# INDUCTION OF THE SITE I PHOSPHORYLATION *IN VIVO* IN *SACCHAROMYCES CARLSBERGENSIS*

Tomoko Ohnishi

Johnson Research Foundation, University of Pennsylvania,
Philadelphia, Pennsylvania 19104.

Received September 9, 1970

## Summary

From the studies on the phosphorylating efficiencies of the isolated mitochondria, it was found that site I phosphorylation is missing in *S. carlsbergensis* cells. This energy coupling site can be induced *in vivo* by the aeration of the cells under non-growing condition, as in case of site I deficient *Candida utilis* cells. The "g=1.94" EPR signal of iron-sulfur proteins in the NADH dehydrogenase region was shown to be lacking in the submitochondrial particles prepared from the cells both before and after the aeration. This clearly demonstrates that the "g=1.94" EPR signal in the NADH dehydrogenase region is not required for the energy coupling at site I. A possible mechanism for the induction of site I phosphorylation in the present system is discussed.

From the direct measurement of the redox level of the electron carriers in *S. cerevisiae* cells starved by 18 hour aeration, Chance (1) observed three cross over points in their respiratory chain. He concluded that like animal mitochondria, *S. cerevisiae* mitochondria possess three phosphorylation sites. Subsequent studies on the phosphorylation efficiencies in the isolated mitochondria and submitochondrial particles from the non-aerated cells grown aerobically under various growth conditions showed the absence of site I phosphorylation in both *S. cerevisiae* (2-5) and *S. carlsbergensis* (6). However, Steckhoven (7) and more recently Ghosh and Bhattacharyya (8) reported the presence of site I phosphorylation in mitochondria isolated from *S. carlsbergensis*, non-aerated and aerated respectively, after harvesting and washing cells. In order to resolve this conflict, the phosphorylation efficiencies of the isolated mitochondria from *S. carlsbergensis* were reevaluated in the present investigation. It was found that site I phosphorylation is not present in mitochondria isolated from non-aerated *S. carlsbergensis* cells at the late logarithmic and the early stationary growth phase, grown aerobically in batch culture, containing 2%

glucose as a carbon source. However, this energy coupling site was induced *in vivo* by aeration of the washed cells suspended in phosphate buffer, as in the case for site I deficient *C. utilis* cells grown initially in an iron deficient culture medium (9, 10). It has also been found that in submitochondrial particles from *S. carlsbergensis*, as in *S. cerevisiae*, there is a very small, if any, "g=1.94" EPR signal of the iron-sulfur proteins associated with the NADH dehydrogenase region (11, 12). Aeration of the cells did not induce this signal.

## Materials and Methods

Cells: *S. carlsbergensis*, Hillman Hospital strain number 4228 was supplied by A.K. Ghosh. Cells were grown in a semisynthetic culture medium as described by Ghosh *et. al.* (13) containing 2% glucose as a main carbon source. Cells were grown at 30°C and were harvested at the early stationary phase or in the late logarithmic growth phase. The air was supplied at the flow rate of 250 ml air per min. per liter culture medium through a glass gas washing filter and dispersed with a magnetic stirrer. When the aeration of the cells was required, washed cells were suspended in 50 mM K-phosphate buffer (pH 6.5) at a cell concentration of 1 gr. wet cells per 10 ml suspension and were aerated for about 5 hours at a flow rate of 7 liter air per min. per liter suspension of the cells in the early stationary phase and 7 hours for the cells in the late logarithmic growth phase.

Preparation of protoplasts and mitochondria: Protoplasts were prepared essentially as described previously (6). The snail gut juice used in the present study is Glusulase, which was obtained from Endo Laboratories Inc., at a final concentration of 1 mg protein per ml cell suspension (0.5 gr. wet cells/1 ml suspension). Washed protoplasts were lysed in 0.25 M mannitol, 0.5 mM EDTA (pH 6.8) containing 0.5 mg purified bovine serum albumin per ml with a 5 seconds stirring at a low speed in a Waring blender. The tonicity of the

---

*Abbreviations: EPR, Electron Spin Resonance.

suspension medium was adjusted by the addition of 0.1 volume of 2.5 M sucrose containing 5 mM EDTA (pH 6.8). Mitochondria were fractionated as described before (6), washed twice in 0.6 M mannitol containing 0.5 mM EDTA (pH 6.8) and purified bovine serum albumin (0.5 mg protein per ml).

Preparation of submitochondrial particles: Mitochondria were suspended in 0.3 M mannitol, 20 mM Tris-maleate buffer (pH 7.2) at a final protein concentration of about 10 mg per ml and were disintegrated by sonication for 45 seconds . After eliminating unbroken mitochondria, the submitochondrial particle fraction was obtained by centrifugation at 105,000 x g for 50 min. and washed twice with the sonication medium.

Assays: Respiration was measured with a Clark oxygen electrode. Phosphorylation efficiencies of mitochondria were measured polarographically according to Chance and Williams (14). Electron paramagnetic resonance (EPR) spectra were obtained with a Varian X-band spectrometer (V 4502-13) (100 kHz field modulation, 12 oersted modulation amplitude). Non-heme iron of the submitochondrial particles was extracted in 1N HCl according to King et. al. (15). Iron concentration in the extract was measured according to Kimura and Suzuki (16). Protein concentration of mitochondria and submitochondrial particles was determined according to Krüger and Klingenberg (17).

## Results and Discussions

Fig. 1 shows polarographic traces of the respiration with different substrates in mitochondria prepared from the cells harvested at the early stationary phase. As shown in the first and second traces, ADP/O ratios below 2 were obtained in the respiration with both pyruvate plus malate and exogenous NADH. Respirations with other NAD-linked substrates, such as citrate or ethanol plus semicarbazide, gave the same ADP/O ratios, 1.7 - 2.0, as with pyruvate plus malate. As shown in the third trace, the same ADP/O ratio was obtained coupled with the succinate respiration. The comparison of these phosphorylation efficiencies in the respirations with various substrates shows

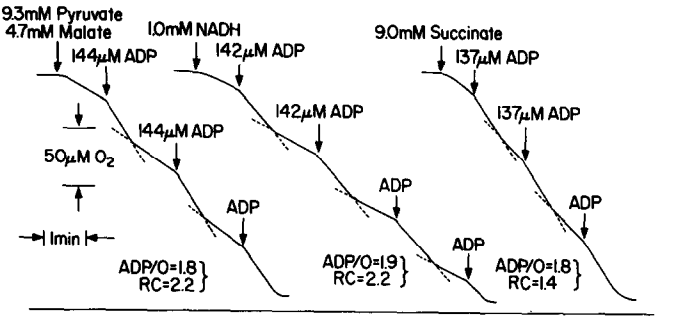


Figure 1: Polarographic traces of the respiration with various substrates of the mitochondria isolated from non-aerated S. carlsbergensis cells. The reaction medium contained 0.6 M mannitol, 20 mM K-phosphate buffer (pH 6.5), 10 mM Tris-maleate buffer (pH 6.5), 0.1 mM EDTA and purified bovine serum albumin (0.5 mg protein/ml). The concentration of mitochondrial protein in the reaction medium was 0.65 mg/ml for pyruvate plus malate, 0.50 mg/ml for the exogenous NADH, and 1.0 mg/ml for succinate respiration. The respiration was measured at 25°C.

---

the absence of site I phosphorylation in these mitochondria, when the cells were not starved by aeration before the isolation of mitochondria. The absence of site I phosphorylation was also observed in the mitochondria isolated from the cells in the late logarithmic growth phase. It is in agreement with the phosphorylation efficiencies in S. carlsbergensis mitochondria reported by Ohnishi et. al. (6) , but not in agreement with the results reported by Steckhofen (7). S. carlsbergensis cells were then subjected to the aeration procedure prior to the isolation of mitochondria, which in an analogous fashion leads to the induction of site I phosphorylation in site I deficient C. utilis cells (9, 10). As described under materials and methods, S. carlsbergensis cells at the early stationary phase were aerated under non-growing condition for four to six hours. During the first 3 hour aeration the endogenous respiration of the cells gradually decreased to about 30 to 40% of the initial rate and stayed at this level until the end of the aeration. Fig. 2 shows the polarographic traces of the respirations with different substrates of the mitochondria isolated from these aerated cells. The respiration with NAD-linked substrates, for example, pyruvate plus malate gave an ADP/O ratio, 2.3 - 2.7, approximately one unit higher than that obtained with exogenous NADH or succinate oxidation. Sim-

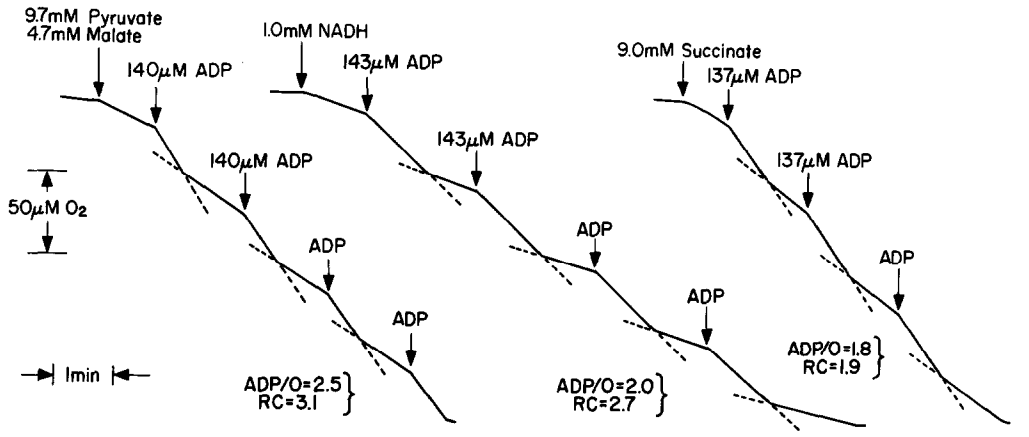


Figure 2: Polarographic traces of the respiration with various substrates of the mitochondria isolated from S. carlsbergensis after aerating the cells for 4.5 hours. The reaction medium was the same as that shown in the legend of Fig. 1. The concentration of mitochondrial protein in the reaction medium was 0.6 mg/ml for pyruvate plus malate, 0.3 mg/ml for exogenous NADH, and 0.8 mg/ml for succinate respiration.

ilarly the induction of the site I phosphorylation was demonstrated in the mitochondria isolated from the cells harvested at the late logarithmic growth phase. In this case a longer aeration was needed to decrease the endogenous respiration than the cells in the stationary phase. This confirmed the presence of site I phosphorylation in S. carlsbergensis mitochondria reported by Ghosh and Bhattachryya (8), since they routinely aerated their cells before the preparation of mitochondria. These observations demonstrate the induction of site I phosphorylation in S. carlsbergensis mitochondria during aeration of the whole cell suspension, similar to the induction of site I phosphorylation by the aeration of C. utilis cells in the absence of added iron, which were grown initially at a low iron concentration and had no energy coupling at the site I (10). These observations show that S. carlsbergensis cells are genetically competent to perform a functional energy-conservation at site I. The induction system of the site I phosphorylation in S. carlsbergensis is also an example demonstrating that rotenone or piericidin A sensitivity of the respiration is not needed for the occurrence of the energy coupling at site I (C.F. 9, 18, 22), since the respiration with NAD-linked substrates

in the mitochondria isolated from site I induced S. carlsbergensis cells is insensitive to these respiratory inhibitors.

A close correlation has been suggested between the occurrence of site I phosphorylation and the presence of the "g=1.94" EPR signal of iron-sulfur proteins associated with the NADH dehydrogenase region (11, 12, 19). EPR signals obtained by the reduction with different substrates in the submitochondrial particles prepared from non-aerated cells are shown in Fig. 3. In order to prevent the reduction of iron-sulfur proteins in the NADH dehydrogenase region by the reversed electron transport from succinate or glycerol-1-phosphate, the EPR signals were obtained in the uncoupled particles with 33 μM pentachlorophenol. Spectrum A was obtained by the addition of both succinate and glycerol-1-phosphate. Spectrum B was obtained by the further addition of NADH to sample A or only with NADH. Comparing the line shape and the intensity

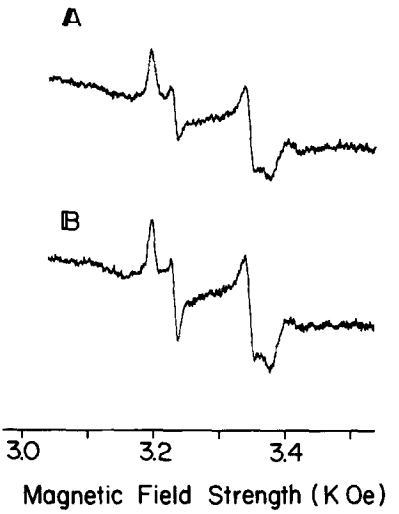


Figure 3: EPR spectra of the submitochondrial particles isolated from the non-aerated S. carlsbergensis cells, reduced with various substrates. Cells were grown in a culture medium containing 10 μM $FeCl_3$. Submitochondrial particles (72.5 mg protein/ml), suspended in 0.3 M mannitol and 20 mM Tris-maleate buffer (pH 7.2), were pre-incubated with 33 μM pentachlorophenol for about 5 min. in an ice bath. For spectrum A, particles were incubated with both 10 mM succinate and 8.3 mM glycerol-1-phosphate for 10 min. at 25°C. For spectrum B, particles were incubated with 1.7 mM NADH together with succinate and glycerol-1-phosphate. The particle suspension in the quartz tubes with 3 mm inner diameter was frozen in the liquid nitrogen.

of "g=1.94" EPR signal, Fig. 3 shows that in the submitochondrial particles from S. carlsbergensis, there is only a very small, if any, "g=1.94" EPR signal associated with the NADH dehydrogenase region, as in the case of S. cerevisiae (11, 12) and in contrast to C. utilis (12, 19) and mammalian systems (20). Furthermore, there was no intensification of the "g=1.94" EPR signal of the iron-sulfur proteins reduced by NADH, succinate and glycerol-1-phosphate, during the in vivo induction of site I phosphorylation by the aeration of the cell suspension, as shown in Fig. 4 (Spectrum A and B). Observations shown in Fig. 3 and 4 demonstrate that the "g=1.94" EPR signal associated with the NADH dehydrogenase region is not needed for the occurrence of site I phosphorylation, as shown independently in the iron-deficient micrococcus denitrificans (21) and in C. utilis systems (18, 22). No significant "g=1.94" EPR signal was associated with the NADH dehydrogenase of submitochondrial particles from S. carlsbergensis cells even when they were grown in a high iron (50 μM) medium; however the EPR signal of the iron-sulfur proteins

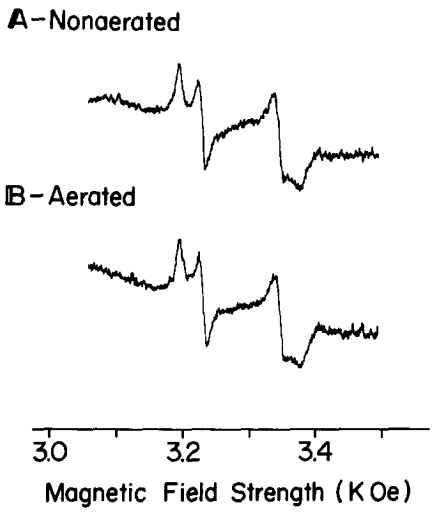


Figure 4: The comparison of the EPR spectra of the submitochondrial particles isolated from the non-aerated (A) and aerated S. carlsbergensis cells (B). Both particle suspensions from the non-aerated cells (A) and from the aerated cells (B) were incubated with 10 mM succinate, 8.3 mM glycerol-1-phosphate and 1.7 mM NADH for 10 min. at 25°C. The protein concentration of the submitochondrial particles was 51 mg/ml in (A) and 53 mg/ml in (B). Cells were initially grown in a culture medium containing 50 μM $FeCl_3$.

of the succinate and glycerol-1-phosphate dehydrogenase region was almost the same in these particles of S. carlsbergensis as those from C. utilis grown in medium of the same iron concentration. Furthermore it is interesting to point out that the concentration of acid-extractable non-heme iron in the submitochondrial particles from S. carlsbergensis was almost the same as that in the C. utilis system, namely about 5 nmoles per mg protein. This concentration did not show an appreciable change during the aeration procedure. What is the mechanism of the site I induction during the aeration process of S. carlsbergensis cells under non-growing condition? It seems to be related to the decrease of the endogenous carbon reserve in the cells, which in turn controls the cytosolic and mitochondrial redox poise. In this context it is very interesting that Pye et. al. (23) recently obtained a preliminary result suggesting the presence of three phosphorylation sites in S. carlsbergensis cells when they are grown in a glucose-limiting condition, but two phosphorylation sites under glucose-excess condition. This was reflected in the growth yields of S. carlsbergensis cells both in batch and in continuous culture. Similarly in C. utilis cells grown in the chemostat, Clegg et. al. (18) demonstrated, by a stepwise increase of the concentration of entering iron, that site I phosphorylation is absent when cells are grown in glycerol-non-limiting condition, but appears when glycerol becomes growth limiting, although they interpreted iron limitation as the cause for the loss of site I phosphorylation rather than glycerol-non-limitation.

Acknowledgement

The author wishes to express her gratitude to Dr. B. Chance for his interest and stimulating advice throughout this investigation, and to Dr. A. Ghosh and Dr. K. Pye for their helpful discussions. Thanks are also due to Mrs. S. Muantongchin for her excellent technical assistance. This research was supported by U.S. Public Health Research Grant GM12202.

## References


1. Chance, B., J. Biol. Chem., 234, 3036 (1959).
2. Vitols, E. and Linnane, A.W., J. Biophys. Biochem. Cytol., 9, 701 (1961).
3. Schatz, G. and Racker, E., Biochem. Biophys. Res. Commun., 22, 597 (1966).
4. Mattoon, J.R. and Balcavage, W.X., Biochim. Biophys. Acta, 153, 521 (1968).
5. Kovaç, L., Biochim. Biophys. Acta, 153, 32 (1968).
6. Ohnishi, T., Kawaguchi, K. and Hagihara, B., J. Biol. Chem., 241, 1597 (1966).
7. Schuurmans-Steckhoven, F.M.A.H., Arch. Biochem. Biophys., 115, 555 (1966).
8. Ghosh, A., and Bhattacharyya, S.N., submitted to Biochim. Biophys. Acta.
9. Light, A., Ragan, C.I., Clegg, R.A. and Garland, P.B., FEBS Lett., 1, 4 (1968).
10. Ohnishi, T. and Chance, B., in the proceedings of the Third International Symposium on Flavins and Flavoproteins, Durham, North Carolina (1969).
11. Schatz, G., Racker, E., Tyler, D.D., Gonze, J. and Estabrook, R.W., Biochem. Biophys. Res. Commun., 22, 585 (1966).
12. Sharp, C.W., Mackler, B., Douglas, H.C., Palmer, G. and Felton, S.P., Arch. Biochem. Biophys., 122, 810 (1967).
13. Ghosh, A., Charalampous, F., Sison, S. and Borer, R., J. Biol. Chem., 235, 2522 (1960).
14. Chance, B. and Williams, G.R., Advan. Enzymol., 17, 54 (1965).
15. King, T., Nickel, K.S. and Jensen, D.R., J. Biol. Chem., 239, 1989 (1964).
16. Kimura, T., and Suzuki, K., J. Biol. Chem., 242, 485 (1967).
17. Krȍger, A., and Klingenberg, M., Biochem. Z., 344, 317 (1966).
18. Clegg, R.A., Ragan, C.I., Haddock, B.A., Light, P.A., Garland, P.B., Swann, J.C., and Bray, R.C., FEBS Lett., 5, 207 (1969).
19. Ohnishi, T., Racker, E., Schleyer, H., and Chance, B., in K. Yagi (Ed.), Flavins and Flavoproteins, Univ. of Tokyo Press, Tokyo, 1968, p. 122.
20. Beinert, H., Heinen, W., and Palmer, B., Brookhaven Symp. Biol., 15, 229 (1962).
21. Imai, K., Asano, A. and Sato, R., J. Biochem., 63, 219 (1968).
22. Ohnishi, T., Panebianco, P., Katz, R., and Chance, B., in preparation (1970).
23. Pye, K., Harison, D. and Mochan, G., personal communication.