Studies on the Energy-coupling Sites of Photophosphorylation

II. TREATMENT OF CHLOROPLASTS WITH NH₂OH PLUS ETHYLENEDIAMINETETRAACETATE TO INHIBIT WATER OXIDATION WHILE MAINTAINING ENERGY-COUPLED EFFICIENCIES

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ABSTRACT

Artificial electron donors to photosystem II provide an important means for characterizing the newly discovered site of energy coupling near photosystem II. However, water oxidation must be completely abolished, without harming the phosphorylation mechanism, for these donor reactions and the associated phosphorylation to withstand rigorous quantitative analysis. In this paper we have demonstrated that treatment of chloroplasts with hydroxylamine plus EDTA at pH 7.5 in the presence of Mg²⁺ followed by washing to remove the amine is a highly reliable technique for this purpose. The decline of the Hill reaction and the coupled phosphorylation during the treatment were carefully followed. No change in the efficiency of phosphorylation (P/e₂ 1.0–1.1) was observed until the reactions became immeasurable. Photosystem I-dependent reactions, such as the transfer of electrons from diaminodurene or reduced 2,6-dichlorophenolindophenol to methylviologen, and the associated phosphorylation were totally unaffected. It is clear that the hydroxylamine treatment is highly specific, with no adverse effect on the mechanism of phosphorylation itself. Benzidine photooxidation via both photosystems II and I in hydroxylamine-treated chloroplasts (electron acceptor, methylviologen; assayed as O₂ uptake) supports phosphorylation with the same efficiency as that observed for the normal Hill reaction (P/e₂ = 1.1). An apparent P/e₂ ratio of 0.6 was computed for the photooxidation of ascorbate.

As an approach to the problem of mapping the location of this site, investigations of the quantitative relationships between electron transport and phosphorylation supported by artificial donors to photosystem II become quite important. This approach calls for a specific and complete inhibition of water oxidation. Yamashita and Butler (31, 32), using their "tris-washed" chloroplasts, and Böhme and Trebst (6), using mildly heat-treated chloroplasts, have already shown that the donor reactions mediated by photosystem II can support phosphorylation with various efficiencies (P/e₂ ratios) depending upon the electron donor used. It is clear, however, that more extensive studies are required, if one is to draw decisive conclusions as to the site of phosphorylation, paying careful attention to possible adverse effects of these treatments or of the electron donors used or both on the machinery of phosphorylation itself. For instance, we have noticed that our chloroplasts are somewhat resistant to tris treatment, and our attempts to totally abolish water oxidation without appreciably impairing the phosphorylation mechanism have not been successful. Even greater difficulties in terms of the inhibition specificity were encountered with the heat treatment.

Hydroxylamine is a potent inhibitor of water oxidation. Cheniae and Martin (8) have shown that its effect on isolated chloroplasts is specific and irreversible, involving a release of Mn from the chloroplast membranes. No inhibition of photosystem I-mediated electron transport was found. The use of hydroxylamine as an electron transport inhibitor for photophosphorylation studies have been shunned because of the ambiguous results one would expect from its possible uncoupling effect as an amine or its ability to serve as an electron donor to photosystem II (22, 29). Wessels (30) did employ NH₂OH in his very early studies on photophosphorylation. The effect of hydroxylamine-O-sulfonate on photophosphorylation has recently been studied by Elstner et al. (11). We have examined the effect of hydroxylamine on chloroplast reactions under various conditions and found a simple method of treating chloroplasts with this amine which allows total inhibition of water oxidation without any detectable damage to the mechanism of phosphorylation. This paper describes details of the method and some preliminary results of photophosphorylation experiments with the hydroxylamine-treated chloroplasts.

MATERIALS AND METHODS

Chloroplast Isolation. Chloroplasts were isolated from commercial spinach (Spinacia oleracea L.). Leaves were washed

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1 This work was supported by Grant GB 22657 from the National Science Foundation.

2 Abbreviations: P/e₂: the ratio of the number of ATP molecules formed to the number of pairs of electrons transported; DAD: diaminodurene (2,3,5,6-tetramethyl-p-phenylenediamine); DCIP: 2,6-dichlorophenolindophenol; MV: methylviologen.
with cold-distilled water and ground in a Waring Blender for 5 sec in a medium consisting of 0.3 m NaCl, 30 mm Tricine-NaOH buffer (pH 7.5), 3 mm MgCl₂, and 0.5 mm EDTA. The homogenate was filtered through eight layers of cheesecloth, and the chloroplasts were sedimented at 2500g for 2 min. The chloroplast pellet was then resuspended in a medium containing 0.2 m sucrose, 5 mm HEPES-NaOH buffer (pH 7.5), 2 mm MgCl₂, and 0.05% bovine serum albumin. After a 45-sec centrifugation at 2000g to remove cell debris, the chloroplasts were spun down again (2000g 4 min) and finally suspended in a few milliliters of the above suspending medium.

**Chemicals.** A stock solution of 0.1 m NH₂OH was made by dissolving the hydrochloride salt in 0.05 M HCl and stored at 0 C. Fresh solutions were prepared every 3 to 4 days. When necessary, the pH of the NH₂OH solution was adjusted to desired pH values immediately before use. D-Ascorbate solution (0.1 M; pH adjusted to 6.5 with NaOH) was stored at -20 C in small, tightly sealed vials. Diamino-dihydrochloride and benzidine dihydrochloride were recrystallized from charcoal-treated aqueous alcoholic and aqueous solutions, respectively, by adding excess HCl at 0 C. The completely colorless crystals thus obtained were stored at -20 C. Fresh aqueous solutions of these compounds were made daily and kept at 0 C during the experiment.

**Hydroxylamine Treatment.** NH₂OH and EDTA (when used) were added to the suspending medium described above, pH adjusted with NaOH to 7.5, and used immediately. The treatment was done in the dark at either room temperature (21 C) or at 0 C as indicated. The Chl concentration during the NH₂OH treatment was approximately 100 μg/ml. Upon the completion of the prescribed treatment period, the chloroplasts were spun down (2000g, 4 min) and washed twice with suspending medium to remove the NH₂OH. The Chl concentrations of final stock suspensions were determined by the method of Arnon (2).

**Electron Transport and Phosphorylation Assays.** The ferricyanide Hill reaction was assayed as O₂ evolution, and the MV Hill reaction as O₂ uptake resulting from aerobic reoxidation of reduced MV. Electron transport from artificial donors to MV was assayed as O₂ uptake (20). A membrane-covered Clark-type oxygen electrode was used for these O₂ assays. When artificial donors were used, the observed rate of electron transport was corrected for the slow rate of dark autoxidation of the donors which ranged from 5 to 20% of the rate in the light. In no case was it necessary to add a H₂O₂ trap to the reaction mixture, since the chloroplast preparations used were free from catalase activity. The intensity of actinic light (600-700 nm) was approximately 600 kergs cm⁻² cm⁻². The reaction temperature was 19 C. Phosphorylation was measured as the residual radioactivity after the extraction of the ³²P-labeled orthophosphate as phosphomolybic acid in butanol-toluene (3). Radioactivity was determined by Čerenkov radiation as described by Gould et al. (14).

**RESULTS**

**Effect of NH₂OH Added in the Reaction Mixture of Phosphorylation.** To test the potency of NH₂OH as an uncoupler, we have examined the effect of NH₂OH added in the reaction mixture on postillumination phosphorylation (Xₑ) (17) and on the steady state phosphorylation supported by the transfer of electrons from DAD to MV (Table 1). In the Xₑ experiments, hydroxylamine was present only during the dark phosphorylation stage. The inhibition of Xₑ thus observed has been shown to be a sensitive indicator of uncoupling (16). The concentrations of NH₂OH used in these experiments are those commonly used for inhibition of O₂ evolution. Clearly, the uncoupler action of NH₂OH is weak, as one would predict from the low basicity of this amine (pKₐ = 6) (13, 18). Weak as it is, this side effect of NH₂OH is definitely undesirable when a rather precise assessment of phosphorylation efficiency is required. As described below, the uncoupling effect of NH₂OH can be completely eliminated by washing the NH₂OH-treated chloroplasts, without relieving the desired inhibition of water oxidation.

**Pretreatment of Chloroplasts with NH₂OH and EDTA.** In all of the following experiments, chloroplasts were treated with NH₂OH under a variety of conditions and then washed twice with a large volume of amine-free suspending medium (see "Materials and Methods") at 0 to 4 C. The data are for these washed chloroplasts.

**Table I. Effect of NH₂OH on Postillumination ATP Formation (Xₑ) and Steady State Photophosphorylation (Test for Uncoupler Action of NH₂OH)**

<table>
<thead>
<tr>
<th>NH₂OH</th>
<th>Postillumination ATP Formation (Xₑ)</th>
<th>Steady State Photophosphorylation (DAD → MV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>electron transport</td>
<td></td>
</tr>
<tr>
<td></td>
<td>μmoles/100 μg Chl</td>
<td>ratio</td>
</tr>
<tr>
<td>0</td>
<td>8.3</td>
<td>2840</td>
</tr>
<tr>
<td>0.1</td>
<td>8.1</td>
<td>2660</td>
</tr>
<tr>
<td>0.2</td>
<td>6.0</td>
<td>2600</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Xₑ experiments were carried out as described before (19). NH₂OH was present only in the dark phosphorylation stage. The reaction mixture for the steady state photophosphorylation (2 ml) contained 0.1 m sucrose, 50 mm Tricine-NaOH buffer (pH 8.0), 2 mm MgCl₂, 0.75 mm ADP, 5 mm Na₃H₂PO₄, 0.5 mm DAD, 1 mm ascorbate, 20 μM MV, 1 μM DCMU, chloroplasts equivalent to 20 μg of Chl, and indicated concentrations for NH₂OH.
Plants were twice with amine-free washed. After chloroplasts on (2 ml) were carried out for the reactions. The concentrations of NH2OH were 5 mM for 0°C and 3 mM for 21°C treatment. EDTA was 1 mM when included. After the treatment, the chloroplasts were spun down, washed twice with amine-free suspending medium, and finally suspended in a few ml of the same medium. These procedures were carried out at 0 to 4°C. The basic ingredients of the reaction mixture (2 ml) were: 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM MgCl2, 0.75 mM ADP, 5 mM Na2HPO4, and chloroplasts containing 40 µg of Chl or 10 µg of Chl for the DAD → MV system. The concentrations of MV and Fc (ferricyanide) were 50 µM and 0.4 mM, respectively. The DAD → MV reaction was run in the presence of 1 mM DCMU and 2.5 mM ascorbate. The concentration of DAD was 0.5 mM. For assay conditions, see "Materials and Methods." The temperatures indicated in the figure are for pre-treatment periods.

Effect of time of hydroxylamine pretreatment of chloroplasts on the Hill reaction and photosystem I-dependent donor reactions. Chloroplasts were pretreated for the indicated periods of time with NH2OH dissolved in the suspending medium (see "Materials and Methods"). The Chl concentration at this stage was approximately 100 µg/ml. The concentrations of NH2OH were 5 mM for 0°C and 3 mM for 21°C treatment. EDTA was 1 mM when included. After the treatment, the chloroplasts were spun down, washed twice with amine-free suspending medium, and finally suspended in a few ml of the same medium. These procedures were carried out at 0 to 4°C. The basic ingredients of the reaction mixture (2 ml) were: 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM MgCl2, 0.75 mM ADP, 5 mM Na2HPO4, and chloroplasts containing 40 µg of Chl or 10 µg of Chl for the DAD → MV system. The concentrations of MV and Fc (ferricyanide) were 50 µM and 0.4 mM, respectively. The DAD → MV reaction was run in the presence of 1 mM DCMU and 2.5 mM ascorbate. The concentration of DAD was 0.5 mM. For assay conditions, see "Materials and Methods." The temperatures indicated in the figure are for pre-treatment periods.

**Fig. 1.** A: Effect of time of hydroxylamine pretreatment of chloroplasts on the Hill reaction and photosystem I-dependent donor reactions. Chloroplasts were pretreated for the indicated periods of time with NH2OH dissolved in the suspending medium (see "Materials and Methods"). The Chl concentration at this stage was approximately 100 µg/ml. The concentrations of NH2OH were 5 mM for 0°C and 3 mM for 21°C treatment. EDTA was 1 mM when included. After the treatment, the chloroplasts were spun down, washed twice with amine-free suspending medium, and finally suspended in a few ml of the same medium. These procedures were carried out at 0 to 4°C. The basic ingredients of the reaction mixture (2 ml) were: 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM MgCl2, 0.75 mM ADP, 5 mM Na2HPO4, and chloroplasts containing 40 µg of Chl or 10 µg of Chl for the DAD → MV system. The concentrations of MV and Fc (ferricyanide) were 50 µM and 0.4 mM, respectively. The DAD → MV reaction was run in the presence of 1 mM DCMU and 2.5 mM ascorbate. The concentration of DAD was 0.5 mM. For assay conditions, see "Materials and Methods." The temperatures indicated in the figure are for pre-treatment periods. B: Effect of varied concentrations of hydroxylamine (in pretreatment medium) on the Hill reaction and photosystem I-mediated donor reactions. The treatment time was 9 min in both 0 and 21°C treatment. For other conditions, see A.

**Fig. 2.** Effect of time of pretreatment of chloroplasts (at 21°C) with hydroxylamine plus EDTA on the Hill reaction, photosystem I-mediated donor reactions, and associated phosphorylation. The reaction mixture for the DCIPH2 → MV reaction contained 0.4 mM DCIP, 2.5 mM ascorbate, 50 mM MV, 1 mM DCMU. For other conditions see Fig. 1A.
Thus it seems that the apparent phosphorylation efficiency of ascorbate photooxidation via Photosystems II and I is close to half of the efficiency when water is photooxidized (see "Discussion"). Also worthy of note here is the fact that with these

\[ \text{NH}_2\text{OH}-\text{treated chloroplasts the ascorbate oxidation contains practically no DCMU-resistant component (Fig. 3). A significant rate of DCMU-insensitive ascorbate photooxidation was observed for tris-washed chloroplasts (32), suggesting that ascorbate could be an electron donor for photosystem I, depending on the integrity of the chloroplast membranes (24).}

Figure 4 shows that benzidine, unlike ascorbate, supports phosphorylation with a P/e ratio of 1.1 which is almost the same as the value for normal noncyclic photophosphorylation involving water oxidation (P/e = 1.0 to 1.2). In this experiment a low level of ascorbate (0.2 mM) was present in the reaction mixture to eliminate the possibility of a cyclic reaction with oxidized benzidine. Nearly identical data (not shown) were obtained without ascorbate, indicating that no significant cyclic electron flow occurred during the short illumination period employed (1–2 min).

Table III. Effect of Ascorbate on Postillumination Phosphorylation (X{sub \text{e}})

<table>
<thead>
<tr>
<th>Addition at Dark Stage</th>
<th>ATP formed (nmoles/100 mg Chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.8</td>
</tr>
<tr>
<td>Ascorbate (3)</td>
<td>8.0</td>
</tr>
<tr>
<td>Ascorbate (30)</td>
<td>7.5</td>
</tr>
<tr>
<td>Methylamine (5)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Thus, ascorbate (3 or 30 mM) but not methylamine (5 mM) supported the phosphorylation when mixed with benzidine and added to the dark reaction mixture. This result also confirms the formation of a noncyclic DHase as measured by benzidine oxidation. Addition of ascorbate (3 or 30 mM) to the assay medium but not methylamine (5 mM) increased the benzidine oxidation indicating the formation of a noncyclic DHase as measured by benzidine oxidation. Addition of ascorbate (3 or 30 mM) to the assay medium but not methylamine (5 mM) increased the benzidine oxidation indicating the formation of a noncyclic DHase as measured by benzidine oxidation.
DISCUSSION

Currently, the most widely employed procedures for inhibiting O₂ production are the tris treatment of Yamashita and Butler (31, 32) and the mild heating of chloroplasts (to 50 C for several minutes) (6, 24). However, both of these methods are not quite satisfactory in dealing with critical experiments on photophosphorylation, as briefly mentioned earlier in this paper. Well coupled chloroplasts are rather resistant to Cl⁻ removal treatment (7) which is also known to inhibit water oxidation (22). Repeated washings with Cl⁻ free media combined with room-temperature treatment did severely suppress the Hill reaction, but their secondary effects were quite appreciable.

In this paper, we have presented a highly effective procedure for abolishing the water-splitting reaction in isolated chloroplasts which can be extremely useful for photophosphorylation studies utilizing artificial electron donors for photosystem II. This procedure involves the treatment of chloroplast with NH₄OH plus EDTA in the presence of Mg²⁺. No signs of uncoupling or inhibition of energy coupling were observed after treated chloroplasts were washed with NH₄OH-free medium. Actually, we found NH₄OH to be a rather poor uncoupler of photophosphorylation, and therefore it is possible that some uncritical phosphorylation experiments may be carried out in the presence of a few millimolar NH₄OH which is sufficient to suppress water oxidation.

The effectiveness of EDTA in facilitating the complete inhibition of water oxidation by NH₄OH suggests an interesting possibility concerning the mechanism of extraction of Mn from the lamellar membranes by NH₄OH. Since EDTA per se has no effect on water oxidation nor is able to release Mn from the membrane (9) (in the absence of Mg²⁺, EDTA-uncoupling [23] gives very high rates of the Hill reaction), the observed effect of EDTA on water oxidation is probably indirect. It seems possible that a portion of the Mn extraction by NH₄OH is reversible, and the binding of released Mn²⁺ by EDTA does not allow the reversal to occur.

The apparent P/eₐ ratio of 0.6 found for theascorbate photophosphorylation in NH₄OH-EDTA-treated chloroplasts is similar to the value of 0.5 Böhm and Trebst (6) found for heat-treated chloroplasts. They interpreted the data to suggest that the donation of electrons by ascorbate may have occurred after one of two sites of phosphorylation, the site which they suggest to be associated with the water-oxidation step. Although this is certainly the simplest and the most attractive interpretation, the validity of the widely used method for computing the electron flux in this donor reaction based on O₂ uptake data may be in error (12). A more comprehensive assessment of this complication is presented in a subsequent publication. In this respect the data for benzidine photophosphorylation may be of more importance. The constant P/eₐ of 1.0 to 1.1 observed over a wide range of benzidine concentrations confirms and greatly strengthens the brief data of Yamashita and Butler (31). These authors, using tris-washed chloroplasts, found a P/eₐ ratio of 0.97 with benzidine (33 μM) as electron donor and NADPH as acceptor. These P/eₐ ratios are indeed very close to that of the normal Hill reaction. It seems unlikely, therefore, that there is a phosphorylation site specifically associated with the mechanism of water oxidation. However, the possibility still remains that an energy conservation reaction is linked to some step of the oxidoreduction reactions on the water-oxidizing side of photosystem II, a step which is involved both in water oxidation and the oxidation of artificial reductants. Research is now in progress testing various donor reactions in NH₄OH-treated chloroplasts in an attempt to locate more precisely the photosystem II-associated site of phosphorylation.

Note. After submission of this manuscript, a paper by J. F. Allen and D. O. Hall (Biochem. Biophys. Res. Commun. 52: 856–862) has appeared in which the authors demonstrated that the aerobic photooxidation of ascorbate by (untreated) chloroplasts involves a nonbiological oxidation of ascorbate by superoxide radicals. Therefore, it is almost certain that the assumption 2e⁻ = O₂ used for computing electron flux and P/eₐ in Figure 3 is incorrect.

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LITERATURE CITED

24. Katoh, S. and A. San Pietro. 1967. Ascorbate supported NADP photo-


