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Mimicking respiratory phosphorylation using purified enzymes

Christoph von Ballmoos [a,*,1], Olivier Biner [a,2], Tobias Nilsson [b,2], Peter Brzezinski [b]

[a] Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland
[b] Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden

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ABSTRACT

The enzymes of oxidative phosphorylation is a striking example of the functional association of multiple enzyme complexes, working together to form ATP from cellular reducing equivalents. These complexes, such as cytochrome *c* oxidase or the ATP synthase, are typically investigated individually and therefore, their functional interplay is not well understood. Here, we present methodology that allows the co-reconstitution of purified terminal oxidases and ATP synthases in synthetic liposomes. The enzymes are functionally coupled via proton translocation where upon addition of reducing equivalents the oxidase creates and maintains a transmembrane electrochemical proton gradient that energizes the synthesis of ATP by the F_1F_0 ATP synthase. The method has been tested with the ATP synthases from *Escherichia coli* and spinach chloroplasts, and with the quinol and cytochrome *c* oxidases from *E. coli* and *Rhodobacter sphaeroides*, respectively. Unlike in experiments with the ATP synthase reconstituted alone, the setup allows in vitro ATP synthesis under steady state conditions, with rates up to 90 $ATP \times s^{-1} \times enzyme^{-1}$. We have also used the novel system to study the phenomenon of "mild uncoupling" as observed in mitochondria upon addition of low concentrations of ionophores (e.g. FCCP, SF6847) and the recoupling effect of 6-ketocholestanol. While we could reproduce the described effects, our data with the in vitro system does not support the idea of a direct interaction between a mitochondrial protein and the uncoupling agents as proposed earlier.

1. Introduction

Proton translocation across biological membranes plays a major role during energy conversion from carbon sources to the universal energy carrier ATP. The highly exergonic hydrolysis of ATP to ADP drives numerous reactions in living cells. According to Peter Mitchell's chemiosmotic theory, reducing equivalents that are accumulated as a result of cellular metabolism are converted into a transmembrane electrochemical proton gradient, which is subsequently utilized by the F_1F_0 ATP synthase to generate ATP from ADP and inorganic phosphate. While the F_1F_0 ATP synthase is found in almost all organisms and its basic mechanism is universally conserved, the enzymes generating the transmembrane electrochemical gradient vary greatly between species. For example, the halophilic archaeon *Halobacterium salinarium* employs the light-driven proton pump bacteriorhodopsin while phototrophic bacteria or plants use photosynthetic reaction centers to charge the membrane. Mitochondria or aerobic bacteria employ a series of H^+-pumping respiratory-chain complexes to charge the membrane for ATP synthesis in a process termed oxidative phosphorylation (for review, see [1–5]). One class of these respiratory complexes is the terminal oxidases, which catalyze the reduction of oxygen to water by quinol or cytochrome *c*, and use part of the free energy released in this reaction to pump protons across the membrane. Terminal oxidases are relatively well characterized and understood in structural and functional details, however, far less is known about the molecular interplay between these enzymes and the ATP synthase.

In an ideal system the membrane is impermeable to protons to ensure a tight coupling between these two reactions, minimizing energy loss during the conversion process. It has been suggested, however that a weak uncoupling activity in mitochondria could be desirable under resting state conditions, where reducing equivalents are high (high NADH) and ATP consumption is low [6]. Under these conditions, the mitochondrial membrane would hyperpolarize and free electron carriers and oxygen could react together to form reactive oxygen species. Membrane hyperpolarization and reactive oxygen species have been considered key factors in degenerative processes as aging and apoptosis [7–10]. While a protein-mediated uncoupling mechanism (UCP1) in brown adipose tissue is well known to create heat during animal hibernation [11], the process might also have a more general role in all tissues for the reasons mentioned above. Regulation by fatty acids and thyroid hormones has been proposed, but the precise mechanism is unclear [8,12–14]. In this matter, classical uncoupling agents like FCCP and SF6847 at low concentrations have been shown to produce a

Abbreviations: FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; Q_1, ubiquinone Q_1; ACMA, 9-amino-6-chloro-2-methoxyacridine; cytc, cytochrome *c*.

* Corresponding author.
E-mail address: christoph.vonballmoos@dcb.unibe.ch (C. von Ballmoos).
[1] Present address.
[2] Contributed equally to the manuscript.

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similar effect that generally is termed "mild uncoupling". Under these conditions, the low dosages of uncoupling agents are sufficient to stimulate respiration (through a proton leak) without the complete abolishment of ATP synthesis activity.

To investigate the questions discussed above, either whole organelles (mitochondria, chloroplasts) or inverted membrane vesicles from bacteria were employed to study coupled enzyme function. While these systems have advantages (correct enzyme orientation, high density protein content), they share the common drawback of not being fully characterized or controllable. For example, many other enzymes are present in these membranes that are also sensitive to the electrochemical proton gradient, the lipid composition of the membrane cannot be controlled and analysis of mutant variants in more than one enzyme type is cumbersome. Very few examples of co-reconstituted isolated respiratory enzymes and ATP synthases are known. A notable exception is the co-reconstitution of an ATP synthase and the archaeal light-driven proton pump bacteriorhodopsin. The experiment was pioneered by Racker and Stoeckenius in 1974 with bacteriorhodopsin and a fraction of the mitochondrial membrane [15] and was crucial for the general acceptance of Mitchell's chemiosmotic theory. The system was revived in the mid-90s by Rigaud and colleagues with the purified ATP synthase from a thermophilic bacillus PS3, performing extensive investigations on reconstitution conditions and energy requirements for ATP synthesis [16,17]. In all these experiments, however, ATP synthesis rates were rather low, usually <5 ATP × s^{-1} × $enzyme^{-1}$, which is far below those measured with whole organelles (e.g. >180 ATP × s^{-1} × $enzyme^{-1}$ for the ATP synthase of *Escherichia coli*) [18].

Here, we present the successful co-reconstitution of purified terminal oxidases from *E. coli* (bo_3) and *Rhodobacter sphaeroides* (aa_3) with the purified ATP synthases from *E. coli* or spinach chloroplasts. After addition of an electron source to initiate proton pumping by the oxidase, ATP synthesis was observed at rates up to 90 ATP × s^{-1} × $enzyme^{-1}$ and could be driven under steady-state conditions as long electrons and O_2 are available. We describe the relevant parameters of the setup and utilize it to investigate the impact of mitochondrial uncoupling agents on this minimal system mimicking oxidative phosphorylation.

2. Materials and methods

2.1. Chemicals

Bovine cytochrome *c* and general chemicals were purchased from Sigma-Aldrich, if not otherwise indicated. CCCP and FCCP were from Santa Cruz Biotechnology. SF6847, valinomycin, nigericin and 6-ketocholestanol were purchased from Sigma-Aldrich. All inhibitors were dissolved in anhydrous EtOH. The luciferin-luciferase assay (CLS II) was purchased from Roche-Chemicals. Soybean lipids, Type II-S and 95% PC content were purchased from Sigma-Aldrich and Avanti Polar Lipids, respectively.

2.2. Purification of membrane proteins

The *E. coli* ATP synthase containing a His-tag at the β-subunit was purified as described [19]. Purification of ATP synthase from spinach chloroplasts was performed as described [20]. The *E. coli* bo_3 quinol oxidase was expressed with plasmid pETcyo, containing the sequence for oxidase subunits I–IV, with a His-tag at the C-terminus of subunit II, in strain BL21 (ΔcyoABCDE) in LB medium, and induced with 0.5 mM IPTG, when the culture reached OD_{600} = 0.5–0.6 [21]. The aa_3 oxidase from *R. sphaeroides* was purified as described [22]. For all protein preparations, droplets of the purified enzyme (~30 μl) were snap frozen in liquid nitrogen and stored at −80 °C to avoid repeated freeze–thaw cycles.

2.3. Liposome preparation

Soybean lipids (95% PC) (10 mg/ml) were extensively re-suspended under nitrogen atmosphere in a buffer composed of 20 mM Hepes, pH 7.5, 2.5 mM $MgCl_2$, 25 g/l sucrose (buffer A) by vortexing until they appeared as a homogeneous suspension. Subsequently, the lipids were frozen in LN_2 and thawed in water (30 °C) and vortexed for 10 s. This procedure was repeated 5 times, yielding unilamellar liposomes. The suspension was then extruded using a pore diameter of 200 nm to obtain a homogeneous liposome preparation. As an alternative to the freeze/thaw procedure, subsequent extrusion procedures with 800 nm and 200 nm membranes were performed, yielding similar results.

2.4. Co-reconstitution of ATP synthase and terminal oxidase

Typically, an amount of 480 μl liposome suspension (10 mg/ml) was mixed with 15 μl Na-cholate (from a 20% stock solution, final ~0.6%) to destabilize the liposomes. Proteins were added at desired concentrations from stock solutions and the mixture was incubated for 30 min at room temperature with occasional gentle shaking. The mixture was then applied to a prepacked Sepharose G-25 column (PD-10, GE Healthcare) equilibrated with 25 ml buffer A (see above). Subsequently, 2.4 ml buffer A was added, before the liposomes were collected with a final addition of 1.3 ml buffer A. If not otherwise stated, the obtained proteoliposomes were used directly.

2.5. Determination of concentration

Phosphorus concentration measurements in liposome suspensions were performed as described [23]. A standard curve was prepared for every set of measurements using a standardized phosphorus solution (Sigma).

2.6. Determination of liposome size distribution

The size distribution of liposome samples before and after reconstitution was assayed by Tunable Resistive Pulse Sensing (TRPS) with a qNano device (Izon Science, UK). Liposome samples were diluted into measurement buffer (20 mM Hepes, 50 mM KCl) and applied on top of a stretchable pore (NP200) and traces with at least 1000 particles were recorded and averaged. Calibration was performed with carboxylated polystyrene beads (200 nm). Data analysis was performed with the Izon Control Suite (Izon Science).

2.7. Proton-uptake measurements with ACMA

Sixty μl of proteoliposomes was diluted into 1.5 ml of a buffer composed of 20 mM Hepes–KOH, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl (HMK buffer), mixed with 2 μM 9-Amino-6-Chloro-2-Methoxyacridine (ACMA) and stirred in a 5 ml fluorescence cuvette until a stable baseline was obtained. Proton pumping was initiated by the addition of 2 mM Na-ATP for the ATP synthase or 2 mM DTT/20 μM ubiquinol Q_1 for the bo_3 oxidase. After the reaction had reached an equilibrium, the proton gradient was dissipated upon addition of 10 mM NH_4Cl. Changes in the fluorescence signals were monitored on a Cary Eclipse, using 410 nm and 480 nm as excitation and emission wavelengths, respectively. The slits were set at 5 nm.

2.8. ATP synthesis measurements

Typically, 20 μl of proteoliposomes was added to 470 μl of a buffer composed of 20 mM Tris–PO_4, pH 7.5, 5 mM $MgCl_2$, and supplemented with 0.8 mM ADP (2 μl from a 20 mM stock solution) and 15 μl CLS II luciferase/luciferin solution (10 mg/ml of powder in ddH_2O). If the proteoliposomes contained bo_3 oxidase, 2 mM DTT (from 1 M stock)