STUDIES ON SITE I PHOSPHORYLATION, EPR DETECTABLE IRON-SULFUR PROTEINS, AND PIERICIDIN A SENSITIVITY IN THE IN VIVO INDUCTION SYSTEM OF CANDIDA UTILIS CELLS

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SUMMARY Site I phosphorylation was induced in vivo by aerating C. utilis cells grown under iron-deficient conditions. This induction process was inhibited by cycloheximide. In parallel with Site I phosphorylation, EPR detectable iron-sulfur proteins (at least Center 1 and 2) and piericidin A sensitivity were induced and the induction of these features was also inhibited by cycloheximide. These observations further suggest their close correlation with Site I energy conservation mechanism.

INTRODUCTION

Ohnishi and Chance (1) have suggested a close correlation between Site I phosphorylation and iron-sulfur protein(s) responsible for the "g = 1.94" EPR signal in the NADH dehydrogenase region, from studies using C. utilis cells grown at varying iron concentrations and cells before and after in vivo induction of Site I phosphorylation. In contrast, Garland and his associates reported that iron-sulfur protein responsible for the "g = 1.94" signal is not needed for the occurrence of Site I phosphorylation from the following two observations: 1) when C. utilis cells which have been grown under iron-limited conditions are aerated in the presence of a cytoplasmic protein synthesis inhibitor, cycloheximide, Site I phosphorylation is induced in the absence of both the "g = 1.94" EPR signal and piericidin A sensitivity (2). 2) continuous culture of C. utilis cells at the transition from iron-limited to glycerol-limited growth results in the cells where mitochondria possess Site I phosphorylation and lack "g = 1.94" EPR signal due to iron-sulfur protein in the NADH dehydrogenase region (3). Garland and his coworkers suggested that a small fraction of the non-heme iron proteins which does not show EPR signals may play a role in Site I phosphorylation. From EPR studies at temperatures below 77°K, Ohnishi

et al. (4,5) have recently demonstrated the existence of multiple species of EPR signals due to at least three iron-sulfur components (Centers 2, 3 and 4) in addition to iron-sulfur Center 1 responsible for the "g = 1.94" signal in the Site I region of the respiratory chain in C. utilis mitochondria, analogous to those found in the mammalian systems (6,7). Our previous study (1) has been further extended to Site I induction in the presence and absence of cycloheximide, in connection with the induction of EPR signals detectable at 77 and 19°K. In contrast to Garland's group, we have reaffirmed a close correlation between EPR detectable iron-sulfur protein(s) and Site I energy conservation.

MATERIALS AND METHODS

C. utilis cells were grown in batch culture with a low aeration system (0.75 l air per min per liter culture medium) as described previously (1). Mitochondria were prepared by a combined enzymatic (8,9) and mechanical procedure (10). Submitochondrial particles were prepared by sonication of mitochondria for 30 sec. in 0.3 M mannitol, 20 mM Tris-maleate buffer (pH 7.2), 5 mM K-phosphate buffer (pH 7.2) and 1 mM $MgCl_2$. The respiration was measured with a Clark oxygen electrode. The phosphorylation efficiency of mitochondria was measured according to Chance and Williams (11). EPR spectra were recorded with a Varian X band spectrometer, V 4502-13 at 77 and 19°K The temperature 19°K was obtained by cooling samples with a stream of cold helium gas derived from boiling liquid helium. A thermocouple, gold-cobalt alloy versus platinum, was used for temperature measurements. Reduction level of endogenous pyridine nucleotide in C. utilis mitochondria was measured by Aminco-Chance differential fluorometer. Iron concentration in the culture medium was determined spectrophotometrically in a cuvette with 5 cm light path, using o-phenanthroline (12). Protein concentration of mitochondria and submitochondrial particles were determined according to Krüger and Klingenberg (13).

RESULTS

C. utilis cells were grown in the culture medium containing 0.75 μM iron with a very low aeration system as described in Materials and Methods. The cell res-

piration in the presence of added ethanol showed no piericidin A sensitivity throughout the entire growth phase (c.f. 14, 15). Cells were harvested at the early stationary phase. After washing, cells were divided into three fractions. The first fraction was kept unaerated at 0°C. The second and third cell fractions were suspended in 50 mM K-phosphate buffer (pH 6.5) at a concentration of 1g wet cells per 10 ml, and aerated vigorously with an air flow of 7 liter per min per liter suspension at 25°C for about 6 hours in the absence and presence of cycloheximide (100 μM) respectively. After aeration in the absence of cycloheximide, cell respiration in the presence of added ethanol became sensitive to piericidin A (70 - 80% inhibition); while the respiration of the cells aerated in the presence of cycloheximide was completely insensitive to this inhibitor, confirming the observation reported by Garland and his colleagues (16,17). Coupled mitochondria were prepared from all three cell fractions. As shown in the top part of Fig. 1, ethanol respiration in the presence of semicarbazide showed almost the same respiratory control (Control ratios, 2.2 - 2.7) in mitochondria prepared from the three cell fractions. The rate of ethanol respiration in State 3 was 0.10 - 0.15 μatoms oxygen per min per mg protein in these mitochondria. Mean values of ADP/O ratio and the standard deviations were calculated from the data of 7 separate experiments. As shown in the middle of Fig. 1, ADP/O ratios coupled with ethanol respiration in mitochondria prepared from cells; non-aerated, aerated in the absence and presence of cycloheximide were close to 2, 3 and 2 respectively in a highly reproducible fashion. The presence or absence of Site I phosphorylation was more specifically demonstrated by the energy dependent reversal of electrons from glycerol-1-phosphate to endogenous pyridine nucleotide in State 4 respiration as shown in the bottom part of Fig. 1. In mitochondria prepared from cells aerated in the absence of cycloheximide, endogenous pyridine nucleotide was reduced by the energy dependent reversal of electrons from glycerol-1-phosphate and the reduced pyridine nucleotide was oxidized by the addition of either ADP or uncoupler, 1799 A. By contrast, in mitochondria prepared from cells aerated in the presence of cycloheximide, energy dependent reduction of endogenous pyridine nucleotide was not obtained as in the case of mitochondria prepared from non-aerated cells.

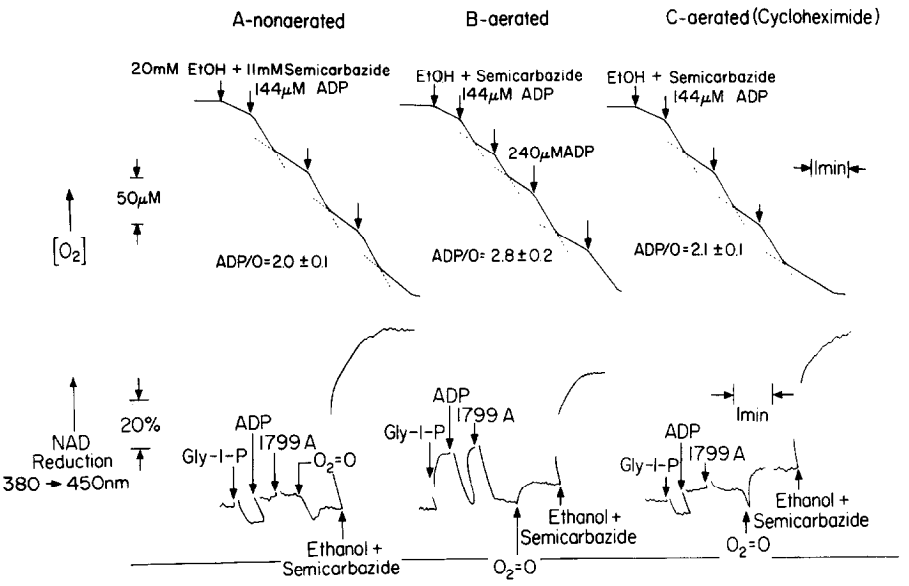

Figure 1. Polarographic traces of ethanol respiration and redox change of endogenous pyridine nucleotide in the glycerol-1-phosphate respiration of mitochondria prepared from C. utilis cells: A, non-aerated; B, aerated; and C, aerated in the presence of cycloheximide (100 μM). Cells were originally grown at 0.75 μM initial iron. The reaction medium of mitochondrial respiration contained 0.6 M mannitol, 0.5 mM EDTA 20 mM K-phosphate (pH 6.8), and bovine serum albumin (1.0 mg protein per ml reaction medium). Mitochondrial protein concentration for the polarographic measurements was (A), 0.92 mg/ml; (B), 1.30 mg/ml; and (C), 1.20 mg/ml. For the fluorometric measuremen oxygen saturated reaction medium was used, with mitochondrial protein concentrations a (A), 4.8 mg/ml, (B) and (C) 4.0 mg/ml.

These observations demonstrate that Site I energy coupling was induced in vivo by the aeration of the cells under non-growing conditions and that this induction process was inhibited by cycloheximide.

As shown in Table I, both pyruvate plus malate and NADH oxidation measured in mitochondria and submitochondrial particles, respectively, were completely insensitive to piericidin A when they were prepared from non-aerated cells. Piericidin A sensitivity was induced by the aeration of the cells under non-growing conditions, as shown by 90-95% inhibition of both pyruvate plus malate and NADH respiration in the aerated cell system. In parallel with the induction process of Site I phosphorylation, induction of piericidin A sensitivity was also completely inhibited by cycloheximide (Table I).

TABLE I. Maximum piericidin A inhibition (%) on pyruvate plus malate and NADH oxidation tested in mitochondria and submitochondrial particles respectively, which were prepared from C. utilis cells: A, non-aerated; B, aerated; C, aerated in the presence of cycloheximide, the same as shown in the legend of Fig. 1.

	A-nonaerated	B-aerated (%)	C-aerated (+cycloheximide)
pyruvate + malate oxidation in mitochondria	0	90 - 95	0
NADH oxidation in submitochondrial particles	0	90 - 95	0

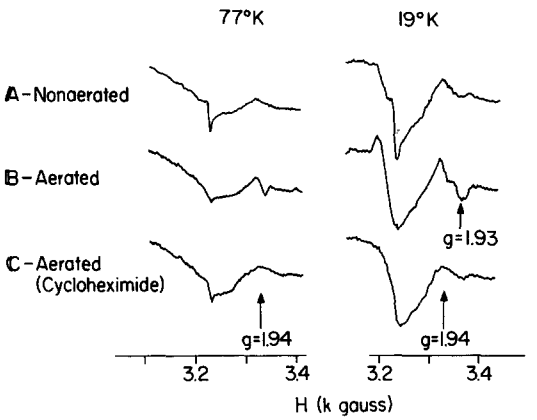

Figure 2. EPR spectra of reduced iron-sulfur proteins in the NADH dehydrogenase region of the submitochondrial particles prepared from C. utilis cells: A, non-aerated; B, aerated; and C, aerated in the presence of cycloheximide (100 μM). Cells were originally grown in the medium containing 0.75 μM initial iron, same as in Fig. 1. EPR spectra were recorded at 77° and 19°K. EPR operating conditions for Varian X band V-4502 spectrometer: modulation amplitude, 12 gauss; microwave power 9.2 mw; microwave frequency, 9.02 GHz; time constant, 0.001 sec; scanning rate, 1 kgauss/min. Protein concentration of these submitochondrial particle suspensions was 37 mg/ml in all three systems. Other experimental conditions are described in the text.

Fig. 2 shows EPR spectra of iron-sulfur proteins in submitochondrial particles prepared from the cells; non-aerated or aerated in the absence or presence of cycloheximide, measured at 77° and 19°K respectively. Samples were frozen in less than

45 seconds in liquid nitrogen after the addition of NADH to the suspension of submitochondrial particles which had been incubated for 5 min in an ice bath with antimycin A (1.6 nmoles/mg protein). Within this time interval, iron-sulfur proteins associated with the glycerol-1-phosphate or succinate dehydrogenase were mostly not reduced. In submitochondrial particles isolated from non-aerated cells, there was almost no detectable "g = 1.94" EPR signal due to iron-sulfur Center 1 in the NADH dehydrogenase region at 77°K. In submitochondrial particles prepared from the aerated cells, a "g = 1.94" EPR signal of significant intensity was obtained, concomitant with the induction of both Site I phosphorylation and piericidin A sensitivity. In submitochondrial particles prepared from cells aerated in the presence of cycloheximide, on the other hand, there was no intensification of the "g = 1.94" EPR signal. When the temperature of EPR measurement was lowered to 19°K, the EPR signal due to iron-sulfur Center 2 was detected at g = 1.93 in the aerated system. This EPR signal due to Center 2 was not found in submitochondrial particles prepared from cells; non-aerated, and aerated in the presence of cycloheximide. Profile of the intensity change of Center 2 signal in system B and C was in parallel with that of Center 1 during the induction process of Site I phosphorylation. EPR signals due to Center 3 plus 4 were too small to be compared among these three systems.

DUSCUSSION

Data presented in this paper demonstrate that Site I phosphorylation, energy dependent reduction of pyridine nucleotide, piericidin A sensitivity, and EPR detectable iron-sulfur proteins are simultaneously induced by the aeration of C. utilis cells which have grown under iron-deficient conditions. The in vivo induction of all these features were inhibited concomitantly by a cytoplasmic protein synthesis inhibitor, cycloheximide (Table II). These observations further strengthen the previous hypothesis presented by Ohnishi and Chance (1), namely, that EPR detectable iron-sulfur protein(s) may play a role in Site I energy conservation. This interpretation seems to contradict with that reported by Garland and his coworkers

TABLE II. Induction of Site I phosphorylation in C. utilis cells grown under iron deficient conditions

	A-nonaerated	B-aerated	C-aerated (+cycloheximide)
Site I phosphorylation	-	+	-
Energy dependent reduction of pyridine nucleotide	-	+	-
Piericidin A sensitivity	-	+	-
EPR detectable iron-sulfur protein(s)	-	+	-

(2). However, their conclusion that the induction of Site I phosphorylation occurred in the cycloheximide inhibited system was based solely on the measurements of ADP/O ratios (reported values, 2.2, 2.2, 2.3 and 2.5). They were not able to obtain energy dependent reversal of electrons from glycerol-1-phosphate to endogenous pyridine nucleotide in this system (18). On the other hand, Haddock and Garland (19) reported ADP/O ratios close to 3 as well as energy dependent reduction of endogenous pyridine nucleotide in mitochondria prepared from sulfur-limited C. utilis cells aerated in the presence of cycloheximide. In this case, however, there is some ambiguity because of a trace of piericidin A sensitivity (reported as less than 10%) induced even in the presence of cycloheximide. It was previously shown that if there is any trace of piericidin A sensitivity, there is always fully acting Site I phosphorylation (1). The second line of the evidence presented by Garland and his coworkers (3) for the occurrence of Site I phosphorylation without accompanying EPR detectable iron-sulfur proteins in the NADH dehydrogenase region is still not conclusive, because EPR spectra have been examined only at 77°K. Sensitivity of EPR measurement is greatly enhanced by further lowering the temperature.

The present study could not identify an iron-sulfur Center which is involved in Site I energy conservation, however, it strongly suggests that at least one of the

"EPR active" iron-sulfur proteins (4-7) in the Site I region plays a role in the Site I energy conservation.

Independent experiments using pigeon heart mitochondria showed that iron-sulfur Center I may be directly involved in energy transduction at Site I (20). Half reduction potential of Center 1 in the presence of ATP is different from that in the absence of ATP (21,22).

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