Oxidative Phosphorylation in Fractionated Bacterial Systems

XXIX. THE INVOLVEMENT OF NONHEME IRON IN THE RESPIRATORY PATHWAYS OF MYCOBACTERIUM PHLEI*

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SUMMARY

Spectrophotometric evidence for the reduction of nonheme iron in the respiratory particles of Mycobacterium phlei was obtained by trapping the metal as ferrous o-phenanthroline. The protein-bound iron-o-phenanthroline complex had an absorption maximum at 510 m\(\mu\), and the extent of reduction of nonheme iron was followed by observing the increase in absorbance at 510 to 540 m\(\mu\) on the addition of the chelating agent. When succinate was used as the electron donor, part of the nonheme iron present in the particles was found to undergo reduction. The amount of nonheme iron undergoing enzymatic reduction was found to increase with increasing concentrations of particle protein, and was about 3 to 4 times the enzymatically reducible cytochrome c in the preparation. Irradiation of the particles did not affect the nonheme iron content. However, irradiated particles did not show nonheme iron reduction. The reduction of nonheme iron, as well as succinate oxidase activity, was restored on the addition of the supernatant fraction to the irradiated particles. This indicated that the light-sensitive site was on the substrate side of nonheme iron on the succinate pathway.

The nonheme iron of the supernatant fraction was not reducible with succinate, but was partially reducible with NAD\(^+\)-linked substrates like \(\beta\)-hydroxybutyrate and malate. The particulate nonheme iron did not appear to be reducible with \(\beta\)-hydroxybutyrate. The particles, however, contained iron that was accessible to reduction with malate. But it was not known whether this iron was respiratory chain-linked or was a component of the bypass enzyme, malate-vitamin K reductase, present in both the particulate and the supernatant fractions.

that a metal, probably nonheme iron, was involved in the oxidation of succinate by electron transport particles isolated from cell-free extracts of Mycobacterium phlei (1, 2). Evidence for the participation of nonheme iron in respiratory pathways, has come primarily from electron spin resonance measurements (3). Although electron spin resonance spectra of the respiratory system of \(M.\) phlei gave indications of the involvement of a component of the nonheme iron type, as evidenced by the appearance of a signal in the \(g = 1.94\) region, no conclusions could be reached from such measurements because the signal was weak and marginal. It was observed, however, that spectrophotometric evidence for the reduction in situ of nonheme iron in these respiratory particles could be obtained by trapping the reduced iron as the o-phenanthroline complex. This technique has permitted a detailed study of the effect of both succinate and NAD\(^+\)-linked substrates on the reduction of nonheme iron. The results presented in this communication indicate that part of the nonheme iron of the bacterial particles is reduced when succinate is used as the electron donor.

EXPERIMENTAL PROCEDURE

Preparation of Particulate and Soluble Fractions—Mycobacterium phlei, ATCC 354, was grown and harvested by procedures previously described (4). The cells were washed with cold water until the wash liquid was free of iron. The cell-free extract was prepared by disrupting the cells in a 10-kc Raytheon sonic oscillator, followed by centrifugation at 20,000 \(\times g\) to remove whole cells and cellular debris (4). The cell-free preparation was separated into particulate and supernatant fractions by centrifugation at 120,000 \(\times g\) for 90 min in a Spinco model L preparative ultracentrifuge. The sedimented particles were suspended in 0.15 M KCl containing 0.01 M MgCl\(_2\), adjusted to pH 7.4 with Tris buffer, pH 7.4 (0.01 m), and centrifuged at 120,000 \(\times g\) for 60 min. The supernatant material from the second centrifugation was discarded and the sedimented particles were resuspended in 0.15 M KCl. Particles washed in this manner required the addition of NAD\(^+\) and magnesium for the oxidation of NAD\(^+\)-linked substrates. To prepare irradiated particles, the cell-free

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extract was exposed in the cold to a series of General Electric lamps (type BLB, “black light”) in a rotary radiation apparatus (5, 6). The particles were isolated from the irradiated extract by the procedure described above.

The supernatant fraction obtained following centrifugation of the cell-free extract at 130,000 × g for 90 min was dialyzed against cold water for 48 hours to remove the endogenous substrates and other impurities. Any precipitate formed during dialysis was removed by centrifugation. For some experiments the dialyzed supernatant fraction was irradiated with ultraviolet light as described above for 6 hours or more to remove the endogenous quinone.

**Estimation of Nonheme Iron**—The nonheme iron content of the particulate and supernatant fractions was determined after deproteinization with trichloroacetic acid in the presence of sodium hydrosulfite as previously described (7).

**Reduction of Nonheme Iron by Substrates**—The reduction of nonheme iron by substrates could be followed spectrophotometrically by allowing the reduced iron to form a complex with o-phenanthroline, since the o-phenanthroline-ferrous iron chelate has an absorption maximum at 510 μm. To estimate the amount of iron reduction it was necessary to determine the extinction coefficient of the protein-bound ferrous iron-o-phenanthroline complex. The extinction coefficient of the complex was determined as follows. The nonheme iron content of a sample of the particles was determined by the procedure previously described (7). The nonheme iron from a known amount of protein was released by the addition of trichloroacetic acid in the presence of hydrosulfite. An aliquot of the iron solution released from the particles was treated with o-phenanthroline under the same conditions as followed for the estimation of nonheme iron (7), and the absorbance of the complex was measured at 510 μm. Since the concentration of iron in the solution was known the extinction could be calculated. By this procedure the molar extinction coefficient of the complex was found to be 7.8 × 10^4 cm⁻¹. This value is about 30% lower than the extinction coefficient calculated from the absorbance values of the complex at 500 μm reported by Mahler and Elowe (8). For the determination of the optical density of the protein-bound iron-o-phenanthroline complex, an aliquot of the particulate preparation with known nonheme iron content was mixed with the chelating agent, the absorbance was adjusted to zero, the iron was reduced by the addition of sodium hydrosulfite, and the absorbance at 510 μm was followed until no further increase occurred. The complex formation was complete in less than 5 min. The molar extinction coefficient calculated in this manner was 7.9 × 10^4 cm⁻¹. Thus, the extinction coefficient of the chelate formed with protein-bound iron was found to be the same as that of ferrous iron-o-phenanthroline. Massay (9), by following a different procedure, was able to show that the protein-bound iron of succinate dehydrogenase formed a complex with o-phenanthroline to give the same extinction as free iron. The extinction coefficient obtained above for the protein-bound iron o-phenanthroline complex was used to calculate the enzymatic reduction of nonheme iron in the M. phlei system.

The absorbance measurements were made in a Beckman DU spectrophotometer. The difference spectra (reduced minus oxidized) were taken in a Cary model 11 recording spectrophotometer. The reduction of cytochrome c was measured as described by Chance and Hagiha (10).

Solutions of o-phenanthroline were prepared fresh by dissolving the chelating agent in ethanol and diluting with water so that the concentration of ethanol in the stock solution was 20% (v/v). Protein was determined by the method of Stadtman, Novelli, and Lipmann (11).

**Preparation of Ferrous Iron-o-Phenanthroline Complex**—A freshly prepared solution of ferrous ammonium sulfate containing 1 atom of iron per ml was mixed with an equal volume of a solution of o-phenanthroline in 20% ethanol (v/v) containing 3 μmoles of the chelating agent per ml. The resulting red-colored solution, containing 500 μmoles of the complex per ml, was used after proper dilution. This solution did not show any increase in color intensity following the addition of more iron or the chelating agent. Ferrous iron forms a 1:3 chelate with o-phenanthroline (12).

**RESULTS**

**Reduction of Nonheme Iron by Succinate**—The ability of metal ions to form colored complexes with sequestering agents has been used in studying the changes in the oxidation-reduction status of the protein-bound metal components of oxidoreductases during electron transport (10, 13). In a previous report (2) it was shown that o-phenanthroline was an effective inhibitor of oxidative phosphorylation in the M. phlei system and that the inhibitory action was reversed by the addition of ferrous iron. This suggested the strong possibility that a metal, probably nonheme iron, served an electron transport function in the M. phlei system. Thus, an attempt was made to determine whether evidence for the reduction of nonheme iron under the conditions of oxidative phosphorylation could be obtained by trapping the reduced iron as the o-phenanthroline complex.

The difference spectrum of the particles on addition of succinate (Fig. 1A) exhibited the reduced bands of cytochrome c and a as previously reported (1). When o-phenanthroline was added to the particles the electron carriers were reduced, the a band of cytochrome c remained in the difference spectrum whereas the β band was covered by the appearance of an absorption peak in the 510 μm region (Fig. 1B). The same spectral band appeared when freshly prepared ferrous iron-o-phenanthroline was added to the succinate-reduced system (Fig. 1C). When ferrous iron-o-phenanthroline was added to the particles which were in the oxidized state, the spectrum exhibited a broad peak in the 510 μm region. However, on subsequent addition of succinate a spectrum similar to that shown in Fig. 1C was obtained. The increase in absorbance (510 to 540 μm) was found to be proportional to the concentration of the complex added, and the extinction value agreed with that given earlier. It may be noted that the absorption band of the complex overlaps the β band of cytochrome c. In calculating the amount of iron reduced the contribution by the β band of cytochrome c was not considered. The absorption due to cytochrome c reduction in this region was shown to be less than one-third of that exhibited at 550 μm (14).

The increase in absorbance observed at 510 μm on the addition of o-phenanthroline appeared to be enzymatic in nature. The peak characteristic for the reduction of nonheme iron did not appear when the substrate or the particles were omitted from the reaction system. However, with the complete system the intensity of absorption at 510 μm was found to be dependent on the concentration of particle protein, and the amount of nonheme iron undergoing reduction was proportional to the concentration of protein (Fig. 2). The relationship between the enzymatically reducible nonheme iron and total nonheme iron as well as the reducible cytochrome c is shown in Table I. The amount of
FIG. 1. Difference spectra of ferrous-o-phenanthroline. The system, consisting of 100 μmoles of Tris-HCl buffer, pH 7.4, 15 μmoles of MgCl₂, 15 μmoles of inorganic phosphate, 25 μmoles of KF, 3 mg of yeast hexokinase (P-L Biochemicals), 10 μmoles of glucose, 2.5 μmoles of ADP, and washed particles (10 mg of protein), was present in a total volume of 3 ml in both the reference and standard cuvettes. After balancing, 100 μmoles of succinate were added to the standard cuvette. A, difference spectrum taken 300 sec later. At this stage, o-phenanthroline (2 × 10⁻⁴ M) was added to both cuvettes. B, spectrum taken 300 sec later. The difference spectrum, C, was obtained 300 sec after the addition of 15 μmoles of ferrous iron-o-phenanthroline to the succinate-reduced system represented by A. The difference spectrum in every case was taken after adjusting the absorbance at 650 μm between 0 and 0.01. The difference spectra were taken in a Cary model 11 recording spectrophotometer.

Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein used</th>
<th>Cytchrome c</th>
<th>Nonheme iron</th>
<th>Ratio of nonheme iron to cytochrome c</th>
<th>Nonheme iron content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg μmoles</td>
<td>μatoms</td>
<td>μatoms</td>
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<td>μatoms</td>
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<tr>
<td>1</td>
<td>13</td>
<td>1.7</td>
<td>5.7</td>
<td>3.4</td>
<td>439</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>2.4</td>
<td>7.7</td>
<td>3.2</td>
<td>720</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>3.2</td>
<td>7.7</td>
<td>2.4</td>
<td>550</td>
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<td>4</td>
<td>13</td>
<td>3.2</td>
<td>11.5</td>
<td>3.6</td>
<td>520</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>4.0</td>
<td>9.6</td>
<td>2.4</td>
<td>441</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>4.8</td>
<td>9.6</td>
<td>2.0</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>5.5</td>
<td>10.3</td>
<td>3.5</td>
<td>704</td>
</tr>
</tbody>
</table>
Effect of o-Phenanthroline Concentration—Information concerning the site of nonheme iron participation in the electron transport chain was obtained by varying the concentration and order of addition of o-phenanthroline. The results indicated that the functional nonheme iron reduction was the addition of o-phenanthroline following partial or complete reduction of the cytochrome pigments. Under these conditions increasing the concentration of the chelating agent did not alter the difference spectrum or the intensity of absorption at 510 mμ due to nonheme iron (Table III). However, when o-phenanthroline was added to the reaction system before the addition of succinate it was observed that the time taken for anaerobiosis (reduction of cytochromes c and a) after the addition of succinate was increased with increasing concentrations of the chelating agent until at high concentrations (6.4 x 10^{-4} mμ) the reduction of cytochromes was completely inhibited. At this concentration range o-phenanthroline was found to inhibit almost completely the oxidation of succinate (9). Nevertheless, the difference spectrum even with high concentrations of the chelating agent exhibited one absorption peak at 510 mμ and the extent of iron reduction was not appreciably altered (Table III). However, as the concentration of o-phenanthroline was increased greater time was necessary for the 510 mμ peak to reach maximum intensity.

Effect of Oxygen on Cytochromes and Nonheme Iron Spectrum—Further indications of the site of interaction of nonheme iron in the electron transport chain was obtained by oxygenation of the reaction system following reduction and trapping with o-phenanthroline. Thus, it would be expected that the electron transport carriers which function on the oxygen side of the site of interaction of the chelating agent would be oxidized by aeration. The reaction was allowed to proceed to anaerobiosis after the addition of succinate, and low concentrations of o-phenanthroline (2 x 10^{-3} mμ) were added to trap the reduced iron. The difference spectrum taken after the addition of the chelating agent was similar to that described in Fig. 1B and exhibited the peaks associated with nonheme iron (510 mμ) and cytochromes c (550 mμ) and a (598 mμ). When the reaction system was shaken with air or oxygenated, the cytochrome peaks at 550 mμ and 598 mμ disappeared. However, the peak at 510 mμ remained intact. If the reaction system was allowed to go anaerobic again the peaks characteristic of the cytochromes reappeared. But the time required for their reappearance was found to be more than twice that observed for the reduction of the cytochromes prior to the addition of the chelating agent. The intensity of cytochrome a was similar to that observed in the absence of the chelating agent. In contrast to the results observed with low concentrations of the chelating agent, with high concentrations of o-phenanthroline, although the cytochromes were oxidized on oxygenation, they remained in the oxidized state and could not be reduced again. The results indicate that the functional nonheme iron of the succinate oxidase pathway in M. phlei is positioned on the substrate side of the cytochromes.

Reduction of Nonheme Iron with β-Hydroxybutyrate or Malate—The role of nonheme iron in the NAD^{+} and malate-vitamin K reductase pathways of M. phlei was examined. In contrast to the coupled activity with succinate, washed particles of M. phlei fail to exhibit significant oxidation or phosphorylation with β-hydroxybutyrate or malate unless the supernatant fraction is added to the system (16). Therefore, it became necessary to find out whether the nonheme iron present in the supernatant fraction was reduced with either of these electron donors. A small fraction of the nonheme iron present in the supernatant

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### Table II

<table>
<thead>
<tr>
<th>Experiment, system, and addition</th>
<th>Total protein</th>
<th>Time required for anaerobiosis</th>
<th>Enzymatic reduction</th>
<th>Ratio of nonheme iron to cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>sec</td>
<td>mμoles</td>
<td>mμatoms</td>
</tr>
<tr>
<td>1. Particles</td>
<td>8.0</td>
<td>412</td>
<td>3.2</td>
<td>10.4</td>
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<tr>
<td>Supernatant fraction</td>
<td>12.5</td>
<td>301</td>
<td>3.5</td>
<td>11.5</td>
</tr>
<tr>
<td>2. Particles</td>
<td>13.0</td>
<td>375</td>
<td>3.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td>22.0</td>
<td>251</td>
<td>4.1</td>
<td>20.2</td>
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</table>

### Table III

<table>
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<th>o-Phenanthroline</th>
<th>Concentration</th>
<th>Time required</th>
<th>Enzymatic reduction</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mμ</td>
<td>sec</td>
<td>mμoles</td>
</tr>
<tr>
<td>After reduction with succinate</td>
<td>0.2</td>
<td>264</td>
<td>3.3</td>
</tr>
<tr>
<td>Before addition of succinate</td>
<td>0.2</td>
<td>353</td>
<td>Not measured</td>
</tr>
<tr>
<td>After addition of succinate</td>
<td>0.4</td>
<td>353</td>
<td>Not measured</td>
</tr>
<tr>
<td>After reduction with succinate</td>
<td>1.6</td>
<td>207</td>
<td>3.3</td>
</tr>
<tr>
<td>Before addition of succinate</td>
<td>1.6</td>
<td>(618)</td>
<td>Trace reduction</td>
</tr>
<tr>
<td>Before addition of succinate</td>
<td>6.4</td>
<td>(910)</td>
<td>No reduction</td>
</tr>
</tbody>
</table>
fraction was reduced with β-hydroxybutyrate (Fig. 3). On addition of the particulate fraction more nonheme iron was reduced; however, the amount reduced was not proportional to the increase in concentration of particulate protein. Furthermore, no increase in the reduction of nonheme iron was observed with increasing concentration of supernatant protein when the particulate protein was kept constant. Thus it was difficult to determine whether the increased iron reduction observed with this substrate originated from the supernatant or the particulate fraction. It is of interest to note that as the particulate protein was increased the ratio of cytochrome c to reducible nonheme iron tended to decrease. Previous studies (2) with metal-chelating agents failed to implicate a metal as a component of the pathway utilized for the oxidation of β-hydroxybutyrate.

The oxidation of malate by M. phlei occurs by two distinct pathways: one pathway requires malate dehydrogenase and NAD+ while the other requires malate-vitamin K reductase and FAD (1, 17). With the soluble malate dehydrogenase, nonheme iron reduction was found to occur (Fig. 4), and was greater than that observed with β-hydroxybutyrate and the soluble fraction. In addition, the particulate nonheme iron was also reduced by malate provided NAD+ was added to the system. However, unlike the results with β-hydroxybutyrate and NAD+, the reduction of nonheme iron with malate was found to be proportional to the particle concentration. The ratio of cytochrome c to nonheme iron reduction tended to remain constant with increase in the concentration of particulate protein, but the magnitude of the ratio (5.0 to 5.5) was much higher than that normally observed with succinate. Oxygen uptake was more susceptible to inhibition by o-phenanthroline with malate as electron donor than with β-hydroxybutyrate.

**Extent of Nonheme Iron Reduction with Different Substrates**—In order to determine whether the reduction of nonheme iron was substrate specific, the effect of combining the substrates on the reduction of iron was tested. When succinate and β-hydroxybutyrate were added together, the reduction of nonheme iron was more than that observed with either substrate alone (Table IV). Although this may indicate that the two substrates reduce nonheme iron in different compartments of the particle, the reduction observed was not additive. When malate was present in the reaction system, regardless of the presence of other electron donors, the reduction of nonheme iron was the same as that obtained with malate alone.

It has been shown that nonheme iron is a functional component of malate-vitamin K reductase and that the reduction of nonheme iron by malate is activated by FAD and vitamin K1 (18). The participation of this enzyme in electron transport was indicated by the observation that when malate was used as the electron donor the time required for the reaction system to reach anaerobiosis was shortened by more than one-half following the addition of FAD. A similar activation by FAD was not observed when either succinate or β-hydroxybutyrate was used as the electron donor. The fact that iron reduction was not found to be additive on the addition of succinate and malate may indicate that the succinate reducible nonheme iron of the particles is also capable of being reduced by the malate-reducible iron of the particles or by the supernatant nonheme iron associated with malate oxidation, but not vice versa.

**Effect of Irradiation on Reduction of Nonheme Iron**—The similarity between the site of action of metal-chelating agents...
(1, 2) and the site sensitive to ultraviolet irradiation (15) on the succinate chain indicated the possibility that these effects might be occurring on the same carrier. It was suggested (2) that irradiation might break the metal-protein bond and release the metal, as has been reported with carboxypeptidase A (19). However, particles isolated from cell-free extracts of M. phlei after prolonged ultraviolet irradiation (4 hours) did not show any appreciable decrease in the content of nonheme iron, nor did the supernatant fraction show any increase in free or nonheme iron content following irradiation. The only observed effect was a 30 to 40% decrease in the sulfhydryl content of the supernatant fraction.

Although irradiation did not result in any significant release of nonheme iron from the particles, the enzymatic reduction of nonheme iron was found to be affected by such treatment (Table V). No significant reduction of cytochrome c or nonheme iron was observed when irradiated particles were used alone. However, in the presence of the unirradiated supernatant fraction both cytochrome c and nonheme iron were reduced, even though the time taken for the system to attain anaerobiosis was greater than that required with unirradiated particles. Reduction was not observed with the system containing irradiated particles and irradiated supernatant material. These findings further support the earlier observations (15) that the supernatant fraction contains a light-sensitive component (or components) which is capable of restoring succinate oxidation following irradiation of the particles. It would appear that the light-sensitive factor differs from nonheme iron and interacts before nonheme iron on the succinate pathway.

The reduction of cytochrome c or nonheme iron was not observed with irradiated particles even in the presence of the supernatant fraction when β-hydroxybutyrate was used as the electron donor. However, when irradiated particles were supplemented

**Table IV**

**Extent of nonheme iron reduction in M. phlei with different substrates**

The reaction system was the same as described in the legend to Fig. 1, and contained 11 mg of particle protein and 1.5 mg of supernatant protein. DPN+ was added with malate and α-hydroxybutyrate. o-Phenanthroline (2 × 10⁻⁴ M) was added after reduction of the respiratory chain had occurred on the addition of the substrate (100 μmoles).

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Time required for anaerobiosis</th>
<th>Enzymatic reduction</th>
<th>Cytochrome c</th>
<th>Nonheme iron</th>
<th>Ratio of nonheme iron to cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>208 sec</td>
<td>4.8</td>
<td>13.5</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>600</td>
<td>3.7</td>
<td>18.0</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>752</td>
<td>3.8</td>
<td>8.9</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Succinate plus malate</td>
<td>207</td>
<td>4.3</td>
<td>18.3</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Succinate plus β-hydroxybutyrate</td>
<td>209</td>
<td>3.8</td>
<td>15.7</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Malate plus β-hydroxybutyrate</td>
<td>582</td>
<td>4.0</td>
<td>18.3</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Succinate plus malate plus β-hydroxybutyrate</td>
<td>285</td>
<td>4.4</td>
<td>19.3</td>
<td>4.4</td>
<td></td>
</tr>
</tbody>
</table>

with vitamin K₃ both cytochrome c and nonheme iron were reduced with this substrate. It had been shown (16) that the ability of irradiated particles to conduct oxidative phosphorylation with NAD⁺-linked substrates was restored only on the addition of naphthoquinones. However, when malate was used as the electron donor the reduction of nonheme iron was observed with irradiated particles even without the addition of vitamin K₃, although addition of the naphthoquinone greatly increased iron reduction. However, no reduction of cytochromes was observed with the irradiated particles in the absence of added naphthoquinone. The possibility that iron reduction under these conditions was mediated by malate-vitamin K₃ reductase, which is unaffected by irradiation (20), cannot be overlooked.

**Discussion**

Both chemical determinations (21, 22) and electron spin resonance spectroscopy (3) have been used in studying the oxidation-reduction status of nonheme iron during electron transport. The appearance of an electron paramagnetic resonance signal at g = 1.94 may not constitute conclusive evidence for a change in the valency state of nonheme iron in the light of the recent report (23) identifying sulfur as the component responsible for the signal. On the other hand, the chemical approach depends on the release of nonheme iron from the protein after interaction with the substrate and determination of the oxidation-reduction status of the released iron. This method, however, is static and does not permit quantitative comparison with the rate of electron flow (24). In addition, it suffers from the added disadvantage that the metal is subject to reduction during release from the protein. Nevertheless, a method of trapping the reduced metal as a colored complex with chelating agents has been attempted by
Succinate → flavoprotein → unknown light-sensitive (360 mN) component → nonheme iron → cytochrome b → cytochrome c → cytochrome a + → oxygen.

Fig. 5. Schematic representation of the succinate pathway of M. phlei

Massey (9) with succinate dehydrogenase and by Yonetani (13) with cytochrome oxidase. The observation that the metal in the succinate oxidase pathway of M. phlei was readily accessible to complex formation with o-phenanthroline (2) has permitted the successful application of the above technique to show that the electron transport chain of M. phlei contains substrate-reducible nonheme iron.

A part of the particulate nonheme iron is reduced when succinate is used as the electron donor. The reduction of nonheme iron increases with increase in the concentration of particle protein and appears to be related to the extent of reduction of cytochrome c. In addition, the amount of nonheme iron reduction appears to be unrelated to the total nonheme iron present in the particles. All these properties indicate that the succinate-reducible nonheme iron may be a direct participant in the transport of electrons from succinate to oxygen. This is further confirmed by the observation that irradiation, which destroys the succinate oxidase activity of the particles, also affects the reduction of nonheme iron. On the addition of the unirradiated supernatant fraction to the irradiated particles both succinate oxidase activity (15) and the reduction of nonheme iron reappear. This, however, indicates that, contrary to the earlier assumption (2), the light-sensitive site on the succinate chain is different from the nonheme iron with P-hydroxybutyrate does not appear to depend on the addition of the supernatant fraction (16). The supernatant fraction has been found to contain nonheme iron reducible with P-hydroxybutyrate or with malate. However, in contrast to the observation with succinate, the reduction of nonheme iron with P-hydroxybutyrate does not appear to depend on the concentration of particle protein. This may indicate that a part of the nonheme iron in the supernatant fraction is accessible to reduction by a reduced electron transport component of the chain. The fact that with P-hydroxybutyrate increased iron reduction is observed even in the presence of succinate lends support to this interpretation. The observation that on irradiation of the particles reduction of nonheme iron fails to occur when P-hydroxybutyrate is used as the electron donor, coupled with the finding that the reduction of nonheme iron requires the addition of vitamin K₁ to irradiated systems, indicates the possibility that the enhanced reduction of nonheme iron observed on the addition of particles to the P-hydroxybutyrate system is due to the fact that the naphthoquinone in the NAD⁺ chain of the particles reduces more nonheme iron in the supernatant fraction.

When malate is used as the electron donor the reduction of nonheme iron is proportional to the concentration of particle protein. This may indicate that the particles contain nonheme iron reducible by malate. However, the fact that both the supernatant fraction and the particles contain malate-vitamin K reductase activity makes it difficult to assess whether the observed nonheme iron reduction is due to the soluble malate-vitamin K reductase which has nonheme iron as the terminal electron transport component (18).

The relationship among nonheme iron, labile sulfide, and sulfhydryl groups has been studied in the M. phlei system (7). In the M. phlei particles, as well as in mammalian particulate preparations, the labile sulfide was found to be much lower than the total nonheme iron content. These results differed from those obtained with homogeneous proteins where the labile sulfide and nonheme iron bear roughly a 1:1 ratio (25). The observation that the sulfhydryl content of the particles was of the same order as the nonheme iron and that most of the sulfhydryl groups in bovine heart mitochondria arise from the structural protein led to the suggestion (7) that most of the nonheme iron may be involved in maintaining the structural integrity of the electron transport system. The present observation that only a small fraction of this nonheme iron is reducible by electron donors and that the reducible iron is of the same order as the labile sulfide content of the particulate system (1) may indicate that as contrasted with the "structural nonheme iron" which is bonded to the structural protein through sulfhydryl groups, the "electron transport nonheme iron" exists in intimate association with sulfide groups. The labile sulfide content of the particulate preparations may be an index of the enzymatically reducible nonheme iron in such preparations.

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