STUDIES ON NON-HEME IRON PROTEINS AND THE PIERICIDIN A BINDING SITE OF SUBMITOCHONDRIAL PARTICLES FROM *CANDIDA UTILIS* CELLS GROWN IN MEDIA OF VARYING IRON CONCENTRATIONS.

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SUMMARY

EPR spectra of iron sulfur proteins associated with succinate dehydrogenase, glycerol-1-phosphate dehydrogenase, NADH dehydrogenase, and, perhaps, the cytochromes *b* and c_1 in submitochondrial particles from *Candida utilis* are presented. The piericidin A sensitive site is assigned to the oxygen side of the iron sulfur protein associated with NADH dehydrogenase. Virtually no correlation of piericidin A sensitivity with the EPR signal of the NADH dehydrogenase-linked iron sulfur protein is indicated from comparison of these parameters as a function of iron concentration in the growth medium.

From comparative studies of Site I phosphorylation and the respiratory chain components in the NADH dehydrogenase segment in mitochondria and submitochondrial particles prepared from *S. cerevisiae* (1-4), *S. carlsbergensis* (5), and *C. utilis* (6-9), it has been suggested that either the non-heme iron EPR signal or the piericidin A binding site, or both, are related to the mechanism of Site I phosphorylation. These possible correlations were further supported by observations of Light *et al.* (10), who showed the disappearance of both Site I phosphorylation and the piericidin A binding site in mitochondria which were prepared from *C. utilis* cells grown in a chemostat with iron limited medium. These authors proposed non-heme iron protein as a site for both Site I phosphorylation and piericidin A binding. Bois and Estabrook (11), using beef heart electron transport particles, suggested that the "g = 1.94" EPR signal is involved in the mechanism of rotenone inhibition. Our previous report (12) described the disappearance of both piericidin A sensitivity and the iron sulfur protein EPR signal associated with NADH dehydrogenase in submitochondrial particles from iron deficient *C. utilis* cells. In the present communication we wish to report studies on submitochondrial particles derived from cells grown at various iron concentrations which indicate that the piericidin A sensitive site and the iron sulfur protein EPR signal associated with NADH dehydrogenase are not directly related to each other.

MATERIALS AND METHODS

C. utilis cells were grown at 30°C in 20-liter vessels equipped with two aeration filters. The flow rate was adjusted to one liter of air per minute per liter medium. The synthetic culture medium (13)(18 liters) was supplemented with $FeCl_3$ at concentrations ranging from 0.7 to 100 μM (manganese was omitted). Cells were harvested in the early stationary phase of growth. Washed cells were suspended in 0.3 M mannitol, 0.5 mM EDTA (pH 6.8) and disintegrated in a colloid mill, following essentially the procedure of Balcavage and Mattoon (2). Mitochondria were isolated as reported previously (5). Submitochondrial particles were derived from mitochondria by sonication in 0.3 M mannitol, 0.5 mM EDTA, 1 mM ATP, 1 mM $MgCl_2$ and 20 mM Tris-HCl buffer (pH 7.4). The respiration was measured with a Clark oxygen electrode. EPR spectra were obtained with a Varian X-Band spectrometer (V4502-13) equipped with 100 KHz field modulation; modulation amplitude was approximately 12 oersteds. The samples were contained in 3 mm inner diameter quartz sample tubes immersed in liquid nitrogen. The cytochrome content of the submitochondrial particles was determined from difference spectra using a Coleman-Hitachi (Model 124) spectrophotometer. Particles were suspended in 0.1 M phosphate buffer (pH 7.2) containing 0.5 percent Na-deoxycholate. Millimolar extinction coefficients used for difference spectra are: cytochrome $(a+a_3)\varepsilon_{mM}$(605-630 mμ) = 24.0 (14), cytochrome $b\varepsilon_{mM}$(562-575 mμ) = 20.0 (15). Non heme iron in submitochondrial particles was extracted in 1 N HCl according to King et al. (16). The iron concentration in the extract and in the culture medium was determined spectrophotometrically using o-phenanthroline (17,18).

RESULTS AND DISCUSSION

EPR spectra of iron sulfur proteins of submitochondrial particles prepared from C. utilis cells under various conditions are shown in Fig. 1. Fig. 1A is an EPR spectrum without addition of substrates and Fig. 1B shows a spectrum obtained by reduction with both succinate and glycerol-1-phosphate after preincubation of particles with antimycin A. Addition of either succinate or glycerol-1-phosphate alone gives rise to a spectrum with a similar lineshape and about half the intensity of that in Fig. 1B. The spectrum obtained by reduction with NADH after pretreatment of particles with piericidin A (1.3 nmoles per mg of protein) is shown in Fig. 1C. The spectrum after all three substrates were added in the presence of antimycin A is shown in Fig. 1D. These spectra clearly illustrate the difference in line shape of the resonance absorption of the various iron sulfur proteins associated with succinate, glycerol-1-phosphate, and NADH dehydrogenases

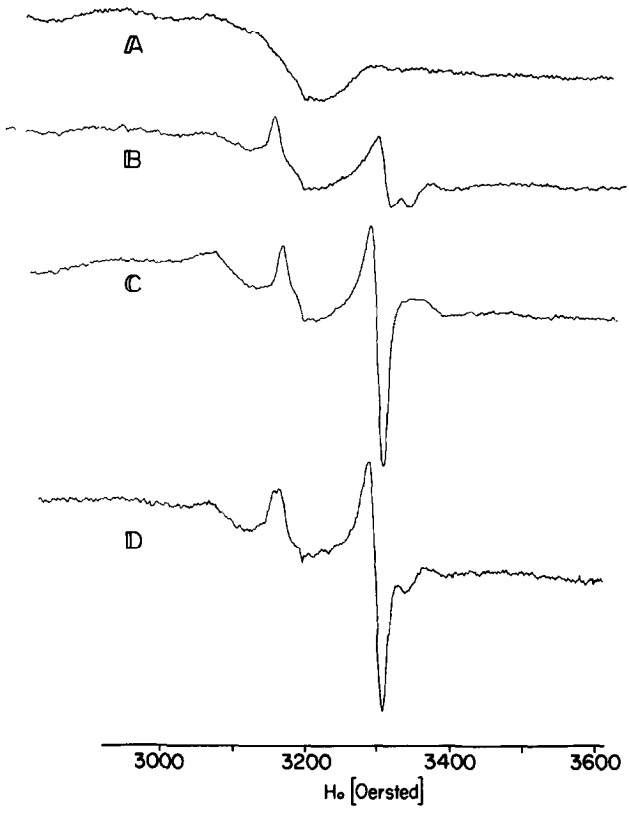

Figure 1. EPR spectra of submitochondrial particles from C. utilis cells reduced by various substrates.
Cells were grown in synthetic medium containing 100 μM $FeCl_3$. Submitochondrial particles (42 mg protein/ml), suspended in 0.3 M mannitol, 10 mM Tris-HCl buffer (pH 7.4), 0.5 mM EDTA, were preincubated with antimycin A (1.2 nmoles/mg protein) for 10 minutes at 0°C for spectra A, B and D. A. The suspension was frozen in liquid nitrogen without addition of substrate. B. Both 17 mM succinate and 17 mM glycerol-1-phosphate were added. C. Particles were preincubated with piericidin A (1.3 nmoles/mg protein) for 10 minutes at 0°C and 17 mM NADH was added. The suspension was frozen in less than 40 seconds. D. Succinate, glycerol-1-phosphate and NADH were added together. The sample was frozen after two minutes.

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and, perhaps, with cytochrome *b* + c_1 in submitochondrial particles from *C. utilis*. In the presence of piericidin A, the non-heme iron species associated with NADH dehydrogenase is reduced almost exclusively, as illustrated clearly by the comparison of the line shapes especially in the low and high field region. The iron sulfur protein associated with NADH dehydrogenase must therefore be located on the substrate side of the piericidin A sensitive site. A similar assignment has been made for mammalian systems

by Tyler et al. (19), Chance et al. (20), and Palmer et al. (21).

In order to study a possible correlation between piericidin A sensitivity and the iron sulfur protein EPR signal associated with NADH dehydrogenase, C. utilis cells were grown in culture media containing iron at various concentrations between 0.7 and 50 μM. Relatively constant growth yields of about 20 gram wet cells per liter were obtained with iron concentration in the range of 2 to 50 μM, but below 1 μM the growth of the cells was found to be limited by iron under our culture conditions (5-8 gram wet cells per liter). Above 3 μM, iron uptake into cells was nearly proportional to initial iron concentration in the medium; about half of the initial iron was taken up. The percentage inhibition of NADH oxidation by piericidin A, the intensity of the iron sulfur protein EPR absorption, the content of bound non-heme iron (Fig. 2A), the cytochrome content (Fig. 2B) in submitochondrial particles prepared from these cells, were measured and are expressed as a function of initial iron concentration in the growth medium.

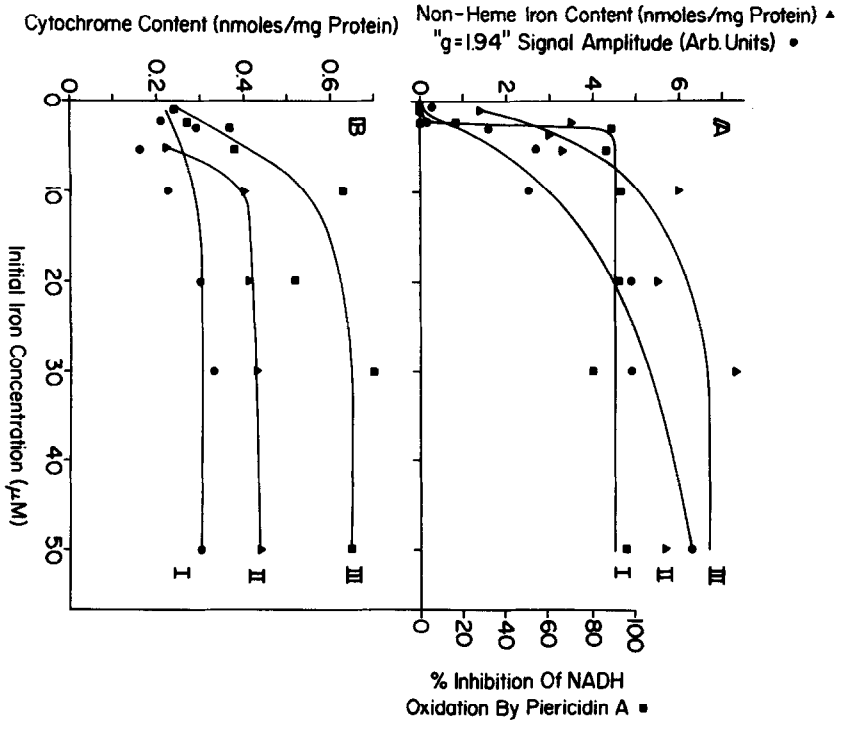

Figure 2. Effect of initial iron concentration in the culture medium on various parameters of submitochondrial particles.
A. Inhibition of NADH oxidation by piericidin A (I), EPR intensity of the NADH dehydrogenase linked iron sulfur protein ("g = 1.94 signal")(II) and content of bound non-heme iron (III). B. Content of cytochrome a + a_3 (I), cytochrome b reducible with succinate (II) and cytochrome b reducible with dithionite (III).

Cells grown in different iron concentrations respired at approximately equal rates with ethanol as substrate. Submitochondrial particles prepared from the cells grown in low iron (0.7-5 μM), however, showed a markedly decreased respiratory rate. The extent of inhibition of NADH oxidation by piericidin A in submitochondrial particles is illustrated in Fig. 2A-I. Below 1 μM iron, NADH oxidation was completely insensitive to piericidin A and at iron concentrations above 3 μM, about 90 percent of the respiration was inhibited by piericidin A.

The intensity of the EPR signal of the iron sulfur protein associated with NADH dehydrogenase was also studied as a function of iron concentration (Fig. 2A-II). In these experiments the iron sulfur protein associated with NADH dehydrogenase was reduced with NADH in the presence of piericidin A as shown in Fig. 1-C. In submitochondrial particles from C. utilis this reduction was fully reversible, while in electron transport particles from beef heart mitochondria this reduction was found to be partly reversible (11). Below 3 μM iron in the culture medium, antimycin A was used to inhibit the respiratory chain. The signal intensity is expressed as peak-to-peak amplitude observed under constant instrumental conditions and normalized for the protein concentration of the samples. The EPR intensity of the iron sulfur proteins associated with NADH dehydrogenase increased steadily in the region from 2 to above 50 μM iron (Fig. 2A-II).

The content of bound non-heme iron in the particles was measured as shown in Fig. 2A-III. It increased more steeply than the EPR signal in the range of iron concentration in the medium from 2 to 10 μM. The maximum bound non-heme iron content was about 7 nmoles per mg of protein, which is much higher than the total heme content (1.9 nmoles per mg of protein). At 1 μM initial iron concentration, 1.4 nmoles of bound non-heme iron per mg of protein was found, whereas almost no iron sulfur protein EPR signal could be detected. Thus, since little correlation of the piericidin A sensitivity with the "g = 1.94" EPR signal and non-heme iron profiles is observed, it appears that very little if any of either the EPR signal or the bound non-heme iron is associated with piericidin A sensitivity.

The relation between cytochrome content and iron concentration is shown in Fig. 2B. As illustrated, the cytochrome $\underline{a} + \underline{a}_3$ content did not vary much over a wide range of iron concentration in the growth medium. Cytochrome b content, however, increased in a similar fashion to the total bound non-heme iron (cf. Fig. 2A). Succinate did not reduce all the cytochrome b which was reducible by dithionite, a phenomenon observed only with cytochrome b. The content of cytochrome c was measured in whole cells,

since most of the cytochrome c is released from these submitochondrial particles during the preparation procedure. At 1 μM iron the cytochrome c content was approximately the same as the cytochrome a + a_3 content, but increased sharply to a three-fold greater content in a narrow range of iron concentration (1-2 μM) remaining constant above 2 μM in the medium under the growth conditions employed.

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