Essential role of ferrous iron in cyanide-resistant respiration in *Hansenula anomala*

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Antimycin A-dependent induction of cyanide-resistant respiration in *Hansenula anomala* was completely blocked by *o*-phenanthroline, α,α'-dipyridyl, or 8-hydroxyquinoline. Pulse-labeling of the cells with [^{35}S]methionine in the presence of both antimycin A and *o*-phenanthroline indicated that the 36-kDa protein previously reported to be involved in cyanide-resistant respiration [(1989) J Biochem 105, 864–866] was formed in mitochondria even under these conditions. The addition of Fe^{2+}, but not Fe^{3+}, ions to these cells in the presence of cycloheximide resulted in the rapid expression of cyanide-resistant respiration activity. These results suggest that in the presence of both antimycin A and *o*-phenanthroline an inactive form of the 36-kDa protein was formed and Fe^{2+} ions converted it to the active form. It is also likely that Fe^{2+} ions are involved in the reaction mechanism of cyanide-resistant respiration.

Cyanide-resistant respiration; Ferrous ion; Chelator

1. INTRODUCTION

Cyanide-resistant respiration, which is catalyzed by a respiratory system 'alternative' to the main cyanide-sensitive cytochrome pathway, has been reported in a variety of organisms [1,2]. Currently, biochemical characterization of the cyanide-resistant oxidase involved in this respiration is still incomplete [3–6] and virtually nothing is known of its molecular properties. We have reported the induction of cyanide-resistant respiration in *Hansenula anomala* both in the presence [7,8] and absence [9] of respiratory inhibitors. We have also provided evidence that a mitochondrial 36-kDa protein is responsible for cyanide-resistant respiration in *H. anomala* [10–12].

This paper reports that in the presence of both antimycin A and *o*-phenanthroline an inactive form of the mitochondrial 36-kDa protein is synthesized de novo in *H. anomala* cells and that incubation of these cells with Fe^{2+}, but not Fe^{3+}, ions leads to the expression of cyanide-resistant respiration, indicating the involvement of Fe^{2+} ions in this respiration.

2. MATERIALS AND METHODS

2.1. *Materials*

Antimycin A and cycloheximide were purchased from Sigma. *o*-Phenanthroline was obtained from Wako Pure Chemical Industries and [^{35}S]methionine (37 TBq/mmol) from American Radiolabeled Chemicals. *Hansenula anomala* LKBY-1 was grown and harvested as described [7].

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2.2 *Preparation of mitochondria*

The mitochondrial fraction was prepared from *H anomala* as previously reported [10] except that 20 mM Hepes-KOH buffer (pH 7.4) containing 0.6 M sorbitol, 5 mM EDTA, 0.5% bovine serum albumin and 0.2 mM phenylmethane sulfonylfluoride was used for the disruption of spheroplasts.

2.3 *Analytical methods*

Cyanide-resistant respiration activity was determined as reported [7] using 1-ml chambers. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis, determination of apparent molecular mass, and autoradiography were carried out as described [11].

3. RESULTS AND DISCUSSION

The addition of *o*-phenanthroline (0.1 mM), a specific chelator for Fe^{2+}, to the assay mixture did not affect both cyanide-sensitive and -resistant respiration in *H. anomala* (data not shown). However, the chelator (0.1 mM) inhibited the antimycin A-dependent induction of cyanide-resistant respiration in this organism completely (Table I). No induction took place either in the presence of α,α'-dipyridyl or 8-hydroxyquinoline, which also chelate Fe^{2+} ions, but tiron, a specific chelator for Fe^{3+} ions, had no effect (data not shown). These findings suggested the involvement of Fe^{2+} ions in the induction.

Our previous work has shown that the antimycin A-dependent induction of cyanide-resistant respiration in *H. anomala* is accompanied by the synthesis of a 39-kDa protein in the cytosol, which is then imported into mitochondria and processed to a 36-kDa protein [10]. Evidence has also been reported that the 36-kDa protein is directly involved in cyanide-resistant respira-

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Table I

Effect of *o*-phenanthroline on the antimycin A-dependent induction of cyanide-resistant respiration in *H. anomala*

Additions	CN^--resistant respiration (nmol O_2/min/mg wet cells)
None	0
Antimycin A	10.6
Antimycin A + *o*-phenanthroline	0
Antimycin A + *o*-phenanthroline + cycloheximide	0

Five ml of cell suspension (A_{600} = 25, 52.4 mg wet cell/ml) in 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose with or without the addition of 10 μM antimycin A, 0.1 mM *o*-phenanthroline, and 20 μM cycloheximide as indicated was aerobically shaken at 30°C for 60 min, and then 20 μM cycloheximide was supplemented. Fifty μl was withdrawn for the determination of cyanide-resistant O_2 uptake activity

tion [11,12]. It was, therefore, of interest to examine the effect of *o*-phenanthroline on the biosynthesis of the 36-kDa protein. For this purpose, the cells were pulse-labeled for 10 min with [^{35}S]methionine in the presence of both antimycin A and *o*-phenanthroline. Mitochondria were then isolated from the cells and analyzed by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography. It was thus found that during the preparation of mitochondria the 36-kDa protein had been formed in mitochondria even under these conditions (Fig. 1, lane 3), as was the case for the cells incubated with antimycin A alone (lane 2). Consequently, it was evident that *o*-phenanthroline inhibited neither the synthesis of the 39-kDa protein nor its processing to the 36-kDa form. The observation that these cells did not exhibit cyanide-resistant respiration activity suggested that the 36-kDa protein produced here is an inactive form and Fe^{2+} ions are required for its conversion to the active form.

To test this possibility, Fe^{2+} ions were added to the washed cells that had been incubated with both antimycin A and *o*-phenanthroline. It was thus found that cyanide-resistant respiration activity did emerge even in the presence of cycloheximide, a potent inhibitor of cytosolic protein synthesis (Table II), indicating that de novo protein synthesis is not required for this activation. The emerged activity corresponded to 76% of that attainable in the cells incubated with antimycin A alone (cf. Table I). The addition of Fe^{2+} ions to the cells that had been incubated with not only antimycin A and *o*-phenanthroline but also cycloheximide did not result in the emergence of cyanide-resistant respiration activity (Table II), because cycloheximide inhibited the synthesis of the 39-kDa (and hence 36-kDa) protein. The Fe^{2+}-induced activation was temperature-dependent and hardly occurred at 0°C. At 30°C the addition of Fe^{2+} caused an immediate increase in the activity,

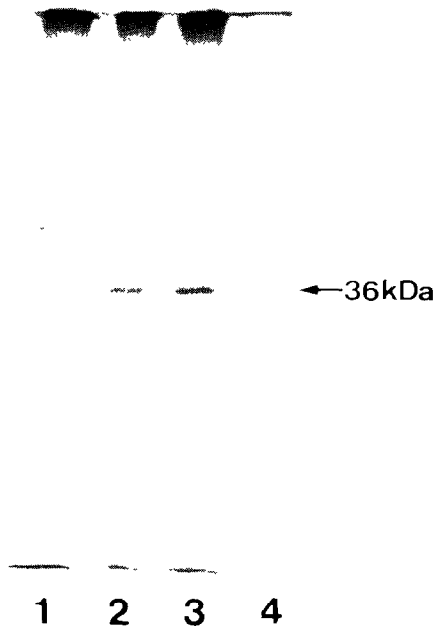

Fig. 1. Incorporation of [^{35}S]methionine into the mitochondrial proteins in the presence of antimycin A and *o*-phenanthroline. To 10 ml of a cell suspension (A_{600} = 25) in 45 mM potassium phosphate buffer (pH 6.5) containing 0.1 M glucose was added 10 μM antimycin A, 0.1 mM *o*-phenanthroline and/or 20 μM cycloheximide as indicated below, and the mixture was aerobically shaken at 30°C. After 35 min, the cells were pulse-labeled with [^{35}S]methionine (0.93 MBq), and 10 min later cold methionine (1 mM) and 20 μM cycloheximide were added, followed by the preparation of mitochondria, which included incubation with Zymolyase 100T at 30°C for 30 min. The isolated mitochondrial sample (60 μg protein) was subjected to SDS-PAGE (11% gel), followed by autoradiography for 7 days. Lane 1, no addition; 2, antimycin A; 3, antimycin A and *o*-phenanthroline; 4, antimycin A, *o*-phenanthroline and cycloheximide.

which reached a plateau in 30–40 min (data not shown). It is to be noted that Fe^{3+} ions did not cause the activation.

It can be concluded that even in the presence of *o*-phenanthroline antimycin A induces the synthesis of

Table II

Effects of ferrous and ferric ions on the cells preincubated with *o*-phenanthroline and antimycin A in the presence or absence of cycloheximide

Pre-incubation	CN^--resistant respiration (nmol O_2/min/mg wet cells)		
	None	Fe^{2+}	Fe^{3+}
Antimycin A + *o*-phenanthroline	0	8.05	0
Antimycin A + *o*-phenanthroline + cycloheximide	0	0	0

One ml of the cell suspension pretreated as indicated in Fig. 1 was shaken at 30°C under air for 45 min with or without the addition of 5 μl of 100 mM $FeSO_4$ or $FeCl_3$ dissolved in 0.01 N HCl. Fifty μl was withdrawn and assayed for the activity

the 39-kDa precursor of the putative alternative oxidase and that the chelator does not inhibit its processing to the 36-kDa mature form, which is, however, enzymatically inactive. The results described above indicate further that Fe^{2+}, but not Fe^{3+}, ions are required for the activation of the inactive form of the 36-kDa protein. This is the first clear indication that Fe^{2+} ions play an essential role in cyanide-resistant respiration. Although Henry et al. [13] have suggested the involvement of iron in biogenesis of the cyanide-resistant respiratory system in the yeast *Saccharomycopsis lipolytica*, their results are at variance with the present report. Only Fe^{3+}, but not Fe^{2+}, ions could activate the inactive protein component, which was not visibly demonstrated but presumed. Lambowitz et al. [14] have recently reported that a mutant of *Neurospora crassa* strain 7301, which is incapable of developing cyanide-resistant respiration activity, contains polypeptides (37 and 36.5 kDa) that can be recognized by a monoclonal antibody to *Sauromatum guttatum* duroquinol oxidase. It is likely that these polypeptides are counterparts of the inactive form of the 36 kDa protein in *H. anomala*. Probably this mutant has a defect in the access of Fe^{2+} to the inactive form of the putative alternative oxidase. Interestingly, we found that α-naphthoquinoline and *m*-phenanthroline, potent inhibitors of non-heme iron-containing metapyrocatechase from *Pseudomonas arvilla* [15], strongly inhibited cyanide-resistant oxygen uptake in *H. anomala* (data not shown).

Finally, we do not yet know the mechanism by which Fe^{2+} ions activate the inactive form of the 36 kDa protein. When α,α'-dipyridyl or 8-hydroxyquinoline was used in place of *o*-phenanthroline, a slight emergence of cyanide-resistant respiration activity was observed by the addition of Fe^{3+} ions, suggesting the possibility that Fe^{3+} ions cause the activation after the reduction within the cell, namely only Fe^{2+} ions have access to the 36 kDa protein. In any case, further studies are still needed to elucidate the molecular and enzymological properties of this putative alternative oxidase.

REFERENCES

[1] Lance, C., Chauveau, M. and Dezengremel, P. (1985) in: Higher Plant Cell Respiration (Douce, R and Day, D.A. eds) pp. 202–247, Springer, Berlin.

[2] Clarkson, A.B , Bienen, E.J., Pollakis, G. and Grady, R.W. (1989) J. Biol. Chem. 264, 17770–17776.

[3] Berthold, D.A., Fluke, D J. and Siedow, J.N. (1988) Biochem. J. 252, 73–77.

[4] Elthon, T.E and McIntosh, L (1987) Proc Natl Acad. Sci. USA 84, 8399–8403

[5] Rasmusson, A.G., Møller, I M. and Palmer, J.M. (1990) FEBS Lett. 259, 311–314.

[6] Rustin, P. (1987) in: Plant Mitochondria (Moore, A I. and Beechey, R.B. eds) pp 37–46, Plenum, New York.

[7] Minagawa, N and Yoshimoto, A. (1987) J. Biochem. 101, 1141–1146.

[8] Minagawa, N. and Yoshimoto, A (1989) Agric Biol. Chem. 53, 2025–2026.

[9] Minagawa, N. and Yoshimoto, A. (1987) Agric. Biol. Chem. 51, 2263–2265.

[10] Minagawa, N., Sakajo, S., Komiyama, T. and Yoshimoto, A. (1990) FEBS Lett., in press.

[11] Yoshimoto, A., Sakajo, S., Minagawa, T. and Komiyama, T. (1989) J. Biochem. 105, 864–866.

[12] Sakajo, S., Minagawa, N , Komiyama, T. and Yoshimoto, A. (1990) J. Biochem., in press.

[13] Henry, M.-F., Bonner W.D and Nyns, E.J. (1977) Biochim. Biophys. Acta 460, 94–100.

[14] Lambowitz, A.M , Sabourin, J R., Bertrand,H., Nickels, R., and McIntosh, L. (1989) Mol. Cell Biol. 9, 1362–1364.

[15] Nozaki, M., Ono, K., Nakazawa, T , Kotani, S. and Hayaishi, O. (1968) J. Biol. Chem 243, 2682–2690.