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Biochimica et Biophysica Acta, 1365 (1998) 3–9

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

ATP synthase—past and future

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Received 27 January 1998; received in revised form 12 March 1998; accepted 12 March 1998

Abstract

This paper gives an overview of a lecture scheduled for the opening of the 10th European Bioenergetics Congress. In this lecture I plan to first reflect on the accomplishments of some of the individuals who were involved in research on the ATP synthase during the past 50 years. Then I will give a brief view of the present information about rotational catalysis by the ATP synthase. This will be followed by a discussion of some results from my laboratory that call for additional experimentation. Finally I will direct attention to other questions about the ATP synthase that should be addressed in future studies. © 1998 Elsevier Science B.V.

Keywords: ATP synthase; Phosphorylation; Photophosphorylation; Rotation; Oxygen 18; Binding change

1. Some views of the past

It is my good fortune to have been involved in research on enzymes for over 50 years; with over half of my efforts directed toward an understanding of how ATP is made. In 1941, when I was in graduate school at the University of Wisconsin, Fritz Lipmann called attention to the central role that ATP plays in the capture and use of energy in biological systems [1]. It was soon widely recognized that synthesis of most of the ATP in animal tissues was coupled to the oxidative steps of the respiratory chain and that in plants energy from light was captured to provide reducing power and to synthesize ATP. In the 1950s and 1960s, how oxidative phosphorylation and photophosphorylation occurred became major problems in biochemistry.

I recall salient contributions by colleagues who have not enjoyed my longevity and thus had the privilege of visualizing the ATP synthase as a rotating molecular machine. Albert Lehninger, around 1950, established that oxidative phosphorylation occurred in mitochondria, and subsequently he contributed much to the field. One of the giants of the field, Efraim Racker, and his associates during the 1960s first identified the F_1-ATPase with the knobs seen in electron micrographs of mitochondrial membranes. Racker's group separated the ATPase from mitochondrial and chloroplast membranes and showed the structural and catalytic similarities of the enzyme from the two sources. This foreshadowed the now recognized distribution of similar ATP synthases throughout nature. Racker and associates reconstituted the capacity for oxidative phosphorylation by recombination of the F_1-ATPase with the F_0 component in the membrane. David Green's group found that mitochondrial membranes could be fractionated to yield five distinct complexes, one of which (complex V) consisted of the ATP synthase. Some of the leading present investigators in bioenergetics came

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PII: S0005-2728(98)00066-8

from Racker's and Green's laboratories, Harvey Penefsky, Yasua Kagawa, Nathan Nelson, Geoffrey Schatz, and Masamutsi Futai were associated with Racker's group. Yousef Hatefi, Alan Senior, Roderick Capaldi and Alexander Tzagaloff worked in Green's laboratory.

In 1953 Cohn reported the discovery that mitochondria capable of oxidative phosphorylation catalyzed a rapid exchange of P_i oxygens with water oxygens [2]. The subsequent use of ^{18}O to probe ATP synthesis in my laboratory was to prove vital for revealing three aspects of the binding change mechanism. In 1955 we found that the overall process of oxidative phosphorylation was rapidly reversible, and that the rate of exchange of P_i oxygens was even faster [3]. It was some 17 years later that we uncovered the important explanation for this behavior, namely the dynamic reversal of formation of tightly bound ATP from ADP and P_i at a catalytic site [4]; the energy-requiring release of the tightly bound ATP was slower than the interconversion rate.

In the latter part of the 1960s Ef Racker brought some of his F_1-ATPase to the University of California at Los Angeles so we could find if it catalyzed the exchange of phosphate oxygens. We found only close to one water oxygen incorporated into each P_i formed during hydrolysis of relatively high concentrations of ATP. Hydrolysis was occurring without oxygen exchange. If we had made measurements at lower ATP concentrations, we would have noted the striking appearance of extra water oxygens into each P_i formed. This we did not discover until about a decade later [5]. The simple but powerful result finds its explanation in the tight retention at one catalytic site of interconverting ATP, ADP and P. Although ATP can bind tightly to one catalytic site, rapid catalysis occurs only when another ATP binds; the isolated ATPase shows pronounced catalytic cooperativity. Such behavior is consistent with the occurrence of strong catalytic cooperativity under conditions for ATP synthesis, as first recognized several years earlier by Kayalar et al. from ^{18}O and ^{32}P exchange experiments with submitochondrial particles [6]. I have always felt that experiments with the intact ATP synthase were more meaningful, but it is satisfying when the isolated F_1 ATPase yields supporting data.

It was in the 1960s that Peter Mitchell began reporting experiments and concepts that were to transform the field. I, and some others, were mistakenly reluctant to accept his suggestion that proton translocation could drive ATP synthesis. I recall a slide Peter showed at a conference in which I was listed as among the last of the investigators to see the light that he was holding aloft. My reluctance arose because it was difficult for me to accept Peter's suggestions that protons were translocated to the catalytic site and were directly involved in ATP formation [7,8]. This difficulty was removed in the early 1970s, when I recognized that data on the insensitivity of ^{18}O exchanges to uncouplers could be explained if the energy from oxidations was used primarily to release a preformed and tightly bound ATP [4]. The remarkable properties of proteins were becoming more widely recognized, so at that time it seemed quite logical that proton translocation could be accompanied by conformational changes that were transmitted to the catalytic site to cause release of ATP [9,10]. That proton translocation could drive ATP synthesis became well established by experiments in Mitchell's and other laboratories, including Jagendorf and Uribe's demonstration of ATP formation induced in chloroplast thylakoids by an acid–base transition [11], and the classical experiment of Racker and Stoeckenius showing ATP synthesis by the ATP synthase energized by light and bacteriorohodopsin [12].

The third aspect of the binding change mechanism, the suggestion of rotational catalysis, depended upon the recognition from experiments in a number of laboratories that the catalytic β subunits of the same composition showed different properties, and also on the further development of our ^{18}O probes. In the late 1970s water highly labeled with ^{18}O became more readily available, and our measurements of ^{18}O in phosphates shifted from conversion of the phosphate oxygens to CO_2 and mass spectrometric measurement of excess ^{18}O in the CO_2, to direct measurement of ^{18}O in phosphate by mass spectrometry or NMR. This had the important consequence that not only the total ^{18}O content but also the portions of P_i containing zero, one, two, three, or four ^{18}O atoms could be measured. We called these ^{18}O isotopomers of P_i. Hackney developed the theory for use of the isotopomer measurements to get details of reaction rates and to assess the homogeneity of catalysis [13,14]. The distributions of ^{18}O isotopomers were measured when limiting substrate concentrations induced considerable intermediate oxygen exchange. The results gave evidence that in ATP synthesis by mitochondria [15] or chloroplasts [13], as well as in ATP hydrolysis by F_1-ATPase [16], all catalytic sites were doing catalysis identically. With what was already known about the binding change mechanism, the only logical explanation to me was that the internal subunits, likely mostly the γ subunit, was interacting with the three catalytic β subunits in sequence, and the simplest way this could be accomplished was by a rotation of the internal portion of the synthase relative to the external α and β subunits [17,18].

2. Gaining the present view of rotational catalysis

Evidence from my laboratory favoring rotational catalysis was presented [19,20] but other results were suggested to negate the possibility [21]. About 6 years ago I marshalled arguments supporting rotational catalysis [18], but to the field I believe it seemed to remain more possible than probable. The case for a rotational catalysis was dramatically improved when Walker and colleagues reported the X-ray structure for the major portion of the F_1-ATPase [22]. In this structure the heterogeneity of the β subunits was striking, and conformed to the required steps for rotational catalysis by the binding change mechanism. I'm sure it is evident to the reader that this was a definitely satisfactory development to me, as stressed in a recent review [23]. Subsequent well-designed cross-linking experiments were conducted in Cross's [24,25] and Capaldi's laboratories [26,27], based on information from the X-ray structure. Disulfide bonds between the γ and β subunits stopped catalysis, and cleaving of the disulfides allowed catalysis accompanied by randomization of the locations of the β subunits with respect to the γ subunit. Such results probably convinced most workers in the field that rotational catalysis occurred. But these experiments did not establish that the subunit movements were kinetically competent. The sophisticated fluorescent measurements in Junge's laboratory added important evidence favoring a rotational mechanism [28]. Any remaining doubts were erased by the brilliant experiment of Yoshida's group, in which the F_1-ATPase was induced to rotate a long, fluorescently labeled actin filament so that the rotation could be observed visually in a microscope [29]. Not only the field of bioenergetics, but also a wider audience now embraced the concept of a splendid molecular machine operating like a rotating motor.

The bioenergetics field can feel a justified pride in the fact that the efforts of many fine investigators have led to the discovery of this unusual enzymatic machine. Award procedures tend to single out a few for special recognition. But all that participated in the long and difficult task of revealing the structure and the properties of the ATP synthase deserve credit for their accomplishments. Although the broad features of the ATP synthase catalysis now appear to be known, it is clearly evident that much still remains to be learned. The balance of this paper will call attention to some important remaining questions; first, some uncertainties as raised by studies from my group, and, second, as evident from other limitations of the present knowledge about ATP synthase.

3. Future studies

3.1. *Catalytic site occupancy*

In both synthesis and hydrolysis of ATP the catalytic rate is slow when only one site is filled under uni-site conditions. An important question is whether rapid catalysis results when substrate(s) add to a second catalytic site, or is the filling of a second and third site required—that is, what are the relative rates of bi-site and tri-site catalysis? Our results suggest that bi-site catalysis is rapid for both synthesis [30] and hydrolysis [31], but more appraisal is needed to find if this is indeed the case.

There is ample evidence that at below micromolar concentrations of ATP, only one catalytic site of F_1-ATPases will bind ATP and this results in a slow hydrolysis rate. Similarly, with ATP synthases below micromolar concentrations of ADP suffice for filling of one catalytic site, with low turnover. When initial velocity measurements are adequately made in the micromolar concentration range, only one apparent K_M is observed for ATP during hydrolysis or for ADP during synthesis, indicative that only two sites need to be filled for near maximal catalysis rates.

With millimolar concentrations, binding of ATP or ADP to a third site may occur with little or modest rate increase. But kinetic measurements are not sufficiently definitive. More direct measurements of catalytic site occupancy are needed.

Two methods for measuring catalytic site occupancy during ATP synthesis have been used in my laboratory and need to be applied more widely. One is based on the measurement of the hexokinase-inaccessible ATP [32]. With the rate of ATP synthesis limited by substrate or energy input, levels of [γ-^{32}P]ATP present are measured. Extrapolation to infinite hexokinase concentration gives an estimate of bound catalytic ATP present. A second approach has been used only for photophosphorylation [30]. With chloroplast thylakoids doing steady state ATP synthesis in the presence of hexokinase to remove medium ATP, a rapid filtration provides an aliquot of the reaction medium for determination of free ADP concentration. With relatively high thylakoid concentrations and sufficient ADP so that the onset of rapid photophosphorylation is just occurring, the amount of any bound ADP+ATP can be determined. The results point to the filling of a second catalytic site as sufficing for rapid catalysis. However, our data are quite limited, and more studies using such an approach could be useful.

To my knowledge no direct measurements have been reported for assessing catalytic site occupancy during ATP synthesis by the mitochondrial ATP synthase. Submitochondrial particles do not allow a filtration–separation as readily as thylakoids, but suitable apparatus or other methods might be devised.

A potentially useful method for direct measurement of catalytic site occupancy during ATP hydrolysis by *Escherichia coli* F_1-ATPase was developed by Weber et al. [33,34]. They replaced a catalytic site tyrosine of *E. coli* F_1-ATPase with tryptophan and correlated activity with nucleotide binding as measured by quenching of the tryptophan fluorescence. They suggested that rapid ATPase activity required filling of three sites. However, as noted elsewhere [23,35], their assay conditions were such that most of the binding was likely to poorly active enzyme forms present. The approach, however, has promise, and additional studies in various laboratories will be awaited with interest.

3.2. Additional insights into the binding change mechanism.

Much is yet to be learned about the catalytic steps of the binding change mechanism. Information on details of the rate and order of substrate binding and release steps are largely lacking. Uncertainties about the nature of intermediate steps remain. The preferred orders of binding and release of substrates and the concentrations and locations of bound ATP, ADP and P_i during rapid net synthesis are largely unknown. Studies on photophosphorylation suggest that the quasi-equilibrium between ADP+P_i is markedly shifted toward ATP during rapid photophosphorylation [30,36]. When rapid photophosphorylation is limited by ADP concentration about half of the catalytic-site nucleotide (measured by hexokinase inaccessibility) is present as ATP. In contrast, with limiting P_i concentration the level of tightly bound ATP is increased to about one per synthase. Such tightly bound ATP rapidly decreases to a lower level when switching off the light and adding ammonia [36] deenergizes the thylakoids. Presence of ADP at a second site and/or high protonmotive force site appears to favor a shift toward ATP at the tight site.

Such possibilities are not readily apparent in depictions of the binding change mechanism as frequently presented (see Fig. 1). Some possible changes that an individual catalytic site undergoes during a complete cycle of three binding changes are shown in Fig. 2. The conversion of a loose site (Form 1) with added ADP and P_i to a tight site (Form 2) in the first binding change is of particular importance. As conformational changes occur groups or conditions responsible for both tight binding and catalysis must surround substrates, e.g., low water activity and charged or H-bonding residues. Some or most of the residues involved for promotion of catalysis and tight binding are likely the same. Thus as tight binding is achieved ADP and P_i may be approaching a transition state or even already present as ATP. The pressure of protonmotive force and/or changes accompanying ADP binding at the newly formed open site (Form 1) may favor shift of the quasi-equilibrium toward ATP. This is indicated in Fig. 2 by the Form 2-S, which is greatly favored during conditions for rapid synthesis. The ATP

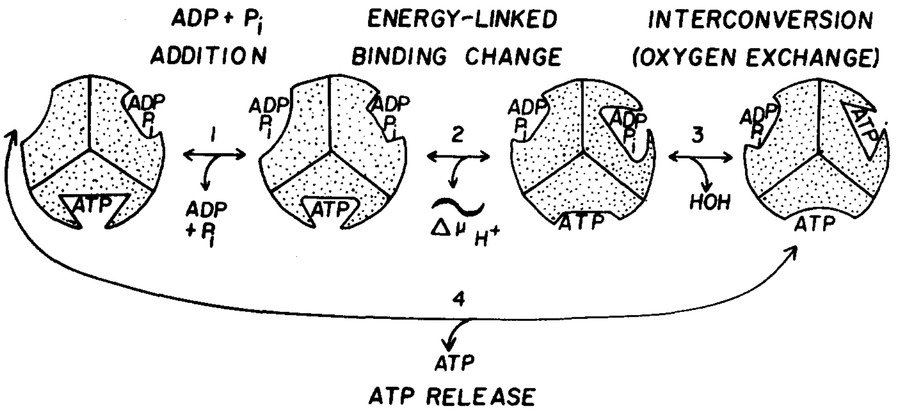

Fig. 1. A depiction of the binding change mechanism as commonly used. Each of the three catalytic sites passes sequentially through the forms indicated in the figure. At any one time all three sites are in different conformations. With each binding change one ATP is liberated.

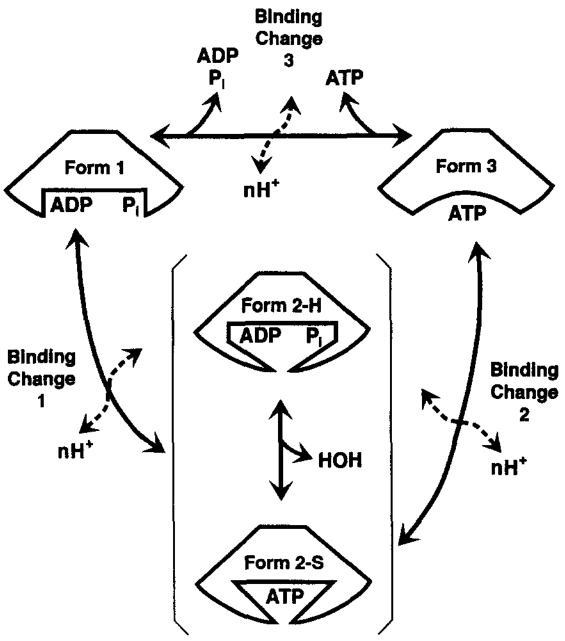

Fig. 2. A depiction of suggested changes that one catalytic site undergoes with three successive binding changes. Synthesis occurs in the counter-clockwise and hydrolysis in the clockwise direction. For rapid ATP synthesis with adequate protonmotive force and substrates, as binding change 1 occurs nearly all of Form 1 with added ADP and P_i is converted to Form 2-S. The next binding change then allows release of ATP, either before (as indicated) or after binding change 3 occurs. Binding change 1 cannot again occur until both ADP and P_i are bound to Form 1. For rapid ATP hydrolysis in the absence of protonmotive force and with adequate ATP present, Form 3 is converted largely to Form 2-H, so that ADP is released in the next binding change. In rapid bi-site synthesis, ADP and P_i add preferentially to Form 1. For rapid bi-site hydrolysis by F_1-ATPase, ATP adds preferentially to Form 3.

liberated during rapid oxidative phosphorylation or photophosphorylation usually has some oxygen derived from water in the terminal phosphoryl group, as a result from rapid reversal of ATP formation in Form 2.

During rapid ATP hydrolysis, the lack of protonmotive force and/or the binding of ATP to the loose site are suggested to favor a shift of the quasi-equilibrium toward ADP+P_i, as designated by the Form 2-H. The next binding change during rapid hydrolysis will then liberate mostly ADP and P_i.

4. Some other uncertainties about ATP synthase

Clarification of the composition and binding loci of the stator needed for the rotational catalysis is well underway (e.g. see Refs. [37,38]), and likely good insight will be available at the time of the European Bioenergetics Congress meeting. Likewise, additional information about the rotation and whether it is stepped or near continuous will be forthcoming. The lack of a three-dimensional X-ray structure for the rest of the F_1-ATPase, and for the entire synthase, seriously limits progress, although other structural and mutational approaches, particularly with the *a* and *c* subunits allow, valuable insights. For example, the demonstration in Dimroth's laboratory of a single occluded Na^+ with a mutant of the Na^+-translocating synthase and other information give evidence for important features of the ion transport [39]. A rotational movement of one of 12 cation binding sites of the *c* subunits to make a connection with a portion of the *a* subunit that results in a transmembrane channel is suggested.

An unsettled problem of considerable interest is the number of protons that need to be translocated for each ATP made. If there are indeed 12 *c* subunits arranged circularly, a mechanism such as that mentioned above would seem to favor translocation of four protons for each ATP made. Results suggesting that considerably more than four protons may be translocated for each ATP cleaved [40] may need further examination. In one of the early suggestions for rotational movement, a variable coupling stoichiometry is envisioned [41], and such a possibility has not been eliminated. One is reminded that uncertainty also remains in the P/O ratio for oxidative phosphorylation [42]. The interest in such problems appears to have diminished but the lack of generally accepted answers is an embarrassment to the bioenergetic community.

There are two other problems that may deserve more attention. One is whether the familiar knobs seen on electron micrographs of coupling membranes represent the structure as in the functional ATP synthase. Three lines of evidence suggest that further evaluation might be helpful. One is the increasing recognition that the δ or OSCP and the *b* subunits are part of a stator between the F_0 and F_1 and should contribute to the observable mass. Another is earlier evidence that the β subunit is readily cross-linked to the *a* subunit of F_0 [43], and that the β subunit appears to be close to the phospholipid bilayer [44]. A third is that when precautions to avoid artifacts are used in electron microscopy the stalks are not seen, as if the enzyme is huddled together in the native configuration [45]. The matter may not, however, be of any major importance to those interested in mechanism. The evidence for rotational catalysis is not dependent upon the appearance of the enzyme in electron microscopy, and that the knobs most often seen arise from the F_1-ATPase seems clear.

A final question to which I would like to direct attention in this lecture concerns the possibility of localized as well as delocalized coupling. That protonmotive force between aqueous phases separated only by a bilayer membrane can drive ATP formation is well established. In some regard it is conceptually simpler to accept this as the only way that electron transport is coupled to ATP synthesis. But I find that evidence for localized coupling has not been convincingly assessed or refuted in the literature. It may be that, as often the case in science, that which is regarded as wrong is simply ignored by most researchers in a field. For example, I find that the evidence from Dilley's laboratory that seems persuasive for some type of localized coupling in chloroplasts (see Ref. [46]) has very few citations and these give little appraisal. Ferguson has diplomatically kept the issue somewhat open and proposed ideas for consideration [47], and other observations [48,49] may merit more consideration. Nature takes advantage of spatial relationships as noted when proximity of enzymes favors metabolic pathways. Possibly in the close folding of the inner membranes of mitochondria and chloroplasts, proton releasing portions of electron-transport enzymes may often be nearly opposite the F_0 portion of the ATP synthase such that a very small intermembrane volume is involved in the coupling of proton translocation to ATP synthesis.

In closing, I want to express my appreciation for the opportunity I have had to undertake research in the field of bioenergetics with many fine colleagues. The combined efforts of many have allowed us to present to the scientific community the major and unusual features of the important and remarkable ATP synthase. Although my time as a research contributor to the field is over, I anticipate years as an interested observer when answers to many of the remaining questions are revealed, and to learn of additional surprises that are likely in store.

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