New perspectives on photosynthetic phosphorylation in the light of a torsional mechanism of energy transduction and ATP synthesis

Sunil Nath · Ravikrishnan Elangovan

Abstract New perspectives on photophosphorylation have been offered from the standpoint of the torsional mechanism of energy transduction and ATP synthesis. New experimental data on the involvement of malate anions in ATP synthesis in an acid-base malate bath procedure has been reported on spinach chloroplast thylakoids as the model system. The data cannot be reconciled with the chemiosmotic theory but has been shown to be naturally explained by the torsional mechanism. The path of malic acid in the acid and base stages of the experiment has been traced, offering further strong support to the new paradigm. Classical observations in the field have been re-interpreted in the light of these findings. A new concept of ion translocation, energy transduction and coupling at the overall physiological level in photophosphorylation has been presented and a large number of novel experimentally testable predictions have been made and shown to arise as logical consequences of the new perspectives.

S. Nath (✉) · R. Elangovan
Department of Biochemical Engineering and Biotechnology,
Indian Institute of Technology, Delhi
Hauz Khas,
New Delhi 110016, India
e-mail: sunil_nath_iit@yahoo.com

S. Nath
e-mail: sunath@dbeb.iitd.ernet.in

Introduction

The molecular mechanism of photosynthetic phosphorylation has inspired an immense amount of research since the pioneering discovery by Arnon and coworkers more than half a century ago that isolated chloroplasts could make ATP in a light-dependent way (Arnon et al. 1954a, b). Yet, amazingly, despite the tremendous amount of experimental and theoretical work, the mechanistic details of the biological energy transduction in photophosphorylation are still not completely understood.

The results of the classical photophosphorylation experiments have generally been interpreted within the framework of the chemiosmotic theory (Mitchell 1966). However, the original landmark experiments also revealed a puzzling anion requirement (Jagendorf and Uribe 1966; Jagendorf 1967) that could not be satisfactorily explained by the chemiosmotic theory. These longstanding problems at the core of such a fundamental physiological process as photophosphorylation were never completely resolved.

One of the current authors has formulated and developed in detail a novel alternative paradigm of ATP synthesis that has become known as the torsional mechanism of energy transduction and ATP synthesis whose realization celebrated its tenth anniversary (Channakeshava 2011; Nath 2010a; Nath 2010b; Villadsen et al. 2011). In this alternative mechanism, both protons and anions (or countercations) play essential roles in the process of ATP synthesis in oxidative and photosynthetic phosphorylation. Recently, using powerful systems biology and engineering approaches, the rationale has been advanced as a unifying concept in bioenergetics and motility through the detailed formulation of the unified theory of ATP synthesis and hydrolysis (Nath 2008; Nath and Nath 2009; Nath 2010b). In this article, the original proposals and their logical

development have been shown to resolve certain fundamental issues in biological energy transduction in general and photophosphorylation in particular and to offer new ways of analyzing, interpreting and unifying the vast amount of experimental data available. The new theory has been shown to make a wealth of experimentally testable predictions. It is hoped that these will prove to be interesting and useful to the large number of experimentalists working in plant biochemistry and biophysics, plant physiology, photosynthesis, and other sub-fields of bioenergetics research.

Experimental methods

The experimental procedures followed for monitoring acid-base transition in chloroplast thylakoids from spinach have been described earlier (Nath 2004; Nath 2008), with the difference that malic acid at the desired concentration was the acid selected for study in this work. The enzymatic procedure for the assay of malic acid described in the literature (Matsuki and Abe 2000; Peres et al. 2008) was adapted and optimized for use and found to yield reliable measurements. Various methods to quench the reaction were tested and subsequently used. In a chemical method, 0.2 ml of 20% (w/v) trichloroacetic acid (TCA) was added to 1.8 ml of the reaction mixture and then centrifuged at 7200g for 10 min at 0°C and a clear supernatant for further analysis was obtained. In physical methods, e.g. micro-centrifugation quenching, the reaction mix was centrifuged at the conditions above and the supernatant collected for analysis. In rapid liquid N_2 quenching with simultaneous centrifugation, the aliquots containing the reaction mix were dipped in liquid nitrogen, taken out after a few seconds and immediately centrifuged without allowing the mix to liquefy. The reaction mix was centrifuged at 30,000g for 15 min at 0°C and the supernatant collected for further analysis. In liquid N_2 quenching with delayed centrifugation, a similar procedure was followed with the exception that the reaction mix was taken out and allowed to be liquefied before centrifugation. In ammonium chloride quenching, a procedure adapted from Smith and Boyer (1976), the acid-base phosphorylation reaction was quenched by adding an equal volume of 0.l5 M NH_4Cl and 50 mM Tris-HCl, pH 8.0 to the reaction mixture.

Detailed description of events occurring during the acid-base malate bath procedure according to the torsional mechanism of energy transduction and ATP synthesis

In the malate bath procedure, which is a modification of the classical acid bath procedure of Jagendorf and Uribe (1966), the isolated spinach thylakoid membranes are subjected to two shocks (Nath 2004; Nath 2008). In the first step, the membranes (pH 7.5) are incubated with buffer of pH 4.5 containing an acid (malic acid in this study) of desired concentration used to investigate the effect of a permeant anion on ATP synthesis. The time for which the membranes are incubated with this acid is termed the acid stage (AS) time. In this stage, proton and anion are allowed to accumulate inside the lumen leaving behind a residual concentration of both species (or of HA) in the external (stromal) medium. In the second stage, after the desired period of acid stage incubation, these ion-loaded membranes are transferred into a base stage buffer of pH 8.3 containing Mg^{2+}, ADP and P_i for the phosphorylation reaction. The time for which the membranes were incubated with the base stage buffer is called the base stage (BS) time. After a desired time of base stage incubation, the reaction is quenched with 20% (w/v) trichloroacetic acid (TCA) or by other physical/chemical methods as given in the Experimental methods section and a profile of the rate of ATP synthesis as a function of the acid concentration for a particular AS and BS time is plotted. It should be noted that membrane-permeant anions (or countercations) are present in every ATP synthesis protocol to date; however, their biological role has not been proposed earlier, and results are still interpreted solely based on the role of protons.

In this study, experiments were conducted on spinach thylakoid membranes as the model system. The chlorophyll content was determined and kept approximately 0.5 mg ml^{-1}. A fixed amount of thylakoid suspension (0.5 ml) with the above chlorophyll content was exposed to 0.4 ml of malic acid buffer (pH 4.5) of desired concentration in the acid stage and ensured the same number of F_1F_O-ATP synthase molecules in each sample. After a specific time of incubation in AS, a total of 0.9 ml of membrane suspension (pH ~4.5) was transferred into 0.9 ml of BS buffer (pH 8.3) and phosphorylation monitored for a BS time. This 1:1 dilution resulted in the establishment of proton and anion electrochemical gradients (i.e., the overall driving force was $\Delta\tilde{\mu}_H + \Delta\tilde{\mu}_A$). The reaction was quenched by a rapid injection of 0.2 ml of 20% (w/v) TCA or by other methods, bringing the total volume to 2 ml. Due to proton accumulation, the pH of the lumen becomes nearly 4.5 and transfer into a buffer of pH 8.3 resulted in the development of a proton gradient. However, it should be emphasized that transition from AS to BS stage also caused an *anion* gradient to be established across the membrane. It should also be noted that the pH jump was kept constant in all cases irrespective of the concentration of the malic acid employed. Discrete translocation of anion and proton therefore occurred along their respective concentration gradients (ΔpA and ΔpH). On the other hand, an ATP synthesis mechanism driven *solely* by ΔpH has been

reported for the chloroplast ATP synthase, ever since the original publication of Jagendorf and Uribe (1966) 45 years ago, which historically has been considered to offer key evidence in favour of the chemiosmotic theory (Mitchell 1966). However, according to the torsional mechanism, both anion and proton are translocated from the lumen to the stroma through the F_O portion of the F_1F_O-ATP synthase complex (through the a-subunit and c-subunit access channels at the a-c interface respectively, where the anion is primary in the direction of ATP synthesis and generates a local electric potential, $\Delta\psi$, and hence *both* ΔpH and $\Delta\psi$ are created by different agents, and are essential and kinetically nonequivalent driving forces in ATP synthesis) located in the stroma lamellae or in the end membranes and margins of the grana in chloroplasts. Proton and anion are returned into the lumen through the coupled operation respectively of cytochrome b_6f and photosystem I in stroma lamellae/end membranes or respectively through cytochrome b_6f and photosystem II in the grana during the light reactions. Thus the electrogenic transport individually of anion and proton was characterized as electroneutral in the overall sense, and the ordered and sequential mode of ion transport involving anions and protons was termed as dynamically electrogenic but overall electroneutral (Nath 2002; Villadsen et al. 2011). The energy stored in the ion gradients was released and transmitted from the ion binding sites in the access channels in F_O to synthesize ATP in the F_1 portion at the catalytic binding sites by a detailed torsional mechanism (Nath et al. 1999; Nath and Jain 2000; Nath 2002; Nath 2008; Nath and Nath 2009).

ATP rates as a function of malic acid concentration

Figure 1 illustrates the results of phosphorylation experiments on spinach chloroplast thylakoids by use of the acid-base malate bath procedure described in the previous section. It shows the variation of the rate of ATP synthesis as a function of the concentration of malic acid in the acid stage. The time of incubation was set at 30 s during the acid stage for all concentrations of malic acid buffer employed. The base stage phosphorylation time measured 30 s in all cases. Conditions with respect to the proton were ensured to be constant for all the measurements. Thus the external pH measured 8.3 for all points and the internal pH was 4.5 or less at increasing acid concentrations from 2 mM to 12 mM. During the fixed incubation time of 30 s, the transport of increasing amounts of acid at higher buffer concentrations can only function to decrease the internal pH, thereby enhancing ΔpH during the phosphorylation in the base stage. This ΔpH is postulated to constitute the sole driving force in chloroplasts according to the chemiosmotic theory (Mitchell 1966). It should be stressed that even if the
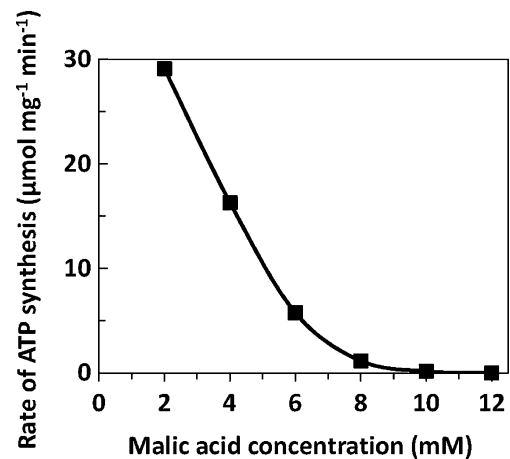

Fig. 1 Relationship of flux (rate) vs. overall driving force in spinach thylakoids. Rate of ATP synthesis as a function of the malate concentration (pH=4.5) in the acid stage of the acid-base malate bath procedure. The time of incubation in the acid stage was kept constant at 30 s, and the phosphorylation rate was measured after 30 s in the base stage in all cases. The pH of the external (stromal) medium was controlled at 8.3 in the base stage in all the experiments

$\Delta\psi$ contributes, it can only increase with increased proton permeation at higher acid concentration, or at most stay constant, since the H^+ alone is presumed to be responsible for providing both ΔpH and $\Delta\psi$ components of the overall protonmotive driving force in the chemiosmotic framework. Thus the presumed driving force (the so-called "protonmotive force") has increased or at most stayed constant with increasing malic acid concentration; yet the rate of ATP synthesis is found to progressively decrease with increasing acid concentration (Fig. 1), a result that could not be fitted into chemiosmotic dogma since an inherently anti-chemiosmotic result/trend is shown by the experiments.

As mentioned above, the results shown in Fig. 1 cannot be explained on the basis of the chemiosmotic theory in which the energy of anion translocation has no role to play in coupling and the energy donation and transduction process of making ATP, and protons alone are hypothesized to provide the driving force responsible for ATP synthesis. However, the pH was kept constant throughout the experiment (pH 4.5 in the acid stage and pH 8.3 in the base stage), and hence the driving force inherently attributable to protons, including any $\Delta\psi$ created by the protons, has not been altered (and has for certain not decreased). Thus, according to chemiosmosis, the rates of ATP synthesis should not have decreased with increasing anion concentration, other things being the same. However, if we consider the concentration gradients of anions and protons during ATP synthesis in the base stage as overall driving forces, as quantitatively modeled by the torsional mechanism (Jain and Nath 2000), we expect the rate to be higher at lower acid concentrations as the concentration

gradient of anions is forced to be kept higher in the malate bath procedure at lower acid concentrations (for fixed BS times) than at higher acid concentrations. Because of this increased driving force due to anions at lower buffer concentrations, the rate of ATP synthesis is higher, i.e. the ATP synthase enzyme molecules turn over more times during the fixed BS time, in agreement with the experimental observations (Fig. 1). It should be stressed that the number of free protons in a thylakoid is extremely small (< 1 at neutral pH) and hence it was problematic to imagine how these protons could drive ATP synthesis. The large capacity for anion and proton translocation revealed by chloroplasts can only be realized if these translocated ions originate from the acid buffer itself, i.e., from HA. In other words, H^+ can be interpreted to be bound by A^-, and HA is distributed within the stromal and lumenal spaces of the chloroplast thylakoid.

The results of the flux-force experiments in this article using malate as the permeant ion (Fig. 1) and earlier with other permeant anions (Nath 2004; Nath 2008; Agarwal 2011) could only be interpreted based on different anion gradients generated at the different acid concentrations used. When the membranes were exposed to a low concentration of acid (e.g. ~2 mM), a large quantity of ions was accumulated inside the lumen during the acid/light stage and only a small amount (and hence a low concentration) was left behind in the stroma. In the presence of high concentration of acid (> 8 mM), the amount left in the stroma was higher. Though 1:1 dilution with the base stage buffer caused ion gradients to develop, the gradients thus formed in the presence of high acid concentration were still lower as compared to the gradients that were established in the presence of low acid concentrations used in the acid stage. Thus a higher rate of phosphorylation was observed at a lower acid concentration in the acid stage. In vivo, a nonequilibrium steady state was maintained because the photosystems, the cytochromes and the F_1F_O molecules were operating together. On the other hand, in vitro, due to the separation of the photosystem/light/acid and ATPase/dark/base stages, the ion gradients formed are run down and thus provide the overall driving force for the process of ATP synthesis as they determine the number of times proton and anion are translocated discretely through their respective access channels in F_O. In these experiments, ΔpH and ΔpA are a measure of the gross driving forces that lead to and can be correlated with the enhanced elementary vectorial translocation of ions across the membranes and thus in turn to increased rates of phosphorylation, i.e. the macroscopic ΔpH and ΔpA determine the number of times the elementary translocation of protons and anions respectively take place in the thylakoid model system.

Tracing of the path of malic acid during the acid-base malate bath procedure

The above-mentioned separation of the light/acid and dark/base stages is a unique property of the plant chloroplast system; such a separation of the oxidation and phosphorylation stages is very difficult to achieve in animal mitochondria and in bacterial systems. This property of separation of the two stages in photophosphorylation can therefore be profitably employed for the design of further experiments to study the decay and build-up of the acid concentrations, for instance by tracing the path of malic acid during the acid-base malate transition. This can be readily done by finding out the malic acid concentration in the external medium at three different stages during ATP synthesis by the malate dehydrogenase assay (Section 2). In the initial state (stage 1), the malic acid added is known and after stopping the reaction by chemical or physical strategies, the final malic acid concentration outside the thylakoid membrane can be readily estimated. After an AS time of 60 s (stage 2; a longer time was chosen compared to that used in Fig. 1 in order to ensure greater penetration of the acid), when malate anions and protons have entered inside, but no ATP synthesis has occurred, the reaction can be quenched by chemical or physical means and the malic acid concentration remaining outside can be found. Finally, the reaction can be stopped after the BS condition (stage 3), i.e. after ATP synthesis, and the malic acid concentration can be determined. A longer BS time of 60 s was used to give sufficient time for the concentration of the acid to be restored. The results of such experiments are shown in Table 1 for initial external concentrations of 2 mM and 5 mM (corresponding to a concentration after reaction of 0.4 mM and 1 mM respectively) and in Fig. 2 (for an initial external concentration of malic acid of 1.5 mM). The pH of the external medium during the acid and base stages was kept fixed at 4.5 and 8.3 respectively in all the experiments.

To summarize, in order to directly correlate the role of malic acid in ATP synthesis, we have estimated the concentration of malic acid in the supertanant after acid stage and base stage in spinach thylakoids. We have started the reaction with a known concentration and amount of malic acid in the solution and upon different stages, the reaction was quenched and analyzed for malic acid concentration in the supernatant after removing the thylakoids by centrifugation. In particular, treatment with strong acids such as trichloroacetic acid and perchloric acid have been routinely used in the past as a general methodology for quenching a reaction and terminating catalysis by dilution into the acidic chemical environment. However, since harsh acid treatment may precipitate enzymes or even

Table 1 Measurement of acid concentration during membrane phosphorylation in spinach thylakoids and tracing of the path of malic acid during the acid-base-malate transition experiment by quenching the reaction at different stages of phosphorylation. Similar results were obtained when the chemical step of addition of 20% (w/v) trichloroacetic acid to quench the reaction was replaced by a rapid physical step that achieved the same effect, e.g. liquid nitrogen quenching with simultaneous centrifugation (see Fig. 2). **a)** Initial concentration of malic acid of 2 mM, corresponding to a final concentration after reaction of 0.4 mM. **b)** Initial concentration of malic acid of 5 mM, corresponding to a final concentration after reaction of 1.0 mM. In both **a)** and **b)** the malic acid concentration (mM) after reaction is tabulated. In each case, the value in the top line corresponds to an acid stage time (AS)=0 s and base stage time (BS)= 0 s, i.e., the quenching agent is added to the thylakoid suspension initially itself to quench the reaction. The middle line represents the situation after AS=60 s and BS=0 s, i.e., the quenching procedure is executed after 60 s of the acid stage, and only the acid/light stage is carried out. The bottom line shows the case after AS=60 s and BS=60 s, i.e., the quenching is accomplished after completion of both acid and base stages of the malate bath procedure, and thus both acid (*light*) and base (*dark*) stages of the phosphorylation reaction are performed. The pH of the medium during the acid and base stages was kept fixed at 4.5 and 8.3 respectively in all the experiments. Values are the mean of two measurements

a) Initial external malic acid concentration=2 mM

Condition	Final malic acid concentration (mM)
AS=0 s, BS=0 s	0.40
AS=60 s, BS=0 s	0.19
AS=60 s, BS=60 s	0.36

b) Initial external malic acid concentration=5 mM

Condition	Final malic acid concentration (mM)
AS=0 s, BS=0 s	0.92
AS=60 s, BS=0 s	0.60
AS=60 s, BS=60 s	0.87

Fig. 2 Measurement of acid concentration during membrane phosphorylation in spinach thylakoids and tracing of the path of malic acid as a function of time during the acid-base-malate transition experiment by quenching the reaction using rapid liquid nitrogen quenching with simultaneous centrifugation at different stages of phosphorylation. The initial concentration of malic acid measured 1.5 mM. Panel 1 corresponds to an acid stage time (AS)=0 s and base stage time (BS)=0 s, i.e., the liquid N_2 quenching with simultaneous centrifugation operates on the thylakoid suspension initially itself to quench the reaction. The second bar (panel 2) represents the situation after AS= 60 s and BS=0 s, i.e., after the quenching procedure upon 60 s of the acid stage, and only the acid/light stage is carried out. The right panels (panel 3–6) depict the case after AS=60 s and as a function of BS time (i.e. after 5, 15, 30 and 60 s of ATP synthesis), and the liquid N_2 quenching with simultaneous centrifugation is executed after both acid and base stages of the malate bath procedure, and thus both acid (*light*) and base (*dark*) stages of the phosphorylation reaction are performed at the times plotted. The pH of the medium during the acid and base stages was kept fixed at 4.5 and 8.3 respectively in all the experiments. The values plotted represent the average of six measurements (see text)

rupture membranes, we have repeatedly tested the efficacy of various alternative chemical and rapid physical quenching methods for our thylakoid system, including microcentrifugal quenching, liquid N_2 quenching with simultaneous as well as delayed centrifugation, and ammonium chloride quenching (see Experimental methods section) on a number of samples. Data obtained using rapid liquid N_2 quenching with a simultaneous centrifugation method for malic acid estimation after 60 s of acid stage and 60 s of acid stage followed by 5, 15, 30 and 60 s of base stage is shown in Fig. 2. Figure 2 plots the mean of data from three different spinach thylakoid preparations and two data points from each preparation. The malic acid concentration was found to increase continuously during the base stage reaching a final concentration of 1.47±0.08 mM after 60 s of base stage time (Fig. 2). We have done control assay without malic acid and found that no ATP synthesis took place without the presence of malic acid. This is the first report that malic acid is transported into and out of thylakoids and is directly coupled to ATP synthesis. Similar
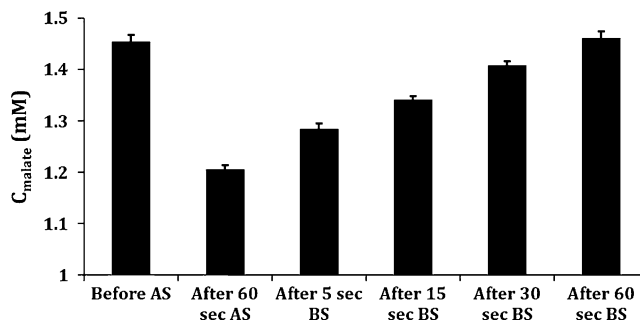
results to those depicted in Fig. 2 were obtained using the ammonium chloride quenching method (data not shown).

The results shown in Fig. 2 cannot be explained by the chemiosmotic theory (Mitchell 1966). Nor is the explanation of Jagendorf and Uribe (1966) tenable that the added organic acid penetrates in its undissociated form, providing a reservoir of dissociable protons, and that the protons are expelled in the base stage and account for the higher rates and yields of ATP synthesis. If that were the case, we would not expect to find the increase observed in external malic acid concentration after the base stage, i.e. between the middle and bottom lines in Table 1 and between the second panel and panels 3–6 in Fig. 2. The results show that malate anions are translocated inward during the 60 s of the acid stage along with a parallel movement of protons and that during the 60 s of the base stage there is progressively (from 0 s to 5, 15, 30 and 60 s in Fig. 2) an outward movement of malate anions and protons along with concomitant synthesis of ATP, as postulated by the torsional mechanism. This explains the observed decrease in malic acid concentration between stages 1 and 2 (Table 1 and Fig. 2) and the subsequent near-complete restoration of the malic acid concentration between stages 2 and 3, i.e. after completion of the base stage (Table 1 and Fig. 2).

Relationship of the new findings with the original report of Jagendorf and Uribe (1966)

In the original report of Jagendorf and Uribe (1966), thylakoids were acidified with succinate (pH 4–5) and diluted rapidly into Tris buffer (pH 8–8.5) and the ATP formed in this acid-base transition was followed. The driving force for the synthesis was postulated to be solely due to the imposed ΔpH. It was mentioned in the original paper that the nature of the acidification buffer played a key role in ATP synthesis. Upon acidification with HCl or glutamate, a low basal level of ATP formation was detected, but upon incubation with 10 mM succinate, the level of ATP synthesis increased 15-fold over the basal level. It was specifically stated that "the function of these acids is far from clear" (Jagendorf and Uribe 1966). In a subsequent publication, the organic anion requirement was termed "puzzling" (Jagendorf 1967) and the important scientific issue was not resolved with finality. As detailed here, the fact that ATP synthesis by the acid-base transition is strongly dependent on the nature of the acid with which the thylakoids are incubated is now readily explained by the differential abilities of these acids to permeate the membrane and create a local electrical potential. It has been shown that the acid bath procedure with dicarboxylic acids such as succinate as the acidic buffer not only leads to the creation of the required ΔpH but also to the establishment of a significant $\Delta\psi$ which is an indispensable driving force for ATP synthesis even in chloroplast thylakoids (Kaim and Dimroth 1999). This was primarily due to the unanticipated high permeability of the energy-transducing membrane for the succinate monoanion. We had proposed the first and only mechanism of ATP synthesis to date that considers the essential role of *both* ΔpH and $\Delta\psi$ and also addresses the indispensable requirement of the electrical potential in torque generation and ATP synthesis (Rohatgi et al. 1998). In contrast, in other mechanisms, termed generically as one-channel (reviewed in Dimroth et al. 2000) and two-channel (Junge et al. 1997; Elston et al. 1998) models, only $\Delta\psi$ or ΔpH respectively is postulated to induce torque and be responsible for ATP synthesis. To illustrate and differentiate the new conceptual thinking of torque generation by F_O in our mechanism from these models, new terminology of the ΔpH-$\Delta\psi$ asymmetric two mutually non-colinear half-access channel model was coined (for a review, see Nath 2002). The fundamental mechanism in both F_O and F_1 was unified, widened in scope, embellished and quantified over the decade (Nath 2002; Nath 2003; Nath 2008; Nath 2010b; Villadsen et al. 2011). The mechanism also postulated a key role for anions (or countercations) in ATP synthesis for the first time with both anions (or countercations such as Na^+ or K^+) and protons each providing ~50% of the energy to synthesize ATP. Various anions were tested in our laboratory over the years for their ability to support ATP synthesis (Nath 2004;

Nath 2008). Based on these experiments and the metabolic chemistry of photosynthetic processes in chloroplasts, malate was found to be the only possible anion that could satisfy the required transport function in the physiological setting. Hence malate is identified here as the physiological anion that is vectorially translocated by PSI or PSII and in the reverse direction by the F_O portion of the F_1F_O-ATP synthase and plays an essential role, along with protons, in synthesizing ATP. It should be noted that succinate was identified as the physiological anion with the corresponding function in animal mitochondria (Nath 2010b). Succinate is a respiratory substrate in various mitochondrial experiments and is present ubiquitously. It is now understandable that its indispensable role in electron transfer could have prevented identification over the decades of its second essential function: as the anion that is cotransported across the inner mitochondrial membrane during the process of oxidative phosphorylation.

We have several comments on one of the reviewers' pertinent query regarding the involvement of OH^- ions in the framework of the torsional mechanism. A brave attempt was made earlier by de Grey (in a bid to devise a model whose "consistency with experimental data greatly exceeds that of the standard chemiosmotic theory") by incorporating trans-membrane OH^- transport into the chemiosmotic theory (de Grey 1999). It should be understood that this idea is essentially contrary to the chemiosmotic proposal of transmission of power by proticity (Mitchell 1966; Nath 2010a; Nath 2010b); in fact, the proposed transmembrane hydroxide transport (de Grey 1999) will nullify much of the proton transport by the electron transport chain and prevent the generation of a protonmotive force of sufficient magnitude. OH^- transport is distinct from organic anion transport and the former cannot explain the observed contribution of organic anions in ATP synthesis. In fact, in animal mitochondria, according to the torsional mechanism, the exchange of OH^- with HPO_4^{2-} occurs in an enzyme complex at a different spatial location (via the phosphate P_i-OH^- antiporter, such that together with the ADP^{3-}/ATP^{4-} exchange by the adenine nucleotide transporter located in the vicinity, the overall process is locally electroneutral, in agreement with electron microscopy data on the ATP synthasome (Chen et al. 2004)) from the proton-dicarboxylic acid monoanion symsequence-port that takes place in the redox complexes I-IV and at the a-c interface of F_O in the ATP synthase, complex V. The OH^- generated by the ATP synthesis reaction $MgADP^- + HPO_4^{2-} \rightarrow MgATP^{2-} + OH^-$ is transported by exchange with inorganic phosphate through the phosphate/OH^- exchanger. Thus, in vivo, where the supply of ATP is regulated by its demand in another organelle, the OH^- is used in the reverse ATP hydrolysis reaction $MgATP^{2-} + OH^- \rightarrow MgADP^- + HPO_4^{2-}$ as a result of which no extra H^+ ion is consumed. On the other hand, in in vitro experiments not coupled to the ATP demand, the transported OH^- will be

neutralized by a proton from the medium, and hence an extra H^+ needs to be accounted for in the stoichiometry in such a case.

Finally, all the above difficulties are resolved by the new paradigm because the *local* electrical potential in the access channels of F_O, $\Delta\psi$ (relevant to the torsional mechanism) has no relationship to the bulk-to-bulk *delocalized* electrical potential postulated by the chemiosmotic theory, $\Delta\varphi$, whether the latter is present or not. In fact only the *bound* ions in the access channels can perform the coupling to rotation and ATP synthesis and thereby ensure that two separate transport systems *function together* as a proton-anion symsequence-porter or a proton-cation antisequenceporter by utilizing the electrochemical potential difference of both protons and anions/countercations, i.e. $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_A$ (or $\Delta\tilde{\mu}_C$). In other words, there is a *direct* intramembrane link between sites on the a-and c-subunits of F_O that is only accessible to H^+ (and coanion A^- or countercation C^+) ions inside their respective half-access channels at the a–c lipid-water interface but not accessible to ions (e.g. H^+) in free solution. Thus neither the delocalized concentration gradients of ions in the bulk aqueous phases (e.g. ΔpH) nor the delocalized membrane potential ($\Delta\varphi$) can *indirectly* link these sites on the a-and c-subunits/systems of the membrane-bound F_O portion of the ATP synthase and effect the coupling of these two independent systems to torque generation and hence ATP synthesis. Furthermore, by seriously considering the properties of *lipophilic* regions of the energy-transducing membrane (e.g. the decrease in hydrophilicity that occurs upon the binding of the A^- and H^+ ions to their binding sites in α-helical regions of a-and c-subunits at the critical a-c interface, causing the trans-bilayer movement and burial of the α-helix in the membrane, thereby leading to the creation of an imbalanced nonequilibrium state favouring electrostatic attraction between charges on a-and c-subunits and finally to rotation of the c-oligomer until a new state of local electrostatic equilibrium is reached) instead of simply dismissing the lipophilic region in cavalier fashion as *mere insulation* (Mitchell 1981), our understanding of biological energy transduction is taken to a higher level by the torsional mechanism.

New concept of coupling at the physiological level and model of the topology and organization of the thylakoid membrane

By incorporating the essential role of the anion in ion translocation and energy coupling in chloroplast thylakoids,
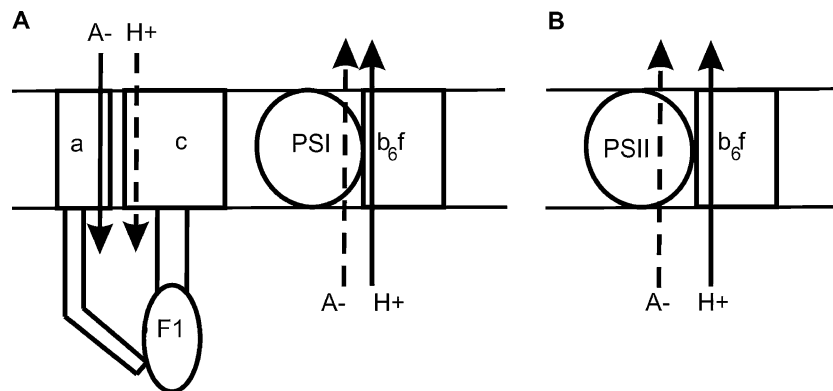

Fig. 3 Ion translocation and energy coupling in photophosphorylation in the chloroplast thylakoid membrane system. Depiction of the overall coupling process in the photosynthetic membrane of higher plants and the elementary anion (A^-) and proton (H^+) translocation events at the photosystem/cytochrome and ATPase coupling sites according to the torsional mechanism of energy transduction and ATP synthesis and the unified theory. Primary translocations are represented by bold arrows and secondary translocations by dashed arrows. The stoichiometries of the photosystems complexes and the presence of supercomplex formation do not alter, in principle, the overall mechanistic picture of coupling depicted in the figure. **a)** In PSI-mediated cyclic phosphorylation in the stroma lamellae, the crucial anion translocation function of photosystem I (apart from its well-established role in catalyzing electron transfer) is a novel prediction of the new paradigm. Further, the physiological anion (A^-) translocated by photosystem I from the stroma into the lumen and then back from the lumen to the stroma by the a-subunit of the F_1F_O-ATP synthase is identified here for the first time as the malate monoanion. Such symsequenceport (or antisequenceport) functions are predicted by the torsional mechanism and the unified theory to be a general property of coupled bioenergetic systems (Nath 2002; Nath 2003; Nath 2004; Jain et al. 2004; Nath 2008; Nath 2010b), and hence mandatory for F-type, V-type, P-type and A-type ATPases. For many systems, the ions cotransported or countertransported are still unknown, and it has proved extremely difficult to identify them because the ion gradients of the cotransported or countertransported species are easily dissipated by various means in the myriad experimental protocols and arrangements employed to date. **b)** The vectorial translocation of eight protons and eight malate anions into the lumen (per four electrons) by the coupled operation of cytochrome b_6f and photosystem II located in the appressed membranes of the grana during the operation of the linear, non-cyclic pathway of electron transfer is shown. PSII also translocates four malate anions into the lumen by secondary transport to balance the four protons deposited into the lumen by the light-mediated splitting of water by photosystem II. In all 12 protons and 12 anions are translocated into the lumen by the non-cyclic pathway per molecule of evolved oxygen

the new concept of coupling at the overall physiological level for the process of photosynthetic phosphorylation and a model of the topology and organization of the thylakoid membrane according to the torsional mechanism and the unified theory is depicted diagrammatically in Figs. 3 and 4. If F represents a monomer of the F_1F_O-ATP synthase, P stands for the PSI monomeric complex, and C delineates a monomer of the cytochrome b_6f dimer, then the ideal, highest flux, and most efficient membrane topology and arrangement, in other words, the optimal design, can be concisely written as the palindrome $(-FPCCPF-)_n$ for the stroma lamellae and end membranes and as $(-P'P'CC-)_n$ for the appressed membranes in the grana stack, where P′ represents a monomer of PSII. Several other novel predictions of the torsional mechanism not given in the detailed legends to Figs. 3 and 4 will now be covered concisely in this section.

According to the model, when light is limiting, both linear and cyclic electron transport pathways with a total requirement of ten quanta operate autonomously and without competition in C3 plants in vivo to provide ATP and NADPH in a 1.5:1 ratio required for the operation of the Calvin cycle (Fig. 4). In one extreme view, there is no need for ATP synthesis by cyclic electron transport involving PSI and only the linear pathway suffices (Heber et al. 1995). In another extreme view, even the basis of the series Z scheme is questioned and it is proposed that PSII reduces $NADP^+$ in a two quanta reaction and PSI is not a part of the linear pathway but only participates in cyclic electron flow (Arnon et al. 1981). It becomes very difficult to explain the classical Emerson enhancement effect (Emerson et al. 1957) and the various antagonistic effects on cytochrome oxidation (Duysens et al. 1961), among other established observations in such a scheme. These
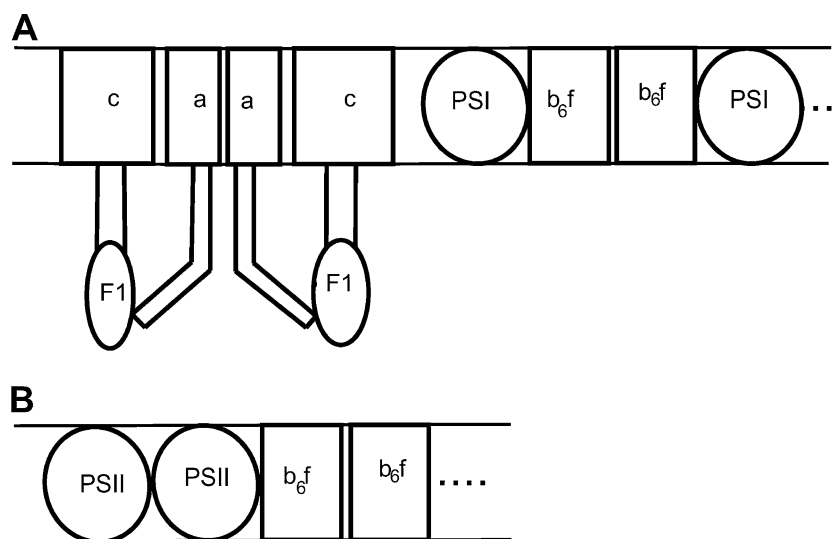

Fig. 4 Model of the topology and organization of **a)** the thylakoid stromal and end membrane lamellae and **b)** the appressed regions of grana, as per the torsional mechanism and the unified theory. Photosystem II complexes located primarily in the appressed domains of the chloroplast grana participate in efficient linear electron transfer from PSII to PSI in local granal microdomains (at a distance of tens of nm), with no need for long-range electron transport between grana and stroma. In all, 8 light quanta, shared equally between the two photosystems, are used in the cooperating PSII-PSI linear e⁻ pathway and 4e⁻ pass through PSII and PSI in series. Water is split only by PSII and one molecule of oxygen is evolved. Two additional light quanta are used for cyclic electron transport coupled solely to ATP synthesis without oxygen evolution or NADPH production in the stroma lamellae. The cytochrome b_6f translocates the proton while PSI or PSII translocates the malate anion from the stroma into the lumen in the cyclic and non-cyclic pathways respectively. Thus, in summary, for photosynthesis under light limiting conditions, balanced operation of the non-cyclic and cyclic pathways leads to the generation of 2 NADPH molecules, the synthesis of 3 ATP molecules and the evolution of one O_2 molecule per 10 quanta of red light. Of these 3 ATP molecules, 2.58 molecules are synthesized by 12 protons and 12 anions deposited into the lumen through coupled cytochrome b_6f and PSII operation by the linear electron pathway in the appressed grana while 0.42 ATP are synthesized by 2 protons and 2 anions contributed by the collaborative action of cytochrome b_6f and PSI in the cyclic pathway in the stroma lamellae. The 14 protons and 14 anions are removed from the lumen and translocated to the stroma by the F_1F_O ATP synthase molecules located in the margins or in the stroma lamellae/end membranes for phosphorylation by the non-cyclic and cyclic pathways respectively. The model is also consistent with the definitively established stoichiometry of 14 c-subunits in the chloroplast ATP synthase by a variety of techniques such as the original reports by contact mode atomic force microscopy (Seelert et al. 2000; Seelert et al. 2003), and subsequently by cryoelectron microscopy of n crystals of complete native F_O (Seelert et al. 2009). The c_{14} stoichiometry of the c-ring in the chloroplast ATP synthase has also been confirmed by the structural work of another group (Varco-Merth et al. 2008). The PSI in the margins contribute electrons via the non-cyclic pathway to reduce ferredoxin (and thereby $NADP^+$). The monomeric PSI (Amunts et al. 2007) in the stromal membrane may permit asymmetry in its lateral interactions with other membrane proteins, e.g. with a monomer of F_1F_O-ATP synthase [which exists as a dimer as shown by elegant experiments (Rexroth et al. 2004)] on one side and a monomer of cytochrome b_6f complex [whose functional unit is also a dimer (Stroebel et al. 2003)] on the other side

problems are avoided in the proposed model. It also bypasses the problems of mobility of large protein complexes and small proteins in a crowded membrane, and the difficulties of long-range electron transfer between the two photosystems. It is also in agreement with long-standing structural information on the lateral asymmetry of the thylakoid membrane (Albertsson 1995). Hence the new paradigm presents a middle ground and shows a way out of the impasse. It makes the novel prediction on theoretical grounds that the fraction of total light quanta absorbed by PSI is ~57% while that absorbed by PSII is ~43%, or approximately in a 60:40 ratio. The simplest way to achieve this and ensure self-regulation of the photosynthesis process is to distribute pigments (e.g., chlorophyll) between PSI and PSII in this ratio (between 57:43 and 60:40). Taking red light at 680 nm as the input energy, the linear pathway evolves one oxygen molecule and produces 2 NADPH and 2.58 ATP molecules (by translocating 12 H^+ and 12 A^- into the lumen) per 8 quanta and works at an overall efficiency of 38.3%. The cyclic PSI-mediated pathway translocates 2 H^+ and $2A^-$ into the lumen and contributes to the synthesis of 0.42 ATP and functions with an efficiency of 4.4%. This leads to an overall efficiency of the light reactions (linear + cyclic), i.e. of photosynthetic energy transduction, of 31.5%. Finally, the ratio of PSI to PSII may vary depending on the organism, growth conditions and light intensity and in this way the ATP:NADPH ratio in the chloroplast may be tuned. The principles outlined in the new paradigm are general and universal and apply not only to F-type ATPases, but also to P-type, V-type and A-type ATPases and to C4 plants as well. A novel prediction of this work to plant V-type ATPases (Lüttge et al. 1981; Lüttge and Smith 1984; Lüttge and Ratajczak 1997; Steiger et al. 1997; Hafke et al. 2003; Fernie and Martinoia 2009) is that the equivalent of the a-subunit in such ATPases also translocates malate anions across the membrane.

Concluding remarks

Like any unified scientific theory that is detailed as well as comprehensive in scope, the torsional mechanism of energy transduction and ATP synthesis offers scientists with a wealth of novel testable predictions. It is hoped that these will prove interesting and useful to the large number of experimentalists working in plant biochemistry and biophysics, plant physiology, photosynthesis, and other subfields of bioenergetics research. It may also lead to a deeper understanding of the molecular mechanism of multidrug transporters that affect crop yields by mediating metabolite transport and also confer herbicide resistance in plants, antibiotic resistance in bacterial infections, and drug resistance in cancer chemotherapy (Holland et al. 2003).

By offering the novel drug target of an anion/countercation binding site in F_1F_O-ATP synthase that is different from the proton binding site, the torsional mechanism may help considerably in the development of new drugs for combating various diseases including cancer and tuberculosis and simultaneously implement effective strategies that circumvent the complex problems of multidrug resistance and cross resistance with existing drugs, thanks to a distinct target and a different micromechanism of action. In the opinion of the author, the work addresses the key elements whose lack of detailed consideration has held back the progress of research in this fundamentally important and interdisciplinary field.

References

Agarwal B (2011) A role for anions in ATP synthesis and its molecular mechanistic interpretation. J Bioenerg Biomembr 43:299–310

Albertsson P-Å (1995) The structure and function of the chloroplast photosynthetic membrane - a model for the domain organization. Photosynth Res 46:141–149

Amunts A, Drory O, Nelson N (2007) The structure of a plant photosystem I supercomplex at 3.4 Å resolution. Nature 447:58–63

Arnon DI, Allen MB, Whatley FR (1954a) Photosynthesis by isolated chloroplasts. Nature 174:394–396

Arnon DI, Whatley FR, Allen MB (1954b) Photosynthesis by isolated chloroplasts. II. Photosynthetic phosphorylation, the conversion of light into phosphate bond energy. J Am Chem Soc 76:6324–6329

Arnon DI, Tsujimoto HY, Tang GM-S (1981) Proton transport in photooxidation of water: A new perspective on photosynthesis. Proc Natl Acad Sci USA 78:2942–2946

Channakeshava C (2011) New paradigm for ATP synthesis and consumption. J Biosci 36:3–4

Chen C, Ko Y, Delannoy M, Ludtke SJ, Chiu W, Pedersen PL (2004) Mitochondrial ATP synthasome: Three-dimensional structure by electron microscopy of the ATP synthase in complex formation with carriers for P_i and ADP/ATP. J Biol Chem 279:31761–31768

de Grey ADNJ (1999) Incorporation of transmembrane hydroxide transport into the chemiosmotic theory. Bioelectrochem Bioenerg 49:43–50

Dimroth P, Matthey U, Kaim G (2000) Critical evaluation of the one-versus the two-channel model for the operation of the ATP synthase's F_o motor. Biochim Biophys Acta 1459:506–513

Duysens LMN, Amesz J, Kamp BM (1961) Two photochemical systems in photosynthesis. Nature 190:510–511

Elston T, Wang H, Oster G (1998) Energy transduction in ATP synthase. Nature 391:510–513

Emerson R, Chalmers R, Cederstrand C (1957) Some factors influencing the longwave limit of photosynthesis. Proc Natl Acad Sci USA 43:133–143

Fernie AR, Martinoia E (2009) Malate. Jack of all trades or master of a few? Phytochemistry 70:828–832

Hafke JB, Hafke Y, Smith JAC, Lüttge U, Thiel G (2003) Vacuolar malate uptake is mediated by an anion-selective inward rectifier. Plant J 35:116–128

Heber U, Gerst U, Krieger A, Neimanis S, Kobayashi Y (1995) Coupled cyclic electron transport in intact chloroplasts and leaves of C3 plants: Does it exist? If so, what is its function? Photosynth Res 46:269–275

Holland IB, Cole SPC, Kuchler K, Higgins CF (eds) (2003) ABC Proteins: From Bacteria to Man. Academic, London

Jagendorf AT, Uribe E (1966) ATP formation caused by acid-base transition of spinach chloroplasts. Proc Natl Acad Sci USA 55:170–177

Jagendorf AT (1967) Acid-base transitions and phosphorylation by chloroplasts. Fed Proc 26:1361–1369

Jain S, Nath S (2000) Kinetic model of ATP synthase: pH dependence of the rate of ATP synthesis. FEBS Lett 476:113–117

Jain S, Murugavel R, Hansen LD (2004) ATP synthase and the torsional mechanism: Resolving a 50-year-old mystery. Curr Sci 87:16–19

Junge W, Lill H, Engelbrecht S (1997) ATP synthase: An electrochemical transducer with rotatory mechanics. Trends Biochem Sci 22:420–423

Kaim G, Dimroth P (1999) ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. EMBO J 18:4118–4127

Lüttge U, Smith JAC, Marigo G, Osmond CB (1981) Energetics of malate accumulation in the vacuoles of Kalanchoë tubiflora cells. FEBS Lett 126:81–84

Lüttge U, Smith JAC (1984) Mechanism of passive malic acid efflux from vacuoles of the CAM plant Kalanchoë daigremontiana. J Membr Biol 81:149–158

Lüttge U, Ratajczak R (1997) The physiology, biochemistry and molecular biology of the plant vacuolar ATPase. Adv Bot Res 25:254–296

Matsuki N, Abe K (2000) Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. Neurosci Res 38:325–329

Mitchell P (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev 41:445–502

Mitchell P (1981) Bioenergetic aspects of unity in biochemistry: evolution of the concept of ligand conduction in chemical, osmotic and chemiosmotic reaction mechanisms. In: Semenza G (ed) Of Oxygen, Fuels and Living Matter, Part 1. John Wiley, New York, pp 30–56

Nath S, Rohatgi H, Saha A (1999) The torsional mechanism of energy transfer in ATP synthase. Curr Sci 77:167–169

Nath S, Jain S (2000) Kinetic modeling of ATP synthesis by ATP synthase and its mechanistic implications. Biochem Biophys Res Commun 272:629–633

Nath S (2002) The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase: A scrutiny of the major possibilities. Adv Biochem Eng Biotechnol 74:65–98

Nath S (2003) Molecular mechanisms of energy transduction in cells: Engineering applications and biological implications. Adv Biochem Eng Biotechnol 85:125–180

Nath S (2004) The torsional mechanism of energy transduction and ATP synthesis as a breakthrough in our understanding of the mechanistic, kinetic and thermodynamic details. Thermochim Acta 422:5–17

Nath S (2008) The new unified theory of ATP synthesis/hydrolysis and muscle contraction, its manifold fundamental consequences and mechanistic implications and its applications in health and disease. Int J Mol Sci 9:1784–1840

Nath SS, Nath S (2009) Energy transfer from adenosine triphosphate: Quantitative analysis and mechanistic implications. J Phys Chem B 113:1533–1537

Nath S (2010a) Beyond the chemiosmotic theory: Analysis of key fundamental aspects of energy coupling in oxidative phosphorylation in the light of a torsional mechanism of energy transduction and ATP synthesis-Invited review part 1. J Bioenerg Biomembr 42:293–300

Nath S (2010b) Beyond the chemiosmotic theory: Analysis of key fundamental aspects of energy coupling in oxidative phosphorylation in the light of a torsional mechanism of energy transduction and ATP synthesis-Invited review part 2. J Bioenerg Biomembr 42:301–309

Peres MFS, Laluce C, Gattás EAL (2008) Colorimetric enzymatic assay of L-malic acid using dehydrogenase from baker's yeast. Food Technol Biotechnol 46:229–233

Rexroth S, Tittingdorf JMV Meyer zu, Schwaßmann HJ, Krause F, Seelert H, Dencher NA (2004) Dimeric H^+-ATP synthase in the chloroplast of Chlamydomonas reinhardtii. Biochim Biophys Acta 1658:202–211

Rohatgi H, Saha A, Nath S (1998) Mechanism of ATP synthesis by protonmotive force. Curr Sci 75:716–718

Seelert H, Poetsch A, Dencher NA, Engel A, Stahlberg H, Müller DJ (2000) Proton-powered turbine of a plant motor. Nature 405:418–419

Seelert H, Dencher NA, Müller DJ (2003) Fourteen protomers compose the oligomer III of the proton-rotor in spinach chloroplast ATP synthase. J Mol Biol 333:337–344

Seelert H, Dani DN, Dante S, Hauß T, Krause F, Schäfer E, Frenzel M, Poetsch A, Rexroth S, Schwaßmann HJ, Suhai T, Vonck J, Dencher NA (2009) From protons to OXPHOS supercomplexes and Alzheimer's disease: Structure-dynamics-function relationships of energy-transducing membranes. Biochim Biophys Acta 1787:657–671

Smith DJ, Boyer PD (1976) Demonstration of a transitory tight binding of ATP and of committed P_i and ADP during ATP synthesis by chloroplasts. Proc Natl Acad Sci USA 73:4314–4318

Steiger S, Pfeifer T, Ratajczak R, Martinoia E, Lüttge U (1997) The vacuolar malate transport of Kalanchoë daigremontiana: A 32 kDa polypeptide? J Plant Physiol 151:137–141

Stroebel D, Choquet Y, Popot JL, Picot D (2003) An atypical haem in the cytochrome b_6f complex. Nature 426:413–418

Varco-Merth B, Fromme R, Wang M, Fromme P (2008) Crystallization of the c_{14}-rotor of the chloroplast ATP synthase reveals that it contains pigments. Biochim Biophys Acta 1777:605–612

Villadsen J, Nielsen J, Lidén G (2011) Bioreaction Engineering Principles, 3rd Edition. Springer, New York, pp 135–137:143–145