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The torsional mechanism of energy transduction and ATP synthesis as a breakthrough in our understanding of the mechanistic, kinetic and thermodynamic details☆

Sunil Nath*

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Hauz Khas, New Delhi 110016, India

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Dedicated to Prof. M.V. Ranganath on the occasion of his 75th birthday.

Abstract

Novel insights into mechanistic, kinetic and thermodynamic details of ATP synthesis by F_1F_0-ATP synthase in oxidative phosphorylation and photophosphorylation have been offered from the perspective of the torsional mechanism of energy transduction and ATP synthesis [Nath, S., Adv. Biochem. Eng. Biotechnol. 85 (2003) 125–180]; [Nath, S., Adv. Biochem. Eng. Biotechnol. 74 (2002) 65–98]. A fundamental energetic analysis of membrane phosphorylation has been performed that highlights the merits of the new paradigm. Biological implications for energy transduction have been discussed in detail. The action of uncouplers and inhibitors of oxidative phosphorylation has been explained by a fresh and completely different rationale. New experimental data that further supports the torsional mechanism has been presented, and the consistency of proposed mechanisms with the laws of thermodynamics has been assessed. A general kinetic analysis of oxygen exchange has been shown to reveal the absence of site-site cooperativity in F_1-ATPase. Details of the nanomechanics of coupling between F_1 and F_0 in ATP hydrolysis-driven proton pumping have been postulated. The original thinking and power of the theoretical approaches embodied in the torsional mechanism have been shown to prove invaluable to unravel the functioning of other biological machines through a new Molecular Systems Biology. The generality and universality of the principles in the theory have been illustrated by taking specific examples of other molecular motors such as myosin and kinesin, revealing the deep underlying unity in diverse energy transduction processes in biology. Changes required in our scientific thinking and industrial technology and particularly in the education of the next generation of biological scientists have been identified as challenging issues that urgently need to be addressed in the near future.

Keywords: ATP synthase; F_1; F_0; Bioenergetics; Molecular mechanism; Torsional mechanism; Binding change mechanism; Chemiosmotic theory; Energy transduction; Energy storage; Ion transport; Coupling; Oxidative phosphorylation; Photophosphorylation; Mitochondria; Chloroplasts; Central dogma; Symsequenceport; Antisequenceport; Dynamically electrogenic but overall electroneutral; Anion–cation coupling; Localized; Delocalized; Uncoupler; Inhibitor; Torsional energy; Cooperativity; Oxygen exchange; Muscle contraction; Rotation-twist-tilt energy storage mechanism; Nanotechnology; Nanomechanics; Myosin; Kinesin; Ncd; Molecular systems biology; Molecular systems engineering

1. Introduction

The detailed molecular mechanism of how the universal biological energy currency, adenosine-5′-triphosphate (ATP) is made has stimulated an immense amount of research and has intrigued some of our best scientific minds; yet it has remained one of the great enigmas of science. The original conception, detailed proposal and logical development and quantification of the torsional mechanism of energy transduction and ATP synthesis during a ∼15-year intensive research effort has greatly contributed to resolve the fundamental issues and has offered new ways of thinking, analysis and interpretation [1–10]. Since this year marks the 75th

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* Tel.: +91 11 26857457; fax: +91 11 26582282.
E-mail address: sunath@dbeb.iitd.ernet.in.

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anniversary of the discovery of ATP [11,12], and 50 years after the proposal of the first hypothesis of oxidative phosphorylation [13], it would be fitting to analyze the difficulties with the old theories [14–17], explain how these difficulties are overcome by the new paradigm, and offer further novel insights into the details of mechanistic, kinetic and thermodynamic characteristics of ATP synthesis. No attempt is made here to quote comprehensive references (for this please consult Refs. [1,2]). Difficulties in extant theories already pointed out or implied by the pioneering research of several groups in the field [18–28] are not taken up in this article. The article focuses on new mechanistic, kinetic and thermodynamic details of phosphorylation in the membrane or membrane-bound F_0 portion, the extramembrane F_1 portion, and the coupling between F_1 and F_0 in the F_1F_0-ATP synthase from the standpoint of the torsional mechanism of energy transduction and ATP synthesis. New insights have been obtained into the functioning of other motor proteins such as myosin and kinesin, indicating the unity of energy transduction principles in fundamental biological processes.

2. Experimental methods

2.1. Acid–base phosphorylation in chloroplast thylakoid membranes

Thylakoid membranes were isolated from market spinach by a method modified from Tripathy and Mohanty [29]. Briefly, 5 g of deribbed spinach leaves were homogenized in 40 ml of ice-cold isolation buffer containing 50 mM pH 7.3 HEPES, 0.4 M sorbitol, 1 mM $MgCl_2$ and 1 mM EDTA. The slurry was strained and centrifuged at 4000 rpm for 7 min at 4 °C. The pellet obtained was suspended in 1 ml of 10 mM Tris, pH 7.5 containing 1 mM EDTA, kept on ice for 15 min and centrifuged at 8000 rpm for 5 min. The pellet containing plastid membranes was suspended in suspension buffer containing 50 mM Tris (pH 7.5), 0.4 M sorbitol, 1 mM $MgCl_2$ and 1 mM EDTA, frozen in liquid nitrogen and stored at −70 °C. Phosphorylation was carried out by the technique of acid–base transition [30,31]. The thylakoid membrane suspension was diluted to a chlorophyll concentration of 0.5 mg/ml with the suspension buffer, and 0.5 ml was injected into a tube containing 0.4 ml of acid stage buffer (succinic acid, pH 4.0) at the required concentration. After 15 s of acidification, the acidified mixture was withdrawn in a syringe and transferred to 0.9 ml of base stage buffer (pH 8.3) containing 100.0 μmol tricine, 5.0 μmol $MgCl_2$, 0.2 μmol ADP, 2.0 μmol P_i and 19.6 μmol NaOH. The phosphorylation was terminated after 15 s by the addition of 0.2 ml of 20% trichloroacetic acid. The contents of the tube were centrifuged and the aliquot of the supernatant was analyzed for phosphate by the microcolorimetric method of Taussky and Shorr [32].

3. Mechanistic, kinetic and thermodynamic details of oxidative and photosynthetic phosphorylation in the energy-transducing membrane or membrane-bound F_0 portion of ATP synthase

3.1. Difficulties with description of membrane phosphorylation based on the chemiosmotic theory

Ultimately, the basis of the chemiosmotic theory [14,15] lies in the realm of classical equilibrium thermodynamics, and the theory expresses the energy transduction relationship: electron transfer $\leftrightarrow \Delta\tilde{\mu}_H \leftrightarrow$ ADP + $P_i \leftrightarrow$ ATP, i.e., the $\Delta\tilde{\mu}_H$ equilibrates both with the respiratory chain and with the ∼ in ATP. Unfortunately, it is impossible to obtain the postulated delocalized $\Delta\tilde{\mu}_H$ of ∼200 mV in the energy-transducing membranes of mitochondria or chloroplasts by using the energy liberated by electron transfer between a pair of neighboring electron transport chain carriers. This is because proton translocation by proton pumps must cease as soon as the value of the potential created becomes equal to the redox potential difference of the neighboring carrier pair at each independently functioning coupling site. (This point has also been discussed in different language in a similar but different context [3]). A carrier pair with sufficiently high redox potential difference for this purpose is not found in either mitochondria or chloroplasts. The above qualitative argument can be quantified in a more graphic way by considering the net redox reaction at pH 7.0,

$$NADH + \tfrac{1}{2}O_2 + H^+ \rightarrow NAD^+ + H_2O \quad (1)$$

with a midpoint potential, E_m, of 1.14 V. For an equilibrium thermodynamics-based delocalized model of coupling, use of the Nernst equation allows an *equivalent* concentration cell to be built with a concentration ratio

$$\frac{c_{out}}{c_{in}} = \exp\left[\frac{E_m F}{RT}\right] \quad (2)$$

of 3.5×10^{18}, a very large number in the context. If we choose a physiological value of ion/solute concentration c_{out} in the aqueous phase on one side of the membrane of 10 mM, then the concentration on the other side of the membrane c_{in} will measure only 2.9×10^{-21} M! Such low solute concentrations can simply not be measured, and with these low values it is impossible for the system to achieve steady state rates of metabolism.

3.2. Resolution of difficulties based on the torsional mechanism of energy transduction and ATP synthesis

Such difficulties do not arise in the torsional mechanism, where the driving forces work at the microscopic molecular level, and the $\Delta\psi$ of ∼60 mV is created by *discrete* elementary translocation events of proton/anion (intensive properties) in the vicinity of the redox/ATP synthase enzyme molecule, i.e., the $\Delta\psi$ is *localized* in nature [1,2,4] (Fig. 1).

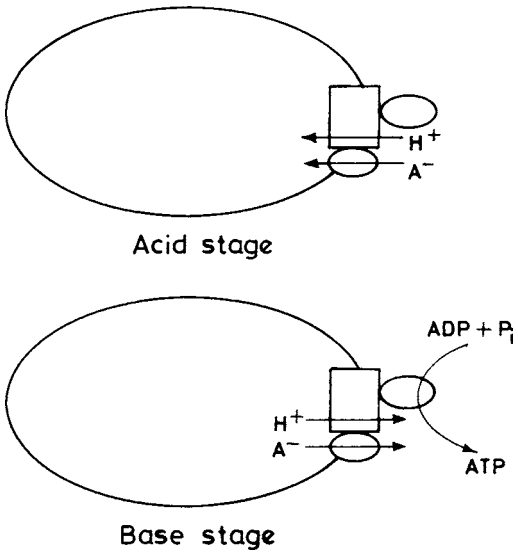

Fig. 1. Schematic representation of the elementary transport events during the acid and base stages of phosphorylation in chloroplast thylakoids.

The macroscopic bulk extensive thermodynamic properties, ΔpH and ΔpA, can however determine the number of times the elementary translocations occur repetitively in experiments carried out on isolated mitochondria or chloroplast thylakoids as model systems. Application of an equation similar to Eq. (2), but with E_m replaced by the diffusion potential, $\Delta\psi$ yields a concentration ratio of approximately 10.0, which is readily achievable by biological systems, requiring a redox energy expenditure of only 60 meV per ion translocated. Further, in the torsional mechanism, a central role is played by storage of internal energy (e.g., as torsional energy) in localized spatial regions of macromolecules existing in long-living (compared to the time for thermal exchange) transient nonequilibrium conformational (or metastable) states. Thus, we can refer to a characteristic space-time structure of the system, i.e., energy storage in a characteristic localized space domain for a characteristic time interval in the context of the torsional mechanism. This stored energy can be utilized through coupling to drive a chemical reaction (e.g., ATP synthesis) in an *antithermodynamic* direction, i.e., in a direction in which it would not spontaneously go. Kinetics [4–6] and *nonequilibrium* thermodynamics [1,10] have been shown to constitute appropriate frameworks to describe these coupled processes.

3.3. New fundamental energetic analysis of membrane phosphorylation

On critically analyzing the relevant bioenergetics literature, it is difficult to dispel the impression of a serious energy crisis. Various long-term experimental research programs using different techniques have found that the measured "protonmotive" force values are of insufficient magnitude to synthesize ATP [33–36]. Yet, a first-principles analysis from a *theoretical* point of view has never been carried out, despite the fundamental importance of the problem. An analysis of this type will greatly clarify the situation and prove very valuable to unambiguously resolve this crucial issue once and for all. From electrochemical and morphological data on rat liver mitochondria, it was calculated that 40,000 H^+ need to be translocated within each mitochondrion to generate a delocalized $\Delta\psi$ of 120 mV [2]. This agrees with the result of similar calculations by other workers [36]. Moreover, it was computed independently from an overall energy balance of ATP synthesis as well as by use of biochemical and stoichiometric data on enzyme complexes in mitochondria [2] that a local $\Delta\psi$ of 60 mV is created by each ion translocation act.

Since proton translocation on the redox side produces a gradient of H^+ ions and in addition creates a (local) electrostatic field, we need to expend 60 + 60 = 120 meV from metabolic energy for translocating the *first* H^+ (assuming no losses, i.e., reversible operation of the redox pumps performing active transport). Neglecting the increase in concentration gradient against which each *subsequent* proton has to be translocated (implying that redox energy requirement against Δc is kept constant at 60 meV for every ion translocation act i = 1 to n), and adding 60 meV from the $\Delta\psi$ (field) part for each translocation act i in such an *electrogenic* process, the reversible redox energy expenditure, E can be expressed as a mathematical series. Thus,

$$E = 120 + 180 + 240 + 300 + \ldots \text{meV} \quad (3)$$

or,

$$E = 60(2 + 3 + 4 + 5 + \ldots) \text{meV} \quad (4)$$

For translocation of 40,000 H^+,

$$E = 60(2 + 3 + 4 + \ldots + 40,001) \quad (5)$$

The series in parentheses in Eq. (5) is an A.P. containing 40,000 terms with a first term of 2 and a common difference of 1. Summing the series gives the important result that $E = 4.80 \times 10^{10}$ meV, an astronomical number, considering that the available redox energy per $2e^-$ in mitochondria measures only 2280 meV. It should also be emphasized that the calculation gives us a most conservative estimate, as it assumes perfect coupling at the sites and further neglects the extra energy consumption needed to work against the increasing concentration gradient of protons with each successive translocation event. Finally, it should be remembered that chemiosmosis postulates a delocalized $\Delta\psi$ of $\sim$200 mV [15] in mitochondria, even larger than that taken here in the calculations. The analysis conclusively demonstrates that it is energetically *impossible* for the respiratory chain to violate electroneutrality to such a large extent and create a delocalized $\Delta\psi$ of $\sim$200 mV across bulk aqueous phases by uncompensated, electrogenic proton translocation.

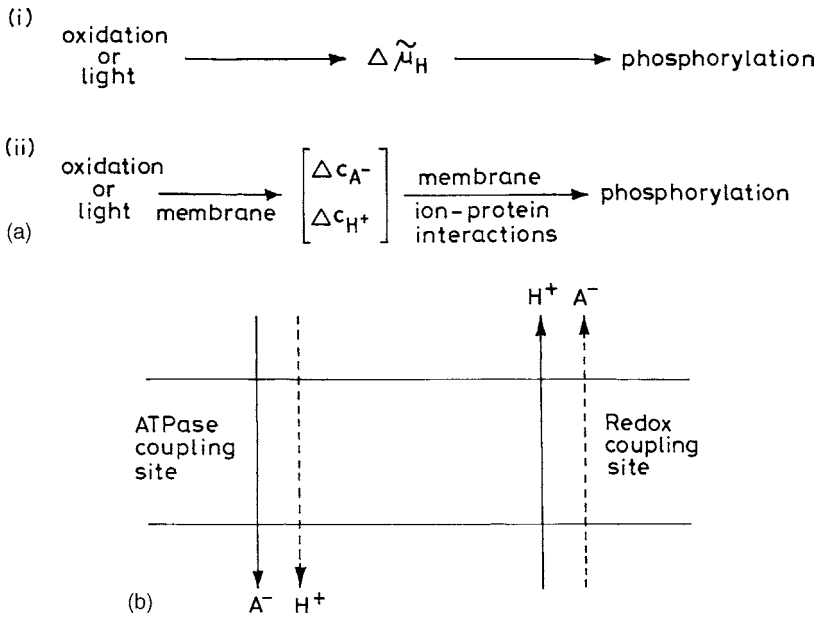

Fig. 2. (a) The central dogma of bioenergetics as described by (i) the chemiosmotic theory, and (ii) the torsional mechanism of energy transduction and ATP synthesis. (b) Elementary ion translocation events at the redox and ATPase coupling sites according to the torsional mechanism. Primary ion translocations are denoted by bold arrows and secondary ion movements by dashed arrows.

3.4. Energy transduction by a dynamically electrogenic but overall electroneutral mode of ion transport as a novel concept in bioenergetics

The torsional mechanism does not suffer from the above serious problems because it proposes that energy transduction takes place by a *dynamically electrogenic but overall electroneutral mode of ion transport* involving membrane-permeable anions and protons through their own, respective ion channels in the membrane (Fig. 1). Thus, the $\Delta\psi$ generated by ion movement is localized in space and is generated *transiently* during the time interval after translocation of the primary ion (the one that disequilibrates the ion distribution) and before translocation of the secondary ion that restores the resting state. No steady state delocalized $\Delta\psi$ is generated in such an (overall electroneutral) mode of ion translocation. A thermodynamic analysis of this process has been made [1]. For the thermodynamic relationship $\Delta\tilde{\mu}_i = 0$ to apply, the two phases on either side of the membrane must be connected, i.e., the channel must be open. However, the constraints [1; Eqs. (11,12)] can be satisfied in another less restrictive way: if, after translocation of an ion of primary species *i*, its channel closes, then no further translocation of *i* can take place because of this physical/kinetic barrier (a closed channel). If the $\Delta\psi$ created due to this translocation act of *i* opens the channel of the secondary ion, as proposed here, then an interesting intrachannel communication and regulation of transport and metabolism develops. After translocation of the secondary ion (i.e., after the passage of both ions in sequence (ion pair)), the channel of the secondary ion closes also. Now, the signal/driver will restart the transport cycle by initiating opening of the channel of the primary ion. This *cation–anion coupling* (via *symsequenceport* or *antisequenceport*) is of central importance for energy transduction as per the torsional mechanism, and both anion and proton (moving down their concentration gradients) contribute ~50% of the energy requirement of ATP synthesis [1–5]. Thus, both ΔpH and $\Delta\psi$ are not created by protons, as assumed in all existing theories of ATP synthesis in bioenergetics to date, but arise from two independent sources, protons and anions respectively, providing us with a new central dogma (Fig. 2a). It should be stressed that though a (transient) $\Delta\psi$ is indeed measured experimentally, no experimental evidence exists that this potential is actually created by the proton. This implies that while the observations provide strong evidence that mitochondria and chloroplasts can utilize energy stored in the form of proton concentration gradients to drive (in the overall sense) ATP synthesis, as pioneered by the chemiosmotic theory, there is, however, no unequivocal experimental evidence that proton gradients constitute the *sole* high energy intermediate in oxidative and photosynthetic phosphorylation.

Thus, in the overall sense, the high-energy intermediates in oxidative phosphorylation and photophosphorylation are the concentration gradients of cations, C^+ (e.g., H^+) and anions, A^-, and not just protons (Figs. 1 and 2). These A^-, H^+ ions interact with specific sites in their respective ion channels in the membrane. This interaction has conformational characteristics, as proposed in consummate detail in the torsional mechanism [1–10], and culminates in the transduction and storage of the energy of the anion and proton gradients as torsional energy in the γ-subunit of the F_1F_0-ATP synthase molecule. The torsional energy is subsequently utilized to synthesize ATP by a novel catalytic

cycle [2,7,8]. The energy of 4 A^- and 4 H^+ ion translocations in sequence is required to make one ATP molecule [1,2,4,9].

Specific mechanistic, thermodynamic and kinetic details can now be added to the above scheme for the first time. The proton, which is primary on the redox side, is pumped out (against its concentration gradient) by the redox complexes first; this event generates a *local* $\Delta\psi$ which is utilized to translocate the counterion (A^-) against its concentration gradient. Thus, two concentration gradients of different species are created (Fig. 2b). On the ATPase side, the anion is primary and is translocated first along its concentration gradient and binds to its binding site in its specific, regulated anion channel; this translocation event creates a local $\Delta\psi$ in the vicinity of the F_1F_0-ATP synthase molecule which is the signal for the proton channel to open. The proton is now translocated along its concentration gradient through its specific ion channel and binds to its Asp (or Glu) binding site, which lies within the electrostatic field created by the previous anion translocation elementary event (Figs. 1 and 2b). In this way, the energies of the discrete anion and proton translocations are added and transduced to a primary rotation of the c-subunit in the F_0 portion of ATP synthase. Thus, according to the torsional mechanism, the local, transient $\Delta\psi$ serves as a *means* for energy transduction. Note that the energies of the discrete elementary steps have been added during energy transduction; however, we are not adding ΔpH and $\Delta\psi$ at the level of the driving force itself. Thus, there should exist an intramembrane link between anion and proton channels that lie close to each other or form a structural/functional union. Finally, if the anion channel itself were to open only after substrate MgADP has bound to the β_C catalytic site in the F_1 portion of ATP synthase (i.e., the driver/controller lies on the ATPase side and is a part of the ATP synthase), as we propose, then the result would be the most optimal and economical machine design conceivable for energy transduction and utilization. The ideal then appears to be the case for bioenergetic systems, fine-tuned by billions of years of evolution.

3.5. Biological implications

The mechanism discussed has great biological implications. It implies that mediation by a *delocalized* $\Delta\psi$ of ~200 mV via a mode of ion transport violating electroneutrality of bulk aqueous phases is not needed, and the so-called "protonmotive" force (with both its components created *solely* by protons) as an *indirect, long-range messenger*, as envisaged by chemiosmosis [1,2,14,15], simply does not exist. Rather, a *direct* and *local* energy transduction takes place by structural/functional association of transporters/channels in the membrane for addition and joint utilization of energy, and for regulation of transport and cell metabolism. Formation of such membrane supercomplexes by a structural union of, and functional intramembrane link/interaction between

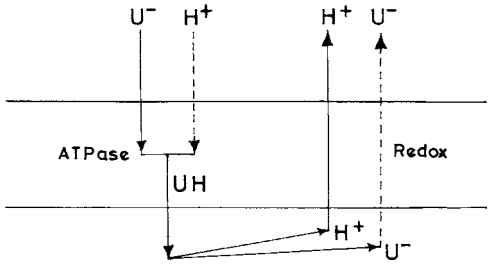

Fig. 3. Uncoupling mechanisms in oxidative phosphorylation according to the torsional mechanism. Uncoupler anion (U^-) competes with substrate anion (A^-) for entry into the anion channel. The uncoupling process involves entry of U^- and H^+ as distinct species through their respective specific, regulated access channels, their recombination (UH) in the vicinity of the proton and anion binding sites in the membrane due to the lipid solubility of the uncoupler U^-, their exit as a single, electrically neutral UH species (thereby interfering with the physiological temporal sequence of ion movements and *disrupting* the provision of energy to F_1F_0 by ~50%), dissociation of UH into U^- and H^+ in the aqueous phase of the mitochondrial matrix, and pumping of the dissociated U^- and H^+ separately by the redox enzyme complexes back to the inner membrane side.

anion and cation channels shall prove to be a general principle for energy coupling in biological systems. The old theories [14,15] were postulated in an era when even the existence of ion channels in membranes had not been revealed by novel electrophysiological techniques. In the light of major subsequent advances in our knowledge of membrane structure and function arising from the research work of a large number of groups spread across the world, these theories require revision. The torsional mechanism offers the next step in this evolution process [1–10].

Further, biological systems possess unique properties and channel structures to conduct electricity in a completely novel way, unrecognized to date. In traditional electrical conduction, oppositely charged ions (if they are both mobile) move in opposite directions; the direction of their migration is determined by the electrical potential gradient. However, in biological systems, opposite charges A^- and C^+ can be translocated in the *same* direction through their own respective ion channels (and not together, but sequentially, e.g., anion followed by cation), such that taken together, an ion pair C^+A^- is translocated and overall electroneutrality is not violated. Finally, the action of inhibitors and uncouplers of oxidative phosphorylation can be explained by a fresh, completely different rationale. An anion or proton inhibitor of F_0 acts by binding to the site meant for the anion/proton and becoming immobile, thereby preventing the mobility of A^-/H^+ through F_0. Uncoupling mechanisms of oxidative phosphorylation are far more complex and subtle than currently believed, as depicted in Fig. 3. Uncouplers are not just proton conductors that interact nonspecifically with membranes and dissipate the proton gradient, as proposed in chemiosmosis. Rather, uncouplers act at a specific proteolipid binding site in the inner mitochondrial membrane and interfere with the establishment of the high-energy conformational (metastable) state of the c-subunit in F_0 [2,3]. An uncoupling anion (U^-) enters through the anion channel (i.e., *separately* from the

proton which enters through its own H^+ half-access channel [2,3,9]), but because of its lipid solubility, approaches close to the proton to form the neutral UH in the vicinity of the A^-/U^- and H^+ membrane binding sites and now moves across the membrane as UH, thereby dissipating the energy of the nonequilibrium conformational state and disrupting energy transduction. It dissociates back in the exit aqueous phase to the uncoupling anion and proton which are both pumped back by the redox complexes, thus uncoupling oxidation from ATP synthesis (Fig. 3).

3.6. New experimental evidence that further supports the torsional mechanism of energy transduction and ATP synthesis

The above points are valid for photophosphorylation also. An impressive body of evidence generated over several decades supports the idea that a transient potential of around 50–100 mV is formed across chloroplast thylakoid membranes upon exposure to light. An equally impressive body of research using various techniques also shows that the magnitude of the membrane potential across the membrane in the steady state is negligible (<10 mV). Further, the kinetics of flash-induced electric potential related absorption changes indicates that the energy of a $\Delta\psi$ (naturally existing or artificially imposed) can be utilized to drive ATP synthesis. All these three major conclusions of the last 50 years are in perfect harmony with the tenets of the torsional mechanism [2]. With the great amount of mechanistic knowledge arising from the formulation of the torsional mechanism, discriminating experiments were designed to see if conditions could be found where the rate of ATP synthesis *decreases* even though the so-called driving force in the chemiosmotic framework *increases* or at least stays constant. Such a region is predicted to be found by the torsional mechanism. Yet, such a result could be explainable within the framework of the torsional mechanism by taking into consideration the flows of anion and proton along their respective concentration gradients.

Fig. 4 depicts the results of such a phosphorylation experiment on spinach chloroplast thylakoids. The figure illustrates the variation of the rate of ATP synthesis as a function of the concentration of the succinate buffer in the *acid stage* for the isolation and incubation conditions described in Section 2. The time of incubation was set at 15 s during the acid stage for *all* the concentrations of buffer (acid) employed. The base (phosphorylation) time measured 15 s in all cases. Conditions vis-à-vis the proton were ensured to be constant for all the measurements. Thus the pH_{out} measured 8.3 for all points and pH_{in} was 4.0 or less at increasing acid concentrations from 2 to 14 mM. During the fixed incubation time of 15 s, the penetration of increasing amount of acid at higher buffer concentrations can only serve to decrease the inside pH, thereby increasing ΔpH during the base stage phosphorylation. This ΔpH is presumed to constitute the major part of

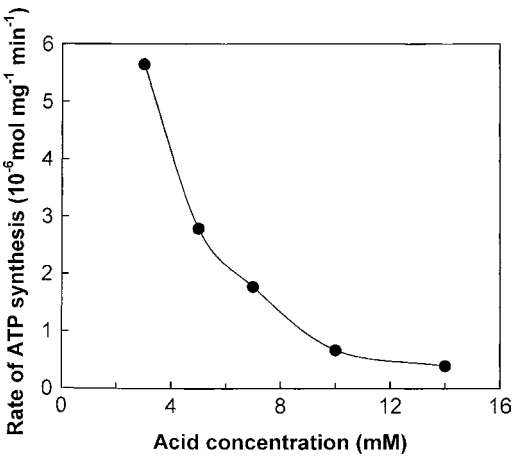

Fig. 4. Measured rate of phosphorylation as a function of the succinate buffer concentration in the acid stage for the acid–base transition experiment. The time of incubation in the acid stage was kept constant at 15 s, and the rate was measured after 15 s in the base stage in all cases. pH_{out} was controlled at 8.3 for all the runs.

the driving force in chloroplasts according to chemiosmotic theory. Even if $\Delta\psi$ contributes, it can only increase with increased proton permeation at higher acid concentrations, or stay constant. Thus the presumed driving force (the so-called "protonmotive" force) has increased (or at most stayed constant); however the rate of phosphorylation has progressively *decreased* with increasing acid concentration, which is a very nonchemiosmotic result. In an overall electroneutral transport of an ion pair into buffered media in a small system, it is not relevant to consider the overall bulk $\Delta\tilde{\mu}_H$ (or the delocalized $\Delta\psi$) due to protons alone as the driving force. However, if we consider the concentration *gradient* of anions and protons during phosphorylation in the base stage as the overall driving forces (as quantitatively modeled by the torsional mechanism [4,5]), we expect the rate to be larger at the lower buffer concentrations as the concentration gradient is forced to be kept higher in the experimental procedure at lower buffer concentrations (at fixed base stage time) than at higher buffer concentrations. Due to the increased concentration gradient of both ions 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 base stage reaction time, in agreement with the observations (Fig. 4). A corollary of these results is that the number of *free* protons in a thylakoid is very small (<1.0 at neutral pH) and therefore the large number of protons and anions translocated must come from the buffer itself. In other words, the H^+ can be interpreted to be bound by buffer A^-, distributed within the inner thylakoid space.

A diagrammatic representation of the basis of the acid–base phosphorylation procedure is given in Fig. 5. Note that the reference level itself is lowered by the acid–base transition (bold arrow). Recently, we have shown by kinetic experiments that both H^+ and A^- are accumulated in the lumen during the acid stage, and both H^+ and A^- come out into

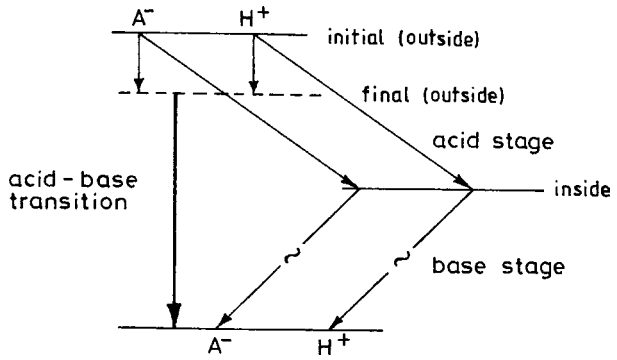

Fig. 5. Schematic diagram interpreting the basis of the acid bath phosphorylation procedure. The lowering of the reference level by means of the acid–base transition is shown by the bold arrow. The independently functioning energy transduction devices in the respective ion channels on the ATPase side are represented by the ∼ symbol.

the stroma during base stage phosphorylation (Figs. 1 and 2), and that the efflux of these ions is linked in time synchrony to the synthesis of ATP (Nath, in press).

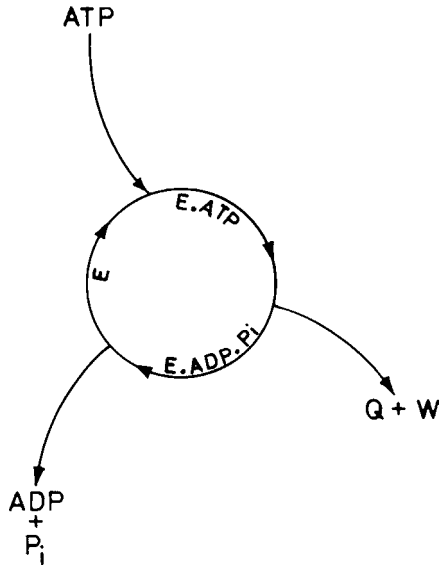

Fig. 6. The overall energy balance for performance of useful work by an enzymatic system undergoing a cyclic isothermal process.

4. Mechanistic, kinetic and thermodynamic details of ATP synthesis/hydrolysis in the extramembrane F_1 portion of ATP synthase

4.1. Consistency of proposed mechanisms in F_1 with the laws of thermodynamics

According to the fundamental tenets of the binding change mechanism, in the synthesis direction, energy is required primarily for release of tightly bound ATP from the catalytic site of F_1. Further, the elementary step of ATP formation from ADP and P_i at the catalytic site of the enzyme occurs spontaneously and does not require energy input from ion translocations [1,2,4,16,17]. Conversely, in the reverse hydrolysis mode, energy release is virtually confined to the binding step, E + ATP → E.ATP and this energy performs useful work; the step involving the actual hydrolysis (E.ATP ↔ E.ADP.P_i) is isoenergetic and does not release energy. According to the torsional mechanism, every elementary step in synthesis requires energy; further, the energy required to perform external useful work (e.g., lifting of a load over a certain distance by muscle) is released during the E.ATP → E.ADP.P_i step, and this energy released must have been stored in the ATP. Thus ATP is indeed a high-energy molecule with respect to ADP + P_i [1,2]. Fig. 6 represents the physical situation for performance of useful work by an ATP-hydrolyzing molecular machine.

We define the molecular machine as the system (i.e., the system boundary is the circle in Fig. 6), and carry out an *overall* energy balance. For the isothermal, cyclic process mediated by the enzyme, all thermodynamic property changes are necessarily zero, and the binding energy changes (e.g., those that occur during the E + ATP → E.ATP binding step) are *internal* to the system; therefore, *binding energy release due to the binding step cannot perform useful external work, W*, contradicting the fundamental tenet of the binding change mechanism. In fact, free ATP enters the system, free (ADP + P_i) leave it and the difference between their energies/enthalpies must equal *W* (neglecting losses), or (*Q* + *W*) in general, i.e., heat released from the system plus work done by the system (Fig. 6). This equation/energy balance constitutes the most general statement of the first law of thermodynamics for open systems in steady state [1]. Moreover, the free ATP by itself has no binding energy associated with it. Thus, in a cyclic isothermal process, the energy that was employed to perform useful work (muscle contraction) must have been *stored* in the ATP molecule with respect to ADP + P_i, as proposed by the torsional mechanism [1–9]. The enzyme serves as a key to unlock this stored energy during the ATP hydrolysis elementary step, and this primarily enthalpy change upon hydrolysis is transduced to useful work [1].

4.2. Kinetic analysis of oxygen exchange experiments reveals absence of site-site cooperativity in F_1-ATPase

The presence of site–site cooperativity during ATP synthesis and hydrolysis constitutes the third fundamental tenet of the binding change mechanism [1,2,6,16,17,37]. We have shown the absence of cooperativity by analysis of a general kinetic scheme for ATP synthesis [6], and by various mechanistic and kinetic arguments [1,2]. However, this tenet of the binding change mechanism arose chiefly from *oxygen exchange* studies on F_1-ATPase, and a kinetic scrutiny of oxygen exchange experiments [37] can throw light on this important issue. A fundamental kinetic analysis of ^{18}O exchange from labelled $H^{18}OH$ during ATP hydrolysis by F_1-ATPase

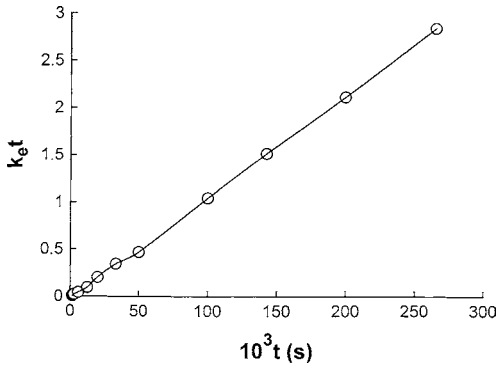

Fig. 7. Kinetic analysis of oxygen exchange experiments revealing the absence of site–site cooperativity in ATP hydrolysis by mitochondrial F_1-ATPase.

leads to the general result.

$$\ln\left[\frac{1 - X_{\mathrm{Ai}}}{1 - X_{\mathrm{A}}}\right] = k_e t \tag{6}$$

where X_{A} is the fraction of ^{18}O (of $H^{18}OH$) in P_i at time t, X_{Ai} is the fraction of water oxygens incorporated in P_i at time 0^+ (= 0.25 for the experiments in ref. [37]), k_e is the rate constant of exchange, and t is the time of exchange. Inspection of the derived Eq. (6) reveals the important point that since $k_e t$ occurs as a product, any difference in the extent of oxygen exchange indicates either a difference in the kinetic constants of the exchange reaction itself, or a difference in the time of exchange. The binding change mechanism assumes the former, while the torsional mechanism postulates the latter. Thus, the torsional mechanism considers the time available for exchange as the appropriate *time scale* for analysis of the kinetics of oxygen exchange. This can be readily tested. If k_e, the rate constant for oxygen exchange, varies with substrate ATP concentration, then site–site cooperativity exists; if k_e is constant over the entire range of substrate concentration, then no cooperativity exists. The measured steady state velocities as a function of substrate ATP concentrations from 0.11 to 5000 μM [37] were employed to estimate t at each ATP concentration, and the measured $^{18}O/P_i$ at each concentration was used to calculate $k_e t$ from Eq. (6). The results have been plotted in Fig. 7; the data yielded an almost perfect straight line (R^2 = 0.999) with a constant slope (k_e) of 10.4 s^{-1} over the entire substrate concentration range, i.e., *over five decades of ATP concentration*. The results of the kinetic analysis of oxygen exchange (Fig. 7) conclusively prove the *absence of site-site cooperativity* during ATP hydrolysis by mitochondrial F_1-ATPase, as predicted by the torsional mechanism [1,2,4,6–9].

5. Mechanistic details of coupling between F_1 and F_0 portions of ATP synthase

Due to the inviolability of microscopic reversibility, it should be possible to reverse the rotation-twist-tilt energy storage mechanism proposed for the synthesis mode [2,3] and the direction of rotation of the γ- and c-subunits, and thereby obtain the molecular mechanism of ATP hydrolysis-driven H^+ pumping along with detailed nanomechanics of coupling and energy transmission between F_1 and F_0. Yet, the mechanism must work in the face of constraints imposed by the *physical construction* of the components of the nanomechanical device (e.g., α-helices). For instance, twist can be induced in the α-helices only if they are subjected to torque in one direction (since the handedness of the α-helices in γ or c remains unchanged), irrespective of whether the process is ATP synthesis or ATP hydrolysis. This is therefore a very stringent test for the torsional mechanism. Thus, looking at the right handed c-subunits from the F_1 side, a counterclockwise torque on the top will increase twist in the c-subunit, primarily in the C-terminal helix. However, both N- and C-terminal helices (i.e., both TMH-1 and TMH-2) participate in the process of tilt [2,3], i.e., they work in concert as a unit.

During steady state ATP hydrolysis, the details of F_1–F_0 coupling are illustrated schematically in Fig. 8. Due to ATP hydrolysis in F_1 because of the conformational change of the catalytic site from β_{DP} to β_{TP} (i.e., T → L transition) [1,2,7], looking from F_1, the top of γ rotates 120° clockwise (while the bottom remains more or less stationary) and twists. The clockwise movement of the bottom of γ (in steps of 15° for H^+ translocation through each access half-channel, or (15 + 15) = 30° for proton translocation across the membrane) (Fig. 8) untwists γ, i.e., relieves its high-internal energy state. This sense of rotation indeed causes the required conformational changes if the bottom part of the α-helix of the γ-subunit forms a left-handed coil or, the possibility that we strongly favor and propose, if it forms a left-handed coiled coil with another constituent α-helix of γ. This is also borne out by the structural data [24]. The untwisting of γ due to clockwise movement of its lower part causes the top of c-subunit to rotate counterclockwise about its own axis (c_t versus c_b), and to twist and tilt, while its bottom part, b, remains almost fixed (Fig. 8). Now, upon proton translocation through access half-channels and electrostatic interactions between residues on the c- and a-subunit (e.g., c-Asp-61 and a-Arg-210, and c-Asp-61 and a-His-245) [2,3,5,9], the c-ring rotates clockwise by 15° steps about the central axis of F_1F_0. This rotation of the c-ring causes the bottom of the c-subunit helices to rotate counterclockwise about their axes with respect to the top, c_t (i.e., counterclockwise rotation of c_b versus c_t), leading to untwisting of the twisted c-subunit helices, release of their torsion, and attainment of their original, resting state conformation (Fig. 8). Further details are given in the legend (Fig. 8) as concisely as possible due to space limitations. From the above analysis it is possible to appreciate the great importance of the large free energy barrier at the hydrophilic-hydrophobic a–c interface (right interface in the panels in Fig. 8) as envisaged in the torsional mechanism right from inception [3,5,9], and the critical nature of the *timing* of the H^+-translocation elementary steps and the conformational changes.

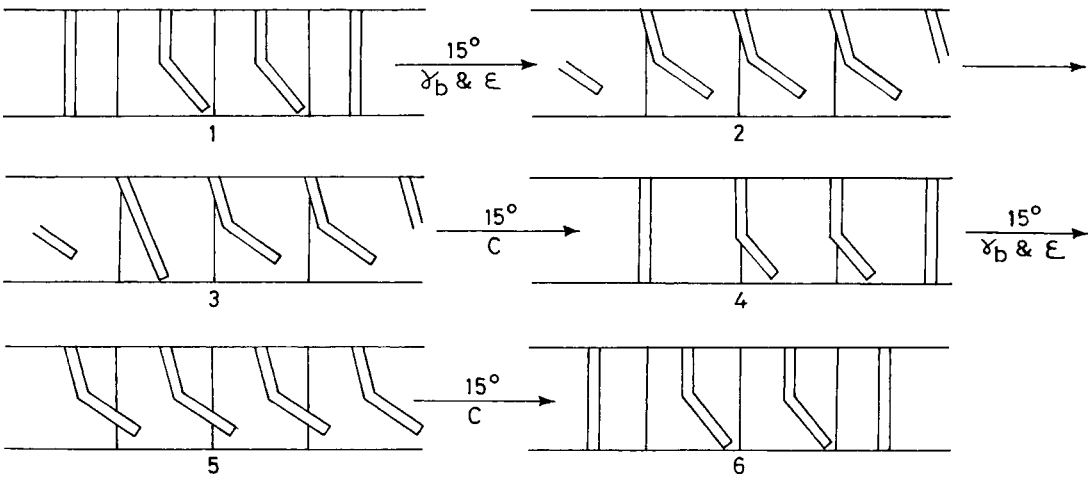

Fig. 8. Rotation-twist-tilt energy storage and transmission mechanism coupling F_1 and F_0 during ATP hydrolysis-driven ion pumping. The bent c-subunits in the diagram also adopt a twisted conformation. H^+ enters through the cytoplasmic access half-channel between steps 1 and 2 (or at 2) and exits from the periplasmic access half-channel between steps 4 and 5 (or at 5). The detailed sequence of events looking from the F_1-side after ATP hydrolysis has taken place in site 2 [1] and the top of γ-subunit has rotated clockwise 120° (due to release of stored energy and changes in electrostatic potential upon the hydrolysis elementary step) and the bottom of γ has rotated clockwise by 15° and untwisted follow the following cycle: 10 c-subunits' TMH helices twist and tilt; two already twisted c-subunit helices tilt → proton binding site on c-subunit is exposed to inlet (cytoplasmic) access half-channel → proton binds to c-Asp-61/equivalent → that c-subunit untwists and straightens → a–c electrostatic interactions take place (e.g., between a-Arg-210 and c-Asp-61 (unprotonated), labeled "Leading" in Fig. 4 of Ref. [2]) → c-rotor rotates 15° clockwise → 10 twisted c-subunit helices untwist and untilt and the resting state is gained, but there exists a twisted helix (one of two twisted c-subunits) at the right a–c interface, and consequently a high free energy barrier [2,3,9] which requires further γ_b movement before the barrier can be crossed → bottom of γ (γ_b) rotates 15° clockwise and untwists → 10 c-subunits' TMH helices twist and tilt; the two already twisted helices of c-subunits facing and interacting with the a-subunit tilt → this tilting of the c-subunit at the right a–c interface exposes the bound proton on that c-subunit Asp-61 binding site to the exit (periplasmic) access half-channel in hydrophilic environment → proton unbinds → a–c electrostatic interactions take place (e.g., between a-His-245 and c-Asp-61, labeled "Protonated" in Fig. 4 of ref. [2], which loses its proton to become unprotonated → c-rotor rotates 15° clockwise → 10 c-subunits' TMH helices untwist and untilt; two twisted c-subunit helices untilt and the resting state is reached, but there exists no access for proton to its binding site on the c-subunit from the inlet (cytoplasmic) access half-channel in this configuration of the c-rotor → γ_b rotates 15° clockwise and untwists, and the cycle repeats.

6. Application of the general principles to elucidate aspects of the molecular mechanism/thermodynamics of other molecular motors

6.1. Myosin–actin system of skeletal muscle

A novel molecular mechanism for the contractile cycle of the myosin II–actin system of skeletal (striated) muscle (the rotation-twist-tilt energy storage mechanism of muscle contraction) has already been proposed [1,39]. A detailed mechanical analysis of the molecular mechanism has also been performed [1]. According to this mechanism, the enthalpy change upon ATP hydrolysis in *one* of the myosin heads (∼35.1 kJ/mol, i.e., 5.8×10^{-20} J or 58 pN-nm per double-headed myosin molecule under physiological conditions) is stored as a small increase in twist in the coiled coil of the S-2 region of the molecule, with its subsequent untwisting causing the power strokes. Since the myosin molecule has two identical heads, and the coiled coil S-2 region is common to both heads, upon untwisting, the stored twist energy is distributed equally between the heads, in our conception. Hence the energy available per myosin head is 2.9×10^{-20} J or 29 pN-nm. Now we will estimate the maximum force generated during the power stroke using the principle of conservation of energy. According to our mechanism, the distance moved by the actomyosin complex in an elementary force generating step is 5.3 nm. If $F_{z,max}$ is the maximum force generated during the power stroke, we have

$$F_{z,max} \times 5.3\,\text{nm} = 29\,\text{pN-nm} \quad (7)$$

Therefore, $F_{z,max}$ = 5.5 pN. From experimental data using single molecule spectroscopy, the myosin head moves along actin in regular steps of 5.3 nm [1,39] and the force generated in an isometric contraction is 5.7 pN, which can only be greater than or equal to $F_{z,max}$. From these experimental data, the maximum energy of the power stroke is 5.7 pN × 5.3 nm = 30 pN-nm per myosin head or 3×10^{-20} J/myosin head. Since the experimental (3×10^{-20} J/myosin head) and calculated values (2.9×10^{-20} J/myosin head) of the maximum available energy for the performance of the power stroke agree perfectly with each other, we conclude that our conception is correct and that both myosin heads of a myosin molecule bind to actin filaments and both heads execute power strokes and perform useful work. If this had not been the case, we would have expected the measured values of the isometric force per myosin head to be approximately double (i.e., ∼11.4 pN), which has not been found experimentally. Alternatively, 50% of the ATP hydrolysis energy would have been dissipated as heat, i.e., the maximum efficiency would never have exceeded 50% under any condition, which is not supported by many experiments. Thus the presence of

the second myosin head increases the efficiency (defined as power stroke work/energy available from ATP hydrolysis) of the muscle contraction process considerably (by a factor of ∼2).

As discussed above, both myosin heads of a myosin molecule bind to actin filament. Now the question arises whether they bind to actin binding sites of the same filament or different filaments. Since the spacing between two actin binding sites measures 5.46 nm and the maximum thickness of the myosin head is 6.3 nm, it is unlikely, in our view, due to steric hinderance that the myosin heads bind to the same actin filament. Moreover, in the thick filament, we have rotational symmetry with three myosin molecules, i.e., six myosin heads (emerging every 14.3 nm of axial distance along the sarcomere) surrounded by six actin filaments in a hexagonal array. This suggests that each myosin head binds to one actin filament. Further, from experimental data on frog skeletal muscle, there are two actin filaments per myosin filament. Thus we have [{2 × (1/5.46)}/(6/14.3)] = ∼0.9 actin binding sites per myosin head, i.e., approximately in a 1:1 ratio. Hence, from this calculation, we expect each actin binding site to be occupied by a myosin head. This also shows that the two myosin heads of a single myosin molecule bind to actin binding sites on *different* actin filaments.

If our conception above is correct, then, since the longest dimension (length) of a myosin head measures 19–20 nm, and the S-2 rod measures 6.5 nm, the distance from the surface of a myosin filament to the surface of an actin filament should be <25.5–26.5 nm. From the hexagonal geometry of the skeletal muscle fiber, we see that at any cross-section three myosin filaments form an equilateral triangle with an actin filament at its centroid. Taking the distance between two myosin filaments as 50 nm from electron microscopy data, we find that *l*, the length of the altitude of the triangle is 43.3 nm. Hence the distance of the centroid of the triangle from the center of the thick myosin filament = 2*l*/3 = ∼29 nm. Since the radius of myosin thick filament measures 5–7 nm, the distance from the surface of myosin thick filament to actin filament (neglecting the thickness of the thin actin filament in order to obtain a conservative estimate) measures at most 22–24 nm, which is well within the upper limit of 25.5–26.5 nm. Hence, these calculations based on the structure of the muscle superlattice also support our proposal of the two myosin heads binding to two different actin filaments. Moreover, the presence of a flexible hinge at the head–tail junction of myosin [1,39] will ensure such binding without much deformation in the structure of each myosin head. We have shown the requirement for partial flexibility and partial rigidity at the S-1–S-2 hinge by an engineering analysis of the system [1]. In fact, such flexibility at the S-1–S-2 hinge is essential from the mechanistic point of view also, and the rotation-twist-tilt energy storage mechanism of muscle contraction provides an explanation for the double-headed structure of the myosin and the importance of the nature of the hinge at the molecular level, as well as for the structure present at the (higher) level of the myosin–actin superlattice.

6.2. Kinesin-microtubule cargo-carrying motor protein system

To understand the movement of cargo-carrying kinesin towards the plus end of microtubules by the rotation-twist energy storage mechanism of kinesin motility, we can start the cycle with both the free kinesin heads (i.e., not bound to microtubule) containing bound MgADP. The heads get bound to adjacent α and β-tubulin subunits of the microtubule primarily due to ionic interactions. Thus, kinesin head binding is an intrinsic property of each tubulin subunit. These conserved residues as seen from our multiple sequence alignment of tubulin sequences are the negatively charged residues at the C-terminal of α and β-tubulin (e.g., for α, the sequence EGMEEGEFSE, and for β, the sequence EGMDEMEFTE, i.e., the residues α: 411–420 and β: 402–411 for *Drosophila melanogaster*). The kinesin head's highly conserved primarily positively charged residues involved in this ionic interaction with tubulin are K-141, K-159, K-240, L-248, K-252, Y-274, R-278, K-281 and R-284 (rat brain kinesin numbering). Although these residues are separated from each other in their primary sequence, they are close to each other on the surface of the kinesin in three-dimensional space. These residues constitute the conserved microtubule binding interface of kinesin head. Head 1 binds strongly with β-tubulin, while head 2 binds weakly with α-tubulin due to the intrinsically asymmetric interactions of the identical kinesin heads with the tubulin αβ heterodimer of the microtubule track. Due to this inherently weak head 2–α-tubulin interaction (compared to the head 1–β-tubulin interaction energy and the binding energy of MgADP to kinesin), head 2 is not able to release the bound MgADP; hence the pocket for MgATP binding is never available in head 2 throughout the entire catalytic/motility cycle.

After release of bound MgADP from head 1 by head 1–β-tubulin interactions, MgATP binds to its vacant binding site in head 1 since the binding energy of MgATP to kinesin head is greater than the head 1–β-tubulin interaction energy. This binding energy causes a conformational change in the kinesin head 1, and this head gets unbound from β-tubulin (note that head 2 is still bound to α-tubulin). After its unbinding and detachment from β-tubulin, the kinesin head (head 1) has the proper conformation for ATP hydrolysis to occur in the head. Due to release of the stored energy in ATP upon the elementary chemical hydrolysis step, a conformational change takes place (the chemical stroke), and head 1 rotates counterclockwise (looking from the C-terminal cargo side of kinesin) *about the hinge* (which is common to both the arms of the V-shaped kinesin molecule). P_i is released from head 1 upon hydrolysis and the head rotates and binds (as described above) to its next β-tubulin site on the microtubule track 8 nm away (head to head distance). Bound MgADP is released from head 1 upon its binding to β-tubulin due to the kinesin head–β-tubulin interactions. Part of the energy obtained on ATP hydrolysis is utilized to move head 1 forward and the rest is stored in the neck coiled coil of kinesin

molecule as elastic strain energy, primarily as twist. Thus the neck coiled coil helps in storing the twist energy before it is utilized for moving the other head (head 2) forward from one α-tubulin binding site to the next α-tubulin binding site by a physical stroke.

For rotation of the rear head (head 1) from the β-tubulin to the next β-tubulin binding site driven by the chemical energy of ATP hydrolysis, the axis of rotation passes through the other head (head 2 bound to the α-tubulin site on the microtubule) and is perpendicular to the microtubule track. Similarly, for the α- tubulin to the next α-tubulin movement of head 2 for the step caused physically by stored twist energy, the axis of rotation passes through the head bound to β-tubulin (head 1) and is perpendicular to the track. The radius of the arc taken by the head in the chemical and physical strokes remains almost constant throughout the motion and is approximately equal to the distance between the two heads in the relaxed state of the kinesin molecule and measures ∼4–5 nm.

For storage of elastic strain energy primarily as twist energy in the kinesin molecule, what should be the *mechanical constraints* on the degree of freedom of the constituents of the molecule? When the head (head 1) detached from β-tubulin site by MgATP binding energy moves ahead to find its next β-tubulin site about the defined axis, the other arm of the kinesin neck is not allowed to rotate *freely* about its *own* axis. However, the kinesin head weakly bound to the α-tubulin subunit (head 2) can rotate a little about its arm (i.e., it can only undergo constrained rotation) which strains and weakens the bound kinesin head's interaction with α-tubulin, making it easier for the stored twist energy to detach this head in the subsequent physical step. In addition, there also exist constraints on the degree of freedom of the hinge due to the coiled coil above it and the cargo at the C-terminal of kinesin. Due to the above loads acting on the hinge in the vertical downward direction, the hinge is not allowed to rotate freely when the head rotates. Thus, when the head rotates by ∼180° about the defined axis, the hinge does not sweep by the same angle; rather it rotates by a very small angle. The smaller this angle, the greater the twist stored in the system of the V-shaped kinesin molecule. However, there is no constraint on the linear motion of the hinge along the track in the plus direction. The same mechanical constraints apply during the physical powerstroke driven by the release of the stored twist energy. Thus, during the untwisting of the strained molecule, if the hinge had been allowed to rotate freely, then the stored energy would have been dissipated and would not have been used to detach the kinesin head weakly bound to α-tubulin and move it ahead by 8 nm (head to head distance) in the physical stroke.

Since the kinesin head bound to β-tubulin is a strong interaction (relative to the kinesin head bound to α-tubulin), the stored twist energy is not sufficient to detach the head bound to β-tubulin; it can only strain the head–β-tubulin interactions and weaken it to a small extent (since the interactions are strong). This makes it somewhat easier for the MgATP binding energy to overcome the slightly weakened interactions of the strongly bound head 1 with β-tubulin in a subsequent step and unbind head 1 from the β-tubulin site. Therefore, upon release of twist, the already strained weakly bound head 2 to α-tubulin detaches and rotates ∼180° in a clockwise direction (viewed from the C-terminal cargo end of kinesin) and binds to its next α-tubulin site 8 nm away (head to head distance). This is reinforced by considerations of microscopic reversibility (twist was stored by a counterclockwise rotation of one head, therefore untwisting must cause a clockwise rotation of the other partner head). Since the constraints on the degree of freedom of the various constituents of the system remain the same, the untwisting will cause head rotation in the opposite sense. The clockwise rotation of the rear head (head 2) of the strained kinesin molecule will cause the forward movement of the entire machinery along the track towards the plus end, with the centroid of the molecule moving forward 4 nm in this physical sub-step.

In the chemical stroke, the system is getting strained from its relaxed state, whereas in the physical stroke, the system is trying to regain its relaxed state from the strained state. Thus we expect the physical stroke to be faster than the chemical stroke. The acceleration of the physical step would be the same or greater than that in the chemical step depending on how much energy is lost in detaching one head (head 2) and straining the other head (head 1). In this way, one cycle is completed and, at the cost of one ATP molecule, the whole kinesin machinery along with cargo moves forward by 8 nm towards the plus end of the microtubule. It should be pointed out that movement of each head by 8 nm advances the centroid of the kinesin molecule by only 4 nm, and steps of each of the two heads in quick succession results in an overall 8 nm movement of the centroid (4 nm + 4 nm), and the movement of the kinesin molecule from one αβ-tubulin heterodimer to the next. An identical rotation-twist energy storage molecular mechanism applies to the motility of the ncd motor, with one extra turn in the ncd neck coiled coil (compared to kinesin) constituting the relaxed state of ncd, thus accounting for movement of the ncd motor towards the minus end of the microtubule.

The rotation-twist energy storage mechanism of kinesin/ncd motility is consistent with the thermodynamics. The *maximum* force generated by kinesin is ∼6.5 pN in the chemical powerstroke and 6.5 pN (or more) in the physical stroke. Due to the energy of a single ATP hydrolysis (58 pN-nm), the distance moved by the center of mass of the kinesin molecule is 8 nm, as discussed above. Thus, the maximum useful work done in the chemical and physical strokes together works out to be ∼6.5 pN × 8 nm = 52 pN-nm. The energy used for breaking kinesin head–α-tubulin interactions plus the energy used to strain the kinesin head–β-tubulin interaction measures (58–52) = 6 pN-nm. Thus the overall mechanical efficiency of kinesin measures 52 × 100/58 = ∼90%. It should be emphasized that the "loss" of ∼10% is loss in the sense that this energy is not employed for the performance of useful external work; however this bond breakage/straining is necessary to ensure the *processivity* of the kinesin motor on

the microtubule track. Until now, the kinesin field has primarily been interested in the hand-over-hand and the inchworm models and considerable time and effort has been spent by several research groups in trying to find out which one of the two models is correct, but as so often happens in science, this has led to increasing disagreements and greater confusion. We should not forget that the true mechanism of motility may lie elsewhere (in some hidden cave, or on some other intellectual plane) and try to keep our mind scientifically open to explore other alternative hypotheses. By working out the molecular mechanism to the last detail, we believe that the rotation-twist energy storage mechanism of kinesin motility solves the problem of how molecular motors move processively on microtubules and hence we think it merits serious consideration by researchers in the important fields of motility and bioenergetics.

The research that has led to the formulation of the torsional mechanism highlights the significance of functional properties arising in biology from *interactions* at the intermolecular and intramolecular level and may be termed, or subsumed under the general term, *Molecular Systems Biology*. The approaches of this Molecular Systems Biology can be employed to understand the functioning of other biological molecular machines, such as the bacterial flagellar motor [38] and the myosin–actin system of muscle [1,39] as well as to discover general design principles of biological molecular machines, and apply them to analyze, design, develop and fabricate novel macroscopic and even molecular devices using a Molecular Systems Engineering [1]. The great intellectual value of the torsional mechanism (and the other rotation-twist-tilt or rotation-twist energy storage mechanisms for diverse molecular motors) to me has been their provision of a fascinating, real biological machine-model that elucidates the details *within the black box* and reveals exactly what happens at each elementary step and how, is capable of quantitative predictions, and offers a refreshing, new way of looking at biological systems using physicochemical and engineering approaches. I believe that the mechanisms have led to a number of novel and original insights into how energy is released, conserved, distributed, transduced, stored, and utilized in fundamental biological processes. However, it is my view that, despite the achievements, we stand only at the beginning (rather than towards the end) of the *changes* that the new paradigm has the power to bring about, both in our scientific thinking as well as in our industrial technology, and, what is paramount, in the education of the next generation of biological scientists for future innovation and progress. So there's much more still to be done.

7. Conclusions

Mechanistic, kinetic and thermodynamic aspects of one of the most fundamental processes in biology – the synthesis of ATP by oxidative phosphorylation and photophosphorylation – have been developed in consummate detail. An in-depth bioenergetic analysis of phosphorylation in the membrane or membrane-bound F_0 portion of ATP synthase has been carried out, and various proposed mechanisms have been subjected to close scrutiny. It has been proved from first principles that some of the old theories are not satisfactory from the energetic viewpoint and that it is very difficult, if not impossible, to generate the delocalized driving forces of such a large magnitude as envisaged by means of a purely electrogenic process violating electroneutrality of bulk aqueous phases. The new paradigm of the torsional mechanism of energy transduction and ATP synthesis has been shown to readily overcome the serious problems inherent in other theories. The great implications for biological energy transduction have been pointed out, and further new mechanistic, kinetic and thermodynamic details have been developed and incorporated into the torsional mechanism. A fresh, completely different rationale has been proposed to explain the action of uncouplers and inhibitors of oxidative phosphorylation. New experimental data that lends further support to the torsional mechanism has been presented. It has been pointed out that our knowledge of membrane structure and function has increased very substantially, thanks to the contributions of several research groups all over the world, and that this explosion of knowledge necessitates revision of old theories and outlooks in a continuum of research work. The torsional mechanism has been viewed as the new thinking and the next step in this evolution process.

A detailed thermodynamic and kinetic analysis of mechanisms proposed for the extramembrane F_1 portion of ATP synthase has been performed. In particular, the consistency of proposed mechanisms with the laws of thermodynamics has been scrutinized. The torsional mechanism has been shown to pass this test of consistency successfully; however, the central and fundamental tenets of certain older proposals have been proved to flagrantly violate the first law of thermodynamics. These arguments have been shown to be so strong, and the analysis so basic, that the conclusions are unassailable. A general kinetic analysis of oxygen exchange experiments has been carried out for the first time and reveals the absence of site-site cooperativity in F_1-ATPase. Mechanistic details of the nanomechanical coupling and transmission between F_1 to F_0 for ATP hydrolysis-driven H^+ pumping have been derived. The efficacy of this solution has been seen as a validation of the rotation-twist-tilt energy storage model proposed within the torsional mechanism for the reverse process of ion translocation-driven ATP synthesis. The torsional mechanism has been shown to take into detailed consideration and to bring together chemical, electrical and mechanical elements of the biological system. It has therefore been regarded as a breakthrough in our understanding, and as a potentially powerful unifying theme in biology. Thinking and approaches similar to that embodied in the torsional mechanism have been predicted to prove invaluable in solving the molecular mechanism of other interacting biological molecular machines through a new *Molecular Systems Biology*, as well as for evolving novel macroscopic engines and

molecular devices in nanotechnology by means of a *Molecular Systems Engineering*. The underlying unity in seemingly disparate energy transduction processes has been brought to light by solving the molecular force generation/motility problems for the case of myosin–actin and kinesin/ncd motors. Changes needed in our scientific thinking and industrial technology, and most important, in the education of a new generation of biological scientists for future innovation and progress have been identified as some of the major and urgent challenges before the community. This will require considered vigorous action in the near future. The opportunities are of great, even unprecedented magnitude, and there are very good reasons to be optimistic.

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