HYDROGEN PEROXIDE AS THE PRODUCT OF AUTOXIDATION OF FERREDOXIN: REDUCED EITHER CHEMICALLY OR BY ILLUMINATED CHLOROPLASTS

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1. Introduction

In isolated spinach chloroplasts, ferredoxin catalyzes both cyclic and non-cyclic electron transport, coupled to phosphorylation [1]. The terminal acceptor of the non-cyclic electron flow can be either $NADP^+$ or, in the absence of $NADP^+$, oxygen. This paper describes experiments to demonstrate: (1) that the primary product of non-cyclic electron flow to oxygen is hydrogen peroxide; (2) that the hydrogen peroxide is a direct product of oxidation of ferredoxin, and not of the flavoprotein, ferredoxin-NADP reductase; (3) that hydrogen peroxide is also the product of oxidation of purified ferredoxin after its chemical reduction with sodium dithionite.

Experiments by Arnon et al. [2] showed that on reduction of ferredoxin by illuminated chloroplasts one molecule of oxygen was evolved per four molecules of ferredoxin reduced, in agreement with the observation that spinach ferredoxin accepts one electron when it is reduced [3]. On reoxidation of the ferredoxin in the dark, one molecule of oxygen was taken up per four molecules of ferredoxin reoxidized.

Arnon et al. [1] proposed that the mechanism of non-cyclic phosphorylation in the absence of $NADP^+$ involved the photoreduction of ferredoxin and its reoxidation by oxygen. Oxygen was presumed to be reduced to water by ferredoxin in these experiments although the only net product measured was ATP. However other workers have reported that ferredoxin stimulates oxygen uptake by illuminated chloroplasts in a Mehler type reaction [4, 5]. We have reinvestigated these experiments using a chloroplast preparation with low catalase activity, and find that with this preparation ATP formation is accompanied by oxygen uptake and hydrogen peroxide formation.

2. Materials and methods

Broken washed spinach chloroplasts (P_1S_1) or pea chloroplasts (P_1S_2) were isolated essentially as described by Whatley and Arnon [6]. The chlorophyll concentration was measured by the method of Arnon [7]. ADP, $NADP^+$, NADPH and horse liver catalase were obtained from Boehringer Corporation Ltd. Spinach ferredoxin was prepared by the method of Cammack, Rao and Hall [8]. Ferredoxin-NADP reductase from lettuce chloroplasts was a gift of Dr. J. Neumann, Department of Botany, University of Tel Aviv, Israel.

Simultaneous measurements of oxygen and pH changes were made in a Rank oxygen electrode cell fitted with a combined glass electrode through the lid of the reaction vessel. The reaction mixture was maintained at a temperature of 20° and was illuminated by a slide projector (300 W) through a heat filter and a filter transmitting light between 540 nm and 740 nm.

Photophosphorylation was measured by pH increase, in a reaction mixture of low buffering capacity. Nishimura, Ito and Chance [9] have shown that between pH 8.2 and 8.5 the ratio of ATP formed per protons consumed is 1.0. Proton consumption was measured by calibrating the pH change with aliquots of standard HCl. Anaerobic spectrophotometric measurements were made in a Unicam SP 800 spectrophotometer, using a 1 cm light path cuvette similar to that described by Dixon [10]. Solutions in the cuvette were bubbled with purified argon, except when additions of oxygen were made.

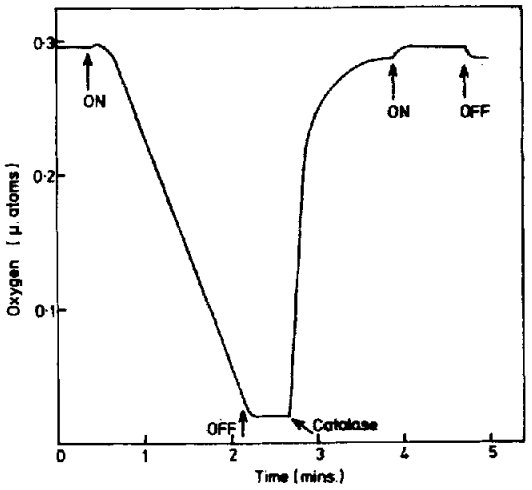

Fig. 1. Oxygen uptake on illumination of spinach chloroplasts in the presence of ferredoxin. The reaction mixture contained, in a total volume of 3 ml: chloroplasts (P_1S_1) equivalent to 100 μg chlorophyll; 60 μmoles KCl; 6 μmoles $MgCl_2$; 1.5 μmoles potassium phosphate; 0.5 μmoles ADP and 170 nmoles ferredoxin, final pH 8.4. Approx. 2.400 units of catalase were added as indicated. The rate of oxygen uptake was 102 μatoms / mg chlorophyll / hr.

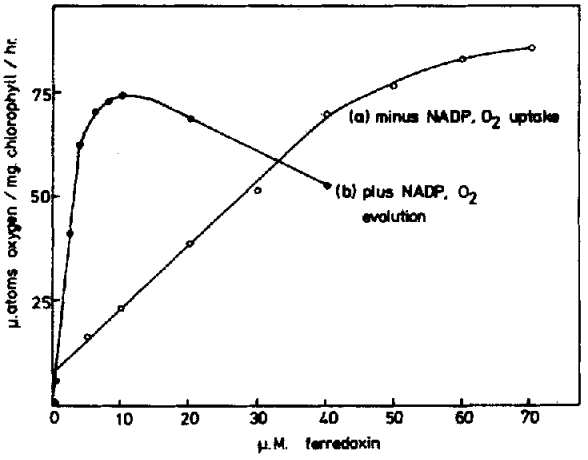

Fig. 2. Effect of ferredoxin concentration on oxygen uptake in the absence of $NADP^+$ (curve *a*) and oxygen evolution in the presence of $NADP^+$ (curve *b*) by illuminated chloroplasts. The reaction mixture contained in a total volume of 3 ml: chloroplasts (P_1S_1) equivalent to 100 μg chlorophyll; 250 μmoles *N*-tris(hydroxymethyl)methylglycine, pH 8.4, 10 μmoles NH_4Cl, 3 μmoles sodium azide; spinach ferredoxin as indicated and 4 μmoles $NADP^+$ (curve *a* only).

3. Results and discussion

Fig. 1 shows that on illumination of spinach chloroplasts with added ferredoxin oxygen was taken up. Uptake ceased in the dark and on addition of catalase an equal quantity of oxygen was evolved. Since catalase causes the decomposition of hydrogen peroxide with the production of oxygen it is concluded that the uptake of oxygen in the light was associated with the formation of hydrogen peroxide. On subsequent illumination after the addition of catalase (fig. 1) a small amount of oxygen was evolved and on switching off the light a similar amount of oxygen was taken up. This was presumably associated with the reduction and reoxidation of ferredoxin, as in the experiments of Arnon et al. [2]. Our experiments show that the initial product of ferredoxin-catalyzed electron transport is hydrogen peroxide and not water. This was apparently not detected by Arnon et al. [2] owing to the presence of catalase activity in their isolated chloroplasts. The chloroplasts used in our experiments showed only a very low level of endogenous catalase acitivity.

When photophosphorylation coupled to the oxygen uptake was measured it was found that one molecule of ATP was formed per atom of oxygen taken up. This ratio was obtained in the presence of 2 mM azide, added to inhibit the low endogenous catalase activity of the chloroplasts. When $NADP^+$ was used as terminal electron acceptor with these chloroplasts the $P/2e^-$ ratio obtained was 1.0. ($NADP^+$ reduction was measured by absorption at 340 nm and ATP formation by the method of Hagihara and Lardy [11]). This suggests that when oxygen was the terminal electron acceptor one atom of oxygen was taken up per two electrons transported. These results can be explained by the reaction scheme:

$$2\,Fd_{ox} + H_2O + ADP + P_i \xrightarrow{h\nu} 2\,Fd_{red} + 2H^+ + \tfrac{1}{2}O_2 + ATP$$

$$2\,Fd_{red} + 2H^+ + O_2 \rightarrow 2\,Fd_{ox} + H_2O_2$$

net result:

$$H_2O + \tfrac{1}{2}O_2 + ADP + P_i \xrightarrow{h\nu} H_2O_2 + ATP\,.$$

Preliminary experiments with *Anabaena cylindrica* ferredoxin and with bacterial type ferredoxin from

Clostridium pasteurianum and *Chloropseudomonas ethylicum* indicate that hydrogen peroxide is also formed when these ferredoxins are oxidized.

Similar results are obtained with artificial electron acceptors such as methyl viologen or FMN that are reduced in the light by chloroplasts and are autoxidized with the formation of hydrogen peroxide.

Fig. 2 (curve *a*) shows that the rate of oxygen *uptake* is dependent on ferredoxin concentration up to 50 μM. By contrast the oxygen *evolution* with $NADP^+$ as acceptor (curve *b*) requires only 10 μM ferredoxin for the maximum rate. This suggests that it is the ferredoxin which is autoxidized to form hydrogen peroxide in the experiment of fig. 1 and not some other component of the electron transport chain.

An alternative explanation might be that hydrogen peroxide is formed by autoxidation of the flavoprotein, ferredoxin-NADP reductase. However if this were the case the rate of hydrogen peroxide formation might be expected to show the same dependence on ferredoxin concentration as $NADP^+$ reduction.

Pea chloroplasts have a relatively more water soluble ferredoxin-NADP reductase than spinach and this fact forms the basis of an experiment to test the alternative explanation. With spinach chloroplasts the rates of ferricyanide reduction, $NADP^+$ reduction and ferredoxin-dependent oxygen uptake were all similar. However, with washed pea chloroplasts (deficient in the reductase) the rates of ferricyanide reduction and ferredoxin-dependent oxygen uptake were similar but the rate of $NADP^+$ reduction was considerably lower, unless ferredoxin-NADP reductase was added. This experiment indicates that the ferredoxin-dependent

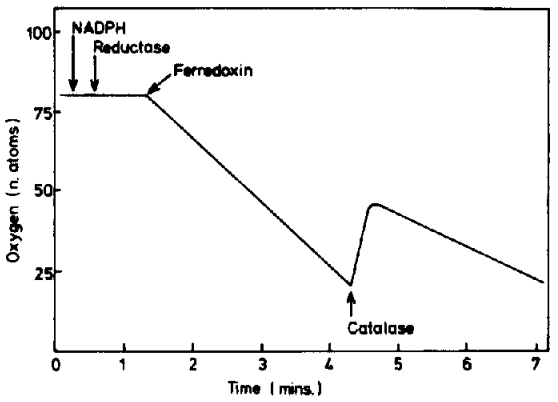

Fig. 3. Oxygen uptake and formation of hydrogen peroxide by ferredoxin in the presence of ferredoxin-NADP reductase and NADPH. The reaction mixture contained in a total volume of 2 ml: 80 μmoles tris-Cl, pH 8.0; 0.5 μmole NADPH; 24.5 units ferredoxin-NADP reductase; 7.5 nmoles ferredoxin and approx. 2.400 units of catalase were added as indicated.

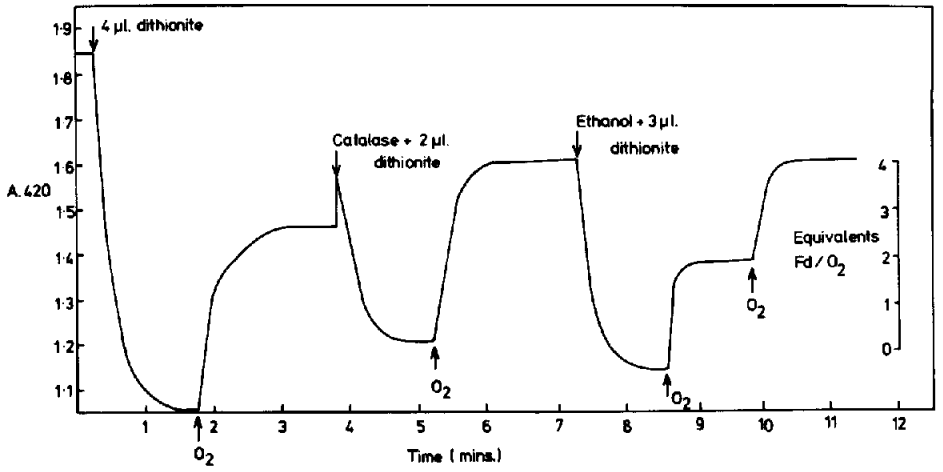

Fig. 4. Reduction and reoxidation of spinach ferredoxin measured by change in absorption at 420 nm. The reaction mixture contained, in a total volume of 2.1 ml: 50 μmoles tris-Cl, pH 8.5 and 0.42 μmole ferredoxin. Aliquots of 48 mM sodium dithionite, 96 nmoles oxygen in the form of oxygen saturated water, 7.800 units catalase and 174 μmoles ethanol were added as indicated. Reaction temperature was 25°.

oxygen uptake does not involve the reoxidation of ferredoxin-NADP reductase by oxygen.

The data presented in fig. 3 show that the reductase is not autoxidizable. When the purified isolated enzyme was reduced by treatment with excess NADPH in air no oxygen uptake was observed. However, when spinach ferredoxin was added oxygen uptake occurred and on the further addition of catalase the rate of oxygen uptake was halved. This indicated that the reaction sequence was NADPH → reductase → ferredoxin → oxygen; and was not NADPH → reductase → oxygen.

The oxidation of reduced ferredoxins by oxygen in the absence of chloroplasts or of the reductase was also investigated. A solution of ferredoxin in an anaerobic cuvette was partially reduced by addition of sodium dithionite solution; there was no excess dithionite present. The reduction was followed spectrophotometrically by the change in absorption at 420 nm (fig. 4). A quantity of oxygen-saturated water was then added to the cuvette. The subsequent reoxidation of ferredoxin showed two phases; (1) an initial rapid phase corresponding to the reoxidation of approximately two molecules of ferredoxin per molecule of oxygen, consistent with the formation of hydrogen peroxide and (2) a slower phase corresponding to the reoxidation of a further two molecules of ferredoxin and probably due to the subsequent decomposition of the hydrogen peroxide by traces of metal ions. When this process was repeated in the presence of catalase, four molecules of ferredoxin were rapidly oxidized per molecule of oxygen. Finally in the presence of a large excess of catalase plus ethanol, to remove hydrogen peroxide without the release of oxygen [12], only two molecules of ferredoxin were reoxidized per molecule of oxygen. This shows that the initial product of the reduction of the oxygen by reduced ferredoxin is hydrogen peroxide and not water.

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