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Topical Review

Energy Coupling to ATP Synthesis by the Proton-Translocating ATPase

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Summary. This review summarizes recent work on energy coupling to ATP synthesis by the reversible, proton-translocating ATPase of mitochondria, chloroplasts, and bacteria. In the first sections, this enzyme is distinguished from other ATP-linked ion transport systems, and progress in the biochemical analysis is discussed. There is at present a reasonably consistent idea of the overall structure of the enzyme, and one can begin to assign specific functional roles to individual subunits of the complex. The latter half of this review deals with mechanisms of energy coupling, about which there is clear divergence of opinion. An "indirect coupling" model would allow for the possibility that H^+ translocation transmits energy for ATP synthesis by driving the enzyme through a sequence of conformational states, so that H^+ translocated need not take part in the chemistry of ATP synthesis. By contrast, a "direct coupling" mechanism would specify that H^+ translocated must participate in the chemical reaction by combining with oxygen from phosphate during the synthetic step. Such discussion is preceded by an outline of the "proton well," since this idea forms the basis of one direct coupling model. In addition, it is suggested that the idea of a proton (ion) well may be of more general significance to the analysis of ion-coupled transport, because it includes the postulate that mechanistically significant ion binding can occur within the profile of the electric field. A proton (ion) well can be derived from both kinetic and equilibrium treatments, and from mechanistic considerations in fields as distinct as biochemistry and neurophysiology. As a result, it illustrates how further advances in formulating mechanisms of energy coupling might profit by a merger of technique and perspective from areas that have as a common goal an understanding of how large proteins catalyze movements of small molecules across a membrane.

Key words chemiosmotic theory · proton-translocating ATPase · membrane potential · proton well · oxidative phosphorylation · ion-coupled transport

Introduction

This article reviews certain aspects of the reversible, proton-translocating ATPase, the membrane-bound enzyme that catalyzes the final step in all oxidative and photosynthetic phosphorylations, in mitochondria, chloroplasts, and bacteria. It is not my choice to attempt comprehensive review of all important topics in this field, since this single enzyme has been the subject of work spanning decades [4]. Instead, I have exercised an editorial license in choosing areas for emphasis and in selecting experiments to illustrate specific points. For example, it will become apparent that major weight is given to studies of this enzyme in bacterial systems. I believe there are good reasons for this. The studies in bacteria have been underway for just over ten years and are only now reaching a maturity that offers purified enzyme, isolated subunits, a variety of informative mutants, the nucleotide sequence of relevant genes, and a technical repertory that can examine this "pump" in both physiological and biochemical settings. Even the near future promises to be embarrassingly rich in new information, and it is certain that conclusions derived from work with the prokaryote example will be of wider significance. Because this general field anticipates extraordinary advances shortly, there have appeared recently a number of articles which, together, define the current status in great detail [4, 5, 14, 18, 19, 25, 33, 35, 40, 58, 62, 76, 88]. This present summary will focus sharply only on questions that have not received specific comment elsewhere.

ATP-Linked Ion Transport Systems

It now appears as if all membranes have associated with them at least one ATP-linked ion transport system. This generalization is surely true of prokaryote and eukaryote plasma membranes and also seems to hold for most (if not all) membranes of eukaryote organelles. As indicated by Table 1, these "pumps" may be assigned to one of two categories, using any of several criteria. So far as is known, there is only one example of a Type I enzyme – the reversible, proton-translocating ATPase of mitochondria (F_0F_1), chloroplasts (CF_0F_1) and bacteria (BF_0F_1). (This enzyme may also be in certain other eukaryote organelles [1].) Judging from well-characterized examples, all other ion-translocating enzymes fall into a second catagory, Type II. In this second class one would

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Table 1. Enzymes that link ion transport to ATP hydrolysis or ATP synthesis

	Classification[a]	
	Type I	Type II
Examples	F_0F_1	Na^+/K^+-ATPase H^+/K^+-ATPase Ca^{2+}-ATPase H^+-ATPase Etc.
Distinguishing features		
Ions transported	H^+	H^+, Na^+, K^+, Ca^{2+}
No. polypeptides[b]	Many	Few
Periferal	5	None
Integral[c]	3	3 (at most)
Intermediates	None	Phosphoprotein
Direction of reaction	ATP hydrolysis ATP synthesis	ATP hydrolysis

[a] This classification does not consider the various ion, amino acid, and sugar transport systems, so far described only in bacteria, that seem to be driven by ATP or by a "high energy" compound in rapid equilibrium with ATP [33].

[b] There is likely an evolutionary relationship within the Type II catagory; some [31] have extended this to include the Type I enzyme, since the combined mass of its two major periferal proteins ($\alpha+\beta$) is about 100,000 daltons, which is very close to the mass of the major integral membrane protein of the Type II enzymes. Other broad similarities are also noted in that report.

[c] As discussed in a later section, the smallest of the Type I integral membrane proteins is highly lipid soluble. A low molecular weight "proteolipid" may also be present in the Na^+/K^+-ATPase [20] and the Ca^{2+}-ATPase [65]. While the role of the proteolipid subunit in the Type I enzyme is firmly established (*see* Fig. 2 and accompanying text), the function of proteolipids found associated with other ATPases remains controversial [46, 65].

place the Na^+/K^+-ATPase of animal cell plasma membranes [9], the H^+/K^+-ATPase of gastric mucosa [70], the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum [12], the H^+-ATPase of fungi [10], and even a K^+ pump in *Escherichia coli* [41].

These two classes are most readily distinguished by ion specificity and overall protein structure. Thus, the Type I enzyme (F_0F_1) catalyzes only H^+ transport, but this simple reaction is mediated by a relatively complex physical structure that incorporates at least eight distinct polypeptides, including both periferal and integral membrane protein. By contrast, a variety of ions is transported by Type II enzymes, a versatility accompanied by a simple physical structure, requiring no more than three different proteins (usually one or two), all integral to the membrane. These classes are also distinguished mechanistically, by the presence or absence of a phosphorylated intermediate. Although the site of phosphorylation on the gastric H^+/K^+-ATPase is not known, the β carboxyl of aspartate accepts phosphate for the Na^+/K^+-ATPase, the Ca^{2+}-ATPase, and the H^+-ATPase of *Neurospora* [9, 11, 12]. But the search for phosphoprotein (or other covalent intermediates) has not been successful for the F_0F_1 enzyme [4], and recent work strongly argues against its presence [87].

Table 1 also includes an important physiological criterion. It is likely that *in vivo* all Type II enzymes couple ATP hydrolysis to ion transport, and that reversal of ATPase activity is of experimental but not physiological significance [83]. The Type I enzyme, however, operates physiologically as either an ATP synthase or an ATPase, depending on circumstance. In many bacteria this enzyme catalyzes ATP synthesis during oxidative phosphorylation, but in others it behaves as a true ATPase, extruding H^+ to maintain the electrical and chemical (pH) gradients needed for other work functions [33].

Finally, it should be noted that one feature is shared by the Type I and Type II systems – each links ATP to the transport of an inorganic cation. As yet there is no convincing example of an anion-translocating ATPase, and one wonders if this deficiency is instructive. One line of reasoning [54, 58] suggests that there is a necessary and direct interaction between the various substrates during transport by both Type I and Type II systems, as the active center receives reactants and discharges products. It is easy to imagine associations of transported cations and the anionic nucleotides and phosphate, not so easy to describe such interactions if all participants are negatively charged. If true, this idea implies that the listing shown in Table 1 is nearly complete, for it gives examples of the ATP-driven transport of most inorganic cations.

Structure of the F_0F_1 ATPase

An analysis of the proton-translocating ATPase has been pursued in a variety of organisms, and while there are problems peculiar to the eukaryote form (especially, the coordination between synthesis and assembly of subunits derived from different cellular compartments [85]), the structure of the prokaryote enzyme can illustrate elements common to all examples of this Type I enzyme. There have been two favored systems for structural work in bacteria. One has been a thermophilic organism, PS3, exploited by Kagawa and his associates to provide the first example in which the coupling between ATP hydrolysis and H^+ movements could be documented in a biochemically defined system of minimal composition [37, 82]. The second, of course, has been *E. coli*, a target for both biochemical and genetic studies [14, 19, 25, 88]. The work with these and other bacteria suggests the overall organization shown in Fig. 1. The

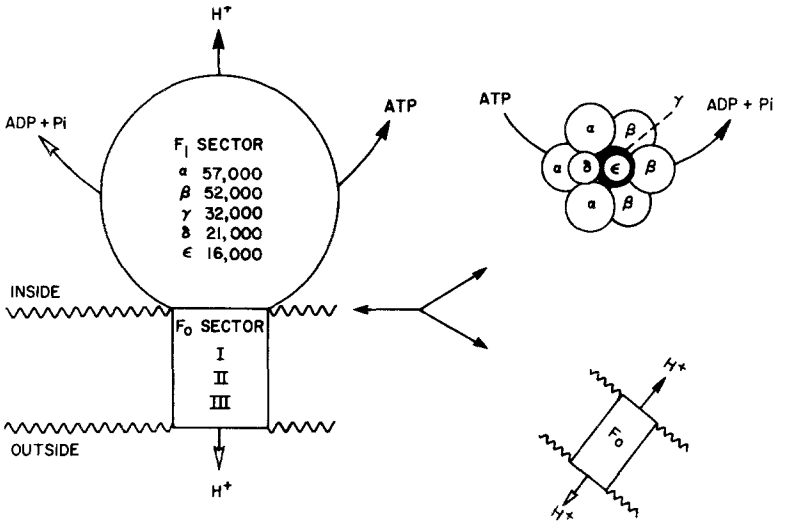

Fig. 1. Overall structure of the proton-translocating ATPase. The molecular weights assigned to F_1 subunits $\alpha-\varepsilon$ are those estimated from gel electrophoresis of the *E. coli* enzyme [summarized in ref. 19]. In the diagram at the top right [redrawn from ref. 6], F_1 has been rotated so that the surface interacting with the F_0 sector faces out of the page

enzyme is built from two major functional units, termed F_0 and F_1. F_1 is by far the larger, massive enough to appear in electron micrographs as a complex (95 Å diameter) with subunits arranged in a hexagonal array [37]. All examples of F_1, not just the bacterial cases, have five distinct polypeptides, named α through ε in order of decreasing molecular weight. These five polypeptides represent periferal or extrinsic membrane proteins, since the entire F_1 complex can be reversibly dissociated from the membrane at low ionic strength. One important observation is that this physical separation is paralleled by a functional division: the isolated (now soluble) F_1 sector catalyzes ATP hydrolysis, while the F_0 sector, containing integral membrane protein, mediates proton movements. The H^+ permeability of such stripped membranes (or liposomes with only F_0) may be elevated 100-fold, and this has provided the basis for a reliable assay of F_0 [61,80] that now complements the ease with which F_1 activity is measured.

Since it behaves as a soluble enzyme when released from the membrane, F_1 has been the more readily studied of the two sectors. It is of high molecular weight, in bacteria typically 350,000–380,000 daltons, and most likely contains one copy each of the minor subunits, γ, δ and ε. There is still some dispute over the relative abundance of the major subunits, α and β – there are at least two of each, possibly three. At the moment this disagreement is most apparent when comparing the prokaryote and eukaryote enzymes. Those working with mitochondrial and chloroplast F_1 consider $\alpha_2\beta_2\gamma\delta\varepsilon$ as the more reasonable estimate of stoichiometry [62, 76], whereas the weight of evidence from studies in bacteria strongly favors a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ [19, 35, 91]. The subunit relationships shown in Fig. 1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) represent the ratios first assigned by Kagawa's group after analysis of the thermophilic enzyme in bacteria [91] and also provide the most reasonable stoichiometry compatible with cross linking studies of *E. coli* F_1 [6]. Overall, the model shown in Fig. 1 [from ref. 6] suggests that the three α and β subunits each lie on opposite sides of a rough hexagon, and that the entire F_1 complex might be organized about the γ subunit (dark circle).

One objective of the biochemical analysis has been to assign specific function to each of the F_1 subunits. The general pattern is best summarized as follows. Each of the α and β subunits has a nucleotide binding site; that on α shows the higher affinity [16, 25, 63] and may contribute to a site(s) not directly involved with ATP hydrolysis by F_1 [25, 36, 39]. But alone, neither of the major subunits will catalyze ATP hydrolysis. In *E. coli*, for example, restoration of ATPase activity by recombination of isolated subunits requires both α and β, as well as γ [16]. The smallest subunits, δ and ε, appear to provide the necessary links between the larger ones and the F_0 sector, so that all five F_1 proteins are needed in reconstitution of the coupling between ATP hydrolysis (synthesis) and H^+ movements [25, 35]. A prominant role for the γ subunit has also been proposed. This molecule is required when ATPase activity latent to $\alpha+\beta$ is expressed (*E. coli*). In addition, although binding of δ and ε does not alter H^+ translocation by F_0, when γ is also present, H^+ movements are clearly suppressed (PS3) [90]. Thus, γ may act as both an organizing element to the overall structure of F_1 (Fig. 1), and as a functional "gate" that stands between H^+ movements through F_0 and the nucleotide binding sites of F_1 [35].

The integral membrane proteins of F_0 have been less easily studied. As shown in Fig. 1, highly purified

examples from bacteria usually have three distinct polypeptides: I, II and III (in order of decreasing mass) [19, 21, 35]. Three (or four) subunits are assigned to the chloroplast CF_0 [62], and the mitochondrial form may have more [76]. Clearly, the minimal composition of F_0 is not yet established, and it may differ even among bacterial examples [19]. For example, the F_0 sector from the thermophilic organism can be further resolved, with elimination of the largest protein [81]. But preparations from *E. coli* always have three proteins [8, 21, 22], and here the biochemical inference is strengthened by genetic studies, since the *unc* operon has three genes whose products affect the activity of F_0.[1] Considering these uncertainties, it is not surprising that the stoichiometry of F_0 polypeptides is still unsettled. Nevertheless, all agree on an important point – that the smallest one, protein III, is present in significant molar excess over the others [19, 73, 79].

Much attention now centers on this smallest subunit, for there is a reasonably good chance that the overall activity of F_0 will be understood largely in terms of the properties of this single protein. The clearest support for this assertion comes from work showing that H^+ conductance of liposome membranes is elevated by subunit III purified from chloroplasts [78]. (Such experiments may not be successful in bacterial systems [24].) Other, more general support for this educated guess is outlined by Fig. 2. The protonophoric activity of F_0 can be suppressed in three ways (Fig. 2*A*). The first is by a rebinding of F_1 (or certain of its subunits). The second is by a genetic modification, while the third reflects chemical modification, most importantly by N,N′-dicyclohexylcarbodiimide (DCCD), an inhibitor of both H^+ flow through F_0 and ATP synthesis or hydrolysis catalyzed by the F_0F_1 enzyme. It was these last observations that first pointed to some specific role for

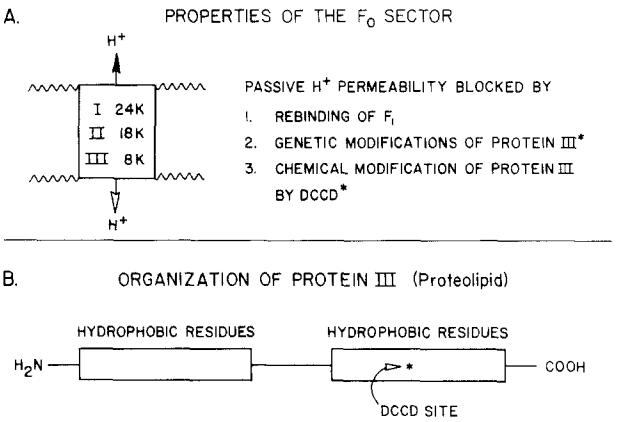

Fig. 2. Properties of the F_0 sector. (*A*) Nomenclature of F_0 polypeptides follows that of Nelson [62]; subunits *I*, *II* and *III* are also known as χ, Ψ and Ω [19] or *a*, *b* and *c* [8, 24]. Subunit molecular weights are from gel electrophoresis of the *E. coli* enzyme (*I*, *II*) [19] or from the amino acid sequence (*III*) [74]. (*B*): A more complete discussion of the amino acid sequences of various subunits *III* is given by [74]

proteins in F_0 [54], and we now believe that these inhibitions by DCCD can follow modification of only a single amino acid residue in subunit III.

In principle, a good deal is known about protein III. Sebald and Wachter, in collaboration with a number of groups, have determined the amino acid sequence of subunit III from three mitochondrial sources (beef heart, yeast and *Neurospora*), from spinach chloroplasts and from both *E. coli* and the thermophilic PS3 [74]. Each of these sequences has the pattern outlined in Fig. 2*B*. The protein (72–81 residues) contains three distinct regions. At about the center is a 12–15 residue stretch enriched for amino acids with hydrophilic side chains. This is flanked on either side by two sister regions, of roughly equal length, each having an unusually high content of hydrophobic amino acids. (The overall preponderance of hydrophobic residues confers a lipid solubility to the molecule, hence its alternative name as the "proteolipid" subunit.) The distribution of polar and nonpolar residues immediately suggests that each of the hydrophobic domains spans the hydrocarbon core of the bilayer with the central hydrophilic region at one surface and the N- and C-termini at the other surface of the membrane. In addition, other work implies interactions between multiple copies of the proteolipid, possibly even between the sister domains of a single molecule. Thus, (i) there are likely many copies of subunit III per F_0 (perhaps as many as $10/F_0$ in *E. coli* [19]); (ii) for all examples of F_0, titration with DCCD shows maximal inhibition of H^+ conduction (or F_0F_1 ATPase activity) when only a small fraction of all proteolipid molecules is modified (the modified fraction is thought to be about one part in six [19, 73, 79]); and (iii) mutation in

[1] Genes coding for all eight "readily" isolated BF_0F_1 proteins can be assigned to the *unc* (or *atp*) operon of *E. coli* [8, 13, 14], and biochemical studies give no strong evidence that additional protein is required during reconstitution of activity. It comes as somewhat of a surprise, then, that the nucleotide sequence of this operon [27, 28, 71] indicates one additional component – a hydrophobic protein with mass of about 14,000 daltons. Gay and Walker [28] have proposed that this ninth component (from gene "1") might catalyze assembly of one or more BF_0F_1 complexes, and consistent with such a catalytic role is the finding that a codon used rather frequently in this newly described gene specifies a *t*RNA of low abundance in *E. coli* [28]. Currently, the nucleotide sequence [27, 28, 71] and biochemical analysis (summarized in [19]) suggest the following arrangement of genes for BF_0F_1 proteins (the number of polypeptides finally residing in BF_0F_1 is shown in parentheses): promoter: 1(?):I(2):III(10):II(2):δ(1):α(3):γ(1):β(3):ε(1). Thus, the newly described "pilot" protein would be translated first, followed next by proteins of F_0, and finally by F_1 components. This striking arrangement does suggest that the nucleotide sequence and gene order may offer clues to the assembly process.

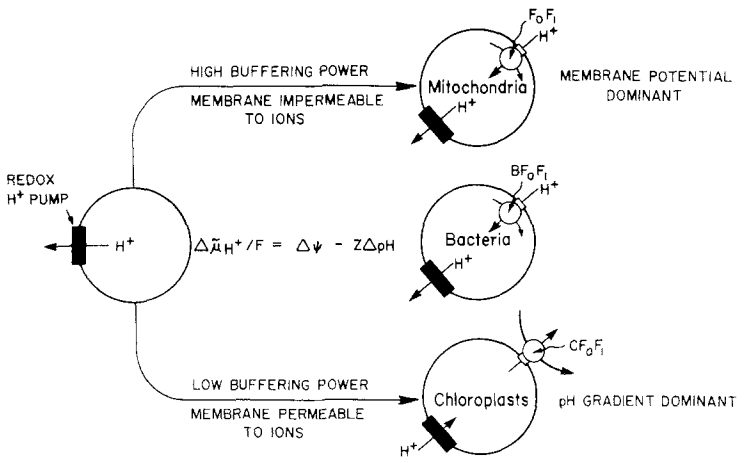

Fig. 3. Physiological driving forces for ATP synthesis. Note the different polarities to H^+ translocations across mitochondrial and bacterial membranes and across the chloroplast thylacoid discs. The limiting cases shown give only the likely physiological conditions. In mitochondria, oxidative phosphorylation can occur when the $-Z\Delta$pH parameter is dominant [59]; chloroplast CF_0F_1 will synthesize ATP in response to an entirely electrical driving force [30]

the N-terminal hydrophobic region alters the reactivity of the residue in the C-terminal domain that is modified by DCCD [86]. Consequently, it is reasonable to argue that several (six ?) proteolipid subunits contribute to an oligomer that forms a channel for access of external H^+ to the active center of the intact F_0F_1. Moreover, this putative channel appears to have good selectivity for H^+ over other cations – even at pH 7–8, aerobic growth (bacteria) that requires oxidative phosphorylation is not materially affected by 0.1 M Na^+ or K^+. Thus, it might also be argued that within the channel represented by F_0 protons move along chains of hydrogen bonds (giving specificity to H^+) provided by either some form of bound water [60], or by resident amino acids. The latter view seems more popular at the moment, since the residue modified by DCCD is capable of accepting or donating H^+ – in all cases, DCCD reacts with the carboxyl of aspartic (*E. coli*) or glutamic (all other forms) acid to form a stable N-acyl urea [74, 75]. Perhaps most significant in this context, F_0 fails to conduct H^+ when this acidic residue is replaced by a neutral amino acid (glycine for aspartic acid, in *E. coli*) [34]. Thus, there is very strong evidence that subunit III participates directly in H^+ conductance through F_0, and the early speculation (*see* [54]) that F_0 behaves as a channel or carrier for H^+ has become supported by more direct considerations.

ATP Synthesis Driven by Electrical and Chemical Gradients

The overall steps in oxidative and photosynthetic phosphorylation are outlined by Fig. 3, where a proton pump coupled to electron transport (box) directs the flow of H^+ to establish and maintain a difference in the electrochemical potential for H^+ ($\Delta\tilde{\mu}_{H^+}/F$) across a "coupling" membrane. The electrochemical H^+ gradient is usually expressed in electrical units, as

$$\Delta\tilde{\mu}_{H^+}/F = \Delta\psi - Z\Delta\text{pH} \quad (1)$$

where $\Delta\psi$ represents the electric component (membrane potential), and $-Z\Delta$pH the contribution made by the chemical potential or pH gradient (Z gives the values of $2.303\,RT/F$).

Although each of the separate terms in Eq. (1) contributes to the conservation of energy first dissipated by electron transport, their relative size is determined by general properties of the system. Suppose, for example, that buffering power is high on either side of a membrane that is impermeable to all ions. In that limiting case (Fig. 3, top), extrusion of H^+ soon charges membrane capacitance so that at final equilibrium neither internal nor external pH is much changed. Under the usual conditions, mitochondria nearly approach this extreme, having a large membrane potential but little pH gradient [59, 69]. Alternatively, the membrane may be permeable to ions in the system (except H^+ and OH^-) and buffering power may be low. In this case (Fig. 3, bottom), the electric component is eventually suppressed when charge moved as H^+ is compensated by charge moving passively (in this example) on some other chemical species. Consequently, as H^+ pumping continues, buffering power is compromised and the final state is characteristically dominated by the chemical (pH) gradient. Here, one might cite chloroplasts as the biological example [52, 69]. Note that bacteria form an intermediate class (Fig. 3, center), being able to approach either extreme, depending for the most part on the value of external pH [17, 66][2].

Figure 3 also shows that each system considered contains the proton-translocating ATPase. In each

[2] The adaptability of bacteria reflects the control of internal pH near pH 7.5, so that the chemical potential dominates when outside pH is low, but the membrane potential is the larger at neutral or alkaline pH. By contrast, the complementary work with animal cells [23] shows an indifference to the precise value of internal sodium during maintenance of the electrochemical sodium gradient, because of high permeability to K^+.

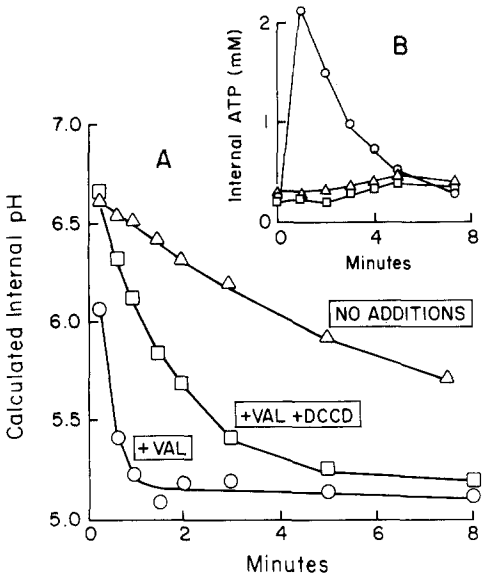

Fig. 4. ATP synthesis driven by a pH gradient. Washed cells of *S. lactis* were suspended in 0.1 M potassium phosphate, pH 8, along with 1.5 μM ^{14}C-salicylic acid. After addition of sulfuric acid to lower outside pH to 3.5, samples were taken for measurement of internal ATP and determination of salicylate accumulation. The distribution of salicylate was used in the calculation of internal pH. Further details are found in the text and in [49]

case, as the return of protons completes their circulation across a membrane, potential energy transiently stored as $\Delta\tilde{\mu}_{H+}/F$ is finally recovered in chemical form as ATP. It is likely that each of these ATPases will have one or more features that mark it as specifically suited to its individual environment, but the summary given above shows that we believe these are fundamentally the same enzyme and that structural and mechanistic solutions to the problem of ATP synthesis will be identical in each case. Clearly, the broad scale used in Fig. 3 emphasizes (i) that such ATP synthesis must take advantage of either the electric gradient (mitochondria), the pH gradient (chloroplasts), or some variable mixture of the two (bacteria); and (ii) that studies of the coupling between H^+ movements and ATP synthesis might profit from work designed to illustrate possible interactions between the electrical and chemical components of net driving force.

It is possible to study these interactions in a quantitative way, using the anaerobe, *Streptococcus lactis* [48]. Because this cell contains BF_0F_1 but no redox-linked proton pumps, it is an example of those bacteria that normally employ this ATPase in the hydrolytic mode (Table 1). Nevertheless, the synthetic reaction is readily demonstrated, and Fig. 4 shows how the coupling between H^+ movements and ATP synthesis can be studied under conditions where electrical and chemical gradients are controlled. In that work, cells were suspended in a potassium-based medium, buffered at pH 8, and treated in one of three ways. In all cases, sulfuric acid was added at zero time to impose a large pH gradient (about 4 pH units). But when only acid was added, net inward driving force on H^+ was limited by formation of an H^+-diffusion potential. As a result, ATP synthesis did not occur, and net acidification of the cell was relatively slow. In the other samples, however, the ionophore valinomycin allowed compensatory movements of potassium that kept the membrane potential near the potassium equilibrium potential. Once the membrane potential was controlled, the imposed pH gradient supported ATP formation in parallel with a pronounced acceleration of H^+ entry, and a comparison with cells exposed to the inhibitor, DCCD, showed that a substantial fraction of the enhanced H^+ flux must be directly coupled to ATP synthesis. This approach provides the most convincing demonstration in bacterial systems that this enzyme participates in a transmembrane current flow [47, 49]. Furthermore, such studies have suggested an important conclusion regarding the overall kinetics of the reaction: that total driving force ($\Delta\tilde{\mu}_{H+}/F$) is the major determinant of BF_0F_1 activity (measured as an H^+ flux), not the absolute value of either membrane potential or pH gradient [48].

This observation merits a closer look, as illustrated by the following argument. We know that either electrical or chemical gradients will support ATP synthesis (Fig. 3) and that at final equilibrium the change in the "phosphate potential" (as $\Delta G'_{ATP}/F$) will be predicted by the steady-state values of $\Delta\tilde{\mu}_{H+}/F$, weighted only for the stoichiometry of the reaction, nH^+/ATP. But during the approach to final equilibrium if electrical and chemical gradients of the same thermodynamic weight elicit different rates of ATP synthesis, we might be encouraged to think of models in which the two driving forces have separate targets – one possibly a carboxyl group that could respond to protonation, but the other a protein whose dipole moment allows it to reorient in the presence of an electric field. Alternatively, if equivalent gradients yield identical rates we are obliged to consider the idea that there is a single rate-limiting element, in some way responsive to both the electrical and chemical components of net driving force.[3]

These alternative possibilities are distinguished by

[3] The observation of a consistent rate equivalence over an appreciable range would be difficult to reconcile with the idea of separate mechanistic events responsive to either voltage or ion concentration(s) [32]. But the converse is not true, and the next section outlines how a common mechanistic step could be differentially sensitive to the two driving forces. The simple alternatives suggested here are nonetheless quite useful in introducing the experimental findings that follow.

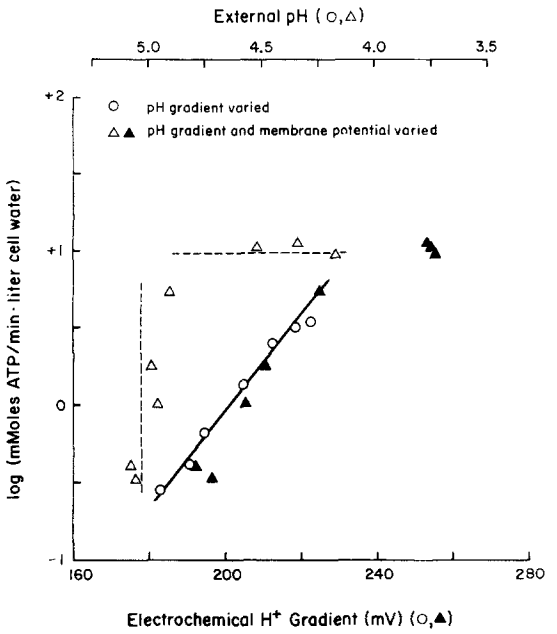

Fig. 5. Initial rates of ATP synthesis after imposition of electrical and chemical gradients. Initial rates of ATP formation were measured after sulfuric acid was added to washed cells of *S. lactis* suspended in a potassium-based medium at pH 8 in the presence of valinomycin. To vary the membrane potential, cells were at the same time diluted into media where choline partially replaced potassium. Final external pH is shown on the upper abscissa; the membrane potential was calculated as the potassium equilibrium potential (E_K), using measured internal and known external levels of the cation. Further details are given in [50]. The interpretation of this experiment (*see* text) assumes that potassium flow by way of the ionophore is very much faster than H^+ current by way of BF_0F_1, so that potassium remains at equilibrium. The assumption is supported by recent studies [*unpublished*] showing that during hydrolysis of ATP at rates comparable to these shown here (for synthesis), E_K is the same as the membrane potential measured by the distribution of tetraphenylphosphonium, provided that valinomycin is present. (The ^{3}H-tetraphenylphosphonium used was a generous gift of H.R. Kaback.)

experiments shown in Fig. 5. As in the earlier example (Fig. 4), washed cells of *S. lactis* were suspended in a potassium-based medium at pH 8, along with valinomycin. Cells were then subjected to one or both of two protocols. In the first instance (circles), acid was added to impose a chemical gradient of varying size; but because outside potassium was held constant, the membrane potential did not change. In the second case (triangles) a standard quantity of acid was added, yielding a fixed pH gradient, while the membrane potential was altered as outside potassium varied. Because inside pH was the same in both experiments, initial rates of ATP synthesis could be expressed as a function of either decreasing external pH (increasing outside H^+ concentration) or increasing total driving force.

The data in Fig. 5 suggest three conclusions. First, there is no required correlation between rate and external H^+ concentration, even though H^+ may be considered a reactant (dotted lines). Instead, the only necessary correlation is found when the electrochemical activity of H^+ is described explicitly (solid line). Second, these studies might give information about the coupling ratio, nH^+/ATP. For example, if stoichiometry is $2H^+$/ATP one might expect rate to rise with the square of the effective proton concentration, or to increase 100-fold for every 60-mV increment in net driving force. In fact, the slope of the solid line in Fig. 5 suggests a coupling ratio of 1.8 ± 0.1 H^+/ATP, and this does agree with other tests of stoichiometry in *S. lactis* [49, 51]. Third, and most interesting, it is clear that identical rates are found when equivalent electrical or chemical driving force is imposed (*compare* circles and closed triangles). (For technical reasons this conclusion is most strongly supported by these particular experiments, although work with similar design has been reported in other systems [30, 52, 84].) Thus, it becomes attractive to consider models of "energy coupling" that accommodate this quantitative interconversion of electrical and chemical driving forces.

Energy Coupling and the Proton Well

One plausible solution to this general problem has been advanced by Mitchell [53], and the following analysis gives a simplified treatment of that initial suggestion. Figure 6 recalls that the proton-translocating ATPase is built from two fundamental units – an F_1 sector that has ATPase activity in the isolated state, and an F_0 sector that behaves as an H^+ carrier or H^+ channel when free of F_1. However, *fast* H^+ flux through F_0 is easily measured only when F_1 is removed (Fig. 3), so that in the native enzyme (F_0F_1) the F_0 sector may be physically blocked at one end and converted into what Mitchell has termed a "proton well". It is further presumed that at some point within F_0 incoming H^+ interacts with specific chemical groups to initiate ATP synthesis by reversal of the ATPase activity associated with isolated F_1. With these premises in mind, it is appropriate to outline factors that influence the concentration of H^+ within this local compartment.

The lower diagram of Fig. 6 describes these factors by showing increasing acidity (decreasing pH) within F_0 as a function of some fractional distance, x, from the outer surface. The simple cases discussed are those in which the value of $\Delta\tilde{\mu}_{H+}/F$ is the same and where a constant electric field spanning membrane thickness has been assumed. In case A the membrane potential is considered clamped at zero, so that the concentration of H^+ is the same at any point within F_0, given by the value of external pH. But in case B the effect of a steady-state membrane potential is introduced.

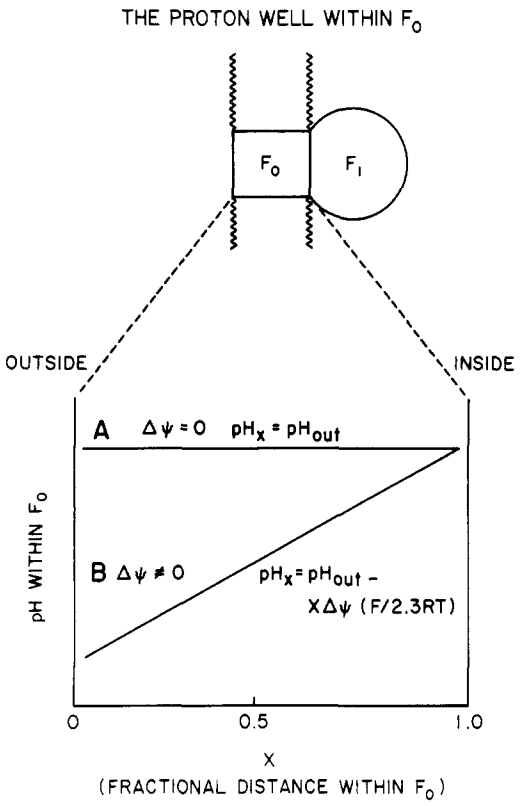

Fig. 6. The proton well within F_0. Vertical distance on the ordinate represents decreasing pH. This is shown as a function of increasing fractional distance, x, from the outer surface of the membrane. The diagram is only slightly modified from [48]. Mitchell [53, 55] has presented a more detailed discussion of the proton well

There, as H^+ moves inward, energy dissipated by H^+ movement down the electric field is conserved as an increased chemical activity, so that H^+ becomes distributed asymmetrically in the steady state and accumulates at the negative pole towards the inner surface of the membrane. In both cases A and B, H^+ is distributed isopotentially throughout F_0 because of the mismatch in probabilities for H^+ entrance and exit on either side. However, the chemical activity of H^+ is quite different in the two instances; H^+ concentration within F_0 becomes equal for A and B only after the full thickness of the membrane is crossed. Thus [referring to Eq. (1)], the proton well converts the $\Delta\psi$ term into its equivalent as $-Z\Delta$pH at the interface between F_0 and F_1. In one sense then, F_0 would itself act as a transducer, although this initial transduction is very different from that which eventually occurs during ATP synthesis. It is specifically the idea that F_0 is physically blocked (which limits the purely dissipative net flow of H^+) that allows this latter transduction to be formulated in strictly chemical terms [55, 57]. Consequently, the idea of a proton well should not be seen as a way to avoid the problem of energy transduction [60], but as one way to place very specific restraints on allowed mechanism.

It should be of interest to those working with "coupled" transport systems that this analysis has been approached from a somewhat different perspective in the field of neurophysiology. Woodhull [89], for example, used the formalism of chemical rate theory to describe how H^+ (or any ion) might bind within a channel. In that case, real data and the general theory were in agreement if it was assumed that blockage of a Na^+ channel by H^+ was due to entry of H^+ from the outside. Thus, in the scheme below, where either external or internal H^+ might bind to some site x within a channel

$$H^+_{out} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H^+_x \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H^+_{in}$$

the ratios of voltage-dependent rate constants k_2/k_1 and k_{-2}/k_1 had to be close to zero. Since the net reaction $H^+_x \rightarrow H^+_{in}$ can be considered to describe the eventual dissipative transfer of H^+, uncoupled to the performance of work ("slippage"), this simplification is equivalent to the assumption made by Mitchell in description of the proton well: with these restraints, the electrochemical potential of H^+ is constant with distance from the outside, so that H^+ within a channel (or in F_0) is always at equilibrium (isopotential) with the external phase. Use of either the equilibrium assumption or the modified kinetic treatment then leads to the same expression for the dependence of pH_x on voltage inside the channel (Fig. 6). In this context one further comment seems appropriate. The kinetic theory (with the assumptions discussed above) gives the following alternate expressions for p, the probability that H^+ is *not* bound to some site x in a channel (or that a "carrier" is not protonated, etc.) [89]:

$$p = \frac{K_D}{[H^+]_o \exp(\alpha) + K_D} = \frac{K_D \exp(-\alpha)}{[H^+]_o + K_D \exp(-\alpha)} \qquad (2)$$

where K_D is the dissociation constant for H^+ at site x (found at zero membrane potential), $[H^+]_o$ is the external concentration of H^+, and α gives the value of $x\Delta\psi F/RT$ (x and $\Delta\psi$ as in Fig. 6). These alternate forms show that voltage may be seen as either altering the concentration of H^+ at site x (i.e., the term $[H^+]_o \exp(\alpha)$), or as changing the dissociation constant at site x due to variations in the structure of its neighborhood (i.e., $K_D \exp(-\alpha)$). Such distinctions may become important in other situations, as in discriminating between "permissive" ($[H^+]$ altered) and "instructive" (K_D affected) events (*see* [42]), but for the moment the former position is preferred, since it more simply accommodates the smooth rate transi-

tions found when both $[H^+]_o$ and voltage are varied (Fig. 5, and other examples cited below). Moreover, in the later discussion of ATP synthesis it will be useful to talk of the F_0 sector as a device that alters the chemical reactivity of H^+.

This sort of analysis turns out to be of use in a number of contexts. Most importantly, it describes how an electric gradient can be converted into a chemical signal, so that the electrostatic force represented by a voltage drop and the phenomenological "force" represented by a concentration difference can each affect a single mechanistic step (e.g., protonation). But in addition, this point of view explicitly suggests that critical ion binding sites might lie within the profile of the electric field and that such energy minima might be identified by studying the kinetic response to changing membrane potential and ion concentration. For example, after simplification of the general kinetic formulation, Woodhull [89] showed that the Na^+ channel of frog nerve also behaves as a proton well, with a preferred H^+ binding site one quarter of the way into the membrane ($x=0.25$, Fig. 6). Similarly, Schwab and Komor [72] suggest rate limiting H^+ binding half way through the membrane for H^+/glucose cotransport by *Chlorella* ($x=0.5$), while the data shown in Fig. 5 [50] indicate that the rate limitation of ATP synthesis is not found until H^+ moves all the way down the electric field ($x=1$). A distinctive feature of such interpretations is the general postulate that mechanistically significant ion binding can occur *within* the electric field, and this contrasts with the more usual formulations [26, 32, 68], which find it convenient to model the primary ion binding step as a voltage-independent, interfacial reaction. While each of these views may be germane to an understanding of ion-coupled reactions, it should be apparent that each signals the discipline of its origin. Thus, the proton (ion) "well" has roots in the more general idea of ligand conduction (*see* [58]), and the perceived need to examine events in terms of specific chemical groups moving from donor to acceptor. In simple cases, it should not be surprising that this leads to a formalism similar to that used by neurophysiologists concerned with ions binding in channels. Other perspectives reflect different beginnings. For example, restricting association-dissociation reactions to a lipid-water interface is appropriate to the analysis of ionophore-mediated ion flux, where it is physically sensible to consider the hydrocarbon core of the membrane as the major barrier to charge transit [43]; but it is not all clear that this is the reasonable point of departure for cases in which ions move through protein [32]. Quite obviously, adapting a general description to any single example will require decisions regarding the likely balance between the permissive and instructive effects of voltage, and statements about the relative importance of interfacial and intramembrane or intraprotein ion binding (*see*, for example, the treatment by Läuger [42]).

Mechanism

This field does not suffer from lack of speculation concerning the mechanism of ATP synthesis (*see* [18, 76] for summary comments), and although the description by Kozlov and Skulachev [40] is perhaps the most comprehensive, there are two specific models usually cited as representative of the two most radically different solutions to the problem. The most detailed account of an "indirect coupling" model has been provided by Boyer and his colleagues [3, 67]. In this case it is thought that H^+ translocation drives the enzyme through a sequence of conformational states, with the concommitant release of previously formed, tightly bound ATP from one site, and binding of ADP and phosphate at an alternate site. This represents a direct attack at understanding the roles of the multiple nucleotide binding sites that characterize all forms of F_1. Also, by suggesting that H^+ translocation occurs out of phase with ATP formation (which requires only the presence of bound ADP and phosphate), this model can rationalize much of what is known about the efficient exchange of oxygen between water and phosphate that is catalyzed by the enzyme. (Others, however, offer an entirely different explanation for this exchange [38, 40].) While the indirect coupling model fails to clearly specify a role for the proton, the same cannot be said of the alternative suggested by Mitchell [55, 57]. Instead, this latter or "direct coupling" model demands an intimate and stoichiometric association between H^+, ADP, and phosphate. It seems appropriate to outline the fundamental elements of this alternate view, since it draws on an assumption noted in the earlier discussion of the proton well and since recent developments make it appear more reasonable and less speculative than when first presented.

The diagram of Fig. 7 emphasizes that the direct coupling model gives pride of place to the chemistry of interactions between small molecules (and the origins of the proton well reflect this concern). The protein itself plays no instructive role in energy transduction, as it does in the indirect model of Boyer, but only specifies the ionic species admitted to the active center and their geometric organization once there. Thus, it is imagined that as ADP and phosphate enter the active site between F_0 and F_1, they leave behind two protons (not shown in the diagram). It is further assumed that the F_0 sector behaves as a proton well,

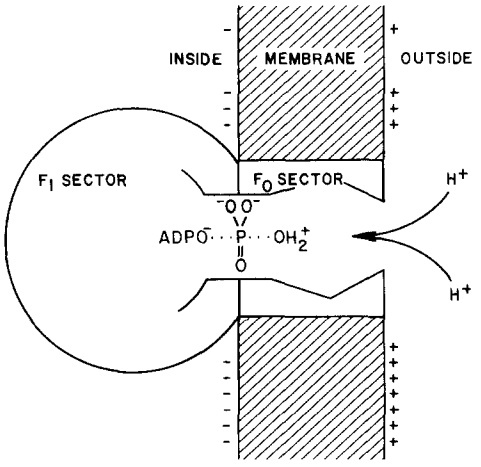

Fig. 7. The direct coupling between H^+ and ATP formation. The diagram is modified from Mitchell [55, 57] to show the proposed structure of a bipyramidal transitional complex during ATP synthesis. The formation of the complex may be promoted by association between two of the equatorial oxygens and the tightly bound Mg^{2+} now known to be an obligatory ligand for F_1 [77]. As shown, the reaction describes an S_{N_2} mechanism [55]. Although Mitchell [52] considers the general scheme as compatible with an S_{N_1} mechanism, this latter would appear to predict generation of a reactive metaphosphate; the scrambling of stereochemistry expected in that case is not found [87] (when ATP hydrolysis is examined)

converting the electrochemical proton gradient into a pool of H^+ concentrated at the interface between F_0 and F_1. Finally, at the active site, binding of ADP and of phosphate is arranged so that reactants are aligned perpendicular to the plane of the membrane, allowing the terminal oxygen atom of phosphate to accept $2H^+$ from F_0. Such protonation would yield an intermediate species (Fig. 7) in which the central phosphorous atom lies poised between two leaving groups – an inner $ADPO^-$ and an outer oxonium group, OH_2^+. Dissociation of the oxonium group with an electron would then generate water along with newly formed ATP. Thus, two positive charges move inward, to finally reside in water, and in the same step two acid equivalents disappear from the medium and appear on the inside, having been left behind as ADP and phosphate first entered the active site. In the net, the coupling ratio would be $2H^+/ATP$.[4]

[4] $2H^+/ATP$ is usually given as a requirement of this model, but higher stoichiometry is possible, depending on the specificity of the ion exchange reactions (anitport) taking place in the F_1 sector [57]. Note also that naming the enzyme is now determined by the sidedness of water transfer. If water is first directed to the inside, the reaction is one of cation (H^+) transfer. But if water appears first on the outside, the primary reaction is anion (O^{2-}) transport. Although water permeability is generally high in the lipid phase (so that this distinction may be trivial), special arrangements might be needed to remove water from within protein around the active site. If F_0 encloses water (*cf.* [60]) this sector could represent an H_2O conductor as well as an H^+ conductor. The behavior of gramicidin offers a possible example [45].

The model described in Fig. 7 was highly speculative and decidedly provocative when it first appeared [2, 3, 55, 56]. However, it is clearly consistent with some newer findings. It does appear as though the F_0 portion in the intact complex behaves functionally as a proton well (Figs. 5 and 6, and text). Also, the stereochemistry of the reaction (as described for ATP hydrolysis) dictates a transitional complex in which there is an in-line relationship between phosphorous and the attacking and leaving groups [87]. Finally, a stoichiometry of $2H^+/ATP$ is required by the direct coupling model, and although there is still no consensus with regard to the coupling ratio for the mitochondrial enzyme [7, 44, 58], a stoichiometry of $2H^+/ATP$ is supported by work in bacterial systems [49, 51, 64]. These are all observations showing that the direct coupling model remains a viable alternative to the idea of an indirect coupling, and in evaluating new information it will be important to recall the features that make these two views so different. As noted earlier, the direct coupling model has roots in the idea of ligand conduction; this does require appropriate changes of structure, as specific groups (including the nucleotides and phosphate) are translocated through protein, but such conformational states are denied a specific role in the transduction step *per se*. The indirect model, however, incorporates the attractive idea that the flux of H^+ drives modifications of protein structure that transiently store potential energy for use at some later step or at some distant location. Both physical and biochemical techniques show that conformational changes accompany nucleotide binding [15, 36], ATP hydrolysis or synthesis (*see* [76]), or even imposition of an electric field [29]. The problem is not to identify changes in protein structure, but to place them in their proper relationship to the movements of the small molecules involved.

Conclusion

I have tried to keep two overall objectives in mind during these discussions of energy transduction catalyzed by the proton-translocating ATPase. On the one hand, the summary of recent structural work was intended to indicate that one stage of the biochemical analysis is nearing completion. One can begin to assign specific functional roles to the individual proteins (or their combinations) and shortly there should be sufficient quantity of purified enzyme, from a variety of sources, to attempt a detailed physical description of the behavior of these large molecules during the reaction. At the same time, I have tried to indicate that understanding the interactions between the large and small molecules may require a

merger of technique and perspective from fields as traditionally distinct as biochemistry and neurophysiology. Movement of a charged particle through a protein is central to questions of energy transduction, and this same topic has been probed with different tools in other areas.

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