Energy-linked Reactions in Photosynthetic Bacteria

IX. P\textsubscript{i}-PP\textsubscript{i} EXCHANGE IN RHODOSPIRILLUM RUBRUM*

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SUMMARY

Chromatophores of Rhodospirillum rubrum catalyze a P\textsubscript{i}-PP\textsubscript{i} exchange reaction in the dark. This reaction was inhibited by uncouplers of phosphorylation and ADP, and was stimulated by oligomycin. An energized state is therefore participating in this reaction. Methylene diphosphonate and fluoride which are inhibitors of the membrane-bound inorganic pyrophosphatase also inhibited and thus the reaction appears to be catalyzed by the pyrophosphatase. The K\textsubscript{m} for P\textsubscript{i} was high (48 mM).

For comparison, the PP\textsubscript{i}-P\textsubscript{i} exchange reaction catalyzed by yeast inorganic pyrophosphatase was studied. Very high P\textsubscript{i} and Mg\textsuperscript{2+} concentrations were required and, as expected, uncouplers had no effect on the reaction with the yeast enzyme. Under similar conditions the ratio of the exchange reaction to the hydrolytic reaction was considerably greater in chromatophores than with the yeast enzyme and this probably reflects the contribution of the energized state.

The mechanism of the exchange in chromatophores is postulated to be due to the dynamic reversal of the energy-linked hydrolytic reaction

\[
\text{inorganic PP}_i \xrightleftharpoons{\text{pyrophosphatase}} \text{2P}_i + \sim \chi
\]

where \(\sim \chi\) represents the energized state in general.

Rhodospirillum rubrum is a unique organism that can couple the synthesis of inorganic pyrophosphate to light-induced electron transport (1). The importance of PP\textsubscript{i} in energy conservation is emphasized by the fact that PP\textsubscript{i} can serve as an energy donor for energy-linked transhydrogenation (2, 3), cytochrome reduction (4, 5), succinate-linked NAD\textsuperscript{+} reduction (6), proton uptake (7), and an energy-linked shift in the membrane-bound carotenoid spectrum (8).

We have also observed that PP\textsubscript{i} can drive ATP synthesis (9, 10) and during these studies, a PP\textsubscript{i}-P\textsubscript{i} exchange reaction associated with chromatophores was noted. This exchange reaction, occurring at a very low rate, previously had been observed by Horio et al. (11). Some years ago, Cohn (12) reported that soluble yeast inorganic pyrophosphatase also catalyzed a slow PP\textsubscript{i}-P\textsubscript{i} exchange. Since enzyme-induced exchanges usually have been found to reflect the basic catalytic mechanism of a reaction and often result from an actual step in the over-all mechanism (13), we have studied this reaction in chromatophores and reexamined the reaction with the yeast enzyme. We found that high phosphate concentration was required for both enzymes and that high magnesium was required for the yeast enzyme. The chromatophore reaction was inhibited by uncouplers of phosphorylation but as was expected, these compounds had no effect on the soluble yeast enzyme. Under similar conditions, the ratio of the exchange reaction to the hydrolytic reaction was considerably greater in chromatophores than with the yeast enzyme and this probably reflects the participation of the energized state in the chromatophore-catalyzed reaction. These observations are consistent with the idea that the exchange results from a dynamic reversal of the hydrolytic reaction.

EXPERIMENTAL PROCEDURES

Materials— Cultures of R. rubrum, S-1, were grown and chromatophores were prepared as previously described (9). Chemicals were obtained primarily from Sigma Chemical Co. and isotopes (\textsuperscript{32}P\textsubscript{i} and \textsuperscript{32}PP\textsubscript{i}) were obtained from New England Nuclear. Yeast inorganic pyrophosphatase (EC 3.6.1.1) was from Worthington Biochemicals. We are indebted to Dr. P. C. Hamm of the Monsanto Co. for gifts of m-Cl-CCP\textsuperscript{3} and S-13.

Methods—The reaction mixture for the chromatophore exchange reaction, unless otherwise noted contained 50 mM Tris-Cl (pH 8.0), 2 mM MgCl\textsubscript{2}, 10 mM \textsuperscript{32}P\textsubscript{i}, 0.67 mM PP\textsubscript{i}, and usually about 23 \mu g per ml bacteriochlorophyll as chromatophores. The reaction was carried out at 25\textdegree and terminated by the addition of trichloroacetic acid to 50%.

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requirement for MgPPi as substrate.

phore ATP-linked reactions and this may be a reflection of the
concentration is significantly higher than is required for most chromato-
gram identification of the product of the reaction.

The optimal Mg2+ concentration for optimal activity at PPi
was not a nucleotide. (b) The product was hydrolyzed by
the enzyme. The rate of the reaction was 12.4 μmoles
per mg of bacteriochlorophyll per hour and 11.5 μmoles of PPi
were hydrolyzed for each μmole of MgPPi, formed in this experiment.
This rate was about one-half that found for PPi-driven
ATP synthesis (10) but as will be shown later, the exchange
reaction requires a higher F1 concentration for optimal activity
and the rate observed is less than the maximum rate.

Identification of MgPPi.—The loss of the Mg2+-labeled product
upon exhaustion of the added PPi made it probable that the product
was indeed MgPPi. However, since there are several possible labeled products including several bound nucleotides,
we identified the product by its characteristics in several ways.
(a) The product was not absorbed on charcoal and consequently
was not a nucleotide. (b) The product was hydrolyzed by
yeast inorganic pyrophosphatase which in the presence of Mg2+
is absolutely specific for PPr. (c) The product was labile during
the PPi-Pi exchange reaction and the hydrolytic reaction using
Fig. 1. Time course of the PPi-Pi exchange. The reaction
mixture was as described under "Experimental Procedures."
Bacteriochlorophyll was 21 μg per ml. Curves 1, 0.33 mM PPi;
Curves 2, 0.67 mM PPi. □ and ○, MgPPi; △ and □, PPi hydro-
yzed.

RESULTS

Time Course of PPi-Pi Exchange.—In Fig. 1, the time course of the
PPi-Pi exchange activity and the hydrolytic reaction using
two levels of PPi is compared. Note that the MgPPi formed be-
gan to decline before the hydrolysis of the added PPi was com-
pleted, as would be expected if the labeled product was hydro-
yzed by the enzyme. The rate of the reaction was 12.4 μmoles
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is absolutely specific for PPr. (c) The product was labile during
treatment at 100°C in 1 N HCl for 7 min. (d) Fig. 2 illustrates a
thin layer chromatogram identifying the product of the reaction
as MgPPi.

Requirements of Reaction.—The probable substrate for the
soluble inorganic pyrophosphate of R. rubrum has been identified
as the MgPPi complex by Klemme and Gest (14) and in this
respect resembles the enzymes from mammalian cells (15) and
Escherichia coli (16). In addition Klemme and Gest found
that the bacterial enzyme was activated by free Mg2+. Free
Mg2+ appears to be required for the PPi-Pi exchange reaction
in that 2 to 3 mM Mg2+ was required for optimal activity at PPi
concentrations of less than 1.5 mM. The PPi concentration had
little effect on the Mg2+ requirement. No reaction was observed
with Mn2+ and Co2+ which support partial activity (24%) of the
inorganic pyrophosphatase.† The optimal Mg2+ concentration
is significantly higher than is required for most chromatophore ATP-linked reactions and this may be a reflection of the
requirement for MgPPi as substrate.

The pH optimum of the exchange reaction was 8.1 with 59% activity at pH 7.0 and 66% at pH 9.0. This is very similar to
the PPi-driven ATP synthesis reaction catalyzed by these chromatophores (10).

The exchange reaction has a high requirement for Pi, for optimal
activity which cannot be satisfied with our assay conditions
due to the limitations of separating small amounts of MgPPi
from large amounts of MgPi. We earlier (10) modified theMartin
and Doty (17) procedure to be able to use higher concentrations
of MgPi but even so it is difficult to handle concentrations greater
than 20 to 30 mM. The inset in Fig. 3 illustrates the Pi require-
ment and Fig. 3 is a Lineweaver-Burk presentation of the data.

EFFECTS OF INHIBITORS.—The energy-linked characteristics of the
exchange reaction are revealed in Table I by the effect of un-
couplers on the reaction. The uncouplers m-CCP, S-13, and
quinacrine inhibited the exchange reaction at concentrations
which were effective in inhibiting phosphorylation whereas the
electron transport inhibitor, antimycin a, had no inhibitory
effect. The energy-transfer inhibitor of ATP-linked reactions,
The reaction was as described under "Experimental Procedures" except it included 3 μg per ml of oligomycin and 29 μg of bacteriochlorophyll per ml. The reaction time was 4 min. The white light intensity, 2.5 ergs cm⁻² s⁻¹, was measured with a Kettering radiometer model 68. The [³²P] incorporated in Reaction 3 is due to light-induced [³²P]PPᵢ synthesis, not exchange activity.

<table>
<thead>
<tr>
<th>Reaction number</th>
<th>Condition</th>
<th>[³²P] incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dark</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Dark + PPᵢ</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Light</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>Light + PPᵢ</td>
<td>123</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Total [³²P] uptake</th>
<th>After hydrolysis [³²P] uptake</th>
<th>Inhibition by ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPᵢ</td>
<td>130</td>
<td>77</td>
<td>53</td>
</tr>
<tr>
<td>PPᵢ, oligomycin, 4 μg/ml</td>
<td>80</td>
<td>2</td>
<td>78</td>
</tr>
<tr>
<td>ADP, 0.33 mM</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>ADP, oligomycin</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PPᵢ, ADP</td>
<td>130</td>
<td>77</td>
<td>53</td>
</tr>
<tr>
<td>PPᵢ, ADP, oligomycin</td>
<td>96</td>
<td>12</td>
<td>84</td>
</tr>
</tbody>
</table>

The reaction mixture contained 25 mM Tris (pH 8.0), 2.33 mM MgCl₂, 10 mM PPᵢ, 1 mM PPᵢ, 10 mM glucose, 1 unit of hexokinase, and 21 μg per ml of bacteriochlorophyll. The reaction time was 6 min. One aliquot of the reaction mixture was hydrolyzed for 20 min at 100° in 1 N H₂SO₄ before assay for esterified [³²P].

**Effect of Light on PPᵢ-Pᵢ Exchange**

Light-induced electron transport has been observed to markedly stimulate ATP-PPᵢ exchange activity in R. rubrum chromatophores (10, 18). In contrast, light did not (Table II) stimulate the PPᵢ-Pᵢ exchange reaction. Sixty nanomoles of [³²P]PPᵢ were incorporated due to the PPᵢ-Pᵢ exchange reaction (Reaction 2) while light-induced PPᵢ synthesis incorporated 94 nanomoles (Reaction 3). Thus, for light to have any stimulating effect, Reaction 4 should be greater than the sum of these reactions. Since it is less than the sum, this means that either (a) light inhibited the exchange reaction by 44%, or that (b) PPᵢ inhibited the light-induced synthesis of PPᵢ by 27%, or that (c) there is a common rate-limiting step in both pathways of [³²P]PPᵢ formation. It is apparent that the

**Effect of ADP on PPᵢ-Pᵢ Exchange**

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**FIG. 3. Effect of Pᵢ.** The reaction mixture contained 20 mM Tris (pH 8.0), 1 mM PPᵢ, 2.33 mM MgCl₂, and 24 μg per ml of bacteriochlorophyll. Pᵢ concentrations are given in the inset. Reaction time was 6 min. Since Pᵢ is a product of the hydrolytic reaction, the Pᵢ concentration was continually changing during the experiment. The Pᵢ plotted is the average amount present during the time course of the reaction. The units of velocity (v) are micromoles of [³²P] uptake/3 ml of reaction mixture. Thus 1/V = 4 is equivalent to 35 rmoles per hour per mg of bacteriochlorophyll.

**TABLE I**

**Effect of inhibitors on PPᵢ-PPᵢ exchange**

The reactions were performed as described under "Experimental Procedures." Photophosphorylation was performed as previously described (10).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPᵢ-Pᵢ exchange</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>m-Cl-CCP, 3 μM</td>
<td>95</td>
</tr>
<tr>
<td>S-13, 1 μM</td>
<td>90</td>
</tr>
<tr>
<td>Quinacrine, 30 μM</td>
<td>78</td>
</tr>
<tr>
<td>Oligomycin, 2 μg/ml</td>
<td>0+</td>
</tr>
<tr>
<td>Antimycin a, 0.33 μM</td>
<td>0</td>
</tr>
<tr>
<td>Methylene diphosphonate, 0.3 mM</td>
<td>43</td>
</tr>
<tr>
<td>Fluoride, 10 mM</td>
<td>90</td>
</tr>
</tbody>
</table>

* Stimulation, 27%.

oligomycin, stimulated the reaction. Fluoride (9) and methylene diphosphonate (10) inhibit the membrane-bound inorganic pyrophosphatase and other PPᵢ-linked reactions in chromatophores. The effect of these inhibitors therefore is consistent with the idea that this is an energy-linked exchange reaction catalyzed by the membrane-bound inorganic pyrophosphatase.

**Effect of Light on PPᵢ-Pᵢ Exchange**—Light-induced electron transport has been observed to markedly stimulate ATP-PPᵢ exchange activity in R. rubrum chromatophores (10, 18). In contrast, light did not (Table II) stimulate the PPᵢ-Pᵢ exchange reaction. Sixty nanomoles of [³²P]PPᵢ were incorporated due to the PPᵢ-Pᵢ exchange reaction (Reaction 2) while light-induced PPᵢ synthesis incorporated 94 nanomoles (Reaction 3). Thus, for light to have any stimulating effect, Reaction 4 should be greater than the sum of these reactions. Since it is less than the sum, this means that either (a) light inhibited the exchange reaction by 44%, or that (b) PPᵢ inhibited the light-induced synthesis of PPᵢ by 27%, or that (c) there is a common rate-limiting step in both pathways of [³²P]PPᵢ formation. It is apparent that the

**Effect of ADP on PPᵢ-Pᵢ Exchange**—The interaction of the PPᵢ-Pᵢ exchange reaction with the adenine nucleotide-linked energy conservation system is shown by the data presented in Table III. ADP inhibited the exchange reaction by 32%, and this inhibition was eliminated by oligomycin indicating that this inhibition is due to ATP synthesis.

**PPᵢ-Pᵢ Exchange with Yeast Inorganic Pyrophosphatase**—Cohn (12) during studies on the phosphate-water exchange reaction catalyzed by yeast inorganic pyrophosphatase, observed that this enzyme catalyzed a PPᵢ-Pᵢ exchange that occurred at about 0.17% the rate of the hydrolytic reaction whereas the [³²P]H₂O exchange occurred at about 500 times this rate. We have studied this reaction in order to compare it with the chromatophore-catalyzed reaction.

**Preliminary studies on the effect of Pᵢ concentration on this exchange reaction are presented in Fig. 4.** The product of the hydrolytic reaction (Pᵢ) is one of the substrates for the exchange reaction and therefore the Pᵢ concentration is continually changing through the course of the reaction. Just as we did with the chromatophore reaction, we have plotted the average amount of Pᵢ present during the time course of the reaction in what we believe to be a representative experiment. An estimate obtained from a double reciprocal plot of the data presented in Fig. 4 gave a Kᵢ value of over 100 mM. However, for some
reversed electron transport (6), proton uptake (7), and the can serve as an energy donor for transhydrogenation (2, 3),

The hydrolysis of PPi can lead to the formation of the energized state, as evidenced by the fact that PPi-driven energy-linked reactions, the PPi-Pi exchange reaction (9, 10) is evidence that the membrane-bound inorganic pyrophosphatase is the enzyme involved in catalyzing these energy-linked reactions. The hydrolysis of PPi can lead to the formation of the energized state, as evidenced by the fact that PPi can serve as an energy donor for transhydrogenation (2, 3), reversed electron transport (4-6), proton uptake (7), and the

reason which we have not yet determined, this value varied considerably between experiments and therefore this number should be taken only as an approximation.

Cohn (12) found that a high Mg2+ concentration (50 mM) was optimal for the [32P]PPi-H2O exchange and we found that 40 mM Mg2+ was optimal for the PPi-Pi exchange reaction whereas the hydrolytic reaction was somewhat inhibited by Mg2+ greater than 6 mM under our conditions (4 mM PPi, 10 mM Pi), reaching 24% inhibition at 40 mM. This opposing effect of Mg2+ on exchange versus hydrolysis increased the ratio of the exchange to hydrolysis to approximately 1% under these conditions which is severely limited by the Pi concentration.

Comparing the rate of the exchange to the rate of the hydrolytic reaction with the chromatophore enzyme and the yeast enzyme at 10 mM Pi and optimal Mg2+ for each reaction, we found that approximately 1 μmole was formed/100 μmole PPi hydrolyzed with the yeast enzyme, whereas the chromatophore enzyme catalyzed the exchange at almost 10 times this rate. As expected, the exchange reaction catalyzed by the soluble yeast enzyme was not inhibited by uncoupling agents. It seems probable that the greater rate of the chromatophore reaction reflects the participation of ~χ in the reaction.

**DISCUSSION**

The PPi-PPi exchange reaction is a new energy-linked reaction that may be useful in studying energy-transduction in chromatophores. Indeed, this reaction could be described as PPi synthesis driven by PPi hydrolysis and thereby would be another facet of the PPi-driven ATP synthesis which we previously described (10). However, at this point in our understanding the reaction appears analogous with the ATP-Pi exchange and thus we prefer to describe it as an exchange reaction. Energy-linked reactions involving PPi seem to be unique in that so far they have been observed only in K. rubrum and Rhodopseudomonas viridis (19). The lack of effect of some inhibitors of photosynthetic ATP formation on photosynthetic PPi formation (1, 20) coupled with the observation that inhibitors of the membrane-bound inorganic pyrophosphatase also inhibit PPi-driven energy-linked reactions, the PPi-Pi exchange reaction (9, 10) is evidence that the membrane-bound inorganic pyrophosphatase is the enzyme involved in catalyzing these energy-linked reactions. The hydrolysis of PPi can lead to the formation of the energized state, as evidenced by the fact that PPi can serve as an energy donor for transhydrogenation (2, 3), reversed electron transport (4-6), proton uptake (7), and the

**Fig. 4. Effect of Pi on the exchange reaction with yeast inorganic pyrophosphatase.** The reaction mixture contained 50 mM Tris-Cl (pH 7.4), 33 mM MgCl2, 3 mM PPi, and 0.63 μg of enzyme in 1.5 ml. Reaction time was 6 min and with 10 mM Pi present; 11 μmoles of PPi were hydrolyzed.

**Fig. 5. Effect of Pi on light-driven PPi formation.** The reaction mixture contained 50 mM Tris-Cl (pH 8.0), 0.33 mM MgCl2, 1.67 mM succinate, and 15 μg of bacteriochlorophyll per ml. The reaction was illuminated for 30 s with 2.5 × 104 ergs cm−2 s−1 of white light.

The above mechanism for the exchange is the same as can be written for the light-driven synthesis of PPi and if this is true the optimal conditions for the exchange should be essentially identical for the light-driven synthesis. One notable exception is that the PPi requirement for the light-driven synthesis is low compared to the exchange reaction. Fig. 5 illustrates the PPi requirement for this reaction and the concentration for one-half maximal activity is less than 3 mM as compared with 48

where ~χ represents the energized state in general. If we take the free energy of hydrolysis of PPi as −5 Cal, the equilibrium of the pyrophosphatase reaction (not including ~χ) is far toward the right, as written, and the rate of the back reaction under steady state conditions would be low. It is so low in chromatophore that we have not been able to measure the rate in the presence of an uncoupler. By including the energized state (~χ), as a product of the hydrolytic reaction in the equation, the ratio of the back reaction would be increased considerably. It is primarily the energy-linked characteristics of the reaction which support this mechanism. Uncouplers, which dissipate ~χ, inhibit the reaction; oligomycin, which stabilizes ~χ, stimulates the reaction; and ADP inhibits, apparently due to the ~χ being removed during the synthesis of ATP (10) since the inhibition is prevented by oligomycin. The high Kₘ of Pi also supports this concept. This mechanism also suggests that conditions which enhance the energized state (~χ) should enhance the exchange reaction just as dissipation of ~χ inhibited the reaction. The fact that we did not observe stimulation with light (Table II) which would be expected to enhance ~χ, as was found for the ATP-Pi exchange reaction (10, 18), is not in agreement and remains unexplained.

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1 ATP did not stimulate the PPi-Pi exchange reaction either (unpublished results). This can be rationalized however, since the ADP formed would inhibit (Table III) thus possibly counterbalancing any stimulation.
mm for the exchange reaction. Thus there are differences in the requirements for the exchange reaction and the light-driven synthesis of PPI which cannot be explained at this time on the basis of the above mechanism. We are planning experiments to study \(^{32}\)P-labeled H\(_2\)O-PPI exchange which we may gain further insight into the mechanism of these reactions.

The rate of the reaction under favorable conditions (high \(P_i\)) is significant when compared with the rate of other PPI-linked reactions. The rate of the reaction taken from Fig. 1 was 12 \(\mu\)moles of \(\text{PPI}\) per mg bacteriochlorophyll per hour \((P_i = 10 \text{ mM})\). The \(V_{\text{max}}\) at saturating \(P_i\) concentration calculated from the data in Fig. 3 was 35 \(\mu\)moles per mg of bacteriochlorophyll per hour which is about the rate of light-induced PPI synthesis as illustrated in Fig. 5. The data presented in Fig. 1 also show that under these conditions, about 12 moles of PPI are hydrolyzed per mole of \(\text{PPI}^\text{32}\) exchanged. This stoichiometry is comparable to that found for the PPI-driven transhydrogenase reaction (22) and for the PP\(_i\)-driven ATP synthesis (10) which we have previously described.

Yeast inorganic pyrophosphatase has been reported to catalyze a PPI-P\(_i\) exchange (13) although Sperow et al. (23) have recently reported that they could not observe the exchange. Josse (24) could not demonstrate an exchange reaction with the inorganic pyrophosphatase of Escherichia coli even though a net reversal of the reaction was achieved by trapping PPI. We have confirmed and somewhat extended Cohn's observation that the yeast enzyme does catalyze the exchange. We observed that very high \(P_i\) and \(Mg^{2+}\) concentrations are required for the reaction. The \(K_m\) was high but a large variation in this value between different experiments was found and the reason for this was discussed under “Results.” This high \(P_i\) requirement is not surprising since it is consistent with the results of Sperow et al. (23), who found that \(P_i\) reacted only very weakly with the enzyme. It appears that if optimal conditions would be experimentally achieved, the exchange reaction and net reversal of the hydrolytic reaction would be easily observable. In toto, these results suggest that the exchange results from a net reversal of the hydrolytic reaction.

The molecular weights of soluble inorganic pyrophosphatase from several photosynthetic bacteria have been reported (25) and in general they range from 60,000 to 100,000. The membrane-bound enzyme has not been solubilized but if we assume

\(^{1}\) A low \(P_i\) requirement for this reaction also can be calculated from the data of Nishikawa et al. (21).

REFERENCES

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