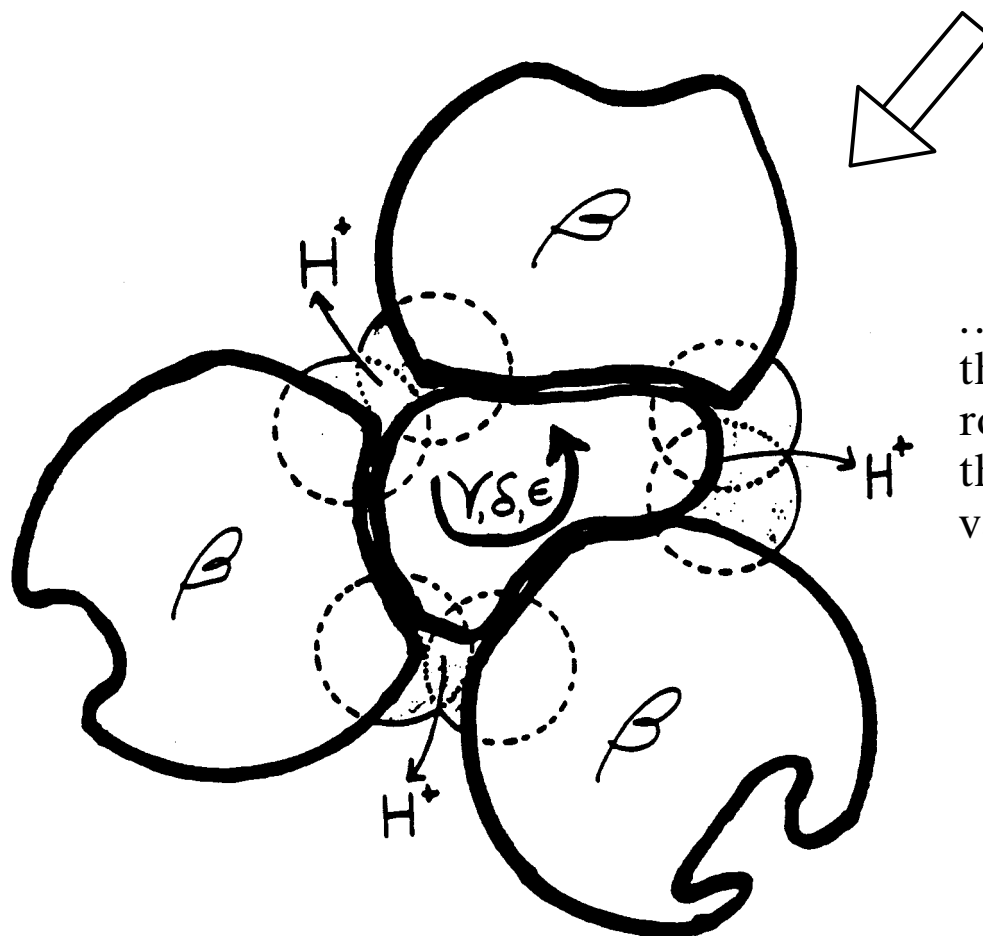
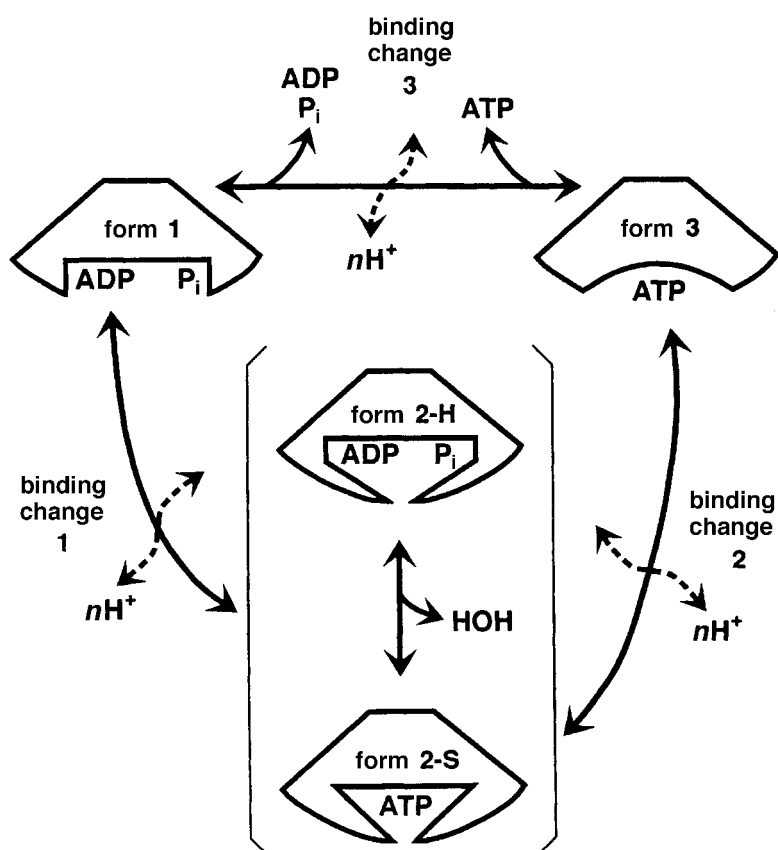


The recognition of binding changes of catalytic sites of the ATP synthase ...



... was followed by the postulate of a rotational catalysis that has now been validated.

## Energy, Life, and ATP (Nobel Lecture)\*\*

Paul D. Boyer\*

### Overview

I have a deep appreciation for the unusual and unexpected chain of events that has brought me the Nobel Award. It is my good fortune to be a spokesman for a considerable number of outstanding researchers in the field of bioenergetics, whose efforts have revealed an unusual and novel mechanism for one of nature's most important enzymes. Over 50 years ago a vital cellular process called oxidative phosphorylation was demonstrated. The process was recognized as the major way that our bodies capture energy from foods to be used for a myriad of essential cellular functions, but how it occurred was largely unknown. The intervening years have seen much progress. Today I will tell you how contributions of my research group in the 1970s led to new hypotheses that helped overcome the limitations of old paradigms which were no longer applicable. We gained further support of the hypotheses and clarified other aspects of the process in the 1980s and early 1990s. Then—as John Walker, my corecipient, will relate<sup>[123]</sup>—the X-ray structural data from his group became available. The structural information about the catalytic portion of the enzyme for the phosphorylation supported the most novel and least accepted aspect of our hypotheses. Now on this occasion, John and I can tell you how a truly remarkable molecular machine accomplishes the oxidative phosphorylation that was left unexplained for over half a century.

A key player in the process is called ATP, the abbreviation for adenosine triphosphate. At the time I was a graduate student, Fritz Lipmann<sup>[1]</sup> recognized the broad role ATP played in biological energy capture and use. The adenosine portion, for our purposes, can be regarded as a convenient handle to bind the ATP to enzymes. It is the three phosphate groups attached in a row, particularly the last two, that participate in energy capture. When the energy stored in ATP

is used, the terminal anhydride bond is split, forming adenosine diphosphate (ADP) and inorganic phosphate ( $P_i$ ). The resynthesis of ATP, coupled to energy input, is catalyzed by an enzyme called ATP synthase, which is present in abundance in intracellular membranes of animal mitochondria, plant chloroplasts, bacteria, and other organisms. The ATP made by your ATP synthase is transported out of the mitochondria and used for the function of muscle, brain, nerve, kidney, liver, and other tissues, and for transport and for making a host of compounds that the cell needs. The ADP and phosphate formed when ATP is used return to the mitochondria, and ATP is made again using the energy from the oxidations. I estimate that the net synthesis of ATP is the most prevalent chemical reaction that occurs in your body. Indeed, because plants and microorganisms capture and use energy by the same reaction, and the amount of biomass is large, the formation and use of ATP is the principal net chemical reaction occurring in the whole world. This is obviously a very important reaction. How does it occur?

All living cells contain hundreds of large, specialized protein molecules called enzymes. These catalyze the hundreds of chemical reactions that are necessary for the cell to function. Among these are the reactions by which energy is captured by the mitochondria, which are packed into muscle, brain, and other cells. Inside the mitochondria and imbedded in its membranes are enzymes that catalyze oxidation of the food you eat. They essentially burn it, using oxygen and producing carbon dioxide and water, in a series of small steps, each catalyzed by a special enzyme. The oxygen you are breathing now is carried by the hemoglobin of your red blood cells, then it reaches the mitochondria where it oxidizes iron atoms that are part of a specialized enzyme, which in turn oxidizes other enzymes in a respiratory chain. The blood stream carries the carbon dioxide produced to the lungs for exhaling. The sequence of oxidations liberates protons and promotes a charge that tends to force protons across the membrane. Similarly, in chloroplasts light energy is coupled to the formation of proton-motive force. This proton-motive force, as shown by the 1978 nobelist Peter Mitchell,<sup>[2]</sup> causes protons (hydrogen ions) to be translocated through the ATP synthase accompanied by formation of ATP. The important and very difficult question that remained unanswered for many years was how the ATP synthase uses the proton-motive force to make ATP.

[\*] Prof. P. D. Boyer  
Department of Chemistry and Biochemistry  
University of California at Los Angeles  
611 Circle Drive East, Los Angeles, CA 90095-1570 (USA)  
Fax: (+1) 310-206-7286  
E-mail: pdboyer@ucla.edu

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## ATP Synthase

First I will summarize what is now known about the ATP synthase, then convey aspects of how this knowledge was attained. The enzyme uses a novel mechanism that has catalytic steps different from any that had been seen before with other enzymes. A sketch that depicts the enzyme function is available on the Nobel Foundation internet site.<sup>[124]</sup> A similar sketch was provided in a recent paper from Richard Cross's laboratory.<sup>[3]</sup> The ATP synthase has three copies each of large  $\alpha$  and  $\beta$  subunits, with three catalytic sites located mostly on the  $\beta$  subunit at the interface of the  $\alpha$  and  $\beta$  subunits. A  $\gamma$  subunit core and smaller  $\delta$  and  $\epsilon$  subunits complete a portion known as  $F_1$ , with a subunit composition in order of decreasing size designated as  $\alpha_3\beta_3\gamma\delta\epsilon$ . This portion of the enzyme was first isolated in the laboratory of a splendid investigator, Efraim Racker, and shown to act as an ATPase.<sup>[4]</sup> Several leading investigators in the bioenergetic field were trained in Racker's laboratory. The  $F_1$ -ATPase<sup>[\*]</sup> catalyzes ATP hydrolysis, but not ATP synthesis. The rest of the enzyme, which is imbedded in the membrane, is known as  $F_0$ ; in *E. coli* the  $F_0$  contains a large subunit *a*, two copies of a subunit *b*, and probably 12 copies of a much smaller *c* subunit. The  $F_0$  of the mitochondrial enzyme is much more complex. The designation  $F_1F_0$ -ATPase is sometimes used in the literature for the complete ATP synthase.

During net ATP synthesis the three catalytic sites on the enzyme, acting in sequence, first bind ADP and phosphate, then undergo a conformational change so as to make a tightly bound ATP, and then change conformation again to release this ATP. These changes are accomplished by a striking rotational catalysis driven by a rotating inner core of the enzyme, which in turn is driven by the protons crossing the mitochondrial membrane. I share the view that revealing the mechanism of the ATP synthase is a fine achievement of modern biochemistry. I am also keenly aware that this achievement comes from the sum of the research of many members of the bioenergetics community, who deserve a major share in the recognition of the accomplishment. But the

Nobel awards tend to make heroes of only one or a few of those responsible. It is my good fortune to be addressing you today because my research group, strongly dependent on the information provided by others, gained the first insights into three unusual features of the ATP synthase catalysis. These unusual features are energy-linked binding changes that include release of a tightly bound ATP, sequential conformational changes of three catalytic sites to accomplish these binding changes, and a rotary mechanism that drives the conformational changes. These features had not been recognized previously in enzymology.

## Early Probes

In the mid 1950s, some twelve years after receiving my Ph.D., some experiments on how ATP is made were conducted in my laboratory. One concerned the capture of energy in glycolysis. We found that the oxidation of glyceraldehyde 3-phosphate could occur without the participation of inorganic phosphate,<sup>[5]</sup> suggesting participation of an acyl enzyme intermediate. Extension of these experiments, and salient findings in Racker's group,<sup>[6]</sup> demonstrated that a sulfhydryl group on the enzyme was acylated and the acyl enzyme was cleaved by inorganic phosphate to form 1,3-diphosphoglycerate, which in turn transferred a phosphoryl group to ADP to make ATP. The demonstration that two covalent intermediates, the acyl enzyme and the phosphorylated substrate, preceded ATP formation made it seem logical to seek for similar intermediates in oxidative phosphorylation. As we and others learned years later, this was not a useful approach.

Of more relevance to ATP synthase were experiments with  $^{18}\text{O}$  and  $^{32}\text{P}$ , initiated because of the demonstration by Mildred Cohn that mitochondria would catalyze a rapid exchange of phosphate oxygen atoms with those of water.<sup>[7]</sup> We found from the  $^{32}\text{P}$  experiments that the overall reaction of oxidative phosphorylation was dynamically reversible.<sup>[8]</sup> The  $^{18}\text{O}$  experiments revealed the striking finding that the exchange of inorganic phosphate oxygen atoms with water was occurring even more rapidly. As illustrated in Figure 1, we attributed this to the formation of a covalent intermediate, which was then cleaved by inorganic phosphate. We tried unsuccessfully

[\*] Abbreviations used for the  $F_1$ -ATPase from various sources: from heart mitochondria MF<sub>1</sub>, from chloroplasts CF<sub>1</sub>, from *E. coli* EcF<sub>1</sub>, and from Kagawa's thermophilic bacterium TF<sub>1</sub>.



*Paul D. Boyer was born on July 31, 1918 in Provo, Utah. He received a B.S. degree in chemistry from Brigham Young University in 1939, and a Ph.D. in biochemistry from the University of Wisconsin in 1943. After two years at Stanford University on a war research project for stabilization of serum albumin, he became an Assistant Professor at the University of Minnesota, where he initiated studies on oxidative phosphorylation. Since 1963 he has been a Professor in the Department and Biochemistry at the University of California, Los Angeles, where he also served as founding Director of the Molecular Biology Institute. In 1955 he was a Guggenheim fellow for studies in Sweden and received the American Chemical Society Award in Enzyme Chemistry. He has honorary doctorate degrees from the Universities of Stockholm, Minnesota, and Wisconsin. He received the Rose Award of the American Society of Biochemistry and Molecular Biology in 1989 and shared the 1997 Nobel Prize in Chemistry.*

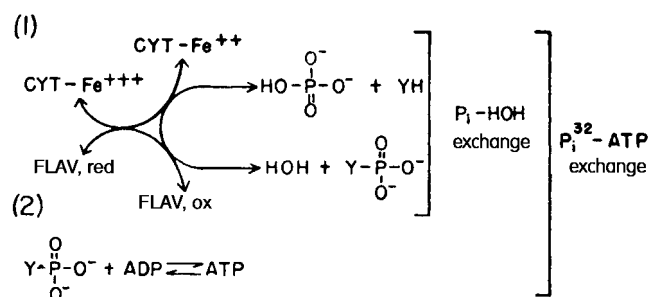


Figure 1. Exchange of phosphate oxygen atoms between  $^{32}\text{P}_i$  and ATP as well as between  $\text{P}_i$  and HOH for the overall reaction  $\text{ADP} + ^{32}\text{P}_i \rightleftharpoons \text{ATP} + \text{HOH}$  along with the dynamic reversal of oxidative phosphorylation (adapted from ref. [7]). In this early study covalent intermediates were proposed to explain why oxygen exchange was more rapid than reversal of the overall reaction. FLAV = flavin, CYT = cytochrome.

to separate out fractions from mitochondria that would catalyze the first step leading to the formation of an intermediate in oxidative phosphorylation. It was some sixteen years later that we found the simple explanation that no intermediate was formed, and that the rapid  $^{18}\text{O}$  exchange resulted from the rapid and reversible formation of a tightly bound ATP.

In the 1960s we embarked on another, only partially successful, series of experiments. By using  $^{32}\text{P}$  as a sensitive tracer, we found in mitochondria a  $^{32}\text{P}$ -labeled protein that was an intermediate between inorganic phosphate and ATP. We identified this as a previously unrecognized phosphorylated protein, with a phosphoryl group attached to a histidine residue. We mistakenly thought we had identified an intermediate in oxidative phosphorylation, but subsequently found it to be an intermediate in GTP or ATP formation by the succinyl CoA synthetase of the citric cycle.<sup>[9]</sup> We were reaching for a gold but got a bronze instead.

## $^{18}\text{O}$ Exchanges and a New Concept

For several years we mostly studied other problems, including taking a look at active transport in *E. coli*. This study gave evidence for an intermediate and unidentified energized state,<sup>[10]</sup> but we did not characterize this state or pay enough attention to the rumblings coming from Peter Mitchell's laboratory. It was difficult for me to accept proton-motive force as a driving agent for ATP formation when I could not visualize a logical way this could occur. But the lure of the ATP synthase continued, and we tried to get leads with photophosphorylation by spinach thylakoid membranes as well as oxidative phosphorylation by heart mitochondria. The use of the  $^{18}\text{O}$ -exchange measurements to study the process provided a crucial insight. The types of exchange that can be measured are readily understood with the aid of the diagram in Figure 2. The box represents a catalytic site. ADP and  $\text{P}_i$  can bind and be converted into a tightly bound ATP. The water formed freely interchanges with medium water. Reversal of this reaction results in the incorporation of one water oxygen atom into the bound  $\text{P}_i$ . If the  $\text{P}_i$  can tumble freely at the catalytic site, when bound ATP is again formed

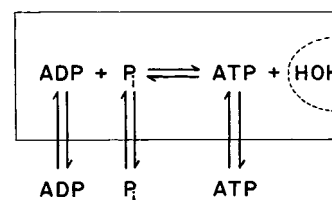


Figure 2. Binding, interconversion, and release steps for oxygen exchanges.

there are three chances out of four that it will contain a water oxygen atom. Various exchanges of phosphate oxygen atoms with water oxygen atoms are measurable, as shown in Table 1. The oxygen exchanges thus provide sensitive probes of reaction steps that otherwise might be hidden.

Table 1. Exchanges of phosphate oxygen atoms with water oxygen atoms catalyzed by ATP synthase.

Exchange	Measurement
intermediate $\text{P}_i \rightleftharpoons \text{HOH}$	hydrolysis of $[\gamma\text{-}^{18}\text{O}]\text{ATP}$ and determination of $^{18}\text{O}$ in $\text{P}_i$ formed
intermediate $\text{ATP} \rightleftharpoons \text{HOH}$	synthesis of ATP from $[\text{P}_i\text{-}^{18}\text{O}]\text{P}_i$ and determination of $^{18}\text{O}$ in ATP formed
medium $\text{P}_i \rightleftharpoons \text{HOH}$	determination of loss of $^{18}\text{O}$ from $[\text{P}_i\text{-}^{18}\text{O}]\text{P}_i$ when $\text{P}_i$ binds, undergoes exchange, and returns to the reaction medium
medium $\text{ATP} \rightleftharpoons \text{HOH}$	determination of loss of $^{18}\text{O}$ from $[\gamma\text{-}^{18}\text{O}]\text{ATP}$ when ATP binds, undergoes exchange, and returns to the reaction medium

The  $^{18}\text{O}$  probes revealed a puzzling aspect, namely that the intermediate  $\text{P}_i \rightleftharpoons \text{HOH}$  exchange was unusually insensitive to uncouplers of oxidative phosphorylation. As shown in Figure 3, even though the potent uncoupler S-13 allowed oxidation to proceed without net ATP synthesis, the rapid exchange of phosphate and water oxygen atoms continued. The significance of this was not grasped for some time. But one day, while listening to a seminar that I did not understand, the oxygen-exchange data churned in my mind. It became clear to me that the results could be explained if the energy from oxidations was not used to *make* the ATP molecule, but instead was used to bring about a *release* of a tightly bound ATP. The reversible formation of the tightly bound ATP molecule could continue at the catalytic site without involving proton-motive force, and give rise to the uncoupler-insensitive oxygen exchange. We now had a new concept for oxidative phosphorylation and were anxious to call it to the attention of the field. The editors of the *Journal of Biological Chemistry* declined the opportunity to publish this new concept. I used the privilege of my recent membership in the National Academy of Sciences<sup>[11]</sup> (Figure 4) to publish this first feature of what was to become the binding change mechanism of ATP synthesis. Independently, Slater's group, based on the presence of tightly bound nucleotides on the isolated  $\text{F}_1\text{-ATPases}$ , also suggested that energy input might be involved in their release.<sup>[12]</sup>

Our feeling that the new concept was valid was strengthened by companion studies with the ATPase activity of muscle

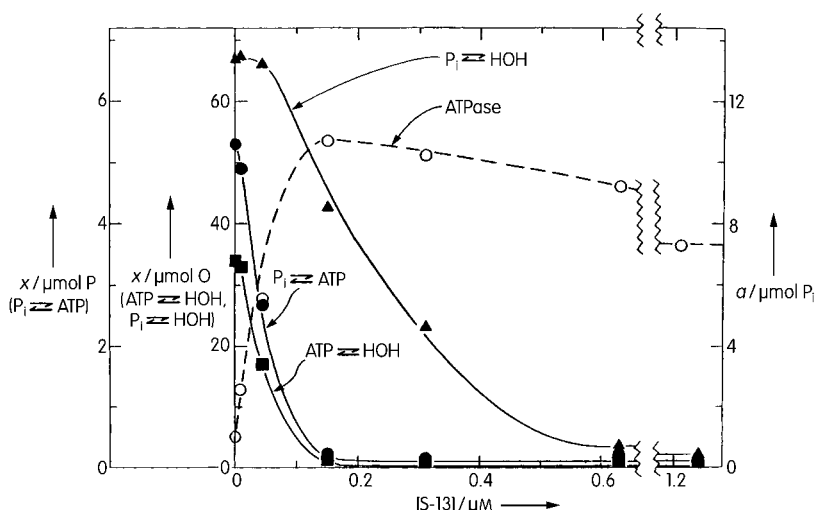


Figure 3. The insensitivity of phosphate oxygen atom exchange to an uncoupler of oxidative phosphorylation as compared to other measured reactions. Various uncouplers gave similar results; in this experiment an uncoupler known as S-13 was used.  $a$  = ATPase activity, given in micromoles of released  $P_i$ ;  $x$  = exchange, given in micromoles of  $P$  ( $P_i \rightleftharpoons ATP$ ) or  $O$  ( $ATP \rightleftharpoons HOH$ ,  $P_i \rightleftharpoons HOH$ ).

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#### A New Concept for Energy Coupling in Oxidative Phosphorylation Based on a Molecular Explanation of the Oxygen Exchange Reactions

(protein conformational change/uncouplers/mitochondria)

PAUL D. BOYER, RICHARD L. CROSS, AND WILLIAM MOMSEN

The Molecular Biology Institute and The Department of Chemistry, University of California, Los Angeles, Calif. 90024

Contributed by Paul D. Boyer, June 25, 1973

**ABSTRACT** The  $P_i \rightleftharpoons HOH$  exchange reaction of oxidative phosphorylation is considerably less sensitive to uncouplers than the  $P_i \rightleftharpoons ATP$  and  $ATP \rightleftharpoons HOH$  exchanges. The uncoupler-insensitive  $P_i \rightleftharpoons HOH$  exchange is inhibited by oligomycin. These results and other considerations suggest that the relatively rapid and uncoupler-insensitive  $P_i \rightleftharpoons HOH$  exchange results from a rapid, reversible hydrolysis of a tightly but noncovalently bound ATP at a catalytic site for oxidative phosphorylation, concomitant with interchange of medium and bound  $P_i$ . Such tightly bound ATP has been demonstrated in sub-mitochondrial particles in the presence of uncouplers,  $P_i$ , and ADP, by rapid labeling from  $^{32}P$ , under essentially steady-state phosphorylation conditions. These results lead to the working hypothesis that in oxidative phosphorylation energy from electron transport causes release of preformed ATP from the catalytic site. This release could logically involve energy-requiring protein conformational change.

Figure 4. The beginning of the binding change mechanism. Portion of the publication presenting a new concept for oxidative phosphorylation.<sup>[11]</sup>

myosin. Data from my and from Koshland's laboratories<sup>[13, 14]</sup> had shown that myosin could catalyze both a medium  $P_i \rightleftharpoons HOH$  and an intermediate  $P_i \rightleftharpoons HOH$  exchange. It seemed possible that myosin might be able to spontaneously form a tightly bound ATP from medium ADP and  $P_i$ . Experiments showed this to be the case.<sup>[15]</sup> This and other salient properties of myosin had also been revealed in contemporary studies by Trentham and associates.<sup>[16, 17]</sup> Importantly, the oxygen exchange could be quantitatively accounted for by the rate of formation and cleavage of the bound ATP.

Not all bioenergeticists readily accepted the concept that a prime function of energy input was to bring about the release of a tightly bound ATP. For example, Mitchell preferred a mechanism in which the protons migrated to the catalytic site and induced the formation of ATP from ADP and  $P_i$ . It

seemed logical to me that proton translocation was linked to ATP release indirectly through conformational changes in the protein.<sup>[18]</sup> Without my being informed, my publication was accompanied by a rebuttal from Mitchell,<sup>[19]</sup> and I thus presented a more complete model for conformational coupling.<sup>[20]</sup> With time this indirect manner in which proton translocation drives ATP formation has become generally accepted, but this does not detract from Mitchell's salient recognition of proton-motive force as a means of capturing energy for ATP synthesis and active transport.

#### Catalytic Cooperativity

We were now launched on an exciting period of research. As we probed mitochondrial oxidative phosphorylation further by  $^{32}P$  and  $^{18}O$  isotope exchanges, some puzzling aspects emerged. For example, when submitochondrial particles capable of oxidative phosphorylation were hydrolyzing ATP, a lively medium  $ATP \rightleftharpoons HOH$  exchange occurred. Removal of product ADP stopped this exchange (Figure 5), although the reversal of ATP hydrolysis was still occurring on the enzyme. Somehow the lack of

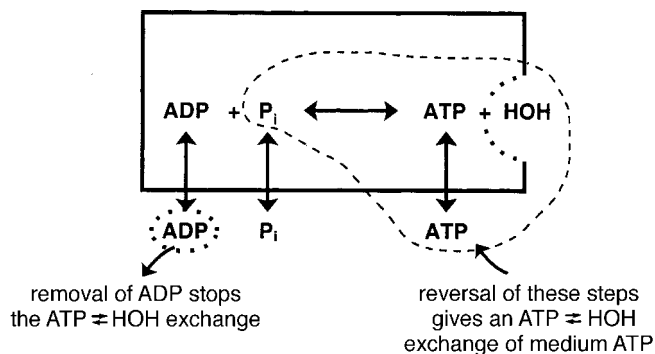


Figure 5. Catalysis of the synthesis and hydrolysis of ATP by sub-mitochondrial particles. Removal of medium ADP stops the medium  $ATP \rightleftharpoons HOH$  oxygen exchange.

medium ADP to bind to the enzyme was stopping the release of ATP. It was not apparent how this could occur if the simple scheme of Figure 2 was used to explain the oxygen exchanges. Similarly, during net synthesis of ATP, removal of the medium ATP stopped the medium  $P_i \rightleftharpoons HOH$  exchange. An explanation for why these oxygen exchanges were blocked, and for other related observations, was suggested by one of my graduate students, Celik Kayalar from Turkey. Celik said he could account for these results if the catalytic sites had to work cooperatively, so that ATP could not be released from one site unless ADP and  $P_i$  were available to bind at another site, or that  $P_i$  could not be released from one catalytic site unless ATP were available to bind at another catalytic site. Celik, together with Jan Rosing from Holland, demonstrated and characterized sequential and cooperative participation of

catalytic sites with the synthase in submitochondrial particles capable of or during oxidative phosphorylation.<sup>[21, 22]</sup> In addition, their results gave evidence that the binding changes accompanying proton translocation also promoted the tight binding of  $P_i$ .

Adolfson and Moudrianakis suggested that site-site cooperativity might occur with the separated  $F_1$ -ATPase, based on the observation that a tightly bound ADP was released when ATP was cleaved by a bacterial  $F_1$ -ATPase.<sup>[23]</sup> Experiments in my laboratory revealed that, as we had found with the ATP synthase, a strong cooperativity of catalytic sites occurs with the isolated ATPase. When the  $MF_1$  hydrolyzes relatively high concentrations of ATP, the  $P_i$  formed contains only slightly more than the one water oxygen atom required for the hydrolysis (Figure 6). But as the ATP concentration is

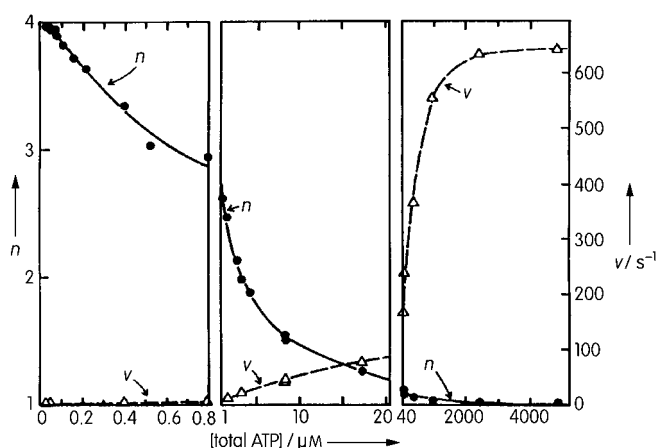


Figure 6. Effect of decrease in ATP concentration on the number of water oxygen atoms incorporated in each phosphate released ( $n = O$  pro  $P_i$ ) and on net hydrolysis velocity  $v$  as catalyzed by the  $MF_1$ -ATPase (adapted from ref. [10]).

lowered, an instructive change occurs. The hydrolysis velocity is of course lowered, but the number of water oxygen atoms appearing in each  $P_i$  formed increases to almost four. It can be calculated that nearly 400 reversals of bound ATP hydrolysis occur before the  $P_i$  formed is released.<sup>[10]</sup> The bound ADP and  $P_i$  formed cannot be released until ATP is available to bind at another catalytic site.<sup>[\*]</sup>

Experiments had now made it seem likely that an unexpected catalytic cooperativity was a prominent feature of the ATP synthase. At that time the prevailing view was that the enzyme had only two catalytic sites, and a diagram

depicting a bi-site mechanism appeared in my 1977 review article in the *Annual Review of Biochemistry*.<sup>[25]</sup> We recognized, however, that if the enzyme were found to have three catalytic sites, a tri-site mechanism, as currently known to occur, would be likely.<sup>[22, 26]</sup> The crucial point was that a tightly bound ATP could not be released until ADP and  $P_i$  bound at a second or a second and third catalytic site and the binding changes driven by proton translocation occur. This positive cooperativity meant that a low substrate concentrations, during either net synthesis or hydrolysis of ATP, a tightly bound ATP should still be present at a catalytic site. We undertook experiments to find if this was so. These tests were made with submitochondrial particles<sup>[27]</sup> or chloroplast thylakoids<sup>[28]</sup> so that net ATP formation was occurring with ADP concentrations far below the apparent  $K_m$  of ADP for maximal phosphorylation rates. About one tightly bound ATP committed to net ATP formation was found on each synthase. Such data give good evidence that strong positive catalytic cooperativity takes place under conditions where ATP synthesis is actually occurring. Additional findings from our and from other laboratories consistent with or favoring the catalytic cooperativity are summarized elsewhere.<sup>[29]</sup>

In 1982 Feldman and Sigman demonstrated that the  $CF_1$ -ATPase or the ATP synthase on chloroplast thylakoids, which have a tightly bound ADP in the catalytic site, would slowly form an equilibrium concentration of bound ATP from relatively high concentrations of medium  $P_i$ .<sup>[30, 31]</sup> Their characterization of this single-site catalysis supported our concept of tight ATP formation without coupling to proton-motive force. Factors that promote formation of ATP at catalytic sites of myosin and  $F_1$ -ATPases likely include the very tight preferential binding of ATP and, as suggested by deMeis,<sup>[32]</sup> low water activity.

Also in 1982, the acceptance of catalytic cooperativity by the field was considerably enhanced by the determination in Penefsky's laboratory of the rate constants for the interconversion, binding, and release steps for  $MF_1$  exposed to ATP at molar concentrations less than the molarity of the enzyme, conditions that gave what was termed uni-site catalysis.<sup>[33, 34]</sup> An additional important contribution from the same laboratory was the demonstration that the hydrolysis of a trinitrophenyl-ATP bound at a single catalytic site was markedly increased by the binding of a second trinitrophenyl-ATP.<sup>[35]</sup>

A number of researchers subsequently found a slow uni-site catalysis with different  $F_1$ -ATPases. However, an inability to see a definite uni-site catalysis with  $TF_1$  raised the question as to whether the cooperativity we had observed was a general phenomenon of  $F_1$ -ATPases.<sup>[36]</sup> That slow uni-site catalysis was indeed occurring was demonstrated by the increase in intermediate  $P_i \rightleftharpoons HOH$  exchange as ATP concentration was lowered.<sup>[37]</sup> For a number of years there appeared to be a general acceptance that a slow uni-site rate occurs and that the catalytic rate is markedly accelerated when ATP binds to additional sites. It was thus somewhat surprising when a quite recent claim appeared that  $MF_1$  depleted of bound nucleotides did not show a slow uni-site catalysis.<sup>[38]</sup> However, this claim is not experimentally sound; slow uni-site catalysis occurs with either native or nucleotide depleted  $MF_1$ .<sup>[39, 40]</sup>

[\*] We missed obtaining this striking result about a decade earlier. At that time Efraim Racker came to my laboratory with some  $F_1$ -ATPase so we could find if it catalyzed an oxygen exchange when ATP was hydrolyzed. We conducted the reaction at a relatively high ATP concentration, and found the incorporation of only one water oxygen atom, as required for the cleavage reaction. Harvey Penefsky made a similar observation.<sup>[24]</sup> If we had measured what happened as the ATP concentration was lowered, we would have revealed the catalytic site cooperativity then. But we had no reason to suspect that the enzyme catalysis for each substrate cleaved would change so dramatically with substrate concentration. The strong catalytic cooperativity that we later demonstrated had not been described previously for any other enzyme.

## Related Experiments

My laboratory group at this time also had an experimental interest in the  $\text{Na}^+/\text{K}^+$ -ATPase that Prof. Skou presents.<sup>[125]</sup> There was uncertainty whether the phosphoryl group that became attached to the enzyme as an intermediate in the catalysis was on a glutamyl or an aspartyl residue. We developed a borohydride reduction method that established that the group was attached to an aspartyl residue.<sup>[41, 42]</sup> We also discovered that the enzyme in the presence of  $\text{K}^+$  and  $\text{Mg}^{2+}$  catalyzed a rapid exchange of oxygen atoms of  $\text{P}_i$  with water oxygen atoms, attributable to a dynamic reversal of enzyme phosphorylation.<sup>[43]</sup> This was and remains<sup>[44]</sup> a useful way to probe this step of the reaction sequence. The related  $\text{Ca}^{++}$ -activated sarcoplasmic reticulum ATPase was likewise found to catalyze a rapid  $\text{P}_i \rightleftharpoons \text{HOH}$  exchange.<sup>[45]</sup>

Our attention was also directed toward the capacity of yeast pyrophosphatase to catalyze a  $\text{P}_i \rightleftharpoons \text{HOH}$  exchange.<sup>[46]</sup> We revealed that this exchange was due to a reversible formation of an enzyme-bound pyrophosphate,<sup>[47]</sup> and the details of the exchange process were elucidated.<sup>[47, 48]</sup> It was important to us that rapid mixing experiments showed that the rate of formation and cleavage of the bound pyrophosphate accounted for the oxygen exchange.<sup>[47]</sup> As mentioned above, this was shown previously for the bound ATP and oxygen exchange catalyzed by myosin. For the pyrophosphatase exchange, Hackney developed a theoretical analysis of the distribution of  $^{18}\text{O}$ -labeled species of  $\text{P}_i$ <sup>[49]</sup> that was to serve us well in studies we had underway on oxidative phosphorylation. The  $\text{P}_i \rightleftharpoons \text{HOH}$  exchange catalyzed by the sarcoplasmic reticulum ATPase was also shown by rapid mixing and quenching experiments to result from the dynamic reversal of the formation of the phosphorylated enzyme intermediate.<sup>[50]</sup> Such results, and the demonstration by Wimmer and Rose that the  $\text{ATP} \rightleftharpoons \text{HOH}$  exchange catalyzed by mitochondria resulted from the reversible cleavage of the terminal  $\text{P}-\text{O}-\text{P}$  bond,<sup>[51]</sup> gave us confidence that in oxidative phosphorylation and photophosphorylation the oxygen exchanges we observed were due to the reversible hydrolysis of tightly bound ATP.

## The Number of Catalytic Sites

Meanwhile studies in other laboratories were revealing the subunit stoichiometry of the  $\text{F}_1$ -ATPase. As noted in a review by Penefsky covering literature up through 1978,<sup>[24]</sup> considerable controversy remained. The difficulty of obtaining satisfactory molecular weights and subunit quantitation made it hard to get a clear choice between the presence of two or three copies of the major  $\alpha$  and  $\beta$  subunits. Reports that measurements with  $\text{EcF}_1$  and  $\text{TF}_1$  isolated from bacteria grown on  $^{14}\text{C}$ -labeled amino acids<sup>[52, 53]</sup> favored a stoichiometry of  $\alpha_3\beta_3\gamma_1$  seemed convincing to us. Reports on the composition of  $\text{CF}_1$  strongly supported the presence of three each of the large subunits.<sup>[54]</sup> On the basis of these and other developments, the field soon widely accepted the composition of  $\text{F}_1$ -ATPases as  $\alpha_3\beta_3\gamma\delta\epsilon$ . All of our further experiments have been based on such a stoichiometry of subunits.

The number of nucleotide binding sites on the enzyme remained controversial until about a decade ago. Both  $\alpha$  and  $\beta$  subunits were shown to have nucleotide binding sites. Reports in 1982 for  $\text{MF}_1$ <sup>[55]</sup> and in 1983 for  $\text{EcF}_1$ <sup>[56]</sup> gave good evidence for the presently accepted values of six potential nucleotide binding sites per enzyme. However, as late as 1987 claims were still made for only three nucleotide binding sites on  $\text{CF}_1$ <sup>[57]</sup> and four for the liver  $\text{F}_1$ .<sup>[58]</sup> Subsequent data for  $\text{CF}_1$ <sup>[59, 60]</sup> and the liver enzyme,<sup>[61]</sup> as well as the highly conserved sequence of the  $\beta$  subunits, support the present view that all  $\text{F}_1$ -ATPases have six nucleotide binding sites, although differing considerably in affinity.

Chemical derivatization studies, such as those in Bragg's laboratory<sup>[62]</sup> and summarized in reviews<sup>[29, 63]</sup> showed that all three  $\beta$  subunits, although with identical amino acid sequence, had distinctly different chemical properties. Such heterogeneity was a prominent reason why we considered it likely that all three  $\beta$  subunits passed through different conformations during catalysis. The participation of all three  $\beta$  subunits in a cooperative, sequential manner was supported, but not proven, by observations (over twenty are given in an earlier review)<sup>[29]</sup> that derivatization of only one site per enzyme would nearly or completely block catalysis. We were also impressed by studies in Futai's laboratory showing that one defective mutant  $\beta$  subunit stopped catalysis,<sup>[64]</sup> and by related mutational studies in Senior's laboratory<sup>[65]</sup> that favored the participation of three equivalent  $\beta$  subunits for catalysis.

There has, however, been considerable delay in reaching a general acceptance that three catalytic sites participate in an equivalent manner. A single catalytic and two regulatory sites has been proposed.<sup>[66, 67]</sup> Various models with only two catalytic sites have been suggested,<sup>[68–73]</sup> as well as a 1991 model with four functioning catalytic sites arranged in two alternate pairs.<sup>[74, 75]</sup> A 1989 review by Tiedge and Schafer<sup>[76]</sup> stresses symmetrical considerations and favors equivalent  $\beta$  subunit participation. Various models, and a 1991 review favoring a two-site model,<sup>[77]</sup> were appraised in a review prepared in 1992, in which I attempted to consider any experiments not in harmony with the binding change mechanism.<sup>[29]</sup> The conclusion I reached is that very likely three sites participate in an equivalent manner. Subsequent events<sup>[78]</sup> have strengthened this conclusion, although some doubts of which I am not aware may remain. The probability that three sites participate equivalently has guided experiments in my laboratory since the presence of three  $\beta$  subunits first seemed likely.

## Rotational Catalysis

Toward the end of the 1970s we initiated experiments that led to the postulation of the third feature of the binding change mechanism. The presence of three copies of the major  $\alpha$  and  $\beta$  subunits and single copies of the  $\gamma$ ,  $\delta$ , and  $\epsilon$  subunits made it unlikely that all three  $\beta$  subunits could have identical interactions with single copy subunits. In particular, interactions with the larger  $\gamma$  subunits seemed likely to be crucial. McCarty's laboratory had reported that, with chloroplasts, light increased the reactivity of SH groups on the  $\gamma$  subunit

and that modifications in the  $\gamma$  subunits increased the leakage of protons across the coupling membrane.<sup>[79, 80]</sup> This and other evidence suggested that the  $\gamma$  subunit interacted strongly with the catalytic  $\beta$  subunit. The growing information about the synthase gave a base for the interpretation of additional experiments with  $^{18}\text{O}$  that were underway in my laboratory.

Water highly labeled with  $^{18}\text{O}$  had become more readily available, and by NMR spectroscopy, as demonstrated by Cohn,<sup>[81]</sup> or mass spectrometry we could measure what we designated as the  $^{18}\text{O}$  isotopomers of  $\text{P}_i$ , containing 0, 1, 2, 3, or 4  $^{18}\text{O}$  atoms. Then when ATP synthesis or hydrolysis occurs with highly  $^{18}\text{O}$ -labeled substrates, under conditions where appreciable oxygen exchange occurs, the distribution of isotopomers formed can be measured. If all the catalytic sites involved behave identically, the distributions of  $^{18}\text{O}$  isotopomers would conform to a statistically predicted pattern. The results observed in a typical experiment for hydrolysis of  $^{18}\text{O}$ -ATP by  $\text{F}_1$ -ATPase are given in Figure 7.<sup>[82]</sup> They show that the distribution of isotopomers conforms very closely to that expected for identical behavior of all catalytic sites. The data rule out the possible participation of two types of catalytic sites. As shown by one example in Figure 7, this would give a markedly different distribution of isotopomers. Importantly, experiments with the net ATP synthesis by chloroplast and mitochondrial ATP synthases also showed that all catalytic sites behave identically.<sup>[83–85]</sup> The tests were sensitive and revealing; if steps of substrate binding, interconversion, or release or their concentration dependencies differed among catalytic sites, this should have been revealed in the  $^{18}\text{O}$  experiments.

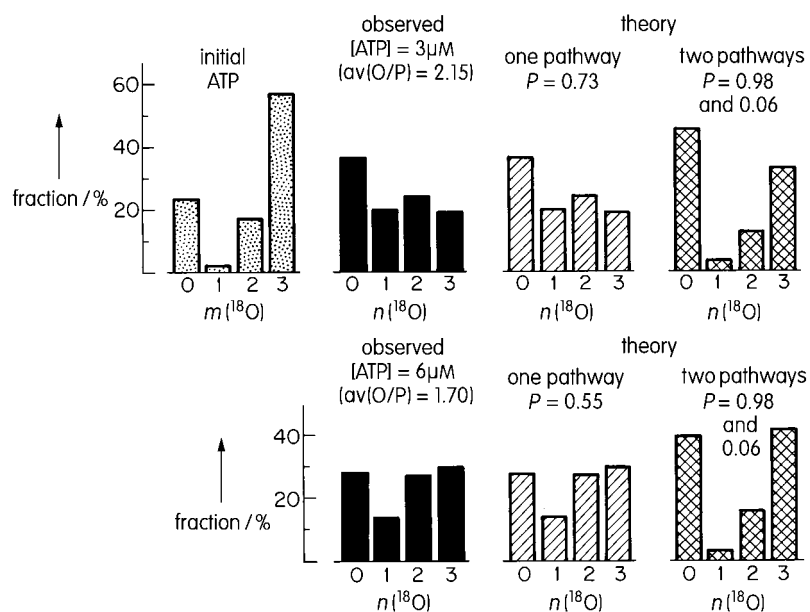


Figure 7. Distribution of  $^{18}\text{O}$  isotopomers of  $\text{P}_i$  formed from  $[\gamma\text{-}^{18}\text{O}]\text{ATP}$  by  $\text{MF}_1$ -ATPase hydrolysis at two relatively low ATP concentrations (top:  $3\ \mu\text{M}$ , bottom:  $6\ \mu\text{M}$ ). The observed average number of water oxygen atoms incorporated (O/P ratio) and the distribution of species with 0 to 4  $^{18}\text{O}$  atoms are shown. Also shown is the theoretical distribution for one pathway as expected if the probability  $P$  for exchange instead of release of bound  $\text{P}_i$  was 0.73 (with  $3\ \mu\text{M}$  ATP) and 0.55 (with  $6\ \mu\text{M}$  ATP). This is compared to the expected distributions if two pathways were operative, one with a high and one with a low probability of exchange, that would give the observed total amount of oxygen exchange.  $m(^{18}\text{O})$  = number of  $^{18}\text{O}$  atoms per  $\gamma$ -phosphate group on ATP;  $n(^{18}\text{O})$  = number of  $^{18}\text{O}$  atoms per product  $\text{P}_i$ . Adapted from ref. [82].

I was again confronted with unexplained results. Although it might be possible to bring a similar residue or residues on minor subunits into contact with each of the three  $\beta$  subunits, the interactions would not be expected to be identical. The situation might be analogous to the family of serine proteases, where markedly different sequences can appropriately position a serine residue. But the resulting proteases do not conduct their catalyses identically. To me, there seemed only one way that all catalytic sites could proceed sequentially and identically, with modulation by one or more single-copy, minor subunits. This was by a rotational catalysis, in which large catalytic subunits moved rotationally around a smaller asymmetric core. Such consideration, together with what was known about the structure of the enzyme, resulted in the postulate of rotational catalysis, presented at Gordon Research Conferences and elsewhere.<sup>[86–88]</sup> A sketch of our view as presented at that time is shown in Figure 8.<sup>[88]</sup> The internal

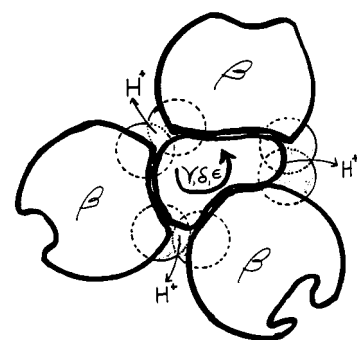


Figure 8. A sketch of possible rotational catalysis as used in 1980 for presentations and discussions.

core was likened to a cam shaft that modulated the conformation of the  $\beta$  subunits. The probability that the core was asymmetric was strengthened when data on the amino acid sequence became available;<sup>[89]</sup> this gave no indications of possible tripartite symmetry of the minor subunits.

Later other suggestions were made of possible rotational features in the ATP synthase catalysis. Increased information about the structure of the  $\text{F}_0$  portion of the synthase made some type of circular motion in the  $\text{F}_0$  attractive. Cox et al. suggested rotational movement of circularly arranged  $c$  subunits.<sup>[90, 91]</sup> Hoppe and Sebald visualized an oligomeric core of  $c$  subunits rotating against subunit  $a$  or  $b$ ,<sup>[92]</sup> a suggestion that still seems pertinent. Mitchell proposed a rotational model that exposed catalytic sites to a proton channel through the  $\gamma$  subunit.<sup>[93]</sup>

The homogeneity of catalysis demonstrated by the  $^{18}\text{O}$  technique also, to my mind, ruled out postulates, as mentioned earlier, that only two  $\beta$  subunits were involved in catalysis, with the other serving a regulatory function. Considerations of the need for symmetry in subunit



interactions made it unlikely that two sites could alternate in catalysis identically. Their interactions on one side could not be identical with those on another side at the same stages of catalysis.

We attempted some assessments of subunit positional interchange as required by a rotational catalysis. After labeling of one  $\beta$  subunit with radioactive dicyclohexylcarbodiimide (DCCD), the  $\text{MF}_1$ -ATPase still retained some activity. A different  $\beta$  subunit reacted with 2-azido-ATP. After catalytic turnover, the reactivity toward DCCD and 2-azido-ATP was randomized, as expected if a change in relative position and conformation had occurred.<sup>[94]</sup> In another approach, we observed that a mild cross-linking of subunits stopped catalysis, and that cleavage of the cross-linker restored activity.<sup>[95]</sup> There was a report from another laboratory that cross-linking of the  $\beta$  and  $\gamma$  subunits did not stop catalysis,<sup>[96]</sup> which I regarded as inconclusive.<sup>[29]</sup> None of these experiments were as edifying as those that came later from other laboratories (see below). It seemed apparent that an adequate evaluation of the possibility of rotational catalysis would need to await the knowledge of the three-dimensional structure of the  $\text{F}_1$ -ATPase. In a review I prepared in the spring of 1992, I summarized the case for rotational catalysis at that stage.<sup>[29]</sup> This included the need for a second attachment between  $\text{F}_0$  and  $\text{F}_1$  to act as a stator, and the suggestion that present evidence indicated that the  $\delta$  subunit of the *E. coli* enzyme, or the analogous oligomycin sensitivity conferring protein (OSCP) of mitochondria, could help serve this function, a prediction that has found support in recent experiments.<sup>[97, 98]</sup> Attachment of a stator to the exterior of an  $\alpha$  subunit might be partly responsible for the asymmetry of the  $\alpha$  subunits, an asymmetry that is retained during catalysis.<sup>[99]</sup> This may be analogous to the symmetry of the internal rotation of a motor not being disrupted upon bolting of the motor to a bench.

The occurrence of a rotational catalysis was dramatically supported by the X-ray structure for the major portion of  $\text{MF}_1$ , attained by Abrahams, Leslie, Lutter, and Walker.<sup>[100]</sup> This structure served as the base for innovative demonstrations of rotation in the laboratories of Cross,<sup>[3, 101, 102]</sup> Capaldi,<sup>[103–105]</sup> and Kagawa.<sup>[97]</sup> Sabbert et al. demonstrated rotation by sophisticated fluorescent techniques,<sup>[106, 107]</sup> and Noji et al. demonstrated rotation visually.<sup>[108]</sup> Such developments allowed me to title a recent review “The ATP Synthase—A Splendid Molecular Machine”.<sup>[78]</sup> These more recent aspects of the ATP synthase story are more appropriately the subject of my able corecipient John Walker’s lecture. But before you have the opportunity to hear from him, I want to discuss some additional important and unsettled facets of the ATP synthase catalysis.

### Some Additional Aspects

Acceptance of the binding change mechanism over the past two decades has been fostered by clarification of a number of unusual aspects of the synthase action, some of which are mentioned here. The number and properties of nucleotide binding sites needed clarification. With the use of the 2-azido-

ATP, introduced for studies with  $\text{F}_1$ -ATPases by Abbot et al.,<sup>[109]</sup> we established where catalytic and noncatalytic sites resided with the  $\text{F}_1$ -ATPase from different sources.<sup>[59, 110–112]</sup> The characteristics of the inhibition of the  $\text{F}_1$ -ATPase by  $\text{Mg}^{2+}$  and tightly bound ADP—which had harassed our, and many other, earlier studies—were established.<sup>[113, 114]</sup> A role for the noncatalytic nucleotides in enabling the inhibition to be overcome was uncovered.<sup>[115, 116]</sup>

A direct estimation of how many catalytic sites were filled during photophosphorylation was accomplished.<sup>[117]</sup> The results gave evidence that near maximal rates of ATP synthesis were attained when a second, and not a second and a third, site were loaded with substrates. The consideration of these results, other earlier data, and recent experiments on site filling in  $\text{MF}_1$  have led to refinements in how I consider the binding change mechanism to operate. Salient points from earlier data are that the rate of ATP formation during uni-site catalysis is much slower than the rate of ATP formation when rapid photophosphorylation is occurring, and that during photophosphorylation about one tightly bound ATP per synthase is present. In previous depictions of the mechanism (Figure 9), after a binding change a site is depicted as having a

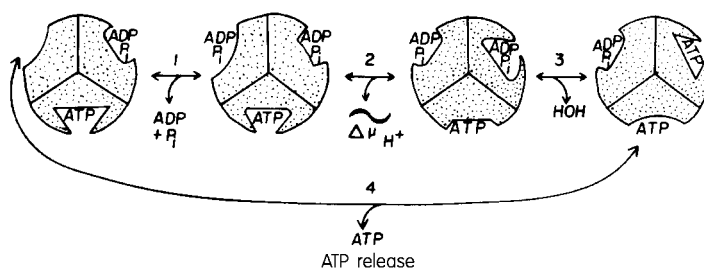


Figure 9. A typical tri-site model for cooperativity including tightly bound ADP and  $\text{P}_i$  as intermediates.

tightly bound ADP and  $\text{P}_i$  that is being reversibly converted into tightly bound ATP while waiting for the next binding change. We now propose that during active net ATP synthesis the interconversion of sites is as depicted in Figure 10. As a site to which ADP and  $\text{P}_i$  have added is converted into a tight site, the capacity for the rapid formation of the terminal covalent bond in ATP is also acquired, such that essentially all the bound ADP and  $\text{P}_i$  are converted into bound ATP. A site with tightly bound ADP and  $\text{P}_i$ , as in Figure 9, is not an intermediate. The next rapid binding change brings about the release of the ATP to the medium.

All ATP made by oxidative phosphorylation<sup>[118]</sup> or photophosphorylation<sup>[84, 119]</sup> contains about 0.4–1.1 water oxygen atoms. This means that some rapid reversal of ATP formation has occurred. Indeed, during net oxidative phosphorylation by mitochondria, rapid reversal of the overall process is demonstrated by  $^{32}\text{P}$  measurements.<sup>[9, 118]</sup> Thus, it seems likely that the rapid incorporation of some water oxygen atoms results from the reversal of a binding change step of Figure 10. The much slower uni-site catalysis that can occur in the absence of medium nucleotides may occur with the forms indicated in the lower portion of Figure 10. With time, the  $\text{P}_i$  drops off, and the remaining synthase retains a tightly bound ADP. This is the

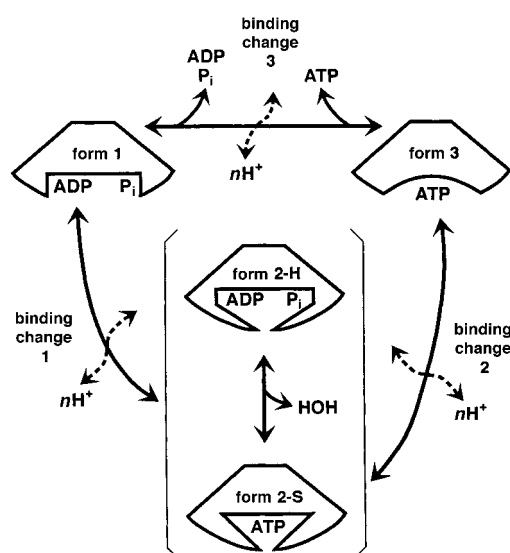


Figure 10. A proposal of how a catalytic site on the ATP synthase is modified by successive binding changes. When ADP +  $P_i$  add to create form 1, and adequate proton-motive force is present, both rapid formation and tight binding of ATP arise during binding change 1. Most of the site assumes the conformation of form 2-S, and the ATP becomes loosely bound in binding change 2. When ATP adds to form 3, and no proton-motive force is present, both rapid formation and tight binding of ADP +  $P_i$  arise during binding change 2. Most of the site assume the conformation of form 2-H, and the ADP +  $P_i$  become loosely bound during binding change 1. Both site occupancy on forms 1 and 3 and proton-motive force modulate the quasiequilibrium of form 2.

ADP that in presence of  $Mg^{2+}$  results in a strong inhibition of ATPase activity. However, when proton-motive force is applied, such tightly bound ADP is released to the medium without delay in the first binding change.<sup>[120]</sup>

Other recent experiments pertinent to site occupancy during ATP hydrolysis by  $MF_1$  were based on competition between ATP and trinitrophenyl-ATP (TNP-ATP). They revealed that TNP-ATP could bind strongly to a third catalytic site for ATP which had a dissociation constant  $K_d$  in the millimolar concentration range. The near maximal ATPase rate was attained at considerably less than 1 mM ATP.<sup>[121]</sup> This result, and further characterization of the transition from uni-site to multi-site catalysis and initial velocity measurements, are best explained by the filling of only two catalytic sites being necessary for near maximal rates of ATP hydrolysis.<sup>[40]</sup> Interestingly, ADP had a considerably higher affinity than ATP for the third empty site of the  $MF_1$ . Our present hypothesis about catalytic site occupancy during rotational catalysis is depicted in Figure 11. During rapid synthesis one site has a bound ATP, and a site to its left (as viewed from above, the  $F_1$  portion of the synthase) can preferentially bind ADP and  $P_i$ . When adequate proton-motive force is present, rapid ATP synthesis ensues. The filling of a third site with ADP and  $P_i$  at higher substrate concentrations results in little rate acceleration. During net ATP hydrolysis, when proton-motive force is weak or absent, the preferential binding of ATP to a site to the right of the tight ATP site can result in a near maximal hydrolysis rate. Filling of the third site at millimolar concentrations of ATP gives little rate acceleration. Nature appears to have designed a way

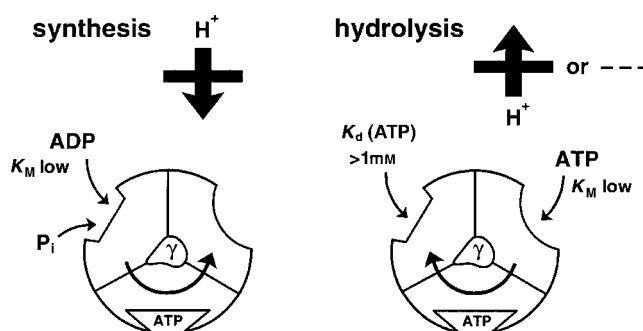


Figure 11. A proposal that near maximum rates of hydrolysis by  $F_1$ -ATPase or synthesis by ATP synthase occurs with the filling of only two sites.

that ATP synthesis occurs with ADP addition to a site that has low affinity for ATP, helping to obviate ATP inhibition of its own synthesis.

The recognition of the principal features of the ATP synthase catalysis creates many opportunities for gaining a better understanding of this remarkable enzyme. I will be an interested spectator in these developments. I believe that societies will, and should continue to, devote some of their resources to basic scientific research, even if the only return is the satisfaction that comes from the knowledge of how living processes occur. An additional justification is that such knowledge underlies past and future gains for attaining a healthy life. As summarized by Ernster,<sup>[122]</sup> the oxygen we use to make ATP is also a toxic substance, resulting in production of harmful free radicals. The mitochondrion is particularly susceptible to such damage, and knowledge of the enzymes involved in energy capture and use may give insight into, and help find how to prevent, unwanted damage.

*A final acknowledgment: I am exceptionally fortunate to have been a biochemist over the past decades when so much has been accomplished in my field. Participation in a series of researches that has revealed an unusual rotational catalysis by a vital enzyme has been warmly gratifying. I am indebted to the society that has made this possible, to my wife, Lyda, for her devotion and guidance given freely to help me and our children find our way, and to the universities and government agencies that provided the environment and the financial support for my researches.*

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