Histidyl Residues Mediate Electron Transport in Plant Mitochondria*

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Plant Mitochondria, Histidine Modification, Electron Transport, Spectral Studies

The effect of chemical modification of histidyl residues using diethyl pyrocarbonate (DEP) on plant mitochondrial electron transport was studied. Mitochondrial membranes from potato tubers were isolated and electron flow from NADH to oxygen, NADH to ferricyanide and ascorbate to oxygen were monitored in presence and/or absence of DEP. Measurements were made at various concentrations of DEP and at different pHs either by using an oxygen electrode or spectrophotometrically. The results show that DEP inhibits flow of electrons from NADH to oxygen, however partial electron transport from NADH to ferricyanide and ascorbate to oxygen was unaffected. Maximum inhibition was observed at pH 6.5. The time course of the DEP action revealed a biphasic nature of inhibition. Effects on the levels of reduction of cytochromes b and c by DEP during electron transport indicated that histidyl residues may be present or are at cytochrome b, which are being modified.

Introduction

Diethyl pyrocarbonate (DEP) modifies specifically histidine residues at pH 6.5 in model systems and in several enzymes [1]. Histidine is one of the important constituents of membrane proteins including those of plant mitochondrial membranes [2] and can participate in proton translocation. In chloroplasts, modification of the thylakoid membranes and photosystem II particles by DEP demonstrated the involvement of histidine residues of the water-oxidizing complex in the electron transport from water to photosystem II reaction centre [3]. DEP has recently been used to modify histidine residues in F$_6$F$_7$-ATPase complex of bovine-heart submitochondrial particles leading to inhibition of ATPase activity and stimulation of passive proton conduction. The inhibition of ATPase activity was correlated with the modification of a histidine residue in the F$_7$ inhibitor protein which regulates the catalytic activity of the ATPase [4]. No effect of histidine modification on electron transport in mitochondria has been reported so far.

Abbreviations: DEP, diethyl pyrocarbonate; NADH, nicotinamide adenine dinucleotide (reduced); EDTA, ethylenediamine tetracetic acid; UQ, ubiquinone; MES, 2-morpholinoethanesulfonic acid; HEPES, N-2-hydroxyethylenediamine tetraacetic acid; UQ, ubiquinone; NADH, nicotinamide adenine dinucleotide (reduced); EDTA, ethylenediamine tetracetic acid; UQ, ubiquinone; MES, 2-morpholinoethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Cyt, cytochrome.

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The protein composition of the energy transducing, inner membrane of mitochondria is known [5]. The electron transport chain has been resolved into four multicomponent complexes, NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), ubiquinol-cytochrome c oxidoreductase (complex III or bc$_1$ complex) and cytochrome c oxidase (complex IV) [6]. These complexes from bovine-heart mitochondria have been well characterized and can be reassembled into a functional respiratory chain [7]. In plant mitochondria, of the four electron transporting complexes, the structure of cytochrome oxidase is perhaps the best understood [2]. As in other mitochondria it consists of a number of subunits with binding sites for copper ions and haem groups. A model for the arrangement of subunit I and II has been proposed recently [8]. According to this model, subunit I consists of 12-membrane spanning regions with portions of the polypeptide exposed on either side of the membrane. Three of these membrane-embedded segments contain conserved histidine residues and have been proposed to provide binding sites for copper ions and haem groups. Also the subunit III of cytochrome oxidase has been shown to contain 13 histidine residues believed to be involved in proton translocation. Conserved histidines are also found in the cytochrome bc$_1$-complex: Four in Cyt b, each pair of them probably holding a haem group [9, 10], two within the peptides which bind the FeS-cluster in the Rieske protein (s. ref. [10]), and one in cytochrome c$_1$ binding to the haem (s. ref. [10]). Also soluble...
cytochrome c contains such a histidine. It was of interest therefore to see if chemical modification of any of these histidines leads to an effect on the electron transport.

By using the histidine selective reagent DEP, we present evidence to show that histidine residues are involved in the electron transport proteins of the plant mitochondrial membrane.

**Materials and Methods**

Mitochondria were isolated from potatoes (*Solanum tuberosum*) using the basic centrifugation procedure of Bonner [11] with certain modifications. The plant tissue was homogenized in extraction buffer containing Tris-HCl (50 mM) pH 7.5, sucrose (0.3 M), MgCl₂ (5 mM), EDTA (1 mM) and 2-mercaptoethanol (5 mM). The ratio of tissue to medium was 1:2. The homogenate was filtered through 8 layers of muslin and filtrate centrifuged at 1000 × g for 10 min. The supernatant was centrifuged again at 16,000 × g for 15 min and the pellet was dissolved in resuspending medium containing potassium phosphate buffer (50 mM) pH 7.2, sucrose (0.3 M) and EDTA (1 mM). The mitochondria so prepared were fully uncoupled — addition of uncouplers did not change the electron flow. The rate of electron transport was determined by the amount of oxygen consumed using an oxygen electrode (Hansatech). Protein content was determined by Peterson's method [12].

The assay mixture for the complete reaction from NADH/succinate to oxygen contained in a total volume of 1 ml potassium phosphate (50 mM) pH 7.2, sucrose (0.3 M), MgCl₂ (10 mM), KCl (10 mM) and mitochondrial membrane equivalent to 0.2–0.5 mg protein. The concentrations of NADH was 1 mM when the oxygen electrode was used, and 0.25 mM when the spectrophotometer was used. Inhibitors used were sodium azide (10 mM), antimycin A (2 μg/ml) and DEP (3.45 mM or as indicated).

The assay mixture for the partial reaction of NADH to ferricyanide in a total volume of 1 ml contained potassium phosphate (50 mM) pH 7.2, sucrose (0.3 M), MgCl₂ (10 mM), KCl (10 mM), azide (20 mM), ferricyanide (1 mM) and mitochondrial membrane equivalent to 0.2–0.5 mg protein. The reaction was initiated by NADH (0.25 mM). Oxidation of NADH was monitored at 340 nm.

The partial reaction of ascorbate with oxygen was monitored using 1 ml assay medium containing mitochondrial protein (0.2–0.5 mg), potassium phosphate (50 mM) pH 7.2, sucrose (0.3 M), MgCl₂ (10 mM) and KCl (10 mM). The reaction was initiated by NADH (1 mM). This reaction was then inhibited by antimycin A (2 μg/ml) and restored by ascorbate (1 mM). The recovered reaction was then inhibited by azide (10 mM). The reaction was monitored using the O₂ electrode.

To study the modification of the mitochondrial membrane by DEP, the above reactions were carried out in the presence and absence of DEP (Sigma Chemical Co.). DEP was added to the desired concentrations in ethanolic solutions not exceeding 2% ethanol in the assay. The controls contained 2% ethanol but no DEP. Ethanol at this concentration had no effect on the rate of electron transport.

For spectral analysis, two sets of experiments were conducted. In one case DEP (3.45 mM) was added during the reaction and then the spectra were monitored. In the second case mitochondria were first incubated with DEP (3.45 mM) and then the reaction was initiated by NADH. Spectra were then monitored.

**Results and Discussion**

Fig. 1 shows that the electron flow of the plant mitochondrial membranes is inhibited by DEP in a concentration and time-dependent manner. The in-

![Fig. 1. Concentration and time dependence of DEP inhibition.](image-url)
Fig. 2. Effect of pH on DEP inhibition of mitochondrial electron transport. Concentration of DEP used was 3.45 mM. 50 mM buffers were used. ○——○ MES, △——△ potassium phosphate, ●——● HEPES.

Inhibition at pH 6.5 appears to be biphasic. The pH dependence of inhibition (Fig. 2) showed that DEP is most effective at pH 6.5. At this pH DEP is known to modify specifically the histidyl residues of proteins [13–15]. We, therefore, conclude that the inhibition of electron flow is due to the modification of one or more histidyl residues of the electron transport proteins. The possibility that the inhibition of electron transport observed could be due to the modification of 
$F_0$–$F_1$ ATPase histidyl residues [4] was ruled out as the experiments were conducted with uncoupled mitochondrial membranes. The biphasic nature of inhibition suggests that there are two types of histidyl residues modified by DEP, one which is easily accessible, and another which is less accessible.

In order to localize the site of inhibition by DEP, studies of partial electron transport were carried out. Table I shows that the electron transport from NADH to O$_2$ was totally inhibited by DEP but the partial reactions, from NADH to ferricyanide and from ascorbate to O$_2$, remained unaffected. The addition of ferricyanide to DEP-treated mitochondria wherein NADH oxidation was fully inhibited resulted in complete recovery of NADH oxidation. Similarly oxygen consumption with NADH as the donor which was fully inhibited by DEP could be completely restored by the addition of ascorbate. This electron transport is fully sensitive to azide addition indicating that we were monitoring the mitochondrial oxygen consumption requiring cytochrome oxidase. These partial reaction studies suggested that the site of DEP inhibition may be between ubiquinone and cytochrome c since ferricyanide can accept electrons from ubiquinone and ascorbate can donate electrons to cytochrome c [16].

A study of cytochrome spectra (Fig. 3 and Table II) showed that addition of NADH to mitochondria initiates electron transport and induces peaks at 550 nm (Cyt c) and 560 nm (Cyt b). Addition of azide which blocks cytochrome oxidase shows both cytochromes in a reduced state (Fig. 3, curve C). If DEP is added to such mitochondria the Cyt b absorption is lost but that of Cyt c (550 nm) still remains (Fig. 3, curve D). This indicates that when azide blocks oxidation of Cyt c through cytochrome oxidase both, cytochromes b and c remain in the reduced state. However, if DEP is added subsequently, the absorption due to reduced b is lost indicating that it is oxidized but in the presence of DEP it cannot be reduced by NADH via ubiquinol. Therefore DEP must be blocking reduction of Cyt b. When mitochondria are preincubated with DEP, and NADH is added subsequently we do not see any peak related to cytochrome reduction (Fig. 3, curve E), indicating that DEP must be blocking electron transport prior to the reduction of Cyt b.

Table I. Partial reactions.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>% Inhibition</th>
<th>Artificial acceptor/donor</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) NADH to ferricyanide:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>azide</td>
<td>86</td>
<td>ferricyanide</td>
<td>80</td>
</tr>
<tr>
<td>NADH</td>
<td>DEP</td>
<td>100</td>
<td>ferricyanide</td>
<td>100</td>
</tr>
<tr>
<td>B) Ascorbate to oxygen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>azide</td>
<td>77</td>
<td>ascorbate</td>
<td>0</td>
</tr>
<tr>
<td>NADH</td>
<td>DEP</td>
<td>100</td>
<td>ascorbate</td>
<td>92</td>
</tr>
</tbody>
</table>
Table II. Presence or absence of the peaks due to reduced cytochrome b or c in the presence of different treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>550 nm (Cyt c)</th>
<th>560 nm (Cyt b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) control</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(B) NADH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(C) B + azide</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>(D) C + DEP</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>(E) DEP + NADH</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(F) E + ascorbate</td>
<td>+</td>
<td>small peak</td>
</tr>
<tr>
<td>(G) F + dithionite</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The spectral data together with the data on partial electron transport show that DEP affects the middle part of the respiratory chain, i.e., the cytochrome bc1-complex, and not the cytochrome oxidase, although both complexes contain numerous histidines as possible targets, as discussed in the introduction. The question is which histidines of the polypeptides in the cytochrome bc1-complex [9, 10] are modified. Since the reduction of cytochrome b and not of cytochrome c/c1 (Fig. 3) by NADH is inhibited, a histidine residue, either directly on cytochrome b, or on a protein involved in reduction of cytochrome b may be changed. The latter possibility is more probable, because chemical reduction of cytochrome b is still possible in the presence of DEP. The target may be the Rieske protein which has two histidines in the vicinity of its FeS-cluster. A widely accepted concept for explaining H+ translocating turnover of the cytochrome bc1-complex is the “Q-cycle mechanism” [17]. In this concept the Rieske FeS-protein is involved in cytochrome b reduction by oxidizing ubiquinol to a semiquinone which then reduces cytochrome b [18].