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Current Opinion in Biotechnology

Dynamics in redox metabolism, from stoichiometry towards kinetics

Koen JA Verhagen, Walter M van Gulik and Sebastian Aljoscha Wahl

Redox metabolism plays an essential role in the central metabolic network of all living cells, connecting, but at the same time separating, catabolic and anabolic pathways. Redox metabolism is inherently linked to the excretion of overflow metabolites. Overflow metabolism allows for higher substrate uptake rates, potentially outcompeting other microorganisms for the same substrate. Within dynamically changing environments, overflow metabolism can act as storage mechanism, as is shown in many recently described processes. However, for complete understanding of these mechanisms, the intracellular state of the metabolism must be elucidated. In recent years, progress has been made in the field of metabolomics to improve the accuracy and precision of measurements of intracellular and intercompartmental metabolites. This article highlights several of these recent advances, with focus on redox cofactor measurements, both fluorescence and mass spectrometry based.

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Introduction

The redox metabolism is commonly studied and interpreted under steady state conditions, where the transfer of electrons and metabolites inside the cell is balanced. Natural environments, as well as incompletely mixed bioreactors are mostly not at steady-state, requiring flexibility of metabolism, including redox metabolism. The transfer of electrons between different reactions of the metabolic network is facilitated by electron carriers such as NADH, NADPH, $FADH_2$, quinones and ferredoxins. Each electron carrier fulfils different functions within the cell, with NADH being used primarily for catabolism, such as respiration and fermentation. NADPH is the cofactor used for anabolism like amino acid and lipid synthesis [1]. This separation of electron carriers allows for additional functionality within the network, as ratios of the reduced and oxidized forms of the different cofactors can vary independently. For example, it is well established that NADPH/NADP ratios are higher compared to NADH/NAD to generate higher thermodynamic driving forces in the reductive direction in anabolism.

Engineered as well as natural environments are frequently dynamic – with perturbation timescales in the order of seconds to regime changes in the order of hours. Dynamics can arise from fluctuations in the availability of carbon source(s), electron donor(s) resp. acceptor(s). To obtain a stable metabolism the redox state within the cell has to be balanced using available electron sinks and sources.

The mechanisms behind overflow metabolism, such as the Crabtree effect in yeast, and their link to redox imbalances have been extensively studied under steady state conditions [2–4], where electrons are essentially channelled towards fermentation products and excreted from the cells. Next to the competitive advantage of a higher growth rate, also higher substrate uptake rates are achieved, potentially outcompeting other microorganisms for the same substrate. Such advantages can become even more relevant in dynamically changing environments where substrate is only available for short periods of time. So far only a few dynamic systems have been studied with respect to sudden changes of the redox state. Under dynamic conditions, the temporary storage of electrons can be an essential mechanism to obtain a competitive advantage by increasing metabolic flux [5•]. Electron overflow has been observed for various prokaryotes and eukaryotes in response to substrate pulses, resulting in excretion of compounds which cannot easily be further metabolized or are even toxic, such as ethanol or lactate [6].

While these phenomena can be explained from a 'strategic' point of view, it is not clear how such behaviours are regulated on a metabolic level. Here, accurate intracellular measurements are required that can capture the fast dynamics of redox metabolism.

With the low concentrations and high turnover rates of intracellular redox cofactors, the measurements are

challenging, especially for dynamic systems [7]. Further complexity arises from subcellular compartmentation of these metabolites within eukaryotes. Compartmentation allows the establishment of different environments within a cell, thereby providing a wider range of thermodynamic possibilities [8]. Different ratios of NADH/NAD in different compartments can allow for simultaneous oxidizing and reducing environments within the same organism. Unfortunately, limited quantitative data are available for compartment specific concentrations. In addition, metabolite channelling may overcome thermodynamic constraints by creating local high concentration environments near enzyme reaction centres, enabling thermodynamic pathway feasibility which may not be observed from the measured overall cellular metabolite concentrations [9,10].

Here, we will review recent studies providing insights into the dynamics of the redox metabolism upon changes in the environment, discuss the relevance of the understanding of the redox metabolism for the design and optimization of microbial production strains and processes, and describe how technical challenges in measuring redox states in different cellular compartments can be approached.

Stoichiometry and dynamics of redox coupling – a link between overflow and storage mechanisms

Recent works have analysed the impact of dynamically fluctuating environments in the context of (very) large-scale microbial cultivations. Within large-scale reactors gradients in substrate, oxygen and pH will occur due to mass-transfer limitations (poor mixing) [11]. A microorganism traveling through these gradients will experience fluctuations in substrate and oxygen supply. Such fluctuations can be mimicked using scale-down approaches, such as two reactor STR/PFR systems [12•] or microfluidic cultivations [13•].

During anaerobic cultivation products such as acetate and ethanol are excreted. These contain electrons that could not be transferred to an external acceptor and consequently 'store' a significant amount of electrons in the extracellular space. Such an excretion of electrons may also occur under aerobic conditions, known as overflow metabolism. Electron (as well as carbon and energy) overflow allows for higher growth rates under protein allocation limiting conditions [3,14••,15]. Yeast, grown under glucose limiting conditions and subjected to aerobic and anaerobic cycles, showed cyclic excretion and (re)consumption of electrons. Ethanol is produced under anaerobic conditions, and reconsumed when oxygen becomes available [16]. Under such dynamic conditions, ethanol could be regarded as a storage pool that is generated under electron excess conditions and consumed when other donors (like glucose) are depleted. A similar behaviour was observed for *Corynebacterium glutamicum*. When cultivated in a glucose fed two-compartment reactor system, lactate production is observed under O_2 limiting conditions, which was reconsumed once oxygen was present [17]. In this sense, fermentation products can be seen as storage metabolites.

Accepting the view that fermentation products can also be interpreted as (external) storage metabolites, overflow metabolism between different organisms can be seen in a more common framework. For example, *Plasticicumulans acidivorans* grown in batch culture with acetate as carbon source accumulates intracellular PHA instead of biomass. This mechanism circumvents a limitation in the respiratory capacity — if acetate is directly converted to biomass, the oxygen requirement per mol acetate consumed is much higher compared to PHA synthesis. Using PHA as electron sink, the substrate can be consumed at a much higher rate because electron transfer to an external donor via respiration is not required. Thus, PHA is storage acts as an overflow valve, generating a competitive advantage at the expense of biomass yield [18,19]. This is mechanistically similar to overflow metabolism in yeast, with the difference that the produced overflow metabolite in yeast is excreted. This has the advantage of a much larger space to store but with the disadvantage of being shared with putative other organisms. Therefore, *P. acidivorans*, with its intracellular storage of PHA, is especially able to prevail over its competitors within environments with short-term fluctuating availability of substrate.

Another example of intracellular overflow metabolism can be found in cyanobacteria. Cyanobacteria utilize glycogen as carbon storage metabolite during light/dark cycles. Knock-out of glycogen synthesis however has shown that glycogen also has an important function as storage of ATP to maintain energy homeostasis when excess ATP is produced at the photosynthetic electron transport chain [20]. Cyanobacteria incapable of storing glycogen will instead produce extracellular organic acids as overflow products to balance their energy homeostasis, thereby preventing unnecessary reduction of redox cofactors and ensuing obstruction of the photosynthetic electron transport chain.

The 'art' of dynamic redox balancing is exemplified by so-called phosphate accumulating organisms (PAOs), such as Candidatus *Accumulibacter phosphatis*. These organisms play a crucial role in biological phosphate removal within wastewater treatment systems [21]. PAOs are grown in a cyclic anaerobic/aerobic environment with volatile fatty acids, such as acetate, as substrates which are fed during the anaerobic phase. Acetate catabolism under anaerobic conditions can only generate a limited amount of Gibbs free energy, and is thus not favourable. In contrast, under aerobic conditions, full oxidation of

acetate generates a much higher amount of ATP [22]. PAO's are able to internalize acetate rapidly and carry carbon and electrons to the aerobic phase using a storage polymer, polyhydroxybutyrate (PHB). However, two challenges need to be tackled: (1) Redox: PHB carries more electrons per amount of carbon compared to acetate, (2) Energy: conversion of acetate to PHB requires ATP. PAO metabolism is adapted to these challenges by utilizing intracellular storage pools such as glycogen and polyphosphate that serve as sources for electrons in the form of NADH and energy as ATP, respectively. Such a variety of storage pools allow for a flexible, dynamic balancing of redox and energy metabolism under anaerobic conditions. How cells have implemented the regulation and optimization of these different sources and sinks is still unknown. The achieved accumulation of PHB from extracellular acetate then serves as carbon and electron source during the aerobic phase, both for biomass synthesis as well as refuelling of the intracellular glycogen and polyphosphate pools [5•,23].

These examples (see also Table 1) show that dynamic balancing of electron sinks (and sources), including intracellular storage as well as overflow products, facilitate the maximization of substrate uptake rates, potentially generating a competitive advantage. Quantitative descriptions of the metabolic processes, including the metabolic and genetic regulation, are limited to model organisms like *Saccharomyces cerevisiae* [3], *Escherichia coli* or *Lactococcus lactis* [24]. Additionally, only a few dynamic conditions have been studied, like diurnal cycles [25]. First modelling studies for microbial communities are available [26], delivering comprehensive insights to the putative competitive advantages of dynamic and flexible redox metabolism.

Redox coupling as metabolic engineering strategy

Commonly, products of interest are either more reduced or more oxidised than the substrate. Consequently, redox couplings and interactions are basically inevitable and have to be taken into account [31] in metabolic engineering. Such a redox coupling can be used to link biomass synthesis with the product pathway. This strategy can especially be exploited for products that generate a surplus of ATP (often referred to as catabolic products) and several approaches can be used [32]: (1) changing the cofactor specificity of either the product pathway or the catabolic pathway [33–35], (2) utilizing transhydrogenases to interconvert different electron carriers [36], (3) enforcing (electron and carbon) flux to the product by eliminating other electron sinks (like by-product pathways to glycerol or ethanol, but also respiration) [37], (4) providing external secondary sources of electrons, such as formate, to increase intracellular reducing power [38•]. Additionally, in the case of eukaryotes, compartmentation has to be taken into account.

Many commodity chemicals, relevant for the production of biofuels or polymers, are more reduced than the carbon sources, such as sugars, which are used in these bioprocesses. This means that additional reducing power has to be generated to drive the respective product pathways. An example of one of these commodity chemicals is 1,4-Butanediol (BDO). Implementation of the BDO pathway in *E. coli*, however, without additional metabolic engineering, shows that insufficient reducing power is available to produce BDO [39]. To provide this additional reducing power, Yim *et al.* [39] eliminated fermentation pathways towards ethanol, lactate, formate, succinate and in addition enabled growth at micro-aerobic conditions allowing for limited respiration and thus limited transfer of electrons to oxygen.

An example for redox pathway engineering in eukaryotes is the implementation of an efficient isobutanol production pathway in yeast. The isobutanol production pathway branches off from the intermediate 2-ketoisovalerate of the valine synthesis pathway which is localized in the mitochondria. The mitochondrial localization requires additional transport steps for the product but also redox cofactors. The synthesis of isobutanol from pyruvate requires additional input of 4 electrons, in the form of 1 NADPH and 1 NADH [40]. With the implementation of the first steps of the valine pathway in the cytosol the theoretical yield for isobutanol on glucose is 1 mol/mol compared to 0.63 for the mitochondrial localization [41].

Table 1

Examples of dual overflow and storage metabolism in a range of processes under dynamic culture conditions, sorted by time scale of the dynamics

Time scale	Organism	IC/EC electron acceptor	Culture conditions	Reference
30 s–20 min	*S. cerevisiae*	Extracellular	Aerobic/Anaerobic cycles	[16,27]
100 s	*C. glutamicum*	Extracellular	Aerated STR, unaerated plug-flow loop	[17]
360 s	*P. chrysogenum*	Intracellular	Aerobic, Repetitive substrate pulse	[28]
400 s	*S. cerevisiae*	Intracellular	Aerobic, Repetitive substrate pulse	[29,30]
6 hour	Candidatus *Accumulibacter phosphatis*	Intracellular	Aerobic/Anaerobic cycles, Repetitive substrate pulse	[22,23]
12 hour	*P. acidivorans*	Intracellular	Aerobic, Repetitive substrate pulse	[19]

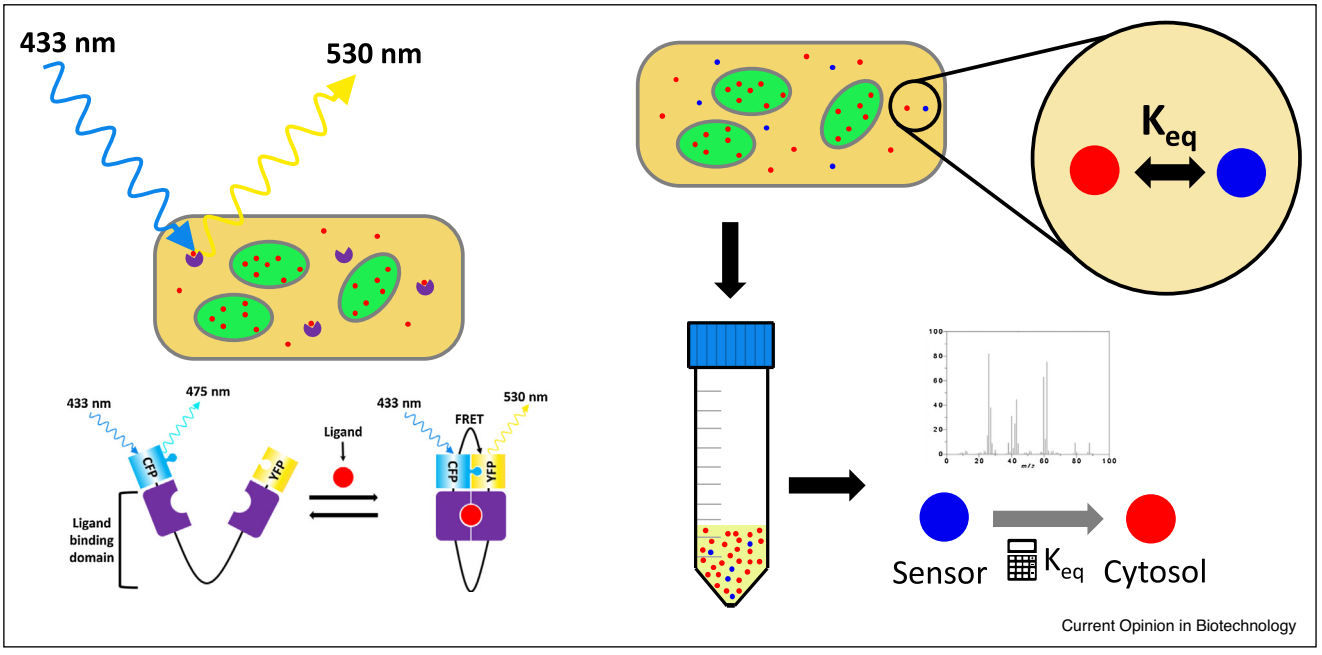
The observed low yields are caused by competition of multiple cellular pathways for the required electrons in the form of NADH and NADPH. By knocking out genes transcribing for pyruvate decarboxylase and glycerol dehydrogenase, ethanol and glycerol production were blocked, and NADH produced in the glycolysis is forced into the isobutanol pathway, essentially achieving redox coupling between the catabolic glycolysis and the isobutanol production pathway. In addition, competing pathways, such as valine and 2,3-butanediol biosynthesis, were eliminated to further improve the production of isobutanol [40]. In addition, the cofactor specificity of the NADPH dependent enzymatic step was changed to NADH, allowing for further redox coupling [41].

Measurement challenges

Accurately monitoring dynamic changes in the redox cofactor ratios is essential for understanding their overall role within the metabolism [42]. Current techniques used for quantification of NAD(P)H, $FADH_2$ and quinones rely on liquid chromatography–mass spectrometry (LC–MS including approaches using isotopically labelled NAD(P)H/$FADH_2$/quinones as internal standard improve measurement accuracy [43]), enzymatic assays or fluorescence [44•,45–48]. However, because of the unstable nature of NAD(P)H, $FADH_2$ and quinones, especially during sample preparation, accurate measurement of the *in-vivo* redox ratios is challenging. *In-vivo* measurements using fluorescence can be applied to circumvent sample preparation, but this technique does not differentiate between NADH and NADPH and additionally suffers from high signal to noise ratios. The redox charge of ferredoxins can be measured using near infrared spectroscopy, although this does present additional challenges in deconvolution of the measured signal [49].

In addition, eukaryotic cells differ from prokaryotic cells in that they have separated different parts of their metabolic reaction network in compartments, with different redox ratios. Canelas *et al.* [50] performed a thermodynamic analysis of the glycolytic reactions using whole cell as well as compartment specific NAD/NADH ratio measurements. With whole cell measurements, glycolysis appeared thermodynamically not feasible, which clearly showed the impact of redox factor compartmentation generating different potentials. To

Figure 1



Left: Cytosolic fluorescence-based sensor (adapted from Ref. [61]): This sensor works based on the principle of Förster resonance energy transfer (FRET), that is, energy is transferred from a donor to an acceptor fluorophore. The energy transfer only occurs when both fluorophores are in proximity of each other, and the distance is influenced by binding of the ligand. The fluorescence signals of YFP versus CFP are subsequently used to evaluate the ligand concentration [61]. Right: Cytosolic equilibrium-based sensor: By overexpressing an enzyme catalysing a close to equilibrium reaction, equilibrium between metabolites of interest and measurable pools can be established. From the equilibrium constant (K_{eq}) and involved measured metabolite concentrations, the metabolite of interest can be determined. With the equilibrium reaction expressed only in the cytosol, and the measured metabolites (blue) exclusively present in the cytosol, the compartment specific concentration of the metabolite of interest (red) can be determined [50].

assess the actual redox ratio driving the various intracellular redox reactions, the compartment-specific NADH/NAD$^+$ and NADPH/NADP$^+$ ratios have to be determined. This presents an additional challenge for measuring the actual intracellular redox ratios in eukaryotic cells.

To address these issues, fluorescent biosensors can be used. These biosensors, composed of fluorescent proteins and allosteric binding domains, change in fluorescent signal based on the binding of NADH to the binding domain (Figure 1). The fluorescence output signal is changed upon binding due to conformational changes in the sensor complex [51,52]. These biosensors are utilized as screening methods using FACS, allowing for high-throughput screening of large mutant libraries for increased pathway activity [53,54]. In addition, NADPH biosensors have also been employed to study the influence of cellular processes on NADPH availability [55] and dynamic changes in NADPH concentrations [56]. However, a disadvantage of these biosensors is that a fluorescence microscope is required to provide a live readout of the single cell biosensor fluorescence, and thus these biosensors cannot be used to monitor the redox ratios in cells in larger cultivations, such as reactors.

Another option is to utilize so-called equilibrium-based sensor reactions [50] (Figure 1). By overexpressing an enzyme catalysing an equilibrium-based reaction, a near equilibrium can be established between different metabolite pools involved in the reaction. From the equilibrium constant and involved measured metabolite concentrations, an undetermined metabolite concentration can be determined. To be sure that such a reaction operates close to equilibrium it should have a high capacity compared to the *in-vivo* reaction rate. Sometimes native reactions can be used for this purpose [57]. If not, a heterologous enzyme can be expressed to act as sensor reaction; however, it should be verified that this does not interfere with the metabolism. An example is the expression of mannitol-1-phosphate dehydrogenase from *E. coli* in *S. cerevisiae*, converting fructose-6-phosphate and NADH into mannitol-1-phosphate and NAD$^+$ and vice versa, which appeared to be essentially a dead end reaction in *S. cerevisiae* [50]. If this reaction is expressed exclusively in a specific compartment, and the measured metabolites are exclusively present in this specific compartment, then the compartment specific concentration of the undetermined metabolite could be determined. Several of these sensor reactions have been developed, both for measuring the cytosolic NADH/NAD$^+$ and NADPH/NADP$^+$ ratios [50,58]. Using mannitol-1-phosphate dehydrogenase expressed in the cytosol as sensor reaction highly dynamic changes in the cytosolic NADH/NAD$^+$ ratio, as result of glucose and combined glucose/acetaldehyde pulses to a steady state glucose limited chemostat, could be measured [50]. Disadvantage of this sensor reaction based technique is that only population average signals can be obtained. Any single cell variation in redox ratios cannot be observed.

The ultimate aim is to be able to measure the complete spatial and temporal state of the entire metabolism. The described sensors, both equilibrium-based and fluorescence-based, are able to provide these measurements, but are limited to only a select amount of metabolites. The current GC–MS and LC–MS techniques are able to measure the full scope of the metabolism within a discrete temporal space, but are unable to provide the spatial property of metabolites of the cell, especially important within eukaryotic cells. However, even with future improvements in resolution and accuracy of MS technology, the spatial property of the metabolism will not be resolved using regular extraction techniques. Promising developments to resolve this issue are made in the field of single-cell metabolomics [59••]. MALDI-MSI (matrix assisted laser desorption/ionization mass spectrometry imaging) is utilized to sample the spatial distribution of metabolites within samples. With a lateral resolution down to 1.4 μm [60], this technique was utilized to analyse single-cell organisms with subcellular resolution. Because of the need of sample preparation, this technique, as of yet, cannot be utilized to measure single-cell and subcellular metabolome changes under dynamic conditions. However, with future developments in metabolome quenching techniques and organelle purification, MS imaging may well help provide full inside into the actual metabolic spatial and temporal state of single cells.

Concluding remarks

Redox cofactors, available redox sinks and sources generate relevant couplings within the metabolic networks and influence the range of reactions that are thermodynamically feasible within the cell. As highlighted, many intracellular storage processes can be considered as overflow processes, similar to fermentative pathways, which are essentially extracellular storage processes. Mechanisms redirecting electron flows, like overflow metabolism or intracellular storage pools, can accelerate metabolic fluxes and increase competitiveness under many dynamic conditions. At the same time electron storage incellular or extracellular can increase metabolic flexibility [23].

Future research will have to consider the limitations imposed by the redox metabolism on the metabolism as a whole. Especially in compartmented systems, the quantification of the *in vivo* subcellular redox ratios is of paramount importance for realistic modelling and understanding of cellular metabolism and for design of novel strains and processes. Further developments in the field of single cell metabolomics may help to provide insight in the metabolic spatial and temporal state of single cells and their subcellular components. In addition, many

enzyme cofactor specificities, especially for non-model organisms, are unknown and thus more enzymatic assays are required for elucidation of novel enzymes for metabolic engineering. Furthermore, additional measurements and computational predictions of electron transfer between compartments through protein-protein complexes [62] or redox shuttles, as well as membrane processes, such as electron bifurcation [63], are essential to gain insight into dynamic switches within the redox metabolism.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Koen JA Verhagen: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Walter M van Gulik:** Writing - review & editing. **Sebastian Aljoscha Wahl:** Conceptualization, Writing - review & editing, Supervision.

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