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A role for anions in ATP synthesis and its molecular mechanistic interpretation

Bhawana Agarwal

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Abstract ATP, the 'universal biological energy currency', is synthesized by utilizing energy either from oxidation of fuels or from light, via the process of oxidative and photo-phosphorylation respectively. The process is mediated by the enzyme F_1F_0-ATP synthase, using the free energy of ion gradients in the final energy catalyzing step, i.e., the synthesis of ATP from ADP and inorganic phosphate (P_i). The details of the molecular mechanism of ATP synthesis are among the most important fundamental issues in biology and hence need to be properly understood. In this work, a role for anions in making ATP has been found. New experimental data has been reported on the inhibition of ATP synthesis at nanomolar concentrations by the potent, specific anion channel blockers 4,4′-diisothiocyanostilbene-2, 2′-disulphonic acid (DIDS) and tributyltin chloride (TBTCl). Based on these inhibition studies, attention has been drawn to anion translocation (in addition to proton translocation) as a requirement for ATP synthesis. The type of inhibition has been quantified and an overall kinetic scheme for mixed inhibition that explains the data has been evolved. The experimental data and the type of inhibition found have been interpreted in the light of the torsional mechanism of energy transduction and ATP synthesis (Nath J Bioenerg Biomembr 42:293–300, 2010a; J Bioenerg Biomembr 42:301–309, 2010b). This detailed and unified mechanism resolves long-standing problems and inconsistencies in the first theories (Slater Nature 172:975–978, 1953; Williams J Theor Biol 1:1–17, 1961; Mitchell Nature 191:144–148, 1961; Mitchell Biol Rev 41:445–502, 1966), makes several novel predictions that are experimentally verifiable (Nath Biophys J 90:8–21, 2006a; Process Biochem 41:2218–2235, 2006b), and provides us with a new and fruitful paradigm in bioenergetics. The interpretation presented here provides intelligent answers to the unexplained existing results in the literature. It is shown that mechanistic interpretation of the experimental data requires substantial addition to available conceptual foundations such that present concepts, theories, and mechanisms must be revised.

Keywords ATP synthesis · ATP synthase · F_0 · Anions · Torsional mechanism · Chemiosmotic theory

Abbreviations

DIDS	4,4′-diisothiocyanostilbene-2, 2′-disulphonic acid
TBTCl	tributyltin chloride
DCPIP	2,6-dichlorophenolindophenol
ΔpH	pH gradient
$\Delta\psi$	electric potential gradient

Introduction

The energy from oxidation of fuels or from light is captured, stored and transported in the form of the chemical compound adenosine triphosphate (ATP) by the

B. Agarwal
Department of Biochemical Engineering and Biotechnology,
Indian Institute of Technology,
Delhi, Hauz Khas,
New Delhi 110 016, India

B. Agarwal (✉)
Biotechnology and Bioengineering Centre,
Department of Physiology, Medical College of Wisconsin,
8701 Watertown Plank Road,
Milwaukee, WI 53226, USA
e-mail: bagarwal@mcw.edu

B. Agarwal
e-mail: bhawanaa@gmail.com

process of oxidative and photo-phosphorylation respectively (Nath 2010a, b). ATP synthase plays a key role in carrying out this process by using the free energy of ion gradients. ATP synthase is located in the cytoplasmic membranes of prokaryotes and in the energy-transducing membranes of mitochondria and chloroplasts of eukaryotes as a prototype of *Escherichia coli* with similar structure and function in different species. This multi-subunit complex belongs to the F-type ATPase enzyme with distinct extramembranous and transmembrane domains termed F_1 and F_0 with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ and ab_2c_{10-12} respectively. F_1 and F_0 are connected via a central stalk constituted by γ–ε subunits and a peripheral stalk constituted by the hydrophilic portion of the two b subunits of F_0 and the δ subunit of F_1. The ion access channels are formed by the interacting regions of a and c subunits in F_0, while the catalytic binding sites are predominantly located in the β subunits of F_1 at the α–β interface.

Intensive research has been carried out over time to deduce the working of this molecular machine, with progress largely achieved for the hydrolysis mode of ATP; it is only during the last few years that single molecule studies have been planned to understand the detailed molecular mechanism in the ATP synthesis mode. The acceptance of the central chemiosmotic dogma as a "universal mechanism", which signifies *indirect* and *delocalized* coupling by uncompensated, electrogenic translocation of protons across the two bulk aqueous phases (Mitchell 1961), has emerged as a topic of great debate and controversies (Williams 1961, Green 1981; Slater 1987) and should rather be seen, based on strong experimental (Rottenberg and Solomon 1969; Massari and Azzone 1970; Azzone and Massari 1971; Massari et al. 1972; Massari and Pozzan 1976; Kaim and Dimroth 1999; Nath 2004, 2006a, b, 2008; Chen et al. 2004; Junge et al. 2009) and theoretical (Nath 2010a, b and references therein) reasons as a *localized* and *direct* coupling phenomenon. Moreover, in recent years, it has been shown that succinate permeates the membrane and generates an electric potential that provides part of the driving force for ATP synthesis (Kaim and Dimroth 1999; Dimroth et al. 2000; George and Dimroth 1999) these results ostensibly inconsistent within the framework of the chemiosmotic theory. Experiments on ATP synthesis driven by an artificially generated ΔpH (Hind and Jagendorf 1963; Jagendorf and Uribe 1966), and by an artificially generated external electric field pulse or diffusion potential (Witt et al. 1976; Kinnally and Tedeschi 1976; Fischer and Gräber 1999) have been carried out also but those studies could not infer anything about the mechanism of action of anion in the coupling phenomenon. During the last 15 years, a unified molecular mechanism of ion translocation in F_0 (Jain and Nath 2000; Jain and Nath 2001; Nath and Jain 2002; Nath 2002, 2003, 2004), ionmotive torque generation in F_0 (Rohatgi et al. 1998; Jain and Nath 2000; Nath and Jain 2002; Nath 2002, 2003, 2004), torque transmission from F_0 to F_1 (Nath et al. 1999, 2000; Nath 2002), energy storage in the enzyme (Nath et al. 1999; Nath and Jain 2000, 2002; Nath 2002, 2003, 2004), conformational changes in F_1, and a novel catalytic cycle of ATP synthesis (Nath et al. 2000; Jain and Nath 2001; Nath 2002, 2003) called Nath's torsional mechanism of energy transduction and ATP synthesis has been formulated in consummate detail, with the specific introductory existence of a specific, regulated anion half-access channel in the a-subunit of the F_0 portion of ATP synthase at the a-c interface, in close vicinity of a proton half-access channel.

The mechanism of anion-proton symsequence-cotransport coupled energetically to ATP synthesis has been pioneered by Nath's torsional mechanism of energy transduction and ATP synthesis (Jain and Nath 2000, 2001, 2002; Nath 2002, 2003, 2004, 2006a, b). The present study was attempted to rationalize the new conceptual thinking of energy coupling through the intervention of coupled transport of proton and anion to overcome the lacunae and inconsistencies present in the previous theories. Moreover, the role of anions has also emerged from previous work by other groups, but the study conducted here helps in consolidation of this role and in clarifying the mechanism of ATP synthesis at the molecular mechanistic level. Our experimental evidence in favor of this novel scenario is discussed. In particular, new experiments showing inhibition of ATP synthesis by potent, specific anion channel blockers such as DIDS (4,4′-diisothiocyanostilbene-2, 2′-disulphonic acid) and TBTCl (tributyltin chloride) at as low as nanomolar concentrations are presented. The inhibitors also tested specificity of the access channels in F_0 for different anions. A mixed type of inhibition has been found in this work and a kinetic scheme that explains the data and the type of inhibition has been proposed. The torsional mechanism has been shown to be consistent with the mixed inhibition scheme obtained for the ATP synthesis process in the membrane-bound F_0 portion of the ATP synthase enzyme. The mechanistic approach taken to infer the results of inhibition proved quite valuable for resolving difficulties associated with previous theories (Slater 1953; Williams 1961; Mitchell 1961; Mitchell 1966). Such a detailed mechanism that has resolved the problems, inconsistencies, and ambiguities in the first theories, and has the power to make novel predictions already exists (Nath 2010a, b and the references therein). Finally, the torsional mechanism itself appears to be a useful theory/framework for guiding future experimentation and logical thought in the field.

Materials and methods

Isolation of thylakoid membranes

Thylakoid membranes were isolated from spinach (*Spinacia oleracea*) leaves purchased from the local market by the method of Tripathy and Mohanty (Tripathy and Mohanty 1980). Spinach leaves were purchased and kept refrigerated in darkness to maintain chloroplast activity. Leaves were first deveined and a total of about 50 g of spinach leaves were washed with distilled water, kept cooled at 4°C, soaked on filter paper and then homogenized in a Waring blender for 40 s together with the isolation buffer (0.4 M Sorbitol, Tris 0.05 M, EDTA 1 mM, $MgCl_2$ 1 mM, adjusted to the pH 7.3) in the ratio of 1:8 w/v of spinach leaves to isolation buffer. The mixture obtained was filtered through 8 layers of cheese-cloth and one layer of miracloth. The filtrate obtained was centrifuged at 1170 g for 7 min at 4°C. The supernatant obtained was discarded and the pellet which contained chloroplast, broken cell wall, starch grains was suspended in hypotonic TE buffer (Tricine 0.01 M and EDTA 1 mM, pH 7.5) in the ratio of 5:1 w/v of spinach leaves taken initially and kept in ice for 15 min. The suspension was again centrifuged at 4650 g for 5 min at 4°C. The supernatant was carefully discarded and the pellet containing thylakoid membranes along with cell debris and a starch layer was dissolved in a minimal volume of hypertonic suspension buffer (Sorbitol 0.4 mM, Tris 0.05 M, EDTA 1 mM and $MgCl_2$ 1 mM, pH 7.5) in 5:1 w/v of spinach leaves and finally stored at −80°C until used (for a maximum of 24 h).The whole procedure was carried out in the dark.

Estimation of chlorophyll content

The chlorophyll content was measured according to the method of Arnon (Arnon, 1949). 100 μl of the thylakoid membrane suspension was suspended in 20 ml of 80% (v/v) acetone and filtered in the dark through Whatman no.1 filter paper. The optical density of the filtrate was determined at 652 nm against a blank of 80% (v/v) acetone in a quartz cuvette and multiplied by a factor of 5.8 to give the chlorophyll concentration in mg/ml. The chlorophyll concentration was adjusted to 0.5 mg/ml with suspension buffer and was used for carrying out phosphorylation as described next.

Measurement of ATP synthesis

The estimation of the rate of ATP synthesis was done by phosphorylation of ADP by the technique of 'acid-base' transition (Jagendorf and Uribe 1966). The method was based on microcolorimetric estimation (Taussky and Shorr 1953) of removal of phosphorus from the mixture, which has been utilized for the formation of ATP from ADP. The classical, 'acid-base' transition scheme involves two steps: acidic stage and basic stage. In the acid stage (AS) 0.5 ml of thylakoid membranes were incubated with 0.9 ml of acid (pH 4.0) for the desired time in order to accumulate the acid of interest. This was then transferred to 0.9 ml basic stage (BS) buffer (Tricine 100 μmoles, Adenosine diphosphate 0.2 μmoles, KH_2PO_4 2.0 μmoles, $MgCl_2$ 5.0 μmoles, and NaOH 19.6 μmoles, pH 8.3) by 1:1 dilution, consequently allowing diffusion through the membrane from inside to out according to the concentration gradient of the species for catalytic synthesis of ATP via the ATP synthase complex. The reaction was terminated by the addition of 200 μl of 20% (w/v) trichloroacetic acid (TCA). For the control, TCA was added in the base stage (prior to incubation with thylakoid membranes). The whole suspension (2.0 ml) was taken in two different tubes (1 ml each) and centrifuged for 10 min at 7270 g. Transfer of thylakoid membranes was carried out with a 1 ml glass syringe attached with a 20-gauge cannula, and the entire procedure was carried out in complete darkness. Inhibition studies were carried out with inhibitors DIDS and TBTCl added during the preparation of base stage buffer at different concentrations required.

Estimation and calculation of rates of ATP synthesis

The rate of ATP synthesis was calculated using an ATP kit and the luciferin-luciferase assay and is measured in μmoles of ATP produced (mg of chlorophyll)$^{-1}$ min^{-1}. The measured concentration of ATP produced was subtracted against the sample blank for each reading.

Estimation of Cl^-

To measure the amount of chloride transported per molecule of ATP synthesized, chloride was estimated by the mercury thiocyanate method using KCl as a standard (Florence and Farrar 1971). 15.1 g of Fe $(No_3)_3.9H_20$ was dissolved in 45 ml of 72% perchloric acid and diluted to 100 ml with water. Saturated solution of mercury thiocyanate in ethanol was prepared. Iron (III) nitrate and mercury (II) thiocynate (2 ml each) were added into 500 μl of the sample aliquot obtained after acid–base phosphorylation and diluted to a volume of 25 ml. Absorbance of the reaction mixture was measured against the reagent blank at 460 nm. Standard was prepared by adding different amounts of 1 μg chloride/ml (in the form of KCl) and 2 ml each of iron nitrate and mercury nitrate solutions were added to the total volume of 25 ml.

Results and discussion

Effect of anion (succinate and chloride) on rate of ATP synthesis

The effect of anion on biochemical rates of ATP synthesis was assessed by the method introduced by Jagendorf and Uribe (materials and methods). Chloride was found to be the physiological anion in ATP synthesis in spinach thylakoid membranes, as physiological rates were observed with this anion being used in the form of HCl at an optimal concentration of 0.5 mM (Nath 1994; Mehta 2004). In these experiments, both acid and base stage times were optimized to measure 15 s. The translocation of charged anion species was postulated by the torsional mechanism to occur through anion access pathways present in the F_0 portion of ATP synthase in coupling with proton half-access channels (Jain and Nath 2000, 2001; Nath and Jain 2002; Nath 2002, 2003, 2004, 2006a, b).

Figure 1 and Fig. 2 show the rate of ATP synthesis as a function of acid concentration with which the thylakoids were incubated in an acid stage. Both acid stage and base stage were kept at 15 s in all cases. Further, the stromal pH was also kept constant during the base stage at 8.3. Thus, a concentration gradient with respect to protons cannot decrease with increased acid concentrations employed in the experiment. Thus the observation of decreased rates with increased succinic acid cannot be explained by conventional theories such as chemiosmosis. However, the increase in succinate concentration results in a large amount of succinate remaining in the stromal side and hence the concentration gradient of anion during the base stage is progressively reduced with increase in HA concentration

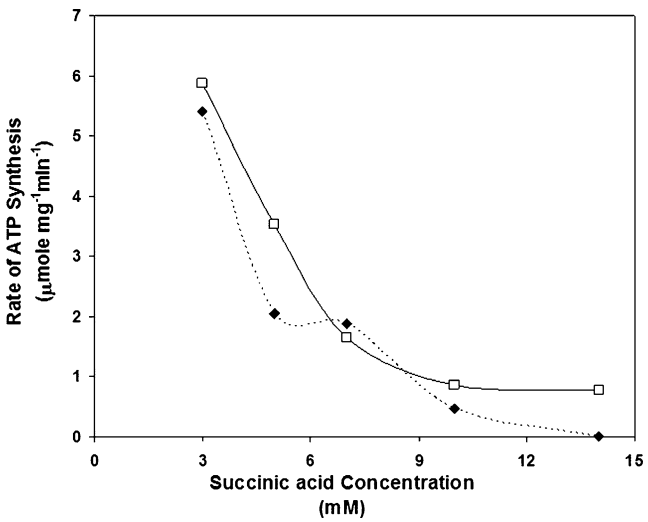

Fig. 1 Rate of ATP synthesis as a function of various concentrations of succinate for AS and BS measured at 15 s. ATP rates measured in duplicate: (□) and (■)

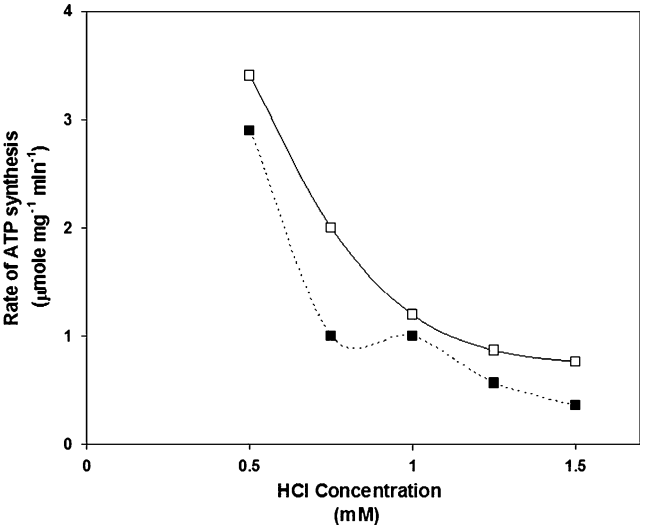

Fig. 2 Rate of ATP synthesis as a function of various concentrations of HCl for AS and BS of 15 s. ATP synthesis rates measured in duplicate: (□) and (■)

during the incubation stage. Hence, the trends obtained in Fig. 1 and 2 are logically explained by simple principles of transport phenomena.

Additionally, in order to elucidate the contribution of chloride (anion) in ATP synthesis in our system, an experimental approach was designed to study the response of anion channel blockers DIDS and TBTCl (also an ionophore) on ATP synthesis. First, experiments were performed with different concentrations of inhibitors and substrate HCl, at its optimum concentration of 0.5 mM, at various acid and the base stage incubation times. No consistent observations were made at high inhibitor concentrations between 50 µM and 100 µM at which they had been tried earlier, and found inhibitory with other systems. In order to find the optimal inhibitory concentration of the inhibitors in our thylakoid model system, the rates of ATP synthesis were measured at inhibitor concentrations between 1 nM and 50 µM. Consistently the inhibitory range with DIDS was located between 1 nM to 10 nM.

The profiles of rate of ATP synthesis with DIDS at concentrations between 1 nM and 10 nM with 0.5 mM HCl as a function of base stage times, as compared to rates observed in control, i.e., in the absence of DIDS are shown in Fig. 3. Comparison of profiles showed a decrease in rates of ATP synthesis with a successive increase in DIDS concentration up to 8 nM at every base stage time studied (Fig. 3).

The rates of phosphorylation observed with the other anion channel blocker TBTCl at 10 nM concentration also exhibited a similar decline in measured rates. An average value of decline in measured rates over the entire range of base stage times (15 s–75 s) was found to be greater than

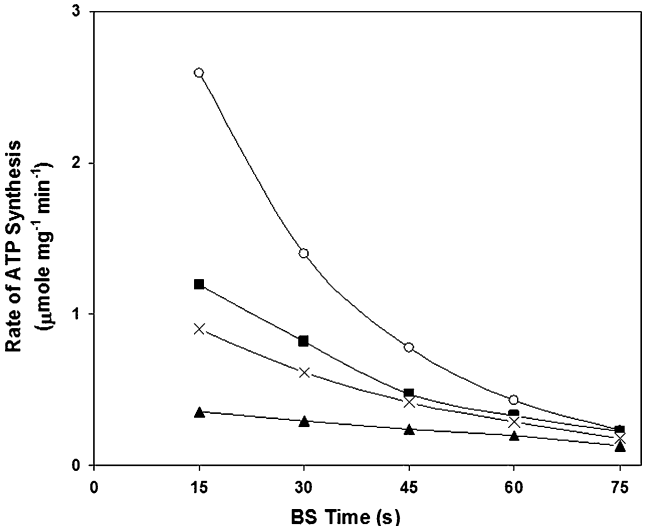

Fig. 3 Effect of different concentrations of DIDS added to the base stage buffer on the rate of ATP synthesis with 0.5 mM HCl concentration. The acid stage time was kept constant at 15 s. The base stage times ranged between 15 s and 75 s. DIDS concentrations: 0 nM (○); 4 nM (■); 6 nM (×); 8 nM (▲)

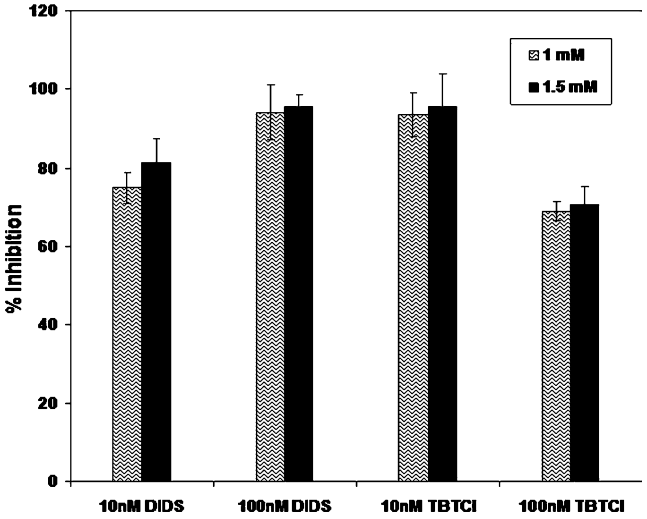

Fig. 4 Percentage inhibition of rate of phosphorylation (with reference to control) at different concentrations of inhibitors (DIDS and TBTCl) used in the base stage buffer. Experiments were made at two different concentrations of succinate (light -1mM and dark-1.5 mM) as the acid stage buffer. The acid and base stage times were kept constant at 15 s and 15 s respectively

65%. However, the rates were completely abolished at other lower concentrations of TBTCl, suggesting the irreversible inhibition by TBTCl at the same DIDS binding site.

Classical studies of Jagendorf and colleagues had also identified succinate as an anion supporting high rates of ATP synthesis (Jagendorf and Uribe 1966). Hence, further experiments were conducted with succinate as the permeant anion. The above result in terms of finding inhibitory range of DIDS for ATP synthesis by the thylakoid model system with HCl at 0.5 mM was also found to be consistent in experiments performed by taking succinate at its optimum concentration found (1 mM) in place of chloride. Though the nature of inhibitors used are reported to be highly specific for chloride, the inhibition trend found was exactly similar to what was found with chloride along with DIDS and TBTCl at 10 nM.

As shown in Fig. 4, we worked with a higher concentration of succinate to check for its ability to reduce the affinity of binding of DIDS, as would be the case if the sites for succinate and DIDS overlapped with each other. Despite TBTCl's ability to exhibit some different mode of inhibition, the same test could also hold good for TBTCl, as binding of both inhibitors may occur at the same site. Further, almost similar inhibition suggested the negligible substitution of bound inhibitor at a higher concentration of succinate (1.5 mM) at all the inhibitor concentrations studied (Fig. 3). This confirmed the inability of succinate to substitute for the inhibitor and relieve its inhibitory effect, probably owing to the non-specificity of succinate to this model system. Several other experiments conducted, however, indicated that at higher concentration, chloride could relieve DIDS inhibition by possessing the ability to substitute for the inhibitor, again pointing to chloride as the physiological anion in our system and confirming reversible inhibition at its binding site.

To further examine whether inhibitors shared the same or had separate binding sites on the enzyme, combinations of different inhibitor concentrations were tested, keeping the total inhibitor concentration constant at 10 nM. Increase in measured rates were observed with an increase in TBTCl concentrations, and a consequent decrease in DIDS concentrations (Fig. 5). Thus, because of a non-specificity of

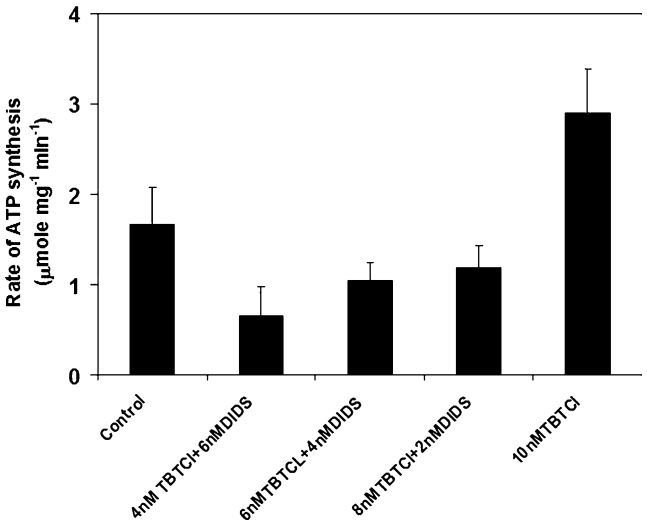

Fig. 5 Effect of combinations of different concentrations of inhibitors employed during the base stage time of phosphorylation with 0.5 mM HCl. The total concentrations of the inhibitors were kept constant at 10 nM. The acid and base stage times were 15 s and 30 s respectively

succinate to compete with DIDS at the reversible binding site, Fig. 5 shows accelerated rates because of a decrease in DIDS concentration. If the binding sites for DIDS and TBTCl had been different, then we would have expected inhibition at 10 nM TBTCl also. These results suggested the same binding sites for reversible and irreversible binding of DIDS and TBTCl respectively. Succinate binds at the substrate site, which partially overlapped the reversible DIDS binding site, and also had to interact when TBTCl bound at the same binding site irreversibly. At higher (greater than 10 nM) concentrations of TBTCl also, further-fold rate enhancement was found, which requires a more detailed and microscopic explanation.

The above observation in terms of inhibitory and enhanced rates could be explained satisfactorily only after the type of inhibition was found with the inhibitor (DIDS) and substrate (HCl) concentrations.

A shortcoming of the chemiosmotic theory: Phosphorylation in presence of anion and its explanation

The acid concentration dependence of the rate of ATP synthesis elucidated the role of anion in ATP synthesis. The physiological rate of ATP synthesis was observed with different mono and dicarboxlic acids and also with an inorganic acid, HCl which were incorporated in the acid stage buffer, AS (materials and methods), When compared for the same time of incubation in acid and base stage buffers, rates observed with the monocarboxlic acids were found lesser than the rates observed with dicarboxlic acids due to delocalization of charge and hence more permeability of the negatively-charged dicarboxlate ion (charged singly) across the membrane. The consequent electrogenic transport of counter ion (in this case a proton, or anion if the primary ion of transport was the proton) made the process overall electroneutral in the acid stage. Further, accumulation of proton and anion inside the lumen of the membrane resulted in a lesser amount of ions left outside i.e., in the stroma. This along with 1:1 dilution in BS led to establishment of the gradients of proton and anion. Proton gradient was developed due to a transition in pH from 4.0 to 8.3. The pH jump was kept constant irrespective of the concentration of anion employed. The anion gradient was largely dependent on the initial concentration of the source of anions taken to expose the membranes in the acid stage.

The above further rules out chemiosmosis theory as in the framework of this mechanism anions have no role to play in the coupling process and both the driving forces ΔpH and $\Delta\psi$ of ATP synthesis are generated by proton only. While, in the view of the torsional mechanism, both Δp proton and Δp anion contribute their own part in generating $\Delta\psi$ that would take place due to discrete translocation of anion and proton through their respective half-channels.

Both anion and proton traversed across the membrane through their respective anion and proton half-access channels present in a- and c- subunit respectively at the a–c interface of the membrane. Moreover, the concentration of the acid used was largely dependent on the solubility of anion in the anion half-access channel. It was reasoned that chloride could have more solubility in the anion access channel of the a- subunit at the a–c interface and hence HCl was required in lower concentration (0.5 mM) than succinic acid (1 mM). Both of these acids showed the maximum rates of ATP synthesis. The result could not be fit into the chemiosmotic framework wherein only the role of protons has been emphasized and was only interpreted based on a different anion gradient generated at different acid concentrations employed. As predicted by the torsional mechanism, in order for the process to sustain physiologically electroneutral, transport of an anion down the concentration gradient along with a proton is considered equally mandatory. Both anion (chloride or succinate) and proton move in a coupled way through their respective half-access channels in the F_0 portion of ATP synthase that constitute the anion-proton symsequence-cotransporter to maintain the 1:1 stoichiometry of anion-proton transport as a most preferred case as per the framework of the torsional mechanism.

Though, both succinate and chloride supported the high rate of ATP synthesis. The experimental observation of a sharp exponential decline in rate of ATP synthesis with increase in base stage phosphorylation time (or with increase in succinic acid concentration), the buffering nature of succinate in acid stage, sucinate as a weak organic acid may interfere negligibly in maintaining the ionic stability of chloroplast that also support the observed ATP rates at in the high concentration range (~1 mM to ~14 mM) of the acid employed, it constitutes as one of the intermediates of the known mitochondrial energy metabolism (and probably the same way it may also participates in energy metabolism of other model systems of chloroplast and bacteria): all these reasoned succinate as the physiological anion for *in vivo* synthesis of ATP with the working models of chloroplast, mitochondria or bacteria. Though chloride (in the form of HCl) also supported the synthesis of ATP with the model system of isolated thylakoids but the small concentration of HCl employed (0.5 mM) stands negligible in front of the high concentration of chloride (~150 mM) present in the cytoplasm as a principal anion in chloroplast and in other plant systems and thus chloride cannot be treated as a physiological anion to account for *in vivo* synthesis of ATP but could give high *in vitro* ATP rates with isolated thylakoids that are devoid of any chloride content which was present in cytoplasm of the chloroplasts.

The anion access half-channels would support the entry to and exit from the thylakoid lumen of membrane permeable anions that is followed by the transport of protons through its respective half-access channels, along their concentration gradients. Anion and proton half-access channels are present in the a- and c- subunits respectively. Thus energies of both delocalized $\Delta pH/\Delta pA$ and localized $\Delta\psi$ created by anion and proton are utilized for phosphorylation by binding to their respective sites in the vicinity of the F_0 complex. The presence of 'coupled symsequence- cotransport' has not only been suggested to be essential for maintaining overall electroneutral transport but also for donating half the energy for synthesizing ATP by a catalytic cycle involving conformational changes in F_1 that have been spelled out in great detail in Nath's torsional mechanism of energy transduction and ATP synthesis.

The elementary steps of translocation of ions, as has been emphasized in the torsional mechanism, can only occur as long as $pH_{in} < pKa < pH_{out}$. If pHout < pKa the ions would bind in the vicinity of ATP synthase (Nath and Jain 2002; Nath 2002; Rohatgi et al. 1998) but would not exit unless the discrete translocation of ions across the membranes can take place. Though enough ΔpH can still exists, that provides the gross or macroscopic driving force, it would not allow the transport to take place. Hence, gross ΔpH or ΔpA cannot drive ATP synthesis though they determine the number of times the elementary translocation of proton and anion can take place. This differs from the chemiosmotic theory according to which the gross ΔpH in the bulk phase is solely responsible for making ATP in plant chloroplasts.

Coupled transport of anion and proton for phosphorylation: Characterization of mixed inhibition with the potent, specific anion channel blocker (DIDS)

New experimental evidence of biochemical studies on the inhibition of ATP synthesis by a specific and potent anion channel blocker, 4,4′-diisothiostilbene 2,2′-disulfonate (DIDS) accounted for a detailed investigation of the molecular mechanism of energy transduction in the membrane bound F_0 potion of F_1F_0 ATP synthase. The result thus obtained (Fig. 6a and b) could not be explained by the classical gross model of chemiosmosis that relies on protons as the only source of energy for ATP synthesis but was found in agreement with framework of the torsional mechanism of energy transduction and ATP synthesis. Inhibition with DIDS was characterized by monitoring the phosphorylation in the presence of chloride as the source of anion by adding different concentrations of HCl (0.5 mM to 1.4 mM) in the acid stage that could give the measurable rates of ATP synthesis, and a nanomolar concentration range of DIDS between 1 nM to 10 nM was incorporated in the base stage. The result was thus inferred from the Dixon plot made between the inverse of the rates at particular substrate concentrations and different inhibitor concentrations. The inhibition was found to be the mixed type, which was further resolved into predominately competitive at low HCl concentrations (0.5 mM to 0.75 mM) and predominately uncompetitive at other higher HCl concentrations (1 mM to 1.4 mM) studied while the minor components of the mixed inhibition would be characterized as uncompetitive and competitive respectively in the above case. In a typical mixed inhibition scheme, by the virtue of competitive and uncompetitive inhibition, the characteristic binary enzyme-inhibitor (EI) complex and ternary enzyme-substrate-inhibitor (ESI) complex would respectively be formed. The substrate for which the inhibitor is competitive can remain in an unbound state and thus would characterize the formation of the EI complex while the uncompetitive inhibition is classified as long as the substrate for which the inhibitor is uncompetitive remains bound to its site on the enzyme (ESI). The presence of competitive and uncompetitive components of a mixed inhibition clearly signifies the role of both proton and anion in phosphorylation. It was of interest to notice that though the inhibitor (DIDS) employed was a specific anion channel blocker, it showed an effect on ATP synthesis, which in the framework of chemiosmosis should only be energetically linked to one species, i.e., a proton. However, the result can satisfactorily be explained in the light of the torsional mechanism that highlights the role of a anion in giving ~50% of its energy in the coupling process and the other ~50% coming from a proton. It could only be possible if both proton and anion move in a coupled way and binding/unbinding of one ion would precede the binding/unbinding of the other.

Any mechanistic molecular level explanation of the above found competitive and uncompetitive inhibition would depend on the primary and secondary activities of the ions in an ordered and sequential way. In our mode of study, the inhibitor DIDS was added at the start of the base stage phosphorylation time. Moreover, since the inhibitor used in our experiments serve as a potent anion channel inhibitor, we logically expected DIDS to bind at the surface of the anion exit half-access channel at the stromal side of the thylakoid membrane and not interfere in entry of anion through the other half entry half-access channel. However thus mentioned primary activity of anion would interfere with movement of proton as the secondary ion in the step-by-step sequential mechanism of transport of anion and proton. As a consequence of which binding of the inhibitor to the anion half-access pathway that along with the proton access pathway forms a rigid intra-membranous link to facilitate the coupled anion-proton transport would also occlude the transport of proton.

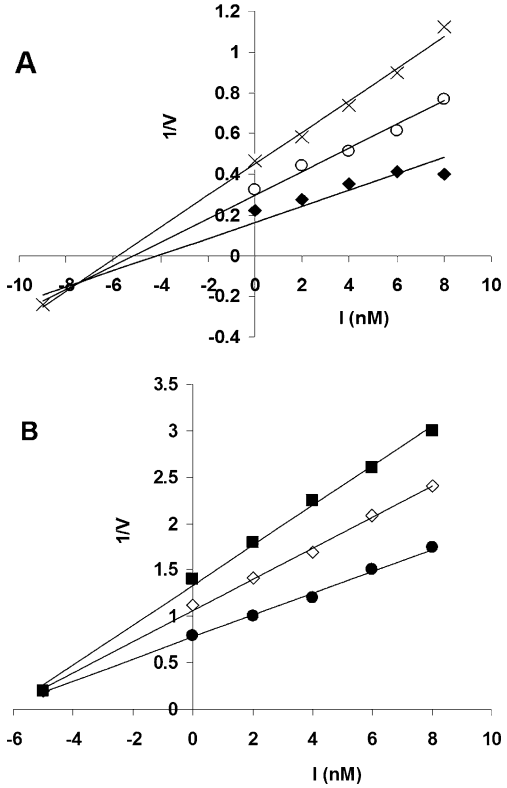

Fig. 6 Dixon plot of inverse of rates (V^{-1}) against inhibitor concentration (I) for ATP synthesis by spinach thylakoid showing mixed (competitive and uncompetitive) inhibition. Inhibition is mixed competitive when the intersection of lines is found above the negative X-axis with substrate increase in inverse direction i. e. from bottom to top. Inhibition is uncompetitive when intersection is found below the line denoted by the X-axis in the first two-quadrant. Inhibiton was studied with HCl and DIDS concentrations ranged between 0.5 mM to 1.25 mM and 2 nM to 7 nM, respectively. HCl concentrations: **(a)** 0.5 mM (♦); 0.625 mM (○); 0.75 mM (×) **(b)** 1 mM (●); 1.25 mM (◊); 1.4 mM (■)

Thus, after both chloride and proton have bound to their respective binding sites on the a- and c- subunits respectively, the inhibition would be competitive with respect to chloride if the Cl^- unbinds from its site but cannot come out of the a- subunit anion exit access channel due to the block caused by the binding of DIDS to its site at the surface of the a-subunit anion access channel. If the two ions translocate in a coupled way and the proton cannot unbind from its site following unbinding and further inhibition in the exit of Cl^- out in the stromal medium (that occur at high stromal chloride concentration around each anion exit half-access channel) would summarize the uncompetitive inhibition. However, the minor associated components of the mixed inhibition at each HCl concentration were the result of the statistical heterogeneity in the ionic distribution of the ions around the large population of the exit half-access channels present, depending on the amount of thylakoids present in the sample.

Determination of Cl^-/ATP ratio

After the role of chloride (anion) was critically examined and validated, attempts were made to find out the stoichiometry of chloride transported per molecule of ATP synthesized. Again no such report is available in the literature on measurement of the amount of chloride traversed that could account for ATP produced because of ignorance of the role of the anion in ATP production.

In order to obtain further information on the correlation of the rate of ATP synthesis and amount of chloride (anion) transported, the rate of ATP synthesis was measured and simultaneously the amount of chloride that came out in the external medium was also estimated (Material and Methods) as a function of BS phosphorylation time.

The amount of chloride that came out into the external stromal medium was converted into the rate of chloride transported per minute through the total number of F_1F_0 molecules that were present in the initial volume of thylakoid sample taken, i.e. in 0.25 mg of chlorophyll. It was correlated with the rate of ATP synthesized per mg of chlorophyll per minute to estimate the amount of chloride transported per molecule of ATP produced in 15 s basic stage time (Fig. 7).

The trends of both the rates were found to follow the same kinetics (Fig. 7). However, the result of such an experiment showed us very high rates of chloride transported (1.5 μmoles/mg chlorophyll per 15 s) in 15 s base stage time at which the maximum ATP rate was observed. The Cl^-/ATP ratio came out to be 3.3–4.1, which is in the range of the structural and thermodynamic measurements (Stock et al. 1999; Van Walraven et al. 1996; Turina et al. 2003). According to the torsional mechanism proton and chloride translocation should follow 1:1 stoichiometry. Further, it has been well established in the literature that ~4 protons are transported through the proton channel to synthesize a molecule of ATP (Van Walraven et al. 1996; Turina et al. 2003). If we correlate the ratio of chloride translocated per molecule of ATP produced from our experimental result, which was equal to 3.3–4.1, to that with the number of protons required to synthesize a molecule of ATP (i.e. ~4, that came from the literature), it almost followed a 1:1 stoichiometry of transport. Hence, it has emphasized the electroneutral transport of proton and counter ion. The resolution of discrepancies in the measurement of this ratio by structural and thermodynamic methods may require further research. Furthermore, depending on the number of c subunits, the exact number of proton and chloride traversed is again controversial and can only be resolved after probing the structure of F_0 in detail.

The above is only consistent with the torsional mechanism of energy transduction and ATP synthesis. According to the torsional mechanism, ~half the energy comes from

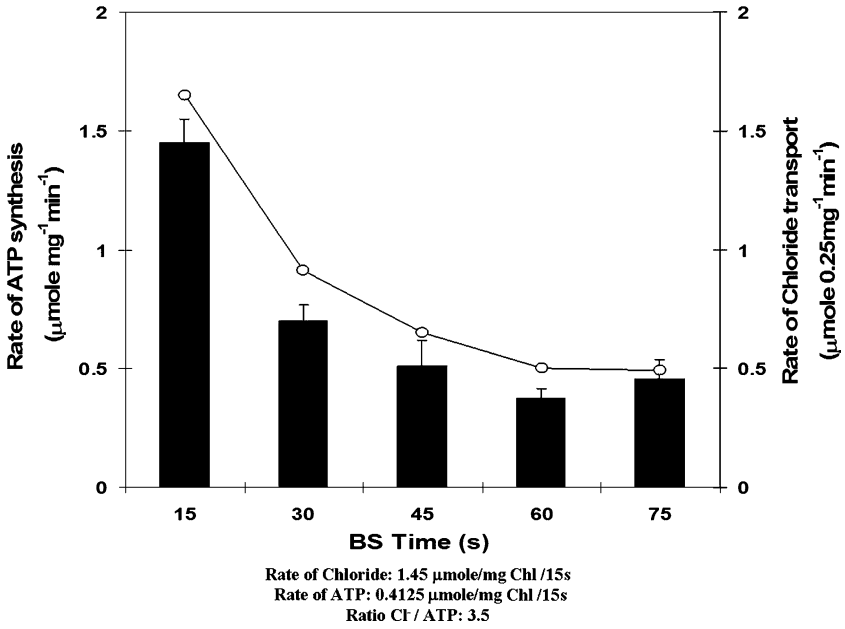

Fig. 7 Comparison of profiles of rate of ATP synthesis (○) and rate of chloride transported (bar) as a function of BS time

anion translocation and other ~half comes from proton translocation through their specific half-access channels in the F_0 portion of ATP synthase. Both these access channels are present close to each other and form an intramembrane link, which facilitate the coupled transport of proton and chloride and help in additive utilization of their energies that drive ATP synthesis (Nath 2002, 2003, 2004).

Implications for role of proton and anion and the consequence of binding of an anion channel blocker on phosphorylation in the light of the torsional mechanism

The inhibitor (DIDS) used was a potent chloride channel blocker but it consistently showed an effect on the rates of ATP synthesis in presence of succinate also. Thus, it has further validated the role of anions in phosphorylation and the presence of anion access channels with specificity for some particular anions (succinate and chloride) that were shown to support the rates.

When events involved were scrutinized, it was experimentally found that proton alone could not traverse inside without the anion. Moreover, binding of DIDS blocked the passage of anion movement. This prevention would lead to following consequences: Firstly, because of the inability of an anion to come out, a proton that traversed inside the membranes will also not exit (if anion is the primary ion to leave the membrane first), or even if the proton would come out (if proton being the primary ion), due to the high proton gradient present across the membrane, the positive charge thus created in the external medium would not be balanced in the absence of the counter ion (anion/succinate).

In either case, blocking of the anion access pathway will not favor the proton to exit. Secondly, as per the torsional mechanism, half of the energy in phosphorylation comes from anions while the other half comes from protons. So even if for instance we assume that binding of DIDS would not inhibit the proton transport, half the energy that comes from anions was blocked from being incorporated in ATP. Hence, other than imparting a physical role in maintaining electroneutrality only, the anion also acts actively to provide energy by linking to the protein in the membrane (anion access channel) in the F_0 portion of ATP synthase. This is in opposition to what has been suggested in the framework of the chemiosmotic hypothesis wherein the membrane acts only as an insulator in separating two sides. Also the role of anion had not been anticipated in the older framework. In the torsional mechanism, binding and release of proton and anion bring step-by-step sequential changes in membrane conformation. Since, subunits ‘c’ and ‘a’ constitute proton and anion access pathways respectively, binding of proton and anion in those respective domains of the F_0 portion cause rotation of the subunit c-rotor by 15–18°. (Rohatgi et al. 1998; Jain and Nath 2000; Nath 2002). In the torsional mechanism it has also been predicted that the anion-binding site resides in the Arg-210 of helix 4 of the a-subunit at the a-c interface of the F_0 portion of the ATP synthase and in its immediate vicinity c-Glu/c-Asp-61 constitutes the proton-binding site. Thus the new mechanism highlights the role of both ΔpH and $\Delta\Psi$ using two mutually non-collinear half channels. Anion and proton flow through their respective half channels along their concentration gradient. Binding of anion in the vicinity of the F_0 complex creates a localized $\Delta\Psi$ that is followed by the transport of protons that cause the overall change in $\Delta\Psi$. The energies released during each binding and unbinding

steps rotates the c-rotor by ~15°. During each ~15° rotation of the c-rotor the ion-protein energy is first stored as twist energy in helices of the c-rotor. Simultaneous untwisting of the c-rotor drives the rotation of the ε-subunit and some part of the γ-subunit that leads to conversion of twist energy to torsional energy that gets stored in the γ-subunit. Thus energies of both delocalized ΔpH and localized ΔΨ is utilized by the enzyme in phosphorylation by binding of the proton within the localized field created by the transport of anion.

Keeping the above discussion and the results of inhibition patterns of Fig. 4 in mind, the only model that could explain all our experimental findings is Nath's torsional mechanism of energy transduction and ATP synthesis. This mechanism specifies the detailed events taking place and their temporal order during ATP synthesis. A unique tenet of this mechanism is the significance and energetic role accorded to membrane-permeable anion translocation (apart from proton/cation translocation) in providing the total energy needed for the production of ATP. The binding and unbinding of anion and proton translocations in their respective access channels, the coupled nature of such transport, and the *local* energy transduction taking place has been described in great detail in the mechanism. According to the mechanism, after chloride unbinds from its binding site, inhibitor binds to its place without allowing time for the proton to move out, and hence leads to inhibition. However, at high concentrations of inhibitor, inhibitor could also bind to the free enzyme (as per the virtue of mixed inhibition). At high concentrations of substrate in the acid stage (for a fixed incubation time), a greater concentration of chloride remained outside the thylakoid membranes, and thus there existed more competition between chloride and inhibitor for the same binding site on the enzyme. This led to more number of cycles per unit time and hence showed enhanced rates.

We could not find any way to explain the role of anions for ATP synthesis identified in this work by currently accepted models, such as the chemiosmotic theory. In a sense, this is clearly understandable, because chemiosmotic dogma and other models of energy coupling only consider the proton as the coupling ion, and do not invoke any role for other ions in energy coupling. A recent debate (Tedeschi 2005a, b; Jain et al. 2004) has pointed out other long-lasting difficulties, inconsistencies, and ambiguities in the first theories. However, no way forward has been suggested (Tedeschi 2005a, b). Revision of the first theories along the lines described in detail in the torsional mechanism provides a way out of the present impasse, and resolves the problems by its new molecular systems biology ideas and approaches once and for all (Jain et al. 2004). Finally, powerful electrophysiological techniques of the kind recently described (Feniouk et al. 2004; Franklin et al. 2004; Vijayvergiya et al. 2004; Lill et al. 1987; Schönknecht et al. 1988; Zachariae et al. 2003) can also help to resolve the problem experimentally.

Conclusions

A role for anions in ATP synthesis has been experimentally found. The new experimental data reported on the inhibition of ATP synthesis at nanomolar concentrations by the potent anion channel blockers 4,4′-diisothiocyanostilbene-2, 2′-disulphonic acid (DIDS) and tributyltin chloride (TBTCl) showed the importance and need to look at ATP synthesis in F_0, and revealed the requirement of permeant anions in bioenergetic coupling of ATP synthesis along with protons. At the molecular level it showed coupled and sequential transport of anion and proton and also characterized the type of inhibition. A mixed type of inhibition has been found; the results have been shown to be satisfactorily explained by considering the inhibition to be uncompetitive with respect to proton and competitive with respect to anion. The experimental data and the type of inhibition found have been shown to be consistent with Nath's torsional mechanism of energy transduction and ATP synthesis.

The data thus collected with DIDS and TBTCl inhibition seemed interesting, notably because both inhibitors appear very potent. Though, it was beyond the scope of this work, one may also suggest exploring the possibility of interacting these inhibitors with and inhibiting ATP synthase directly by using purified ATP synthase reconstituted in liposomes, or by using radioactive affinity labels of DIDS, or perhaps by using radioactive DIDS and TBTCl. But according to authors' view point working with the purified system may not reflect the real picture of replicating the data *in vivo* (as given here with the isolated thylakoid model) and also following others could be considered to have obvious disadvantages, like (a) during the course of the process of purification the enzyme may lose one or more subunit(s) (b) penetration of a radio labeled anion into the cell membrane is very slow and hence would lead to poorly labeled enzyme (c) it would further lead to the oxidation of only externally exposed surfaces of the membrane and may not penetrate to bind with the 'a–c' subunit where ATP synthase anion channels are supposed to be located (d) radioisotopes have short half lives and show slow specific radioactivities of the labeled product. Further, the class of trialkyltin compounds exhibits chloride/hydroxide exchange activity that leads to an interpretation even more difficult especially if chloride is present in the reaction mixture. Nevertheless, the results of the experiments suggest here that the transport of chloride is closely linked with ATP synthesis.

The concepts presented in this and previous communications (Nath 2010a, b and references therein) permit a consistent and unified treatment of essential aspects of ATP synthesis. Indeed, it is even possible to make predictions of mechanistic details of ATP synthesis in the F_0 portion of the ATP synthase, and to offer detailed microscopic explanations of such data in the light of the torsional mechanism. This is certainly a very satisfying outcome in bioenergetics, membrane biology, biochemistry and biophysics.

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