FACTORS CONTROLLING THE OCCURRENCE OF SITE I PHOSPHORYLATION IN *C. UTILIS* MITOCHONDRIA

Tomoko OHNISHI
Johnson Research Foundation, Medical School, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

Received 15 June 1972

1. Introduction

Evidence in favor of a role for iron–sulfur protein(s) in the energy conservation at Site I has been provided by Garland and his colleagues [1, 2] and by Ohnishi and Chance [3].

However, a serious question about the direct involvement of iron–sulfur proteins in Site I energy conservation has been raised by the following observations of Katz et al. [4, 5]: i) *C. utilis* cells acquire rotenone (or piericidin A) sensitivity and Site I energy conservation, on the transition from logarithmic to stationary phase of growth due to carbon depletion, irrespective of the iron concentration in the culture medium; ii) in a chemostat, an increase of the concentration of carbon source without changing the iron concentration, results in the disappearance of rotenone sensitivity.

It has been known that there are two different NADH dehydrogenases located in the cristae (the inner mitochondrial membrane) of *C. utilis* mitochondria [6, 7]: one is located on the inner surface of the cristae and oxidizes NADH generated in the matrix space; the other is located on the outer surface of the cristae and oxidizes cytoplasmic NADH [7]. The former NADH oxidation pathway is piericidin A sensitive and associated with phosphorylation at Site I, while the latter NADH oxidation pathway is piericidin A insensitive and gives P/O ratio 2, showing no energy coupling at Site I. These two NADH oxidation pathways in the inner mitochondrial membrane can be distinguished from the NADH oxidation system located on the outer membrane of mitochondria, from their B-stereospecificity [6] and antimycin A sensitivity.

In the present study, it is shown that observations reported by Katz et al. [4, 5] reflect a by-pass mechanism at the mitochondrial level between a phosphorylating and non-phosphorylating electron transfer pathway at Site I region for the oxidation of NADH generated in the matrix space of mitochondria. Thus, the non-phosphorylating pathway is used when cells are growing rapidly under optimal growth condition. This control mechanism can be clearly distinguished from the previously proposed correlation between iron–sulfur proteins in the NADH dehydrogenase region and Site I energy conservation in mitochondria [1–3].

2. Materials and methods

C. utilis cells were grown aerobically at 28° in two different aeration systems: i) In 6 l batches in a fermentor jar (New Brunswick Model-F). Very efficient aeration was assured by bubbling air at a flow rate of 2 l per min per liter medium and operating the impeller at about 600 rpm. ii) In a 20 l vessel equipped with two aeration filters (A.H. Thomas 5/51-C) with low aeration (0.75 l air per min per liter medium as previously described [3]. In both systems cells were grown in Galzy and Slonimski's synthetic media [8] with some modifications [3]. When the cells were harvested at the logarithmic growth phase, the culture suspension was quickly cooled and 100 μM cycloheximide was added to the cell suspension

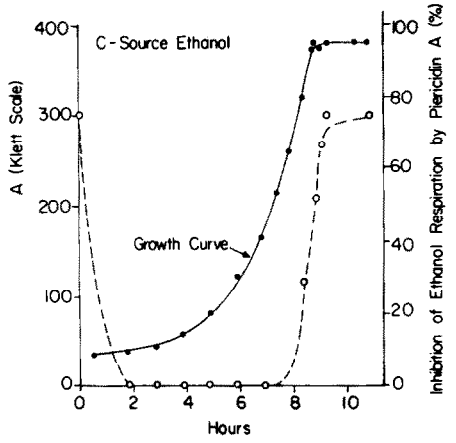

Fig. 1. Effect of growth phase on the piericidin A sensitivity of ethanol respiration in *C. utilis* cells grown under conditions of high aeration. Culture medium contained 0.25% ethanol and 50 μM Fe SO_4. Composition of other components in the medium is the same as described before [3]. Respiration was measured with cells washed in 50 mM K-phosphate buffer, pH 6.8. (——): Turbidity of the culture expressed in Klett absorbance units; (- - - -): maximum percent inhibition of ethanol respiration by piericidin A.

throughout the harvesting, washing and enzyme treatment procedures. Mitochondria and submitochondrial particles were prepared as previously described by Ohnishi and Chance [3]. Respiration was measured with a Clark oxygen electrode.

3. Results and discussion

Piericidin A sensitivity of ethanol respiration of *C. utilis* cells was reexamined using cells grown in batch with a sufficient supply of air as described in Materials and methods. Piericidin A sensitivity of innoculum cells disappears in less than two hour's culture in fresh medium. Ethanol respiration exhibits no sensitivity towards piericidin A during the exponential growth phase and it appears again before reaching the stationary phase. In this experiment the concentration of oxygen in the culture medium was kept above 80% of air saturation throughout the growth time and doubling time of cell population was about 2 hr. The same phenomena were observed even with lower aeration, as long as oxygen supply is not rate limiting for cell growth, extending the findings by Katz [4].

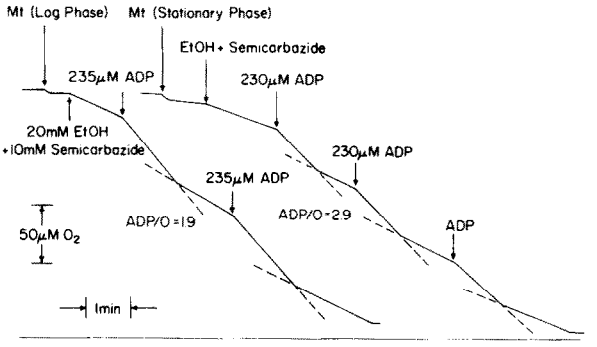

Fig. 2. Respiratory pattern of ethanol oxidation in *C. utilis* mitochondria prepared from cells in logarithmic and stationary growth phase. Reaction medium contained 0.6 M mannitol, 10 mM Tris-maleate buffer, pH 6.8, 20 mM K-phosphate buffer, pH 6.8 and 0.1 mM EDTA. Protein concentration of mitochondria: log phase mitochondria, 0.7 mg/ml; stationary phase mitochondria, 0.9 mg/ml. Respiration was measured at 25°.

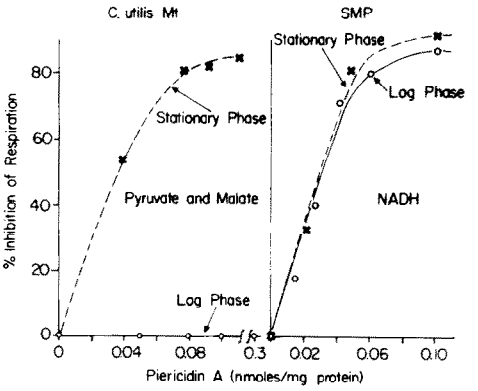

Fig. 3. Effect of piericidin A on pyruvate + malate oxidation in mitochondria and on NADH oxidation in submitochondrial particles, which are prepared from cells either in logarithmic or stationary growth phase.

Coupled mitochondria were prepared from cells harvested at either logarithmic or stationary growth phase as seen in fig. 2. ADP/O ratios 1.9 and 2.9 were obtained coupled with ethanol respiration in mitochondria prepared from log-phase and stationary-phase cells, respectively, showing that Site I phosphorylation is not functioning in mitochondria prepared from log-phase cells, as suggested by Katz et al. [5].

Pyruvate + malate oxidation in mitochondria pre-

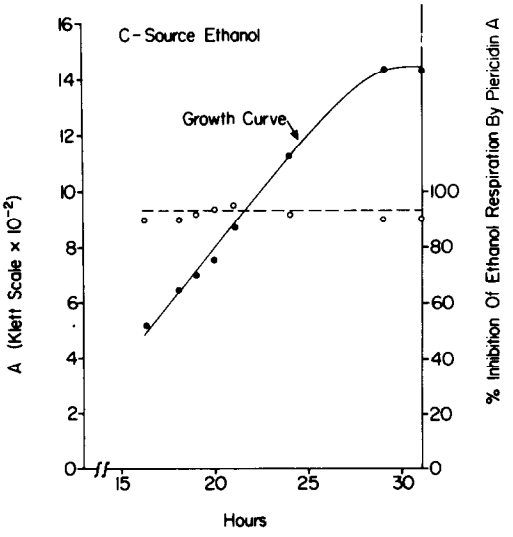

Fig. 4. Piericidin A sensitivity of ethanol respiration in *C. utilis* (harvested at different stages of growth) when the cells were grown in a low aeration system. Concentration of ethanol and iron in the culture medium was 1.5% and 50 μM, respectively.

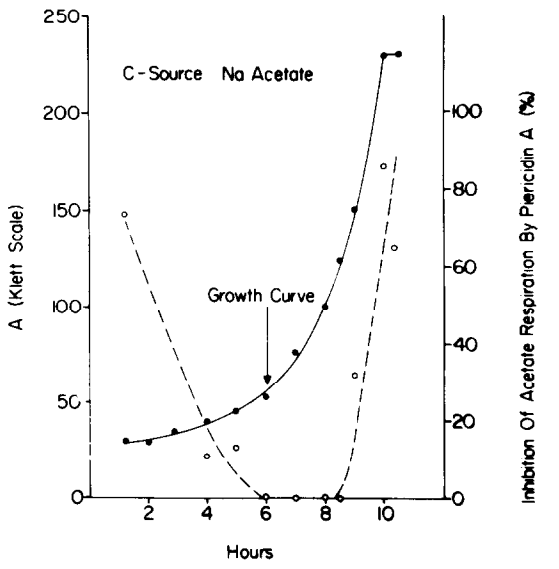

Fig. 5. Piericidin A sensitivity of acetate respiration in *C. utilis* cells at different growth phase. Cells were grown with an efficient aeration in a medium, pH 5.8, containing 0.04 M sodium acetate as the main carbon source. All other growth conditions were the same as in the legend to fig. 1.

pared from log-phase cells was completely insensitive to piericidin A while in stationary-phase mitochondria, pyruvate + malate respiration was inhibited by about 90% in the presence of piericidin A (fig. 3). However, NADH, oxidation in submitochondrial particles prepared from log-phase mitochondria showed almost identical sensitivity towards piericidin A to that of NADH oxidation in submitochondrial particles from stationary-phase mitochondria (fig. 3). This strongly suggests that in mitochondria of the exponentially growing cells, the piericidin A sensitive component and most probably the Site I phosphorylation machinery are present. However, NADH generated in mitochondria is oxidized via an alternate electron transfer pathway which is piericidin A insensitive and non-phosphorylating at Site I. This bypass mechanism is not conducted by the direct exchange of the reducing equivalents from internally generated NADH to extramitochondrial NAD, since (pyruvate + malate)-ferricyanide reductase activity in log-phase mitochondria is completely inhibited by antimycin A (c.f. [7]). Because of the reversed sidedness of the two NADH dehydrogenases in mitochondria and submitochondrial particles, NADH dehydrogenase which was localized towards the inner surface of the cristae is shifted to the outer surface of submitochondrial particles. The NADH dehydrogenase system for the oxidation of external NADH in mitochondria was suggested to be either solubilized or dislocated during the preparation of submitochondrial particles [7]. Thus in submitochondrial particles NADH is oxidized solely via the Site I phosphorylating electron transfer chain. Under conditions of high aeration, the minimal iron concentration due to iron contaminants in the culture medium is sufficient to obtain piericidin A sensitive NADH oxidation in submitochondrial particles.

In contrast to the system described above, ethanol respiration of *C. utilis* cells grown with a very low aeration system as described previously [3] shows piericidin A sensitivity even in cells growing in the presence of excess carbon source (fig. 4). In this system apparent doubling time of the cell population is about 5 hr, due to a very slow supply of oxygen.

From the cells grown under the low aeration in the culture medium containing less than 1.1 μM

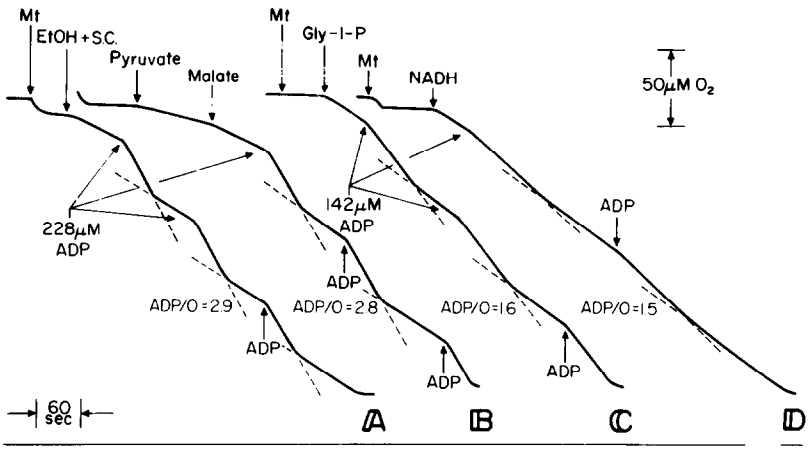

Fig. 6. Traces of the respiratory pattern with different substrates in mitochondria prepared from *C. utilis* cells after being grown in acetate-culture medium. The cells were harvested at stationary growth phase. Protein concentration of mitochondria is 1.0 mg/ml for traces A and B; 0.53 mg/ml for traces C and D. The concentration of substrates added are: 17 mM ethanol + 10 mM semicarbazide, 5 mM pyruvate + 5 mM malate, 10 mM glycerol-1-phosphate and 2 mM NADH as pointed with arrows in the figure. Other conditions are the same as in the legend to fig. 2.

initial iron, mitochondria deficient in both Site I phosphorylation and piericidin A sensitivity were obtained (c.f. [3]). In this case NADH oxidation tested in submitochondrial particles is also insensitive to piericidin A in contrast to the NADH oxidation in submitochondrial particles prepared from the log-phase mitochondria described above. Therefore the by-pass mechanism of Site I phosphorylation in mitochondria can be distinguished from the intrinsic effect of iron deficiency which causes the lack of energy transducing component(s) at Site I. In this connection Fukami et al. [9] have reported that acetate-limited growth of *C. utilis* cells results in the loss of the external rotenone insensitive NADH dehydrogenase. It was found, however, that in the culture medium containing acetate as a main carbon source *C. utilis* can grow exponentially only in the medium with pH higher than around 5.5. Acetate respiration of exponentially growing cells is completely insensitive to piericidin A, as in the case of ethanol grown cells (fig. 5). In addition, the presence of the piericidin A insensitive and Site I non-phosphorylating external NADH oxidation pathway was also demonstrated as shown with trace D in fig. 6. Lowering the pH (most probably due to inhibitory effect of acetic acid) causes a much longer lag-phase and decreased growth rate. Under these sub-optimal growth conditions, acetate respiration of the cells exhibits piericidin A sensitivity, even when cells are growing with excess carbon source and with excess oxygen supply. This explains the inability of *C. utilis* cells to grow in acetate-culture medium (pH 5.0), which contains piericidin A or rotenone [9].

These results suggest that when *C. utilis* cells are growing exponentially with an optimal growth rate, Site I phosphorylation is not utilized even if the energy coupling machinery is present. This phosphorylation site is utilized only when cells are growing under sub-optimal growth conditions.

The discovery of this by-pass mechanism provides a basis for understanding the many conflicting reports relating to the presence or absence of Site I phosphorylation in yeast cells cultured under a variety of conditions. However, the importance of this mechanism for the growth cycle of the organism and the molecular basis for its regulation remain to be elucidated.

Acknowledgements

This research was supported by Research Grant GM 12202 from the U.S. Public Health Service. The author would like to express her gratitude to Dr. B. Chance for his encouragement and advice throughout this work. Thanks are also due to Mr. R. Katz for his stimulating discussions, to Mrs. S. Muantongchin for her excellent technical assistance, and to Dr. T.P. Singer for his generous gift of piericidin A.

References

[1] P.A. Light, C.I. Ragan, R.A. Clegg and P.B. Garland, FEBS Letters 1 (1968) 4.
[2] P.B. Garland, Biochem. J. 118 (1970) 329.
[3] T. Ohnishi and B. Chance, in: Flavins and Flavoproteins, ed. H. Kamin (University Park Press, Durham, North Carolina, 1971) p. 681.
[4] R. Katz, FEBS Letters 12 (1971) 153.
[5] K. Katz, L. Kilpatrick and B. Chance, European J. Biochem. 21 (1971) 301.
[6] T. Ohnishi, G. Sottocasa and L. Ernster, Bull. Soc. Chim. Biol. 48 (1966) 1189.
[7] G. Von Jagow and M. Klingenberg, European J. Biochem. 12 (1970) 583.
[8] P. Galzy and P.P. Slonimski, Compt. Rend. 245 (1957) 2423.
[9] M.H. Fukami, P.A. Light and P.B. Garland, FEBS Letters 7 (1970) 132.