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ATP Synthesis in Oxidative Phosphorylation: A Direct-Union Stereochemical Reaction Mechanism

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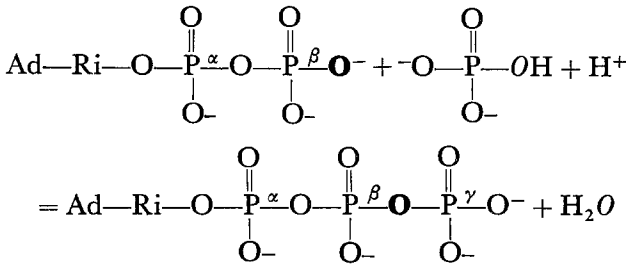
The Experimental Foundation for Formulating a Direct-Union Reaction Mechanism for ATP Synthesis

A fundamental understanding of oxidative phosphorylation will involve *chemical reaction mechanisms*. Since chemical reaction mechanisms describe in detail the making and/or breaking of chemical bonds, before a meaningful reaction mechanism can be formulated for a given reaction, the actual bonds involved must be known. This applies to all chemical reactions, including the chemical reactions in oxidative phosphorylation.

There is virtually universal agreement that in oxidative phosphorylation it is substrate inorganic phosphate (P_i) which forms a covalent chemical bond with some "first" polyatomic partner (of either high or low molecular weight) to give a phosphorylated entity. It is agreed that an atom which is a constituent of P_i engages an atom which is a constituent of some polyatomic partner to form a covalent bond. There is no question *whether* substrate P_i reacts to form a covalent bond with a "first" partner, but there is a fundamental question as to the *identity* of the "first" partner. In certain formulations of what is known as the "chemical hypothesis",[1] the "first" partner is *other than ADP*, and is arbitrarily designated "X". The covalent bond between "X" and substrate P_i could involve either the phosphorous atom or one of the oxygen atoms which are constituent to P_i. If such formulations were correct, ADP would clearly *not* be the "first" polyatomic chemical partner to react with P_i, but would be instead a later partner reacting with a covalent chemical "derivative" of P_i in a subsequent reaction to form the pertinent P–O bond in ATP. The various forms of the "chemical hypothesis" represent attempts, in terms of classical chemistry, to reckon, on the one hand, with the thermodynamically "downhill" energetics of respiratory oxidation-reduction, and on the other hand, with the thermodynamically

"uphill" energetics of ATP synthesis. In the various formulations, the covalent chemical derivatives of P_i are intrinsically (or, upon oxidation, become) "high-energy" species with which ADP can react spontaneously to form ATP.

However, in the overall phosphorylation chemistry of oxidative phosphorylation, what has been actually experimentally observable over many years of investigation is given simply by equation (1). It is



Equation (1)

specifically an oxygen atom which is a constituent of the β-phosphate group of ADP which ultimately engages in a covalent P–O bond with the phosphorous atom which is constituent to substrate P_i.[2] This P–O bond is the pertinent bond formed in ATP. Also, it is specifically an oxygen atom from P_i which ultimately ends up in H_2O.[2] These observations are the observations which are experimentally definitive at the level of bond making/breaking in the overall phosphorylation chemistry of oxidative phosphorylation.

The above experimentally definitive observations are insufficient of themselves to mechanistically implicate any polyatomic chemical partner reacting with P_i *other than ADP itself.* This does not mean that the data definitively rule out the possibility that some partner "X" covalently participates with P_i prior to ADP. What it does mean is that the data are simply insufficient to mechanistically implicate any such participation of "X". Indeed, for "X" to ever become mechanistically implicated, additional positive experimental data would have to demonstrate the existence of a covalent bond in a discrete "X"-derivative of P_i. The "X"-derivative of P_i would clearly have to be demonstrated as participating in oxidative phosphorylation proper.

In spite of many intensive investigations over a period of two decades, there is no experimentally definitive evidence for any chemical derivative of P_i other than the ADP-derivative of P_i, which is ATP itself, in the phosphorylation chemistry of oxidative phosphorylation.[3] The fact that the long-term search for an "X"-derivative of P_i has been unsuccessful clearly supports the very real possibility that there are in fact *no* "X"-derivatives of P_i participating in ATP synthesis and that ATP synthesis is occurring simply by a

direct-union of ADP and P_i giving ATP plus H_2O. Indeed such a formulation is the *minimum speculative* formulation in terms of the actual experimental data at the level of bond making/breaking. In its strictly literal sense, equation (1) states that an oxygen atom which is a constituent of the β-phosphate group of ADP directly forms a covalent bond with the phosphorous atom of substrate P_i (where P_i is enzyme-bound as inorganic phosphate), while one of the oxygen atoms which is a constituent of substrate P_i breaks its covalent bond with phosphorous to directly give rise to an H_2O molecule. This strictly literal description, which is in terms of P–O bond-making and bond-breaking specifics, permits formulation of a chemical reaction mechanism for ATP synthesis in terms which are mechanistically classical. To us, a nucleophilic substitution reaction at the substrate inorganic phosphate phosphorous center is strongly indicated for the direct-union reaction. This would mean that the ATP synthesis reaction is amenable to mechanistic analysis using well-established fundamental principles applicable to nucleophilic substitution reactions at phosphate phosphorous centers in general.

Mechanistic Problems Engendered by the Isotopic Exchange Data

Historically, workers in oxidative phosphorylation have had the difficult problem of formulating an enzyme-catalyzed reaction mechanism to achieve ATP synthesis, while at the same time explaining the well-known isotopic exchanges which accompany ATP synthesis.[4,5] The isotopic exchange are:

1. $ATP–{}^{32}P_i$
2. $ATP–ADP({}^{14}C)$

} Rates approximate the net ATP synthesis rate

3. $ATP–H_2{}^{18}O$
4. $P_i–H_2{}^{18}O$

} Rates much faster than net ATP synthesis rate

All of these isotopic exchanges are inhibited by oligomycin and by uncouplers of oxidative phosphorylation and are therefore recognized as being intimately related to the mechanism of ATP synthesis in oxidative phosphorylation.[4,5] The relatively rapid $ATP–H_2{}^{18}O$ oxygen exchange has a special prominence, because it, unlike the other isotopic exchanges, is unique to oxidative phosphorylation systems, occurring rapidly in no other type of system.

The $ATP–{}^{32}P_i$ and $ATP–ADP({}^{14}C)$ exchanges and their relative rates can be quite simply explained by overall dynamic reversibility of ATP synthesis. As seen in equation (1) viewed in its overall sense, ATP cleaves (either by a direct hydrolysis or by some indirect cleavage) to ultimately give ADP and P_i. These unlabelled species can be replaced on the enzyme by $ADP({}^{14}C)$ and/or ${}^{32}P_i$. When ATP

re-forms, ^{14}C and/or ^{32}P will be incorporated into ATP. This simple explanation applies irrespective of whether a direct-union reaction mechanism is involved in ATP synthesis or not. It depends solely on overall dynamic reversibility of ATP synthesis, whatever the mechanism. For this reason, the ^{14}C and ^{32}P exchanges have never posed a problem in the phosphorylation chemistry of oxidative phosphorylation. For the very same reason, however, the ^{14}C and ^{32}P exchanges have not provided a fruitful insight into the mechanism proper of ATP synthesis in oxidative phosphorylation.

By contrast to the ^{14}C and ^{32}P exchanges, the rapid ^{18}O exchanges cannot in any simple way be explained solely by dynamic reversibility of overall ATP synthesis. The ATP synthesis mechanism proper becomes critical here, including the obvious question of whether or not a direct-union reaction mechanism is involved. When viewed in its strictly literal sense, equation (1) would indicate that when $H_2{}^{18}O$ reacts to directly hydrolyze ATP to give ADP plus P_i, ^{18}O would be directly incorporated into P_i. If the ^{18}O-labelled P_i were to remain stereospecifically bound to the enzyme, resynthesis of ATP (the microscopic reverse of hydrolysis of ATP) would presumably expel ^{18}O from the ^{18}O-labelled P_i, and thus no ^{18}O would be incorporated into ATP. On the other hand, if the ^{18}O-labelled P_i fully dissociates from the enzyme and rebinds in a new steric orientation, resynthesis would incorporate ^{18}O into ATP. However, this latter sequence is a type of ATP-P_i exchange, and could thus occur no faster than the observed ATP-$^{32}P_i$ exchange.[6] The ^{18}O exchange from $H_2{}^{18}O$ into ATP actually can occur at a rate more than ten times as fast as the rate of the ATP-$^{32}P_i$ exchange.[6, 7]

Perhaps in part because of the seeming inability of a direct-union reaction mechanism for ATP synthesis to account for the rapid ATP-$H_2{}^{18}O$ oxygen exchange, some workers have continued to entertain a belief that ATP synthesis may not be occurring by a direct-union reaction mechanism but instead by a complex non-direct-union mechanism involving one, or perhaps a series of, covalently bonded phosphorylated intermediates. The rapid ATP-$H_2{}^{18}O$ oxygen exchange, although still unexplained in any simple way, may be, according to such workers, an expression ultimately of the complex mechanism of ATP synthesis whose mechanistic details remain obscure.

However, as we have strived to make clear, the experimentally definitive data are insufficient to implicate any phosphorylated entity other than ATP in oxidative phosphorylation. From this consideration we conclude that the resolution of the seeming "dilemma" of the rapid ATP-$H_2{}^{18}O$ oxygen exchange requires not a rejection of a direct-union reaction mechanism and the espousal of a more complex

mechanism leaving the required explanation still obscure, but instead it requires adherence to the only mechanism for ATP synthesis in oxidative phosphorylation which is sufficiently experimentally founded. To us, a satisfactory mechanistic explanation of the rapid ATP-$H_2{}^{18}O$ oxygen exchange will consequently be in terms of a direct-union reaction mechanism for ATP synthesis in oxidative phosphorylation. Rather than finding the ATP-$H_2{}^{18}O$ oxygen exchange a "dilemma," we have found it to be the most revealing feature of our direct-union reaction mechanism. The significance of the ATP-$H_2{}^{18}O$ oxygen exchange emerges only within the framework of fundamental phosphorous reaction stereochemistry and reaction mechanisms. We deal with these matters of fundamental phosphorous chemistry in the next section as a prelude to the presentation of our proposed direct-union reaction mechanism for ATP synthesis in oxidative phosphorylation.

Fundamental Phosphorous Stereochemistry Relating to Reaction Mechanisms

A direct-union reaction mechanism in which ADP and P_i give ATP plus H_2O implicates to us a nucleophilic substitution reaction at the inorganic phosphate phosphorous center. It is generally accepted that nucleophilic substitution reactions at phosphate phosphorous centers can proceed by way of unstable pentacovalent reaction intermediates having trigonal bipyramidal geometry.[8,9] A classical trigonal bipyramid structure is depicted in Fig. 1 and described in the accompanying legend, including features such as apical and equatorial bonds. We formulate the ATP synthesis reaction mechanism in terms of such classical trigonal bipyramidal unstable reaction intermediates. In nucleophilic substitution reactions at phosphate phosphorous centers, a nucleophile classically attacks the tetrahedral phosphorus atom to give a trigonal bipyramidal structure in which the attacking nucleophile is apical. Exit of the leaving group is also classically from an apical position. In other words, groups both enter into and exit from trigonal bipyramidal unstable reaction intermediates classically by way of the long, weak, relatively reactive *apical* bonds.

Unstable trigonal bipyramidal phosphorous reaction intermediates can have structural non-rigidity, i.e., capacity for pseudorotation.[9,10] Pseudorotation of a trigonal bipyramid is depicted in Figure 1 and described in the legend.

Pseudorotation has been found to be governed by certain electronic rules[11]:

1. Electron-withdrawing groups prefer apical bonding.
2. Electron-releasing groups prefer equatorial bonding.

Some examples of these rules for oxygen groups bonded to pentacovalent phosphorous are:

$-OH_2^+$ (oxonium) groups prefer apical bonding.
$-O^-$ groups prefer equatorial bonding.
–OH groups have relatively no bond orientation preference.

These fundamental matters concerning phosphorous stereochemistry and reaction mechanisms constitute a critical part of our proposed direct-union reaction mechanism for ATP synthesis in oxidative phosphorylation.

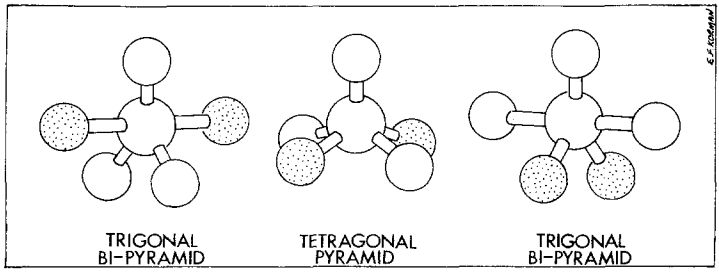


Figure 1. Classical trigonal bipyramidal geometry (see the structure on the extreme left).

The central atom has five atoms (or groups) bonded to it. Two of the atoms (shaded) are each bonded by a long, relatively weak *apical* bond and together are linear with the central atom. Three of the atoms (unshaded) are each bonded by a short, relatively strong *equatorial* bond and they form, together with the central atom, the equatorial plane. The apical bonds are perpendicular to the equatorial plane.

Pseudorotation of a trigonal bipyramid structure. In the structure at the extreme left, the pair of shaded atoms each start out apically bonded. By a process of bond-bending the shaded atoms become, in the structure at the extreme right, equatorially bonded. At the same time, in the original structure at the extreme left, the lower pair of equatorially bonded atoms (unshaded) become by the bond-bending process each apically bonded in the structure at the extreme right. The structure in the center is a tetragonal (square-base) pyramid and is the transition state halfway between the two trigonal bipyramidal structures. In structures having pairs of atoms (or groups) indistinguishable from each other (as pairs), the trigonal bipyramidal structure seemingly undergoes a 90° rotation, i.e., pseudorotates.

Description of the Enzyme-Catalyzed ATP Synthesis Reaction Mechanism

We are now prepared to formulate our proposed direct-union chemical reaction mechanism for the ATP synthesis reaction in oxidative phosphorylation.[12] In Fig. 2, which will describe the dynamic stereochemical reaction mechanism pictorially, we will designate substrate inorganic phosphate as HPO_4^{-2} since that protonation state is predominant in free solution at physiological pH. We will designate ADP as $ADPO^-$ to focus attention upon the pertinent oxygen atom of ADP directly involved in the reaction mechanism. The substrate oxygen atoms will be numbered for reference purposes.

In our ball-and-stick diagram, the formal double bond of P_i will be represented by one stick (σ-bond) and a pair of "sausage" π-clouds. In the enzyme active site we will invoke Mg^{2+} and proton-donor groups. We will not depict the chemical structures of the proton-donor groups (which we suggest could be imidazole groups[12]) but will depict only the pertinent protons. All substrates are considered to be tightly and stereospecifically bound to the enzyme during the entire catalysis.[13] The legend to Fig. 2 outlines some of the details of the enzyme-catalyzed chemical reaction mechanism. The proposed reaction mechanism encompasses features such as the direct-union of ADP and P_i to give ATP plus H_2O, fundamental phosphorous stereochemistry, tight stereospecific substrate binding during catalysis etc., which have been introduced in this and in previous sections as a prelude to its formulation.

Essence of the Reaction Mechanism

Pseudorotation, which commences with ADPO being apically bonded in reaction intermediate (I), results in ADPO becoming equatorially bonded in reaction intermediate (II). Since equatorial bonds are shorter and stronger than apical bonds, the pseudorotation constitutes *equatorial capture of ADPO* which is the essence of the proposed reaction mechanism.

Role of the Pair of Oxonium groups in the Pseudorotation

$ADPO^-$ is an excellent group to apically *leave* from the pentacovalent phosphorous center. Thus, ADPO has an inherently high preference for apical bond orientation at such a center. For ADPO to be equatorially captured, a pair of groups originally equatorially bonded must have a preference for apical bonding at least nearly equal to that of ADPO. In such an originally equatorially bonded pair, a single oxonium group (paired with some non-oxonium oxygen group) probably does not itself have sufficient preference for apical bonding for pseudorotation to be permitted. However, *two* oxonium groups, *acting together as a pair*, undoubtedly would have a combined preference for apical bonding which is sufficient for pseudorotation to be permitted, and thus for equatorial capture to be effected.

The Rate-Limiting Step

Since $ADPO^-$ is an excellent leaving group from the pentacovalent phosphorous center, it is conversely a poor entering group (i.e., it is a relatively weak nucleophile) at such a center. From that consideration, attainment of reaction intermediate (I) is probably the rate-limiting step in the chemical reaction mechanism.

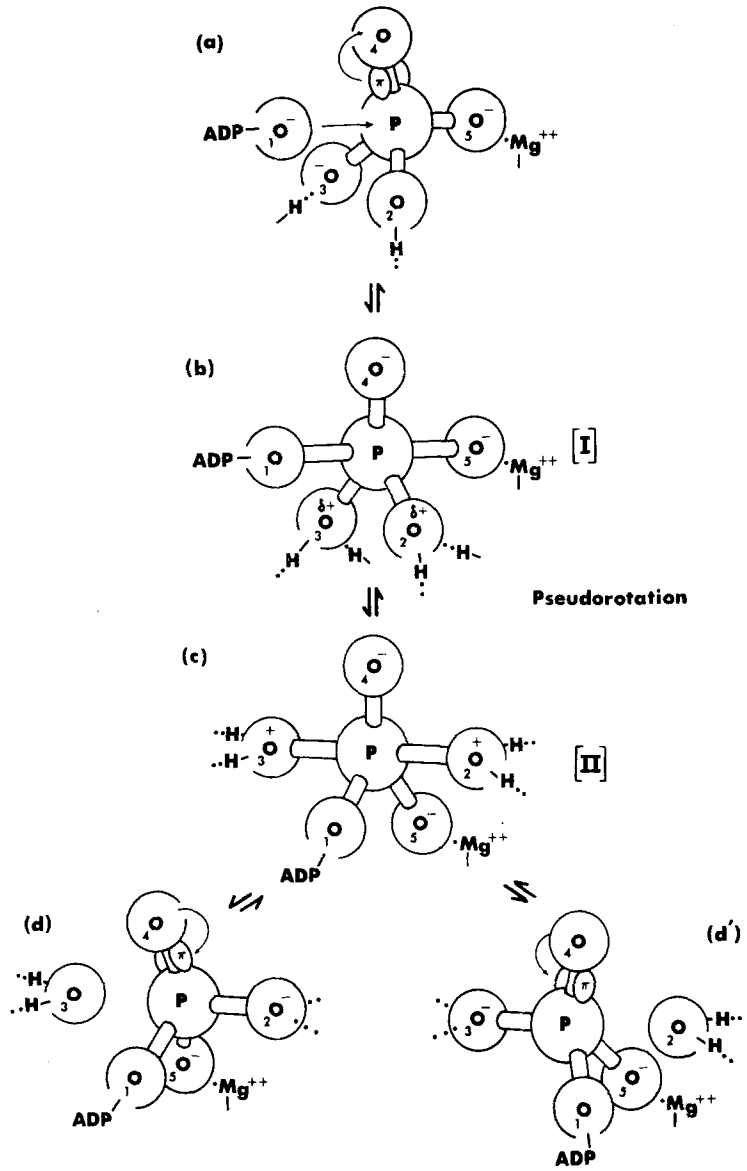


Figure 2. A proposed stereochemical reaction mechanism for ATP synthesis in oxidative phosphorylation.

In stage (a), tetrahedral HPO_4^{-2} binds tightly to the enzyme active site by a 3-point attachment: (1) the H of ($_2$O–H) H-bonds to an atom in the active site; (2) $_3O^-$ binds to a proton-donor group via a H-bond; and (3) $_5O^-$ coordinates Mg^{2+}. $_4O$ is depicted as doubly bonded to the P center of P_i and no binding function is specified for it.

The tight binding (unspecified) of $ADPO^-$ together with P_i geometrically positions $_1O^-$ to interact with the P center of P_i. Any prohibitive charge-charge repulsion is removed by substrate binding to the enzyme. The interaction of $_1O^-$ and P constitutes a classically apical nucleophilic "attack" by $ADPO^-$ upon the P center of P_i. Tetrahedral P_i transforms to unstable trigonal bipyramidal reaction intermediate (I), depicted in stage (b). $_2O$, $_3O$,

Reaction Mechanism Stereochemistry

In the proposed reaction mechanism, the entering ADPO group has a 90° geometrical relationship with the leaving H_2O group at the phosphorous reaction center, which constitutes a *retention* stereochemistry path at that center.[14] The retention stereochemistry path is absolutely fundamental to the equatorial capture-apical dehydration reaction mechanism for ATP synthesis.

Mechanistic Explanation of the Isotopic Exchanges

We can now explain the isotopic exchanges which accompany the ATP synthesis reaction in oxidative phosphorylation in terms which are rigorously *mechanistic*. We explain the ATP-$^{32}P_i$ and ATP-ADP(^{14}C) exchanges in terms of the dynamic reversibility of the overall ATP synthesis mechanism, as described earlier above. By contrast, we explain the rapid ATP-$H_2{}^{18}O$ oxygen exchange as occurring simply and directly via the "double-oxonium" reaction intermediate (II) which is on the mechanistic path proper to ATP and which is at the heart of the equatorial capture of ADPO in that mechanistic path. If $H_2{}^{18}O$ apically attacks ATP (see Fig. 2, d or d') leading to the "double-oxonium" reaction intermediate (II), in which one of the two coapical oxonium groups is now ^{18}O-labelled, followed by a Walden *inversion* type of expulsion of the unlabelled apical oxonium group, ^{18}O is directly incorporated into ATP. In this direct invertive exchange, ATP is never cleaved, i.e., the pertinent P–O bond formed in ATP synthesis is never broken.[15] The ATP-$H_2{}^{18}O$ oxygen exchange does not involve overall reversal of the ATP synthesis reaction mechanism and thus does not traverse the rate-limiting step. Therefore, the ATP-$H_2{}^{18}O$ oxygen exchange would clearly be expected to occur at a rate significantly faster than the rates of the ATP-$^{32}P_i$ and ATP-ADP(^{14}C) exchanges.

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and $_4O$ assume equatorial bonding in going to (I). $_2O$ carries the H it brought into the active site, while $_3O$ acquires the H it was initially H-bonded to. At the same time, $_4O$ develops a negative charge.

In the active site, the trigonal bipyramidal geometry of (I) positions the equatorial $_2OH$ and $_3OH$ groups each into proximity to a proton-donor group. The proton-donor groups are geometrically disposed and sufficiently acidic to strongly H-bond to the $_2OH$ and $_3OH$ groups imparting partial oxonium character ($-OH_2^{\delta+}$) to those latter groups. (I) is now structured to permit pseudorotation to give reaction intermediate (II) depicted in stage (c). As (II) develops from (I) and the pair of partial oxonium groups become apically bonded, they develop essentially full oxonium character ($-OH_2^+$) in (II). (II) is called the "double-oxonium" reaction intermediate.

(II) can favorably "collapse" by classically apically losing either one (see stage d) or the other (see stage d') of the oxonium groups as H_2O, while "returning" catalytic protons from the non-leaving oxonium group back to the enzyme active site. The loss of the H_2O transforms (II) to a tetrahedral phosphate group. The phosphate group thus formed has a π-bond between $_4O$ and the P center, and a full σ-bond between the P center and the $_1O$ oxygen atom. The pertinent P–O bond is formed and a molecule of ATP is achieved.

The inversion stereochemical reaction mechanism which we have deduced for the rapid ATP-$H_2{}^{18}O$ oxygen exchange is mechanistically classical. In principle, such an invertive exchange reaction mechanism would lead to racemization of a population of asymmetric reaction centers. Of course, the γ-phosphate phosphorous reaction centers in the ATP molecules are not normally asymmetric, and thus racemization cannot readily be measured. Nevertheless, in terms of its ability to catalyze the invertive ATP-$H_2{}^{18}O$ oxygen exchange reaction, the ATP synthesis enzyme has a "racemase" character. At the heart of the "racemizing" activity is the "double-oxonium" reaction intermediate (II), which is indispensable to effect equatorial capture of ADPO.

We explain the rapid P_i-$H_2{}^{18}O$ oxygen exchange with an inversion reaction mechanism analogous to that for the ATP-$H_2{}^{18}O$ oxygen exchange. In the P_i-$H_2{}^{18}O$ case, P_i acts as an analog of ATP, to give, upon attack of $H_2{}^{18}O$, a reaction intermediate having "double-oxonium" geometry analogous to reaction intermediate (II).

Implications Concerning Overall Oxidative Phosphorylation

The prominent ATP-$H_2{}^{18}O$ oxygen exchange, which heretofore seemed to be a "dilemma," emerges upon our analysis as exceptionally "meaningful" in that it arises directly from a reaction intermediate which is at the heart of equatorial capture of ADPO and thus indispensably on the mechanistic path proper to ATP. Since the ATP-$H_2{}^{18}O$ oxygen exchange can now be accounted for in *meaningful mechanistic terms* within the framework of a direct-union nucleophilic substitution reaction mechanism for ATP synthesis, an impediment to acceptance of such a direct-union reaction mechanism has been removed. The granting of such a direct-union nucleophilic substitution reaction mechanism for ATP synthesis has some very profound implications concerning the overall mechanism and energetics of oxidative phosphorylation. It immediately means that the ATP synthesis reaction is mechanistically "self-contained" and "separate" from the oxidation-reduction chemistry. Although "separate" in the sense that each has its own reaction mechanism identity (i.e., nucleophilic substitution as distinct from oxidation-reduction), the phosphorylation chemistry and the oxidation-reduction chemistry are nevertheless "coupled." The "coupling" must perforce be by way of the protein in its role as catalyst to both chemistries.

In the ATP synthesis reaction mechanism we alluded to the enzyme in its catalytic role. Proton-donor groups in the enzyme effect protonation of oxygen atoms of substrate P_i as a principal feature of the enzyme catalysis. The "double-oxonium" reaction intermediate

(II), which is at the mechanistic heart of both equatorial capture of ADPO and also of the ATP-$H_2{}^{18}O$ exchange reaction mechanism, involves four protons. The protonic events during catalysis are dependent upon proper proton geometric positioning and acidity. Our direct-union formulation implies that the requisite proton geometric positioning and acidity arise in the ATP synthesis active site upon respiratory electron transfer towards O_2 in active sites elsewhere in the enzyme architecture. Although we have not here included a mechanistic picture for the oxidation-reduction chemistry, we expect that chemistry to also involve protons. Protonic events very likely are salient mechanistic features of the oxidation-reduction chemistry.[16] Just as the proper geometric positioning and acidity of protons needed to catalyze the ATP synthesis reaction in the ATP synthesis site arise upon electron transfer towards O_2 in the oxidation-reduction sites, so, too, the proper geometric positioning and acidity of protons needed to catalyze reversed electron transfer in the oxidation-reduction sites arise upon reverse of ATP synthesis, i.e., upon ATP hydrolysis, in the ATP reaction site. The role of the enzyme is to provide a molecular architecture containing both the ATP synthesis site and the oxidation-reduction sites. As molecular architecture, the enzyme, in its state of tight binding to the substrates, will perforce undergo geometric changes in ensemble with the substrates as the latter obviously undergo geometric changes in transforming from reactants to products during catalysis, as is depicted, for example, in our stereochemical reaction mechanism. The geometric changes in the enzyme molecular architecture (conformational changes[17]), concomitant with the catalysis in one active site of a chemical reaction which is thermodynamically overall "downhill", geometrically positions protons of proton-donor groups (among other parameters) in the other active site to catalyze a chemical reaction which is thermodynamically overall "uphill". Thus, the enzyme, acting as a conformational system, weds the two chemistries, each of which nevertheless retains its separate mechanistic identity. In conformationally marrying the two chemistries, the enzyme "couples" their overall thermodynamic parameters. In terms of our view, a fundamental understanding of oxidative phosphorylation will involve *dynamic structural chemistry* applying not only to the substrates in the two chemistries but also to the enzyme to which the substrates are tightly bound.

Acknowledgements

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