REVISTA ESPAÑOLA DE FISIOLOGIA, 34, 481-490. 1978

ATPase, an Electron Carrier and Energy Transducer: The Missing Link in Oxidative Phosphorylation?

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(Received on August 25, 1978)

E. SANTIAGO and N. LOPEZ-MORATALLA. *ATPase, an Electron Carrier and Energy Transducer: The Missing Link in Oxidative Phosphorylation?* Rev. esp. Fisiol., 34, 481-490. 1978.

A mechanism for oxidative phosphorylation is proposed. ATP synthesis would take place on a catalytic site of ATPase constituted by two irons with ligands ATP, ADP, and Pi. The interchange of ATP with ADP and Pi makes possible a redox cycle between two potentials permitting the energy transduction.

In the past few decades oxidative phosphorylation has been the subject of many discussions and different theories have been advanced in an effort to help our understanding of what has been considered one of the most elusive problems of modern biochemistry. A recent review (1) has analyzed in detail the present day state of this crucial question.

It is now firmly established that the synthesis of ATP is catalyzed by mitochondrial ATPase when properly coupled to electron flow (20). The same enzyme complex is also known to catalyze a series of other reactions including the hydrolysis of ATP and several exchanges which are considered as «partial reactions» in the process of oxidative phosphorylation (15).

The recent finding in our laboratory (16) that soluble ATPase from rat liver mitochondria undergoes redox reactions, accompanied by profound reversible changes of its properties, has led to the detection of one mole of FAD and six g atoms of iron per mole of F_1-ATPase (23). These facts, and the further data here reported, together with the abundant information available in the literature regarding oxidative phosphorylation have permitted the elaboration of a rather simple and unifying model which may offer a reasonable explanation for the phenomenon of energy transduction at the molecular level.

It is not at all surprising that iron could catalyze the removal or the addition of a molecule of water. This type of reaction takes place, for example, in the transformation of citrate to isocitrate through a combined succesive dehydration and hydration; the role of iron in this reaction catalyzed by the enzyme aconitase

is now well established (8). In the synthesis of ATP, the presence ATPase ensures the removal of water, i.e., the abstraction of an OH^- and of an H^+ from ADP and P_i. The question now arises of how this reaction takes place, and in such a way as to satisfy all the ensuing energy changes. Furthermore, the mechanism of such a reaction must necessarily explain the coupling of electron flow to the formation of a pyrophosphate bond.

The oxidation of Fe (II) produces the simultaneous ejection of a proton and the binding of ligand OH^- according to the reaction

$$\left[Fe^{II}\ (OH_2)_6\right]^{2+} \underset{e^-}{\overset{e^-}{\rightleftharpoons}} \left[Fe^{III}\ (OH_2)_5\ (OH^-)\right]^{2+} + H^+$$

It is also well known that the standard reduction potential of the couple Fe^{+++}/Fe^{++} is strongly influenced by the nature of the ligands. Moreover, the following generalization has also been established: ligands with higher affinity for the reduced form of the metal make the standard reduction potential more positive, whereas ligands with higher affinity for its oxidized form make the standard reduction potential more negative. For a detailed discussion of these general concepts see CHABEREK and MARTELL (3).

If the assumption is made that tightly bound nucleotides ATP and ADP in ATPase (10) could be bound as ligands of the iron atoms, ATP and ADP may consequently affect the standard reduction potential of the enzyme.

Materials and Methods

Mitochondria were isolated by the method of HOGEBOOM (12). Protein determination was carried out following the technique of LOWRY *et al.* (18). F_1-ATPase was prepared from rat liver mitochondria by the procedure of LAMBETH and LARDY (13). ATPase activity was determined essentially as described by PULLMAN *et al.* (22) in the absence of an ATP generating system. Aliquots of the F_1-ATPase were preincubated for 5 minutes at 30° C in 0.8 ml of a medium containing 50 μmoles of Tris-acetate, pH 7.4, and 3 μmoles of $MgCl_2$. The reaction was initiated by the addition of 3 μmoles of sodium ATP, pH 7.4, dissolved in 0.2 ml of distilled water. The incubation was continued for 2 minutes and stopped by the addition of 0.1 ml of 50 % trichloroacetic acid. Inorganic phosphorus was determined according to FISKE and SUBBAROW (7). Reagent and enzyme blanks were determined in each experiment.

Results and Discussion

Effect of ADP and ATP on the reduced and oxidized forms of ATPase. It has been demonstrated that the reduction of ATPase causes an increase of its hydrolytic activity, together with a loss of its sensitivity to bicarbonate stimulation (16). The effect of ADP on the kinetic properties o ATPase in the presence of the reducing agent dithionite has been studied. It may be seen in table I that ADP showed

Table I. *Effect of ADP and ATP on the activity of the reduced form of ATPase, and on its sensitivity to bicarbonate stimulation.* The activity has been referred to 5 μg of purified proteins. Number of experiments, 10. Data represent mean ± S.D.

Additions	ATPase activity (nmol × min^{-1})		
	Control	+1 mM ADP	+1.5 mM ATP
None	67±2	41±2	77±2
10 mM HCO_3^-	140±3	90±2	167±3
30 μM Dithionite	171±3	69±2	210±4
30 μM Dithionite + 10 mM HCO_3^-	172±3	107±3	215±4

Table II. *Effect of ADP on the reduction of the FAD component of F_1-ATPase.*
Aliquots from a solution of dithionite (35 mg/ml) were added to a suspension of F_1-ATPase containing approximately 2 mg of protein in 3 ml of 100 mM Tris-acetate, pH 7.5. Fluorescence spectrophotometer Perkin-Elmer 204, sensitivity setting 9. Excitation ligth, 450 nm.

	Relative fluorescence at 535 nm Volumes of dithionite solution added (µl)							
	0	4	8	12	30	50	100	200
F_1-ATPase	100	91	80	75	56	35	0	0
F_1-ATPase + 2 mM ADP	100	98,5	92	91	85	80	45	0

its well known inhibitory effects on the hydrolytic activity of ATPase (9); at the same time it affected the kinetic properties of the enzyme elicited by dithionite. The enzyme was not activated by the reducing agent and its sensitivity to bicarbonate stimulation was preserved. These effects of ADP lead us to suggest that a hindrance of the reduction of ATPase through a decrease in its standard reduction potential might have taken place.

Further support for the idea that ADP might induce a decrease in the standard reduction potential comes from the following observation: As shown in table II greater amounts of dithionite were needed for the reduction of ATPase when ADP was present. The reduction of ATPase was followed by the changes in fluorescence which are specific to the FAD moiety of the enzyme (23). Measurements of the standard reduction potentials of ATPase are now being attempted in our laboratory.

It has also been observed that free ATP (ATP in excess of Mg-ATP) behaved as an activator of the hydrolytic activity of ATPase (table I). The activating effect of this nucleotide was higher in the presence of dithionite; these results suggest that free ATP could have a higher affinity for the reduced form of the enzyme, or expressed in other terms, free ATP could cause a shift in the standard reduction potential of ATPase towards less negative or more positive values.

The iron redox cycle of ATPase in oxidative phosphorylation. In the light of the previous theoretical discussion and the results reported above together with data from a vast number of different research groups, it is possible to postulate a mechanism which could explain the process of energy transduction by ATPase at a molecular level (fig. 1).

According to this proposal two atoms of iron in ATPase should be involved in the synthesis of each molecule of ATP to account for the simultaneous flow of two electrons. Each set of two irons could have ATP, ADP and Pi as ligands bound more or less strongly according to the state of oxidation. The following steps can now be envisaged. *Step I.* ATPase with ligands ATP, ADP and Pi receives a pair of electrons from an adequate donor of the respiratory chain and the set of 2 Fe^{+++} becomes 2 F^{++}. *Step II.* ATPase donates a pair of electrons to an adequate acceptor of the chain. Simultaneous with the transformation of 2 Fe^{++}

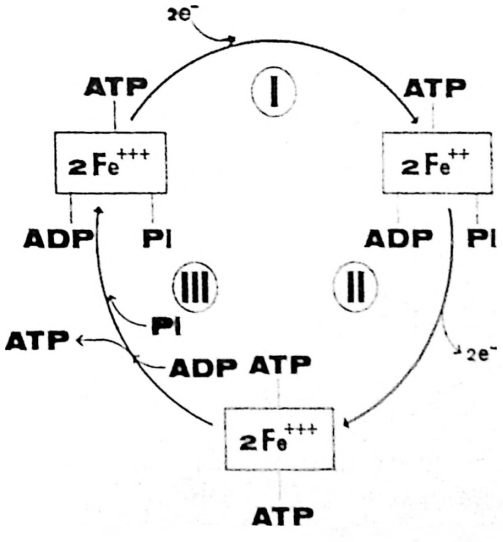

Fig. 1. *The iron redox cycle of ATPase.*

into 2 Fe^{+++} a reaction takes place in which an OH^- is removed from a molecule of Pi, a proton ejected from ADP, and a molecule of ATP is synthesized. (The detailed chemical mechanism of this reaction will be discussed below.) *Step III.* ATPase liberates a molecule of ATP, and binds both a molecule of ADP and a molecule of Pi. This exchange would be favored by the different affinities of the oxidized and reduced forms of ATPase for ADP and ATP. At this point ATPase would be ready to initiate a new redox cycle.

The electron donors would be the reduced forms of carriers of the respiratory chain located at the more negative end of each one the three gaps of potential (1). The electron acceptors would be the oxidized carriers located at the less negative end of those gaps (1). The exchange of electrons between components of the respiratory chain and ATPase should take place more or less isopotentially. ATPase, as suggested by the results reported above, may exist in two forms with different standard reduction potentials depending upon the replacement of a ligand ATP by ADP and Pi. With this property ATPase would fulfill the requisite considered by DE VAULT (5) as essential for an energy transducer; this author has affirmed that such a catalyst should act between two potentials; it should also work in a cycle. More recently LOSADA (17), following a different and independent reasoning, has pointed out the existence of two energy transducing redox systems exhibiting two redox couples of alternating potentials; those which energize their reduced form, lowering the potential of the pair, and those which energize their oxidized form, increasing the potential of the pair.

Detailed chemical mechanism of the synthesis of ATP. Figure 2 shows the proposed detailed chemical mechanism for the synthesis of ATP linked to the iron redox cycle of ATPase. Two of the six coordination bonds of each iron would be formed by functional groups of the enzyme, possibly sulfide ions belonging to sulfhydryl groups. The presence of up to 12 sulphurs in ATPase, eight as sulfhydryl groups, and the rest suggested as two disulfide bonds has been reported by SENIOR (24). The remaining coordination bonds of the set of two irons in the reduced state would be occupied by ATP, ADP, Pi and HX as indicated. The release of two electrons, one from each iron, would induce a series of electron shifts bringing about the synthesis of ATP. A nucleophilic attack by ligand ADP to the atom of phosphorus of ligand Pi would be facilitated by the displacement of electrons of this latter ligand towards the atom of iron in the new state of oxidation. The second atom of iron would attract the electrons from a ligand indicated as X. (The possible nature of this ligand will be discussed below.) As a consequence of this process, two protons would be eliminated, and accepted by specific groups B: (Lewis bases) of the enzyme. The abstraction of these protons by the enzyme would undoubtedly favor the phosphorylation reaction. The shortening of the atomic radius of the iron atoms when passing from Fe^{++} to Fe^{+++} would cause a conformational change of the protein, perhaps promoting the expulsion of these protons towards the outer face of the membrane; this seems to be an obliged requirement in order to regenerate the Lewis bases (B:) for the next oxidative phosphorylation cycle. The electron shifts would tend to weaken the bond between the right hand side iron and the formerly tightly bound ATP; the left hand side iron would also release one of the bonds which belonged to ligand ADP before its reaction with ligand phosphate of the right hand side iron. Two free OH^- would remain in the interior of the mitochondrion. The pH gradient thus formed between both sides of the membrane

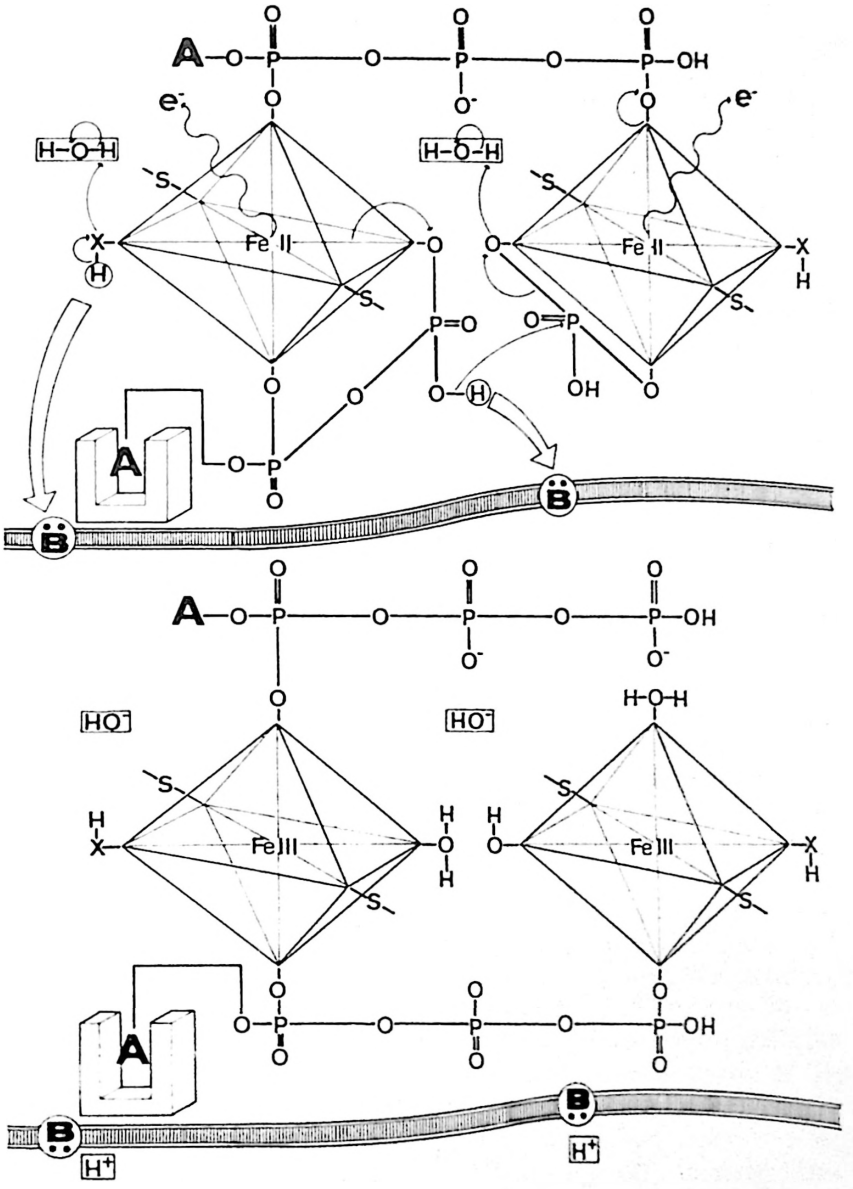

Fig. 2. *Chemical mechanism of oxidative phosphorylation.*
A stands for adenosine. In the lower part of the figure, A would be occupying its recognition site on the enzyme.

disappears as a consequence of the ATP^{4-}:ADP^{3-} exchange and the entry of a $H_2PO_4^-$.

As a consequence of the conformational change a separation of the two iron atoms could take place, forcing a rotation of them around the axes S-Fe-S since they are now bridged by the newly synthesized molecule of ATP (fig. 3). This rotation would also cause the liberation of the ATP from the recognition site of the adenosine moiety. The recognition site would now be ready to accept a new molecule of ADP. This new ADP would

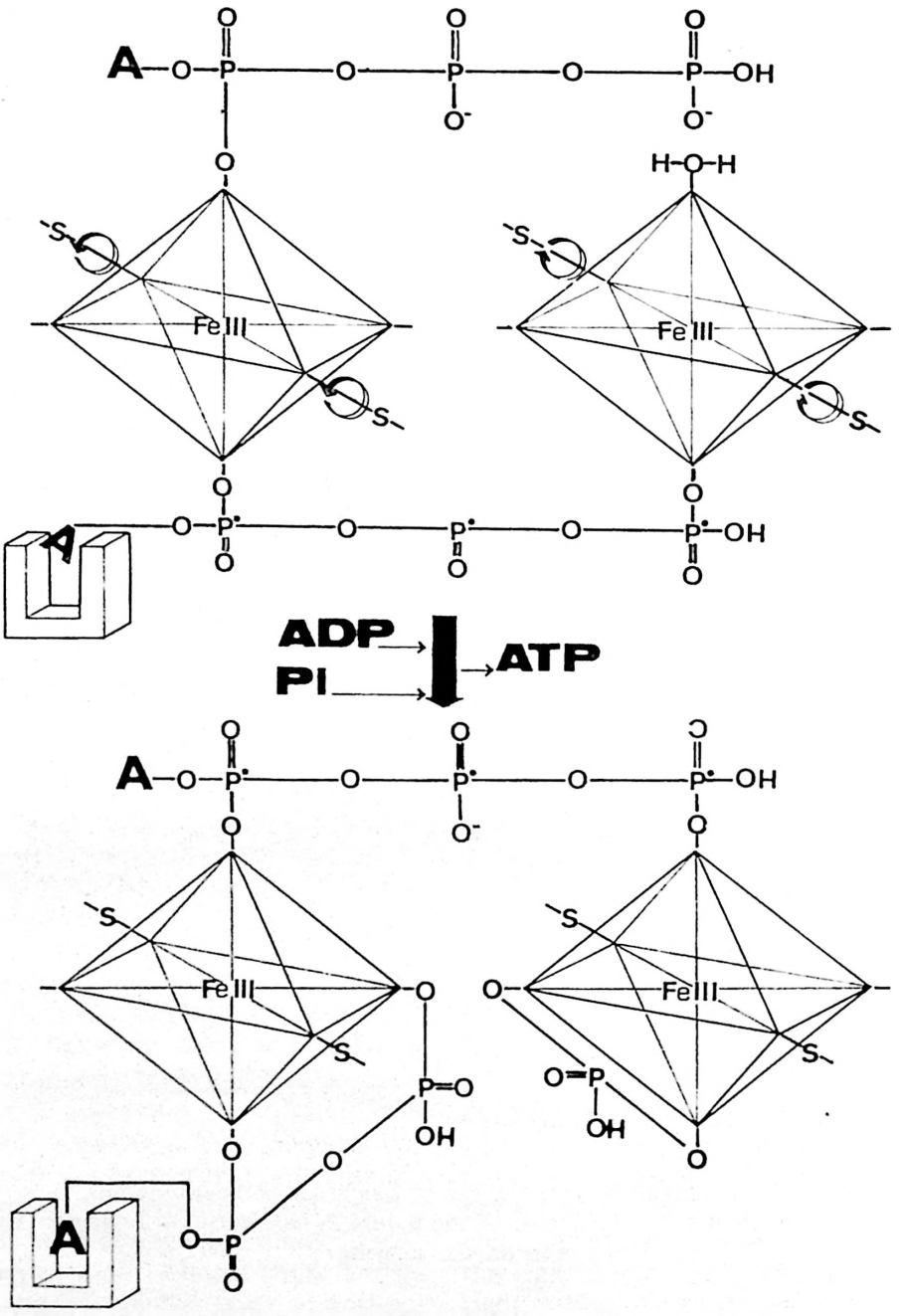

Fig. 3. *Sequential release of tightly bound ATP in the process of oxidative phosphorylation.*

now become a bidentate ligand of the iron on the left. At this point each one of the two irons would have completed a turn of 180°.

The presence of up to six atoms of iron in ATPase would be consistent with the operation of three cycles by three independent sets of iron pairs in the traversing of two electrons along the respiratory chain. Unpublished preliminary results

from our laboratory suggest that ATPase could operate within the three potential gaps of the respiratory chain. All that remains to be postulated is a different chemical structure for ligand X in each set of iron atoms. X could probably represent either components of the respiratory chain which could directly interact with the catalytic centers of ATPase, or functional groups of ATPase capable themselves of undergoing redox reactions.

Justification of the redox cycle in the light of available experimental data. The oxidative phosphorylation model here proposed may offer a coherent answer to the many questions posed by a wealth of experimental results obtained by different research groups. It also offers a possibility of bringing together many features of the main theories which have been advanced so far in an effort to explain the mechanisms of oxidative phosphorylation. In order to avoid an endless reference list only the data deemed as more relevant to these questions will be now considered.

1. According to the model proposed the H^+/site ratio (i.e. the number of protons ejected per pair of electrons traversing each of the energy-conserving sites of the respiratory chain) originated in one of the steps of the redox cycle is equal to 2 (fig. 2). These protons should be added to those originated by other sources, such as the proposed «loops» or through other suggested membrane Bohr effects recently reviewed (1). The latter point would be in agreement with recent findings which clearly demonstrate that, after careful measurements, values of H^+/site ratios of 3 and even as high a 4 can be obtained with intact mitochondria (2). 2. The observation made by ORT *et al.* (19) that ATP formation by chloroplast thylakoids precedes the establishment of a proton gradient or of a transmembrane electrochemical potential is consistent with the new model. 3. The iron redox cycle of ATPase might also offer a satisfactory answer to the problem of how the energy of electron flow can be used for purposes other than ATP synthesis. The transport of cations such as Ca^{++} to the interior of the mitochondrion (14) might serve as an example of this. It will suffice to replace the bidentate Pi ligand on the right hand side iron by two molecules of water (fig. 2). Obviously in this case ATP would not be synthesized, but two H^+ would still be transferred to the exterior of the mitochondrion. The pH gradient thus generated could be used for the transport of cations, instead of the obliged transport of nucleotides when phosphorylation is taking place. 4. The control of electron flow in respiring mitochondria, which is known to depend on the concentration of ADP (4), also appears to be reasonably explained by the new model. Ligand ADP would raise the standard reduction potential thus allowing the isopotential transfer of electrons from the donor to ATPase— the initial step of the postulated redox cycle. 5. The reverse flow of electrons, a process which requires the energy supplied by the hydrolysis of ATP (6), could also be explained through the operation of the redox cycle. The ADP produced in the catalytic center as a result of the ATP hydrolysis would cause a shift in the standard reduction potential towards more negative os less positive values necessary to donate the electrons at potentials more negative than those at which they were received. This reasoning could also explain the mechanism of energy dependent transhydrogenation catalyzed by ATPase (6). 6. The so-called «partial reactions» of oxidative phosphorylation (15) —ADP-ATP, ATP-Pi, ATP-$H_2{}^{18}O$ and Pi-$H_2{}^{18}O$ exchanges, and the ATPase activity— which are known to take place in the absence of a net flow of electrons along the chain could be interpreted in the light of the new model. A series of electron shifts in a set of two iron atoms duly justifies the exchanges

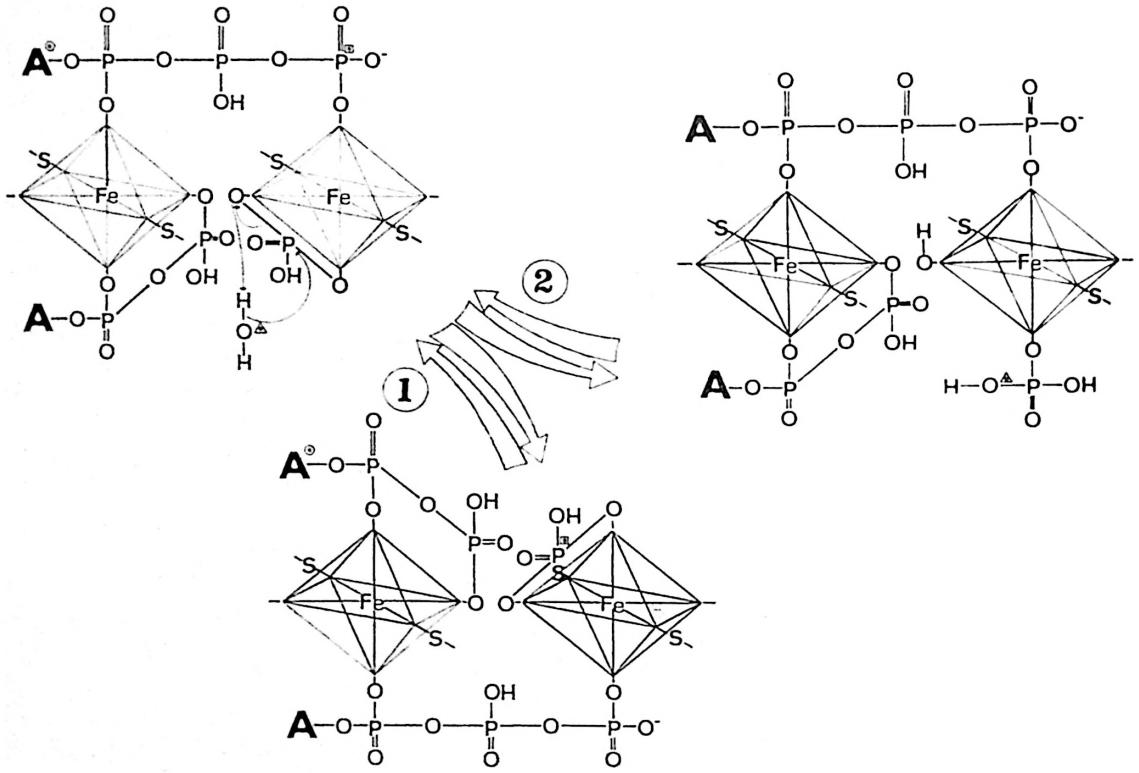

Fig. 4. *Mechanism of the so-called «partial reactions» of oxidative phosphorylation.*

observed experimentally. As shown in figure 4 the reversibility of reaction 1 would explain the ATP-ADP exchange. Reversibility of reaction 2 would explain the oxygen exchange between phosphate and water. The ATP-Pi exchange would be possible through reaction 1 followed by the free interchange of ligand phosphate with phosphate of the medium. The ATP-H_2O exchange could be explained through the association of reactions 2 and 1, together with an interchange of ligand ATP with ATP of the medium. The hydrolyzing activity of ATPase would be favored by Mg^{++} which would form a chelate with ligand ATP. This hydrolyzing activity would entail a continuous exchange of ligands ADP and Pi with ATP. 7. The observation made by PHELPS *et al.* (21) that low concentrations of bathophenanthroline inhibits the activity of soluble ATPase, and, moreover, that this inhibition is antagonized by ATP, led them to suggest the possibility that bathophenanthroline displaces ATP from a binding site with a regulatory function. Those authors (21) reported that no iron was detected in ATPase although in a note added to the article they state that: «the presence of iron in F_1 cannot be eliminated at this time». Since bathophenanthroline is a strong iron chelator these results would support our suggestion that iron could be the nucleotide binding site. 8. The finding of HARRIS *et al.* (10) that soluble ATPase contains up to 3 molecules of ATP and 2 molecules of ADP tightly bound to each enzyme complex would seem to support the type of interactions suggested by our model. The stoichiometry found would also be consistent with the presence of three catalytic centers each constituted by a set of two iron atoms. 9. The sug-

gestions of HARRIS *et al.* (11) that energy might be used to cause a release of tightly bound ATP would also support the dynamic aspects of the model described above; the oxidation of the set of iron atoms would induce not only the formation of a pyrophosphate bond leading to a molecule of ATP which would remain as a ligand, but also the release of the formerly tightly bound ATP. 10. The observations which led BOYER (1) to propose his «alternating site model» might also be satisfactorily interpreted at a molecular level through the dynamic mechanism now proposed which implies the conjoint rotation of the two irons of each catalytic center and the release of the adenosine moiety of the newly synthesized ATP from its recognition site (fig. 3).

In the light of the model proposed it now seems that Slater's early ideas (25) of the participation of a chemical intermediate in oxidative phosphorylation are not to be abandoned; furthermore, this view should be reexamined under a new perspective: the chemical intermediate would be the reduced form of ATPase with ligands ADP and Pi, ligands which would be committed to the synthesis of ATP.

Similar models to the one now postulated as the basis for the mechanism of oxidative phosphorylation could also contribute to our understanding of other energy transduction systems.

Acknowledgements

The authors are deeply indebted to Professor I. Rasines and Professor J. Iñiguez for their valuable comments and criticisms, and especially to Professor F. Ponz for his encouragement and helpful advice during the realization of this work. The participation of M. J. López-Zabalza, A. J. Iriarte, J. Huamán and M. Preciado in some of the experiments is gratefully acknowledged. Many thanks are also given to Mrs. Diana Dawid for her patience and comprehension in correcting the English version of the manuscript.

Resumen

Se propone un mecanismo para explicar a nivel molecular el proceso de fosforilación oxidativa. La síntesis de ATP tendría lugar en un centro catalítico de la ATPasa constituido por dos átomos de hierro, con ligandos ATP, ADP y Pi. El intercambio de una molécula de ATP por ADP y Pi hace posible un ciclo redox entre dos potenciales, permitiendo de este modo la transducción de energía.

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