Energy-linked Reactions in Photosynthetic Bacteria

IX. P$_1$-PP$_i$ EXCHANGE IN RHODOSPIRILLUM RUBRUM*

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SUMMARY

Chromatophores of Rhodospirillum rubrum catalyze a P$_1$-PP$_i$ 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 $K_m$ for P$_1$ was high (48 mM).

For comparison, the PP$_i$-P$_i$ exchange reaction catalyzed by yeast inorganic pyrophosphatase was studied. Very high P$_1$ and Mg$^{2+}$ 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 to the overall reaction.

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

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

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$_i$ in energy conservation is emphasized by the fact that PP$_i$ can serve as an energy donor for energy-linked transhydrogenation (2, 3), cytochrome reduction (4, 5), succinate-linked NAD$^+$ reduction (6), proton uptake (7), and an energy-linked shift in the membrane-bound carotenoid spectrum (8).

We have also observed that PP$_i$ can drive ATP synthesis (9, 10) and during those studies, a PP$_i$-P$_i$ 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$_i$-P$_i$ 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 overall mechanism (13), we have studied this reaction in chromatophores and reexamined the reaction with the yeast enzyme. We found that a 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 (PP$_i$ and $^{32}$PP$_i$) were obtained from New England Nuclear. Yeast inorganic pyrophosphatase (EC 3.6.1.1) was from Worthington Biochemicals. We are indebted to Dr. P. G. Heytler of the E.I. duPont de Nemours Co., and to Dr. P. C. Hamm of the Monsanto Co. for gifts of m-Cl-CCP$^i$ 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$_2$, 10 mM $^{32}$Pi, 0.57 mM PP$_i$, and usually about 20 $\mu$g per ml bacteriochlorophyll as chromatophores. The reaction was carried out at 25°C and terminated by the addition of trichloroacetic acid to 5%. $^{32}$PP$_i$ formed was determined as previously described (10).

PP$_i$ hydrolysis was determined in the reaction mixture by using $^{32}$PP$_i$ and unlabeled P$_i$. In this case the $^{32}$PP$_i$ formed was determined (10).

The exchange reaction catalyzed by yeast inorganic pyrophosphatase was measured in the following reaction mixture: 50 mM Tris-Cl (pH 7.4); 40 mM MgCl$_2$; 10 mM PP$_i$; 2 to 4 mM PP$_i$; and sufficient enzyme to give 25 to 35% hydrolysis of the PP$_i$ during the assay period. The reaction was terminated and assayed for $^{32}$PP$_i$ as above.

* This work was partially supported by National Science Foundation Grant GB-10388. Contribution No. 523 of the Charles F. Kettering Research Laboratory.

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The abbreviations used are: m-Cl-CCP$^i$, m-chlorocarbonyl cyanide phenylhydrazone; S-13, 5-chloro-3-tert-butyl-2'-chloro-4'-nitrosalicylanilide.
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 P1-driven ATP synthesis reaction catalyzed by these chromatophores (10).
PPi synthesis incorporated 94 nmoles (Reaction 3). Thus, for light to have any stimulating effect, Reaction 4 should be greater than Reaction 3 (Table II). Light-induced electron transport has been observed to markedly stimulate ATP-Pi exchange activity in R. rubrum chromatophores (10, 18). In contrast, light did not stimulate the PPi-Pi exchange activity in R. rubrum chromatophores (10). The PPi-Pi exchange reaction is due to light-induced \(^{32}\)PPi synthesis, not exchange activity.

### Table I

**Effect of inhibitors on P\(_i\)-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 (\mu)M</td>
<td>95</td>
</tr>
<tr>
<td>S-18, 1 (\mu)M</td>
<td>90</td>
</tr>
<tr>
<td>Quinacrine, 30 (\mu)M</td>
<td>78</td>
</tr>
<tr>
<td>Oligomycin, 2 (\mu)g/ml</td>
<td>0(^+)</td>
</tr>
<tr>
<td>Antimycin a, 0.33 (\mu)M</td>
<td>0</td>
</tr>
<tr>
<td>Methylene diphosphonate, 0.3 (\mu)M</td>
<td>43</td>
</tr>
<tr>
<td>Fluoride, 10 (\mu)M</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 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-Pi exchange activity in R. rubrum chromatophores (10, 18). In contrast, light did not (Table I) stimulate the PPi-Pi exchange reaction. Sixty nanomoles of \(^{32}\)Pi were incorporated due to the PPi-Pi exchange reaction (Reaction 3) while light-induced PPi synthesis incorporated 94 nmoles (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 \(^{32}\)PPi formation. It is apparent that the PPi-Pi exchange has quite different characteristics from the ATP-Pi exchange.

**Effect of ADP**—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 \(^{32}\)O\(\Pi\) 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 P\(_i\) concentration on this exchange reaction are presented in Fig. 4. The product of the hydrolytic reaction (P\(_i\)) is one of the substrates for the exchange reaction and therefore the P\(_i\) 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 P\(_i\) 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 \(K_m\) value of over 100 \(\mu\)M. However, for some
reversed electron transport, proton uptake, and the
process driven energy-linked reactions, the PPi-Pi exchange reaction
involves PPi hydrolysis which is catalyzed by the soluble yeast organic pyrophosphatase. The reaction mixture contained 50 mM Tris-Cl (pH 8.0), 0.33 mM MgCl2, 1.67 mM aminocitrate, and 15 μg of bacteriochlorophyll per ml. The reaction was illuminated for 30 s with $2.5 \times 10^4$ ergs cm$^{-2}$ s$^{-1}$ of white light.

The PPi-Pi 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 of 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 R. 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 carotenoid absorption band shift (8) which is an indication of membrane energization.

These studies do not elucidate the mechanism of this reaction although they suggest that it may be represented by

$$PP_i = 2P_i + \sim\chi$$

where $\sim\chi$ 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 $\sim\chi$) 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 ($\sim\chi$), as a product of the hydrolytic reaction in the equation, the rate 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 $\sim\chi$, inhibit the reaction; oligomycin, which stabilizes $\sim\chi$, stimulates the reaction; and ADP inhibits, apparently due to the $\sim\chi$ being removed during the synthesis of ATP (10) since the inhibition is prevented by oligomycin. The high $K_m$ of Pi also supports this concept. This mechanism also suggests that conditions which enhance the energized state ($\sim\chi$) should enhance the exchange reaction just as dissipation of $\sim\chi$ inhibited the reaction. The fact that we did not observe stimulation with light (Table II) which would be expected to enhance $\sim\chi$, as was found for the ATP-Pi exchange reaction (10, 18), is not in agreement and remains unexplained.

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 Pi requirement for the light-driven synthesis is low compared to the exchange reaction. Fig. 5 illustrates the Pi requirement for this reaction and the concentration for one-half maximal activity is less than 5 mM as compared with 48

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 counter-balancing any stimulation.
mm for the exchange reaction. Thus there are differences in the requirements for the exchange reaction and the light-induced synthesis of Pi which can not be explained at this time on the basis of the above mechanism. We are planning experiments to study 3H-labeled H2O-Pi exchange which may give further insight into the mechanism of these reactions.

The rate of the reaction under favorable conditions (high P1) is significant when compared with the rate of other Pi-linked reactions. The rate of the reaction taken from Fig. 1 was 12 μmoles of 32P Pi formed per mg of bacteriochlorophyll per hour (P1 = 10 mM). The V_max at saturating P1 concentration calculated from the data in Fig. 3 was 35 μmoles per mg of bacteriochlorophyll per hour which is about the rate of light-induced Pi synthesis as illustrated in Fig. 5. The data presented in Fig. 1 also show that under those conditions, about 12 moles of Pi are hydrolyzed per mole of 32P Pi exchanged. This stoichiometry is comparable to that found for the Pi-driven transhydrogenase reaction (22) and for the Pi-driven ATP synthesis (10) which we have previously described.

Yeast inorganic pyrophosphatase has been reported to catalyze a Pi-Pi exchange (13) although Sperow et al. (23) have recently reported that they could not observe the exchange. Jesse (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 Pi. We have confirmed and somewhat extended Cohn’s observation that the yeast enzyme does catalyze the exchange. We observed that very high Pi and Mg2+ 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 Pi requirement is not surprising since it is consistent with the results of Sperow et al. (23), who found that Pi 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

4 A low Pi requirement for this reaction also can be calculated roughly from the data of Nishikawa et al. (21).
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