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Piericidin A Sensitivity, Site 1 Phosphorylation, and Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase during Iron-limited Growth of *Candida utilis**

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SUMMARY

It has been reported that cells of *Candida utilis*, grown in continuous culture under iron-limited conditions, develop site 1 phosphorylation, without the appearance of piericidin sensitivity and without changes in the iron-sulfur centers of NADH dehydrogenase, on aeration in the presence of cycloheximide, as well as on increasing the supply of iron during growth. These findings were reinvestigated in the present study. The parameters and properties followed during these transitions were sensitivity of NADH oxidation to piericidin, presence or absence of coupling site 1, EPR signals appearing on reduction with NADH or dithionite, the specific activities of NADH oxidase, NADH-ferricyanide reductase, and NADH-5-hydroxy-1,4-naphthoquinone (juglone) reductase, and the kinetic behavior of NADH dehydrogenase in the ferricyanide assay. Monitoring the rates of oxidation of NADH in submitochondrial particles with artificial oxidants, observing the kinetics of the ferricyanide assay, and measuring the concentration of iron-sulfur centers elicited by EPR permitted ascertaining the type of NADH dehydrogenase present and its relative concentration in different experimental situations. It was found that on gradually increasing the concentration of iron during continuous culture (transition from iron-limited to iron- and substrate-limited growth), as well as on aeration of iron-limited cells, coupling site 1, piericidin sensitivity, NADH-ferricyanide activity, and iron-sulfur centers 1 and 2 increased concurrently, with concomitant decline of NADH-juglone reductase activity. Cycloheximide prevented all these changes. Iron-sulfur centers 3 plus 4 underwent relatively little increase during these transitions. It is concluded that in both of these experimental conditions a replacement of the type of NADH dehydrogenase present in exponential phase cells by that characteristic of stationary phase cells occurs and that the appearance of site 1 phosphorylation, piericidin sensitivity, and iron-sulfur centers 1 plus 2, all associated with the latter enzyme, is a consequence of this replacement. No evidence was found for the development of coupling site 1 without the appearance of piericidin sensitivity.

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It has been reported (2) that *Candida utilis* cells grown in continuous culture at the transition between iron-limited and substrate-limited growth possess energy coupling site 1 but lack piericidin sensitivity. The same workers found that cells grown under conditions of iron limitation lack both site 1 and sensitivity to piericidin, but on aeration of such cells in the absence of substrate but in the presence of cycloheximide site 1 appears, without the acquisition of piericidin sensitivity (2). Cells endowed with phosphorylation site 1 but lacking sensitivity to piericidin were also said to lack the EPR signal at g = 1.94 (3, 4) characteristically seen at and below 77 K on reduction of NADH dehydrogenase preparations from mammalian (5, 6) and stationary phase *C. utilis* cells (7) with substrate. This last observation may seem surprising in view of the fact that coupling site 1 is intimately associated with one of the iron-sulfur centers of NADH dehydrogenase (8, 9), unless one considers that the component involved in site 1 is iron-sulfur center 2 (10, 11), which is only seen at temperatures around 13 K, while the observations of Clegg *et al.* (3) were conducted at 77 K.

These reports of the apparent dissociation of site 1 from piericidin sensitivity and the EPR-detectable components of NADH dehydrogenase were debated by Ohnishi *et al.* (12–15). Working with batch cultures at varying iron concentrations, they noted a good correlation between the appearance of piericidin sensi-

tivity and site 1 (12), a close relation between a small fraction of the g = 1.94 signal detected at 77 K and site 1 (13), but no correlation between the presence of this signal and piericidin sensitivity (14). Moreover, when the experiments of Clegg and Garland (2) on the effect of aerating iron-limited cells were repeated (but with cells grown in batch culture) (15), piericidin sensitivity, site 1, and iron-sulfur centers 1 and 2 (detected at 13 K) all appeared on aeration and cycloheximide prevented the appearance of all these properties and components, in contradiction to the earlier report (2). The significance of these observations was questioned (2, 4) on the ground that cells grown in batch culture represent a mixed population, rather than purely iron-limited cells.

Although it is admittedly difficult to reconcile observations on yeast grown batchwise and in continuous culture, another impediment to the resolution of these discrepancies is that the two laboratories used different observation temperatures and instruments of different sensitivity in their EPR studies. Moreover, neither group attempted to correlate their results with the type and level of NADH dehydrogenase present in the different growth conditions, although it is well known that the binding site of piericidin, coupling site 1, and the EPR-detectable centers discerned are all located on NADH dehydrogenase (16). The importance of this omission became apparent when it was found that log and stationary phase cells of *C. utilis* possess different types of NADH dehydrogenase and that only the latter contains centers 1 and 2, coupling site 1, and is inhibited by piericidin (1, 7).

It seemed clearly desirable to re-examine the basic observations on the apparent dissociation of coupling site 1 and piericidin sensitivity and of both from the EPR-detectable components of NADH dehydrogenase, with simultaneous determination of the type and level of NADH dehydrogenase present, using cells grown in continuous culture and monitoring EPR signals under optimal conditions. The present paper is an account of such an investigation.

EXPERIMENTAL PROCEDURE

The strain of *C. utilis* used in our previous studies (1, 17) was maintained on agar plates (2% agar, 1% yeast extract, 1% bactopeptone, 5% glucose, all w/v). Cells were grown by continuous culture in the medium of Light and Garland (18), except that $MnCl_2$ and $FeCl_3$ were omitted from the metal solution and the concentration of glycerol was 110 mM. A standardized (19) 10 mM solution of $FeCl_3$ in 1 N HCl was added to the growth medium as required. The concentration of total iron in the growth medium, in the absence of added iron, was found to be 0.12 μM by the bathophenanthroline procedure (20). The iron concentrations prevailing during growth noted in the figures to follow represent added iron and must, therefore, be corrected by the value above to arrive at total iron concentration.

The chemostat consisted of a Mini-Ferm M-1000 vessel (0.7-liter working volume, Fermentation Design, Inc., Allentown, Pa.) thermostated at 30 ± 1°. The growth medium was delivered at 0.12 liter per hour by means of a peristaltic pump (model 203, Scientific Industries, Inc., Mineola, N. Y.). The pH of the culture was monitored continuously with a steam-sterilizable Ingold electrode, coupled by means of a Corning model 10 pH meter and a Calex model 415-212 volt sensor (Calex, Inc., Alamo, Calif.) to operate either a solenoid valve or to activate a peristaltic pump (Coleman I-V pump). These, in turn, served for the automatic addition of 2 N KOH, maintaining the culture at pH 5.0 ± 0.1 at all times. Air was introduced into the growth vessel at 3 liters per min through a candlewick-type filter immersed in the culture. The outflowing yeast suspension and gases left the chemostat vessel through a side arm and the cell suspension was collected in a reservoir surrounded by ice.

At the onset of operations the appropriate culture medium in the chemostat vessel was inoculated with 1 ml of a stationary phase culture (grown on 1.5% (v/v) ethanol in a shake flask (1)) through a self-sealing rubber septum by means of a hypodermic syringe. After 24 hours growth the flow of growth medium was started and the collection of cells for the experiments to be described was begun after an additional 36 to 48 hours of equilibration period. The cells, collected overnight, were sedimented in a Lourdes Clinifuge at 2000 × *g* and were twice washed with distilled water at 0° by centrifugation.

Dry weight of cells was determined by sedimenting 4 ml of the effluent from the chemostat, washing the cells twice with distilled water by centrifugation, and drying for 24 hours at 100°. In all experiments to be described the dry weight of cells varied linearly with the concentration of iron in the range of 0 to 0.5 μM added iron and was constant at 5.5 mg per ml in the range of 1 to 10 μM added iron. The dry weight of cells did not increase on increasing the flow rate of incoming air beyond 3 liters per min.

Mitochondria were prepared and mitochondrial activities were measured as described by Light and Garland (18) except that 2 mg per ml of bovine serum albumin were present during osmotic breakage, incubation with Glusulase was at 25°, and 1 mg per ml of bovine serum albumin was present in the assay medium in measurements of the oxidation of pyruvate plus malate (12.5 mM), ethanol (20 mM), DL-α-glycerophosphate (5 mM), and NADH (1 mM). In polarographic assays the O_2 electrode was calibrated by the method of Chappell (21) and ADP solutions were standardized by the procedure of Adam (22).

The preparation of ETP_c,[1] measurements of respiratory control, P:O ratio, measurement of the sensitivity to piericidin of mitochondrial oxidations and of NADH oxidation in ETP_c, and assays of NADH oxidase and NADH-ferricyanide reductase activities were as previously described (1, 17). The oxidation of NADH by juglone in ETP_c was measured polarographically (1) but the values given in the present paper are maximal velocities at extrapolated infinite juglone concentration. Protein was determined by the method of Lowry *et al.* (23).

Oxidized, NADH-reduced, and dithionite-reduced ETP_c samples were prepared, maintained in liquid nitrogen, and EPR spectra were recorded at ~13 K and evaluated as in the previous paper (1).

RESULTS

Dependence of NADH Oxidase Activity and Piericidin Sensitivity on Iron Concentration—Fig. 1*A* represents the variation of NADH oxidase activity and of the maximal piericidin sensitivity of NADH oxidation with the concentration of iron. The sigmoidal curve describing the variation in piericidin sensitivity is strongly reminiscent of similar data for the transition of exponential phase cells to stationary phase cells (1). It may also be noted that the degree of inhibition of the oxidation of malate plus pyruvate or of ethanol, at any incoming iron concentration, coincides with the inhibition of NADH oxidation in ETP_c from the same cells.

The curve describing the variation of the specific activity of NADH oxidase in ETP_c with increasing iron concentration is more complex. At relatively low iron concentrations (~0.3 to 0.5 μM), when piericidin sensitivity is nearly absent, NADH oxidase activity is high, and as piericidin sensitivity increases, NADH oxidase activity falls, as in the log → stationary phase transition (1). At very high iron concentrations, however, NADH oxidase activity rises again and at very low (<0.2 μM) incoming iron concentrations both NADH oxidase activity and piericidin sensitivity are low.

Dependence of NADH Dehydrogenase on Iron Concentration—Fig. 1*B* summarizes data for the variation of NADH-ferricyanide reductase and NADH-juglone reductase activities with increasing iron concentration in the medium. It was shown in the previous

[1] The abbreviations used are: ETP_c, inner membrane preparation obtained by shaking yeast cells with glass beads; juglone, 5-hydroxy-1,4-naphthoquinone; Hipip, high potential iron protein.

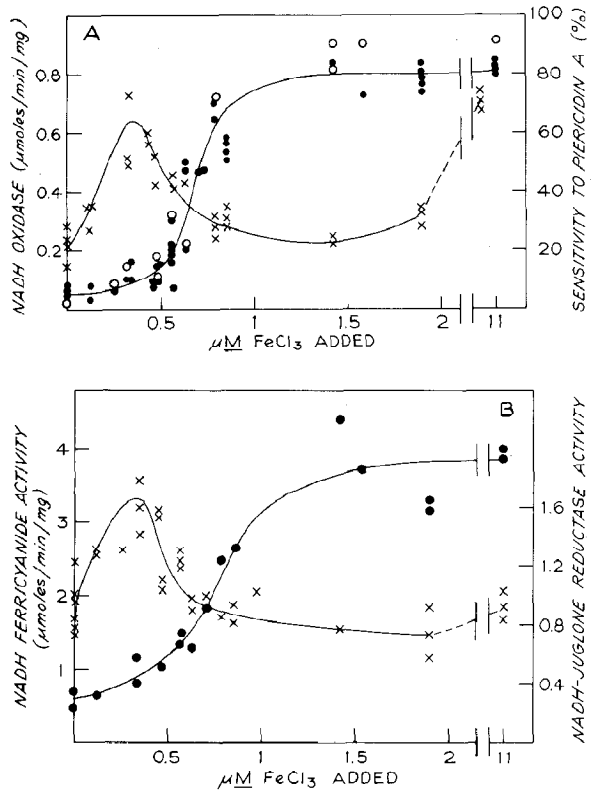

FIG. 1. *A*, variation of piericidin sensitivity and of NADH oxidase activity with iron concentration during the transition from iron-limited to glycerol-limited growth. ●, piericidin sensitivity of NADH oxidation in ETP_c; ○, piericidin sensitivity of State 3 pyruvate + malate oxidation in mitochondria; ×, NADH oxidase activity in ETP_c. Piericidin A was used at a ratio of 300 nmol per mg of protein. *B*, variation of NADH-ferricyanide and NADH-juglone reductase activities with iron concentration. ●, NADH-ferricyanide activity in ETP_c; ×, NADH-juglone reductase activity in ETP_c. Both activities are expressed in micromoles of NADH oxidized per min per mg of protein.

paper (1) that the former activity is characteristic of the type of NADH dehydrogenase present in stationary phase cells of *C. utilis* and the latter of the type occurring in log phase and catabolite-repressed cells. The two curves in Fig. 1*B* are practically superimposable on those in Fig. 1*A*, showing that piericidin sensitivity rises parallel with NADH-ferricyanide activity as the concentration of iron is increased and that NADH oxidase activity rises and falls with juglone reductase activity, as in the log → stationary phase transition (1). It is important for what follows to note that in the critical range of iron concentration (~0.5 to 1 μM) where piericidin sensitivity undergoes the most rapid increase, the type of inner membrane NADH dehydrogenase characteristic of log phase cells (high juglone and low ferricyanide reductase activity) is replaced by the type characteristic of stationary phase cells (high ferricyanide, low juglone reductase). That this is so is further documented in Fig. 2, showing the kinetics of the NADH-ferricyanide assay at various increasing iron concentrations. It is known (1) that the NADH-ferricyanide assay gives biphasic double reciprocal plots in log phase and catabolite-repressed cells but not in stationary phase cells. At very low iron concentrations this assay is clearly biphasic; in the critical range of iron concentrations the biphasicity diminishes as one type of NADH dehydrogenase is replaced by another; at increasing iron concentrations in excess of ~1 μM biphasic plots are no longer seen.

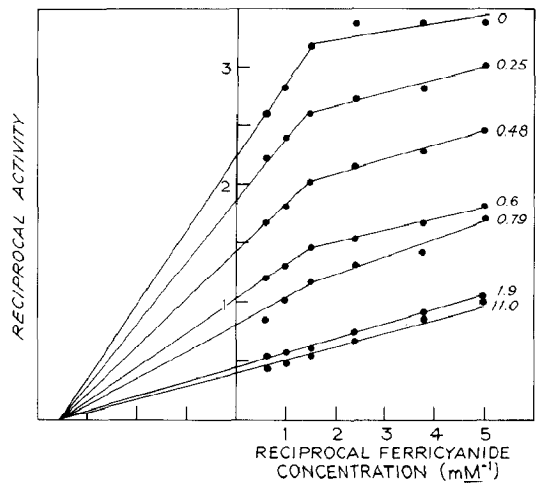

FIG. 2. Double reciprocal plots for NADH-ferricyanide reductase activity in ETP_c. The number assigned to each plot indicates the micromolar concentration of $FeCl_3$ added to the growth medium. The units of the *ordinate* are reciprocal absorbance change at 420 nm per min, and the plots have been normalized to a protein concentration of 0.33 mg of protein per ml.

EPR Signals of NADH Dehydrogenase at Various Iron Concentrations—If the presence of piericidin-sensitive NADH oxidase activity and of site 1 phosphorylation (*cf.* below) are primarily functions of the concentration of the type of NADH dehydrogenase in stationary phase cells of *C. utilis* and in mammalian mitochondria, this should be reflected in the EPR signal of iron-sulfur centers 1 and 2 of NADH dehydrogenase, since these are not seen in log phase cells but are prominent in stationary phase cells (1). This was indeed the case, as shown in Fig. 3 (*solid circles*). In the critical range of increasing iron concentrations a sharp rise in the signal amplitude of centers 1 + 2 is seen in dithionite-reduced ETP_c particles. The increase occurs essentially parallel to the rise in NADH-ferricyanide reductase activity and piericidin sensitivity (Fig. 1, *A* and *B*). In contrast, the concentration[2] of iron-sulfur centers 3 + 4 shows no significant change in the concentration range of 0 to 1 μM incoming iron, which includes the critical range (Fig. 3). This is also in accord with our previous observations (1) that iron-sulfur centers 3 + 4 show much less variation in the log → stationary phase transition or during catabolite repression than centers 1 + 2.

Observations have been reported (24) which make it appear that under some conditions iron-sulfur center 1 may be formed before center 2, whereas we have consistently observed their simultaneous appearance. The observations cited were qualified, however, by the statement that "greater sensitivity (of instrumentation) . . . is required to exclude the presence of center 2" (24). We believe that in our experiment sufficient sensitivity was reached to justify the conclusion that centers 1 and 2 are formed simultaneously under all conditions studied by us.

Fig. 3 (*open circles*) also shows the dependence of the Hipip signal on increasing iron concentration. A protein of this type has been recently isolated from heart mitochondria (25) and the same or a similar protein is functionally linked to succinate oxidation in Complex II but not in soluble preparations from that tissue (26); however, it is not known to function in the NADH oxidase system. The Hipip iron-sulfur signal increases in practi-

[2] Note that in this study, as in previous work (1), we were not able to differentiate between Centers 3 and 4 and, therefore, these centers are considered together.

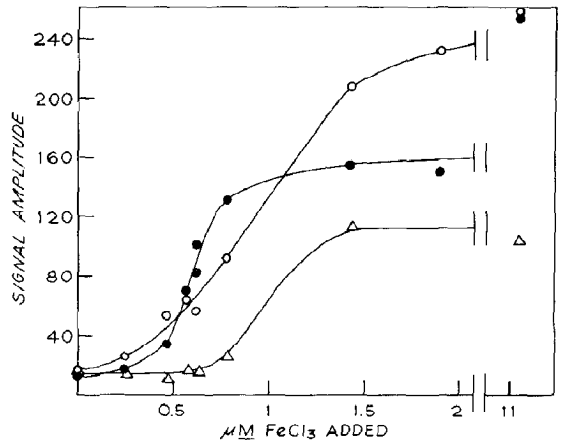

FIG. 3. Plots of the relative heights of the EPR signals of Fe-S centers 1 + 2, 3 + 4, and of the Hipip species in ETP_c *versus* iron content of growth medium. The spectra were recorded and the signal heights were evaluated as in Ref. 1. For the measurement of the intensity of centers 3 + 4, eight spectra of every sample were accumulated in the Nicolet 1020 signal averager. The appearance and quality of the spectra used for the graph were analogous to those shown in Figs. 2, 6, and 7 of Ref. 1. The signal of the Hipip species in oxidized samples was estimated from spectra recorded at 10 K and at 10 mwatt. Under these conditions the signals due to copper and free radicals are saturated. After correction for remaining features of these signals the main slope of the Hipip signal at g = 2.01 was taken as a measure of its intensity. The signal heights of the various species shown in the graph were normalized for the sample with no added iron and are expressed in arbitrary units. If a comparison of signal heights between species is desired, the following ratio of signal heights under standard recording conditions, as observed for the samples with 11 μM added $FeCl_3$ may be used as a basis: Hipip centers 1 + 2:centers 3 + 4 was 30:13:1. ●, centers 1 + 2; △, centers 3 + 4; ○, Hipip.

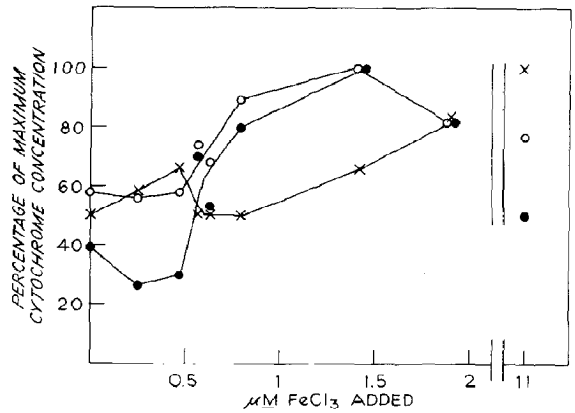

FIG. 4. Plot of the relative intensities of light absorption of cytochromes $a + a_3$, b, and $c + c_1$ in ETP_c at characteristic wavelengths *versus* iron content of growth medium. The light absorption was evaluated from diffuse reflectance spectra recorded at 95 K as in Ref. 1. Absorbances were measured for the dithionite-reduced samples at 605 *versus* 630, 562 *versus* 580, and 548 *versus* 540 nm. The absorption intensities shown are expressed as percentage of the maximal intensity observed in this set of samples. ●, cytochromes $a + a_3$; ○, cytochromes b; ×, cytochromes $c + c_1$.

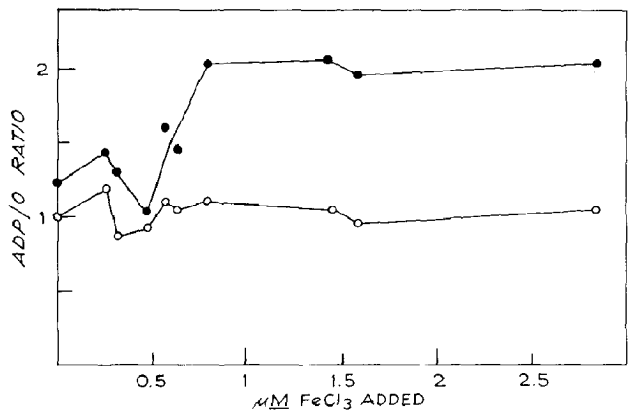

FIG. 5. Appearance of site 1 phosphorylation during the transition from iron-limited to glycerol-limited growth. Mitochondria were assayed polarographically with either pyruvate + malate (●) or α-glycerophosphate (○) as substrate. Assay conditions were as described under "Experimental Procedure."

cally the same range of iron concentration as do the signals of centers 1 + 2. This is also in accord with the fact that the Hipip signal shows a major increase in the log → stationary phase transition (1).

Variation of Cytochrome Content with Iron Concentration—Fig. 4 shows the variation of the apparent content of cytochromes a, b, and $c + c_1$ with iron concentration as determined by reflectance spectra in ETP_c samples reduced with dithionite. Cytochromes $c + c_1$ show relatively little variation, while the content of cytochromes b and, particularly, of cytochrome a increases significantly in the critical range of iron concentration. The curves relating the apparent content of cytochromes a and b to prevailing iron concentration appear to be similar, but opposite in sign, to those characterizing NADH oxidase and juglone reductase activities (Fig. 1, *A* and *B*).

Dependence of Coupling Site 1 on Iron Concentration—With this background we may now examine whether coupling site 1 indeed develops at lower iron concentrations than piericidin sensitivity, as has been claimed (2). Since the appearance of site 1 prior to that of piericidin sensitivity is said to occur in an extremely narrow range of iron concentration, it seemed better to present the data by plotting P:O (*i.e.* ADP:O) ratios against increasing iron concentration, as in Fig. 5, so that the data can be superimposed on similar data for piericidin sensitivity (Fig. 1*A*), in order to minimize errors, rather than to present tabulated data for P:O ratios and piericidin sensitivity at a given iron concentration, as in the report of Clegg and Garland (2). As a further attempt to detect any deviation in the dependence of site 1 and of piericidin sensitivity on iron concentration, the two parameters are plotted against each other in Fig. 6. Inspection of Figs. 5 and 6 reveals no evidence for the dissociation of site 1 phosphorylation and of piericidin sensitivity in the transition from iron-limited to iron- and substrate-limited cells (*i.e.* in the critical range of ~0.5 to 1 μM iron).

Some comments are appropriate on the ADP:O ratios in Figs. 5 and 6. In our hands, mitochondria from *C. utilis* prepared by the Glusulase procedure under the conditions used by Clegg and Garland (2) and Light and Garland (18) never give the theoretical ratios for ADP:O of 3 for malate + pyruvate or ethanol and 2 for α-glycerophosphate oxidation. Rather, the ratios observed are 2 to 2.2 and 1.0 to 1.2, respectively, apparently because of some ATPase action.[3] These ratios did not vary with the respiratory control, so that even when the latter was as high as 4.5, the ADP:O ratio for NAD-linked substrates was 2 to 2.2. This in no way invalidates the data, however, since our comparison is based on the relative increase in the ADP:O ratio for pyruvate + malate oxidation over α-glycerophosphate oxidation

[3] In a report from Garland's laboratory (27) the value of 1.6 is called more realistic than 3.0 for the oxidation of NAD-linked substrates in glycerol-limited (*i.e.* stationary phase) cells.

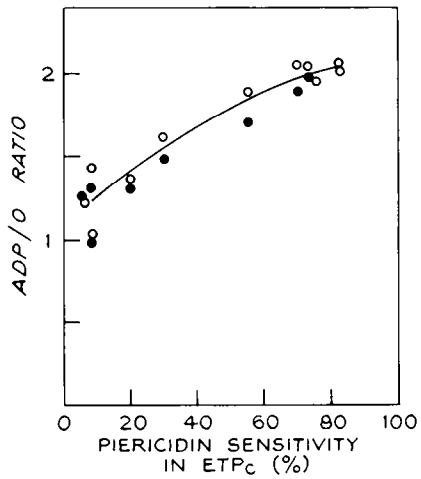

FIG. 6. Appearance of coupling site 1 plotted as a function of piericidin sensitivity. The ADP:O ratios were determined in mitochondria as described under "Experimental Procedure" with either ethanol (●) or pyruvate + malate (○) as substrate.

(which by-passes site 1) (Fig. 5). The increase corresponds clearly to an ADP:O ratio of 1, showing the operation of site 1 in the case of malate + pyruvate (Fig. 5) or ethanol oxidation (Fig. 6) over that observed during α-glycerophosphate oxidation in the same mitochondria.

Events Accompanying Aeration of Iron-limited Cells—Since no evidence was found for the prior appearance of coupling site 1 than of piericidin sensitivity during the transformation of iron-limited to iron- and substrate-limited cells, attention was turned to studying the finding (2) that during aeration of iron-limited cells in the presence of cycloheximide site 1, but not piericidin sensitivity, develops.

Tables I and II summarize the data for the results of aeration of cells grown in the presence of 0.32 μM added iron, in the presence and absence of cycloheximide and of 50 μM added iron. Table I shows that aeration without added iron or cycloheximide results in a major increase in NADH-ferricyanide activity and piericidin sensitivity and a decline in NADH-juglone reductase and NADH oxidase activities. The concentration of iron-sulfur centers 1 + 2 nearly doubles, whereas that of the Hipip signal shows an 8-fold increase (Table II). All these changes are prevented by cycloheximide. In contrast, the concentration of iron-sulfur centers 3 + 4, as measured by signal height, increases little on aeration and this slight increase is not prevented by cycloheximide (Table II). These observations are as would be expected if aeration caused primarily a transition from log type to stationary phase type cells. This interpretation is further supported by the fact (Table I) that aeration abolishes the biphasic kinetics in the NADH-ferricyanide assay.

Aeration in the presence of 50 μM added iron gave substantially the same results as aeration without added iron, except that the rise in the EPR signals of all of the iron-sulfur centers was substantially greater, while the decline in NADH oxidase activity was not evident (Tables I and II). Again, cycloheximide prevented these changes. More importantly, aeration in the presence of iron resulted in the appearance of site 1 phosphorylation, along with piericidin sensitivity, and the appearance of both of these properties was prevented by cycloheximide.

It appears, therefore, that aeration of iron-limited cells in the presence of cycloheximide fails to produce cells endowed with coupling site 1 but lacking piericidin sensitivity. The process of aeration in the absence of oxidizable substrate, with or without added iron, closely simulates the events occurring during the transition of log phase to stationary phase type cells in batch culture (1), as may be expected from the fact that both situations involve a response of *C. utilis* cells to substrate exhaustion. The more pronounced effects of aeration in the presence of excess iron than without iron, as reflected in the increased concentration of the EPR-detectable iron-sulfur centers, undoubtedly reflects the greater availability of iron, the limiting component, for the *de novo* synthesis of the NADH dehydrogenase characteristic of stationary phase cells.

TABLE I

Changes accompanying aeration of iron-limited cells with and without added iron and of cycloheximide

Cells were grown in the chemostat at 0.32 μM added iron concentration under the conditions given under "Experimental Procedure." The cells were collected overnight. A part of the cells (20 g) was used as a control for the preparation of mitochondria and of ETP_c without further treatment. Thirty grams of cells, suspended in 300 ml of 10 mM KP_i buffer, pH 7.5, were aerated for 7.5 hours at 30° with or without 50 μM $FeCl_3$ and 100 μg per ml of cycloheximide as indicated. After aeration, the cells were collected by centrifugation, washed twice with cold distilled water, and mitochondria and ETP_c were prepared.

Property	Control (not aerated)	Aerated without iron or cycloheximide	Aerated without iron in presence of cycloheximide	Aerated with iron, without cycloheximide	Aerated with iron and cycloheximide
Piericidin sensitivity in ETP_c (%)	10	86	9	85	19
Piericidin sensitivity in mitochondria (%)	15			88	22
NADH oxidase[a]	0.34	0.15	0.45	0.34	0.38
NADH-ferricyanide reductase[a]	0.81	1.9	1.3	1.5	1.1
NADH-juglone reductase[a]	1.43	0.45	1.29	0.62	1.14
Biphasic kinetics in ferricyanide assay	Present	Absent	Present	Absent	Present
ADP:O for pyruvate + malate	1.3			2.06	1.4
ADP:O for α-glycerophosphate	0.86			1.05	1.14

[a] Micromoles of NADH oxidized per min per mg.

DISCUSSION

This investigation was initiated to gather additional evidence for the identity of the particular iron-sulfur centers of NADH dehydrogenase involved in coupling site 1. We hoped to take advantage of the observations (2, 3) that the appearance of coupling site 1 may be elicited, without the appearance of piericidin sensitivity or of iron-sulfur center 1 (the only iron-sulfur component detected by EPR at 77 K), by suitable manipulation of the cells, offering thereby an opportunity to identify by EPR studies at 13 K the particular iron-sulfur center which develops coincidentally with site 1. As shown in this paper, however, the observation on the dissociation of site 1 phosphorylation from piericidin sensitivity or from the emergence of iron-sulfur center 1 could not be confirmed. While the present finding that the

TABLE II

EPR-detectable iron-sulfur centers appearing on aeration of iron-limited cells

The preparations used were those described in Table I. ETP_c samples were reduced with dithionite and EPR spectroscopy conducted as in previous work (1).

Treatment	Signal height[a]		
	Centers 1 + 2	Centers 3 + 4	Hipip
	mm/mg protein		
Control (not aerated)	32	16	13
Aerated without iron or cycloheximide	57	18	113
Aerated without iron in presence of cycloheximide	32	22	25
Aerated with iron, without cycloheximide	96	22	156
Aerated with iron and cycloheximide	31	16	8

[a] The signal heights are expressed as in Fig. 3 and the comparison of heights between species is to be done as outlined here.

aeration of iron-limited cells in the presence of cycloheximide prevents the appearance of site 1, piericidin sensitivity, and of iron-sulfur centers 1 + 2 thus confirms the report of Ohnishi *et al.* (15), we agree with Clegg and Garland (2) and Garland (4) that Ohnishi's studies were inconclusive because of the heterogeneity of the cell population in batch cultures. That is why in the present study cells were grown in continuous culture under as nearly as possible the same conditions as used by Clegg and Garland (2). Moreover, by monitoring several parameters which had not been examined in the earlier studies (2, 15), we obtained data for the type of NADH dehydrogenases present and their activities in the various experimental situations. This, in turn, provides a basis for explaining why site 1, piericidin sensitivity, and iron-sulfur centers 1 + 2 develop on aeration of iron-limited cells or in the transition from iron-limited to iron- and substrate-limited cells. In both situations cells possessing the exponential phase type NADH dehydrogenase replace this enzyme with the dehydrogenase characteristic of stationary phase and mammalian cells (1) and, because of this, site 1, piericidin sensitivity, and centers 1 + 2 appear.

There is one other observation on the differential development of site 1, without the appearance of piericidin sensitivity, which has not been dealt with in the present study. Haddock and Garland (28) reported that on aeration in the presence of cycloheximide of *C. utilis* cells, grown in sulfur-limited conditions, site 1 appears, but piericidin sensitivity does not. The experiment is entirely analogous to that involving the aeration of iron-limited cells. In both situations growth is limited by one of the components of the iron-sulfur centers of the enzyme with substrate in excess. If these observations are correct, it seems likely that aeration of sulfur-limited cells, as of iron-limited cells, would cause exhaustion of the substrate and thereby transformation of log phase type to stationary phase type cells, with attendant development of site 1, piericidin sensitivity, and centers 1 + 2. By preventing the replacement of the log phase type enzyme with the stationary phase type, cycloheximide may be expected to block the appearance of both site 1 and of piericidin sensitivity.

Following the completion of this study, we attempted to repeat the observations of Haddock and Garland (28) on the effects of sulfur limitation. Cells were grown under their conditions, but since the concentration of iron was not specified in their paper, 10 μM $FeCl_3$ was included to assure the absence of iron limitation. Under these conditions, with 100 μM sulfur, as K_2SO_4, present in the medium, as recommended (28) for the production of sulfur-limited cells lacking piericidin sensitivity, growth was clearly limited by the amount of sulfur, since the cell yield fell from 5.0 to 3.5 mg per ml, but the sensitivity of NADH oxidation to piericidin was still 65 to 80%. When the sulfur concentration was further lowered to 20 μM, the yield of cells was extremely low (0.8 mg per ml) but sensitivity to piericidin was still evident (40 to 46%). NADH-ferricyanide activity paralleled piericidin sensitivity, with specific activities in the ETP_c of 3.8 to 2.5, respectively, in the experiments quoted, showing the predominance of the stationary phase enzyme. In view of these data, it does not seem feasible to evoke site 1 phosphorylation in the absence of sensitivity to piericidin by the aeration of sulfur-limited cells in the presence of cycloheximide.

Although the present study failed to confirm the observations (2, 4) of the dissociation of coupling site 1 from piericidin sensitivity, this should be by no means taken to suggest that the same iron-sulfur center is involved in the piericidin inhibition site and in coupling site 1, for it is well known (8, 10, 11) that site 1 phosphorylation occurs normally in preparations completely inhibited by piericidin or rotenone.

As noted above, the original aim of this investigation was to search for a correlation between the appearance of specific iron-sulfur centers and the development of coupling site 1. It is doubtful whether this aim has been attained, for while the appearance of both iron-sulfur centers 1 and 2 of the mammalian type NADH dehydrogenase showed a much better correlation with the development of site 1 than iron-sulfur centers 3 + 4, the appearance of site 1 was not selective but involved the replacement of one type of NADH dehydrogenase by another. On the other hand, two findings of considerable interest emerged as a result of this investigation. The first is that *C. utilis* appears to be coded for two different inner membrane-bound NADH dehydrogenases, one which it develops when substrate supply is ample, the other which emerges when substrate supply is limited. Only the latter is coupled to energy conservation site 1. Thus the ATP yield from the oxidation of NAD-linked substrates is higher when substrate supply is limited and, hence, the mammalian type NADH dehydrogenase predominates.

This flexibility in regulating the type of enzyme dealing with an important step in electron transport, to our knowledge, has no counterpart in mammalian cells but bears close analogy to dual succinate dehydrogenases which occur in facultative anaerobes (*e.g.*, *Escherichia coli*, *Saccharomyces cerevisiae*) (29). In the latter instances, the two types of dehydrogenase are under separate genetic control and their biosynthesis and active destruction is controlled by the availability of fermentable substrates and by the supply of O_2 (29). The analogy to the situation in *C. utilis* appears obvious.

The second observation pertains to the type of iron-sulfur centers present and operational in the log and stationary phase types of NADH dehydrogenase. Although only the latter has been obtained in purified form so far,[4] certain predictions may be made concerning the iron-sulfur centers of both enzymes. Thus, it is clear that iron-sulfur centers 1 + 2 and 3 + 4 are all present

[4] Purification of the log phase type enzyme is in progress in this laboratory.

in the stationary phase enzyme. Because centers 3 + 4 undergo relatively little increase in the log → stationary phase transition and during catabolite repression (1, 7), in the transition of iron-limited to iron- and substrate-limited cells, or on aeration of iron-limited cells, it seems very likely that these centers are also present in the log phase enzyme.

The EPR data presented in this paper also contradict the report (14) that no correlation exists between the appearance of iron-sulfur center 1 and piericidin sensitivity as the iron concentration prevailing during growth is increased. The correlation is, in fact, excellent (Figs. 1 and 3).

There are two other aspects of previous studies of the problem which deserve comment. First, our discovery of the replacement of one type of NADH dehydrogenase by another in *C. utilis* during the log → stationary phase transition and during catabolite repression (1) was based on the studies of Katz *et al.* (30), who first reported the changes in piericidin sensitivity and site 1 phosphorylation in these transitions. These fundamental observations were questioned by Ohnishi (31), who claimed that they occur only when growth is slow because of limited O_2 supply. We have shown elsewhere (1, 7) that the observations of Katz *et al.* are verifiable, whether growth is rapid and O_2 supply in excess or not. Had it not been for the correctness of these observations, the interpretation of the data in this paper would have been difficult, if not impossible.

The second point concerns the interpretation of Clegg and Garland (2) and Ragan and Garland (32) based on their reports of the separation of coupling site 1 from piericidin sensitivity, as regards the question whether soluble NADH-ubiquinone reductase, devoid of typical rotenone- and piericidin-sensitivity, is a breakdown product of NADH dehydrogenase. They proposed that, since an NADH dehydrogenase functional in electron transport and site 1 phosphorylation may be observed in the absence of piericidin sensitivity, piericidin-insensitive CoQ reductases from mammalian cells need not be artifacts. The conclusion that piericidin-insensitive NADH-ubiquinone reductases solubilized from mammalian mitochondria result from the breakdown of the native structure of the enzyme does not rest solely, however, on their insensitivity to inhibitors but on a number of other experimental facts (1, 33). In view of the present findings, *viz.*, that piericidin sensitivity is an invariable attribute of NADH dehydrogenase of the mammalian type, previous inferences (2, 32) as to the nature of purified NADH dehydrogenases lacking such sensitivity would seem unwarranted.

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