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Comparative Stabilization of Biological Photosystems by Several Immobilization Procedures

1. ATP Production by Immobilized Bacterial Chromatophores

V. Larreta Garde[1], B. Thomasset[1], A. Tanaka[2], G. Gellf[1], and D. Thomas[1]

[1] Laboratoire de Technologie Enzymatique, Université de Technologie de Compiègne, B.P. 233, F-60206 Compiègne, France

[2] Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan

Summary. Bacterial chromatophores have been isolated from a purple non-sulfur bacterium (*Rhodopseudomonas capsulata*) by sonication and immobilized within various supports. In each case, the activity yield after immobilization and the storage stability (under dark conditions at 4 °C) have been determined. Some preliminary comparative experiments concerning the ATP production in a batch reactor are presented for native and immobilized chromatophores.

Introduction

Numerous biosynthetic pathways involve adenosine triphosphate (ATP) requiring reactions. This cofactor molecule acts as an energy donor to drive, under mild conditions, endergonic bioconversions of high specificity and selectivity. Enzymatic (Marshall 1973; Langer et al. 1976) electrochemical (Fan et al. 1978) photobiochemical (Pace et al. 1976) and microbiological (Tochikura et al. 1967; Murata et al. 1980) methods for ATP regeneration have been described.

A majority of photosynthetic bacteria (Chromatiaceae and Chlorobiaceae) requires a sulfur compound as an electron donor; a minority (Rhodospirillaceae) exhibits a cyclic electron transfer (Pfennig 1978). Mild disruption by sonication of these photosynthetic bacteria yields small fragments of the cytoplasmic membrane which spontaneously form inside-out vesicles (Chromatophores) containing the cell pigments. In the particular case of purple non-sulfur bacteria, these chromatophores, carrying out a cyclic photophosphorylation, constitute an interesting system for the regeneration of ATP: they require only light as an energy source, ADP and inorganic phosphate as substrates (Frenkel 1954).

Immobilization of such chromatophores by inclusion into a polyacrylamide gel (Yang et al. 1976), various alginate gels (Paul and Vignais 1980) and by crosslinking with glutaraldehyde in the presence of albumin (Larreta Garde et al. 1980) in order to improve their stability and facilitate their use in a bioreactor have been described.

Materials and Methods

Preparation of the Chromatophores

Rhodopseudomonas capsulata, strain B 10 (kindly donated by Dr. Vignais, Grenoble) was grown anaerobically under illumination at 30 °C in a R.C.V. medium (Weaver et al. 1975; Hillmer and Gest 1977), supplemented with malate (30 m*M*) and ammonium sulfate (7 m*M*) as carbon and nitrogen sources respectively. Cells were harvested during the late logarithmic phase and resuspended in a HEPES buffer 0.1 M, pH 8 containing $MgCl_2$ 8 m*M*. The bacterial suspension was sonicated five times for 30 s at a power rating of 60 W (sonifier B 12, Branson Sonic Power Co) and centrifuged twice at 5,000 g for 15 min. The chromatophores were collected in the supernatant.

Immobilization Methods

Crosslinked Albumin Film. Of a solution containing 70 mg albumin, 5.4 mg glutaraldehyde in 0.02 M phosphate buffer pH 6.8 0.9 ml were prepared. After 5 min, 0.3 ml of a chromatophore suspension were added. The resulting solution was spread on a flat glass plate. After 2 h, a complete insolubilization had occurred and a 50 μm thick proteic membrane was produced. The membrane was rinsed with a phosphate buffer solution several times.

Crosslinked Gelatin Film. Of phosphate buffer pH 6.8, 0.3 ml of chromatophore suspension and 0.6 ml of a 10% gelatin solution 0.3 ml were mixed and spread on a flat glass plate. After 1 h at room temperature a firm gel had formed and was hardened by dipping for 3 min into a 1% glutaraldehyde solution followed by a thorough rinse with a phosphate buffer solution.

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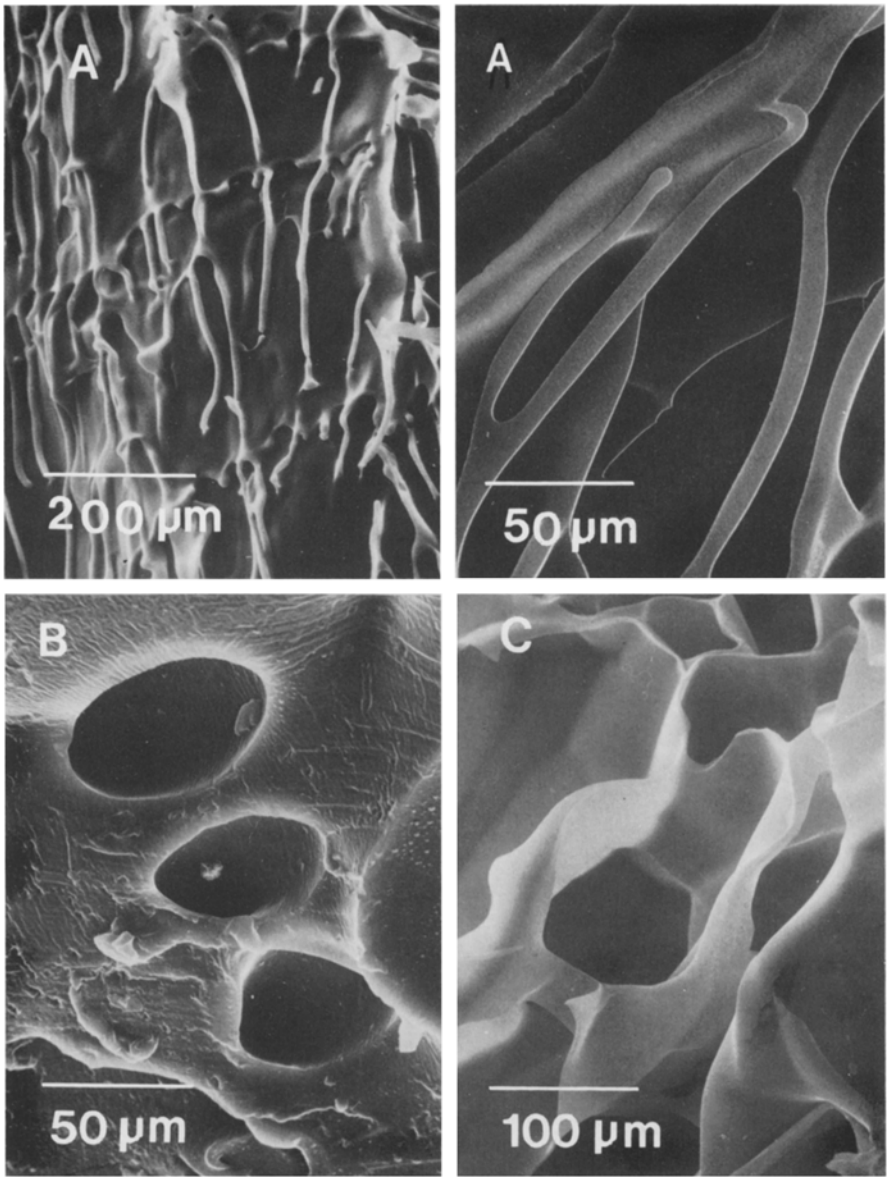


Fig. 1A–C
Scanning electron micrographs of the supports.
A Albumin foam structure
B BSA-polyurethan polymer
C Gelatin foam structure

Crosslinked Albumin Foam Structure. A solution was made by mixing 1 ml of 0.02 M pH 6.8 phosphate buffer, 1.25 ml of a 20% albumin solution, 1 ml of a 1.5% glutaraldehyde solution and 1 ml of chromatophore suspension. (The resulting glutaraldehyde concentration is then 0.35%). This mixture was frozen at −20 °C for several hours and then slowly thawed at 4 °C and rinsed.

Crosslinked Gelatin Foam Structure. The same procedure was used after mixing 0.5 ml of phosphate buffer pH 6.8, 1 ml of chromatophore suspension, 2 ml of a 10% gelatin solution and 0.5 ml of a 1.5% glutaraldehyde solution (The resulting glutaraldehyde concentration is then 0.19%).

Alginate and Carrageenan Gels. The chromatophore suspension was immobilized, according to previously published procedures, inside calcium alginate gel (Hackel et al. 1975; Kierstan and Bucke 1977; Ohlson et al. 1979) and inside potassium carrageenan (Takata et al. 1979). Sodium alginate and carrageenan were kindly donated by the "Centre de Recherches CECA-F 50500 Carentan Baupte".

Photocrosslinkable Resin. Immobilization of chromatophores with a photocrosslinkable resin (ENT 4000) was carried out using the method described by Fukui et al. (1976). Of a 65% ENT 4000 aqueous solution 1.5 g were mixed with 0.1 ml of a 10% benzoin isobutylether solution in polyethylene glycol and 1 ml of chromatophore suspension. The mixture was spread on a sheet of transparent polyester and illumated for 3 min.

Urethan Polymer. The chromatophore suspension was immobilized by mixing with urethan prepolymer PU 3 according to the procedure of Fukushima et al. (1978). Urethan and photocrosslinkable prepolymers were kindly donated by Professor S. Fukui (Kyoto University, Japan). In some cases (mentioned as "polyurethan-BSA method") bovine serum albumin was added to the mixture to give a final concentration of 5% (V/V).

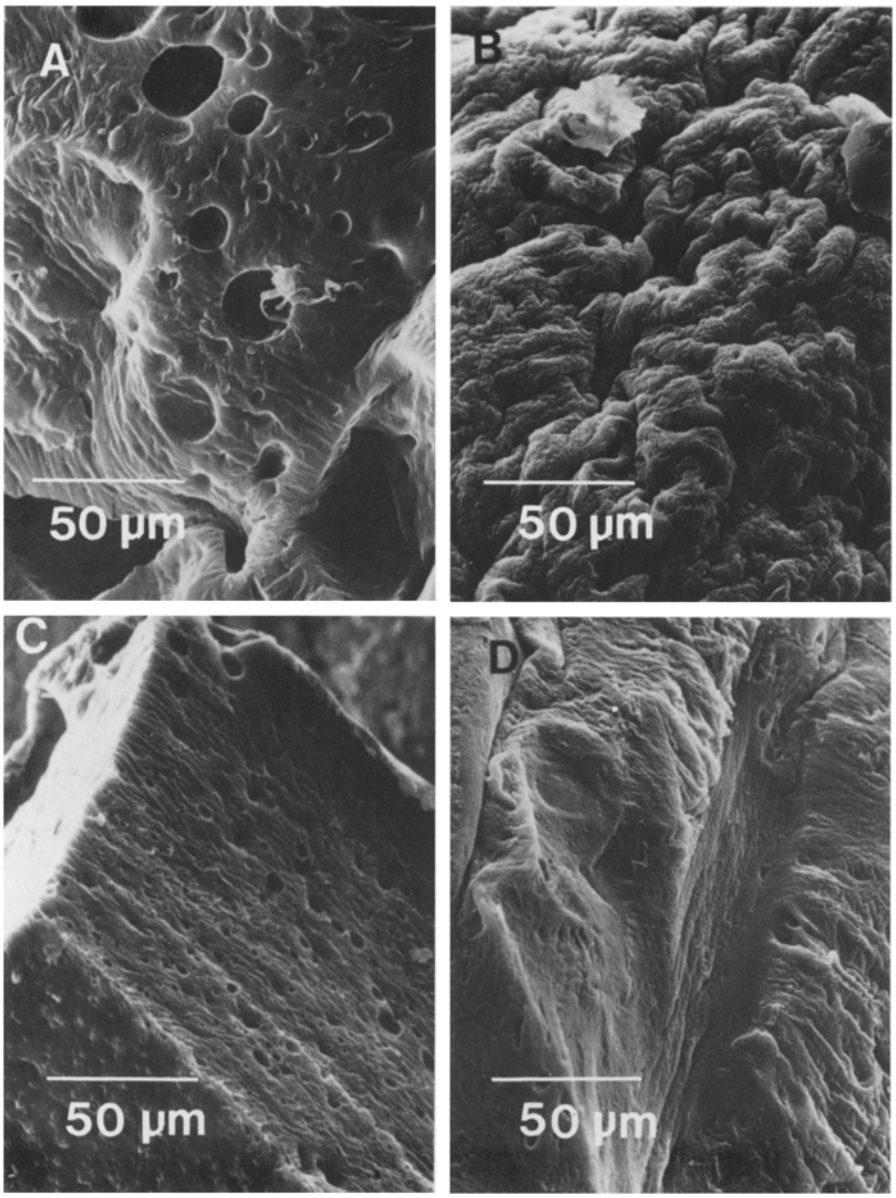


Fig. 2A–D
Scanning electron micrographs of the supports
A Polyurethan polymer
B Calcium alginate gel
C Photocroslinkable resin
D Carrageenan gel

ATP Production

Photophosphorylation was carried out according to Hochman and Carmeli (1977). Illumination was provided by a 150 W flood (approximately 10,000 lux). The reaction medium used was a 0.1 M Hepes – NaOH buffer pH 8 containing 8 m*M* $MgCl_2$, 17 m*M* glucose, 2.5 I.U. ml^{-1} hexokinase and the chromatophore suspension diluted to a final concentration of 2 µM bacteriochlorophyll determined in an acetone/methanol extract according to Clayton (1963).

ATP synthesis was initiated by adding 18 µl/ml of reaction medium of a solution containing 0.2 M ADP and 0.35 M Pi.

Photophosphorylation assays for the measurement of the initial rate of ATP evolution and activity yields after immobilization were done in 5 ml vessels containing 1.75 ml of N_2-flushed reaction medium. After 3 mn incubation at 30 °C under illumination, the reaction was stopped by adding 0.25 ml of 20% trichloracetic acid. ATP production was measured according to Lamprecht and Trautschold (1974) with a NADP-glucose-6-phosphate dehydrogenase system.

For the experiments concerning the total ATP production, a 20 ml batch reactor was used. The reaction was carried out under continuous illumination with the NADP-G 6 PDH system present in the reaction medium, allowing a continuous monitoring of the ATP production through the NADPH absorbance at 340 nm. ATP production was expressed as µ mol. mg $BChl^{-1}$ h^{-1} (BChl: Bacteriochlorophyll).

Scanning Electron Microscopy. All specimens were fixed for 2 h in 3% glutaraldehyde and postfixed for 1 h in 2% osmium tetroxide. These supports were dehydrated with increasing alcohol concentrations, then coated with gold and examined on a CAMECA M E B 07 scanning electron microscope under 30 kV.

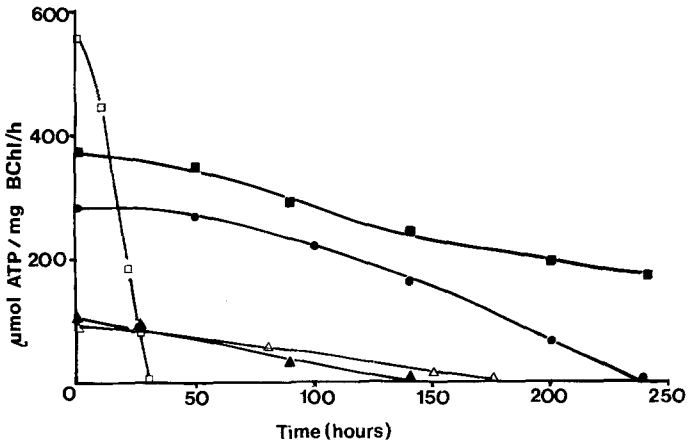
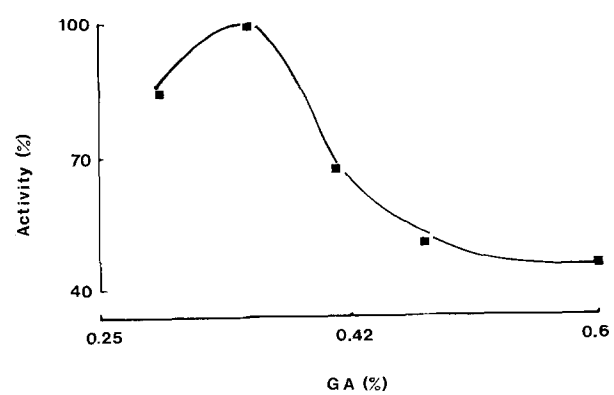


Fig. 3. Photophosphorylation rate as a function of time of storage under dark conditions at 4 °C for the native chromatophores (–□–□–) and for the chromatophores immobilized into the albumin foam structure (–■–■–), the polyurethan polymer (–●–●–), the photocrosslinkable resin (–△–△–), the calcium alginate gel (–▲–▲–)

Table 1. ATP production (µmoles · mg $BChl^{-1} \cdot h^{-1}$) by the native and immobilized chromatophores. (BChl: Bacteriochlorophyll)

Immobilization method	Support	ATP µmol · mg $BChl^{-1} \cdot h^{-1}$	Activity yield %
Native chromatophores		560	100
Entrapment	ι-Carrageenan gel	16	3
	Ca^{2+} Alginate gel	91	16
	Photocrosslinkable resin	95	17
	Urethan polymer	263	47
Cocrosslinking process	BSA film	160	29
	Gelatin film	219	39
	Foam structure BSA	369	66
	Foam structure gelatin	235	42



Results and Discussion

Scanning Electron Microscopy

Albumin foam structure presents a continuous surface with homogeneous thin sheets separated by long threads (Fig. 1A). Contrary to albumin polymers, the gelatin foam structure has a macroporous architecture (Fig. 1C). The structure has continuous, solid membranous walls with separated individual pores (from 10 to 50 µm in diameter) typical of a gel form. BSA-polyurethan polymers (Fig. 1B) reveal a typical spongy structure very similar to the structure of the polyurethan polymer (Fig. 2A). However, in the case of the BSA-polyurethan mixture, the absence of micropores can be noted. The calcium alginate gel (Fig. 2B) shows a rugged aspect. The photocrosslinkable resin (Fig. 2C) constitutes an homogeneous film with numerous internal micropores, unlike the carrageenan gel (Fig. 2D).

Activity Yield and Storage Stability

The rate of ATP synthesis was comparatively measured for the native and immobilized chromatophores and their stability was studied at 4 °C under dark conditions. It has been checked that in absence of light or in the presence of $2.5\ 10^{-6}$ M CCCP (carbonylcyanide m chlorophenylhydrazone), the ATP synthesis was inhibited (95% inhibition in both cases). Carrageenan and alginate gels are mild immobilization procedures allowing for instance the growth of bacterial cells in situ (Wada et al. 1979). The storage stability of the chromatophores was improved after entrapment inside these polysaccharide gels (Fig. 3) but the photosynthetic activity was largely denatured during the immobilization process. Similar results were obtained with the photocrosslinkable resins (Table 1). Entrapment inside polyurethan gels and the different cocrosslinking methods gave more satisfactory results for the activity yield and the storage stability. In the case of the bovine serum albumin foam structure (crosslinking at subzero temperature), it was important to optimize the glutaraldehyde concentration (Fig. 4). This optimal value (0.35% V/V) for the activity yield gave rise to the preparation with the highest ATP production (data not shown) contrary to what has been observed with immobilized thylakoids (Cocquempot et al. 1980). It is noteworthy to mention that after one month's storage at 4 °C, the chromatophores immobilized inside the BSA foam structure still kept 20% of their initial phosphorylating activity.

◀ Fig. 4. Relative photophosphorylation rate as a function of glutaraldehyde concentration for the chromatophores immobilized into the albumin foam structure. (The 100% value corresponds to 310 µmol ATP/mg $BChl^{-1} \cdot h^{-1}$)

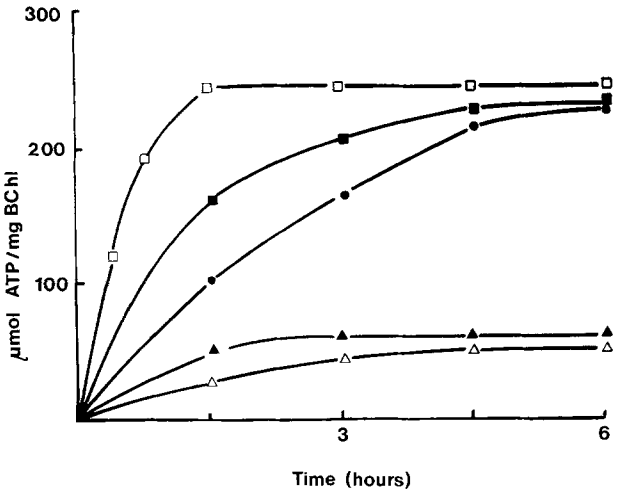


Fig. 5. Total ATP production as a function of time in a batch reactor. Hexokinase was added in solution. Same symbols as in Fig. 3

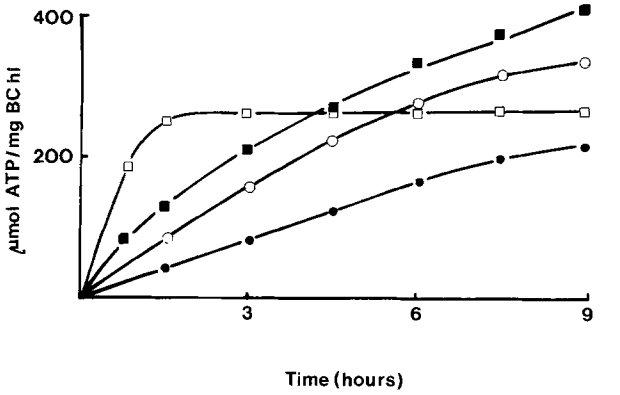


Fig. 6. Total ATP production as a function of time in a batch reactor. Hexokinase was coimmobilized with the chromatophores into the albumin foam structure (–■–■–), the urethan polymer, (–●–●–), the BSA-urethan polymer (–□–○–). For the experiment with native chromatophores (–□–□–) Hexokinase was added in solution as in Fig. 5

ATP Production. ATP production was measured in a batch reactor (20 ml) under continuous illumination as previously described (Materials and Methods). After several hours, the ATP concentration reaches a plateau (Fig. 5). Addition of fresh ADP to the reaction mixture restores the ATP synthesis, although at a lower rate. This result suggests that the chromatophores are still active and that there is a thermodynamic equilibrium limiting the ATP production. Immobilization of the enzyme hexokinase (ATP consuming) inside the support together with the chromatophores improves the ATP production by the chromatophores immobilized in the BSA foam structure and in the polyurethan-BSA polymer as compared with the native chromatophores (Fig. 6). These results indicate that, from the various experimental methods, the BSA foam structure and the polyurethan-BSA method seem the most appropriate for further studies. We are now comparatively investigating the kinetics of the ATP production for the native and immobilized chromatophores.

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