THE MOLECULAR MECHANISM OF OXIDATIVE PHOSPHORYLATION*

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Current scientific interest in the molecular mechanism of respiratory chain oxidative phosphorylation is witnessed by the multitude of attractive working hypotheses which have appeared in the literature during the last eight years. But in addition to discussions of electron transfer1, 2 and possible “energy-rich” intermediates,3-11 a satisfactory mechanism must also account for the following well-established facts: (1) the tight coupling between electron transport and phosphorylation; (2) the observed P:O ratio; (3) the crossover points of Chance and Williams; (4) the coupling factors; (5) the action of uncouplers; (6) the inhibitors of electron transport and phosphorylating oxidation; (7) the data on phosphohistidine; (8) the reversal of electron transport by adenosine 5'-triphosphate (ATP).

Unfortunately, none of the molecular mechanisms proposed to date seems to account for all these facts. The only exception appears to be the chemi-osmotic hypothesis of Mitchell12 which, because of its inherent complexity, is rather difficult to prove or disprove. While the observed phosphorylation of adenosine 5'-diphosphate (ADP) in pH-jump experiments is consistent with Mitchell's theory, it is also understandable in terms of the conventional theories involving “energy-rich” intermediates. For example, if electron transport in mitochondria produces local pH-changes, then a sudden pH-change could also induce electron transport along the respiratory chain with concomitant phosphorylation through the conventional “energy-rich” intermediates. Chance and Mela found that the initial rate of hydrogen ion formation is much slower than the rate of electron transport in submitochondrial particles.13

In this paper, a new attempt is made to formulate a molecular mechanism of oxidative phosphorylation consistent with most of the known facts of the mitochondrial system. The mechanism is suggested by experimental results on model oxidative phosphorylation systems obtained in our laboratory during the last five years, encouraged by the work of Boyer’s group on phosphohistidine,11 and prompted by the possibly naïve hope that all biological phenomena are understandable in simple chemical terms.

It has previously been shown that aerobic oxidation of ferroheme in solutions of N,N-dimethylacetamide (DMAC) containing imidazole, the imidazolium salts of inorganic orthophosphate (P_i), and adenosine 5'-phosphate (AMP) or ADP produces ATP.14 If the nucleotides are omitted and the P_i is labeled with P^{32}, a radioactive “energy-rich” intermediate product can be detected which has hydrolytic, paper-chromatographic, and electrophoretic behavior indistinguishable from that of synthetic 1-phosphoimidazole.15 The principal mechanism of this model reaction may be summarized as follows.

When the dimethyl ester of ferroheme is oxidized by molecular oxygen in pure, anhydrous DMAC solution, the radical ion O_2^- is produced,

\[ \text{O}_2 + e^- \text{(ferroheme)} \rightarrow \text{O}_2^- . \] (1)
Since imidazole has been found to react with $O_2^-$ in the above solution very rapidly to form hydroperoxide ion, $HO_2^-$, and the radical-complex imidazolyl-ferriheme, the initial oxidation of ferrohemochrome by $O_2$ in DMAC solution containing imidazole may be represented by

$$O_2 + \text{HN}N\text{Fe}^II \rightarrow HO_2^- + \text{N}N\text{Fe}^III$$  \hspace{1cm} (2)

The evidence for the existence of these radical intermediates has been obtained from electron-spin resonance studies.\textsuperscript{16}

In the presence of $P_i$ (or AMP), the above radical-complex may further react to form the phosphoimidazolyl (or AMP-imidazolyl) radical,

$$\text{Fe}^III \text{N}N\text{OH} + H_3PO_4 \rightarrow \left[\text{Fe}^III \text{N}N\text{PO}_4\right]^-$$  \hspace{1cm} (3)

which can subsequently be reduced by another ferrohemochrome molecule either to eliminate $H_2O$ to produce 1-phosphoimidazole, or to eliminate imidazole and regenerate $P_i$ according to equations (4) and (5), respectively.

$$\text{N}N\text{PO}_4^- + H_2O$$  \hspace{1cm} (4)

$$\text{N}N\text{NH} + HPO_4^-$$  \hspace{1cm} (5)

The 1-phosphoimidazole can then phosphorylate AMP first to ADP, then to ATP as reported previously.\textsuperscript{17}

In the light of these results on model systems, a molecular mechanism which involves the reaction between covalently bonded radicals with mitochondrial phospholipids is now formulated. It will be shown that in spite of the enormous bulk, most of the oxidative phosphorylation data reported from different laboratories during the last 12 years can be given a unified interpretation in terms of the proposed mechanism.

Proposed Mechanism.—The above experimental results in DMAC solution suggest that a similar radical mechanism may be operative in the phospholipid-protein membrane of mitochondria, with the difference that the corresponding imidazolyl-ferriheme radical cannot diffuse away to cause biological damage but is covalently bonded to the protein of cytochromes as part of the histidine side chain. In addition, if the responsible cytochrome is bound to the membrane in such a manner that the substituted imidazole is adjacent to the phosphodiester group of a phospholipid molecule, $(RO)(R'O)PO_4^-$, the imidazolyl radical may react rapidly with the latter to form a phosphoimidazolyl radical which can then be reduced to the
"energy-rich" intermediates II or III as illustrated in Figure 1. In Figure 1, the direction of electron transport under normal conditions is represented by the heavy arrows, and the in vitro mid-point potentials in volts at pH 7 for different electron

![Diagram of oxidative phosphorylation](image)

Fig. 1.—Proposed molecular mechanism of oxidative phosphorylation. The intermediates in brackets represent covalently bonded radicals. Compounds I, II, and III represent "energy-rich" intermediates from flavin, cytochromes c (or c) and a (or a3), respectively. CFα, CFβ, CFγ, CFδ represent enzymes or specific coupling factors for various transphosphorylation reactions. The numbers in parentheses represent approximate mid-point potentials in volts at pH 7.
carriers are indicated by the numbers in parentheses. The "energy-rich" inter-
mediates II or III can presumably react with P₁ in the presence of a specific enzyme
or coupling factor to give compound V, which can in turn react with ADP in the pres-
ence of another enzyme or coupling factor to give ATP and regenerate the phos-
pholipid VII for the next round of oxidative phosphorylation.

Similarly, a flavinyl radical can presumably also react with phospholipid to form
a covalently bonded phospholipid-flavinyl radical which is tentatively assumed
to be subsequently further oxidized to the "energy-rich" intermediate I. Com-
pound I can then react with P₁ in the presence of its specific enzyme or coupling
factor to give compound IV, which can in turn react with ADP to form ATP and
regenerate the phospholipid VI.

Discussion.—In addition to the indirect evidences from the model system, the
proposed mechanism in Figure 1 is also consistent with the other well-established
facts on respiratory chain oxidative phosphorylation mentioned above. These
will now be discussed in the listed order.

(1) Coupling between electron transport and phosphorylation: Although the
experimentally determined in vitro mid-point reduction potentials of the electron
carriers may differ appreciably from the corresponding in vivo values, it is generally
believed that the differences are not large enough to offset the sequence of increasing
reduction potentials from nicotinamide-adenine dinucleotide⁺ (NAD⁺) to O₂ as
depicted in Figure 1 which allows efficient electron transport along the chain. That
electron transport is necessary for phosphorylation is obvious not only for thermo-
dynamic reasons but also from a mechanistic point of view, since it is the transfer of
electrons which gives rise to the radical intermediates and then changes them to the
"energy-rich" compounds I, II, and III. The more puzzling requirement of
phosphorylation for electron transport becomes understandable if we recall that
unless ADP and P₁ are present in sufficient concentration to discharge the inter-
mediates I, II, and III continually, the electron carriers will be left in these "energy-
rich" states, each with considerably lower mid-point reduction potential than its
neighboring carrier on the substrate side of the respiratory chain. Such a modified
sequence will prevent efficient electron transport in the normal direction. In
aqueous solution, ferriheme has a mid-point reduction potential of about −0.12 v
at pH 7. By complexing with strong coordinating ligands such as pyridine or
imidazole, its mid-point reduction potential can be raised by as much as 0.3 or
0.4 v. Conversely, since the phosphorylated imidazole group is a much weaker
coordinating ligand for the Fe(II)-state than the original imidazole group, one
could expect the mid-point reduction potential of a (or a₆) in compound III to
be < 0.26 v, and that of a₁ (or c) in compound II to be < 0.08 v. Likewise, the mid-
point reduction potential of flavin in compound I could for structural reasons be
< −0.32. Consequently, unless the concentrations of ADP and P₁ are sufficiently
high and that of ATP sufficiently low so that compound I, II, and III are discharged
continually by the mitochondrial enzyme system, normal electron transport cannot
continue. Thus regulation of the ratio [ADP] [Pi]/[ATP] controls the rate of
respiration.

(2) P:O ratio: Inspection of the mid-point reduction potentials of oxygen at
pH 7 as indicated in the following diagram shows that O₂ serves more efficiently
as a 2- or 4-electron than 1- or 3-electron acceptor:
In the respiratory chain, once the bound \( \text{O}_2 \) has extracted an electron from the Fe(II) of heme \( a \) (or \( a_6 \)), it has a strong tendency also to extract a second electron from the coordinating imidazole group, thereby converting the latter to the highly reactive imidazolyl radical which could immediately attack the neighboring phospholipid to form the phospholipid-imidazolyl radical. In the subsequent reduction by cytochrome \( c_1 + c \), the phospholipid-imidazolyl radical is first reduced to compound III with elimination of \( \text{OH}^- \), followed by the formation of compound V and the reduction of the Fe(III) of heme back to Fe(II). Consequently, one ATP is formed at heme \( a \) (or \( a_6 \)) for every pair of electrons transferred. The same P:O ratio is expected at the heme \( c_1 \) (or \( c \)) phosphorylation site where compound II is formed. At the flavinyl site, the phosphorylation mechanism proposed in Figure 1 is similar except that the phosphoflavinylic radical is oxidized by heme \( b \) to the “energy-rich” intermediate I, and hence also produces one ATP per two electrons transferred. Consequently, over-all P:O ratio expected from the proposed mechanism is 3.

(3) **Crossover points:** If ATP is formed according to the scheme in Figure 1 from ADP, \( P_1 \) compounds, I, II, and III through enzyme-controlled transphosphorylation reactions, then depletion of ADP will cause accumulation of compounds I, II, and III. Since the mid-point reduction potentials of FP, \( c_1 \) (or \( c \)), and \( a \) (or \( a_6 \)) in these respective “energy-rich” states should be much lower than in their normal states, they could fail to oxidize their respective neighboring carriers on the substrate side of the chain. Consequently, the difference spectrum of the ADP-depleted system may show crossover points between NAD and FP, between \( b \) and \( c_1 \) (or \( c \)), and between \( c \) and \( a \) as observed in the ingenious pioneering work of Chance and Williams.19

(4) **Coupling factors:** Because of the fact that the triesters of phosphoric acid are generally much more reactive than the monoesters,20 and that 1-phosphoimidazole rapidly hydrolyzes in even very weakly acidic solutions,21 it may be inferred by analogy that “energy-rich” intermediates such as compound III should be much more susceptible to hydrolysis than even 1-phosphoimidazole and hence unsuitable to function as a free-energy transducer at an exposed site. Protecting this reactive group by a second lipid layer is hardly satisfactory, since the latter would also hinder the elimination of water or \( \text{OH}^- \) and prevent the subsequent reaction with \( P_1 \). The only conceivable protection which satisfies all the necessary requirements is to combine the reactive part of compound III with a specific enzyme or coupling factor (CF\(_5\)) which catalyzes the transphosphorylation of \( P_1 \) by compound III in preference to the hydrolysis of the latter. Similarly, the enzyme, CF\(_{a_6}\), which catalyzes the transphosphorylation of ADP by compound V may also be regarded as a dual-function coupling factor.

If any of these endogenous coupling factors is leached out during the preparation of the so-called electron-transport particles, ETP, from mitochondria,22 the corresponding “energy-rich” intermediate will be susceptible to rapid hydrolysis. Consequently, compounds I, II, and III will be spontaneously discharged to re-
store the mid-point reduction potentials of the respective electron carriers to their normal values for efficient electron transport without phosphorylation. By complexing the electron-transport particles with coupling factors, the phosphorylating properties of the former can be restored.

These interpretations are entirely consistent with the mechanism proposed in Figure 1. Moreover, according to the proposed mechanism, the enzymes CF₀ and CF₄ should exhibit ATPase activity in the presence of specific phospholipids.

(5) Uncouplers: When the phosphoimidazolyl radical in our model system is reduced, it could either eliminate water to form 1-phosphoimidazole according to equation (4), or eliminate imidazole to regenerate P₁ according to equation (5). The success of the model oxidative phosphorylation experiments shows that elimination of water in DMAC solution according to equation (4) is favored. Presumably, in normal respiratory chain oxidative phosphorylation, the H₂O (or OH⁻) is also preferentially eliminated from the mitochondrial membrane to form compound II or III with the help of specific enzymes.

Molecules such as 2,4-dinitrophenol, dicumarol, etc., may, because of their lipid solubility, penetrate the mitochondrial membrane and react with compounds I, II, or III to form phenolic ester of the corresponding phospholipid such as compound VIII below:

\[
\text{VIII}
\]

But if this happens, then when the product of reaction between VIII and the imidazolyl radical is subsequently reduced, the elimination of dinitrophenol may be much less favorable because of its hydrophobic nature. If it is indeed even less favorable than the elimination of the imidazole group, the latter reaction will take place with the consequence that the cytochrome potential will be restored to its normal value for further efficient electron transport without phosphorylation having taken place according to equation (6):

\[
\text{(6)}
\]

It is also of interest to note that according to this interpretation, P₁ is not necessary for the uncoupling of respiratory chain by dinitrophenol, an inference which is in agreement with the work of Slater.²⁴

(6) Inhibitors: In terms of the proposed mechanism, inhibitors of electron transport could be molecules which complex with the electron carriers and thereby
prevent electron transport either by increasing the energy barrier for electron transfer between neighboring carriers, or by drastically changing the reduction potential of the carriers, or both. On the other hand, inhibitors of specific enzymes or coupling factors in Figure 1 should behave as the inhibitors of phosphorylating oxidation reported in the literature, e.g., oligomycin, atractylate, etc.

(7) Phosphohistidine: The isolation of $^32P$-labeled phosphohistidine by Boyer et al. stimulated many interesting speculations on its role in oxidative phosphorylation. Recently, Bieber and Boyer concluded that phosphohistidine probably does not serve as an intermediate in the formation of ATP by oxidative phosphorylation in mitochondrial particles, because the observed rate of labeling of phosphohistidine from $P_i$ during oxidative phosphorylation did not reach its maximum specific radioactivity when or before the rate of ATP labeling was maximal. Slater et al. had previously reached a similar conclusion.

However, according to the mechanism in Figure 1, the "energy-rich" intermediates I, II, and III are formed through reactions of the flavoprotein or cytochromes with the phosphodiester groups of the respective phospholipids, not with the $P_i$ which was initially labeled in Bieber and Boyer's experiment. Consequently, any $P_i$-labeled phosphohistidine isolated by their procedure had to originate from subsequent transphosphorylation reactions which need not bear strict sequential relationships to the labeling of ATP from $P_i$ during oxidative phosphorylation.

In addition, if oligomycin, atractylate, etc., are indeed specific inhibitors of coupling factors such as CF$_s$ and/or CF$_d$ in Figure 1, they would be expected to inhibit the labeling of ATP from $P_i$ but not the labeling of phosphohistidine from $P_i$, as was also observed by Bieber and Boyer.

(8) Reversal of electron transport by ATP: Since catalysts do not alter chemical equilibrium, the same enzymes which catalyze the transformations from I, II, and III to VI and VII, respectively, in Figure 1, must also catalyze the reverse transformations. Thus, in the presence of an excess of ATP at high concentration, the following reverse reactions may take place:

$$K_7 \quad \text{ATP} + \text{VII} \rightleftharpoons \text{ADP} + \text{V}, \quad (7)$$

$$K_8 \quad \text{V} + c^{\text{III}} \rightleftharpoons P_i + c^{\text{III}^*}, \quad (8)$$

where $c^{\text{III}}$ represents the Fe(III)-state of cytochrome $c_1$ (or $c$), and $c^{\text{III}^*}$ its "energy-rich" form, i.e., compound II, and $K_7$, $K_8$ the equilibrium constants of reactions (7) and (8), respectively.

Combining equilibria (7) and (8) we get

$$\frac{[\text{ADP}][P_i]}{[\text{ATP}][\text{VII}][c^{\text{III}^*}]} = K_7K_8. \quad (9)$$

The presumably much lower mid-point reduction potential of $c^{\text{III}^*}$, relative to $c^{\text{III}}$, could cause electrons to flow in the NAD direction, provided that ATP or other "energy-rich" intermediates are present in sufficient concentration and that the O$_2$ side of the respiratory chain is intercepted, e.g., by cyanide.

Making the simplifying approximation that practically all the reduced cyto-
Chromes are in the unphosphorylated form \( c^I \) or \( a^I \), we obtain from (9) the relationship

\[
\frac{[c^{III}]}{[c^I]} + \frac{[c^{III}]}{[c^I]} = B \left( 1 + K_7 K_8 \frac{[\text{ATP}]}{[\text{ADP}] [P_1]} \right),
\]

(10)

where \( B \) represents the concentration ratio \( [c^{III}] / [c^I] \).

Equation (10) states that the ratio of the equilibrium concentrations of oxidized to reduced cytochrome \( c_1 \) (or \( c \)) increases with the ratio \( [\text{ATP}]/([\text{ADP}] [P_1]) \).

A similar expression can be written down for the flavinyl phosphorylation site. Both uncoupler and inhibitors are expected from the proposed mechanism to interrupt this type of reverse electron transport through the relevant path.

These conclusions appear to be consistent with published experimental data on the reversal of electron transport by ATP.\(^{29-32} \) But further investigation is necessary before the validity of the present interpretation can be definitely established.

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