Inhibition of Cyanide-Resistant Respiration in Pea Cotyledon Mitochondria by Chloroquine¹

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Received for publication May 18, 1981 and in revised form January 11, 1982

ABSTRACT

The action on mitochondrial respiration of a ubiquinone analog, chloroquine, has been studied using purified mitochondria from the cotyledons of germinating peas (Pisum sativum L. var. Homesteader). Chloroquine at 3 millimolar did not inhibit malate or succinate oxidation at pH 7.2, but it did inhibit malate (but not succinate) oxidation at pH 8.2. Cyanide-resistant respiration was also inhibited.

The implications of these experiments on the role of ubiquinone in the cyanide-resistant respiratory pathway and on the location of the alternate oxidase are discussed.

A topic of recent interest in plant mitochondrial studies has been the inhibition of electron transport by inhibitors acting at or near the level of ubiquinone (9). The stimulus for these experiments has, to a large degree, been the suggestion that ubiquinone acts as the branch point in the cyanide-resistant respiratory pathway (4, 6, 7). This subject has been reviewed recently (2).

The inhibitor that has been used in these studies is the ubiquinone analog dibromothymoquinone. Dibromothymoquinone has been shown to inhibit both cyanide-sensitive and cyanide-resistant respiration. The mode of dibromothymoquinone action is generally believed to be the blockage of ubiquinone oxidation (1), and it has been concluded that ubiquinone is involved in cyanide-resistant respiration (9).

The present study reports the effects of another inhibitor of mitochondrial reactions, chloroquine.

MATERIALS AND METHODS

Plant Material. Pea seeds (Pisum sativum L. var. Homesteader) were soaked in tap water for 6 h. Following this, damaged and nonimbibed seeds were discarded. The remaining seeds were planted in horticultural grade vermiculite and were germinated at 27°C in the dark. The cotyledons were harvested 6 d after the onset of imbibition.

Isolation of Mitochondria. The procedures used were identical to those reported in an earlier study (5).

Respiratory Measurements. O₂ consumption was determined polarographically, as described previously (5). When chloroquine was used, 30 µl of an aqueous stock solution were added to the 3 ml of assay medium. New chloroquine stock solutions were prepared daily.

Protein. Protein was measured as described previously (5).

Chemicals. All chemicals were of reagent grade and, with the exception of the following, were from Fisher Scientific Co.: chloroquine, ADP, Tes, and cysteine (Sigma Chemical Co.); BSA (Calbiochem); and salicyldihydroxaminic acid (Aldrich Chemical Co.).

RESULTS

The effects of chloroquine on succinate oxidation and malate oxidation by isolated pea cotyledon mitochondria are shown in Figures 1 and 2. Chloroquine had only a small effect on the state 3 respiration rate at either pH 7.2 or 8.2 when succinate was used as the respiratory substrate. The state 4 rate was accelerated at pH 7.2, and, at pH 8.2, state 4 respiration and respiratory control were obliterated. At pH 7.2, the effect of 3 mM chloroquine on malate oxidation was qualitatively similar to the effect on succinate oxidation. However, at pH 8.2, the situation was different. Almost

Fig. 1. Inhibition of succinate oxidation by chloroquine. Mitochondria were prepared from 6-d-old cotyledons by the zonal technique and were assayed polarographically (see "Materials and Methods"). The final concentration of chloroquine was 3 mM. The numbers alongside the traces are the uptake in nmol O₂/min - mg protein. The circled numbers refer to the RC ratio.

1 Supported by the Natural Sciences and Engineering Research of Canada in the form of a grant (to M. S. S.) and a postgraduate scholarship (to T. W. J.).
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We have shown previously (5) that mitochondria from pea cotyledons at this stage of development have cyanide-resistant respiration rates of between 15 and 20% of the total succinate oxidation rate. In the present study, we have found that chloroquine will inhibit this cyanide-resistant respiration. Figure 3 shows the effect of different concentrations of chloroquine on the cyanide-resistant respiration.

![Graph showing inhibition of malate oxidation by chloroquine](image1)

**FIG. 2.** Inhibition of malate oxidation by chloroquine. Mitochondria were isolated from 6-d-old cotyledons by the zonal technique and were assayed polarographically (see "Materials and Methods"). The final concentration of chloroquine was 3 mM. The numbers alongside the traces are the \( \text{O}_2 \) uptake in nmol \( \text{O}_2/\text{min} \cdot \text{mg protein}. \) The circled numbers refer to the RC ration.

![Graph showing inhibition of cyanide-resistant respiration by chloroquine](image2)

**FIG. 3.** Inhibition of cyanide-resistant respiration by chloroquine. Mitochondria from 6-d-old cotyledons were prepared by the zonal procedure and were assayed polarographically (see "Materials and Methods"). The respiratory substrate was succinate, and the pH was 7.2. During the second cycle of state 3 respiration, KCN was added to give a concentration of 0.3 mM. Following this, an aqueous chloroquine solution was added.

![Chemical structures](image3)

**FIG. 4.** Chemical structures of duraquinone, menadione, chloroquine, and coenzyme Q10.

complete inhibition of malate oxidation occurred at 3 mM chloroquine, while 30% inhibition was observed at 0.1 mM chloroquine (not shown).

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**DISCUSSION**

Inhibition of cyanide-resistant respiration by chloroquine has not been reported previously. The structural similarity of chloroquine to ubiquinone (Fig. 4), as well as the previously reported mode of action on beef-heart submitochondrial particles (1), suggests that chloroquine inhibits the alternate oxidase by competing with reduced ubiquinone for an enzyme site.

Chloroquine is a unique inhibitor in that it inhibits the internal NADH oxidizing system, but it does not inhibit the succinate oxidizing system (1). It has also been shown to uncouple photosynthetic phosphorylation (3). In the present work, also, chloroquine has been found to inhibit malate but not succinate oxidation and to affect phosphorylation.

Experiments were done at two pH values, inasmuch as it has been reported (1) that, at pH 7.2, chloroquine does not pass through the inner mitochondrial membrane. For that reason, most previous experiments using chloroquine have been conducted with
inverted (inside out) electron transport particles. About 75% inhibition of the NADH oxidation of these particles can be obtained with a 33 mM concentration of chloroquine (1). Crane (1) also reports that there is another inhibition site in right-side-out particles evidenced at 100 mM chloroquine. No evidence for this site was found in the intact pea cotyledon mitochondria.

The inhibition by chloroquine raises some interesting questions concerning the location of the alternate oxidase. Since inhibition of the alternate oxidase occurred at pH 7.2, it appears that the alternate oxidase may be located on the outside of the inner membrane. However, some increase in respiration took place at pH 7.2 (Fig. 1), indicating perhaps that a small amount of chloroquine was passing through the inner membrane. Also, because relatively high concentrations of chloroquine were required to inhibit the alternate oxidase, one could argue that the inhibition of the alternate oxidase was being effected by a small amount of chloroquine passing through the inner membrane. However, inasmuch as malate oxidation was not inhibited at pH 7.2 by chloroquine but was dramatically inhibited at pH 8.2, it seems unlikely that much chloroquine was passing through the inner membrane at pH 7.2. An experiment conducted to compare the chloroquine inhibition of succinate-supported cyanide-resistant respiration at pH 7.2 and 8.2 was complicated by the fact that the alternate oxidase itself was considerably inhibited at pH 8.2 in the absence of chloroquine. No evidence could be found, though, for greater inhibition by chloroquine of the alternate pathway at this pH. Therefore, the possibility remains that the alternate oxidase may be located on the external surface of the inner membrane. This is in contrast to the suggestion (8) that the oxidase is on the inner side of the mitochondrial inner membrane.

Since cyanide-insensitive respiration was inhibited by chloroquine, we can ask why succinate oxidation was not also affected. The reason may be because of the control mechanism through which electrons are shunted to the alternate pathway. Cyanide-insensitive respiration does not operate during state 3 respiration with many types of plant mitochondria (2). However, it does operate at maximal capacity during state 4. Our own work (5) has shown that, in pea cotyledon mitochondria, the cyanide-resistant pathway operates only to a small extent during state 3 respiration. Therefore, if chloroquine affects the cyanide-resistant respiratory pathway, an inhibition would only be expected in state 4. Such an inhibition cannot be observed because of the uncoupling caused by chloroquine.

When considered in the context of the results of other researchers (1, 4), the chloroquine inhibition experiments supports the involvement of ubiquinone in cyanide-resistant respiration.

**LITERATURE CITED**