Biochemistry & Physiology: Open Access

Reynafarje, Biochem Physiol 2014, 3:2
DOI: 10.4172/2168-9652.1000128

Research Article Open Access

The Electromechanical Mechanism of ATP Synthesis in the Presence of *In Vivo* Concentrations of Oxygen

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Abstract

The synthesis of ATP is undoubtedly the most important phenomenon that occurs in living organisms. The following experimentally determined facts are mechanistically significant. 1) Net synthesis of ATP only occurs during the extremely fast respiratory process in which cytochrome aa_3 undergoes net oxidation. 2) The hyperbolical processes of electron flow and O_2 reduction to water precede the sigmoidal process of ATP synthesis. 3) The exergonic process of O_2 consumption controls the level of ADP and the endergonic process of ATP synthesis, not vice versa. 4) The extent and rates of electron flow and O_2 uptake are the same in the presence or absence of ADP. 5) The rates of O_2 uptake and ATP synthesis are orders of magnitude higher in the presence of *in vivo* levels of O_2 than under state-3 metabolic conditions in the presence ~230 μM O_2. 6) The KM of cytochrome aa_3 for O_2 is close to 30 μM not below 0.5 μM. 7) The ATP/O ratio is not constant but changes from near zero to 3.4 exquisitely depending on the redox potential and the relative concentrations of cytochrome aa_3, O_2 and ADP. 8) Net ejection of H+ only occurs during the reduction of cytochrome aa_3 and the slow phase of O_2 uptake. It is concluded that the free energy responsible for the synthesis of ATP is not the protonmotive force but the structural changes that induced by the flow of electrons occur at the levels of cytochrome aa3 and ATP synthase.

Keywords: H^+ uptake; H^+ ejection; Cytochrome aa_3 oxidation; O_2 uptake; ATP synthesis

Abbreviations: ΔE_h: Redox Potential Difference; $\Delta\mu H^+$: Proton Electrochemical Difference; ΔG_P: Phosphorylation Potential Difference; F_1 and F_0: Hydrophilic and Hydrophobic Parts of the ATP Synthase; γ and β: Subunits of F_0

Introduction

To this day, the extent and rates of O_2 consumption and ATP synthesis are generally determined in reactions initiated by adding a large amount of ADP to oxidized mitochondria respiring in the presence of respiratory substrates and ~230 μM O_2 [1]. Under *in vivo* conditions, however, the concentration of O_2 inside the cell is no higher than 70 μM [2], and the process of ATP synthesis begins not with a sudden increment in ADP concentration but with the binding of O_2 to mitochondria already charged with ADP.

In reality, the net synthesis of ATP only occurs during the very short and extremely fast period of oxidative phosphorylation in which O_2 is hyperbolically reduced to water at the level of the cytochrome aa_3. The process of ATP synthesis begins with the oxidation of cytochrome aa_3 by O_2 that, driven by a net gradient of O_2 concentrations, enters the mitochondria already charged with ADP [3,4]. Thus, in these experiments the synthesis of ATP was initiated by adding from 0.23 to 60 μM O_2 to reduced forms of mitochondria in the presence of different levels of ADP (near zero to 300 μM).

The processes of all, electron flow, H^+ uptake, H^+ ejection, cytochrome aa_3 oxidation, O_2 uptake and ATP synthesis were determined from the first milliseconds to the end of the process of oxidative phosphorylation. It was found that a strict kinetic and thermodynamic correlation between O_2 uptake and ATP synthesis only occurs during the elusive and extremely fast initial phase of the respiratory process, which in classic oxygen-pulse experiments [5] was commonly considered to be an "experimental artifact". It is concluded that, regardless of the experimental conditions, the fundamental form of energy involved in the endergonic process of ATP synthesis is not the free energy of a proton gradient but the structural changes that induced by the free energy of electron flow occur at the levels of cytochrome aa_3 and ATP synthase [6-8].

Experimental Procedures

Materials

Cytochrome c oxidase from bovine heart embedded in liposomes was prepared as previously reported [9]. Rat liver mitochondria (RLM) and sub-mitochondrial particles (SMP) were prepared as described in a previous publication [10]. The standard reaction mixture, at 25°C, contained 200 mM sucrose, 50 mM KCl, 10 mM Na-KPi, pH 7.05, 2 mM $MgSO_4$, 5.0 μl of a mixture of luciferin/luciferase (a product of Bio Orbit) dissolved in 5.0 ml of standard medium, and either 5 mM NADH, 10 mM succinate or 100 μM cytochrome c plus 10 mM ascorbate.

Equipment

A Luminometer made by Man-Tech Associates. Inc. was used to detect the presence of ATP in reaction mixtures. A fast responding O_2 electrode [11], a pH electrode and its reference electrode were fitted inside the airtight-closed chamber of the luminometer to simultaneously determine the processes of O_2 uptake, H^+ translocation, and ATP synthesis. A stirring devise placed at the bottom of the chamber was used to mix the components of the medium. The electrical outputs of all, luminometer, fast responding O_2 electrode and pH electrode were fed into a multi-channel recorder running at a rate of 2 cm/second.

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Received February 05, 2014; **Accepted** March 03, 2014; **Published** March 10, 2014

Citation: Reynafarje BD (2014) The Electromechanical Mechanism of ATP Synthesis in the Presence of *In Vivo* Concentrations of Oxygen. Biochem Physiol 3: 128. doi: 10.4172/2168-9652.1000128

Calibrations

The extent of ATP synthesis was calculated by comparing the recorded size of the trace with a standard curve prepared by adding from 0.001 to 100 μmoles of ATP to standard reaction mixtures containing either isolated cytochrome aa_3 or heat-denatured forms of mitochondria [12]. A plot of the intensity of light emission versus ATP concentration resulted in a straight line that intercepted the coordinates at the near origin. The very small fraction of ATP used by the luciferin/luciferase reaction during the process of light emission was insignificant under current experimental conditions. The rates of ATP synthesis were determined during the steepest portion of the sigmoidal process of ATP synthesis [4]. The amount of O_2 consumed was determined by subtracting the amount of O_2 consumed at any point of the reaction from the amount of O_2 added and comparing the size of the trace with the size of a standard curve obtained by adding O_2 to anaerobic standard-reaction mixtures [13]. The magnitude of ΔGp was evaluated by determining the difference between the ratio of products and substrates at the beginning and the equilibrium of every reaction [14]. Thus, in the process of ATP synthesis:

$$DG_p = RT \ln [ATP]^a [S]^b/[ADP]^c [Pi]^d [O_2]^e [SH2]^f - RT \ln K_{eq}$$

in which, $RT \ln K_{eq}$ is the standard free energy change of ATP hydrolysis at equilibrium. S and SH_2 represent, respectively, the oxidized and reduced forms of the respiratory substrates. The coefficients of ATP, S, ADP, PI, O_2, and SH_2 are represented by *a, b, c, d* and *f,* respectively. Because the changes in substrate concentration that occur during the actual synthesis of ATP are practically negligible, the value of ΔGp was calculated considering that the SH_2/S ratio is 1.0. The standard free-energy change of NADH oxidation was considered to be -52.6 kcal/mol and that of ATP hydrolysis equal to +7.3 kcal/mol.

Methods

Reactions were initiated by injecting different forms of mitochondria into a tightly closed chamber containing a standard reaction mixture in the presence of respiratory substrates and close to 230 μM O_2. After a period of incubation of about 25 min, when every trace of O_2 and ATP completely disappeared from the medium, the oxidative phosphorylation process was initiated by injecting from 0.10 to 60 μM O_2 to anaerobic and fully reduced suspensions of mitochondria. The consumption of O_2, the uptake of scalar H^+, the ejection of vectorial H+, and the synthesis of ATP were recorded from the first milliseconds to the end of the entire process of oxidative phosphorylation. The possibility of a contamination of the medium with the ATP synthesized by the activity of enzymes such as adenylate kinase or nucleoside monophosphate kinase was discarded because in the absence of O_2 there were no traces of ATP [15].

Results

Kinetic and thermodynamic correlation between the oxidative phosphorylation processes of O_2 consumption and ATP synthesis

Figure 1 shows the simultaneously determined processes of O_2 consumption and ATP synthesis in a reaction initiated by adding 2.3 μM O_2 (4.6 nmols O) to an anaerobic and fully reduced suspension of RLM (0.15 mg protein) in the presence of ADP, NADH and succinate. The figure shows the following novel facts:

a) Net synthesis of ATP only occurs during the initial phase of the polyphasic process of O_2 consumption [16-18].

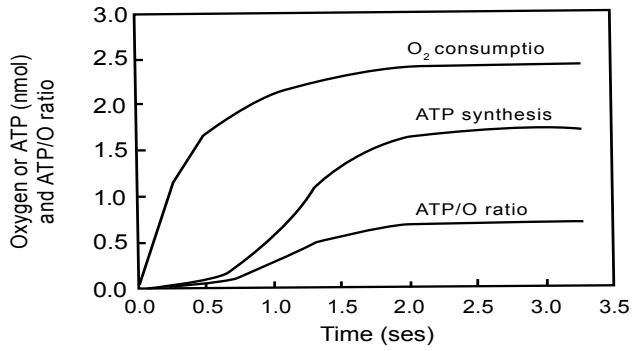

Figure 1: The processes of O_2 consumption and ATP synthesis are polyphasic in nature. The reaction was initiated by adding 4.6 nmols of O (2.3 μM O_2) to a fully reduced suspension of RLM (0.15 mg of protein) in the presence of 300 nmols of ADP and 5 mM of each NADH and succinate.

b) The hyperbolical processes of electron flow and O_2 consumption precede the sigmoidal process of ATP synthesis. The amount of O_2 consumed during the first milliseconds of the reaction accounts for more than 36% of the amount of O_2 initially present. The amount of ATP formed during the same period only accounts for less than 10% of totally formed.

c) The initial rate of O_2 consumption is higher than 1,700 nmols O min^{-1} mg^{-1} of protein.

d) The rate of ATP synthesis during the fastest portion of the reaction is close to 750 nmols min^{-1} mg^{-1} protein.

e) The net synthesis of ATP ceases when the amount of O_2 consumed is only 53% of the initially present (2.42 out of 4.6 nmols O).

f) Under this *in vivo* concentration of O_2 the ATP/O ratio changes from near zero to a maximum of 0.71 (1.71/2.42).

Effect of ADP concentration on the amount of O_2 consumed during the process of ATP synthesis

Data presented in Figure 2 show that the amount of O_2 consumed during the first phase of the respiratory process, which is directly involved in the process of ATP synthesis, is not at all affected by the initial concentration of ADP. Thus, in reactions catalyzed by homogenates of whole liver in the presence *in vivo* levels of O_2 (0.46 to 18.4 nmols O) the extent of O_2 consumed during the synthesis of ATP increases from 0.22 to 7.9 nmols O, whether the level of ADP is nil (less than 2.3 nmols of only endogenous) or 250 nmols of externally added.

For the same extent of O_2 consumption, the extent of ATP synthesis increases from 0.22 to 9.4 nmols in the presence of 250 nmols of added ADP, and from only 0.001 to 0.16 nmols in the presence of endogenous ADP (<2.3 nmols). The ATP/O stoichiometry increases from 0.003 to 0.02 in the presence of endogenous ADP and from 0.91 to 1.2 in the presence of 250 nmols of ADP.

Effect of O_2 concentration on the K_M of cytochrome aa3 for O_2

Data presented in Table 1 and Figure 3 show that the K_M of cytochrome aa_3 for O_2 is close to 30 μM, i.e. orders of magnitude higher than that observed under classic state-3 metabolic conditions in the presence of ~230 μM O_2 [17]. Although, the K_M of cytochrome aa_3 for O_2 is independent of the ΔE_h, the form of mitochondria (SMP or homogenate or whole liver) and the concentrations of O_2 (0.115 to 10

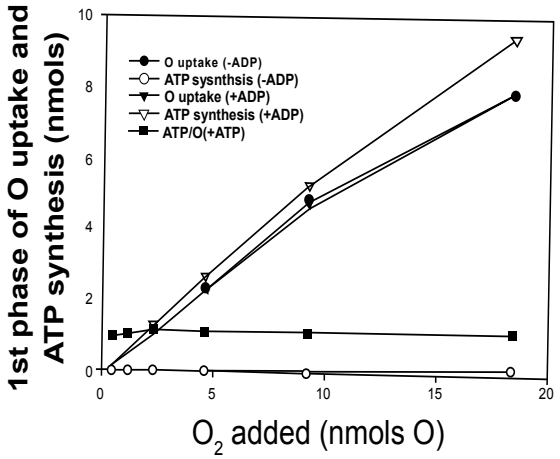

Figure 2: The respiratory process of O_2 consumption controls the phosphorylative process of ATP synthesis. Reactions were initiated by adding from 0.46 to 18.4 nmols of O (0.23 to 9.2 µM O_2) to homogenates of Pig liver (10 mg of protein) in the presence of 5 mM NADH, 10 mM succinate and either less than 2.3 nmols of endogenous ADP or 250 nmols of externally added ADP. The extent of the net synthesis of ATP was calculated at the end of the hyperbolical phase of O_2 consumption (Figure 1).

O2 added (nmoles O)	Liver homogenates	Sub-mitochondrial particles
	(µmol min-1 mg-1 protein)	
0.23	3.509	
0.575	8.475	
1.15	16.667	
2		6.02
2.3	32.258	
2.5		7.41
4.6	58.824	
5		13.69
7.5		19.61
9.2	83.33	
10		24.39
20		38.46

Table 1: Correlation between O_2 concentration and rates of O_2 consumption. Reactions were catalyzed by either 10 mg protein of homogenates of Pig liver or 0.1 mg protein of SMP in the presence of 5 mM NADH and 10 mM succinate. The rates of O_2 consumption were determined during the initial and hyperbolical phase of O_2 consumption. Values are averages of at least 2 determinations performed in the presence or absence of externally added ADP (Figure 2).

µM), ADP (<2.3 or 250 nmols) and cytochrome aa_3 (0.1 or 10 mg of protein), the maximal rates of O_2 uptake exquisitely depend on all these factors. Thus, in the same range of O_2 concentrations the V_{max} of O_2 consumption is 105 µmoles min^{-1} mg^{-1} protein in reactions catalyzed by homogenates of whole liver, and 500 µmoles min^{-1} mg^{-1} protein in those catalyzed by SMP.

Effect of ΔE_h, O_2 and ADP concentration on the rates of ATP synthesis

Data in Figure 4 show that the rates of ATP synthesis in reactions catalyzed by RLM in the presence of either NADH or cytochrome c, depend on all, the ΔE_h and the initial concentrations of O_2 (0.46 to 12.5 µM) and ADP (25 or 100 µM). The rates of ATP synthesis in the presence of extremely low levels of O_2 are identical in the presence of cytochrome c than in the presence of NADH. In the range of O_2 concentrations from 0.46 to 11 µM, the rates of ATP synthesis are higher in the presence of cytochrome c and 100 nmols of ADP than in the presence of NADH and 25 nmols of ADP, i.e., higher at the lowest than the highest ΔE_h. Only at high levels of both O_2 and ADP the rates of ATP synthesis can attain values that are up to 3.6 times higher in the presence of NADH than in the presence of cytochrome c.

Effect of the ΔE_h and the concentrations of O_2 and ADP on the ATP/O stoichiometry

Data in Figure 5 show that the value of the ATP/O stoichiometry is not constant [19,20] but increases from 0.1 to 3.4 intricately depending on the all, the ΔE_h and initial concentrations of O_2 (0.23 to 15 µM) and ADP (25 or 100 µM). The results presented in Figure 5 show that under *in vivo* levels of O_2 the ATP/O ratio can be up to 10 times higher in the presence of cytochrome c and high levels of ADP (100 nmols) than

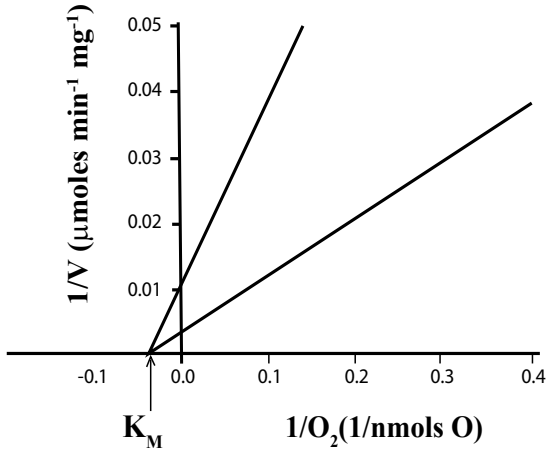

Figure 3: The KM of cytochrome aa_3 for O_2 is close to 30 µM. Reactions were catalyzed by either 0.1 mg of SMP protein or 10 mg of homogenates of Pig liver protein, in the range of O_2 concentrations from 0.23 to 20 nmols O, in the presence of 5 mM NADH, 10 mM succinate and either the absence or presence of 250 nmols of externally added ADP (Table 1).

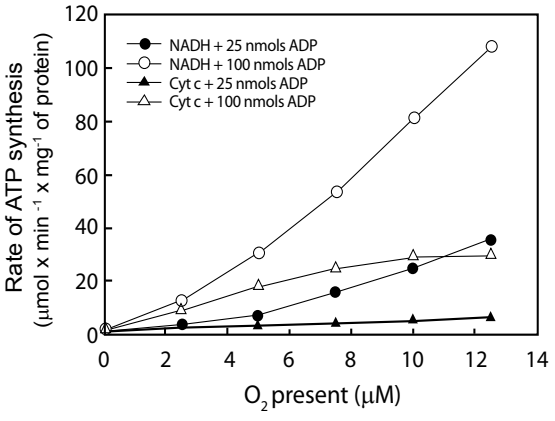

Figure 4: The rates of ATP synthesis depend on all, ΔE_h, degree of reduction of the mitochondrial membrane and initial levels of O_2 and ADP. The rates were determined during the steepest portion of the sigmoidal process of ATP synthesis (Figure 1) in reactions initiated by adding from 0.5 to 12.5 µM O_2 to RLM (0.15 mg protein) in the presence of either 25 or 50 nmols of ADP, 5 mM NADH or 100 µM cytochrome c plus 10 mMascorbate.

in the presence of NADH and low levels of ADP (25 nmols). Only at high levels of both O_2 and ADP the ATP/O stoichiometry can be close to 2.4 times higher in the presence of NADH than in the presence of cytochrome c.

Effect of the relative concentrations of O_2 and cytochrome aa_3 on the amount of O_2 consumed during the process of ATP synthesis

Figure 6 shows the effect of the relative concentrations of O_2 and cytochrome aa_3 on the respiratory process of O_2 consumption that is directly involved in the process of ATP synthesis. The extents of O_2 and H^+ uptake were measured at the end of the hyperbolical phase of O_2 consumption (Figure 1) in oxygen-pulse experiments initiated by adding from 0.23 to 30 μM O_2 to fully reduced suspensions of isolated cytochrome aa_3 (0.2 to 2.3 nmols) embedded in liposomes. Maximal values of O_2 and H+ uptake are only attained at an O/cytochrome aa_3 ratio of 20.0 At any O/cytochrome aa_3 ratio lower or higher than 20 the extents of both O_2 and H^+ uptake are greatly impaired. The H^+/O uptake-ratio, however, is always 2.0. The mechanistically significance of these results is discussed.

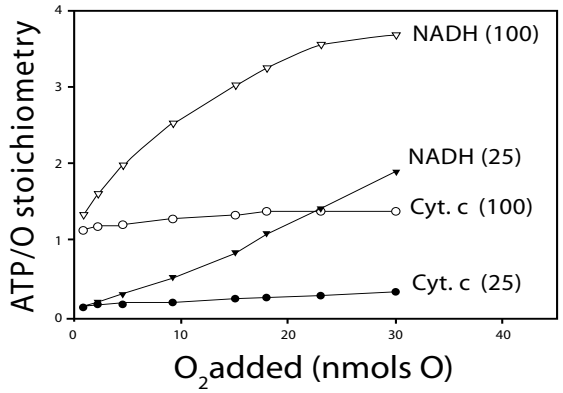

Figure 5: The ATP/O stoichiometry changes depending on all, ΔE_h and initial levels of O_2 and ADP. The ATP/O ratio was evaluated by determined the extents of ATP synthesis and O_2 consumption at the moment in which both the hyperbolical process of O_2 consumption and the sigmoidal of ATP synthesis cease. The experimental conditions were like those described for Figure 4.

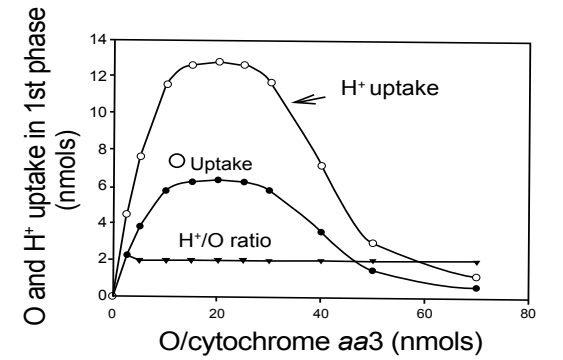

Figure 6: Maximal rates and extents of O_2 and H^+ uptake are limited by the O_2/cytochrome aa_3 ratio. Extents of O_2 and H^+ uptake were determined during the hyperbolical phase of O_2 uptake in reactions initiated by adding from 2.76 to 18.4 nmols of O (1.38 to 9.4 μM O_2) to fully reduced suspensions of isolated cytochrome aa_3 (0.2 to 2.3 nmols) embedded in liposomes in the presence of 10 mMascorbate and 60 μM cytochrome c plus 10 mMascorbate.

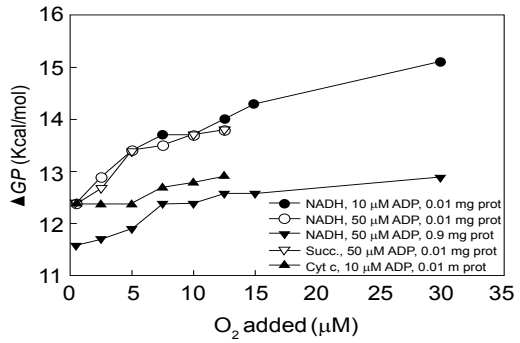

Figure 7: The ΔGp attains maximal efficiency in the absence of proton gradients. The ΔGp was determined in reactions initiated by adding from 0.23 to 30 μM O_2 to frozen/thawed and inverted vesicles from SMP (0.01 or 0.9 mg) in the presence of each 5 mM NADH, 10 mM succinate or 100 μM cytochrome c plus 10 mMascorbate, supplemented with either 10 or 50 nmols of ADP.

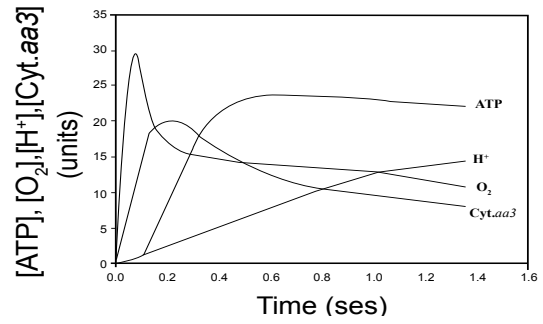

Figure 8: Time courses of the processes of H^+ ejection, O_2 consumption, cytochrome aa_3 oxidation and ATP synthesis. Reactions were initiated by adding 9.2 nmols O to fully reduced samples RLM (3.5 mg protein) in the presence of 5 mM NADH and 50 μM ADP. Every unit in the y-axis represents 0.24 nmols of O, 3.37 nmols of H^+ ejection, 0.186 nmols of ATP, and a ΔA of 1.2×10^{-4} at 606-630 nm.

Effect of protein (cytochrome aa_3) concentration on the magnitude of the phosphorylation potential (ΔGp)

Data in Figure 7 provides experimental evidence that, regardless of ΔE_h and ADP concentration, the magnitude of the ΔGp is an exquisite function of the relative concentrations of O_2 and protein, i.e. O/cytochrome aa_3 ratio. In the presence of 0.01 mg of SMP protein the efficiency of ΔGp increases from 12.4 to 13.8 kcal per mol of O_2 when, in the presence of either NADH or succinate, the concentration of O_2 increases from 0.23 to 12.5 μM. In the presence of 0.01 mg of SMP protein and cytochrome c plus ascorbate the ΔGp increases from 12.4 to only 12.8 kcal per mol. Distinctly, in the range of O_2 concentrations from 0.23 to 30.0 μM the magnitude of the ΔGp increases from 12.4 to 15.1 kcal/mol in the presence of 0.01 mg of protein and from only 11.6 to 12.8 kcal/mole in the presence of 0.9 mg of SMP protein. It is evident that the magnitude of the ΔGp is a sensitive function of the O_2/protein or O_2/cytochrome aa_3 ratio.

Kinetic and thermodynamic correlation between H^+ ejection, O^2 consumption, cytochrome aa_3 oxidation and ATP synthesis

Data in Figure 8 shows the time course of the respiratory processes of H^+ ejection, O^2 consumption, cytochrome aa_3 oxidation and ATP synthesis in reactions initiated by adding 9.2 nmols O to fully reduced suspensions of 3.5 mg of RLM protein in the presence of NADH and

50 μM ADP [4]. Note that the net synthesis of ATP ceases at the time that extremely fast phases of O^2 consumption and cytochrome aa_3 oxidation cease. The extent of H^+ ejection is not related to the synthesis of ATP but increases coinciding with the reduction (not the oxidation) of cytochrome aa_3 and the slow phases of O^2 consumption and ATP hydrolysis.

Data in Table 2 demonstrate that, in reactions catalyzed by fully reduced cytochrome aa_3 embedded in liposomes, the extent of H^+ ejection increases from 2.4 to 27.6 when the concentration of cytochrome aa_3 increases from 0.2 to 2.3. On the contrary, the extent of H^+ ejection decreases from 27.6 to 2.4 when the O/cytochrome aa_3 ratio increases from 15.9 to 250. The number of H^+ ejected per atom of O^2 consumed is the exclusive function of the concentration of cytochrome aa_3, increasing from 0.06 to 0.75 when the concentration of cytochrome aa_3 increases from 0.2 to 2.3, in such a way that the H^+/cytochrome aa_3 ratio is always 12.0.

Discussion

To this day the consensus is that, regardless of the ΔE_h and the actual concentrations of protein (cytochrome aa_3), O_2 and ADP, the processes of O_2 consumption and ATP synthesis maintain at all times a strict kinetic and thermodynamic correlation. Consequently, maximal rates of O_2 consumption and ATP synthesis are generally determined in reactions initiated by adding ADP to oxidized mitochondria respiring in the presence of abnormally high levels of O_2 [1].

Under *in vivo* conditions, however, the synthesis of ATP begins not with a sudden increment in ADP concentration but with the net diffusion of O_2 from the cytosolic to the matrix side of nearly anaerobic and fully reduced mitochondria already charged with ADP. The experimental results presented in this study provide the unmistakable evidence of the following mechanistically significative facts:

1. A strict kinetic and thermodynamic correlation between O_2 consumption and ATP synthesis only occurs during the elusive and extremely fast initial phase of a polyphasic process of oxidative phosphorylation. As previously demonstrated [4,16] the actual synthesis of ATP takes place during the first milliseconds of the process of oxidative phosphorylation, precisely coinciding with the period in which cytochrome aa_3 undergoes net oxidation (Figures 1 and 8).

2. The exergonic and hyperbolical processes of electron flow and O_2 consumption precede the endergonic and sigmoidal process of ATP synthesis. Data in Figure 1 contradicts the textbook assertions that "electrons do not usually flow through the electron transport chain to O_2 unless ADP is simultaneously phosphorylated to ATP", and that "the most important factor in determining the rate of ATP synthesis is the level of ADP" [21]. In reality the most important factor in controlling the extent and rates of ATP is not the level of ADP but the relative concentrations of O_2 and cytochrome aa_3.

Cytochrome aa_3 (nmols)	O/cytochrome aa_3 (ratio)	H^+ ejection (nmols)	H^+/O ejection (ratio)	H^+/cytochrome aa_3 (ratio)
0.2	250	2.4	0.06	12
0.6	62.5	7.2	0.19	12
1.2	30	14.4	0.39	12
2.3	15.9	27.6	0.75	12

Table 2: Correlation between the maximal ejection of vectorial H^+ and the relative concentrations of O_2 and cytochrome aa_3.
Reactions were catalyzed by adding in vivo levels of O_2 to fully reduced suspensions of cytochrome aa_3 embedded in liposomes. The extent of H^+ ejection was determined at the end of every reaction. Values are average of at least 2 experiments.

3. The level of ADP has no effect on the extent of O_2 consumed during the actual process of ATP synthesis. Since 1956, when Chance and Williams [1] described the classic state-3 metabolic state of mitochondria, it is firmly believed that the extent and rates of O_2 consumption are controlled by the level of ADP and the process of ATP synthesis. Although the binding of ADP to oxidized mitochondria induces a reduction on the inner mitochondrial membrane and cytochrome aa_3, thus facilitating the rates of O_2 consumption, the truth is that under *in vivo* levels of O_2 (<60 μM) the level of ADP has absolutely no effect on the rates of O_2 uptake (Figure 2). In fact, the exergonic processes ofelectron flow, cytochrome aa3 oxidation and O_2 reduction to water control the level of ADP and the endergonic process of ATP synthesis, not vice versa [18].

4. The K_M of cytochrome aa_3 for O_2 is close to 30 μM. Data presented in Table 1 and Figure 3 demonstrate that, regardless of the ΔE_h, the form of mitochondria (SMP or homogenate or whole liver), and the concentrations of O_2, ADP and protein (cytochrome aa_3), the K_M of cytochrome aa_3 for O_2 is from 60 to 600 times higher than previously reported values [17]. The reason for this discrepancy is that the K_M for O_2 was previously determined at the end of reactions that occurred in the presence of abnormally high levels of O_2 when the rates of O_2 uptake were not the exclusive function of O_2 concentration and did not obey first order kinetics [17]. The real K_M of cytochrome aa_3 for O_2 can only be determined the instant in which O_2 binds to fully reduced cytochrome aa_3 and the rates of O_2 consumption depend, exclusively, on O_2 concentration strictly obeying first order kinetics. Distinctly, the maximal rate (V_{max}) of O_2 consumption is a delicate function of the ΔE_h and the O_2/cytochrome aa_3 ratio, decreasing when this ratio is either lower or higher than 10 (Figure 4).

5. The rates of ATP synthesis are orders of magnitude higher in the presence of *in vivo* levels of O_2 than under classic state-3 metabolic conditions. The consensus is that maximal rates of ATP synthesis can only be attained under state-3 metabolic conditions in the presence of nearly 230 μM O_2 [1]. Distinctly, data in Figure 4 show that the rates of ATP synthesis change intricately depending on ΔE_h and actual concentrations of cytochrome aa_3, O_2 and ADP. Thus, at *in vivo* levels of O_2 between zero and 10 μM the rates of ATP synthesis are significantly higher in the presence of cytochrome c and high levels of ADP than in the presence of NADH and low levels of ADP. Only at high levels of both, O_2 and ADP, the rates of ATP synthesis can attain values that are more than 3.6 times higher in the presence of NADH than in the presence of cytochrome c alone. Obviously, the actual rates of O_2 consumption and ATP synthesis are orders of magnitude higher in the presence of *in vivo* levels of O_2 than under classic state-3 metabolic conditions in the presence of ~230 μM.

6. The ATP/O stoichiometry is not constant but normally changes from near zero to a maximum of 3.4. For the first time, the results presented in Figure 5 provide clear experimental evidence that the ATP/O stoichiometry is not constant but changes depending on the energy demands of the cell and the magnitude of the ΔE_h and the relative concentrations of O_2 and ADP. Under resting conditions, when the free energy of electron flow is just enough to maintain the homeostasis of the cell and the intra mitochondrial concentrations of O_2 and ADP are very high, the ATP/O stoichiometry is most likely no much higher than 1.0. Only under conditions of maximal energy expenditure, like intense physical exercise, the ATP/O ratio can attain a value of ~3.4.

Considering that the ATP/O stoichiometry in the presence of NADH is always 3.0 [19,20-23], the impressive assertion has been made that the "cell energy cycle may turn over at rest as much as half

an adult's body weight in ATP per day, and many times more during physical exercise or work" [24]. If this assertion were consistent with the fact, the efficiency of the cell to generate useful forms of energy would be abnormal. In reality, the processes of O_2 consumption and ATP synthesis are controlled by brain chemoreceptors and reflexes [22] and the ATP/O stoichiometry normally changes from near zero to a maximum of about 3.4.

7. The amount of O_2 consumed during the processes of ATP synthesis is limited to a narrow range of O_2 per cytochrome aa3 ratios. Data in Figure 6 demonstrate that the amount of O_2 consumed in strict kinetic and thermodynamic correlation with the process of ATP synthesis exquisitely depends on the concentrations of O_2 and cytochrome aa_3. Whether the process of O_2 consumption takes place in the absence or presence of ADP, the amount of O_2 consumed in the process of ATP synthesis depends on the O_2/cytochrome aa_3 ratio.

At O_2/cytochrome aa_3 ratios lower than 10 the free energy of electron flow is reduced due to limitations in O_2 concentration. At O_2/cytochrome aa_3 ratios higher than 10, the free energy of electron flow is most likely reduced by the excess of O_2 and the impairing concentrations of O_2 radicals.

8. The magnitude of the phosphorylation potential (ΔGp) is an exquisite function of the O_2/cytochrome aa_3 ratio. As shown in Figure 6, data in Figure 7 demonstrate that the magnitude of the ΔGp is an exquisite function of the O/cytochrome aa_3. These results provide experimental evidence that, regardless of ΔE_h and the concentrations of respiratory substrates and ADP, the actual concentrations of O_2 and cytochrome aa_3 are the most fundamental factors in the process of ATP synthesis.

9. The vectorial ejection of H^+ is neither kinetically nor thermodynamically related to the process of ATP synthesis. The consensus is that the vectorial ejection of H^+ precedes the process of ATP synthesis [5,25-27]. However, data in Figures 1 and 8 provide experimental evidence that the net synthesis of ATP follows rather than precedes the respiratory processes of cytochrome aa_3 oxidation and O_2 consumption. Regardless of the level of ADP, the process of H^+ ejection takes place coinciding not with the oxidation but the reduction of cytochrome aa_3.

10. The mechanisms of ATP synthesis and ATP hydrolysis are kinetically and thermodynamically different. It is firmly believed that the main form of energy involved in the process of ATP synthesis is the protonmotive force of H^+ ejection [5], and that the mechanisms of ATP synthesis and ATP hydrolysis are the same [28-30].

The results of this study demonstrate, however, that inverted vesicles from SMP can generate ΔGp with maximal efficiency in the absolute absence of a protonmotive force, Δp (Figure 7). It is thus mechanistically significative that the catalytic sites of the F1-moiety are kinetically dependent on each other during the sigmoidal synthesis of ATP but are kinetically equivalent during the hyperbolical hydrolysis of ATP [12]. In fact, while the endergonic process of ATP synthesis depends on the process of O_2 consumption, the exergonic process of ATP hydrolysis is independent of this process.

The hypothetical scheme depicted in Figure 9 considers that the electromechanical mechanism of ATP synthesis that occur at the levels of the γ and β subunits of the ATP synthase may be the exclusive function of the free energy of electron flow [31,32]. During the endergonic process of ATP synthesis the free energy of electron flow induces a counterclockwise rotation of the γ subunit that is tightly coupled to the clockwise rotation of the β subunits and the consequent synthesis of ATP. Distinctly, during the hydrolysis of ATP, the clockwise rotation of the γ subunit is not coupled to the clockwise rotation of the β subunits and the process of ATP synthesis.

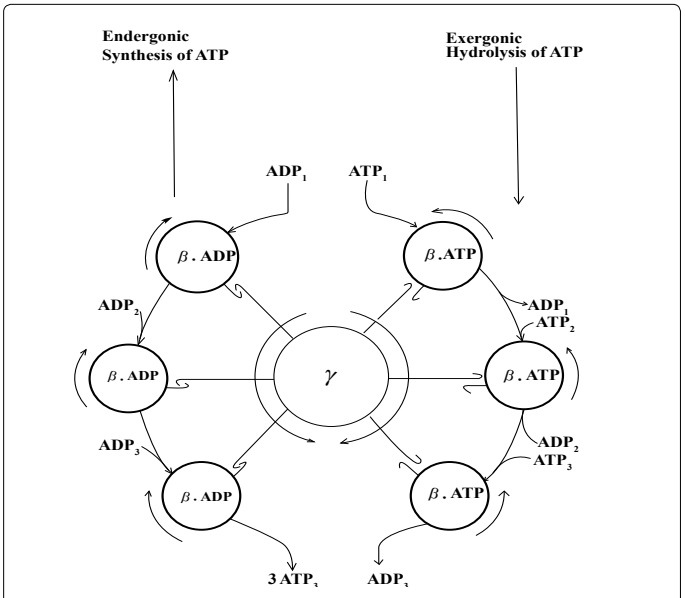

Figure 9: Hypothetical scheme showing the reverse rotation of the γ and β sub-units of the ATP synthase during the processes of ATP synthesis and ATP hydrolysis.

The validity of this interpretation is substantiated by the experimental evidence that the rates of ATP synthesis in guinea pigs native to high altitudes [33] are higher than in those from see level, and much lower in cancer derived AS30D hepatocytes than in normal hepatocytes (unpublished observations).

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