MECHANISM OF ELECTRON TRANSPORT AND ENERGY CONSERVATION IN THE SITE I REGION OF THE RESPIRATORY CHAIN

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I. INTRODUCTION

The mechanism of electron transfer and energy conservation in the Site I region has proven to be one of the most difficult problems to solve in the study of the respiratory chain, mainly due to three reasons: (1) the Site I segment of the respiratory chain contains many iron-sulfur components (as shown in Scheme I) and its labile structure hindered isolation and reconstitution studies of the respiratory components in this segment of the chain; (2) because of the low in situ concentration of the NADH dehydrogenase in comparison to that of the cytochrome chain, and because of its lack of readily measurable absorption bands in the visible region, optical
respiratory chain components, energy coupling sites and the reaction sites of respiratory inhibitors. The iron-sulfur centers (Fe–S) are numbered for identification. The (Fe–S) and (Fe–S) in the NADH dehydrogenase region are designated according to Orme-Johnson et al. and Ohnishi et al., respectively. (Fe–S) and (Fe–S) are iron-sulfur centers with the half-reduction potentials around 30 mV and 0 mV, respectively. (Fe–S) and (Fe–S) are iron–sulfur centers associated with the succinate dehydrogenase, the (Fe–S) signals are detectable at 77°K, while the (Fe–S) signals are detectable only below 30°K. (Fe–S) is that originally reported by Rieske et al.

techniques which have been useful for the study of Sites II and III have had rather limited merit for the study of Site I; (3) EPR techniques were also considered to have a limited applicability for studying mechanisms of electron transfer and energy conservation in the Site I region, because it was believed that EPR-detectable iron–sulfur proteins accounted for only 10 to 20% of the chemically determined total iron–sulfur proteins, until more iron–sulfur signals were observed using temperatures below that of liquid nitrogen. On the other hand, yeast has provided a very useful biological system for elucidating the importance of iron–sulfur proteins for both electron transport and energy conservation in the Site I region of the respiratory chain. In this review, attention will be focused on the development along this line of biological approach, and then extended to more recent progress on newly identified multiple iron–sulfur centers and their relationships to the mechanism of energy conservation at Site I.

II. COMPARATIVE STUDIES OF ELECTRON TRANSPORT AND ENERGY COUPLING IN THE SITE I REGION AMONG DIFFERENT YEAST STRAINS

Table I lists some characteristics of electron transport and energy coupling at the Site I region in various yeast strains. In studies with isolated, loosely coupled Saccharomyces cerevisiae (baker’s yeast) mitochondria, Vitols and Linnane obtained identical P/O ratios with either succinate or endogenous NAD-linked substrates and attributed this apparent lack of Site I phosphorylation to damage during the preparation of the mitochondria. However, Ohnishi et al. reported the same...
TABLE I

DIFFERENCES AMONG THE NADH–CYTOCHROME SEGMENTS OF THE RESPIRATORY CHAIN IN VARIOUS YEAST

N.T. = not tested. Q = ubiquinone.

<table>
<thead>
<tr>
<th></th>
<th>Site I*</th>
<th>Rotenone sensitivity*</th>
<th>&quot;g = 1.94&quot; in the NADH dehydrogenase region*</th>
<th>Flavin</th>
<th>Q, number of side-chain isoprenoid units</th>
<th>−SH (NADH–flavin) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. carlsbergensis</td>
<td>−***</td>
<td>−</td>
<td>−</td>
<td>N.T.</td>
<td>6^23</td>
<td>N.T.</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>−***</td>
<td>−</td>
<td>−</td>
<td>FAD^{19,32,33}</td>
<td>6^35</td>
<td>−^26</td>
</tr>
<tr>
<td>C. utilis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FMN and FAD^{33}</td>
<td>7 and 9^26</td>
<td>+^23</td>
</tr>
<tr>
<td>E. magnusii, yeast type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N.T.</td>
<td>9 or 10^23</td>
<td>N.T.</td>
</tr>
<tr>
<td>Animal tissue</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FMN^{34}</td>
<td>9–10^{37,38}</td>
<td>+^39</td>
</tr>
</tbody>
</table>

* +, present; −, absent.

** +, mercurial sensitive; −, mercurial insensitive.

*** This point will be discussed in more detail in Section IX.
phosphorylation efficiencies even in tightly coupled mitochondria prepared from *Saccharomyces carlsbergensis* by the mild procedure of disrupting protoplasts by osmotic shock. The lack of Site I phosphorylation in *Saccharomyces* mitochondria was subsequently confirmed by many investigators. On the other hand, all three phosphorylation sites were reported to be present even in loosely coupled mitochondria isolated from different strains, *Candida utilis* and yeast-type *Endomyces magnusii*. Ohnishi *et al.* succeeded in preparing tightly coupled *C. utilis* mitochondria which showed respiratory control in the oxidation of both endogenous NAD-linked substrates and exogenous NADH. They demonstrated the presence of Site I phosphorylation in the oxidation of endogenous NADH and the absence of Site I phosphorylation in the oxidation of exogenous NADH. They also showed that the endogenous NADH oxidation in *C. utilis* mitochondria was inhibited by rotenone similar to that in mammalian mitochondria while exogenous NADH was oxidized via a rotenone-insensitive pathway as in the case of either endogenous or exogenous NADH oxidation in *Saccharomyces* mitochondria. These observations suggested a close correlation between the occurrence of Site I phosphorylation and the presence of the rotenone inhibition site, a correlation which was also suggested in mammalian systems by Ernster *et al.* and Schatz and Racker. A further correlation between these two features (Site I energy coupling and rotenone sensitivity) and the presence of the "g = 1.94" EPR signal arising from iron–sulfur proteins associated with the NADH dehydrogenase region of the respiratory chain was provided from comparative studies in various strains of yeast. The iron–sulfur protein responsible for the "g = 1.94" EPR signal in the NADH dehydrogenase region is absent in submitochondrial particles of *S. cerevisiae* or *S. carlsbergensis*. On the other hand, it is present in *C. utilis* submitochondrial particles which in this respect are very similar to mammalian submitochondrial particles.

Butow and Racker also proposed that energy coupling at Site I occurs at the level of iron–sulfur protein, based on their observation that inhibition of NADH oxidation by the iron chelator o-phenanthroline was released by ADP plus Pi or by the uncoupler in mammalian submitochondrial particles. These observations implied a close correlation among the occurrence of Site I energy conservation, rotenone sensitivity, and EPR-detectable iron–sulfur proteins in the Site I region.

Thus comparative studies among different yeast strains promised to provide a useful approach to understanding the electron transport and energy conservation in the Site I region of the respiratory chain. However, a more detailed comparison of the electron transport system of *Saccharomyces* mitochondria and that of *C. utilis* and mammalian mitochondria showed the additional differences presented in the last three columns of Table I: (a) FAD rather than FMN is the flavin component of the NADH dehydrogenase of *Saccharomyces*; (b) the reduction of flavin by NADH is not inhibited by mercurials in *Saccharomyces* submitochondrial particles indicating that no mercurial-sensitive –SH groups are involved in the electron transport between NADH and flavin; (c) the Q component in *Saccharomyces* is Q₅ whereas *C. utilis* and mammalian systems contain higher homologues.
III. LOSS OF SITE I ENERGY CONSERVATION AND ROTENONE SENSITIVITY BY IRON- OR SULFUR-LIMITED GROWTH OF C. UTILIS CELLS

This biological approach became more promising when Light et al.\textsuperscript{40} and Garland\textsuperscript{41} reported the following observations: (1) Both Site I phosphorylation and piericidin A (or rotenone) sensitivity were lost when the growth-limiting component of the continuous culture of \textit{C. utilis} cells was shifted from the carbon source (glycerol) to iron. (2) These conversions were reversed \textit{in vivo} by aerating the cells (grown in the iron-limited medium) in the presence of added iron under non-growing conditions. (3) It was subsequently demonstrated that cells grown in sulfur-limited continuous culture were also deficient in Site I phosphorylation and piericidin A sensitivity\textsuperscript{42}. This conversion was likewise reversed \textit{by} \textit{in vivo} induction procedures. From these findings Garland and co-workers\textsuperscript{40}-\textsuperscript{42} proposed that iron–sulfur proteins may play a role in Site I energy conservation as well as in piericidin A sensitivity. Since no change occurred in the flavin and Q species during the Site I “on and off” transition in \textit{C. utilis} cells, these differences were regarded as a species difference and considered not to be directly related to the presence or absence of Site I energy conservation or the piericidin A-sensitive site. This dependence of the presence of Site I in \textit{C. utilis} cells on the iron concentration of the medium was confirmed by Ohnishi \textit{et al.}\textsuperscript{43,44} using cells grown in the batch culture. These investigators further suggested a close correlation between Site I phosphorylation and iron–sulfur proteins responsible for the \textit{g = 1.94} EPR signal in the NADH dehydrogenase region, from EPR studies using \textit{C. utilis} cells grown at varying iron concentration and cells before and after \textit{in vivo} induction of Site I phosphorylation (Fig. 1).

![Fig. 1. Correlation between phosphorylation at Site I and the normalized peak-to-peak amplitude of \textit{g = 1.94} EPR signal (measured at 77 °K) of iron–sulfur protein in the NADH dehydrogenase region of the respiratory chain in \textit{C. utilis} submitochondrial particles. Amplitudes of \textit{g = 1.94} signals are normalized for the same instrumental conditions and protein concentration of the samples. Arrows show simultaneous changes in these two parameters during aeration of resting cells, which have grown in low-iron medium\textsuperscript{44}. The P/O ratios were measured with isolated mitochondria, using 20 mM ethanol \textit{plus} 15 mM semicarbazide as a substrate.](image-url)
IV. EPR-DETECTABLE IRON-SULFUR PROTEINS AS ELECTRON CARRIERS IN THE RESPIRATORY CHAIN

Beinert and Palmer\textsuperscript{45} previously showed that in beef heart submitochondrial particles the \textquotedblleft $g = 1.94$\textquotedblright iron-sulfur proteins were reduced or oxidized at a rate not grossly different from that of the cytochromes, suggesting their role as electron carriers in the respiratory chain. This suggested role was questioned by several workers. Yamashita and Racker\textsuperscript{46} indicated non-involvement of iron-sulfur proteins in the respiratory chain of beef heart mitochondria from their observation that iron-sulfur protein\textsuperscript{7} in the cytochrome $b, c_1$ region was not required for the reconstituted succinate oxidase activity. Light \textit{et al.}\textsuperscript{40} and Garland\textsuperscript{41} proposed that iron-sulfur protein is not essential for the electron flow in the region between NADH and (Q, cytochrome $b$) because the mitochondrial respiratory rate with NAD-linked substrates was not altered when the continuous culture was shifted from carbon-limited (iron-excess) to iron-limited medium although the concentration of iron–sulfur proteins in the submitochondrial particles decreased by more than 20-fold. Clegg \textit{et al.}\textsuperscript{42} also reported that the presence of EPR signals at 77 °K in the \textquotedblleft $g = 1.94$\textquotedblright region is not obligatory for fully active NADH or glycerol 1-phosphate oxidation activity. These questions were examined by Ohnishi \textit{et al.}\textsuperscript{48} using submitochondrial particles prepared from \textit{C. utilis} cells grown under controlled growth conditions. As described by Ohnishi and Chance\textsuperscript{44}, the intensity of EPR signals arising from iron-sulfur proteins in the NADH dehydrogenase region varied dramatically as a function of iron concentration in the culture medium when \textit{C. utilis} cells were grown under a specified low aeration system\textsuperscript{49}. It was found that phosphate, magnesium and cytochrome $c$ were required for obtaining the maximal rate of NADH oxidation in \textit{C. utilis} submitochondrial particles prepared from the cells grown in either low- or high-iron medium. When cells were grown in a high-iron (50 \textmu M) medium the maximal rate of NADH oxidation by submitochondrial particles, 1.2–1.6 \textmu atoms O \cdot min\textsuperscript{-1} \cdot mg protein\textsuperscript{-1}, was much higher than the maximal respiratory rate with NAD-linked substrates measured in intact mitochondria (0.1–0.2 \textmu atom O \cdot min\textsuperscript{-1} \cdot mg protein\textsuperscript{-1}). When cells were grown in the low-iron medium (1.1 \textmu M), the respiratory rate of NAD-linked substrates in mitochondria were not altered, but the maximal rate of NADH oxidation in submitochondrial particles was about 10 times diminished, in parallel with an approximately 10-fold decrease in the intensity of the \textquotedblleft $g = 1.94$\textquotedblright EPR signal arising from iron–sulfur proteins in the NADH dehydrogenase region (Table II).

Another example of concomitant change in the maximum NADH oxidation rate and the intensity of \textquotedblleft $g = 1.94$\textquotedblright EPR signal was observed when oxygen tension in the culture medium was changed. With much higher oxygen tension (steady level of O$_2$ concentration; 15–20 \% of air saturation) using an oxystat\textsuperscript{50}, the rate of NADH oxidation in submitochondrial particles decreased to 0.4 \textmu atom O \cdot min\textsuperscript{-1} \cdot mg protein\textsuperscript{-1} with a parallel decrease of the EPR signal in the NADH dehydrogenase region even when cells were grown in high-iron medium (50 \textmu M). In contrast, the rate of glycerol 1-phosphate oxidation and the relative intensity of \textquotedblleft $g = 1.94$\textquotedblright EPR signal associated with this dehydrogenase region were almost unchanged in all submito-
TABLE II

PARALLELS BETWEEN THE MAXIMUM OXIDATION RATE OF NADH AND GLYCEROL 1-PHOSPHATE, AND THE RELATIVE INTENSITY OF "g = 1.94" EPR SIGNALS (MEASURED AT 77 °K) ASSOCIATED WITH THE CORRESPONDING DEHYDROGENASE

*C. utilis* submitochondrial particles were isolated from cells grown in low- and high-iron culture medium, respectively. Preparation and reaction medium contained 0.3 M mannitol, 10 mM Tris-maleate buffer (pH 7.2), 1 mM MgCl₂ and 5 mM potassium phosphate buffer (pH 7.2). Concentration of the following additions: NADH, 1.5 mM; glycerol 1-phosphate, 10 mM; cytochrome c, 1 μM.

<table>
<thead>
<tr>
<th>Initial iron concn in the culture medium (μM):</th>
<th>1.1</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation rate (μatoms O₂ min⁻¹ mg protein⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH + cytochrome c</td>
<td>0.10-0.12</td>
<td>1.20-1.55</td>
</tr>
<tr>
<td>Glycerol 1-phosphate + cytochrome c</td>
<td>0.17-0.24</td>
<td>0.15-0.25</td>
</tr>
<tr>
<td>Relative intensity of &quot;g = 1.94&quot; signal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase region</td>
<td>1</td>
<td>10-13</td>
</tr>
<tr>
<td>Glycerol 1-phosphate dehydrogenase region</td>
<td>1</td>
<td>1-2</td>
</tr>
</tbody>
</table>

Submitochondrial particles prepared from *C. utilis* cells grown under the above described conditions (cf. Table II). Only a further extensive decrease of the iron concentration in the culture medium resulted in the diminished content of iron–sulfur protein in the glycerol 1-phosphate (or succinate) dehydrogenase and cytochromes in *C. utilis* mitochondria. These observations suggest that the dehydrogenase is the rate-limiting step in either NADH or glycerol 1-phosphate oxidation in submitochondrial particles under conditions presented in Table II and a proportionality exists between the maximum oxidation rate and relative intensity of EPR signals arising from iron–sulfur proteins in the respiratory chain. Thus, it is strongly suggested that “EPR-active” iron–sulfur proteins are required for electron transport in yeast submitochondrial particles. This could not be shown in the mitochondrial respiration with NAD-linked substrates, since the rate-limiting step is presumably localized in the region of the NAD-linked substrate dehydrogenases which precede the NAD dehydrogenase in the mitochondrial respiratory chain.

It is interesting to point out that NADH–K₃Fe(CN)₆ reductase activity of the submitochondrial particles (V) which is almost 20 times higher than the overall NADH oxidation activity also decreased several fold when the iron concentration in the culture medium of *C. utilis* is lowered from 50 to 1.1 μM. Clegg recently reported that the isolated NADH–K₃Fe(CN)₆ reductase from iron-deficient cells showed a specific activity (measured at a fixed K₃Fe(CN)₆ concentration) which is only about 10% of that of the NADH–K₃Fe(CN)₆ reductase of iron-sufficient cells. These observations are in agreement with the previous finding by Beinert et al. who demonstrated the parallel decrease of NADH–K₃Fe(CN)₆ reductase activity (Vmax) and the intensity of "g = 1.94" EPR signal on partial inactivation of the isolated NADH
dehydrogenase, indicating the involvement of iron–sulfur protein responsible for the “\(g = 1.94\)” signal in the NADH-K\(_3\)Fe(CN)\(_6\) reductase activity.

As will be described in the next section, an increasing number of iron–sulfur proteins (centers) has been recently detected in both yeast and mammalian mitochondria by the measurement of the EPR absorbance of samples at temperatures below 77 °K. This technique greatly increased the sensitivity of the EPR measurement of iron–sulfur proteins and has made possible the measurement of the redox level of individual iron–sulfur centers in different metabolic states, such as in State 4, State 3, or the uncoupled state. Under these conditions, iron–sulfur centers show a response similar to other well established components of the respiratory chain. Information from these studies provides us with further evidence to support the proposition that multiple iron–sulfur centers act as members of the respiratory chain.

V. MULTIPLE IRON–SULFUR CENTERS IN THE SITE I REGION AND THEIR CHARACTERISTICS

Some bacterial ferredoxins and other enzymes containing iron–sulfur centers have been known to exhibit measurable EPR absorbance signals only at temperatures close to that of liquid helium. Thus EPR signals of iron–sulfur proteins in \(C.\) \textit{utilis} mitochondria have been examined at temperatures between that of liquid nitrogen (77 °K) and liquid helium (4.2 °K), in order to establish a more definite correlation between the presence or absence of EPR-detectable iron–sulfur proteins and Site I energy conservation. Previously unrecognized multiple EPR signals arising from iron–sulfur centers in the Site I region have been detected in \(C.\) \textit{utilis} submitochondrial particles at temperatures below 30 °K. Similar iron–sulfur proteins which show measurable EPR signals only at lower temperatures were subsequently found in mammalian systems in three laboratories, namely, by Ohnishi \textit{et al.} (see Fig. 2), Orme-Johnson \textit{et al.}, and by Albracht and Slater. Orme-Johnson \textit{et al.} resolved multiple EPR signals and identified four separate iron–sulfur centers (Centers 1–4) in the anaerobic NADH–Q reductase, by a stepwise reduction with measured quantities of reducing equivalents. They reported \(g\) values of the prominent peaks of individual iron–sulfur centers in the NADH–Q reductase as follows: Center 1 (2.022, 1.938, 1.923), Center 2 (2.054, 1.922), Center 3 (2.101, 1.886, 1.864) and Center 4 (2.103, 1.863). These investigators also showed the relative oxidation–reduction potentials of iron–sulfur centers as in the order of 2 > 3 > 4 > 1. From double integration of the EPR signals, each iron–sulfur center (probably 2 or 4 iron atoms per center) was estimated to take up a number of electrons approximately equal to the molarity of FMN in the NADH–Q reductase preparation. Ohnishi \textit{et al.} resolved EPR signals arising from iron–sulfur centers in \(C.\) \textit{utilis} mitochondria and submitochondrial particles by the anaerobic titration procedure of Dutton and Wilson \textit{et al.}. The EPR spectra of \(C.\) \textit{utilis} submitochondrial particles at different oxidation–reduction potentials are presented in Fig. 3. Individual EPR signals are
designated according to Orme-Johnson et al.\textsuperscript{1}. The characteristics of the EPR absorption spectra arising from individual iron–sulfur centers in the Site I region of \textit{C. utilis}, such as line shape, field position of prominent absorption peaks, temperature and power profile, are similar to those observed in beef heart systems. The values of

![EPR spectra of iron–sulfur proteins](image)

**Fig. 2.** EPR spectra of iron–sulfur proteins in the NADH dehydrogenase region of the respiratory chain in \textit{C. utilis} and bovine heart submitochondrial particles (SMP) at temperatures below 77 °K. Iron–sulfur proteins in these particles were reduced with NADH in the presence of piericidin A. Submitochondrial particles (56.1 mg of protein per ml) isolated from \textit{C. utilis} cells which were grown in a synthetic medium containing 25 \textmu M FeCl\textsubscript{3} were previously incubated with piericidin A (0.5 nmoles per mg of protein) for 10 min in an ice bath. Bovine heart submitochondrial particles (38.4 mg of protein per ml) were previously incubated with piericidin A (1.2 nmoles per mg of protein) in a similar fashion. In both cases NADH oxidase activity was inhibited up to 98\% and thus about 1 min was allowed prior to anaerobiosis. Both suspensions were frozen in liquid nitrogen in less than 40 s after the addition of 1.7 mM NADH. EPR operating conditions were: field modulation, 100 kHz; modulation amplitude, 12 gauss; microwave power, 9.2 mW; microwave frequency, 9.02 GHz; time constant, 0.001 s; scanning rate, 400 gauss/min; temperature, 77, 43, 27, 23, 18, or 4 °K as indicated. Relative instrument gain is shown in the figure\textsuperscript{2}.
Fig. 3. EPR spectra of iron–sulfur proteins in *C. utilis* submitochondrial particles as measured at 18 °K. *C. utilis* submitochondrial particles were suspended at approximately 25 mg of protein per ml in a medium containing 0.3 M mannitol, 5 mM potassium phosphate buffer (pH 7.2), 1 mM MgCl₂, and 50 mM morpholinopropane sulfonic acid buffer (pH 7.2). Oxidation–reduction potentials were measured potentiometrically⁹⁹. Oxidation–reduction mediators added were 40 μM phenazine methosulfate, 40 μM phenazine ethosulfate, 63 μM duroquinone, 6 μM pyocyanine, 6 μM resorufin, 58 μM phenosafranine, 56 μM benzyl viologen, 108 μM methyl viologen, and 25 μM 2-hydroxynaphthoquinone. The oxidation–reduction potential of the suspension system was lowered by stepwise additions of small aliquots of 0.1 M NADH solution. Oxidation–reduction potentials shown in the figure are relative to the standard hydrogen electrode. The EPR operating conditions were: modulation amplitude, 12 gauss; microwave power, 20.5 mW; microwave frequency, 9.02 GHz; time constant, 0.001 s; scanning rate, 1 kgauss/min; temperature, 18 °K. The ordinate is the first derivative of the microwave absorption in arbitrary units. Small Roman letters are placed along the spectra vertically above or below the field position of the resonances typical for various components according to Orme-Johnson *et al.*¹.
half-reduction potentials of iron–sulfur Centers 1, 3 + 4, and 2 are —240 mV, —210 mV and —50 mV, respectively. Ohnishi et al. extended these studies to pigeon heart mitochondria and submitochondrial particles. Typical oxidation–reduction titration curves are shown in Fig. 4. In both C. utilis and pigeon heart

![Graph showing oxidation-reduction potential dependence of EPR signals arising from different iron-sulfur centers in pigeon heart mitochondria.](image)

Fig. 4. The oxidation–reduction potential dependence of EPR signals arising from different iron–sulfur centers in pigeon heart mitochondria. Pigeon heart submitochondrial particles were suspended at the protein concentration of 23.1 mg/ml in a medium containing 0.25 M sucrose and 40 mM morpholinopropane sulfonic acid buffer (pH 7.2). Oxidation–reduction mediators added were 25 μM dianimodurene, 48 μM phenazine methosulfate, 48 μM phenazine ethosulfate, 62 μM duroquinone, 6 μM pyocyanine, 6 μM resorufin, 25 μM 2-hydroxynaphthoquinone, 60 μM phenosafranine, 72 μM benzyl viologen and 130 μM methyl viologen. EPR spectra were recorded by a Varian X-band spectrometer (V 4502) at 77, 18.8 and 8.8 °K. Microwave power for EPR measurements at 77 and 18.8 °K was 20.5 mW, while it was 120 mW for the measurements at 8.8 °K.

mitochondria, Center 1 appears to be composed of at least two iron–sulfur centers with similar EPR spectra and half-reduction potentials (n value of the redox titration is considerably below 1). EPR signals arising from Centers 3 and 4 are overlapped and these two centers have slightly different half-reduction potentials, which could not be measured separately by potentiometric titration procedures due to the overlap of their EPR spectra. The thermodynamic profile of iron–sulfur centers in pigeon heart mitochondria is presented in Scheme II. Although Center 2 has a half-reduction potential close to that of Q or cytochrome b, it is located on the substrate side of the rotenone inhibition site. In C. utilis submitochondrial particles, Center 2 is not reducible with succinate or glycerol 1-phosphate, while it is partially reducible with succinate in the mammalian system. This can be explained by the observation that the respective half-reduction potential values are —50 mV and —20 mV. There is a large difference between the half-reduction potentials of low-potential
iron–sulfur centers (Centers 1, 3 and 4) and a high-potential iron–sulfur center (Center 2). This suggests that the energy transduction at Site I is associated with the transfer of electrons across this potential span. In this connection, Singer and Gutman\textsuperscript{9} indicated the location of Site I energy coupling between iron–sulfur Center 1 and Center 2 from the following two observations: (1) the occurrence of energy-dependent electron reverse from Center 2 to NAD in the rotenone-inhibited, NADH-pretreated submitochondrial particles\textsuperscript{62} and (2) the absence of phosphorylation coupled to electron transfer from NADH to K$_3$Fe(CN)$_6$ via Center \textsuperscript{125,52.}

VI. INVOLVEMENT OF IRON–SULFUR CENTER(S) IN SITE I ENERGY COUPLING, SUGGESTED BY \textit{IN VIVO} INDUCTION STUDIES OF SITE I IN \textit{C. UTILIS} CELLS

As described in Section II, Ohnishi and Chance\textsuperscript{44} have suggested a close correlation between Site I phosphorylation and iron–sulfur protein(s) responsible for the \textquotedblleft g = 1.94\textquotedblright\ EPR signal in the NADH dehydrogenase region. In contrast, Garland and co-workers reported that the iron–sulfur protein responsible for the \textquotedblleft g = 1.94\textquotedblright\ signal is not needed for the occurrence of Site I phosphorylation from the following two observations: (1) when \textit{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 while neither the \textquotedblleft g = 1.94\textquotedblright\ EPR signal nor piericidin A sensitivity\textsuperscript{41} are induced; (2) continuous culture of \textit{C. utilis} cells at the transition from iron-limited to glycerol-limited growth produces cells where mitochondria possess Site I phosphorylation and lack the \textquotedblleft g = 1.94\textquotedblright\ EPR signal arising from the iron–sulfur protein in the NADH dehydrogenase region\textsuperscript{47} as measured at 77 °K. Thus, Garland and co-workers suggested that a small fraction of the non-heme iron proteins which does not show detectable EPR absorbance may play a role in Site I phosphorylation.

Since the existence of at least five iron–sulfur centers (Centers 1a, 1b, 2, 3 and 4) in the Site I region of the respiratory chain in \textit{C. utilis} mitochondria have been demonstrated to have characteristic EPR absorption spectra at temperatures below
Ohnishi et al.\textsuperscript{53} examined the induction of iron–sulfur proteins responsible for these multiple EPR signals and correlated with the Site I induction in the presence and absence of cycloheximide using C.\textit{utilis} cells grown in the batch culture. It was observed that the induction of Site I phosphorylation, the appearance of the iron–sulfur EPR signals arising from both Center 1 and Center 2, and piericidin A sensitivity were concomitantly inhibited by cycloheximide (Table III), in contrast to the result reported by Garland\textsuperscript{41}. The absence of Site I phosphorylation in the cycloheximide-inhibited system was demonstrated not only by an ADP/O ratio of less than 2 in NAD-linked substrate oxidation in tightly coupled mitochondria, but also by the lack of energy-dependent reversal of electrons from glycerol 1-phosphate dehydrogenase to endogenous NAD. Garland\textsuperscript{41} and Ragan and Garland\textsuperscript{64} concluded that the induction of Site I energy conservation occurred in the presence of cycloheximide, based on only a small increase in ADP/O ratio, although they could not demonstrate significant energy-dependent reversal of electron flow. These observations further strengthened the previous hypothesis presented by Ohnishi and Chance\textsuperscript{44}, namely, that EPR-detectable iron–sulfur protein(s) may play a role in Site I energy conservation. On the other hand, Haddock and Garland\textsuperscript{42} demonstrated the induction of Site I phosphorylation from ADP/O ratio close to 3 as well as energy-dependent reduction of endogenous pyridine nucleotide in tightly coupled mitochondria prepared from sulfur-limited C.\textit{utilis} cells aerated in the presence of cycloheximide. In this case, they also reported that \textquoteleft\textquoteleft g = 1.94\textquoteright\textquoteright EPR signals arising from iron–sulfur proteins tested at 77 °K was not induced in the presence of cycloheximide, thus EPR-detectable iron–sulfur protein was considered to be unnecessary for Site I energy conservation\textsuperscript{41,64}. However, more recently at the 8th FEBS Meeting in Amsterdam (September, 1972) Garland \textit{et al.}\textsuperscript{65} reported the following result: \textquoteleft\textquoteleft When the sulfur-limited cells are aerated in the presence of cycloheximide, EPR signals arising from iron–sulfur Center 1 were detected at 21 °K\textquoteright\textquoteright. This shows the

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Site I phosphorylation</th>
<th>Iron–sulfur centers</th>
<th>Piericidin A sensitivity</th>
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</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonaerated</td>
<td>–</td>
<td>–</td>
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<td>Aerated with added cycloheximide</td>
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<tr>
<td>Sulfur deficiency</td>
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<tr>
<td>Nonaerated</td>
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<td>Aerated with added cycloheximide</td>
<td>+</td>
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induction of Center 1 concomitant with the induction of Site I phosphorylation, although a small contribution from Center 2 signal cannot be excluded until the temperature profile of prominent peaks in this systems is measured. Although Garland and co-workers obtained variable results in these in vivo induction studies, they have most recently concluded that EPR-measurable iron–sulfur protein may play an important role in Site I phosphorylation in agreement with Ohnishi et al.\(^4^4\). The approach using phenotypic variants of \textit{C. utilis} cells thus strongly suggests that at least one of the “EPR-active” iron–sulfur proteins in the Site I region plays a role in the Site I energy conservation.

VII. SITE I BYPASS MECHANISM FOUND IN \textit{C. UTILIS} MITOCHONDRIA

Katz\(^5^0\) and Katz \textit{et al.}\(^6^6\) raised a question about the suggested role of iron–sulfur proteins in Site I energy coupling from the following observations: (1) \textit{C. utilis} cells, grown in batch using an oxystat (steady oxygen concentration in the medium; 15–25\% of air saturation), acquire both rotenone sensitivity and presumably Site I energy conservation on the transition from logarithmic to stationary growth phase due to the depletion of carbon source, even in the high-iron medium; (2) in continuous culture, an increasing in the level of the carbon source without changing the iron concentration (50 \(\mu\text{M}\)) results in the disappearance of rotenone sensitivity. These observations suggested that upon shifting the chemostat growth conditions from carbon-limited to iron-limited condition\(^4^1\)–\(^4^7\), a loss of Site I energy coupling and rotenone sensitivity was caused by the transition from carbon-limited to carbon-excess condition, rather than by iron limitation.

Ohnishi\(^6^7\) extended the studies by Katz \textit{et al.} and demonstrated the absence of functional Site I energy conservation in log-phase mitochondria from the measured ADP/O ratios coupled with the NAD-linked substrate respiration. Piericidin A sensitivity of NAD-linked substrate oxidation measured in intact mitochondria parallels the presence or absence of Site I phosphorylation, but the oxidation of NADH by submitochondrial particles was sensitive to piericidin A for both log-phase and stationary-phase cells. This strongly suggests that in mitochondria from both log-phase and stationary-phase cells, the piericidin A-sensitive component and most probably the Site I phosphorylation machinery are present. However, NADH generated in the mitochondria from log-phase cells is oxidized via an alternate electron transfer pathway (most probably \textit{via} the external NADH oxidation pathway) which is piericidin A insensitive and does not involve Site I. It has been shown that there are two different NADH dehydrogenases located in the cristae of \textit{C. utilis} mitochondria\(^2^3\),\(^6^8\): one is located on the inner surface of the cristae and oxidizes NADH generated in the matrix space (internal pathway); the other is located on the outer surface of the cristae and oxidizes cytoplasmic NADH (external pathway). The internal NADH oxidation pathway is piericidin A sensitive and associated with phosphorylation at Site I, while the external NADH oxidation pathway is piericidin
A insensitive and shows no energy coupling at Site I. The Site I bypass mechanism does not involve the direct exchange of the reducing equivalents from internally generated NADH to extramitochondrial NAD, since pyruvate plus malate or ethanol–ferricyanide reductase activity in the log-phase mitochondria is completely inhibited by antimycin A. On preparation of submitochondrial particles, NADH dehydrogenase which was present in the cristae space is shifted to the outer (external space) surface of submitochondrial particles because of the reversal of membrane orientation, and the mitochondrial NADH dehydrogenase for the oxidation of cytoplasmic NADH was suggested to be either solubilized or dislocated during the sonication of mitochondria. Thus, in submitochondrial particles, NADH is oxidized solely via Site I of the electron transfer chain. It was further demonstrated that the carbon level of the culture medium is not the parameter which directly controls this bypass mechanism. If the oxygen supply is lowered to slow down the growth rate in the log phase, both piericidin A sensitivity and Site I energy coupling can be seen even in mitochondria from cells growing in the excess of carbon source. Moreover, when *C. utilis* cells are grown in an acetate medium, exponential growth with a doubling time of 1.5–2.0 h can be achieved only at pH values more alkaline than 5.5. Under this optimal growth condition, both the rotenone-sensitive site and Site I are bypassed, and cells can grow in the culture medium containing rotenone or piericidin A. On the other hand, if the pH of the culture medium is more acid than pH 5, the growth rate of the cells is very slow and the cells always respire via a piericidin A-sensitive and Site I-operative respiratory pathway even when they are growing with excess carbon level and sufficient supply of air. Thus, these cells are not capable of growing in the presence of rotenone in the culture medium although isolated mitochondria show the presence of both external and internal NADH oxidation pathways, which is contrary to the observation reported by Fukami et al., for continuous culture. These observations indicate 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. Thus, energy coupling at Site I is utilized only when cells are growing under suboptimal growth conditions. By contrast, if iron concentration in the culture medium is lowered below a certain level, mitochondria deficient in both Site I energy coupling and piericidin A sensitivity are obtained. In this case, NADH oxidation measured in submitochondrial particles is also insensitive to piericidin A, in contrast to NADH oxidation in submitochondrial particles prepared from exponentially growing cells. Therefore, the intrinsic effect of iron deficiency, which causes the lack of energy-transducing components at Site I, can be distinguished from the bypass mechanism of Site I phosphorylation functioning in mitochondria.

As described in the early part of this section, Katz et al. proposed that a loss of both Site I phosphorylation and piericidin A sensitivity in *C. utilis* cells grown under iron or sulfur limitation in a chemostat was caused by the carbon excess rather than the iron or sulfur deficiency. This point was tested by Light and Garland who examined piericidin A sensitivity and Site I energy coupling in *C. utilis* cells grown in
a chemostat when the growth was limited by components other than iron, sulfur and carbon source, namely, magnesium, phosphate or nitrogen source (all correspond to carbon-excess condition). In all cases, mitochondrial NAD-linked substrate respiration showed piericidin A sensitivity and Site I energy conservation, suggesting that continuous culture is a "suboptimal" condition for the growth cycle of the cells. Only iron or sulfur limitation caused the loss of Site I energy coupling in \( C. \) utilis cells growing in a chemostat. These results further strengthened the possible role of iron–sulfur proteins in Site I energy conservations.

VIII. IRON–SULFUR CENTER 1a AS AN ENERGY-TRANSUDING COMPONENT AT SITE I

Wilson and Dutton\(^{71,72}\) provided evidence that cytochrome \( b_\tau \) and \( a_3 \) are directly involved in energy transduction at Site II and III, respectively, from the phosphate potential dependence of their half-reduction potentials. This experimental approach has been applied to Site I, in combination with low-temperature (\(<77\, ^\circ\)K) EPR techniques.

Yeast mitochondria were found unsuitable for studying direct interaction between externally added ATP and endogenous respiratory chain carriers, probably due to poor accessibility of added ATP to the mitochondrial respiratory chain. For example, the energy-dependent shift of the half-reduction potential of cytochrome \( b_\tau \) and reversed electron transfer from glycerol 1-phosphate dehydrogenase to endogenous NAD were observed in \( C. \) utilis mitochondria, but they can be demonstrated only by internally produced high-energy states, but not by added ATP (cf. Sato et al.\(^{73}\)). Thus, this experimental approach was followed using pigeon heart mitochondria.

In order to determine which of the multiple iron–sulfur centers in the Site I region has an energy-dependent half-reduction potential, the effect of ATP addition on the oxidation–reduction level of individual iron–sulfur centers was examined on "potential-clamped" pigeon heart mitochondria\(^3\). In a series of experiments, pigeon heart mitochondrial suspensions in the presence of suitable redox mediators were equilibrated at potentials ranging from \(-460\) to \(+100\, \text{mV}\) and then ATP was added. Under these conditions almost no effect of ATP was observed on the redox level of Centers 3, 4 and 2. When the potential was set at values where Center 1 (\( E_{m7.2} = -305\, \text{mV}\)) was partially or almost completely reduced, ATP addition caused a partial oxidation of Center 1 (cf. Fig. 5) and this oxidation was inhibited by either uncoupler or oligomycin. Experimentally only \(50 \pm 10\%\) of the Center 1 signal is observed to be affected by ATP addition. This ATP-affected center has been designated as Center 1a. These observations suggested that iron–sulfur Center 1a has a half-reduction potential which is dependent on the phosphate potential (lowering of \( E_{m7.2}\) on the addition of ATP). The iron–sulfur Center 1a is thus proposed to be involved in the energy transduction process at Site I of the respiratory chain\(^3\), corresponding to cytochrome \( b_\tau \) and \( a_3 \) in Sites II and III, respectively.
Fig. 5. The effect of ATP addition on the EPR signal of iron-sulfur Center 1 in pigeon heart mitochondria. Pigeon heart mitochondria were suspended at a protein concentration of 10 mg/ml in a medium containing 0.3 M mannitol, 50 mM morpholinopropane sulfonate (pH 7.2). To this suspension, 67 μM phenazine ethosulfate, 33 μM duroquinone, 20 μM pyocyanine, 20 μM 2-hydroxynaphthoquinone, 78 μM phenosafranine, 74 μM benzyl viologen and 133 μM methyl viologen were added to act as oxidation-reduction mediators. The oxidation-reduction potential of the suspension was lowered to -438 mV by the addition of aliquots of freshly prepared dilute solution of dithionite. An aliquot (0.3 ml) of the suspension was transferred anaerobically to an EPR tube and frozen in liquid isopentane at 113 °K (Spectrum A). After addition of 3.7 mM ATP, which was previously bubbled with argon to remove oxygen, the oxidation-reduction potential of the suspension was readjusted to the same potential and an aliquot of the suspension was quickly frozen (Spectrum B). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (0.8 nmole/mg protein) and oligomycin (0.8 μg/mg protein) were further added to the mitochondrial suspension and an aliquot was frozen in the same fashion (Spectrum C). EPR operating conditions were: modulation amplitude, 12.5 gauss; microwave power, 50 mW; microwave frequency, 9.113 GHz; time constant, 0.3 s; scanning rate, 500 gauss/min; sample temperature, 20 °K.
Although more detailed studies are required, this tentative proposal by Ohnishi et al. is supported by the following independent observations:

1. In State 4 respiration with succinate plus glutamate as substrates, pyridine nucleotide, Centers 3 and 4 are over 90% reduced, but Center 1 is only about 40% reduced.\(^3\)

2. Slater and co-workers\(^74,75\) have reported that in the absence of added redox mediators, the addition of ATP to NADH-reduced phosphorylating submitochondrial particles, causes partial oxidation of all iron–sulfur centers measured, including iron–sulfur Center 1 (Centers 3 and 4 were not measured).

3. The addition of ATP caused partial oxidation of Center 1 in the submitochondrial particles where all iron–sulfur centers in the Site I region were kept reduced either by anaerobiosis with added NADH or by the addition of NADH to the rotenone-inhibited particles.\(^65\).

IX. SITE I ENERGY COUPLING IN *SACCHAROMYCES* MITOCHONDRIA

The existence of Site I energy coupling in *Saccharomyces* (cerevisiae or carlsbergensis) mitochondria has been a matter of controversy for a long time. As summarized in Table IV, Chance\(^76\) observed a cross-over point at the Site I region by the

<table>
<thead>
<tr>
<th>Reference</th>
<th>Site I phosphorylation</th>
<th>Growth conditions</th>
<th>Carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chance(^76)</td>
<td>+</td>
<td>Stationary phase</td>
<td>(Commercial pressed yeast)</td>
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<tr>
<td>Vitols and Linnane(^14)</td>
<td>–</td>
<td>Stationary phase</td>
<td>(Commercial pressed yeast)</td>
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<tr>
<td>Ohnishi and co-workers(^15,16)</td>
<td>–</td>
<td>Early log phase</td>
<td>Lactate</td>
</tr>
<tr>
<td>Schatz and Racker(^17)</td>
<td>–</td>
<td>Stationary phase</td>
<td>(Commercial pressed yeast)</td>
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<tr>
<td>Balcavage and Mattoon(^18)</td>
<td>–</td>
<td>Early stationary phase</td>
<td>Glucose</td>
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<tr>
<td>Kováč et al.(^19)</td>
<td>–</td>
<td>Early stationary phase</td>
<td>Glucose</td>
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<tr>
<td>Light and Garland(^20)</td>
<td>–</td>
<td>Continuous culture (carbon limiting)</td>
<td>Glucose</td>
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<tr>
<td>Schuurmans-Steckhoven(^77)</td>
<td>+</td>
<td>Early log phase</td>
<td>Lactate</td>
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<tr>
<td>Ghosh and Bhattacharyya(^78)</td>
<td>+</td>
<td>Early stationary phase (starved for 3 h)</td>
<td>Glucose</td>
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<tr>
<td>Ohnishi(^28)</td>
<td>–</td>
<td>Early stationary phase</td>
<td>Glucose</td>
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<td>+</td>
<td>Early stationary phase (starved for 3–5 h)</td>
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<td>Glucose</td>
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<td>Glucose</td>
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<td>Mackler and Haynes(^81)</td>
<td>+</td>
<td>Late stationary phase (7–14 h)</td>
<td>Glucose</td>
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direct measurement of mitochondrial pyridine nucleotide and flavin fluorescence using 18-h starved *S. cerevisiae* cells, suggesting the presence of Site I energy conservation in the baker's yeast as in the mammalian systems. Subsequent studies on the phosphorylation efficiencies in the isolated mitochondria or submitochondrial particles showed the absence of Site I phosphorylation when they were prepared from cells either at the log phase or early stationary phase. On the other hand, Schuurmans-Steckhoven reported the presence of Site I phosphorylation in mitochondria isolated from *S. carlsbergensis* cells in the early log phase. Similarly, Ghosh and Bhattacharyya reported Site I phosphorylation in *S. carlsbergensis* which was harvested in the early stationary phase and starved after washing the cells. In order to resolve these conflicts, Ohnishi prepared mitochondria from the cells both before and after aeration of the *S. carlsbergensis* cells and showed the apparent induction of Site I energy coupling by aerating cells under non-growing conditions. More recently, Mackler and Haynes demonstrated that mitochondria from either *S. cerevisiae* or *carlsbergensis* can perform energy coupling at Site I only at the late stationary phase (5–7 h after reaching the stationary phase). These observations strongly suggest the following characteristics of *Saccharomyces* mitochondria: (1) although Garland previously concluded that *Saccharomyces* mitochondria are genetically incompetent to perform Site I energy conservation, they seem to be competent; (2) similar to *C. utilis* systems, a metabolic control mechanism to bypass Site I under the “optimal” growth conditions is also functional in *Saccharomyces* mitochondria; (3) apparently, *Saccharomyces* cells show a slow response to the transition from carbon-excess to carbon-depleted condition, probably because of a high endogenous carbon storage in the cells. However, there are still additional unknown parameters which regulate the bypass mechanism in *Saccharomyces* systems. For example, Pye *et al.* obtained preliminary results indicating the presence of three phosphorylation sites in *S. carlsbergensis* cells when they were grown in glucose-limited conditions and two phosphorylation sites when cells were grown under nitrogen-limited (carbon-excess) conditions. This was reflected in the growth yield of the cells grown in both batch and continuous culture. On the contrary, Light and Garland observed two phosphorylation sites in *S. carlsbergensis* mitochondria prepared from cells growing in a chemostat with glucose-limited (carbon-limited) medium but three phosphorylation sites from the cells grown in ethanol-limited (carbon-limited) medium. Mackler and Haynes also observed different Site I behavior among different strains of *Saccharomyces*. Thus, further investigations are required for establishing more definite and reproducible conditions for the presence or absence of Site I energy conservation in *Saccharomyces* mitochondria. It should also be pointed out that no one has ever demonstrated energy-dependent reversal of electron transfer from glycerol 1-phosphate or succinate to endogenous pyridine nucleotide in *Saccharomyces* mitochondria or NADH-Q reduction coupled with Site I phosphorylation in the submitochondrial particle preparation. *Saccharomyces* systems may provide us with an important line of approach for the mechanism of Site I energy conservation, because the EPR signals arising from low-potential iron–sulfur centers in the NADH dehydrogenase
region have not been detected at 77 °K\(^26,28,29\) and below 77 °K\(^27,82\) in contrast to mammalian\(^1\) or \textit{C. utilis}\(^27,58\) systems. On the other hand, EPR signals arising from iron–sulfur centers in the cytochrome \textit{b, c} region or iron–sulfur centers associated with the succinate dehydrogenase can be detected at and below 77 °K and show very similar characteristics as those in mammalian or \textit{C. utilis} systems\(^82\). \textit{Saccharomyces} mitochondria do not show rotenone sensitivity under any growth conditions so far examined.

X. INHIBITION MECHANISM OF PIERICIDIN A (OR ROTENONE)

As described in Sections II and III, piericidin A (or rotenone) sensitivity was suggested to be closely related to the presence of Site I energy conservation and to the iron–sulfur protein(s) which give rise to the EPR signals in the "\(g = 1.94\)" region. However, three yeast systems have been obtained where Site I energy conservation is present and the accompanying electron transport is piericidin A insensitive: (1) \textit{C. utilis} cells growing in a chemostat at the transition from iron limitation to carbon limitation\(^47\); (2) \textit{C. utilis} cells where Site I energy coupling was induced by aerating sulfur-limited cells in the presence of cycloheximide\(^41,42\); (3) \textit{S. carlsbergensis} and \textit{cerevisiae} under certain growth conditions. These observations provide an evidence that electron transfer through the piericidin A-sensitive site is not obligatory for the occurrence of Site I energy conservation. It should be pointed out, however, that there is no example so far known where rotenone-sensitive respiration does not yield phosphorylation at Site I.

Bois and Estabrook\(^83\) proposed that rotenone reacts irreversibly with the reduced form of iron–sulfur proteins and this may be the mechanism of rotenone inhibition of the NADH oxidation. This mechanism was questioned by Gutman et al.\(^84\), because rotenone can be strongly bound to specific binding sites even in the oxidized state of non-heme iron proteins. Ohnishi et al.\(^27\), Gutman et al.\(^62\) and Albracht and Slater\(^57\) reported that a high-potential iron–sulfur Center 2 as well as low-potential iron–sulfur centers can be reduced with NADH in the submitochondrial particles pretreated with rotenone. Thus, the rotenone inhibition site was suggested to be on the oxygen side of iron–sulfur Center 2. Gutman et al.\(^62\) demonstrated that in the submitochondrial particles pretreated with rotenone, Center 2 remains reduced after exhausting added NADH, although all other iron–sulfur centers are oxidized. This reduced Center 2 can not be oxidized by the forward electron transfer by the electron leak through the rotenone inhibition site, but can be completely oxidized by the energy-dependent reversal of electrons to NAD, on addition of ATP. These observations suggest that the rotenone interaction site is not directly on Center 2, but is in a very close proximity of Center 2, on its oxygen side. Consistent with these results Ohnishi et al.\(^43\) previously reported that iron–sulfur centers in the NADH dehydrogenase region and piericidin A sensitivity are not directly related from the following observation: the relative intensity of the EPR signals arising from iron–
sulfur proteins in the NADH dehydrogenase region and piericidin A sensitivity show different profiles when they are measured as functions of iron concentration in the culture medium of *C. utilis* cells.

Ragan and Garland\(^6\) reported spectrophotometric observations suggesting the presence of low- and high-potential iron–sulfur proteins in the Site I region of the respiratory chain. They proposed that induction of both low- and high-potential iron–sulfur proteins is required for the appearance of piericidin A sensitivity in *C. utilis* cells. This high-potential iron–sulfur protein, however, was characterized as being located between piericidin A and antimycin A inhibition sites, thus it does not correspond to Center 2. Identification of this high-potential iron–sulfur protein by EPR measurement and demonstration of its direct correlation to piericidin A sensitivity are still lacking.

The mechanism of piericidin A or rotenone sensitivity was discussed in great detail by Singer and Gutman\(^9\) in their recent review. It would be summarized as follows: (1) Both lipids and proteins are involved in the strong, noncovalent forces holding these inhibitors at the specific sites. Prior binding of piericidin A at the specific binding sites strongly inhibits the extraction of the NADH dehydrogenase with phospholipase A. This suggests a close proximity of the specific binding site to the phospholipids, which are involved in binding the dehydrogenase to the mitochondrial membrane. (2) Piericidin A and rotenone act competitively on the same site, the former binding more strongly than the latter. The number of specific inhibitor binding sites is two per one respiratory chain. One of these suggested to be a “Type V” –SH group\(^9\) which is located on the oxygen side of iron–sulfur Center 2. However, this relationship between the “Type V” –SH group and rotenone sensitivity does not hold in the *S. cerevisiae* system. *S. cerevisiae* does not show any sensitivity to rotenone inhibition, although the “Type V” –SH group is present in this system. The other inhibitor binding site remains unknown.

In order to define the site of rotenone inhibition more closely, the isolation of a rotenone-resistant mutant would be of great value. However, this approach was not feasible in the *C. utilis* system, because these cells can grow normally even in the presence of high concentration of rotenone\(^42,85\). Fukami *et al.*\(^69\) found that *C. utilis* cells show rotenone-sensitive growth when cells are grown in acetate (pH 5.0) medium, and they reported that in mitochondria prepared from acetate-grown cells in continuous culture, external NADH is oxidized via a rotenone-sensitive pathway. Thus, Garland\(^41\) proposed that the screening for rotenone-resistant mutants poses no difficulty. However, Ohnishi\(^67\) demonstrated that the rotenone-insensitive external NADH oxidation pathway is still present in mitochondria of acetate-grown cells. Rotenone-sensitive or -insensitive growth of *C. utilis* cells is only a reflection of the metabolic control mechanism switching between rotenone-sensitive and -insensitive (Site I phosphorylating and nonphosphorylating) electron transfer pathways depending on the “suboptimal” (below pH 5 in acetate medium) or “optimal” growth conditions (see Section VII), respectively. Hence, the isolation of rotenone-resistant mutant of *C. utilis* cells grown on acetate seems to be less promising.
XI. SUMMARY

1. A biological approach using phenotypic variants of yeast cells under deprived conditions (especially iron- or sulfur-deficient \textit{C. utilis} cells) strongly suggested the important role of iron-sulfur proteins in both electron transfer and energy conservation at Site I.

2. EPR signals arising from many previously unrecognized iron-sulfur centers (Centers 1a, 1b, 3, 4 and 2) associated with the Site I region of the respiratory chain have been characterized using EPR measurements at temperatures between that of liquid nitrogen and liquid helium.

3. The half-reduction potentials of iron-sulfur Centers 1a + 1b, 3 + 4 and 2 have been determined by the potentiometric titration procedure; they are --305 mV, --245 mV, and --20 mV, respectively, in pigeon heart mitochondria, showing a large potential gap between low-potential iron-sulfur centers and high-potential iron-sulfur Center 2.

4. All of these iron-sulfur centers are located on the substrate side of rotenone inhibition site.

5. From the phosphate potential dependence of the half-reduction potential, iron-sulfur Center 1a has been tentatively assigned as an energy-transducing component at Site I, corresponding to cytochrome \( b_1 \) and \( a_3 \) at Sites II and III, respectively.

6. The molecular mechanism of piericidin A and rotenone inhibition still remains unsolved.

7. The Site I bypass mechanism has been described in rapidly growing \textit{C. utilis} cells which can be differentiated from the loss of Site I phosphorylation in iron- or sulfur-limited \textit{C. utilis} cells. The Site I bypass mechanism also seems to be functional in \textit{S. cerevisiae} or \textit{carlsbergensis} mitochondria under similar growth conditions.

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(1) Preparations of reconstitutively active NADH dehydrogenase have been reported by Baugh and King\textsuperscript{86} and by Ragan and Racker\textsuperscript{87}.

(2) Further investigation has revealed that the half-reduction potential of iron-sulfur Center 2 becomes more positive upon addition of ATP. Hence it is suggested that both Center 1a and Center 2 are involved in the energy transducing reaction at Site 1\textsuperscript{88}.

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