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**Mitochondrial respiratory control:
a conceptual perspective on coupling states in mitochondrial preparations.
MITOEAGLE recommendations Part 1**

MITOEAGLE Terminology Group

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Abstract

Clarity of concepts and consistency of nomenclature is a hallmark of the quality of a research field across its specializations, aimed at facilitating transdisciplinary communication and education. As mitochondrial physiology continues to expand, the necessity for improved harmonization of nomenclature on mitochondrial respiratory states and rates has become apparent. Peter Mitchell's protonmotive force across the inner mitochondrial membrane establishes the link between electron transfer and phosphorylation of ADP to ATP, and between the electric and chemical components of energy transformation. This unifying concept provides the framework for developing a consistent terminology on mitochondrial physiology and bioenergetics. We follow IUPAC guidelines on general terms of physical chemistry, extended by concepts of nonequilibrium thermodynamics and open systems. The nomenclature of respiratory states in classical bioenergetics (States 1 to 5 in an experimental protocol) is incorporated into a concept-driven constructive terminology to address the meaning of each respiratory state and to focus primarily on the conceptual 'why' with clarification of the experimental 'how'. LEAK states are evaluated to study arrested respiration, when oxygen consumption compensates mainly for the proton leak. OXPHOS capacity is measured at saturating concentrations of ADP and inorganic phosphate to obtain kinetic reference values for diagnostic applications. The oxidative capacity of the electron transfer system is determined in the ETS state, revealing the limitation of OXPHOS capacity mediated by the capacity of the phosphorylation system. Application of strictly defined terms for comparison of respiratory states is necessary for the development of databases of mitochondrial respiratory control.

Keywords: Mitochondrial respiratory control, coupling control; mitochondrial preparations, protonmotive force, chemiosmotic theory, oxidative phosphorylation, efficiency; electron transfer system, ETS; proton leak, LEAK; residual oxygen consumption, ROX; State 2, State 3, State 4.


- * Does  public expect that biologists understand Darwin's theory of evolution?
- * Do students expect that researchers of bioenergetics can explain Mitchell's theory of chemiosmotic energy transformation?

1. Introduction

Every study of mitochondrial function and disease in tissues and cells is faced with evolution, age, gender, lifestyle and environment (EAGLE) as essential background conditions characterizing the individual patient or subject, cohort, species, tissue and even cell line. As a large and highly coordinated group of laboratories and researchers, the global MITOEAGLE network is uniquely poised to generate the necessary scale, type, and quality of consistent data sets to address this intrinsic complexity. The mission of the MITOEAGLE network aims at developing harmonized experimental protocols and implementing a quality control and data management system to interrelate results obtained in different studies and to generate a rigorously monitored database focused on mitochondrial respiratory function.

Reliability and comparability of quantitative results depends on the accuracy of measurement under well-defined conditions. A conceptually meaningful framework is required to relate the results carried out by different research groups. Vague or ambiguous terminology can lead to confusion and may relegate valuable signals to wasteful noise. For this reason, measurements must be expressed in common units on each well-defined attribute for mitochondrial respiratory control. Standardization of nomenclature and technical jargon is an optimistic goal that can improve the awareness of the intricate meaning of divergent scientific vocabulary. The MITOEAGLE Terminology Group aims at accomplishing the ambitious goal to harmonize, unify and thus simplify the terminology in the field of mitochondrial physiology. A focus on coupling states in mitochondrial preparations can be

considered as a first step in the attempt to generate a harmonized and conceptually oriented nomenclature in bioenergetics and mitochondrial physiology.

Mitochondrial preparation  are defined as tissue or cellular preparations in which the plasma membranes are either removed (isolated mitochondria), or mechanically and/or chemically permeabilized (tissue homogenate, permeabilized fibres, permeabilized cells), while mitochondrial functional integrity and to a large extent the mitochondrial structure are maintained.

2. Fundamental respiratory coupling states in mitochondrial preparations

‘Every professional group develops its own technical jargon for talking about matters of critical concern ... People who know a word can share that idea with other members of their group, and a shared vocabulary is part of the glue that holds people together and allows them to create a shared culture’ (Miller 1991).

Mitochondrial respiratory control is exerted in a mt-preparation by experimental conditions defined as respiratory states. Coupling states in mt-preparations depend on the exogenous supply of fuel substrates and oxygen to support the electron transfer system (Fig. 1). In coupling states the phosphorylation of ADP to ATP is stimulated or depressed which causes an increase or decrease of electron flow linked to oxygen consumption in ‘controlled’ states. Alternatively, coupling of electron flow and phosphorylation is disengaged by uncouplers, and the corresponding ‘uncontrolled’ state is characterized by high levels of oxygen consumption without performance of biochemical work (Fig. 2). Such uncoupling is different from switching to mitochondrial pathways which involve less than three coupling sites with electron entry into the Q-junction bypassing Complex I (Fig. 1; including a bypass of CIV by alternative oxidases, not shown). This may be considered as a switch of gears (stoichiometry) rather than uncoupling (loosening the stoichiometry).

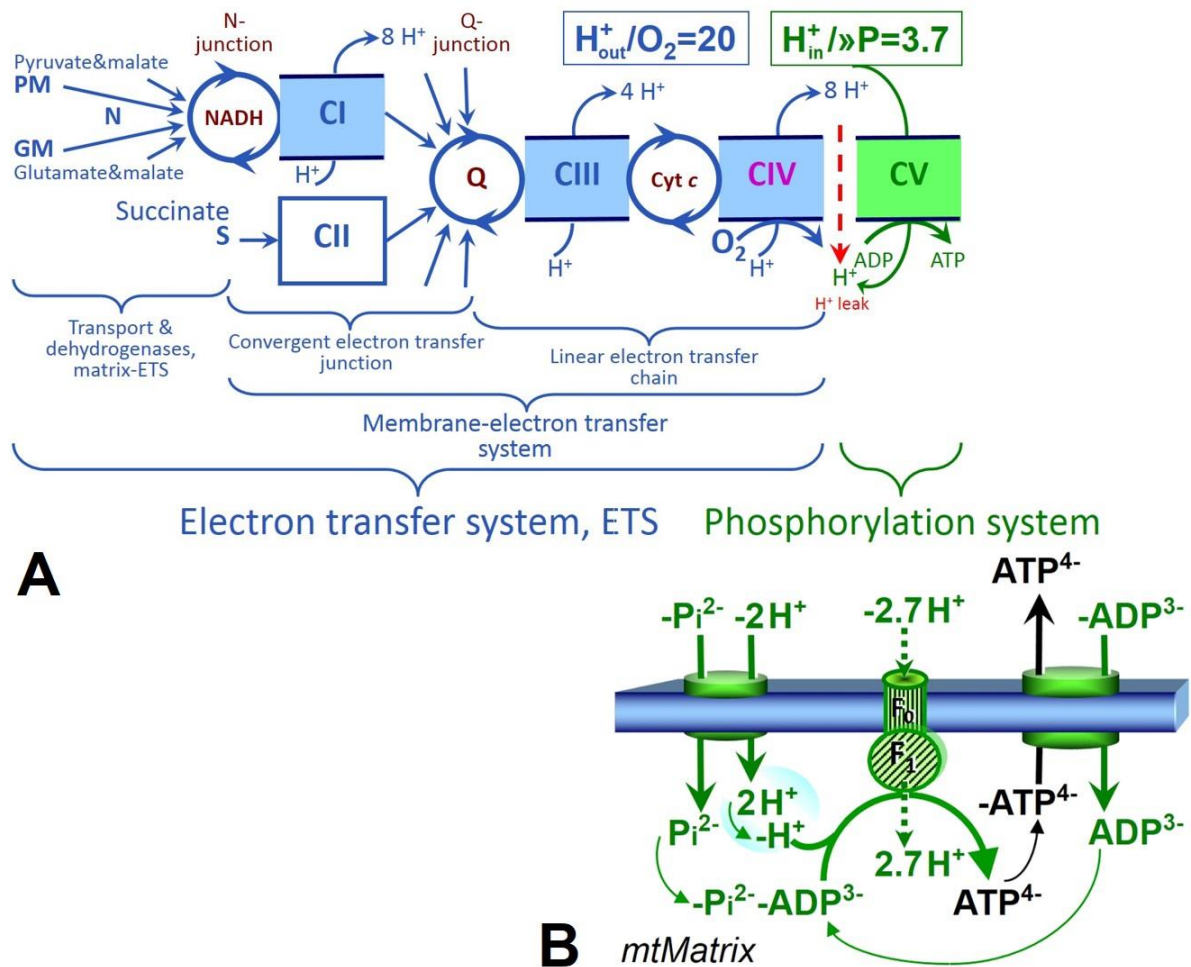


Fig. 1. The mitochondrial respiratory system. In oxidative phosphorylation the electron transfer system (A) is coupled to the phosphorylation system (B). See Eqs. 4 and 5 for further explanation. Modified after (A) Lemieux et al (2017) and (B) Gnaiger (2014).

Phosphorylation, »P: Although *phosphorylation* in the context of OXPHOS is clearly defined as phosphorylation of ADP to ATP, potentially involving substrate-level phosphorylation as part of the tricarboxylic acid cycle (succinyl-CoA ligase), in the matrix (phosphoenylpyruvate carboxykinase) and in the cytosol (pyruvate kinase, phosphoglycerate kinase). ADP is formed in the adenylate kinase reaction, $2 \text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$. In isolated mammalian mitochondria ATP production related to adenylate kinase can be detected in the presence of ADP and without respiratory substrates (Komlódi and Tretter 2017). On the other hand, the term phosphorylation is used in the general literature in many different contexts

(phosphorylation of enzymes, etc.). This justifies consideration of a symbol more discriminative than P as used in the P/O ratio (phosphate to atomic oxygen ratio), where P indicates phosphorylation of ADP to ATP or GDP to GTP. We propose the symbol »P for the energetic uphill direction of phosphorylation coupled to catabolic reactions, and likewise the symbol «P for the corresponding downhill reaction (Fig. 2).

Control and regulation: The terms metabolic *control* and *regulation* are frequently used synonymously, but are distinguished in metabolic control analysis (Fell 1997). Respiratory control may be exerted by (1) ATP demand (Fig. 2), (2) fuel substrate, pathway competition and oxygen availability (starvation and hypoxia), (3) the protonmotive force, redox states, flux-force relationships, coupling and efficiency, (4) mitochondrial enzyme activities and allosteric regulation by adenylates, phosphorylation of regulatory enzymes, Ca^{2+} and other ions including pH, (5) inhibitors (e.g. NO or intermediary metabolites, such as oxaloacetate), (6) enzyme content, concentrations of cofactors and conserved moieties) such as adenylates, NADH/NAD⁺, coenzyme Q, cytochrome *c*); (7) metabolic channeling by supercomplexes, (8) mitochondrial density and morphology (fission and fusion), (9) hormone levels, gender, life style (influencing all control mechanisms listed before), and (10) genetic or acquired diseases causing mitochondrial dysfunction (for reviews see Brown 1992; Gnaiger 1993a, 2009; 2014; Morrow et al 2017).

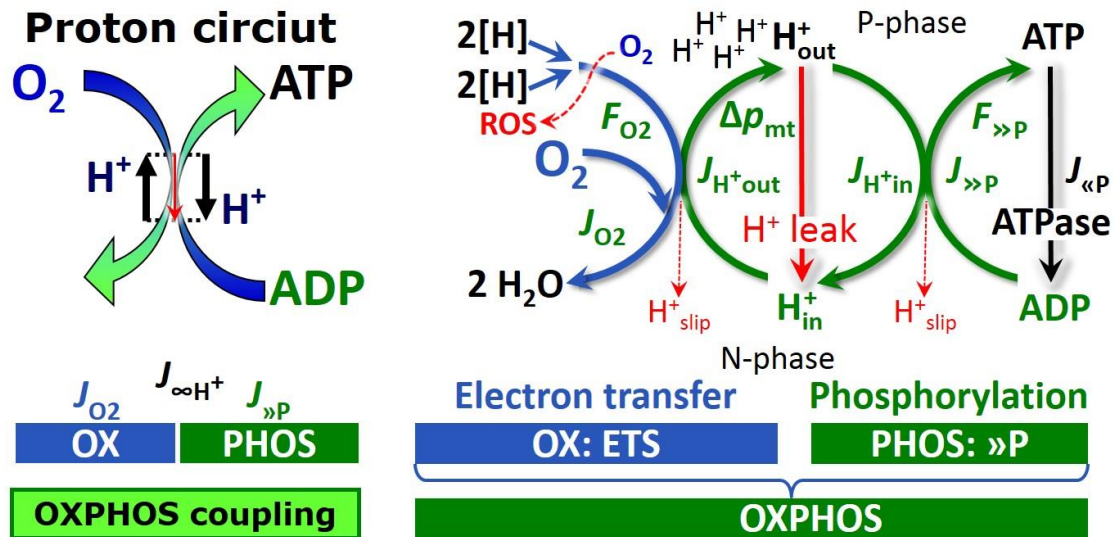


Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, J_{O_2} , is coupled to the phosphorylation of ADP to ATP, $J_{\gg P}$, by the proton pumps of the electron transfer system (ETS) pushing the outwards proton flux, J_{H^+out} . The ATP synthase is driven by the protonmotive force, Δp_{mt} , and inwards proton flux, J_{H^+in} , to phosphorylate ADP to ATP. $2[H]$ indicates the reduced hydrogen equivalents of fuel substrates providing the chemical input force or molar Gibbs energy, F_{O_2} [kJ/mol O_2], of the reaction with oxygen, typically in the range of -460 to -480 kJ/mol. The output force is given by the phosphorylation potential, $F_{\gg P}$ [kJ/mol ADP phosphorylated to ATP], which varies *in vivo* in the range of about 48 to 62 kJ/mol under physiological conditions. Proton turnover, $J_{\infty H^+}$, and ATP turnover, $J_{\infty P}$, proceed in the steady state at constant Δp_{mt} , when $J_{\infty H^+} = J_{H^+out} = J_{H^+in}$, and at constant phosphorylation potential, $F_{\gg P}$, when $J_{\infty P} = J_{\gg P} = J_{\ll P}$. $J_{\gg P}/J_{O_2}$ ($\gg P/O_2$) is two times the ‘P/O’ ratio of classical bioenergetics. The effective $\gg P/O_2$ ratio is diminished by (i) the proton leak across the inner mt-membrane from low pH in the positive P-phase to the negative N-phase, (ii) cycling of other cations, (iii) proton slip of the proton pumps when effectively a proton is not pumped, and (iv) electron leak generating reactive oxygen species, ROS. Modified after Gnaiger (2014).

2.1. Classical terminology for isolated mitochondria

'When a code is familiar enough, it ceases appearing like a code; one forgets that there is a decoding mechanism. The message is identical with its meaning' (Hofstadter 1979).

Five classical states of mitochondrial respiration and cytochrome redox states were introduced by Chance and Williams (1955; 1956). Table 1 explains a protocol with isolated mitochondria in a closed respirometric chamber, defining a consecutive sequence of respiratory states.

State 1 is obtained after addition of isolated mitochondria to air-saturated isoosmotic/isotonic respiration medium containing inorganic phosphate, but no adenylates (i.e. AMP, ADP, ATP) and no fuel substrates.

State 2 is induced by addition of a high concentration of ADP (typically 100 to 300 μM), which stimulates respiration transiently on the basis of endogenous fuel substrates and phosphorylates only a small part of the added ADP. State 2 is then obtained at a low respiratory activity limited by endogenous fuel substrate availability.

State 3 is the state stimulated by addition of fuel substrates while the ADP concentration is still high (Tab. 1) and supports coupled energy transformation in oxidative phosphorylation. 'High ADP' is a concentration of ADP specifically selected to allow the measurement of State 3 to State 4 transitions of isolated mitochondria in a closed respirometric system. A repeated ADP titration re-establishes State 3 at 'high ADP'. Starting at oxygen concentrations near air saturation (c. 200 $\mu\text{M O}_2$), the total ADP concentration added must be low enough (preferentially $<500 \mu\text{M}$) to allow phosphorylation to ATP at a coupled oxygen consumption that does not lead to oxygen depletion during the transition to State 4. In contrast, kinetically saturating ADP concentrations are usually an order of magnitude higher than 'high ADP'.

State 4 is only reached if the mitochondrial preparation is of high quality and is well coupled.

Depletion of ADP by phosphorylation to ATP will then lead to a decline in oxygen uptake in the transition from State 3 to State 4. Under these conditions a maximum Δp_{mt} and high ATP/ADP ratio are maintained. State 4 respiration reflects intrinsic proton leak and ATPase activity.

State 5 is a state obtained after exhaustion of oxygen in a closed respirometric chamber.

Oxygen backdiffusion into the aqueous solution may be a cofounding factor preventing complete anoxia (Gnaiger 2001).

Table 1. Metabolic states of mitochondria (after Chance and Williams, 1956).

State	[O ₂]	[ADP]	[Substrate]	Respiration rate	Rate-limiting substance
1	>0	Low	Low	Slow	ADP
2	>0	High	~0	Slow	Substrate
3	>0	High	High	Fast	respiratory chain
4	>0	Low	High	Slow	ADP
5	<0	High	High	0	Oxygen

2.2. Three fundamental coupling states of mitochondrial preparations and residual oxygen consumption

It has been suggested to extend the differential nomenclature (States 1 to 5) by a concept-driven terminology carrying explicit information on the nature of the respiratory states (Gnaiger 2009). The three fundamental coupling states of mt-preparations can be compared in any mitochondrial pathway control state, i.e. keeping fuel substrates and ETS inhibitors constant while varying adenylate levels and inhibitors of the phosphorylation system (Fig. 1). The terminology must be general and not restricted to any particular experimental protocol or mt-preparation.

Coupling and efficiency: Coupling occurs in an energy transformation between processes, if a coupling mechanism allows work to be performed on the endergonic or uphill *output* process (work per unit time is power; dW/dt [J/s] = P_{out} [W]; with a positive partial Gibbs energy change) driven by the exergonic or downhill *input* process (with a negative partial Gibbs energy change). At the limit of maximum efficiency of a completely coupled system, the (negative) input power equals the (positive) output power, such that the total power equals zero at an efficiency of 1. If the coupling mechanism is disengaged, the output process becomes independent of the input, and both proceed in their downhill direction (Fig. 2).

Proton leak: Proton leak is the process in which protons are translocated across the inner mt-membrane in the direction of the downhill protonmotive force without coupling to phosphorylation. The proton leak flux depends on Δp_{mt} and is a property of the inner mt-membrane.

Proton slip: Proton slip is the process in which protons are only partially translocated by a proton pump and slip back to the original compartment. The proton slip is a property of the proton pump and depends on the turnover rate of the proton pump.

OXPHOS state (Fig. 3): The respiratory state with saturating levels of oxygen, respiratory and phosphorylation substrates, and zero uncoupler, to provide an estimate of the maximal capacity of

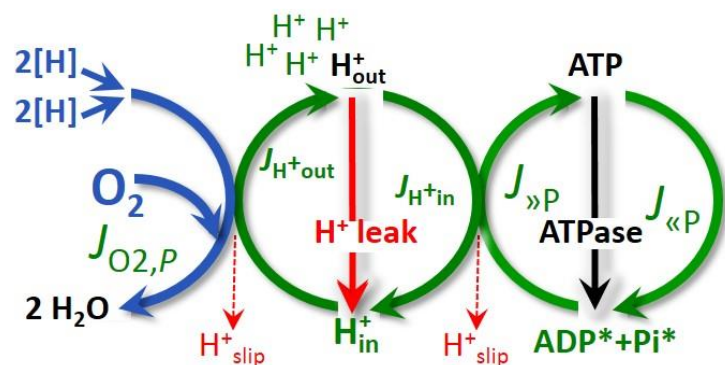


Fig. 3. OXPHOS state when phosphorylation, $J_{\gg P}$, is supported by a high Δp_{mt} , is stimulated by saturating [ADP]* and inorganic phosphate, [Pi]*. Oxygen flux, $J_{O_2,P}$, is highly coupled at a maximum $\gg P/O_2$ ratio, $J_{\gg P}/J_{O_2,P}$ (compare Fig. 2).

OXPHOS in any given pathway control state. Respiratory capacities at saturating substrate concentrations provide reference values or upper limits of performance, aiming at the generation of data sets for comparative purposes. Any effects of substrate kinetics are thus separated from reporting actual mitochondrial capacities, against which physiological activities can be evaluated.

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Puchowicz et al., 2004; Gnaiger, 2001), and even higher ADP concentrations are required particularly in permeabilized fibres to overcome limitations by diffusion and by the tubulin-regulated conductance of the outer mitochondrial membrane (Rostovtseva et al., 2008). In permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks et al., 1998). This implies that >90 % saturation is reached only at >5 mM ADP.

ETS state (Fig. 4): The electron transfer system state or ETS state is defined as the non-controlled state with saturating levels of oxygen, respiratory substrate and uncoupler, as an estimate of the maximal capacity of the ETS. Optimal *exogenous*

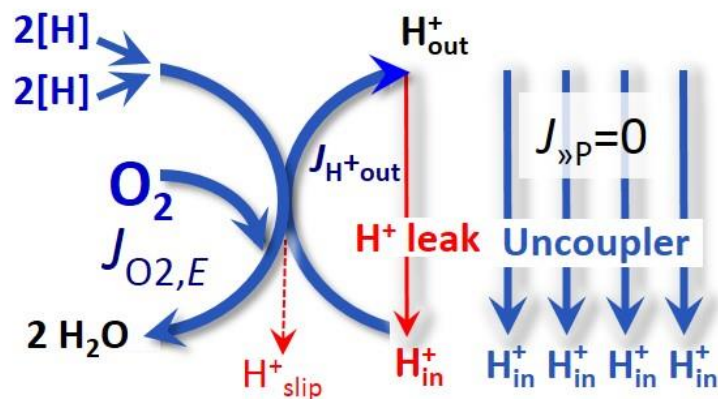


Fig. 4. ETS state when noncoupled respiration, $J_{O_2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{\gg P}=0$ (compare Fig. 2).

uncoupler concentration for maximum oxygen flux provides the condition for measurement of ETS capacity. As a consequence of the nearly collapsed Δp_{mt} , the driving force for phosphorylation is missing and $J_{\gg P}=0$. The abbreviation State 3u is

used frequently in bioenergetics, to indicate the state of maximum respiration without sufficient emphasis on the fundamental difference between OXPHOS capacity (*well coupled* with an *endogenous* uncoupled component) and ETS capacity (*noncoupled*).

LEAK state (Fig. 5): A state of mitochondrial respiration when oxygen flux is maintained at saturating levels of oxygen and respiratory substrates, and zero ATP-turnover without addition of any experimental uncoupler, as an estimate of the maximal proton leak rate.

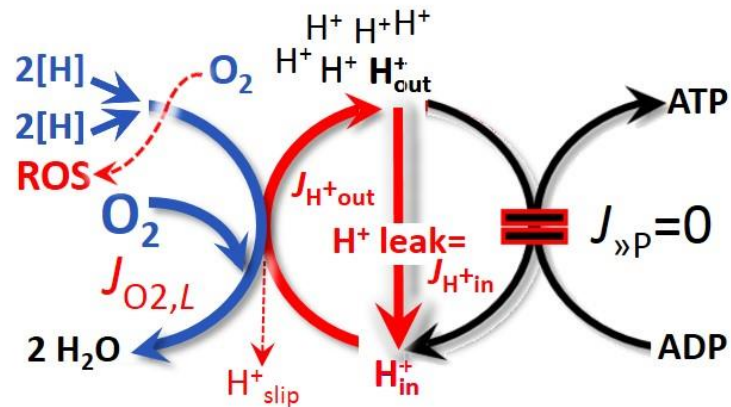


Fig. 5. LEAK state when phosphorylation is arrested, $J_{\gg P}=0$, and oxygen flux, $J_{O_2,L}$, is controlled mainly by the proton leak, which equals J_{H^+in} , at maximum Δp_{mt} (compare Fig. 2).


The LEAK state can be measured either (i) in the absence of adenylates, (ii) after depletion of ADP at maximum ATP/ADP ratio, or (iii) after inhibition the phosphorylation system by inhibitors such as oligomycin (ATP synthase) or carboxyatractyloside (adenylate nucleotide translocase). State 4 represents an overestimation of LEAK respiration if ATPase activity maintains some stimulation of respiration by recycled ADP at $J_{\gg P}>0$. This can be tested by inhibition of the phosphorylation system using oligomycin, ensuring that $J_{\gg P}=0$.

When subtracting LEAK respiration from OXPHOS capacity, the dissipative LEAK component may be overestimated. This can be avoided by measurement of LEAK respiration in a state when the protonmotive force, Δp_{mt} , is adjusted to the slightly lower value maintained in the OXPHOS state. Any turnover-dependent components of proton leak and slip, however, are underestimated under these conditions (Garlid et al 1993).

ROX: Residual oxygen consumption (ROX) is defined as oxygen consumption due to oxidative side reactions remaining after inhibition of the ETS. ROX is not a coupling state but represents a baseline that is used to correct mitochondrial respiration in defined coupling states. ROX is not necessarily equivalent to non-mitochondrial respiration, considering oxygen-consuming reactions in mitochondria not related to ETS, such as oxygen consumption in the monoaminoxidase catalyzed reaction. In the presence of oxygen, ROX is measured either in the absence of fuel substrates or after blocking the electron supply to cytochrome *c* oxidase and alternative oxidases.

3. States and rates

3.1. The steady-state

Steady-state variables (membrane potential; redox states) and metabolic fluxes (*rates*) are measured in defined mitochondrial respiratory *states*. Strict  steady states can be obtained only in open systems, in which changes due to internal transformations (e.g. oxygen consumption) are instantaneously compensated by external flows (e.g. oxygen supply), such that oxygen concentration does not change in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems may satisfy the criteria of pseudo-steady states for limited periods of time, when the changes occurring in the system (concentrations of oxygen, fuel substrates, ADP) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require saturating levels of substrates to be maintained and thus depend on the kinetics of the processes under investigation.

Protonmotive force, Δp_{mt} : The protonmotive force, Δp_{mt} ,

$$\Delta p_{\text{mt}} = \Delta \Psi_{\text{mt}} + \Delta \mu_{\text{H}^+} / F \quad (1)$$

is composed of an electric part, $\Delta \Psi_{\text{mt}}$, which is the difference of charge (electrical potential difference) across the inner mt-membrane, and a chemical part incorporating the Faraday

constant, $\Delta\mu_{\text{H}^+}/F$, which stems from the difference of pH (chemical potential difference) across the mt-membrane. Protonmotive means that protons are moved across the mt-membrane at $\Delta p_{\text{H}_{\text{mt}}}$ maintained across the mt-membrane,

$$\Delta\mu_{\text{H}^+} = -\ln(10) \cdot RT \cdot \Delta p_{\text{H}_{\text{mt}}} \quad (2)$$


where RT is the gas constant times absolute temperature. $\ln(10) \cdot RT = 5.708$ and 5.938 $\text{kJ} \cdot \text{mol}^{-1}$ at 25 and 37 °C, respectively. The Faraday constant, $F = e \cdot N_{\text{A}}$, is the product of the elementary charge, e [C], and the Avogadro (or Loschmidt) constant, N_{A} . F yields the conversion between electric force, $\Delta\Psi_{\text{mt}}$, expressed in joules per *motive* coulomb or volt [$\text{V}=\text{J}/\text{C}$] and chemical force, $\Delta\mu_{\text{H}^+}$, with the unit joules per *motive* mole [J/mol]. $\ln(10) \cdot RT/F = 59.16$ and 61.54 mV at 25 and 37 °C, respectively. For a ΔpH of 1 unit, the chemical potential difference (Eq. 2) changes by $6 \text{ kJ} \cdot \text{mol}^{-1}$ and Δp_{mt} (Eq. 1) changes by 0.06 V . Since F equals $96.5 \text{ (kJ} \cdot \text{mol}^{-1})/\text{V}$, a membrane potential of -0.2 V (Eq. 1) equals a chemiosmotic potential difference, $\Delta\tilde{\mu}_{\text{H}^+}$, of $19 \text{ kJ} \cdot \text{mol}^{-1} \text{ H}^+_{\text{out}}$. Considering a driving force of $-470 \text{ kJ} \cdot \text{mol}^{-1} \text{ O}_2$ for oxidation, the thermodynamic limit of the $\text{H}^+_{\text{out}}/\text{O}_2$ ratio is reached at a value of $470/19 = 24$, compared to a mechanistic stoichiometry of 20 ($\text{H}^+_{\text{out}}/\text{O}=10$).

The protonmotive force is *elevated* in the LEAK state of coupled mitochondria, driven by LEAK respiration at a minimum backflux of protons to the matrix side. Δp_{mt} is *high* in the OXPHOS state when it drives phosphorylation, and *very low* in the ETS state when uncouplers short-circuit the proton cycle.

Forces and flows in physics and irreversible thermodynamics: According to definition in physics, a potential difference and as such the *protonmotive force*, Δp_{mt} , is not a force (Cohen et al 2008). The fundamental forces of physics are distinguished from *motive forces* (e.g. Δp_{mt}) of statistical and irreversible thermodynamics. Complementary to the attempt towards unification of fundamental forces defined in physics, the concepts of Nobel laureates Lars Onsager, Erwin Schrödinger, Ilya Prigogine and Peter Mitchell (even if

expressed in apparently unrelated terms) unite the diversity of ‘isomorphic’ *flow-force* relationships, the product of which links to the dissipation function and Second Law of thermodynamics (Prigogine 1967; Schrödinger 1944). A *motive force* is the change of potentially available or ‘free’ energy (exergy) per isomorphic *motive* unit (force=exergy/motive unit; in integral form, this definition takes care of isothermal and non-isothermal processes). A potential difference is, in the framework of flow-force relationships, an isomorphic force, F_{tr} , involved in an exergy transformation, defined as the *partial* derivative of Gibbs energy, $\partial_{tr}G$, per advancement, $\partial_{tr}\zeta$, of the transformation, tr (the isomorphic motive unit in the transformation): $F_{tr} = \partial_{tr}G/\partial_{tr}\zeta$ (Gnaiger 1993a,b). This formal generalization represents an appreciation of the conceptual beauty of Peter Mitchel’s innovation of the protonmotive force against the background of the established paradigm of the electromotive force (emf) defined at the limit of zero current (Cohen et al 2008).

Molar quantities: ‘The adjective *molar* before the name of an extensive quantity generally means *divided by amount of substance*’ (Cohen et al 2008). The notion that all molar quantities then become *intensive* causes ambiguity in the meaning of *molar Gibbs energy*. It is important to emphasize the fundamental difference between normalization for *amount of substance* B in a system, where $dn_B/dt \cdot V^{-1}$ is the *rate of concentration change* [$\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-3}$], versus normalization for *amount of motive substance*, where $d_r\zeta_B/dt \cdot V^{-1}$ is the volume-specific flux of chemical reaction r. When the Gibbs energy of a system, G [J], is divided by the amount of substance B, n_B [mol], a *size-specific* molar quantity is obtained, $G_m = G/n_B$ [$\text{J} \cdot \text{mol}^{-1}$], which is not an (isomorphic) force. In contrast, when the partial Gibbs energy change, $\partial_r G$ [J], is divided by the motive amount of substance B in reaction r (advancement of reaction), $\partial_r\zeta_B$ [mol], the resulting *intensive* molar quantity, $F_r = \partial_r G/\partial_r\zeta_B$ [$\text{J} \cdot \text{mol}^{-1}$], is the chemical force of reaction r involving 1 mol B (-1 or 1, depending on B being a product or substrate, respectively).

Vectorial and scalar forces and fluxes: In chemical reactions and osmotic or diffusion processes occurring in a closed heterogenous system, such as a chamber containing isolated mitochondria, scalar transformations occur without measured spatial direction but between separate compartments (translocation between the matrix and extramitochondrial space) or between energetically separated chemical substances (reactions from substrates to products). Hence the corresponding fluxes are not vectorial but scalar, and are expressed per volume and not per membrane area. The corresponding motive forces are also scalar, expressed in units [$\text{J}\cdot\text{mol}^{-1}$] as a potential *differences* across the membrane, without taking into account the *gradients* across the 6 nm thick inner mt-membrane (Rich 1993). In a scalar electric transformation (flux of charge or current from the matrix space to the intermembrane and extramitochondrial space) the motive force is the difference of charge, $\Delta\Psi_{\text{mt}}$ [$\text{V}=\text{J}\cdot\text{C}^{-1}$]. For comparison, in a mechanical, vectorial advancement, $d_{\text{me}}\xi$ [m], the unit of the *force* is  N , F_{me} [$\text{N}=\text{J}\cdot\text{m}^{-1}$], and *flow* is the velocity, $v = d_{\text{me}}\xi/dt$ [$\text{m}\cdot\text{s}^{-1}$], such that the flow·force product yields mechanical power, P_{me} [W] (Cohen et al 2008). The corresponding *vectorial flux* (flow density per area) is velocity per cross-sectional area [$\text{s}^{-1}\cdot\text{m}^{-1}$]. The *scalar flux* lacks spatial information in a given volume, such that flux ($\text{m}\cdot\text{s}^{-1}$ per volume [$\text{s}^{-1}\cdot\text{m}^{-2}$]) times force yields volume-specific power, P_{Vme} [$\text{W}\cdot\text{m}^{-3}$].

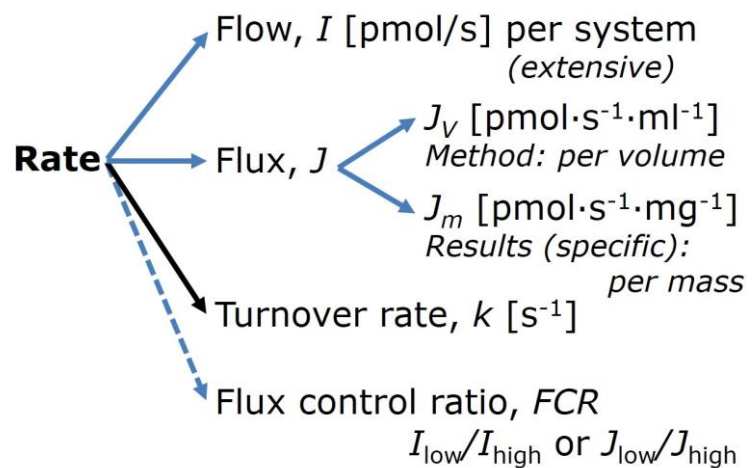
Coupled versus bound processes: Since the chemiosmotic theory explains the mechanism of coupling in OXPHOS, it may be interesting to ask if the electrical and chemical parts of proton translocation are coupled processes. This is not the case according to the definition of coupling given above. It is not possible to physically uncouple the electrical and chemical processes, which are only *theoretically* partitioned as electrical and chemical components (Eq. 1) and can be measured separately. If partial processes (fluxes, forces) are non-separable, i.e. cannot be uncoupled, then these are not *coupled* but are defined as *bound* processes. The electrical and chemical part of Eq. 1 are tightly bound partial forces of the protonmotive force.

Coupling, efficiency and power: In energetics (ergodynamics) coupling is defined as an exergy transformation fuelled by an exergonic (downhill) input process driving the advancement of an endergonic (uphill) output process. The (negative) output/input power ratio is the efficiency of a coupled energy transformation. Power, $P_{tr} = \partial_{tr}G/dt$ [$W=J\cdot s^{-1}$], is closely linked to the dissipation function (Prigogine 1967) and is the product of flow, $I_{tr} = d_{tr}\xi \cdot dt^{-1}$ [$x_{tr}\cdot s^{-1}$] times generalized force, $F_{tr} = \partial_{tr}G/\partial_{tr}\xi$ [$J\cdot x_{tr}^{-1}$] (Gnaiger 1993b).

3.2. Normalization: flows and fluxes

Application of common and generally defined units is required for direct transfer of reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. The term *rate* is too general and not useful for a database (Fig. 6).

Fig. 6. Different meanings of *rate* may lead to confusion, if the normalization is not sufficiently specified. Results are frequently expressed as mass-specific *flux*, J_m , per mg protein, dry or wet



J_m , per mg protein, dry or wet weight, ignoring the fundamental difference between weight and mass. Cell volume, V_{ce} , or mitochondrial volume, V_{mt} , may be used for normalization (volume-specific flux, J_{Vce} or J_{Vmt}), which then must be clearly distinguished from flux, J_V , expressed for methodological reasons per volume of the measurement system.

Extensive quantities: An extensive quantity increases proportional with system size. The magnitude of an extensive quantity is completely additive for non-interacting subsystems, such as mass or flow expressed per defined system. The magnitude of these quantities depends on the extent or size of the system (Cohen et al 2008).

Size-specific quantities: ‘The adjective *specific* before the name of an extensive quantity is often used to mean *divided by mass*’ (Cohen et al 2008). A mass-specific quantity (e.g. mass-specific flux is flow divided by mass of the system) is independent of the extent of non-interacting homogenous subsystems. Tissue specific quantities are of fundamental interest in comparative mitochondrial physiology, where *specific* refers to the *type* rather than *mass* of the tissue. The term *specific*, therefore, must be clarified further, such that tissue mass-specific (e.g. muscle mass-specific) quantities are defined.

Flow, I , per system: In analogy to electric terms, flow as an extensive quantity (I ; per system) is distinguished from flux as a size-specific quantity (J ; per system size) (Fig. 6). Electric current is a flow, I_{el} [$A = C \cdot s^{-1}$], per system (extensive quantity). When dividing this extensive quantity by system size (the cross-sectional area of a wire or area of a membrane), a size-specific quantity is obtained, which is electric flux (electric current density), J_{el} [$A \cdot m^{-2} = C \cdot s^{-1} \cdot m^{-2}$].

Size-specific flux, J : Oxygen flow per muscle increases as muscle mass is increased. Muscle mass-specific oxygen flux should be independent of the size of the tissue sample studied in the instrumental chamber, but volume-specific oxygen flux (per volume of the instrumental chamber, V) should increase in direct proportion to the amount of sample in the chamber. Accurate definition and reference to the *system* is decisive: the experimental system of the muscle, or the instrumental system of the measurement chamber. Volume-specific oxygen flux depends on mass-concentration of the sample in the chamber, but should be independent of chamber volume. If mass-specific oxygen flux is constant and independent of experimental system size (expressed as mass), then there is no interaction between the subsystems. A 1.5 mg and 3.0 mg muscle sample (wet weight) respire at identical mass-specific flux. The complexity changes when whole organisms are studied as experimental models. The well-established scaling law in respiratory physiology reveals a strong interaction of oxygen consumption and individual body mass of an organism, since *basal*

metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific oxygen flux, $V^{\text{[dot]}}_{\text{O2max}}$ or $V^{\text{[dot]}}_{\text{O2peak}}$, is constant across a large range of individual body mass (Weibel and Hoppeler 2005). $V^{\text{[dot]}}_{\text{O2peak}}$ of human endurance athletes is 60 up to 80 ml $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ body mass, converted to $J_{m,\text{O2peak}}$ of 45 to 60 $\text{nmol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ (Gnaiger 2014).

Flux per volume of the instrumental system, J_V : In open systems, external flows (such as oxygen supply) are distinguished from internal transformations (metabolic flow, oxygen consumption). In closed systems, external flows of all substances are zero and oxygen consumption (internal flow), I_{O_2} [$\text{pmol} \cdot \text{s}^{-1}$], causes a decline of the amount of oxygen in the system, n_{O_2} [nmol]. Normalization of these quantities for the volume of the system, V [$\text{ml} = \text{cm}^3$], yields volume-specific oxygen flux, $J_{V,\text{O}_2} = I_{\text{O}_2}/V$ [$\text{pmol} \cdot \text{s}^{-1} \cdot \text{ml}^{-1}$], and oxygen concentration, $[\text{O}_2]$ or $c_{\text{O}_2} = n_{\text{O}_2}/V$ [$\text{nmol} \cdot \text{ml}^{-1} = \mu\text{mol} \cdot \text{l}^{-1} = \mu\text{M}$]. Volume-specific metabolic oxygen flux, J_{V,O_2} , depends on the specific activity and the concentration of the mt-preparation in the measurement system, $\text{mt-preparation}/V$.

Instrumental volume-specific flux, J_{V,O_2} , should be compared with instrumental resolution and is thus relevant mainly for methodological reasons. Normalization for sample concentration, $\text{mt-preparation}/V$, is required for reporting respiratory results, e.g. in terms of respiration per mass, W_{mtprep} (of tissue homogenate or permeabilized fibres, or mitochondrial protein), $J_{\text{O}_2} = J_{V,\text{O}_2}/(W_{\text{mtprep}}/V) = I_{\text{O}_2}/W_{\text{mtprep}}$.

Flow, I , per experimental model: A special case of normalization is encountered in respiratory studies with permeabilized (or intact) cells. If respiration is expressed per million cells, the oxygen flow per measurement system is replaced by the oxygen flow, I_{O_2} , per cell (or per 10^6 cells). Similarly, oxygen flow can be calculated from volume-specific oxygen flux, J_{V,O_2} [$\text{pmol} \cdot \text{s}^{-1} \cdot \text{ml}^{-1}$] (per V of the measurement chamber), divided by the number density of cells, $C = N_{\text{ce}}/V$ [$10^6 \cdot \text{ml}^{-1}$], where N_{ce} is the number of cells in the chamber. Cellular oxygen flow can be compared only between cells of identical cell size. Therefore, further

normalization is important to obtain cell size-specific oxygen flux or mt-marker-specific oxygen flux (Renner et al, 2003).

3.3. Conversion: oxygen, protons, ATP

J_{O_2} is coupled in mitochondrial steady states to proton cycling, $J_{\infty H^+} = J_{H^+out} = J_{H^+in}$ (Fig. 2). J_{H^+out} and J_{H^+in} [$\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$] are converted into an electric flux (per volume), J_{el} [$\mu\text{C}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}=\mu\text{A}\cdot\text{ml}^{-1}$] = J_{H^+out} [$\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$] $\cdot F$ [$\text{C}\cdot\text{mol}^{-1}$] $\cdot 10^{-6}$. F is the Faraday constant (96,485.3 $\text{C}\cdot\text{mol}^{-1}$). At a J_{H^+out}/J_{O_2} ratio or H^+_{out}/O_2 of 20 ($H^+_{out}/O=10$), a volume-specific oxygen flux of 100 $\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ would correspond to a proton flux of 2,000 $\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ or volume-specific current of 193 $\mu\text{A}\cdot\text{ml}^{-1}$.

$$J_{el} [\mu\text{A}\cdot\text{ml}^{-1}] = J_{H^+out} \cdot F \cdot 10^{-6} [\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1} \cdot \mu\text{C}\cdot\text{pmol}^{-1}] \quad (3.1)$$

$$J_{el} [\mu\text{A}\cdot\text{ml}^{-1}] = J_{V,O_2} \cdot (H^+_{out}/O_2) \cdot F \cdot 10^{-6} [\mu\text{C}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}=\mu\text{A}\cdot\text{ml}^{-1}] \quad (3.2)$$

ETS capacity in various human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 $\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ cells (see Gnaiger 2014). At 100 $\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ cells corrected for ROX (corresponding to a catabolic power of -48 $\mu\text{W}\cdot 10^{-6}$ cells), the current across the mt-membranes, I_{el} , approximates 193 $\mu\text{A}\cdot 10^{-6}$ cells or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W.

For NADH- and succinate-linked respiration, the mechanistic »P/O₂ ratio (referring to the full 4 electron reduction of O₂) is calculated at 20/3.7 and 12/3.7 (Eq. 2) equal to 5.4 and 3.3. The classical »P/O ratios (referring to the 2 electron reduction of 0.5 O₂) are 2.7 and 1.6 (Watt et al 2010), in direct agreement with the measured »P/O ratio for succinate of 1.58 ± 0.02 (Gnaiger et al 2000; for detailed reviews see Sazanov 2015; Wikström and Hummer 2012),

$$\text{»P/O}_2 = (H^+_{out}/O_2)/(H^+_{in}/\text{»P}) \quad (4)$$

In summary (Fig. 1),


$$J_{V, \gg P} [\text{pmol} \cdot \text{s}^{-1} \cdot \text{ml}^{-1}] = J_{V, \text{O}_2} \cdot (\text{H}^+_{\text{out}} / \text{O}_2) / (\text{H}^+_{\text{in}} / \gg P) \quad (5.1)$$

$$J_{V, \gg P} [\text{pmol} \cdot \text{s}^{-1} \cdot \text{ml}^{-1}] = J_{V, \text{O}_2} \cdot (\gg P / \text{O}_2) \quad (5.2)$$

Relating the experimental results on isolated mitochondria and proton pumps as molecular machines to energy metabolism of the intact cell, the cellular $\gg P / \text{O}_2$ based on oxidation of glycogen is increased by the glycolytic substrate-level phosphorylation of 3 $\gg P / \text{Glyc}$, equivalent to 0.5 $\gg P / \text{O}_2$. Added to the mitochondrial $\gg P / \text{O}_2$ ratio of 5.4, this yields a bioenergetic cell physiological $\gg P / \text{O}_2$ ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, the energetic cost of which must potentially be taken into account. Taking also into account substrate-level phosphorylation in the TCA cycle, this high $\gg P / \text{O}_2$ ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993b).

4. Conclusions

MITOEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects which are linked to genetic variations, age-related health risks, gender-specific mitochondrial performance, life style with its consequences on degenerative diseases, and environmental exposure to toxicological agents. The present recommendations on coupling control (part 1) will be extended to pathway control of mitochondrial respiration (part 2), substrate-uncoupler-inhibitor-titration (SUIT) protocols and the harmonization of experimental procedures.

 **mitochondria, mt** (Greek mitos: thread; chondros: granule) are small organelles of eukaryotic cells with a double membrane separating the intermembrane space and the matrix with tubular or disk-shaped cristae. Mitochondria maintain their nuclear-independent mtDNA and function as powerhouses and chemoelectric generators in cell respiration or oxidative

phosphorylation. Abbreviation: mt, as generally used in mtDNA. Singular: mitochondrion; plural: mitochondria. Mitochondria contain the cytochrome system and ATP synthase or alternative oxidases, the enzymes of the tricarboxylic acid cycle with several dehydrogenases, fatty acid oxidation, and ion transporters including proton pumps in particular. Mitochondria are the oxygen consuming organelles, where the reduction of O₂ is chemiosmotically coupled to conservation of energy in the form of ATP (oxidative phosphorylation; OXPHOS). The bioblasts of Richard Altmann (1894) are not only the mitochondria as presently defined, but include symbiotic and free-living bacteria. “For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport, vectorial metabolism, and metabolic control mechanisms on a subcellular level” (Ernster and Schatz 1981).

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