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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 area across its specializations, aimed at facilitating transdisciplinary communication and teaching. The expanding field of mitochondrial respiratory physiology can benefit from harmonization of nomenclature on mitochondrial respiratory states and rates. Peter Mitchell's protonmotive, chemiosmotic force across the inner mitochondrial membrane, Δp_{mt} , establishes the link between electron transfer and phosphorylation of ADP to ATP, and between the electric and chemical components of energy transformation ($\Delta \Psi_{\text{mt}}$ and $\Delta p\text{H}_{\text{mt}}$). 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 differential 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 basic meaning of each respiratory state and focus primarily on the conceptual 'why' with clarification of the experimental 'how'. LEAK states are evaluated to study arrested respiration, L , when oxygen consumption compensates mainly for the proton leak. OXPHOS capacity, P , is measured at saturating concentrations of ADP and inorganic phosphate to obtain kinetic reference values for diagnostic applications. The ETS state differentiates the oxidative capacity of the electron transfer system, E , from P , revealing the limitation of OXPHOS capacity by the capacity of the phosphorylation system. Development of databases on mitochondrial respiratory control requires the application of strictly defined terms for comparison of respiratory states.

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

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. Only a large and coordinated network can manage to generate the necessary type, quality and number of consistent data sets to address this intrinsic complexity. The global 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 generate a rigorously monitored database on mitochondrial respiratory function.

Reliability and comparability of quantitative results depends on 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 turn valuable signals into wasteful noise. For this reason, measurements must be expressed in common units on each well-defined attribute for mitochondrial respiratory control. Even if standardization of nomenclature remains a goal of the optimist and is out of reach in the real world, harmonization of the technical jargon will 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 may be considered as a first step in the attempt to elaborate a harmonized and conceptually oriented nomenclature in bioenergetics and mitochondrial physiology.

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 or batteries in cell respiration or oxidative phosphorylation. 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 coupled to conservation of energy in the form of ATP (oxidative phosphorylation; OXPHOS). They belong to the bioblasts of Richard Altmann (1894), which comprise mitochondria and symbiotic as well as free-living bacteria. Abbreviation: mt, as generally used in mtDNA. Singular: mitochondrion; plural: mitochondria. “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).

Mitochondrial preparations, mtprep, are defined as tissue or cell preparations in which the plasma membranes are either removed (isolated mitochondria, imt), or mechanically and/or chemically permeabilized (tissue homogenate, thom; permeabilized fibres, pfi; permeabilized cells, pce), while mitochondrial functional integrity and to a large extent the mt-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 mt-preparations by experimental conditions defined as respiratory states. Coupling states in mt-preparations depend on 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).

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).

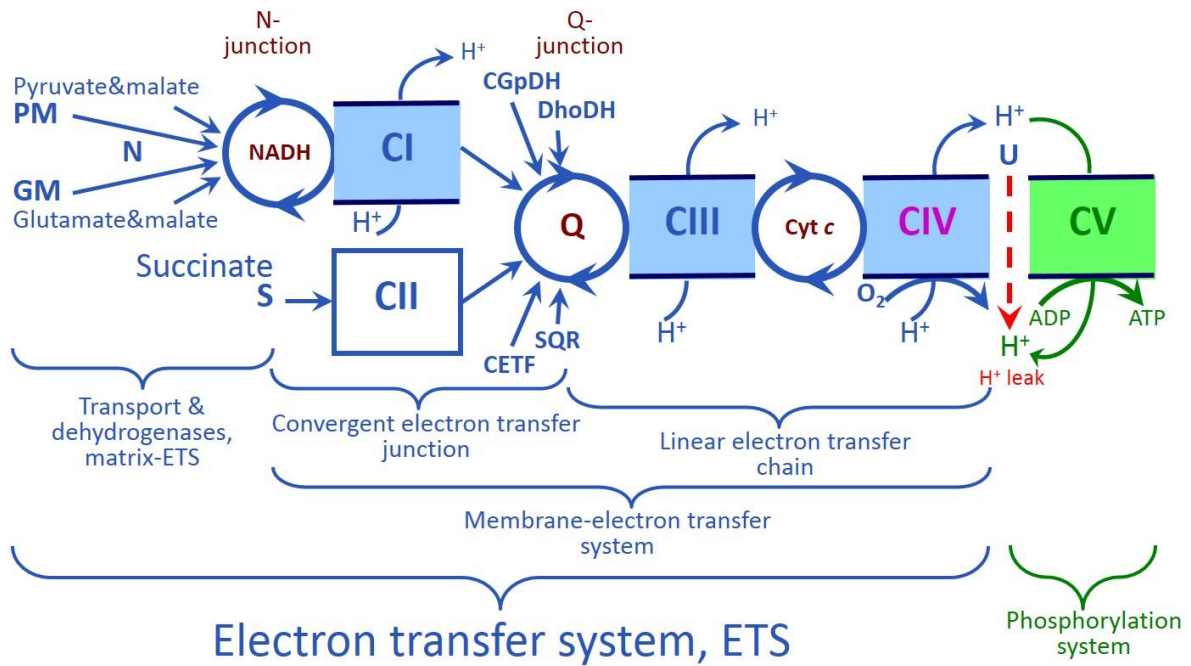
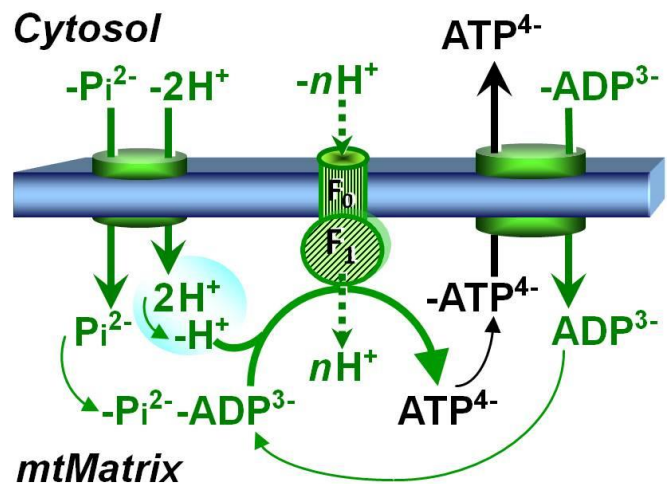


Fig. 1. The mitochondrial respiratory system. In oxidative phosphorylation the electron transfer system (A) is coupled to the phosphorylation system (B). Modified after (A) Lemieux et al (2017) and (B) Gnaiger (2014).



Coupling and efficiency: In an energy transformation, coupling occurs between processes, if a coupling mechanism allows work to be performed on the endergonic or uphill output process (work per unit of time is power; dW_{out}/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).

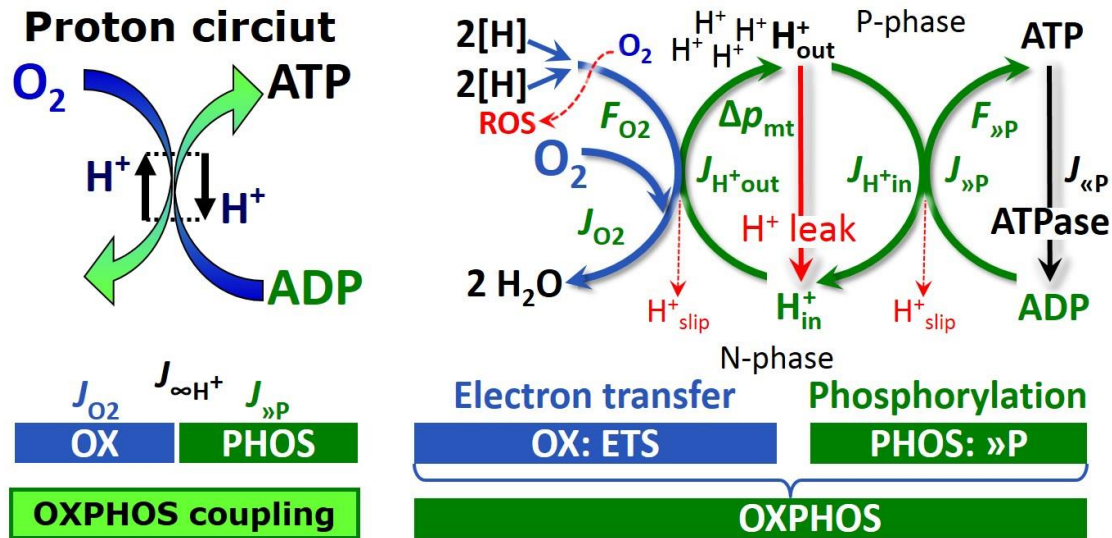


Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, J_{O_2} , is coupled to the phosphorylation of ADP to ATP, $J_{»P}$, by the proton pumps of the electron transfer system (ETS) pushing the outwards proton flux, J_{H^+out} . The ATP synthase is driven by the protonmotive 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, $F_{»P}$ [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, $F_{»P}$, when $J_{\infty P} = J_{»P} = J_{«P}$. $J_{»P}/J_{O_2}$ is two times the 'P/O' ($\gg P/O$) ratio of classical bioenergetics. The effective $J_{»P}/J_{O_2}$ 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, and (iv) electron leak generating reactive oxygen species, ROS. Modified after Gnaiger (2014).

2.1. Classical terminology for isolated mitochondria (imt)

'It is essential to define both the substrate and ADP levels in order to identify the steady-state condition of the mitochondria during the experiment' (Chance and Williams, 1956).

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

State 1 is obtained after addition of imt 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, which stimulates respiration transiently on the basis of endogenous fuel substrates, followed by a low respiratory activity limited by endogenous fuel substrate availability.

State 3 is the state stimulated by addition of fuel substrates while the ADP content is still high and supports coupled energy transformation in oxidative phosphorylation.

State 4 is only reached if the imt 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 confounding 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. This terminology must be general and not restricted to any particular experimental protocol or mt-preparation. This provides a platform to expand the classical respiratory states with the extended constructive framework in the theory of mitochondrial physiology.

LEAK state (Fig. 3): A state of

mitochondrial respiration when oxygen flux is maintained mainly to compensate for the proton leak in the absence of ATP synthesis, in the presence of fuel substrates and oxygen. The LEAK state can be established either in

the absence of adenylates, after depletion of ADP at maximum ATP/ADP ratio, and after inhibition the phosphorylation system by inhibitors such as oligomycin (ATP

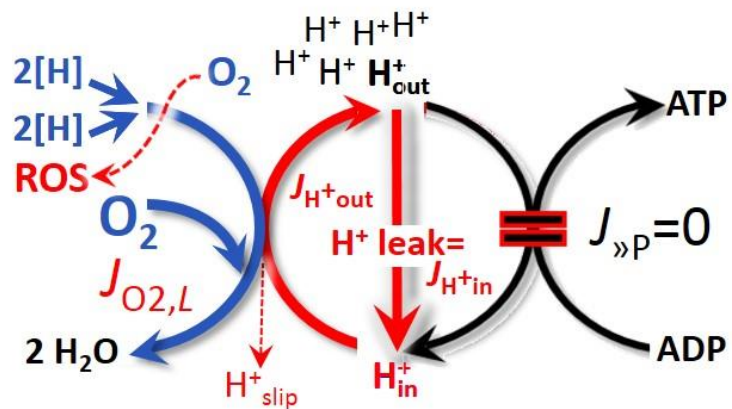


Fig. 3. LEAK state when phosphorylation is arrested, $J_{p=0}$, and LEAK oxygen flux, $J_{O_2,L}$, is controlled mainly by the proton leak, which equals J_{H^+in} , at maximum Δp_{mt} (compare Fig. 2).

synthase) or carboxyatractyloside (adenylate nucleotide translocase). State 4 represents an overestimation of LEAK respiration if ATPase activity prevents final accumulation of ATP and maintains a continuous 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 $J_{\gg P} = 0$.

OXPHOS state (Fig. 4): In mitochondrial physiology and pathology, maximal mitochondrial respiration in the *coupled* state is measured for quantitative determination of oxidative phosphorylation (OXPHOS) capacity (Gnaiger 2009). The OXPHOS state is supported by kinetically

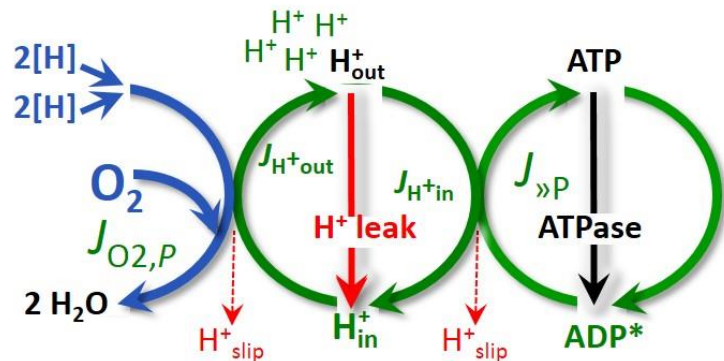


Fig. 4. OXPHOS state when phosphorylation, $J_{\gg P}$, is supported by a high Δp_{mt} , is stimulated by saturating $[ADP]^*$ and $[Pi]^*$, and oxygen flux, $J_{O_2,P}$, is highly coupled at a maximum $\gg P/O_2$ ratio, $J_{\gg P}/J_{O_2,P}$ (compare Fig. 2).

saturating ADP, $[ADP]^*$, and inorganic phosphate concentrations, $[Pi]^*$, in the presence of fuel substrates and oxygen. In contrast, the definition of State 3 lacks a fundamental attribute of OXPHOS capacity. As previously highlighted, 'high ADP' in State 3 is a concentration of ADP specifically selected to allow the measurement of a State 3 to State 4 transitions of isolated mitochondria in a closed respirometric system. Starting at oxygen concentrations near air saturation, the ADP concentration added must be low enough 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, OXPHOS capacity requires evaluation of kinetically saturating ADP concentrations, which are usually an

order of magnitude higher than 'high ADP'. 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).

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. 5): The electron transfer system or ETS state is the noncoupled state at optimal uncoupler concentration for maximum oxygen flux as a measure of ETS capacity, in the presence of fuel substrates and oxygen. 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

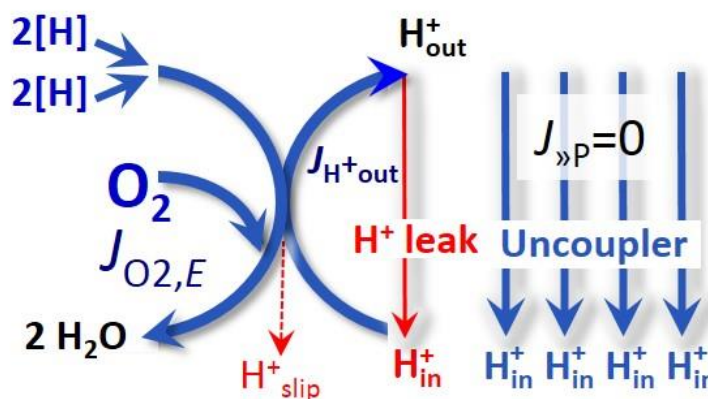


Fig. 5. ETS state when noncoupled respiration, $J_{O_2,E}$, is maximum at optimum uncoupler concentration and phosphorylation is zero, $J_{\gg P}=0$ (compare Fig. 2).

maximum respiration without sufficient emphasis on the fundamental difference between OXPHOS capacity (coupled, with an uncoupled component) and ETS capacity (noncoupled).

ROX: Residual oxygen consumption (ROX) is defined as respiration 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. Strictly 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 (changes in 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} ,

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

is composed of an electric part, $\Delta \Psi_{mt}$, which is the potential difference (difference of charge) across the inner mt-membrane, and a chemical part, $\Delta \mu_{H^+}/F$, which stems from the difference of pH across the mt-membrane. Protonmotive means that protons are moved across the mt-membrane at $\Delta p_{H_{mt}}$ maintained across the mt-membrane,

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

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, $F = e \cdot N_A$, is the product of the elementary charge, e [C], and the Avogadro (or Loschmidt) constant, N_A . F yields the conversion between electric force, $\Delta \Psi_{mt}$, expressed in joules per coulomb or Volt [$\text{V} = \text{J}/\text{C}$] and chemical force, $\Delta \mu_{H^+}$, with the unit joules per 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 ($\text{kJ} \cdot \text{mol}^{-1}$)/V, a membrane potential of -0.2 V (Eq. 1) equals a chemiosmotic potential difference, $\Delta \tilde{\mu}_{H^+}$, of 19 $\text{kJ} \cdot \text{mol}^{-1}$ H^+_{out} . Considering a driving force of -470 $\text{kJ} \cdot \text{mol}^{-1}$ O_2 for oxidation, the thermodynamic limit of the H^+/O_2 ratio is reached at a value of $470/19 = 24$, compared to a mechanistic stoichiometry of 20 ($\text{H}^+/\text{O} = 10$).

The protonmotive force is *maximum* in the LEAK state of well 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 shortcircuit the proton cycle.

Forces and flows in physics and irreversible thermodynamics: According to the 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 the

generalized *motive* forces (e.g. Δp_{mt}) of statistical and irreversible thermodynamics. Complementary to the attempt towards unification of fundamental forces defined in physics, the style of thinking of Nobel laureates Lars Onsager, Erwin Schrödinger, Ilya Prigogine and Peter Mitchell (even if expressed in apparently inconsistent terms) is related to the diversity of conjugated flow-force relationships, the product of which links to the dissipation function and the Second Law of thermodynamics (Prigogine 1967; Schrödinger 1944). A generalized ‘motive’ force is the change of potentially available or ‘free’ energy (exergy) per ‘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, a generalized force, F_{tr} , involved in an exergy transformation, defined as the partial derivative of Gibbs energy per advancement, $d_{tr}\xi$, of the transformation, tr (the motive unit in the transformation): $F_{tr} = \partial_{tr}G/\partial_{tr}\xi$ (Gnaiger 1993a,b). This formal generalization represents an appreciation of the conceptual beauty of Peter Mitchell’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). 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 generalized forces are also scalar, expressed in units $[J \cdot mol^{-1}]$. In a scalar electric transformation (flux of charge or current from the matrix space to the intermembrane and extramitochondrial space) the effective force is the mt-membrane potential, $\Delta\Psi_{mt}$ $[V=J \cdot C^{-1}]$. For comparison, in a mechanical, vectorial advancement, $d_{me}\xi$ $[m]$, the unit of the force is newton, F_{me} $[N=J \cdot m^{-1}]$, and the ‘flow’ is the velocity, $v = d_{me}\xi/dt$ $[m \cdot s^{-1}]$, 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 [$s^{-1}\cdot m^{-1}$]. The scalar ‘flux’ lacks spatial information in a given volume, such that flux (volume-density [$s^{-1}\cdot m^{-2}$]) times force yields volume-specific power, P_{Vme} [$W\cdot m^{-3}$].

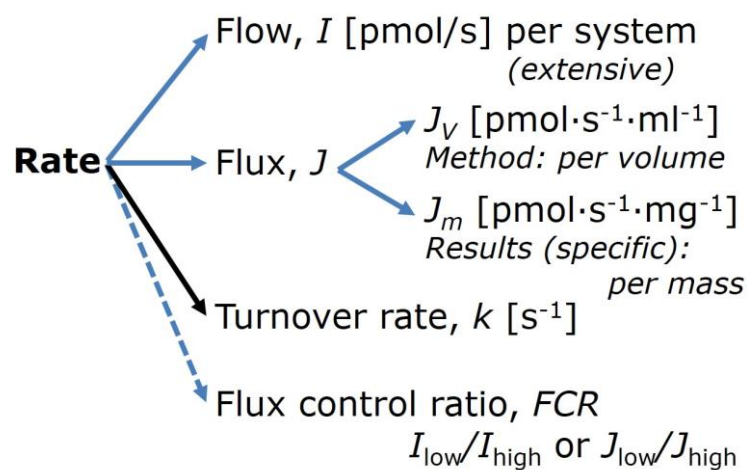
Coupling, efficiency and power: In energetics (ergodynamics) coupling is defined as an exergy transformation fuelled by the advancement of an exergonic (downhill) input process driving the advancement of an endergonic (uphill) output process. The (negative) output/input power ratio is the efficiency 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, $I_{tr} = d_{tr}\zeta \cdot dt^{-1}$ [$x_{tr}\cdot s^{-1}$] times generalized force, $F_{tr} = \partial_{tr}G/\partial_{tr}\zeta$ [$J\cdot x_{tr}^{-1}$] (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

weight, ignoring the fundamental difference between weight and mass. Cell volume or mitochondrial volume 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 chamber.

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 a of fundamental interest in comparative mitochondrial physiology. 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) 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 mass-specific basal metabolic rate does not increase proportionally and linearly with body mass, whereas maximum mass-specific oxygen flux, V_{O2max} , is constant across a large range of individual body mass (Weibel and Hoppeler 2005).

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_{O2} [$\text{pmol}\cdot\text{s}^{-1}$], causes a decline of the amount of oxygen in the system, n_{O2} [nmol]. Normalization of these quantities for the volume of the system, V [$\text{ml} = \text{cm}^3$], yields volume-specific oxygen flux, $J_{V,O2} = I_{O2}/V$ [$\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$], and oxygen concentration, $[O_2]$ or $c_{O2} = n_{O2}/V$ [$\text{nmol}\cdot\text{ml}^{-1} = \mu\text{mol}\cdot\text{l}^{-1} = \mu\text{M}$]. Volume-specific metabolic oxygen flux, $J_{V,O2}$, depends on the specific activity and the concentration of the mt-preparation in the measurement system, mt_{prep}/V .

Instrumental volume-specific flux, $J_{V,O2}$, should be compared with instrumental resolution and is thus relevant mainly for methodological reasons. Normalization for sample concentration, mt_{prep}/V , is required for reporting respiratory results, e.g. in terms of respiration per mass, $W_{mt_{prep}}$ (of tissue homogenate or permeabilized fibres), $J_{O2} = J_{V,O2}/(W_{mt_{prep}}/V) = I_{O2}/W_{mt_{prep}}$.

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, $C=N_{ce}/V$ [$10^6\cdot\text{ml}^{-1}$], where N_{ce} is the number of cells in the chamber. Cellular oxygen flow can be compared only between cells of identical cell size, after normalization to a size-specific oxygen flux or after normalization by a mt-marker (Renner et al, 2003).

3.3. Conversion: oxygen, protons, ATP

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

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

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

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 ($-48 \mu\text{W}\cdot 10^{-6}$ 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 (transmembrane proton flux equivalent to 520 A in an adult).

For NADH- and succinate-linked respiration, the mechanistic $\gg P/O_2$ ratio is calculated at $20/3.7$ and $12/3.7$ (Eq 2), or 5.4 and 3.3 (equivalent to $\gg P/O$ of 2.7 and 1.6 ; Watt et al 2010), in direct agreement with the measured $\gg P/O$ ratio for succinate of 1.58 ± 0.02 (Gnaiger et al 2000),

$$\gg P/O_2 = (H^{+}_{out}/O_2)/(H^{+}_{in}/\gg P) \quad (4)$$

In summary,

$$J_{V,\gg P} [\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}] = J_{V,O_2}\cdot(H^{+}_{out}/O_2)/(H^{+}_{in}/\gg P) \quad (5.1)$$

$$J_{V,\gg P} [\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}] = J_{V,O_2}\cdot(\gg P/O_2) \quad (5.2)$$

Relating the experimental results on isolated mitochondria and proton pumps as molecular machines to energy metabolism of the intact cell, the cellular $\gg P/O_2$ based on oxidation of glycogen is increased by the glycolytic substrate-level phosphorylation of 3 $\gg P/\text{Glyc}$, equivalent to 0.5 $\gg P/O_2$. Added to the mitochondrial $\gg P/O_2$ ratio of 5.4, this yields a bioenergetic cell physiological $\gg P/O_2$ 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 $\gg P/O_2$ 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

With a perspective to extend the present recommendations on coupling control (part 1) to pathway control of mitochondrial respiration (part 2), substrate-uncoupler-inhibitor-titration (SUIT) protocols and harmonization of experimental procedures, MITOEAGLE will be a gateway and milestone 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.

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