Postillumination Adenosine Triphosphate Synthesis in 
Rhodospirillum rubrum Chromatophores

I. CONDITIONS FOR MAXIMAL YIELDS

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MARGARITA LEISER AND ZIPPORA GROMET-ELHANAN
From the Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel

SUMMARY

The very low level of postillumination ATP synthesis in chromatophores was markedly stimulated when permeant anions (thiocyanate or perchlorate) or permeant cations (potassium in the presence of valinomycin) were added to the light stage. Although these compounds stimulated also light-induced proton uptake in chromatophores the pH dependence of both photoreactions was different. Proton uptake peaked at pH 6.5 while the amount of postillumination ATP was maximal when the light stage was carried out around pH 7.7. The increased yield of ATP at the more alkaline pH could not be explained by a slower decay of the high energy state at this pH, since the decay rate was faster at pH 7.7 than at pH 6.5.

The proton concentration gradient which is maintained across the chromatophore membrane in the light was also found to increase when the external pH was raised from 6.0 to 8.0. Only a minimal amount of postillumination ATP was formed when this gradient was below 2.1 pH units, but above this value the ATP yield rose steeply as a function of the increasing pH gradient. In light of these results it is suggested that in order to obtain a high yield of postillumination ATP synthesis in chromatophores two conditions are required: the particles have to be loaded with a sufficient number of protons and a light-induced pH gradient above a certain threshold value has to be maintained across their membrane. The low yield of postillumination ATP in chromatophores and the increase obtained by adding permeating ions, is thus explained by similar variations in the extent of the pH gradient, which exceeded the threshold value only in the presence of the permeating ions.

Illumination of chloroplasts in the absence of ADP and Pi resulted in accumulation of a high energy state or intermediate (Xe) which could be utilized for ATP synthesis in a subsequent dark reaction (1). The light stage of this two-stage or postillumination ATP synthesis in chloroplasts was found to have the same pH optimum as the light-induced H+ uptake (2-4). Also, addition of compounds which can serve as internal buffers markedly increased the extent of H+ uptake as well as the amount of postillumination ATP formation in chloroplasts (5, 6). It has, therefore, been proposed that the energy storage in the light stage of two-stage ATP synthesis resides in the protons concentrated inside the chloroplasts. This view was substantiated by the finding that ATP formation could be driven in total darkness by an artificially produced pH gradient (7).

Although light-induced H+ uptake was observed also in chromatophores (8) tests for postillumination ATP synthesis in these particles gave negative results (9, 10). Another marked difference between chloroplasts and chromatophores was recorded in the effect of various inhibitors on H+ uptake and photophosphorylation. Thus, addition of nigericin + KCl or NH₄Cl resulted in inhibition of H+ uptake but not of photophosphorylation. Therefore, light-induced H+ uptake results in chromatophores in the formation of an electrochemical proton gradient (15) which is composed of a much larger membrane potential component than in chloroplasts and a smaller pH gradient.

According to Mitchell (16) the membrane potential should be neutralized in the presence of permeant anions. Addition of SCN⁻ and ClO₄⁻, which were found to be permeant anions in Rhodospirillum rubrum chromatophores (17), has indeed led to a stimulation of light-induced H+ uptake (18). It was therefore of interest to see if this larger amount of proton storage might be used in enabling chromatophores to carry out postillumination ATP synthesis.

In this communication we demonstrate that postillumination ATP synthesis occurs in chromatophores in the presence of permeant anions as well as in the presence of valinomycin + KCl (19) which were also shown to stimulate proton uptake in these particles (13, 20). The amount of ATP synthesized in this system was similar to that reported in chloroplasts, but the optimal conditions required were found to be different, including a marked difference in the pH optimum of the light stage. These results indicate that the number of protons concentrated inside the chromatophores cannot be the only factor responsible for postillumination ATP synthesis in these particles.
TABLE I
Effect of various additions to light stage on postillumination ATP synthesis

The reaction mixture in the light stage contained in a total volume of 1.5 ml: 1.32 mM Tricine-\(\text{NaOH}, \text{pH} 7.7; 5.3 \text{ mM} \text{MgCl}_2;\)
\(66 \mu\text{M}\) phenazine methosulfate; \(330 \mu\text{M}\) succinate; \(0.5 \text{ mM} \text{KCN};\)
and \(150 \mu\text{g}\) of bacteriochlorophyll. The reaction mixture was illuminated at \(25^\circ\) for 45 s before injection into the dark reaction mixture which contained in a total volume of 0.75 ml: 120 mM Tricine-\(\text{NaOH}, \text{pH} 8.0; 1.06 \text{ mM ADP}; 2.13 \text{ mM sodium phosphate (containing about} 2 \times 10^8 \text{ cpm of}^{32}\text{P}); 20 \text{ mM glucose};\) and 10 units of hexokinase. The dark reaction was stopped after 30 s by adding 0.25 ml of 30% perchloric acid. Appropriate dark controls have been subtracted from all values reported (see text).

<table>
<thead>
<tr>
<th>Additions to light stage</th>
<th>ATP formed (nmol/mg bacteriochlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.3</td>
</tr>
<tr>
<td>NaSCN, 5 mM</td>
<td>9.0</td>
</tr>
<tr>
<td>NaSCN, 20 mM</td>
<td>11.6</td>
</tr>
<tr>
<td>NaClO(_4), 5 mM</td>
<td>10.0</td>
</tr>
<tr>
<td>NaClO(_4), 20 mM</td>
<td>14.0</td>
</tr>
<tr>
<td>NH(_4)Cl, 20 mM</td>
<td>1.2</td>
</tr>
<tr>
<td>KCl, 50 mM</td>
<td>2.6</td>
</tr>
<tr>
<td>KCl + valinomycin, 1 (\mu\text{M})</td>
<td>42.5</td>
</tr>
</tbody>
</table>

EXPERIMENTAL PROCEDURE

The growth of \(R.\ rubrum\) cells and the isolation and storage of chromatophores were as previously described (21). Bacteriochlorophyll was determined using the absorbance coefficient measured as outlined by Gromet-Elhanan (25).

ATP synthesis was measured according to Avron (23) and postillumination (Xe) experiments were performed as in Ref. 24, except for the pH and temperature, which were varied as specified. All of the experiments were run in duplicate which agreed within ±10%. Proton uptake was assayed with a Metrohm combined microglass electrode (type x) and a Radiometer PHM 22 pH meter and the signals were recorded on a Photovolt model 40 recorder. The samples were illuminated by a 500-watt slide projector (with heat filter removed) through 9 cm of water and a combination of a Schott RG 715 filter and a C.S.3-09 Corning filter.

Light-induced changes in the fluorescence of atebrin were measured as outlined by Gromet-Elhanan (23).

Valinomycin was purchased from Calbiochem, hexokinase (from yeast, type VI) from Sigma, and \(p\)-phenylenedianime dihydrochloride from B.D.H.

RESULTS

Confirming earlier observations (9, 10) very small amounts of postillumination ATP synthesis were observed in chromatophores when the light stage contained only an electron carrier and MgCl\(_2\) (Table I). A 5- to 6-fold increase was observed when permeant anions, e.g. SCN\(^-\) or ClO\(_4^-\), were added to the light stage and a 20-fold increase was obtained with valinomycin + KCl. KCl alone had no effect and NH\(_4\)Cl, which under continuous illumination inhibited \(H^+\) uptake but not ATP synthesis (12), decreased the very low amount of ATP synthesized (Table I).

In order to determine the optimal conditions for this postillumination ATP synthesis in chromatophores several complicating reactions, which have been observed in chromatophores but not in chloroplasts, had to be eliminated. Thus, an active dark ATP\(_\text{ase}\) has been reported in chromatophores (26), and the kinetics of synthesis of ATP in the dark stage showed a pronounced decrease after 15 s (Fig. 1) which could be due to this activity. Addition of glucose and hexokinase eliminated this decrease and they were therefore routinely added to the dark reaction mixture.

Another complicating reaction is a dark ADP-P\(_1\) exchange catalyzed by polynucleotide phosphorylase which has recently been reported in chromatophores (26). By the method employed here to measure ATP formation (23) [\(^{32}\text{P}\)ADP] formed via this exchange reaction in the presence of \(^{32}\text{P}\) would be recorded as ATP. This could account for the rather high amounts of 3 to 5 nmol of \(^{32}\text{P}\) esterified per mg of bacteriochlorophyll which we have obtained in nonilluminated controls as compared to less than 1 nmol per mg of chlorophyll observed in chloroplasts (24). To eliminate this complication all samples were heated in 1 \(n\) H\(_2\)SO\(_4\) at 100\(^\circ\) for 10 min (27).

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FIG. 1. Kinetics of synthesis of ATP in the dark stage. Reaction mixtures were as in Table I in the presence of valinomycin and KCl, except that glucose and hexokinase were added only where indicated. Bchl, bacteriochlorophyll.

TABLE II
Effect of acid treatment on the amount of \[^{32}\text{P}\]esterified in two-stage phosphorylation

Reaction mixtures were as in Table I. Where indicated 1 \(\mu\text{M}\) valinomycin and 50 \(\mu\text{M}\) KCl were added to the light reaction mixture. In the columns designated "Dark" both stages were carried out in complete darkness. The numbers in the columns designated "Light" were obtained in experiment carried out as described in Table I.

<table>
<thead>
<tr>
<th>Treatment of samples</th>
<th>[^{32}\text{P}] esterified with system tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dark Light Light-dark Dark Light Light-dark</td>
</tr>
<tr>
<td>None</td>
<td>3.9 6.7 2.8 2.5 42.3 39.8</td>
</tr>
<tr>
<td>1 (n) H(_2)SO(_4) at 100(^\circ) for 10 min</td>
<td>1.2 3.7 2.5 1.4 37.7 36.8</td>
</tr>
</tbody>
</table>

The abbreviation used is: Tricine, \(N\)-tris(hydroxymethyl)methylglycine.
TABLE III

Effect of addition of ADP to dark stage on amount of ATP esterified in two-stage phosphorylation

Table III presents the results of experiments conducted in the presence of valinomycin and KCl, except that ADP was added only where indicated. The data show that ADP addition to the dark stage of phosphorylation caused a significant increase in the amount of ATP formed. The addition of ADP to the dark stage increased the yield of ATP from 43 to 29 nmol per mg of bacteriochlorophyll ( BCHl). The pH optimum for ATP formation in this system was 5.4, which was also the optimum pH for H+ uptake, while in chromatophores it was around pH 8.0 although the pH optimum for H+ uptake was 6.3 as in chloroplasts.

Additions to dark stage | 113.7 | 112.2 | 112.2 | 3.5
---|---|---|---|---
ADP, μM | 1.5 | 0.9 | 1.5 | 0.9

H+ uptake, while in chromatophores it was around pH 8.0 although the pH optimum for H+ uptake was 6.3 as in chloroplasts.
FIG. 3. Kinetics of decay of Xe as a function of the pH in the presence of valinomycin and KCl. The reaction mixtures were as in Fig. 2 in the presence of 1 μM valinomycin and 50 mM KCl. After 45-s illumination the light was turned off for the indicated time interval before injection into the dark reaction mixture. Bchl, bacteriochlorophyll.

FIG. 4. Kinetics of decay of Xe as a function of the pH in the presence of SCN−. Reaction mixtures and conditions were as described in Fig. 3, except that 20 mM NaSCN were added to the light stage.

Increasing with pH and must be large enough at pH 7.7 to counteract the unfavorable decay rate. It has recently been reported (29) that the yield of postillumination ATP formed in chloroplasts was very low when the pH gradient maintained across their membrane was below a certain threshold value. Above this threshold value the ATP yield increased as a function of the increasing pH gradient (ΔpH). Fig. 5 illustrates that the pH gradient across the chromatophore membranes rose as the external pH was varied from 6.0 to 8.0. The pH gradient was measured in this case by the quenching of atebrin fluorescence (30). A similar increase of the ΔpH with external pH was recently observed also by following the quenching of 9-aminacridine. This increase was much steeper in the presence of SCN− so that in this case the ΔpH obtained at pH 7.0 was already higher than that observed in the control at pH 8.0 (Fig. 5). The relationship between the amount of postillumination ATP formed in chromatophores and the light-induced ΔpH is illustrated in Fig. 6. As long as the ΔpH was below 2.1 a very low yield of ATP was obtained, but above this value the ATP yield rose sharply with the increasing ΔpH. It is therefore suggested that in chromatophores as well as in chloroplasts a highly active postillumination ATP synthase can be demonstrated only when the pH gradient rises above a threshold value. But while in chloroplasts, a ΔpH above the threshold value is already obtained when the light stage is carried out at an external pH of 6.5 (29), the required threshold ΔpH in control chromatophores cannot be

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Fig. 5. Dependence of ΔpH on the external pH. The ΔpH was measured by following the light-induced quenching in the fluorescence of atebrin (25). The reaction mixture contained in a total volume of 3 ml: 3.3 mM MgCl₂, 33 μM phenazine methosulfate, 160 μM succinate, and 3.5 μg of bacteriochlorophyll. The pH was fixed with 30 mM of either Tricine-NaOH or Tricine-maleate. Where indicated 20 mM NaSCN were present. The ΔpH was calculated as the difference between the chromatophore internal pH and the external pH (30). The internal volume used in these calculations, 60 μl of H₂O per mg of bacteriochlorophyll, was determined by Schuldiner et al.4

Fig. 6. Postillumination ATP synthesis as a function of the light-induced ΔpH. The reaction mixtures were as described in Fig. 2 and the pH of the light stage was varied between 6.0 and 8.0. ΔpH values were taken from Fig. 5. Each point in the curve relates the ATP yield obtained at a specific pH in the light to the ΔpH recorded at the same external pH in the light in the absence (○) or presence (○) of 20 mM NaSCN.

reached even at pH 8.0 and is surpassed in the presence of SCN− only when the pH of the light stage is above 7.0 (Fig. 5).

**DISCUSSION**

It has been reported by McCarty (19) that addition of valinomycin + KCl in the light stimulates both H+ uptake and postillumination ATP synthesis in subchloroplast particles. In the presence of these compounds, some postillumination ATP synthesis was observed also in chromatophores but the yield was much lower than in chloroplasts (19). The data summarized above demonstrates that such ATP synthesis does occur in chromatophores when either permeant anions or valinomycin + KCl are present in the light. Furthermore, under optimal conditions ΔpH yields approaching those observed in chloroplasts could be obtained in chromatophores, but these conditions required that the light stage should be conducted at a pH which was not optimal for H+ uptake. Thus, in the presence of SCN−, when the pH in the light was raised from 6.5 to 7.5 or even 8.0 the yield of ATP markedly increased (Fig. 2 and Table IV) while the extent of H+ uptake rather decreased (Fig. 2). Moreover, although the number of protons concentrated inside the chromatophores in the light stage in the presence of SCN− at pH 7.5 and in the control at pH 6.0 were identical, the amounts of postillumination ATP formed in the subsequent dark stage were quite different.

These findings indicate that the active synthesis of ATP in the presence of SCN− above pH 7.0 could not be due simply to the presence of a sufficient number of protons inside the chromatophores, because an accumulation of the same number of protons in the control system at pH 6.0 or an even greater number with SCN− at pH 6.5 did not result in an active synthesis of ATP. The additional factor which is required for postillumination ATP synthesis, and was not formed with SCN− below 7.0 or in the control system even at pH 8.0 in the light, seems to be a pH gradient larger than 2.1 pH units. The enhancement of postillumination ATP synthesis by SCN− seems therefore to be due to its ability to stimulate H+ uptake at pH values above the optimal one, where the internal buffer capacity is already below its maximum (31). Under these conditions the same amount of protons concentrated inside the chromatophores will maintain a larger pH gradient across their membrane than under optimal conditions for H+ uptake when the buffer capacity is maximal.

The dependence of postillumination ATP synthesis on a ΔpH above a certain threshold value as well as on a sufficient concentration of protons has been demonstrated by Schuldiner et al. also in chloroplasts (20). In chromatophores the ΔpH was calculated as the difference between their internal pH and the extranl pH in the light stage (Fig. 5). Schuldiner et al. included in their ΔpH calculations also the difference in pH between the light and dark stages (see Fig. 2 in Reference 20), but in chromatophores this pH difference did not seem to contribute to the ATP yield. For instance, at pH 6.0 in the light the large difference between this and the pH of the dark stage failed to improve the low yields of ATP, which seem to follow closely the ΔpH formed in the light stage only (Fig. 6). Indeed in chloroplasts a pH difference imposed in total darkness was found to initiate the so called acid-base phosphorylation (7), while in chromatophores a similar acid-base transition was inactive in driving ATP synthesis unless an additional source of energy was added (32).

Although the amount of postillumination ATP formed at pH 8.0 in the light stage was much larger in the presence of 20 mM NaSCN than in its absence (Table I and Fig. 2), no such difference was observed under continuous illumination (17, 18). In the presence of NaSCN the pH gradient was found to increase at the expense of the membrane potential (18), while the over-all proton motive force remained practically unchanged. It was therefore suggested that under continuous illumination either the membrane potential or the pH gradient can drive ATP synthesis. In the postillumination set up, on the other hand, the membrane potential developed by H+ uptake seems to be much less effective than the pH gradient, since a large yield of ATP was obtained only when the pH gradient was increased at the expense of the membrane potential. The inactivity of this H+ driven membrane potential may be due to its fast decay (19) so that it is largely dissipated by the time the chromatophores come in contact with the phosphorylation reagents in the dark stage.

Unlike the membrane potential formed solely by H+ uptake, a K+ diffusion potential formed in the dark has been proposed to stimulate postillumination ATP synthesis (19). It has been recently reported that addition of valinomycin + KCl to the dark stage, resulted in an increase in the amount of postillumination ATP formed under suboptimal conditions in chloroplasts (29, 33), as well as in chromatophores (34). The large stimulation of postillumination ATP synthesis in chromatophores when valinomycin + KCl were added to the light stage (Table I and Fig. 2) seems therefore to be due to a dual effect: stimulation of H+ in the light and formation of a K+ diffusion potential in the dark. This double effect is, however, complicated since, as illustrated in the accompanying paper (34) the effect of the K+ diffusion potential has a different pH dependence than that of the light induced pH gradient.

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