behavior of the 39 kDa protein described above, it can be concluded that in the presence of antimycin A the 39 kDa precursor is synthesized de novo in the cytosol and then imported into mitochondria during which the processing to the 36-kDa mature form occurs. A comparison of the results in Figs. 2 and 4 demonstrates a good correlation between the increase in cyanide-resistant respiration activity and that in the amount of 36 kDa protein, further supporting the view that this protein is responsible for cyanide-resistant oxygen uptake, that is, the 'alternative oxidase'. It is of interest to note that in cyanide-resistant mitochondria from *Neurospora crassa* 36.5 and 37 kDa polypeptides were recognized by a monoclonal antibody to duroquinol oxidase of *Sauromatum guttatum* [12].

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