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Reconstitution of cyclic electron transport and photophosphorylation by incorporation of the reaction center, cytochrome bc_1 complex and ATP synthase from *Rhodobacter capsulatus* into ubiquinone-10/phospholipid vesicles

Nadia Gabellini [1,*], Zhan Gao [1], Dieter Oesterhelt [1], Giovanni Venturoli [2] and B. Andrea Melandri [2]

[1] *Max-Planck-Institut für Biochemie, Martinsried (F.R.G.) and* [2] *Dipartimento di Biologia, Università di Bologna, Bologna (Italy)*

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The photosynthetic reaction center, the ubiquinol–cytochrome *c* reductase (EC 1.10.2.2) and the ATP synthase (EC 3.6.1.4) were selectively solubilized and isolated from the intracytoplasmic membranes of photoheterotrophically grown *Rhodobacter capsulatus* with *n*-octyl β-D-glucopyranoside. The purified enzymes were co-reconstituted in predetermined stoichiometries in ubiquinol-10 phospholipid vesicles by a two-step procedure. In the first step, reaction centers and ubiquinol-cytochrome *c* reductase were incorporated randomly by detergent dialysis. Cyclic electron transport between these two membrane complexes was selected by including cytochrome *c* in the interior of the proteoliposomes. In the second step, the ATP synthase molecules were incorporated by detergent dilution into the preformed proteoliposomes from the outer face of the vesicles. Analysis of the light-induced redox changes of the cytochromes and of the bacteriochlorophyll dimer, in combination with the effect of specific inhibitors demonstrated that an electron transport pathway closely resembling that of the physiological system was reconstructed in the proteoliposomes. Light-driven ATP synthesis was detected at a maximal rate of 36 nmol ATP/min per mg ATP synthase. Variation in the stoichiometry of the protein components indicated that the rate-limiting reaction was ATP synthesis.

Introduction

Purple photosynthetic bacteria of the genus *Rhodobacter* are one of the most widely studied bioenergetic systems. These bacteria are anoxygenic photoheterotrophs, capable of synthesizing ATP and fixing N_2 and CO_2 photosynthetically [1,2]. Oxygen tension and light intensity regulate the synthesis of their photosynthetic apparatus [3,4], which is located in the invaginations of the cytoplasmic membrane (chromatophores). Through a cyclic electron transport system that involves two membrane-bound complexes, the photochemical reaction center (RC) and the ubiquinol–cytochrome *c* reductase (bc_1 complex), the bacteria convert radiation energy into the electrochemical potential of protons that is used for the synthesis of ATP [5]. The photosynthetic electron transport is well characterized in these organisms [6,7]. The radiant energy is collected by the light-harvesting complexes and is delivered to a special dimer of bacteriochlorophyll *a* $(Bchl)_2$ associated with the RC ($E_{m,7}$ 450 mV) that becomes photoxidized. This reaction is followed by electron transport through a bacteriopheophytin to the primary electron acceptor quinone molecule, $(Q)_A$, with a half-time of 200 ps and to the secondary electron acceptor, $(Q)_B$ ($t_{1/2} = 150$ μs).

* Present address: Fidia Research Laboratories, Via Ponte della Fabbrica 3/A, 35031 Abano Terme (PD), Italy.

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, DCCD, *N*,*N*′-dicyclohexylcarbodiimide; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; octyl glucoside, *n*-octyl β-D-glucopyranoside; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; RC-LH I, reaction center-light harvesting complex I; bc_1, ubiquinol–cytochrome *c* reductase (EC 1.10.2.2); UQH_2, ubiquinol-2; UQ-10, ubiquinone-10; $(Bchl)_2$, bacteriochlorophyll dimer, special pair.

Correspondence: D. Oesterhelt, Department of Membrane Biochemistry, Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, F.R.G.

This process displaces an electron across the membrane and thereby generates an electrical potential difference. The electron is stored on the Q_B molecule until a second turnover produces a fully reduced quinol, giving rise to a two-electron gate mechanism. Finally Q_BH_2 is believed to exchange with a lipid-soluble quinone, thus delivering two reducing equivalents to the UQ pool ($E_{m,7} \approx 90$ mV). The $(Bchl)_2^+$ is rereduced by a water-soluble cytochrome c_2 ($t_{1/2} = 5$ μs).

The electron transport pathway in the bc_1 complex and the mechanism of proton translocation are best explained in analogy to the Q-cycle model proposed for the mitochondrial reductase [8,9]. The ubiquinol is oxidized on the positive side of the membrane (Q_z or Q_o site) through a concerted reaction ($t_{1/2} = 300$ μs) that involves the Fe_2S_2 cluster ($E_{m,7}$ 290 mV) and the low-potential heme of cytochrome b (b_L, $E_{m,7}$ −90 mV). The two electrons enter a branched pathway and two protons are released into the aqueous phase. One electron is transferred to cytochrome c_1 (E_{m7} 260 mV) and on to cytochrome c_2 (E_{m7} 340 mV), located on the positive side of the membrane; the reactions occur with half-times of 300 and 200 μs, respectively. The second electron is transferred from heme b_L to heme b_H (E_{m7} 50 mV) which is supported to be located near the opposite site of the membrane (Q_c or Q_i site). The total reduction to quinol requires the uptake of two protons at the Q_c site. When measured at appropriate redox conditions (E_h about 100 mV), the slowest reactions of the cycle are the reduction of cytochrome b and re-reduction of cytochrome c that occur with similar kinetics ($t_{1/2} \approx 1$–2 ms).

Photosynthetic purple bacteria are also capable of aerobic respiration and, in the presence of oxygen, the cytochrome bc_1 complex is part of a linear electron transport chain of the mitochondrial type [10]. The bc_1 complex of *R. capsulatus* is composed of three subunits that are highly homologous with the corresponding polypeptides of the mitochondrial oxidoreductase [11]. Although the latter complex comprises several additional subunits, it is operationally identical to the prokaryotic enzyme. The bacterial bc_1 complex generates changes of proton motive force during electron transport from ubiquinol to cytochrome c when incorporated into liposomes [12]. A hybrid cyclic electron transport system has been obtained in solution with the reaction center from *Rhodobacter sphaeroides* and the bc_1 complex from mitochondria in the presence of detergents [13]. Generation of a protonmotive force induced by light was demonstrated when the hybrid system was incorporated into liposomes [14].

The availability of the methods to isolate the ATP synthase as well as the cytochrome bc_1 and the reaction center from the same species of photosynthetic bacteria suggested the possibility of reconstructing a completely homologous photophosphorylating system. Until now, reconstitution of photophosphorylation has been achieved by co-incorporation of the light-driven proton pump bacteriorhodopsin and the mitochondrial or bacterial ATP synthase into liposomes [15,16]. Photophosphorylation was also obtained by co-reconstitution of the Photosystem I and the CF_1CF_0 complex from chloroplasts, but the cyclic electron transport was completed with an artificial redox component such as phenazine methosulfate [17].

Here we report the successful co-reconstitution of three membrane complexes from *R. capsulatus* for photophosphorylation.

Materials and Methods

Purification of proteins

A green mutant of *R. capsulatus* [18,11] was grown photoheterotrophically and chromatophores were prepared therefrom according to Ref. 19. The cytochrome bc_1 complex was prepared according to Ref. 20 but modified by omission of cholate. Chromatophores were incubated in 2 M NaBr for 30 min on ice and at 0.3 mg protein/ml, to remove the ATPase. Membranes were then diluted 1 : 1 with 50 mM glycylglycine (pH 7.8) and sedimented by centrifugation. The cytochrome bc_1 complex was selectively solubilized from these membranes by addition of 1% octyl glucoside at 0 °C at a protein concentration of about 1 mg/ml and incubation for 20 min.

The RC-LH I complex was isolated from the non-solubilized membrane fraction above recovered by sedimentation. The pellet was resuspended in 50 mM glycylglycine (pH 7.8)/3% octyl glucoside/0.5% cholate, at about 10 mg protein/ml. After 10 min incubation on ice, the mixture was centrifuged for 1 h at 50000 rpm. The dark green supernatant was subsequently loaded onto six tubes (Kontron – TST 41) containing a linear sucrose gradient (10–40% w/v) in 1% octyl glucoside, 0.25% cholate and 50 mM glycylglycine (pH 7.8). After centrifugation at 36000 rpm for 19 h at 4 °C, the purified RC-LH I was collected from the gradient, frozen in liquid N_2 and stored at −20 °C. The cytochrome bc_1 complex and the H^+-ATPase were stored in the same way, but concentrated with a Centricon-30 (Amicon) to a protein concentration of 2 mg/ml before freezing.

The H^+-ATPase was prepared essentially as the bc_1 complex except that the NaBr wash of the membranes was omitted [21]. The cytochrome c_2 from *R. capsulatus* was purified by ion-exchange chromatography according to Ref. 22.

Preparation of proteoliposomes

Chloroform solutions (10 mg/ml) of L-α-phosphatidylethanolamine (Type V), L-α-phosphatidylcholine (Type IIIB) and L-α-phosphatidylglycerol (Sigma)

were mixed at a ratio of 2 : 1 : 1. Ubiquinone-10 (Sigma) was added to 5 ml of the lipid solution (50 UQ_{10} : 1 RC). After mixing with an equal volume of 50 mM glycylglycine (pH 7.8) and the addition of 1% octyl glucoside (final concentration), the chloroform was removed from the suspension in a rotary evaporator. The phospholipid-ubiquinone-10 suspension was further dispersed by sonication with the mini-tip of a Branson sonifier at maximal output for about 3 min, under argon and at 0 °C. A total of about 6 mg proteins (RC-LH I and bc_1) was then added to the suspension in the desired ratios. Proteoliposomes were prepared by detergent dialysis at 4 °C, against 100 vol. of 50 mM glycylglycine (pH 7.8)/5 mM $MgCl_2$/0.1 mM dithiothreitol for 18 h with three changes of buffer.

Cytochrome *c* from horse heart (Sigma type VI) or cytochrome c_2 from *R. capsulatus*, was then added and proteoliposomes were frozen in liquid N_2 in 1 ml portions and stored at −20 °C. Subsequently proteoliposomes were thawed slowly on ice and sonicated 2 s with the mini-tip of a Branson sonifier at medium output. Finally the proteoliposomes were sedimented by ultracentrifugation. The supernatant containing the soluble cytochrome was discarded and the pellet was washed and resuspended to the original volume in 50 mM glycylglycine (pH 7.8)/5 mM $MgCl_2$.

The H^+-ATPase (2–5 mg/ml) in 1% octyl glucoside and 30% sucrose was inserted into the liposomes in different amounts by detergent dilution. The concentrations of detergent used for incorporation were between 0.1 and 0.5%, which was below the critical micellar concentration of this detergent (0.9%). After 10 min incubation on ice, the RC-LH I, bc_1 and H^+-ATPase liposomes were diluted 10- to 30-times with the photophosphorylation mixture.

The proteoliposomes were layered onto a sucrose density gradient as described in Ref. 12 and centrifuged for 19 h at 300 000 × *g*; the protein concentration of the proteoliposomes which migrated as a single band was determined. The individual concentrations of the RC-LH I, cytochrome *c* and bc_1 complex were determined spectrophotometrically. The insertion of the H^+-ATPase into the proteoliposomes was nearly 100%.

Kinetic spectrophotometry

The kinetics of flash-induced redox changes of the cytochromes and reaction center were measured using a single-beam spectrophotometer with a maximal time resolution of 0.5 ms and a bandwidth of 1.5 nm as described in Ref. 4. Light flashes were provided by a xenon lamp and filtered through a Wratten 88 A filter, giving saturating flashes of 15 μs duration at half-maximal intensity. The signals were averaged utilizing an Olivetti M24 personal computer interfaced with a Data Lab DL 905 transient recorder. Measurements were carried out at 20 °C in 50 mM glycylglycine (pH 7.8)/20 mM KCl/0.5 mM sodium ascorbate/5 μM DAD/5 μM valinomycin in an open cuvette, without stirring.

Photophosphorylation

Photophosphorylation in continous light was measured as described in Ref. 19. The reaction was performed in 1.5 ml of 70 mM glycylglycine (pH 8)/10 mM $MgCl_2$/2 mM ADP with 50 μl of proteoliposomes. The redox poise of the assay was obtained with 0.15 mM sodium succinate or 0.5 mM sodium ascorbate and 5 μM DAD. The tubes were preincubated for 10 min at 30 °C, then 0.1 ml of 0.15 M $NaH_2{}^{32}PO_4$ (10^6 cpm) was added and the reaction was started by turning on a 150 W halogen tungsten lamp placed at a distance of 10 cm from the samples and passing through a red filter. After 10 min incubation at 30 °C, the reaction was stopped by adding 10% trichloroacetic acid. For each series of experiments, control samples were taken at zero time of incubation and after 10 min incubation in the dark. The tubes were centrifuged at low speed and the clear supernatant was assayed for organic radioactive phosphate according to Avron [23]. The photophosphorylation activity was calculated after subtraction of the dark reaction.

ATP synthesis following a short period of illumination was monitored by the luciferin-luciferase assay essentially as described in Ref. 24. The measurement was performed in 2 ml of 50 mM glycylglycine (pH 7.8)/10 mM magnesium acetate, 8 mM potassium phosphate/20 μM ADP/0.8 mM AMP/0.1% BSA/60 μM luciferin (Sigma)/ 0.5 mM sodium ascorbate/5 μM DAD which contained 1.2 mg of crude firefly lantern extract (Sigma, FLE-50) and 100–200 μl proteoliposomes. The bioluminescence was measured under continuous stirring using the photomultiplier of a dual-wavelength spectrophotometer which was protected with a Corning 4/96 blue filter; the actinic light was supplied by a collimated 55 W quartz-halogen lamp screened with two layers of Wratten 88A gelatin filter. The luminescence signals were calibrated by addition of an internal standard.

Other analytical methods

Protein concentration was determined according to Bradford [25]. SDS-PAGE was performed according to Laemmli [26]. The ubiquinol-cytochrome *c* oxidoreductase activity, was measured in an Aminco DW-2 spectrophotometer in 50 mM glycylglycine (pH 7.4) with 20 μM horse-heart cytochrome *c* as electron acceptor ($\varepsilon = 18\ mM^{-1} \cdot cm^{-1}$ at 550–540 nm) and 100 μM ubiquinol-2 added in ethanolic solution as electron donor. The reaction was started by addition of 0.05 nmol bc_1 complex.

Ubiquinone was extracted from the proteoliposomes as described in Ref. 27 and the concentration was determined spectroscopically in ethanol solution (dif-

ferential absorption coefficient, $\Delta\varepsilon = 12.5\ mM^{-1} \cdot cm^{-1}$ at 275 nm for the oxidized minus reduced form).

Cytochrome *b* and cytochrome c_1 content were estimated by difference spectroscopy, using a $\Delta\varepsilon$ of 20 $mM^{-1} \cdot cm^{-1}$ at 561 and 553 nm, respectively [20].

Reaction center concentration was measured with a Shimadzu UV-Vis recording spectrophotometer, using an absorption coefficient of 288 $mM^{-1} \cdot cm^{-1}$ at 800 nm [28].

Results and Discussion

Composition of the purified enzymes

The three membrane protein complexes that together catalyze cyclic electron transport and photophosphorylation were isolated separately from the same photosynthetic bacterial species, *R. capsulatus*. Selective solubilization of the H^+-ATPase from chromatophore membrane was obtained after a short incubation in 1% octyl glucoside. If the F_1-ATPase was removed by NaBr treatment, the bc_1 complex was solubilized by 1% octyl glucoside instead. The more hydrophobic RC-LH I complex was isolated by a second solubilization of the membrane fraction from which the bc_1 had been extracted.

The proteins were analyzed by SDS-PAGE and spectroscopy. As shown in Fig. 1 (lane A), the RC-LH I preparation included the three subunits of the photochemical reaction center, H (28.5 kDa), M (34.4 kDa) and L (31.5 kDa) and the two polypeptides forming the

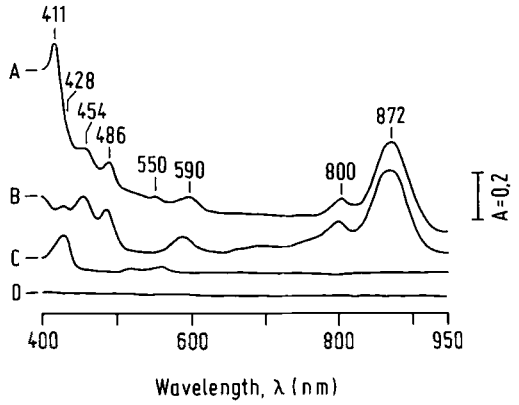

Fig. 2. Absorption spectra of the purified enzymes in proteoliposomes and in detergent micelles. (A) proteoliposomes (2 RC:1 bc_1:3 cytochrome c_2); (B) reaction center light harvesting complex 1; (C) bc_1 complex; (D) ATP synthase. The maxima are due to the following components: 872 nm, LH I; 800 nm, P800 from RC; 590 nm, bacteriochlorophyll *a*; 550 nm, α-peak of cytochrome *c*; 486, 454 and 428 nm, carotenoids; 411 nm, Soret band of cytochromes.

LH complex I (5.4 and 6.5 kDa) [29]. At the top of the gel aggregates of subunits L, M and presumably LH complex I were seen. The sample prepared in 1% octyl glucoside and 0.25% cholate retained the spectroscopic properties of the membrane bound complex as shown in Fig. 2, spectrum B [30]. The use of sodium cholate at low concentrations specifically stabilized the RC-LH I preparation. The bc_1 complex and the H^+-ATPase were isolated without cholate. The omission did not significantly affect their stability, but improved their purity.

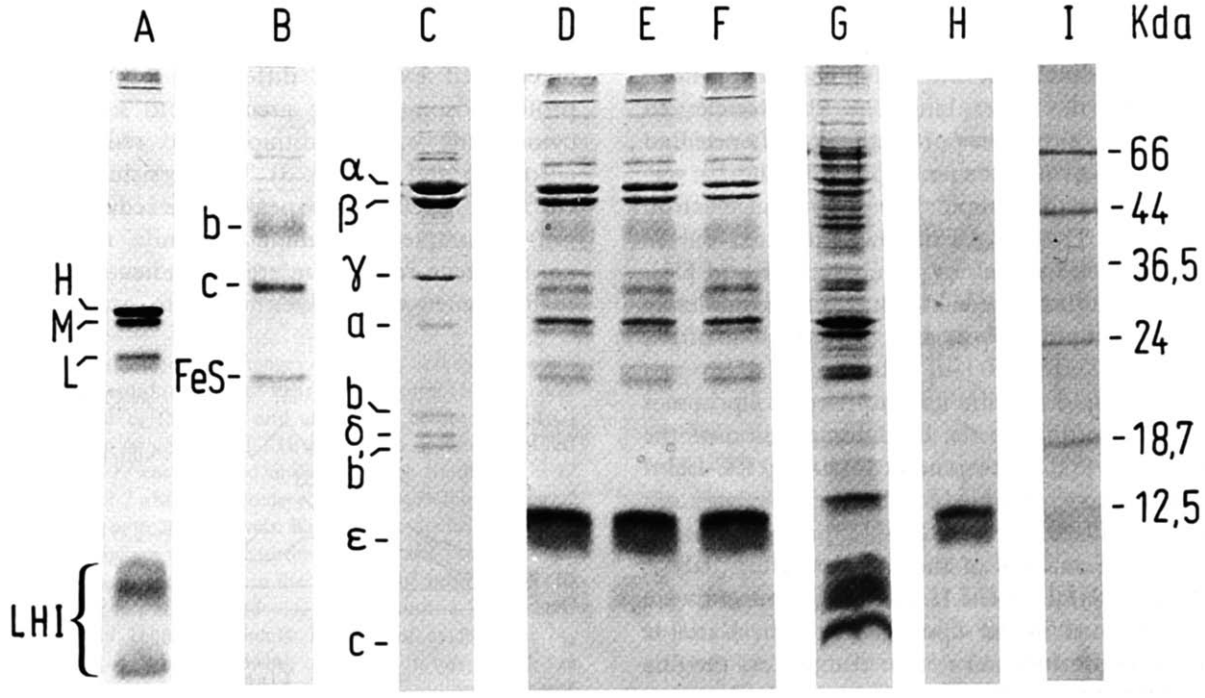

Fig. 1. Coomassie blue staining of a 15% acrylamide SDS gel showing the purified proteins before and after reconstitution in proteoliposomes. (A) RC-LH I (3 μg); (B) b/c_1 complex (2 μg); (C) ATP synthase (10 μg), the diffuse band of subunit c does not stain well with Coomassie blue; (D), (E), (F) proteoliposomes with constant ratio of RC/bc_1/cytochrome *c* (2:1:5 μg) and decreasing ATP synthase content (4, 2 and 1 μg, respectively); (G) chromatophores 36 μg; (H) cytochrome *c* from horse heart (1 μg); (I) molecular weight standard.

The cytochrome bc_1 complex is composed of the three catalytic subunits cytochrome *b* (48.1 kDa), cytochrome c_1 (30 kDa) and FeS protein (21 kDa) (Fig. 1, lane B). A 10 kDa polypeptide that copurified with the bc_1 complex when 0.25% cholate was used in addition to octyl glucoside was found to be the α-polypeptide chain of the LH complex I [11]. The absorption spectrum (Fig. 2C) shows that the purified bc_1 complex is free of photosynthetic pigments. The ubiquinol-cytochrome *c* oxidoreductase activity of the enzyme in detergent micelles, using UQH_2-2 as electron donor, corresponded to a turnover rate of 8 s^{-1} as previously reported [20].

The H^+-ATPase of *R. capsulatus* was recently characterized [21]. As shown in Fig. 1 (lane C) the enzyme is composed of 9 subunits, five of which form the water-soluble F_1 portion (α, β, γ, δ, ε). The four remaining polypeptides form the membrane-spanning part, F_0. Similar to the CF_0 of chloroplasts, the F_0 of this photosynthetic bacterial species includes four subunits. Two of these correspond to subunits a and c of the well-characterized *E. coli* F_0 and the other two are both homologous with subunit b. Low level contamination in the high-molecular-mass region can also be seen, one band in the 70 kDa region is an aggregate of subunit c. The purified ATP synthase is free of photosynthetic pigments (Fig. 2, spectrum D) and retains the ATPase and the ATP–P_i exchange activity [21].

Construction of the photophosphorylating system

For reconstruction of the active system, properties of chromatophores like lipid and coenzyme-Q composition as well as polarity of the lipid vesicles (positively charged inside) were preserved. The water-soluble cytochrome *c* was trapped in the inner lumen of the vesicles to mediate cyclic electron transport with properly oriented RC-bc_1 complexes. An adequate pool of UQ-10 was dissolved in the phospholipids, to shunt the electron transport chain. The H^+-ATPase was inserted in the performed proteoliposomes by detergent dilution [31]; we assumed that this procedure must have completely oriented the hydrophilic F_1 part on the outer side of the proteoliposomes.

The phospholipid mixture used to prepare liposomes was selected according to the lipid composition of the chromatophores [32]. Liposomes containing RC-LH I and bc_1 complex were formed by detergent dialysis at a protein/lipid molar ratio of 1 : 1000 [33]. As determined by spectroscopic analysis of the samples, after dialysis and centrifugation, the RC-LH I and bc_1 complex were completely inserted in the liposomal bilayer. Variable proportions of the two membrane complexes, ranging from 4 to 1 RC per bc_1 complex, could also be incorporated. Standard reconstitutions were performed with 2.5 μM bc_1 complex and 5 μM RC. About 80% of the UQ-10 added to the lipid (200 μM) was recovered after extraction of the proteoliposomes with petroleum ether. This confirmed that the proteoliposomes contained a ubiquinone pool of a size comparable to that present in chromatophores (about 30–40 UQ : 1 RC).

The water-soluble cytochrome *c* or c_2 was trapped within the inner volume of proteoliposomes by freezing and thawing. After removal of external cytochrome *c*, 20% was found associated with the proteoliposomes.

The incorporation of the ATP synthase was achieved simply by diluting the enzyme dissolved in 1% octyl glucoside in a suspension of proteoliposomes containing RC-LH I/bc_1. The best condition for reconstitution was found to be 10 min incubation in 0.1% octyl glucoside before the second dilution to the final detergent concentration (0.01%) was made. Prolonged incubation in detergent significantly decreased photophosphorylation. Variable amounts of enzyme could be inserted into the vesicles by this method (Fig. 1. lanes D, E, F) as measured by the increase in protein content of the proteoliposomes.

The absorption spectrum of the reconstituted system (Fig. 2, A) represents the absorptions of all components and light scattering from the liposomes.

The homogeneity of the proteoliposomes was analyzed on a 0.1–2 M sucrose density gradient [12]. The proteoliposomes were recovered as a discrete and sharp band at the 0.5 M sucrose position, well below liposomes, which were co-centrifuged as a control. The discrete band pattern of the proteoliposomes indicated that they had a homogeneous protein distribution.

Spectroscopic analysis

The orientation of the cytochrome bc_1 complex was determined by redox difference spectroscopy of the proteoliposomes using ascorbate to selectively reduce cytochrome c_1 and dithionite to reduce both cytochrome *b* and c_1 (Fig. 3). The persistence of the cytochrome c_1 spectrum (α peak of the reduced form at 553 nm) in samples containing dithionite, recorded against an ascorbate-reduced reference, indicated that the proteoliposomes were impermeable to ascorbate (Fig. 3,

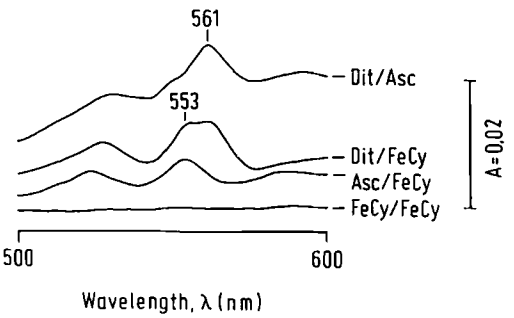

Fig. 3. Redox difference spectra of the bc_1 complex incorporated in lipid vesicles. The α peaks of cytochrome c_1 (553 nm) and of cytochrome *b* (561 nm) are indicated. FeCy, Acs and Dit stand for ferricyanide, ascorbate and dithionite, respectively.

Dit/Asc spectrum), but not to dithionite. Therefore the portion of cytochrome c_1 that remained oxidized after addition of ascorbate was considered to be located on the inner side of the vesicles. In several liposome preparations, this was estimated to be about 30–40% of the total cytochrome c_1.

The kinetics of oxidoreduction of the cyclic electron transport components in the proteoliposomes were analyzed in single-turnover experiments. The redox state of the system ($E_h \approx 100$ mV) was adjusted with ascorbate (0.5 mM) and low concentration of DAD (5 μM), to reduce the high-potential components of the electron transport chain while maintaining the cytochrome b hemes in the oxidized state. This redox poise provided maximal electron transport rates in chromatophores [9,34]. Proteoliposomes containing 2 mol RC and 3 mol cytochrome c_2 per mole bc_1 complex were used for the kinetic analysis shown in Fig. 4. This stoichiometry was similar to that usually found in chromatophore preparations [9].

The oxidation-reduction cycle of the special pair $(Bchl)_2$ in the RC was induced by a trains of 16 flashes and is shown in Fig. 4 (trace A). A rapid oxidation of the special pair is followed by a biphasic re-reduction. The fast phase corresponds to the re-reduction of RC complexes correctly oriented with respect to the cytochrome c_2 trapped in the vesicles. This reaction, which in chromatophores has a half-time of a few microseconds, was quicker than the resolution capabilities of our instruments; therefore, its amplitude was estimated from the maximal amount of RC photooxidized in the presence of the quinone analogue, UHDBT, following a train of 16 flashes (see Fig. 4, trace B). UHDBT inhibits the electron transport from the FeS center to cytochrome c_1 [35]. The slow phase of re-reduction was contributed by those RC complexes that could not react with cytochrome c_2. In this case reduction possibly took place through electron transfer back from the primary electron acceptor Q_B ($t_{1/2}$ about 1.5 s). The amplitudes of the two phases had a ratio of about 1, and from this it was calculated that the RC was randomly incorporated. Proteoliposomes lacking cytochrome c showed only the slow phase of RC re-reduction (not shown). The role of a soluble cytochrome c in mediating fast electron transfer between the RC and bc_1 complex appears to be essential in our reconstituted system, which is different from the suggestion that a direct intercomplex electron transfer can occur [36]. The exposure of $(Bchl)_2^+$ site to the external phase was confirmed by the prompt photooxidation of externally added cytochrome c (Fig. 4E), which was induced by a train of 16 flashes in a sample completely inhibited by antimycin A and UHDBT. The stepwise accumulation of oxidized cytochrome c at each flash confirmed the presence of a chemically active ubiquinone pool in the proteoliposomes [37] which was capable of storing a large number of electrons.

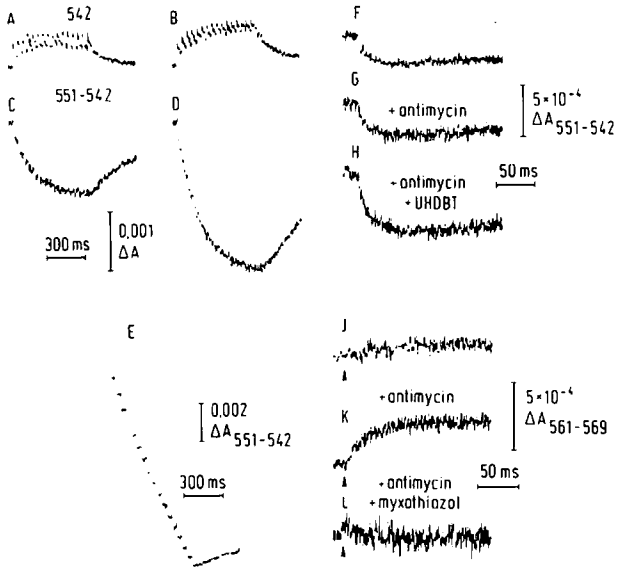

Fig. 4. Light-induced absorption changes of $(Bchl)_2$ and cytochromes. Changes in the absorption spectra of the reaction center (542 nm) and cytochromes c_1 and c_2 (551–542 nm) are shown before (A and C) and after addition of 40 μM UHDBT (B and D). 16 flashes were fired 40 ms apart. Trace E was recorded under the same conditions as D but after addition of 7 μM cytochrome c. Signals were the average of eight measurements with 20 s intervals. Kinetics of cytochrome $c_1 + c_2$ redox changes following a single turnover flash are shown in the absence of inhibitors (trace F) and in the presence of antimycin A (10 μM) and UHDBT (40 μM), respectively, traces G and H. The signals were the average of 16 measurements. Kinetics of cytochrome b_H redox changes (561–569 nm) following one single-turnover flash are shown in the absence of inhibitors (trace J), after addition of 10 μM antimycin A (trace K) and of 3 μM myxothiazol (trace L). Traces were the average of 32 measurements. Arrow indicates the time of the flash. The assay conditions are described under Materials and Methods.

The redox changes of cytochrome c_1 and c_2 following a train of 16 flashes are shown in Fig. 4C. The initial fast oxidation of ferrocytochrome c_2 and c_1 by $(Bchl)_2^+$ is followed by the establishment of a stationary state in which the Fe_2S_2 centers re-reduce the cytochromes. The amplitude of this ferricytochrome c_2 re-reduction is documented with trace D in Fig. 4, which was recorded after addition of UHDBT. The inhibitory effect of antimycin A and UHDBT on the rate of ferricytochrome c_2 re-reduction after a single flash is shown in traces F, G and H. As expected, approximately twice the amount of cytochrome c remained oxidized in the presence of the two inhibitors. The slow re-reduction rate of cytochrome c_1 and c_2 after one flash in proteoliposomes without added inhibitors (trace F) can be tentatively attributed to a competition for the cytochrome binding site on the bc_1 complex by reduced and oxidized cytochrome c_2. It is expected that the large excess of reduced cytochrome c (cf. traces F vs. D) will slow down re-reduction. This is also supported by the degree of oxidation of cytochrome $(c_1 + c_2)$ in the

TABLE I

Specific photophosphorylation activity of proteoliposomes and effect of specific inhibitors and uncouplers

In A the specific activity was calculated from the incorporation of ^{32}P in to ADP during 1 min of illumination; in B it was estimated from the luciferin-luciferase luminescence during 10 s of illumination. Inhibitors and uncouplers were added as specified in the text, photophosphorylation assays were performed as described in Materials and Methods; n.d., not determined.

	Photophosphorylation (nmol ATP/mg H^+-ATPase per min)		Residual activity (%)	
	A	B	A	B
Proteoliposomes	18	18	100	100
Antimycin A	5	6.5	28	34
Valinomycin (50 mM KCl)	0.9	n.d.	5	n.d.
Valinomycin (20 mM KCl)	n.d.	7.7	n.d.	43
Valinomycin + nigericin (20 mM KCl)	n.d.	3.6	n.d.	20
FCCP	0.5	n.d.	3	n.d.
Oligomycin	1.9	2.1	11	12
DCCD	0.2	n.d.	1	n.d.

steady-state level reached during a sequence of 16 flashes (trace C).

The kinetics of cytochrome b_H reduction could be observed in single flash mode only after addition of antimycin A that inhibits the re-oxidation of the cytochrome at the Q_i site (Fig. 4, trace K). In the absence of this inhibitor significant absorption changes were not detected (trace J). This indicated that fast re-oxidation of cytochrome *b* occurred at the Q_i site. In the presence of both myxothiazol, which specifically inhibits the reduction of cytochrome b_L at the Q_0 site [38], and antimycin A, the reduction of cytochrome *b* was completely blocked (Fig. 4, trace L). The half-time of cytochrome *b* reduction was about 18 ms. This was about 10-times slower than observed in chromatophores under optimal redox conditions. The kinetics described are, however, complex and could include slower phases due

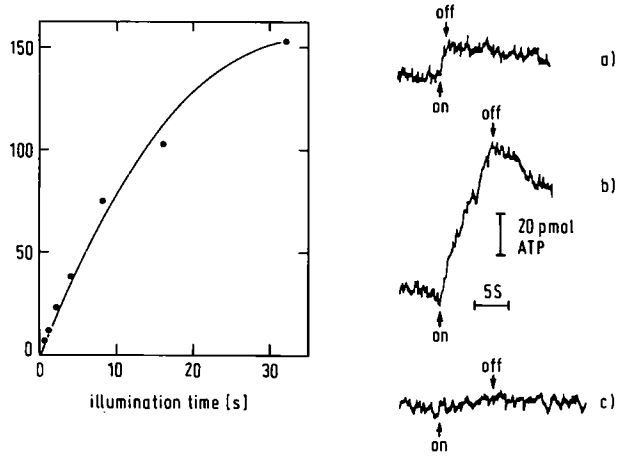

Fig. 5. ATP synthesis following a short period of illumination monitored by the luciferin-luciferase luminescence. A time course of ATP formation is shown. Examples of signals obtained are seen in: (a) after 1 s of light; (b) after 10 s; (c) as in (b) but in the presence of oligomycin (20 μg/ml). Proteoliposomes equivalent to 0.075 μM photooxidizable reaction center contained 25 μg ATP synthase, at a molar ratio of 0.5 : bc_1 complex and 0.25 : RC.

to the electron transport between the complexes which are misoriented and do not interact rapidly with cytochrome *c* trapped inside the vesicles. The turnover of bc_1 complex in steady-state conditions was 50 s^{-1} when measured in the dark with UQ_2H_2 as electron donor and in the presence of FCCP. This turnover rate was 6-times faster than that of the enzyme in detergent micelles and agreed well with the estimated half-time of cytochrome b_H reduction.

Photophosphorylation activity of the reconstituted system

The light-driven ATP synthesis during a short illumination period was measured in proteoliposomes of the type used for the kinetic analysis described above. ATP synthesis could be detected by luciferine luminescence after 1 s illumination (Fig. 5, trace A). The rate of photophosphorylation was linear during the first 10 s (trace B), after this period proteoliposomes had synthesized 75 pmol ATP (Fig. 5). The effect of the ATP synthase inhibitor oligomycin on photophosphorylation is also shown (trace C). The specific activity in the linear range of time dependence is 18 nmol ATP/min per mg H^+-ATPase (Table I) measured at an ADP concentration of 20 μM which is the K_m value of the ATP synthase.

The photophosphorylation at times longer than 1 min could be detected by monitoring the synthesis of radioactive ATP at 2 mM ADP, and at pH 8. These conditions provided good rates of photophosphorylation in chromatophores [19]. Liposomes containing RC-LHI, bc_1, H^+-ATPase and cytochrome *c* at molar ratios of 2 : 1 : 1 : 20 which are shown in Fig. 1, lane D, were tested. The amount of cytochrome *c* present was kept significantly higher than in chromatophores, in order to obtain a fast redox coupling between properly oriented bc_1 and RC complexes. As shown in Fig. 6, the reconstituted system could synthesize ATP for at least 15 min of illumination, but with a non-linear time dependence.

The specific activity of the system in this assay, during the first minute illumination, was again 18 nmol ATP/mg H^+-ATPase per min.

Extrapolation of the value obtained by the luciferase method at the V_{max} is 36 nmol ATP/min per mg. This rate is about 3% of the photophosphorylation activity measured in chromatophores expressed per ATP synthase (based on a 4% ATP synthase content of chromatophores) and about 2% of the photophosphorylation activity of chromatophores expressed per Bchl (assuming 100 Bchl per photosynthetic unit).

The mitochondrial ATP synthase co-reconstituted with bacteriorhodopsin into liposomes of a phospholipid composition similar to that used in this study, synthesized 80 nmol ATP/min per mg [15]. Thus the photophosphorylation activity of the two systems is comparable, since mitochondrial ATP synthases are more active than the bacterial ones [16]. A reason for the much lower activity of artificial systems compared to natural ones is not known and requires experimental investigation.

Proteoliposomes containing RC at molar ratios of 4, 2 and 1 per mol bc_1 complex were supplemented with different concentrations of H^+-ATPase ranging from 0.1 to 1 mol per mol bc_1 complex (see Fig. 1, lanes F, E, D) respectively. The three sets showed similar rates of photophosphorylation when normalized to the same H^+-ATPase concentration. One example is shown in Fig. 7 and demonstrates that the rate of photophosphorylation increases proportionally with the amount of H^+-ATPase incorporated. This indicated that the electron transport reactions were not limiting the overall process.

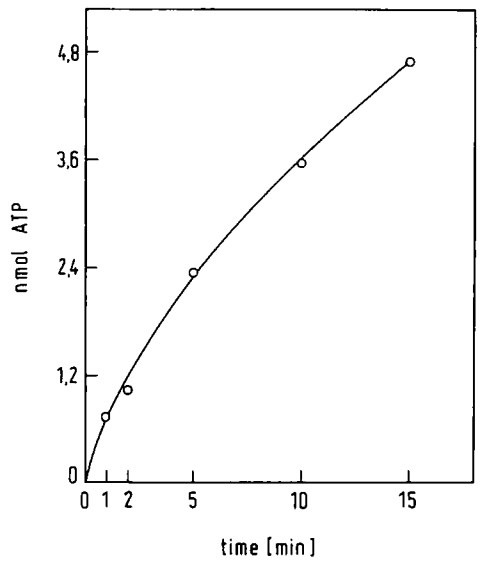

Fig. 6. ATP synthesis following illumination times longer than 1 min. The time-course of ATP formation was calculated from the amount of radioactive organic phosphate produced, as described in Material and Methods. Proteoliposomes contained 50 μg ATP synthase, in a molar ratio of 1 : 1 with the bc_1 and 0.5 with the RC.

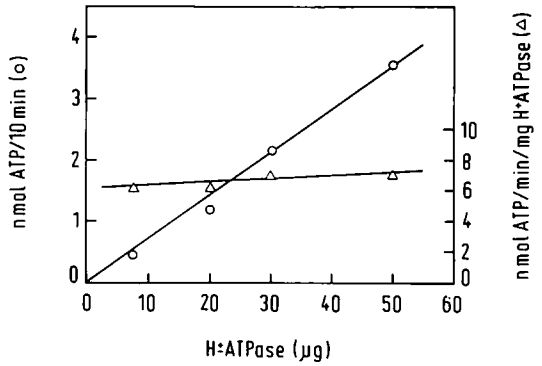

Fig. 7. Photophosphorylation activity of proteoliposomes in relation to the ATP synthase content. The net ATP synthesis after 10 min illumination is shown as well as the specific ATP synthase activity at each enzyme concentration. The proteoliposomes had a fixed stoichiometry of RC to bc_1 complex (4 : 1).

The effect of various inhibitors on photophosphorylation by proteoliposomes is summarized in Table I.

About 30% activity was retained when the bc_1 complex was inhibited by antimycin A (10 μM). Reconstitution experiments of RC-LHI and ATP synthase were performed as a control. Liposomes lacking the bc_1 complex showed photophosphorylation rates equivalent to those obtained with liposomes containing the bc_1 complex inhibited by antimycin A. The nature of the antimycin insensitive photophosphorylation is presently under investigation. In chromatophores it was shown that high concentrations of DAD (100 μM) are required to bypass the antimycin A block [39]; therefore, it is unlikely that the concentration of DAD (5 μM) used to adjust the redox potential in the photophosphorylation assay can bypass the antimycin A block in this system. It is likely that that at redox potentials of about 100 mV and in the presence of a large pool of UQ-10 and ferrocytochrome *c*, the photochemical activity of the RC can generate a membrane potential difference sufficient to drive ATP synthesis [40]. In agreement with this suggestion, the synthesis of ATP was detected in chromatophores inhibited by antimycin A in single-turnover flash measurements [41].

A strong inhibition by valinomycin (5 μM) in combination with 50 mM KCl was observed, suggesting that the electrical component was the major contributor to the formation of the transmembrane electrochemical potential difference in this system. Combinations of valinomycin and of the protonophore nigericin at 20 mM KCl also inhibited photophosphorylation strongly. Also the uncoupler FCCP at 1 μM and the two H^+-ATPase-specific inhibitors DCCD at 0.1 mM and oligomycin at 10 μM eliminated activity.

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