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A research journey with ATP synthase

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These reflections present a perspective of how I and my graduate students and postdoctoral fellows, over a span of many years, arrived at the concept that ATP is made by an unusual rotational catalysis of the ATP synthase. A recent sketch of the structure of this remarkable enzyme is given in Fig. 1. Such a depiction is the culmination of the efforts of many investigators[1]. The two portions of the enzyme are the membrane-imbedded F_0 and the attached F_1 that has three catalytic sites, principally on the large β subunits. ATP is formed when protons pass through the $F_{0,}$ driving the rotation of the ring-shaped cluster of *c* subunits and the attached ε and γ subunits. Other subunits attached to outer portions of the F_0 and F_1 served as a stator. The internal rotary movement of the γ subunit is coupled to sequential changes in the conformation of the catalytic sites. During ATP synthesis these conformational change promote the binding of ADP and P_i, the formation of tightly bound ATP and the release of ATP.

[1] Except for a few instances, mention of important advances in information about the ATP synthase and in related areas of biochemistry are included without specific references. The objective of this contribution is not to provide a review of the field and to recognize priorities for contributions, but to note how various advances impacted on studies by my group.

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Revealing the mechanism of the ATP synthase became a major research goal in the latter part of my long career. This paper recalls how my career developed as related to the remarkable progress in biochemical knowledge. It presents the background and results of fruitful, as well as mistaken approaches that were explored.

The early years--Born and educated through college in Utah, at the age of 21 I entered graduate school in the Department of Biochemistry at the University of Wisconsin in the fall of 1939. The biochemical research and teaching there were excellent. Not until years later did I appreciate all that is necessary to create such a fine scientific environment.

I had had no previous courses or research experience in biochemistry and was uncertain about my career choice. By the end of my first year of graduate study the fascination of biochemical understanding, and the addictive effect of experimental attempts to uncover new knowledge, had firmly launched me towards a career in biochemical research. The Department at Wisconsin was at the forefront of research in nutrition and metabolism. Recent achievements included the identification of nicotinic acid as a vitamin, the irradiation of milk to produce vitamin D, the discovery of a vitamin K antagonist (dicoumarin), and the discovery of lipoic acid as a growth factor for bacteria. At that time incoming graduate students were assigned to a mentor Professor. Both Henry Lardy, from South Dakota, and I joined the group of Professor Paul Phillips whose major interest was in dairy cattle nutrition. Evidence had been obtained that vitamin C might help prevent reproductive difficulties in cattle, and one of my assignments was to find if vitamin C might ameliorate the reproductive failure that occurred in rats with vitamin E deficiency. No benefits of vitamin C were noted, but the rats also showed the striking muscular dystrophy characteristic of vitamin E deficiency. Exploration, together with Henry Lardy, of the possible cause of this dystrophy led me into study of ATP-related enzymes.

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Henry is still active in an exceptionally distinguished career that has included major contributions to the understanding of oxidative phosphorylation.

The milieu at Wisconsin--meetings where students and staff discussed recent research papers, frequent research seminars, and class instructions--introduced me to the wonder of enzyme catalysis. A prominent event was a "Symposium on Respiratory Enzymes" at which the outstanding biochemists Meyerhof, Cori, Ochoa, Lipmann, Kalckar and others contributed (1). From this and other sources I learned that ATP and phosphorylations were central to the capture and use of energy derived from foodstuffs.

Perhaps defective formation of ATP might underlie the muscle dystrophy in my vitamin E deficient rats. One approach was to measure the ability of muscle extracts to make phosphocreatine during glycolysis. No definitive defect from vitamin E deficiency was found, but in the course of these experiments, I noted a stimulation of the transfer of phosphate from 3-phosphoglycerate to creatine by K^+ ions. This was traced to a requirement of K^+ for transfer of the phosphoryl group from 2-phosphoenolpyruvate to ADP. The discovery of the K^+ activation of pyruvate kinase was the first demonstration of a K^+ requirement for an enzyme reaction. The two *J. Biol. Chem.* publications reporting this were the best of several from my graduate studies (2, 3). An understanding of the K^+ activation was attained at the University of Wisconsin some 50 years later by from the X-ray structure of pyruvate kinase (4). The K^+, coordinated to four protein ligands, to an oxygen of the γ-phosphate of ATP and to a water oxygen, apparently provides a requisite positive charge.

Oxidative phosphorylation was discovered only seven years before I started graduate studies. As noted in an interesting introductory chapter by Englehardt in Annual Reviews of Biochemistry (5), ATP was discovered by Lohmann in 1927 and oxidative phosphorylation was

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first demonstrated by Engelhardt and Liubimova in 1932. These salient contributions at that time seemed far from recent to me. And discoveries such as that of cell-free fermentation by Buchner made about 40 years earlier were relegated to the distant past--science after the escape from the Middle Ages. Now, from my present perspective, research of 30 years ago still seems fairly recent and vibrant. Time seems to go much faster, but it is I who has changed while a unit of time has retained its constant value.

An introduction to the properties of proteins. Some 20 amino acids linked in peptide bonds can yield proteins with a truly remarkable diversity of structural properties and ability for specific combination and catalysis. The versatility of proteins is arguably the most important property of matter that has made life possible. Little was known about protein structure when I was a graduate student. As stated in a 1943 textbook of biochemistry (6) "Since the protein molecule is often built up of hundreds, even thousands, of these amino acids, the problem of protein structure is one of almost insuperable difficulty." Since that time, to be an observer as the wondrous properties of proteins have been revealed is one of the finest rewards provided by my profession.

My appreciation of protein structure and function arose in 1943 I joined a small group at Stanford University that was supervised by Murray Luck, founder of the *Annual Review of Biochemistry*. Our nation was at war and Luck's group was asked if they could find how concentrated solutions of human serum albumin, used primarily for the treatment of shock in wounded soldiers, could be heated to inactivate pathogens without denaturing the albumin. The group found that low concentrations of long chain fatty acids or other non-polar anions such as acetyl tryptophan would satisfactorily stabilize the albumin. Albumin preparations used militarily and commercially are still stabilized with small concentrations of acetyl tryptophan.

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As part of these studies, I noted that when albumin solutions were exposed to urea or guanidine hydrochloride, the large viscosity increase accompanying denaturation could be reversed by fatty acid addition--a specific combination was markedly influencing the folding of the protein (7). My interest in protein structure was firmly initiated.

Early studies at Minnesota--In my 17 years at the University of Minnesota I studied of a wide variety of biochemical problems, including such items as the chemistry of α-tocopherol oxidation products, possible formation of antibodies by a refolding of denatured γ-globulins, sulfhydryl groups and enzyme catalysis, and the free energy of hydrolysis of ATP. Mostly my interests have concerned enzymes and over the years names of some 25 different enzymes have appeared in titles of my publications.

An unsettled problem from my graduate studies at Wisconsin was clarified by the demonstration that mitochondria from the muscles of vitamin E deficient rats performed oxidative phosphorylation as well those from as normal muscle (8). Studies in my laboratory (9), and those of my graduate colleague Henry Lardy (10), independently reported that during oxidative phosphorylation oxygen uptake was decreased by the lack of phosphate acceptors. Such respiratory control was the basis for the later development, by Britton Chance and others, of the extensive use of an oxygen electrode to replace the cumbersome Warburg manometric method for measuring rates of oxidative phosphorylation.

More importantly, stimulated by the pioneering studies of Mildred Cohn (11), we initiated studies using the heavy oxygen isotope, ^{18}O, for probing phosphorylation reactions. As noted in later sections, insights into ATP synthase catalysis by my group were crucially dependent upon the use of ^{18}O. The ^{18}O isotope and mass spectrometer facilities were made available by physics professor Alfred Nier--a benefit of a research university and a cooperative

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faculty. Over the years we and others have modified and improved techniques for ^{18}O measurements. Yet studies with ^{18}O remain more laborious than many approaches, and have not been widely used. The lack of familiarity with the ^{18}O measurements probably added to the reluctance of the field to accept our concepts, as they were later developed in the 1970s.

In our early studies with ^{18}O we demonstrated that in the glyceraldehyde 3-phosphate dehydrogenase reaction an oxygen from inorganic phosphate appears in the carboxyl group of the 3-phosphoglycerate formed (12). This was explained by a phosphorolysis of an acyl enzyme intermediate demonstrated by studies of Racker's group (13) and mine (14). The phosphorylation accompanying this oxidative step of glycolysis was a prominent basis for the widely adopted paradigm that a phosphorylated intermediate was likely formed during the oxidative phosphorylation of the respiratory chain. .

In related experiments my group showed that the enzymic catalyses for formation of phosphocreatine from 3-phosphoglycerate occurred with the retention of all 3 oxygens of the phosphoryl group. Thus such phosphoryl transfers do not involve any steps giving exchange of P_i oxygens with water (12). Also we found that syntheses coupled to ATP cleavage, such as formation of glutamine from glutamate and ammonia, occur with transfer of an oxygen from the substrate to P_i (15). No water oxygen is incorporated into the P_i.

Our initial studies of oxidative phosphorylation with ^{18}O revealed an important characteristic of the oxidative phosphorylation process. We incubated mitochondria with P_i labeled with both ^{18}O and ^{32}P and unlabeled ATP in the presence or absence of substrates or of oxidation inhibitors. We were surprised to discover that, in addition to the strikingly rapid exchange of P_i oxygens with water, a quite rapid $P_i \leftrightarrow$ ATP exchange was occurring (16). The reactions of oxidative phosphorylation appeared to be dynamically reversible. The reversibility

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continued even when electron carriers were inhibited or nearly fully reduced. This gave evidence for formation of some type of energized compound or state, independent of oxidation-reduction reactions that allowed the ready reversal of the reaction sequence. We thought this likely was some type of chemical intermediate--the idea of an electrochemical gradient across a coupling membrane was far from our thoughts.

Possibilities arose of pursuing interesting aspects of enzyme catalysis not related to ATP formation. For example, in 1955 while on a Guggenheim fellowship for study in Sweden with Nobelist Hugo Theorell, I noted a previously overlooked shift in the fluorescence of NADH upon binding by a dehydrogenase (17). This gave a new basis for measuring combinations of NADH with enzymes. But the problem was not as interesting as the studies of oxidative phosphorylation that I was also pursuing in the laboratories of Olov Lindberg and Lars Ernster at the Wenner Gren Institute. In an experiment conducted in part in Sweden ^{18}O was used to demonstrate that the terminal bridge oxygen in ATP formed by oxidative phosphorylation came from ADP, not P_i. This and some other research were reported at an International Union of Biochemistry symposium in Japan (18). At that time I was a bit pessimistic about gaining a satisfactory insight into how oxidative phosphorylation occurs. In my contribution I stated "Our basic knowledge of the chemistry involved does not appear adequate for the task, and the problem is likely to be with us for some time. Researchers who undertake indirect approaches to the problem should do so with recognition that their experiments cannot give final answers, and may not even point the way to final solutions". In retrospect, the pessimism seems appropriate.

During the next several years we undertook experiments looking for intermediates in oxidative phosphorylation, particularly by making use of ^{32}P as a tracer. We learned that

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radioactively-induced reactions of phosphorus compounds in highly labeled $^{32}P_i$ could give rise to radioactive impurities that stick to mitochondrial components but that did not behave like intermediates. Most of my publications during this period were from some worthwhile investigations with other enzymes--one needs to keep research funding available. One of my favorite sayings is that most of what you accomplish in research is the coal that you mine while looking for diamonds.

Some of our studies concerned patterns of isotope exchanges at equilibrium with glutamine synthetase using $^{18}0$, ^{32}P and ^{14}C. It soon became apparent that covalent bond cleaving and formation may not be rate limiting in enzyme-catalyzed exchanges. Somewhat surprisingly, adequate rate equations governing exchange reactions of enzymes were mostly lacking. I spent a fair effort in a pioneering development of appropriate relationships (19). To some reviewers these relationships were unexpected and there is an interesting story not told here about what I needed to do to get the publication accepted. Various applications were made by my group. For example, data with glutamine synthetase revealed that the binding of ATP and glutamate was random, and such subtleties as a spatial selectivity of transfer of only one oxygen of the glutamate γ-carboxyl group to phosphate when glutamate and glutamine are readily interconverted at the catalytic site (20). The understanding obtained was useful for later measurements of isotope exchanges that helped in the discovery of compulsory sequential participation of catalytic sites of ATP synthase.

An observation of later interest was that myosin and actomyosin can catalyze an exchange of phosphate oxygens with water oxygens. This can occur with P_i in the medium without added ATP (21) or with the P_i formed from ATP before it is released to the medium

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(22). We did not pursue such observations until about a decade later when we belatedly recognized their potential relationship to the mechanism of oxidative phosphorylation.

The phosphohistidine story--In 1961 it seemed that our searches with ^{32}P had hit pay dirt. We reported that under oxidative phosphorylation conditions a brief exposure to $^{32}P_i$ and solubilization of the mitochondria with concentrated urea and detergent gave a non-dialyzable ^{32}P-labeled substance. The rate of its formation from P_i or ATP, the disappearance in a cold P_i chase, and the effect of inhibitors and reaction conditions were consistent with its being an intermediate in oxidative phosphorylation. Our interest was heightened when my capable associates identified the substance as a phosphorylated histidine residue in a protein (23). This was the first recognition of a phosphohistidine in biochemical systems. The ability to form the bound phosphohistidine in soluble preparations from mitochondria encouraged the possibility that we could characterize details of the formation process. As the research developed, I became over-enthusiastic in regarding the phosphorylated protein as an intermediate of oxidative phosphorylation (24). In retrospect, I should have been more cautious. It was at this stage that my laboratory group moved to UCLA where we joined the Biochemistry Division of the Chemistry Department.

Our continued studies showed that dialyzable substances from mitochondria could modulate the bound phosphohistidine formation, and this led to the recognition that CoA and succinate were particularly effective. We had overlooked the substrate level phosphorylation accompanying the citric acid cycle. We became aware that a Ph.D. thesis at Illinois by Upper (25) had reported evidence of formation of a phosphoenzyme with the *E. coli* succinyl CoA synthetase, and that such formation had been suggested earlier from catalysis of an ADP$\leftarrow\rightarrow$ATP exchange by the synthetase (26). Our further studies showed that the

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phosphorylated protein we had detected was indeed an intermediate in the formation of nucleoside triphosphate (ATP or GTP depending on enzyme source) from P_i by succinyl CoA synthetase (27,28). Our bound phosphohistidine was clearly not an intermediate in oxidative phosphorylation. In Olympic analogy, we were reaching for a gold but were fortunate to have obtained a bronze.

Another decade with little essential progress--At this stage I felt that perhaps I could do more for science by accepting an opportunity to become the initial Director of a Molecular Biology Institute at UCLA. Fortunately this did not prevent reasonable continuation of laboratory studies, although I was not encouraged about the progress we and others were making toward elucidation of the major problem of how cells captured energy from oxidations to make ATP. At that time I of course did not know that a decade later we would be fortunate in developing a new concept for oxidative and photosynthetic phosphorylation.

Meanwhile my group pursued some worthwhile studies with other enzymes and continued a few probes of ATP synthesis that were useful but did not yield or point to breakthroughs. In a more sensitive search for the labeling of unidentified components with ^{32}P, a small amount of rapidly labeled lipid fraction was detected (28). But this labeling was found to continually increase with time, not an expected characteristic of an intermediate. The independence of oxygen exchanges from oxidation-reduction reactions was more firmly established (29). A claim that a localized AMP might be the initial phosphoryl acceptor was refuted and ADP as the initial phosphoryl acceptor more firmly established (30). A sensitive search for possible substances that might transitorily bind an oxygen from P_i on its way to water was negative (31). An exploration of the source of phosphate oxygens in *E. coli and B. subtilis* showed that only a few oxygens that entered with the P_i remained (32). Most of them came

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from water and substrates, undoubtedly by exchange patterns we had been investigating. Other studies gave a welcome observation that laid the base for the later extensive use of chloroplasts by my group: Under appropriate conditions chloroplasts catalyzed rapid $P_i \leftrightarrow$ATP, $P_i \leftrightarrow$HOH, and ATP$\leftrightarrow$HOH exchanges. Like oxidative phosphorylation, photophosphorylation was dynamically reversible and its mechanism could be probed by oxygen exchange measurements (33). From later developments the lack of the exchanges noted previously was likely due to the unusual and particularly strong Mg-ADP inhibition of chloroplast ATPase activity that can occur in the dark but is readily reversed by protonmotive force.

In an attempt to gain more insight about energy coupling we also conducted some studies on active transport by *E. coli.* We obtained convincing evidence that a common energized state or intermediate could drive transport or ATP synthesis (34), a view that had been independently developed by Harold (35) and others. However, unlike Harold, we were reluctant at that time to regard that the energized state was a proton motive force. We were not alone in this reluctance. The field was active, and frequently reviewed. The 1967 (36), 1969 (37), 1971 (38) and 1974 (39) reviews in the *Annual Review of Biochemistry* on electron transport and phosphorylation gave brief and generally negative assessments of Mitchell's proposal. My hesitation in accepting protonmotive force as driving ATP synthesis was based on the lack of satisfying explanations of how proton migration could drive ATP formation.

The mechanism of the ATP synthase remained unclear. As noted in the reviews mentioned above, there were a plethora of hypothetical compounds and reactions suggested for participation in ATP formation. A possibility consistent with our various experiments was that an energized state, not involving oxidation-reduction reactions, was used to drive a reaction in

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which an oxygen from P_i formed water as ADP was phosphorylated to yield ATP. We and others wondered if in some manner energy captured in conformational changes of proteins was involved. Remarkable advances in the recognizing the versatility of protein structure were occurring. The x-ray structure of hemoglobin and other proteins, and the allosteric properties of enzymes suggested the energy requirements for ATP formation might be accommodated in conformational changes of proteins. But we still had no clear idea about how the conformational changes might function.

A new concept--In 1972, the first part of what I later called the binding change mechanism of ATP synthesis came from further considerations of past data, not new experimental findings. While attending a seminar that I did not understand, some puzzling aspects of oxygen exchange measurements were occupying my mind. Our thoughts had been that the major use of captured energy was to make the covalent structure of ATP. The realization struck me that past data could be explained if the major use of energy was not to form the ATP, but to release a tightly bound ATP from the enzyme. Reversible formation of bound ATP at a catalytic site could explain why the exchange of P_i oxygens was less sensitive to uncouplers than net oxidative phosphorylation. For me it was a rare moment of insight, like suddenly reaching a summit on a mountain climb and seeing a beautiful valley spread below. All enzymes have the capacity for ready reaction reversal at catalytic sites and to bind both products and reactants. The reversal of the hydrolysis of ATP by the ATP synthase is no more remarkable than the reversal of simple hydrolyses by many enzymes, except that with the ATP synthase the product ATP is tightly bound. An additional step or steps must intervene for ATP release. This could logically be an energy-requiring conformational change of the catalytic site.

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Richard Cross had joined our laboratory as a postdoctoral fellow. At UCLA he further documented the uncoupler-insensitive oxygen exchange and other aspects. We submitted a paper, "On a new concept for energy coupling in oxidative phosphorylation based on a molecular explanation of the oxygen exchange reactions," to the *J. Biol. Chem.* for consideration. The publication was declined--at that stage our evidence was not strongly convincing. But the concept remained appealing. I had recently been elected to the National Academy, and the paper was published in *PNAS* as the first paper I sponsored for the journal (40). A follow-up paper gave additional details (41).

The presence of multiple binding sites for ADP and ATP on the isolated F_1-ATPase and the ATP synthase had been recognized by Slater's group and others. During catalytic turnover some of these nucleotides exchanged with medium nucleotides, and Slater and associates had also suggested the possibility that energy-requiring release of bound ATP might occur in oxidative phosphorylation (42).

The validity of the concept of the role of a tightly bound ATP was strengthened by our finding that myosin ATPase would spontaneously form a tightly bound ATP from medium P_i (43). The estimated $-\Delta G^0$ of the binding of ATP from our and other data was 12-13 kcal per mole. A corresponding tight binding was anticipated for the ATP synthase. In related experiments, Bagshaw and Trentham had recently shown that the apparent ΔG for the hydrolysis of the bound ATP to bound ADP and P_i was only about -1.3 kcal per mole (44)-- the equilibrium was not far from unity. In a subsequent cooperative study with these investigators an exchange of phosphate oxygens of bound ATP with water was demonstrated to accompany the ATP hydrolysis by myosin (45). The ability to form a bound ATP from P_i by the reversal of ATP hydrolysis readily accounts for the capacity of myosin to catalyze a $P_i \leftrightarrow$ HOH exchange

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we had observed years earlier (21). Later observations characterized how the combination of myosin with actin promotes the release of the tightly bound ATP, a conformational transition analogous to that proposed for the ATP synthase (46).

At this time I contributed a chapter on "Conformational Coupling in Biological Energy Transductions" in which the possibility that changes with ATP synthase were driven by proton motive force was recognized. But my preferred view was still that the conformational changes were driven by some type of interaction with oxidation-reduction enzymes (47).

Recognition of the role of proton motive force -- Peter Mitchell introduced his concept of energy-linked proton translocation in 1961 (48), and in ensuing years he and others continued to present evidence and win converts. By the early 1970's even holdouts like myself were beginning to see the light. It seems probable that the role of proton motive force would not have been recognized for a long time without Mitchell's contributions. In contrast, for some advances, such as the classical genetic understanding that came from the unveiling of DNA structure, the probing of DNA structure was an active area and the advance was imminent.

If proton translocation were coupled to ATP synthesis, I felt it would be accomplished indirectly by protein-linked conformational changes. In contrast, Mitchell proposed that the translocated protons reached the catalytic site and participated directly in the removal of a water molecule. I found his 1974 proposal in *FEBS Letters* (49) unattractive and called attention to some deficiencies in a *FEBS Letters* contribution (50). Without informing me, the journal allowed Mitchell to present a rebuttal following my paper (51). This seemed inappropriate, and the journal agreed to publish my subsequent paper presenting a model of how, through conformational coupling, proton translocation could drive ATP synthesis (52). The suggestions made still seem applicable.

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Over the years Peter and I had extensive correspondence and shared a mutual respect. Although we were looking at essentially the same mechanism we tended to present different pictures of our views. Too often in science there is rancor between those who disagree. An important lesson that I have learned is that more will be accomplished if one can maintain cordial relations in an exchange of interpretations.

Other developments--By the mid-seventies other investigators had provided much welcomed information about the ATP synthase that was quite relevant to mechanism studies. Hatefi and others in David Green's laboratory had shown that the mitochondrial inner membrane could be fractionated to yield separate complexes of the respiratory chain components and the ATP synthase. They-- and particularly Racker[2] and associates-- had separated and characterized the F_1-ATPase. The knobs visible in electron micrographs of mitochondrial membranes were identified with the F_1-ATPase, connected by a stalk to the membrane portion of the synthase. A similar ATPase had been found in a wide variety of organisms. The ATPase was known to have two or three copies of major α and β subunits, and single copies of other smaller subunits. The unusual subunit stoichiometry, and observations in a number of laboratories that modification of one β subunit per enzyme essentially stopped catalysis, raised intriguing questions about mechanism. The portion of the synthase imbedded in the membrane, F_0, was recognized as being involved in proton transport. The addition of F_1-ATPase to F_0 preparations could restore oxidative phosphorylation or photophosphorylation. Either proton gradients or membrane potential sufficed to drive ATP formation. Beechey had

[2]Racker's contributions were outstanding. One of his former associates, Geoffrey Schatz, has provided a splendid memoir of Racker's career (Memoirs, National Academy of Sciences, 1996) available on the Internet from www.nationalacademies.org)

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shown that a buried carboxyl group on a small hydrophobic subunit of F_0, present in multiple copies, readily reacted with dicyclohexylcarbodiimide (DCCD) and that this blocked oxidative phosphorylation.

Although information about the ATPase was becoming extensive, how proton translocation could be coupled to ATP formation remained poorly understood. We were encouraged some by the concept that energy-linked binding changes were involved. Fortunately, at this time we obtained evidence for an unusual catalytic site cooperativity displayed by the ATP synthase and the isolated F_1 -ATPase. There was a feeling in my research group that some important secrets about the ATP synthase were being revealed. This created an ambience that stimulated research efforts. Such occasions are an all too infrequent reward of basic research. They help soften the disappointments of the many experiments that yield little or no helpful information.

Alternating site participation--Many enzymes have more that one catalytic site, suggesting the possibility of a catalytic cooperativity between sites such that catalytic events at one site are promoted by substrate binding at another site. With most multi-catalytic site enzymes, limited or no cooperativity has been observed. In contrast, we found that the ATP synthase showed a nearly complete dependency of continued catalytic steps at one site on the presence of substrate(s) at a second site. This was the first enzyme for which such a striking behavior had been discovered, adding to our interest in the phenomenon.

Our discovery arose from researches by Jan Rosing, a postdoctoral fellow with exceptional experimental skills from Slater's group, and Celik Kayalar, a gifted graduate student. They were symbiotically productive. We devised methods for estimating oxygen exchanges by submitochondrial particles that accompany: a) the binding, exchange and return to

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the medium of P_i; b) the binding, exchange and return to the medium of ATP; c) the binding of P_i, intermediate exchange, and the release of ATP formed and; d) the binding of ATP, intermediate exchange, and the release of the P_i formed. These measurements with ^{18}O were accompanied by measurement of the $P_i \leftrightarrow$ ATP exchange with $^{32}P_i$. The exchange patterns gave evidence that besides promoting ATP release, energy input also increased competent P_i binding. More importantly, the measurements yielded exchange patterns that Kayalar proposed could be explained if the binding of a substrate at one site was necessary for the release of a product from another site.

Whether two or three catalytic sites per enzyme were present was not known at that time. We proposed alternating behavior of two sites, although it was recognized that the results would also be compatible with sequential participation of three sites (53, 54). During net ATP formation or hydrolysis, sites were considered to proceed sequentially through the steps of binding, interconversion of reactants, and release, so that at any one time each catalytic site was at a different stage of the catalysis. The concept seemed attractive, but more evaluation was needed.

David Hackney, a talented postdoctoral fellow from Dan Koshland's laboratory, had joined our group and initiated his excellent experimental and theoretical studies of the oxygen exchanges. We were proposing that that P_i and ADP can bind and reversibly form bound ATP, but that ATP cannot be released until P_i and ADP bind to an additional site. If dynamic reversal of ATP formation at a catalytic site continued in absence of net reaction, then reductions in the concentration of P_i or ADP should increase the amount of intermediate oxygen exchange per ATP made. We were encouraged by a report from a former postdoctoral fellow of our group, Robert Mitchell, that he and his colleagues observed increased intermediate oxygen exchange

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accompanying ATP hydrolysis by submitochondrial particles when ATP concentration was lowered (55). Support for the possibility also came from the observation of Wimmer and Rose that when ATP was exposed to chloroplasts in the light, the ATP showed nearly complete exchange of its oxygens before being released (56). This is as expected if low ADP concentration in the medium prevented the release of the ATP and many reversals occurred before its release

Hackney observed that during net oxidative phosphorylation as either ADP or P_i concentration was decreased, there was a marked increase in water oxygen incorporation into each ATP formed (57). Additional observations made it unlikely that some type of enzyme heterogeneity or hysteresis could explain the exchange patterns. It deserves emphasis that these experiments were performed with submitochondrial particles during net ATP synthesis, giving them relevance to the actual oxidative phosphorylation process.

An interesting possibility was that catalytic site cooperativity might also be found with the isolated F_1-ATPase. Several years earlier, Ef Racker brought some of his purified F_1-ATPase to our laboratory to find if his enzyme would catalyze an intermediate $P_i \leftrightarrow$HOH exchange. We tested this at mM concentrations of ATP and found that the P_i formed contained only close to the one water oxygen necessary for the hydrolysis. But now, with our evidence for cooperativity, it was evident that if reversible ATP formation could occur in the absence of protonmotive force, and if participation of alternating sites was necessary, then the extent of intermediate $P_i \leftrightarrow$HOH exchange with each P_i released should increase as ATP concentrations are lowered. This was found to be so (58) and as ATP concentrations were lowered the number of reversals before the P_i was released approached a limit of over three hundred (59). Tightly

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bound ATP at a single site was undergoing reversible hydrolysis waiting for ATP to bind to another site and promote ADP and P_i release.

The reaction rates and equilibrium characterizing the slow catalysis at a single site were determined in a widely recognized study by Cross together with Grubmeyer and Penefsky (60). They termed this "uni-site catalysis" and their results added considerably to the acceptance by others of alternating site participation. In these studies the K_d for ATP binding to one site of the F_1-ATPase was shown to be near 10^{-12} M (61), indicative of the need for energy input for ATP release and akin to the affinity of ATP for myosin.

The capacity to make bound ATP from medium P_i and ADP/ATP ratio near unity on the enzyme was nicely demonstrated with the chloroplast F_1-ATPase by Feldman and Sigman (62), a contributions that warrants wider recognition. In a slow reaction, needing relatively high P_i concentration, a tightly bound ADP became phosphorylated. Other findings made it probable that this was at the same site as the ADP that was rapidly released in the acid-base transition of thylakoid membranes, and thus that this site was likely where covalent bond formation occurred during photophosphorylation.

In addition results of various investigators established that chemical modification of only one catalytic site effectively stopped catalysis and that each of the three catalytic sites had a different capacity for derivatization. Such behavior agreed with the concept that during catalysis all three catalytic sites were in different conformations and proceeded sequentially through the conformations.

The basis of the ^{18}O exchange--Our studies with ^{18}O are interpreted on the basis that the exchange results from a reversal of the formation of bound ATP from bound ADP and P_i. As covered in the Appendix of a review there is strong support for this interpretation (63). This

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includes demonstrations that the P_i oxygen exchanges catalyzed by the sarcoplasmic reticulum ATPase (64, 65) and pyrophosphatase (66, 67), as well as that of myosin ATPase as mentioned above, result from reversible formation of a phosphorylated enzyme, or enzyme-bound pyrophosphate or ATP respectively.

Probes of initial reaction rates--Other evaluations of our postulates were needed. Rapid mixing and quenching techniques yielded essential information. One objective was to find if a tightly bound ADP on the chloroplast ATP synthase might react with medium P_i to form ATP in the first turnover of the enzyme. We used rapid mixing in an acid-base transition of chloroplast thylakoid membranes, as introduced by Jagendorf and colleagues, to start ATP synthesis in a few milliseconds. We found that the tightly bound ADP was not directly phosphorylated but was rapidly released to the medium and that the first ATP formed came from medium P_i and ADP (68). As substantiated in later experiments, the tightly bound ADP in such chloroplast membranes prior to release is tightly bound at a catalytic site without P_i.

The demonstration that exposure to proton motive force caused the release of a tightly bound ADP from a catalytic site without phosphorylation had important implications for later developments. The tightly bound ADP in presence of Mg^{++} causes potent inhibition of ATPase activity of the ATP synthase and F_1-ATPase. Thus such inhibition in the intact synthase is readily and quickly overcome by proton motive force. When a step of rotational catalysis occurs, the binding site with the tight ADP is opened as if it had an ATP present, while another site is binding ADP and P_i. The properties of the tightly bound ADP also aided interpretation of Walker's 1994 X-ray structure of the major portion of the F_1-ATPase, in which one β subunit has a tightly bound ADP and Mg^{++} present (69).

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Our rapid mixing experiments verified that medium ADP was rapidly bound and phosphorylated as if no phosphorylated intermediates were involved. They provided evidence that during photophosphorylation, in addition to a transitorily bound ATP, about one bound P_i and one bound ADP per enzyme are present and committed to ATP synthesis (70). Such results harmonize with the alternating site model with more than one catalytic site having bound reactants, as required if a tight site is already filled and substrates must initially bind at another site.

Research conferences and the binding change mechanism--Research conferences are important to scientific progress because concepts can be freely discussed and the publication of proceedings often allows inclusion of material not suited for the usual journals. For example, in my contribution to a 1979 conference honoring Ef Racker, I summarized our concepts and considered how to name our suggested mechanism. A name seemed desirable for ease of discussion and to identify the concept in the field. My contribution entitled "The Binding Change Mechanism for ATP Synthesis" was the first publication in which this nomenclature was used (71).

The binding change mechanism at that time included the following concepts: The first compound made from P_i is ATP itself (no intermediates); a principal requirement of energy is not for the formation but for the release of ATP; energy input also promotes the competent binding of P_i; and the sequential participation of catalytic sites so that binding of substrate(s) at one site is necessary for release of product(s) from another site. Two years later, another and even more novel concept of the binding change mechanism was developed, namely the proposal of rotational catalysis. The suggestion that rotation of internal subunit(s) drives the binding

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changes for catalysis was first published in reports from 1981 and 1983 conferences at the University of Wisconsin (72,73). How this concept came about is outlined next.

The proposal of rotational catalysis—In the 1970's highly enriched ^{18}O was available, mass spectrometry techniques for ^{18}O analysis had improved, and Mildred Cohn had introduced an NMR method for measuring ^{18}O in phosphate compounds. David Hackney developed theoretical aspects of ^{18}O measurements relevant to observed distributions of ^{18}O isotopomers of P_i with 0 to 4 ^{18}O atoms per P_i or 0 to 3 ^{18}O atoms per ATP molecule. Measurement of the presence of ^{18}O in ATP formed by photophosphorylation showed a pronounced increase in ^{18}O loss at lower ADP and P_i concentrations (74). More importantly, the distribution of ^{18}O isotopomers corresponded to that statistically expected if all the ATP were produced by the same catalytic pathway. This eliminated the possibility that substrate modulation arose from heterogeneity of the enzyme used and made modulation by control sites unlikely. We now regarded the catalytic site cooperativity of ATP synthase to be reasonably well established.

Companion studies with the F_1-ATPase showed that when highly ^{18}O-labeled ATP was hydrolyzed by F_1-ATPase at different ATP concentrations, the distribution of ^{18}O isotopomers was as expected for a single catalytic pathway (58). At appropriate labeling and substrate concentration ranges, the distribution patterns provided a sensitive test for more than one catalytic pathway. A statistically homogeneous distribution meant that every substrate that reacted faced the same possibilities of proceeding through the same reaction steps. This means that rate constants governing the binding and release of substrate(s), their reversible interconversion, and the release of product were the same. To me, the power of this type of ^{18}O use is unusual and indeed a bit awesome.

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By now essential contributions of other investigators, including Kagawa and associates in their fine studies with the F_1-ATPase from thermophilic bacteria, had established the presence of three catalytic sites with circular distribution of alternating large α and β subunits around a central core. Catalytic sites were regarded as largely on the β subunits, with the core representing the γ and possibly other small subunits. Observations in McCarty's laboratory demonstrated that modifications of -SH groups on the γ subunit markedly affected catalytic capacity of the chloroplast enzyme (75). The capacity of the F_O component for DCCD-sensitive proton transport had been established. These and other findings strengthened our view that conformational changes in the F_O were in some manner transmitted through the stalk to the catalytic sites on the β subunits to drive the binding changes for ATP synthesis.

Catalytic sites on multi-subunit enzymes can be very sensitive to conformational changes in adjacent subunits. Changes in the γ subunit markedly modulated catalysis. How could all three β subunits have identical interactions with the γ subunit? Occurrence of tri-partite symmetry of the γ subunit seemed unlikely. The evidence that all three sites conducted catalysis identically was compelling to me. The more I puzzled about these aspects, the more it seemed that there was only one satisfactory answer. This is that the internal asymmetric core, composed of γ and any other tightly associated minor subunits, would need to move rotationally with respect to the outer ring of catalytic subunits. Such movement would allow identical interactions with β subunits as the rotation drove the sequential conformational changes of catalytic sites. When I first presented this concept to my research group, their acceptance was initially quite reserved (they knew all too well that I could be wrong). With further consideration, they became interested and supportive. Much remained to be explored, and some experimental approaches are summarized in the next few sections.

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Modulation of oxygen exchanges by ATP concentration--The modulation by ATP concentration of the ^{18}O exchange by the mitochondrial F_1-ATPase was more carefully documented (59). The chloroplast F_1-ATPase showed a similar behavior and the distribution of the ^{18}O isotopomers in the P_i formed corresponded to a single catalytic pathway (76). Various wild type and mutant *E. coli* F_1-ATPase likewise showed increased exchange of the P_i formed with lower ATP concentrations. However, the distribution of ^{18}O isotopomers with the *E. coli* enzyme revealed more than one reaction pathway, apparently arising in part from the degree of dissociation of the inhibitory ε subunit (77). Question had been raised whether the F_1-ATPase from a thermophile showed catalytic cooperativity because uni-site catalysis was not readily apparent. A cooperative experiment disclosed the expected modulation of the oxygen exchange, but at a higher range of ATP concentration (78). The ATPase activity of yeast and neurospora mitochondria showed distinct ATP modulation of the oxygen exchange (79). These various results meant that the increase in the extent of oxygen exchange with each P_i formed (which occurs with a decrease in the ATP concentration) is likely a general property of all F_1-ATPases, and supports the probability that all ATP synthases share a common mechanism.

The ATPase of vacuolar membranes has been noted to have a composition resembling that of the ATP synthase. We felt that it should show similar oxygen exchange properties, and measurements demonstrated that this was so (79).

Some other assessments--We devised methods to measure bound reactants during steady-state ATP synthesis. A hexokinase-accessibility method gave a measure of bound ATP, and a rapid dilution of medium $^{32}P_i$ gave a measure of bound P_i committed to form ATP. Measurements during photophosphorylation showed that even at lower substrate concentrations the total of catalytic site bound ATP and committed P_i was greater than one per enzyme, as

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anticipated if the proposed catalytic site cooperativity was occurring. During photophosphorylation, $^{32}P_i$ rapidly labeled catalytic ATP, and then the medium ^{32}P-ATP formed was incorporated much more slowly into the noncatalytic sites (80). When illumination ceased, the catalytic site ATP continued to show ^{18}O exchange (81), meaning that reversible formation of bound ADP was still occurring. Within minutes the P_i dropped off, leaving a tightly bound ADP at the catalytic site (82). Such results helped explain labeling patterns we and others had observed, and supported our concepts of tightly bound ATP as an intermediate and of catalytic site cooperativity.

The insidious MgADP inhibition--Occasionally in biochemical research one encounters a property of a system that seems designed to confuse and thwart the researcher. Such is the case with the inhibition by Mg^{++}, which is dependent on the presence of an ADP bound without P_i at a catalytic site. Clarification of this unusual role of a tight ADP was necessary for an adequate understanding of the proposed binding change mechanism. The F_1-ATPase as conventionally isolated usually has a considerable portion with tight ADP present. In 1975, Moyle and Mitchell reported that mitochondrial F_1-ATPase was slowly inactivated by Mg^{++} (83). Hackney noted the inhibition was slowly reversible by ATP addition (84). Observations in Vinogradov's laboratory showed that the inhibition depended on the presence of tightly bound ADP, and that the Mg-ADP-inhibited form was stabilized by azide (85). Subsequent studies in our and other laboratories revealed characteristics of the inhibition. F_1-ATPases with tightly bound ADP when exposed to Mg^{++} shows little or no initial activity upon ATP addition. Added ATP promotes slow release of the inhibitory ADP from a catalytic site as an increase to a steady-state rate is attained. At steady state, a slow interconversion of active and inactive forms continues. The bound ADP required for inhibition may arise from the cleavage of bound ATP

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or from medium ADP depending upon reaction conditions. P_i and various anions activate by promoting release of the ADP. The inhibitory ADP is at a catalytic site, not at a regulatory site as had been suggested.

Another important result of our continued probing was the recognition that, under some conditions, the presence of ATP at a certain noncatalytic site is necessary for the onset of activity of the chloroplast F_1-ATPase (86). This was the first recognized function for a noncatalytic bound ATP. The action was found to result from acceleration of the release of the inhibitory ADP from catalytic sites that follows the addition of medium ATP (87). With the mitochondrial F_1-ATPase, ATP binding to the noncatalytic site could also accelerate the onset of the Mg-ADP inhibition. Upon addition of ATP and Mg^{++} to the mitochondrial enzyme, an initial burst of activity declines to a slow rate as the Mg^{++}-induced inhibition sets in, then the rate increases to a steady state as the noncatalytic sites slowly bind ATP (88).

From the above it is apparent that complicated rate patterns may be found. It is probable that with all F_1-ATPases, and even under favorable conditions, a fair portion of the enzyme may be in the inhibited form. Many reported and planned experiments may be undermined by an unrecognized occurrence of the Mg-ADP inhibition. A procedure for estimating the portion of the enzyme in the inhibited form, as developed by Murataliev (87), deserves wider application.

As mentioned earlier, with the intact ATP synthase the inhibitory MgADP is quickly removed by exposure to proton motive force. This is akin to the removal of inhibitory imido-ATP that blocks hydrolysis but not synthesis. Chloroplast fragments show a light-activated ATPase that can be maintained by ATP cleavage. For unknown reasons, the activity continues even at higher Mg^{++} concentrations that would readily result in inhibition of the separated F_1-ATPase (89).

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Insights from use of 2-azido nucleotides--By the mid 1980's, the sequence of the ATP synthase subunits was becoming available. An ATP derivative, 2-azido ATP, which serves as a good substrate and upon photolysis becomes covalently attached, was described in Lardy's laboratory. We embarked on studies to find the number, and clarify the location, of ATP and ADP binding sites on the F_1-ATPases. That the ADP needed for the Mg^{++} inhibition was bound at a catalytic site was readily confirmed. The 2-azido-ATP or ADP at catalytic or noncatalytic sites (known to be principally on the β or α subunits respectively) labeled specific tyrosines not far apart on the β subunit. The sites were thus near subunit interfaces. Sites with similar conserved sequences were noted with the mitochondrial, chloroplast and *E. coli* enzymes (90-92). Whether the liver (93) and chloroplast enzymes (see 94 and earlier references) had six nucleotide binding sites remained in question. Our data with the 2-azido nucleotides supported the probability that they, like the mitochondrial enzyme, had six total nucleotide sites (95, 96). Such results added to the already recognized similarity of structure and mechanism of the enzyme from different sources.

We were somewhat surprised to find that derivatization by 2-azido nucleotides of some catalytic or of noncatalytic sites of the chloroplast F_1-ATPase gave rise to multiple catalytic pathways. Measurements of the distribution of ^{18}O isotopomers formed revealed that partially modified enzymes retained some activity that still showed modulation of oxygen exchange by ATP concentration. With more extensive derivatization, the native catalytic pathway disappeared and two weak, but independent, pathways were noted. Clearly some remaining catalytic sites retained weak activity independent from what neighboring sites are doing (97).

Behavior of the ATP synthase in intact mitochondria--Our experiments developing the binding change mechanism had been performed with isolated F_1-ATPases or fragmented

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membranes. The characteristics of the exchange reactions of the ATP synthase under conditions where mitochondria were capable of a high rate of tightly-coupled oxidative phosphorylation were not known. To gain such information, we undertook cooperative experiments with the research group of K. LaNoue, using the ^{32}P and ^{18}O labels (98).

The tightly coupled mitochondria were incubated with oxidizable substrates. When low ADP limited net ATP synthesis, all catalytic steps continued rapidly as concentrations of P_i, ADP and ATP remained unchanged. The expected rapid exchange of medium P_i with medium ATP with ^{32}P was observed. But this rate was only about a fourth of the rate of interconversion of bound P_i and ATP at the catalytic site as measured by ^{18}O. Strikingly, the interconversion rate remained high even when the membrane potential was reduced considerably by dinitrophenol addition. The addition of ADP and a hexokinase and glucose trap resulted in rapid formation of glucose 6-phosphate. The distribution of ^{18}O isotopomers in the glucose 6-phosphate showed a single reaction pathway, even when some uncoupler was added. The rapid reversal of bound ATP formation continued, so that about two reversals at the catalytic site occurred for each ATP released to the medium. Even though this reversal was occurring, and some medium P_i was being formed from bound ATP, the overall reversal to form medium P_i from medium ATP ceased. This can be explained by the lack of import of ATP by the ADP-ATP translocase. During rapid ATP synthesis, unlike the rapid reaction reversal that is occurring at the catalytic site of the ATP synthase, with high ADP in the medium the translocase is a one-way street.

Another important confirmation of alternating site participation came from these experiments with intact mitochondria. In the dynamic state with no added ADP the forward and reverse rates of all steps are equal, so that there is an equal chance that medium P_i that has formed bound ATP will be released to the medium as P_i or as ATP. If release of the bound ATP

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can only occur when another ADP and P_i bind, then the rate that medium P_i forms bound ATP will be twice the rate that it forms medium ATP. A ratio of about two to one for these rates was found when the overall rates were varied up to ten fold by changes in reactant concentrations or temperature. This provides evidence that alternating site participation is occurring under conditions where rapid oxidative phosphorylation is possible.

One aspect of these experiments may be pointing to an important and unrecognized property of the ATP synthase in its native environment. When the membrane potential was reduced by the addition of some uncoupler while ATP was present, not only did net ATP hydrolysis occur but also the rate of reaction reversal at the catalytic site remained high. This was reminiscent of earlier observations with submitochondrial particles that the oxygen exchange accompanying ATP hydrolysis was much greater at higher ATP concentrations than with the separated F_1-ATPase, and was relatively insensitive to uncouplers. In some manner, the capacity for rapid interconversion of bound reactants is retained better with the native membrane-bound synthase. I would still like to know how this is accomplished.

Site filling and catalysis, an unfinished story--It is well recognized that when only one catalytic site on the F_1-ATPase binds ATP or ADP and P_i, a slow interconversion of the substrates occurs. What remains uncertain at the time of this writing is whether a second or a second and a third site must bind ATP for the rapid release of ADP and P_i to occur. For over two decades since slow uni-site catalysis and alternating participation of catalytic sites was recognized, my group and most others felt that the binding of ATP to a second site sufficed for rapid catalytic turnover during net ATP hydrolysis. Because of binding affinities, it was recognized that all three catalytic sites would be filled at millimolar concentrations of ATP, approximating physiological conditions. I proposed that ADP during net synthesis, or ATP

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during net hydrolysis, entered the catalytic cycle by binding to different sites as indicated in Fig. 2. The critical need under conditions favoring rapid synthesis was regarded as the presence of interconverting, tightly bound substrates at Site 2 of Fig. 2 and ADP and P_i at Site 1. Conversely, under conditions for rapid hydrolysis the critical need was the presence of ATP at Site 3. In each case one rotational step would change the site to the tight conformation where covalent catalysis could occur. For both net synthesis or hydrolysis, the presence or lack of ADP or ATP at a third site was regarded as having a minor influence on the rates.

Several years ago, Senior and Weber and colleagues introduced a fluorometric method for estimating the amount of bound nucleotides at catalytic sites. They replaced a tyrosine at catalytic sites with tryptophan, and replaced tryptophans in other locations of the *E.* coli F_1-ATPase (99 and earlier references). The binding of nucleotides at the catalytic sites quenched the fluorescence of the tryptophan and allowed an estimation of the number of catalytic sites filled. They, and Allison's group with the thermophilic F_1-ATPase (100 and earlier references), found that three sites appeared to be filled with nucleotide as near maximal velocity was reached with increase in the ATP concentration. They then assumed that the binding of ATP to a second and to a third site was necessary for rapid net hydrolysis. This I believe will prove to be incorrect. Instead, it still seems likely that although three sites may become filled (probably mostly with ADP), the essential need for rapid hydrolysis is the binding of ATP to a second site. In other words, bi-site activation probably occurs along with tri-site filling. These issues are considered in more detail elsewhere (101).

Evaluations of rotational catalysis--As the research journey proceeded, we attempted some evaluations of whether rotational catalysis indeed occurs. We found that when the chloroplast F_1-ATPase was reacted first with 2-azido-ATP, and then with ^{14}C-DCCD, two

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different β units were derivatized. Thus the DCCD does not label a subunit with tightly bound nucleotide present. The DCCD-labeled enzyme retained weak catalytic activity. This made it possible for us to find if catalysis changed the conformations of the β subunits that determine their chemical reactivity, as would be expected if rotational movement of the γ relative to the β subunit had occurred. When the enzyme was first reacted with DCCD, allowed to perform catalysis with 2-azido-ATP and then the azido-ATP photolyzed, the subunits were randomly labeled (102). To us, this made rotational catalysis likely, but the weak catalytic activity of the DCCD-modified enzyme detracted from the result.

In another approach a bifunctional cross-linking agent that reacted with lysine NH_2 groups, and that had a central cleavable -S-S- linkage, was used. About three cross-links, mostly between the γ and δ subunits and the α subunit, caused loss of two-thirds of the activity, and the activity was recovered when the disulfide bonds were cleaved (103). Although the findings were consistent with rotational catalysis, they were not proof.

In contrast, in the same year (1987) Musier and Hammes reported that a cross-linking of the β and γ subunits did not inhibit catalysis and concluded that rotational catalysis did not occur (104). This at first appeared to be a to be a definitive finding. But examination of their paper suggested some possible experimental uncertainties. Also, possibly the derivatization had uncoupled Ca^{++}-activated hydrolysis from rotation. A more likely possibility was that the long -CH_2- chains in their cross-linkers may have allowed sufficient freedom of movements to not be restrictive. The catalytically induced movements of β subunits that we had observed with the 2-azido experiments (102) still seemed valid. I thus did not abandon the concept of rotation, and looked forward to better evaluations. The concept remained controversial in the field.

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It was becoming clear that structural data could provide the base for critical assessment of rotational catalysis. I was aware that Walker's group was attempting to obtain suitable crystals for X-ray analysis. In the meantime, it seemed that my group might accomplish more by studies that were underway with the 2-azido derivatives and by trying to define the location and function of bound nucleotides. As these and related studies progressed I prepared a comprehensive 1993 review of the status of research on how ATP is made under the title "The binding change mechanism for ATP synthase--some probabilities and possibilities" (63). The literature at that time was regarded as giving strong support to the concepts proposed in the binding change mechanism, with the exception that rotational catalysis was regarded as likely but definitely not established.

Fortunately progress continued in Walker's laboratory (105). This culminated in the 1994 report of the structure of a major portion of the mitochondrial F_1-ATPase (69). Receipt of an advance copy of the report from Walker was an occasion for gratifying emotion. The reported structure showed that the three β subunits were indeed in different conformations, and one had poor nucleotide affinity. The γ subunit was centrally located with structural associations consistent with its rotation driving sequential conformational changes of the β subunits. The authors interpreted their data as strongly supporting the binding change mechanism. Other X-ray studies interpreted as inconsistent with the binding change mechanism (106) appear mistaken.

The availability of high-resolution structural data made more critical assessments of rotational catalysis possible. Richard Cross, my former postdoctoral associate, noted residues in the γ and β subunits that were closely adjacent. When these were replaced with cysteines by mutagenesis good catalytic activity was retained. Oxidation of the -SH groups to form a

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disulfide cross-link blocked catalytic capacity, which was regained when the disulfide was reduced. With the disulfide linkage present, two β subunits that were not cross-linked were replaced by β subunits from an enzyme labeled with radioactivity during growth of the *E. coli.* Cleavage of the disulfide and catalysis resulted in randomization of the position of the γ subunit relative to the labeled β subunits (107). Similar loss of catalytic capacity upon disulfide cross-linking and related salient findings were reported from Capaldi's laboratory (108). Such results and other related findings were considered by the field to establish the occurrence of rotational catalysis.

In the spring of 1997, a stunning visual conformation of rotational catalysis came from the laboratories of Yoshida and Kinosita in Japan. In a novel experimental approach, they attached a long actin side chain with a fluorescent label to the γ subunit and, through inserted histidine residues, attached the modified enzyme to solid support. Upon hydrolysis of ATP, the rotational movement of γ was observable in the microscope (109). Important characteristics of the catalysis were shown, and are still being studied. I remember the thrill when I saw the rotation from a VCR recording that Yoshida kindly sent me. The dramatic experiment has gained wide recognition and removed nearly all doubt about the existence of rotational catalysis. Independently, Junge and associates developed a sophisticated fluorescence polarization technique that showed rotation accompanying ATP cleavage (110). This progress added to the near certainty of rotational catalysis. I was able to include references to their papers in press in a contribution entitled "The ATP Synthase--A Splendid Molecular Machine" that appeared in the 1997 *Annual Review of Biochemistry* (111).

A life style change--By 1994, my research laboratories were essentially closed. ATP synthase, and bioenergetics and enzymology, had yielded center stage to biochemistry related to

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genetics and development. Postdoctoral fellows were no longer seeking my laboratory. It was over 50 years since I had received my Ph.D. My mental capacities seemed to have slipped more than my physical capacities. I felt it would be difficult to continue to be at the research forefront and that unfunded USPHS applicants might make better use of funds than I. So I gave two years of committed research support back to the USPHS and bought a summer home in a mountain valley in Wyoming with accompanying golf and tennis facilities.

In October of 1997, while on my way to back to my winter haven in a home I love in the hills above UCLA, I received that electrifying early morning call informing me that I had been chosen to share the 1997 Nobel Prize in Chemistry. This of course was personally very gratifying. It increased my stature with my grandchildren. More importantly, it was a recognition of the many fine investigators that had contributed to the unveiling of the ATP synthase mechanism. It gave additional meaning to the careers of postdoctoral fellows and graduate students of my group, without whom there would have been no prize. And it has made my life since more vital and interesting. I recommend that if you are going to receive a Nobel prize, have it come late in your career when you no longer have the rewards that come from participation with a fine research group. Also, then you do not need to answer the question, "What research are you going to do now?"

Some final comments--The contributions of many scientists were essential for the gaining of our present insights into the ATP synthase catalysis. They should share in the satisfaction that comes from knowing much about how this important biological catalyst appears to operate. As the scientific enterprise grows ever larger, many fine contributors do not receive the appreciation they deserve. They should share in a pride for what has been achieved.

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The scientific accomplishment of the Twentieth Century that I admire most is the revealing of the multifaceted capabilities of proteins and of their structures that make these capabilities possible. Perhaps I am a bit more infatuated with enzymes than some. I do not know any enzyme that I could not learn to love, although I will admit that some are more attractive than others.

To all who created our stable and prosperous country and its research universities, which made a career such as mine possible, and mostly to my colleagues, I give my thanks.

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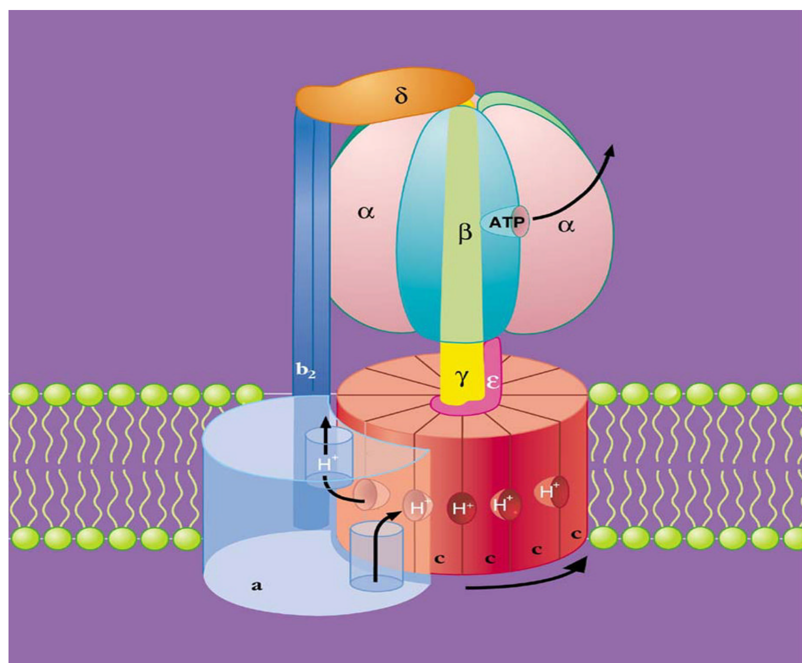

Fig. 1. The ATP synthase. The enzyme from *E. coli* has an F_1 portion with subunits designated as **a**$_3$**b**$_3$**gde**. When separated it acts as an ATPase. The F_0 portion subunits are designated as **ab$_2$c$_{9\text{-}12}$**. The passage of protons, at the interface of the **a** and the ring of the **c** subunits, causes a rotation of the **c** and attached **e** and **g** subunits relative to the rest of the enzyme. The rotation results in changes in the conformation of catalytic sites that promote ADP and P_i binding, ATP formation and ATP release. The mitochondrial and chloroplast enzymes are similar, except the F_0 portion has more subunits. The three catalytic sites are principally on the **b** subunits at an interface with the **a** subunits. The **a** subunits have three non-catalytic sites that bind nucleotides. The figure is from (112).

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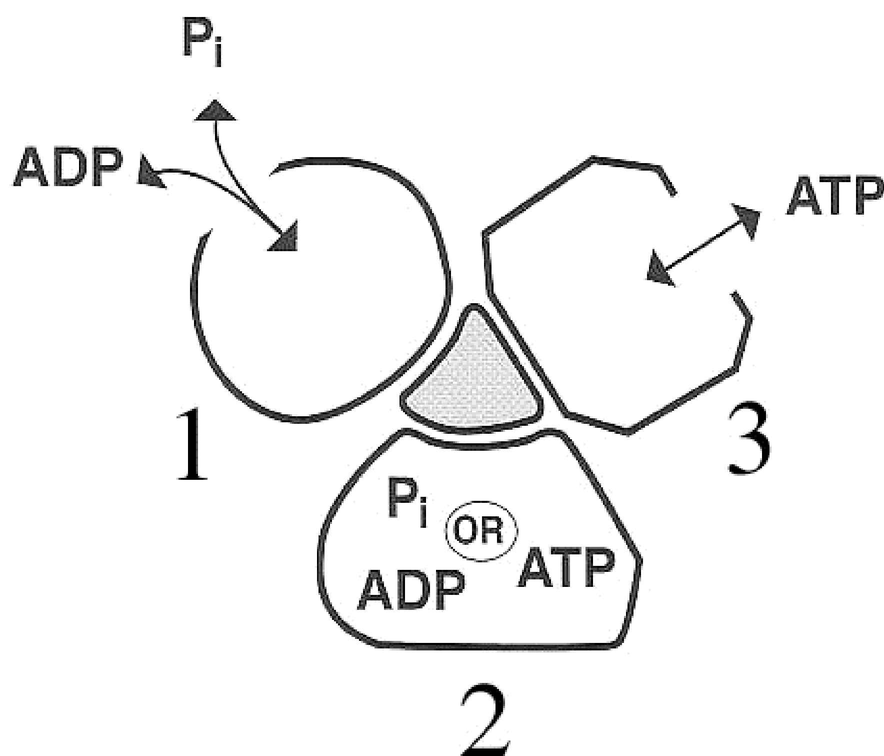

Fig. 2. **A depiction of the three major conformations of catalytic sites for bi-site activation of ATP synthesis or hydrolysis by the ATP synthase**. Three catalytic sites in different conformations are shown with asymmetric interactions to the shaded γ subunit. During catalysis sites are converted sequentially into three different states accompanying rotation of the γ subunit. The sequence for synthesis is 1 → 2 → 3, for hydrolysis it is 3 → 2 → 1. Site 1 binds ADP better than ATP and is the site at which ADP and P_i must be present for rapid synthesis to occur. Site 2 has the ability to catalyze chemical transformation and to be present as a form with ADP and P_i present or with ATP present. ATP can be released from Site 3 during synthesis and must be present at this site for rapid hydrolysis. Figure and legend from (101)

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