On Reconstitution of Bacterial Photophosphorylation In Vitro
(reaction center/coupling factor/phosphorylating inactive membrane/Rhodopseudomonas capsulata)

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ABSTRACT Membranes active in oxidative phosphorylation but inactive in photophosphorylation were isolated from a carotenoid-less and bacteriochlorophyll-less mutant strain of Rhodopseudomonas capsulata. These membranes were uncoupled and recombined with coupling factor from the same strain and with reaction centers from a photosynthetically active revertant strain. The reconstituted system was able to form ATP from ADP and inorganic phosphate under anaerobic conditions in the light. Rates of 2 nmoles of ATP formed per min per mg of bacteriochlorophyll were obtained. A high incorporation of 32P from inorganic phosphate into ATP in the light as well as in the dark was observed, the activity of which was associated with coupling factor. This incorporation occurs both aerobically and anaerobically and appears to be independent of photophosphorylation.

A unique opportunity to attempt definitive reconstitution of bacterial photophosphorylation is afforded by the recent isolation in this laboratory of two mutant strains of Rhodopseudomonas capsulata (1). One of these (Ala−) is essentially devoid of bacteriochlorophyll (Bchl) and unable to grow photosynthetically, but retains capacity for oxidative phosphorylation and dark aerobic growth. The other (Ala+R) is its positive revertant, with a recovered ability to grow photosynthetically and with a Bchl complex that can support photophosphorylation. We have proposed to extend recent suggestions that the two phosphorylation systems share many components (2–4), and are to some extent interchangeable (2), by experiments in which reaction centers from Ala− are incubated anaerobically in light and dark with uncoupled, oxidative phosphorylation competent membranes from Ala− together with coupling factor preparations. In this report we submit evidence that such a reconstitution of photophosphorylation, apparently linked to electron transport through components of the aerobic phosphorylation system, can be achieved.

EXPERIMENTAL

Bacteria and Growth Conditions. The isolation of the mutant strains Ala− and Ala+ of R. capsulata and their properties have been described elsewhere (1). The phototrophic positive strain Ala+ was cultivated anaerobically in the light, the phototrophic negative mutant Ala− aerobically in the dark. The bacteria were harvested at the end of the logarithmic growth phase and stored frozen at −85°C.

Isolation of Membranes. The method described elsewhere (5) was modified in that the aerobic membranes of Ala− were separated from cell debris by centrifugation at 8000 X g. In these membranes and also in those of semiaerobically grown cells, no Bchl was detected by spectrophotometric means, either in the spectrum in vivo or in the methanol extract.

Preparation of Reaction Center. The membranes from the mutant Ala− were washed twice with 50 mM Na-phosphate buffer (pH 7.6) and finally resuspended in the same buffer to a final concentration of 20–25 mg of protein per ml. Sufficient 15% (w/v) lauryl dimethylamine oxide (“Ammonyx LO”, Millmaster Onyx Corp., Jersey City, N.J.) was added to make a final concentration of 3 mg of detergent per mg of protein. The suspension was left in the dark for 30 min and then applied to a linear sucrose gradient (0.5–0.05 M; 11 ml of gradient, 1.0 ml of sample). The tubes then were centrifuged 15 hr at 39,000 rpm in the SW 41 rotor of the Spinco ultracentrifuge (LS 65 B). The crude reaction centers appeared as a gray-blue band below the dissolved bulk Bchl, which appeared at the top of the tubes as a green zone. The reaction center fraction was drawn off and dialyzed twice against 50 volumes of 0.1 M glycerol-glycine buffer (pH 8.0). The elimination of excess detergent resulted in turbidity in the reaction center solution. This suspension was purified in a sucrose gradient as described above. Finally, the preparation of reaction center was dialyzed overnight against glycerol-glycine buffer, and kept frozen until further use.

Preparation of Uncoupled Membranes from the Phototrophic-Negative Mutant. Membranes of the mutant Ala− were treated with EDTA and sonication as described (2, 6). The resultant uncoupled membranes were resuspended in 0.1 M glycyrl-glycine buffer containing 50% glycerol. They were stored at −20°C without significant loss of recombining capacity for at least 3 weeks.

Preparation of Coupling Factor. The coupling factor was obtained from either the EDTA extracts or from an acetone powder prepared with the phototrophic-negative membranes using Tris-ATP solution for its extraction (6). Both extracts were centrifuged at 200,000 X g for 90 min. Then the protein fraction precipitating between 35 and 60% saturation of ammonium sulfate was collected and resuspended in a small volume of 0.1 M glycyrl-glycine buffer plus 50% glycerol, and

Abbreviation: Bchl, bacteriochlorophyll.
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kept at −20° or used immediately. However, we did not test its stability under these conditions and always used it within 24 hr of its preparation.

**Preparation of Reconstituted Particles.** For this purpose uncoupled membranes from A1a− (70 mg of protein) were mixed with 3–5 mg of protein of coupling factor and 5 mg of proteins of reaction center in a standard mixture, which also contained 3.6 mg/ml of bovine serum albumin, 30 μmole/ml of glycyl-glycine buffer, and 3.66 μmole/ml of MgCl₂. The final volume was 2.73 ml. The mixture was incubated for 60 min at 30° and either used as such or centrifuged for 90 min at 200,000 × g, and finally resuspended in glycyl-glycine buffer (“washed particles”).

**Photophosphorylation Assay.** Photophosphorylation was assayed in Warburg type vessels; a gentle stream of oxygen-free N₂ (99.99% pure) was maintained throughout the reaction. The reaction mixture contained, in μmoles: glycyl-glycine buffer, 105; MgCl₂, 15.3; glucose, 28; inorganic phosphate (Pi), 12 (labeled with about 3 to 5 × 10⁶ cpm of ³²P), and either 2.8 mg of ADP (high ADP) or 0.28 mg of ADP (low ADP). Bovine serum albumin was added to 2 mg/ml, and hexokinase was 15 units/ml of final concentration. The final volume was 1.8 ml.

Before addition to the reaction mixture, the bovine serum albumin was mixed with the hexokinase suspension. The mixture was passed through a G-25 Sephadex column (medium) previously equilibrated with 0.1 M glycyl-glycine buffer and eluted with the same buffer to eliminate the ammonium ions present in the commercial hexokinase suspension.

The reaction mixture (1.3 ml) containing glycyl-glycine buffer, MgCl₂, glucose, bovine serum albumin, and hexokinase was pipetted into the main compartment of the vessels, and 0.5 ml of the recombined particles were added. This suspension was first incubated under N₂ at 30° in the dark for 40 min. The reaction was started by tipping in from the side arm 0.18 ml containing the ADP and ³²P. Illumination at saturating intensities was provided by a set of tungsten lamps. The reaction was stopped by addition of 0.5 ml of 50% trichloroacetic acid, and organic phosphate was determined (7).

**Chromatography of the Products.** The aqueous phase, containing the organic phosphate obtained after elimination of the inorganic phosphate (7), was brought to neutrality with NaOH and applied to Whatman no. 7 paper. The alkaline solvent described by Bandurski and Axelrod (8) was used for ascending chromatography, which was usually allowed to develop for 18 hr.

Appropriate standards were used to identify the different products: glucose-6-phosphate, ADP, ATP, and Pi. These compounds were recovered by extraction with the chromatography solvent. The extracts were then evaporated under a stream of hot air. The residue was dissolved in 0.5 ml of water and used as such for determination of 32P.

**Other Methods.** Radioactivity was determined with the liquid scintillation spectrometer Packard Tricarb 574. The solvent used was a mixture of 1 volume of Triton X-100 and 2 volumes of toluol containing 2,5-diphenyloxazol (POPO) and dimethyl 1,4-bis[2-(4-methyl-5-phenyloxazole)]-benzene (dimethyl POPOP). This mixture will tolerate up to 1.0 ml of water with no appreciable quenching (9). Protein was determined by the method of Lowry et al. (10). Bchl concentration was assayed by extraction with acetone–methanol, using a millimolar extinction coefficient of 75 (11).

**RESULTS AND DISCUSSION**

As seen in Fig. 1, formation of ATP, as evidenced by the incorporation of labeled phosphate into glucose-6-phosphate in the presence of the hexokinase coupling system, occurs to a
significant extent when membranes devoid of the capacity to support photophosphorylation are supplemented with a reaction center pigment complex from membranes that can mediate photophosphorylation.

Table 1 shows the requirements of the reaction, indicating that: (i) A substantial amount of the phosphate uptake in the dark is present in the coupling factor fraction. About the same rate is observed with coupling factor alone. (ii) The phosphate uptake in the dark does not involve formation of ATP because hexokinase is without effect on this incorporation, and glucose-6-phosphate is not formed under these conditions. (iii) The light-induced phosphate uptake only occurs when the reaction center fraction is present (complete system.).

It should be noted that higher and lower values than that shown in Table 1 were obtained ranging between 0.4 and 2.5 nmoles of ATP per min/μg of Bchl. In one experiment, with washed and unwashed particles under saturating light intensity for periods of 15 and 45 min, comparable rates of ATP formation were found (Table 2), and the dark controls showed negligible (<3%) incorporation (see Fig. 1). In the presence of KCN (5 × 10^{-4}M) the same rates of photophosphorylation were observed, demonstrating that no oxidative phosphorylation was involved.

Photophosphorylation in a reconstituted system has been shown to occur by the formation of a light-induced membrane potential (12), and the rate obtained was 2.97 × 10^{-2} nmoles of ATP per min/μg of pigment. However, it is likely that the present incorporation is associated with electron transport rather than a result of merely a proton pump mechanism (13) on the following grounds: (i) the phosphate uptake in the light was inhibited by both HOQNO (10^{-4} M) and ω-phenanthroline (2 × 10^{-4} M) and (ii) the amounts of phosphate uptake per unit time induced by light and by the aerobic oxidation of succinate were closely comparable, i.e., rates of aerobic phosphate uptake ranged from 94 to 100% of those of light-induced anaerobic uptake (Table 1). However, chromatographic analysis of the amount of glucose-6-phosphate was not performed for the oxidative phosphorylation experiment.

It is also pertinent to remark that the aerobic membranes, either coupled or not, in the presence or in the absence of added coupling factor, were able to support an O_{2} uptake through the oxidation of either NADH or succinate, thus indicating that the aerobic membranes were able to function in an electron transport process.

Conclusive evidence for light-induced electron transport in the reconstituted particles requires demonstration of actual electron transport, such as correlations of light-induced redox changes in the endogenous cytochromes with phosphate esterification. Nevertheless, the correlations presented above, as well as the demonstration by Jones (3) of electron transport in an essentially identical system, strongly support the contention that our attempts to reconstitute a photophosphorylation system in vitro, linked to electron transport, have been successful.

The large incorporation of labeled phosphate into ADP and perhaps other compounds noted (Fig. 1) persists in the dark controls even under the best conditions (low ADP, long period of incubation; see Table 2) and may be the consequence of an as yet unreported ADP:P_{i} exchange. Thus, a 10-fold decrease in ADP concentration results in a decrease of 98% in phosphate uptake in the dark. This response to ADP concentration, which is not observed for ATP formation, indicates that the mechanisms involved in the labeling of ADP proceed essentially independently of the photophosphorylation process.


