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Chapter 2: Importance of electron flow in microbiological metabolism

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Abstract: This chapter provides an overview of microbial intracellular electron flow and its physiological contribution to metabolism. Oxidative and reductive reactions are involved in most cellular processes, including energy conservation, anabolic biosynthesis, maintenance of cellular redox balance, and antioxidant defense. The respiratory electron transport pathway linked to oxidative phosphorylation is important for ATP synthesis in both aerobic and anaerobic environments. Aerobic respiration employs various types of terminal oxidases, and organisms use the appropriate one(s) according to the environment they inhabit. Electron transport pathways are also functional in anaerobic organisms, where anaerobic respiration and photosynthesis operate. The expression of genes involved in these electron transport pathways are generally governed by regulators that sense oxygen tension or redox status of quinone pool. Finally, the concept and mechanisms of electron bifurcation, which provides strong electron donors with a higher energy level to diverse reducing reactions, has been discussed. We believe that detailed understanding of the flow of electrons would offer novel avenues in basic and translational research.

Keywords: Aerobic respiration, Carbon fixation, Denitrification, Electron bifurcation, Electron transport chain, Ferredoxin, Glycolysis, NADH, NADPH, Photosynthesis, Sulfate reduction

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2.1 Introduction

Many types of oxidative and reductive reactions occur within individual microbial cells to mediate intracellular metabolism and biological activities. These reactions are diverse, with more than 1,800 Enzyme Commission (EC) number entries categorized in the oxidoreductases class (EC 1) in the ENZYME database (Bairoch 2000) till date (October 2019). These redox reactions coordinate to accomplish various physiological demands, such as energy conservation, anabolic biosynthesis, maintenance of cellular redox homeostasis, and antioxidant defense. Intracellular electrons flow through coordinated redox reactions arranged in complicated chains and networks. Understanding the regulated electron flow can form the basis for studies on electron-based bioscience, biotechnology, and biocorrosion as discussed in this book.

With this premise, we elaborate on the biological electron carriers operative in cellular metabolism (Sect. 2.2) and the redox reactions and pathways that govern the total electron flow in microbes (Sect. 2.3). Further, we discuss the ubiquitous energy synthesis system through the respiratory chain (Sect. 2.4). Finally, we will shed light on the unconventional mechanisms of electron transfer carried out by electron bifurcation systems (Sect. 2.5).

2.2 Biological electron carriers

Biological electron transfers are mediated by many kinds of biomolecules, including quinone/quinol, cytochromes, flavins (FMN and FAD), and disulfides. In this section, nicotinamide nucleotide coenzymes (Sect. 2.2.1) and ferredoxin (Sect. 2.2.2) are reviewed as primary electron carriers.

2.2.1 Nicotinamide nucleotide coenzymes: NAD(H) and NADP(H)

Among the various biological electron carriers, NAD(H) and NADP(H) serve as the primary electron acceptor/donor and contribute ubiquitously to most metabolic pathways. Because these two share a similar structure except for the presence/absence of an additional phosphate group, their redox potentials are comparable to each other (E_0' = −320 mV). The oxidized forms, NAD^+ and $NADP^+$, get reduced by the transfer of a hydride ion (H^-) onto the nicotinamide ring. Due to this reaction mechanism, their oxidation and reduction require a simultaneous transfer of two-electrons. The importance

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of this feature would be discussed in further detail for understanding two-electron/one-electron switch in the flavin-based bifurcation system (Sect. 2.5).

Several physiological differences exist between NAD(H) and NADP(H). One of them is their redox ratio; while NADP(H) is generally maintained in a reduced state, NAD(H) remains in more oxidized state. It is reported that in bacterial cells, $NADPH/NADP^+$ ratio ranges from 1.1 to 59 whereas $NADH/NAD^+$ ratio is lower (between 0.032 to 0.27) (Spaans et al. 2015). Another difference occurs in the metabolic pathways in which these two coenzymes work; while NAD(H) is involved in catabolism and a wide range of metabolism, NADPH works as an electron donor in anabolic and biosynthetic pathways such as photosynthesis and fatty acid synthesis (Spaans et al. 2015; Agledal et al. 2010). The highly reduced state of NADP(H) can promote biosynthetic metabolism by driving the NADPH-dependent reduction reactions. The difference in the redox balance between NAD(H) and NADP(H) also forms a key factor for an electron bifurcation system (NADH-dependent reduced ferredoxin $NADP^+$ oxidoreductase in Sect. 2.5.3) in which NADPH and NAD^+ serves as an electron donor and acceptor, respectively.

2.2.2 Ferredoxin

Ferredoxin (Fd) is a small metalloprotein harboring Fe-S cluster(s) as the redox center. Fd can be classified based on the structure of its Fe-S cluster, such as [2Fe-2S], [4Fe-4S], [3Fe-4S], and [7Fe-8S]. In contrast to NAD(H) and NADP(H), an Fe-S cluster in Fd physiologically transfers only one electron at a time. Due to its simple structure and wide distribution among organisms, Fd is presumed to be one of the evolutionarily oldest proteins and to be even more primitive than NAD(H) (Eck and Dayhoff 1966; Hall et al. 1971; Daniel and Danson 1995).

Another characteristic of Fd as an electron carrier is its low redox potential. Although Fds are diverse in their redox potentials ranging from −500 mV to −340 mV, many of them are around −420 mV (Valentine 1964; Tagawa and Arnon 1962; Buckel and Thauer 2018b), significantly lower than those of NAD(P)H. Thus, reduced Fd can provide a reducing power strong enough to drive energetically-unfavored reactions that cannot be driven by NAD(P)H, such as H_2 production, CO_2 fixation, and nitrogen fixation.

2.3 Redox reactions in metabolism

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2.3.1 Carbon metabolism

Glycolysis Conversion of one molecule of glucose to two pyruvate molecules is accompanied by the generation of reducing equivalents ($4e^-$). In the Embden-Meyerhof pathway in many bacteria, the reducing equivalents are transferred to NAD^+ by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), followed by the NADH re-oxidation coupled to the respiratory chain (see Sect. 2.4). In bacteria where respiration is not functional, NADH is oxidized by the donation of electrons to glycolysis products, resulting in fermentation products such as ethanol and lactate. Another type of glycolytic pathway, the Entner-Doudoroff pathway, is operative in some microbes (Chen et al. 2016). This pathway donates electrons not only to NAD^+ but also to $NADP^+$, providing one NADH and one NADPH per one glucose oxidized. Instead of these pathways, glucose can be metabolized also through the pentose-phosphate pathway (Stincone et al. 2015). It should be noted that the provision of NADPH for cellular biosynthetic reactions is one of the important roles of the pentose-phosphate pathway because electrons are donated not to NAD^+ but only to $NADP^+$ in this pathway.

In Archaea, some NAD-reducing reactions in glycolysis are replaced by Fd-reducing reactions. Glyceraldehyde-3-phosphate:Fd oxidoreductase (EC 1.2.7.6) substitutes (or coexists with) NAD-dependent glyceraldehyde-3-phosphate dehydrogenase with the concomitant reduction of ferredoxin in archaeal species, including *Pyrococcus furiosus*, *Pyrobaculum aerophilum*, and *Methanococcus maripaludis* (Reher et al. 2007; Mukund and Adams 1995; Costa et al. 2013). As another example, pyruvate:Fd oxidoreductase (POR; EC 1.2.7.1) substitutes NAD-depending pyruvate dehydrogenase complex (PDH; EC 1.2.4.1, 2.3.1.12, 1.8.1.4), catalyzing oxidative decarboxylation of pyruvate to acetyl-CoA (Furdui and Ragsdale 2000). Fd reduced by these reactions is used for hydrogenation as an electron sink or for driving methanogenesis in these archaea living in anaerobic environments.

Tricarboxylic acid cycle The Tricarboxylic acid (TCA) cycle is a major source of NADH and electron equivalents in many aerobic organisms. In this cycle, NAD^+ is reduced to NADH by isocitrate dehydrogenase (EC 1.1.1.42), 2-oxoglutarate dehydrogenase (OGDH) complex (OGOR; EC 1.2.4.2, 2.3.1.61, 1.8.1.4), and malate dehydrogenase (EC

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1.1.137). In the other oxidative reaction catalyzed by succinate dehydrogenase (EC 1.3.5.1), electrons are transferred not to NAD^+ but to quinone because the reducing power provided by the oxidation of succinate to fumarate is weak (E_0' = 33 mV) (Thauer et al. 1977).

The TCA cycle produces reducing equivalents ($8e^-$ per 1 acetyl-CoA oxidized), but an electron sink is often unavailable in organisms grown in anaerobic environments. In these organisms, an incomplete TCA cycle functions in a "horseshoe" structure, being divided into two halves: an oxidative half-cycle leading to 2-oxoglutarate and a reductive half-cycle to fumarate (Jahn et al. 2007; Marco-Urrea et al. 2011). This incomplete cycle still provides TCA cycle metabolites as important precursors for various biosynthetic processes without producing excess electrons.

Carbon fixation To date, six metabolic pathways have been found to fix CO_2 in autotrophic organisms (Montoya et al. 2012; Berg and Ivanovskii 2009; Berg et al. 2010). All of the fixing pathways require reductants to convert CO_2 into metabolites. While Fd is used in three pathways (including the acetyl-CoA pathway and the reductive TCA cycle) out of the six, the other three pathways (including the Calvin cycle) rely only on NAD(P)H. Because the reduced Fd can more strongly drive the reaction due to its low redox potential than that of NAD(P)H, the Fd-dependent pathways require less ATP equivalents than the Fd-independent pathways.

The reductive TCA cycle is known as a "reversed" version of the TCA cycle, and the two cycles share homologous enzymes and reaction steps. However, the difference between them surfaces especially when they are analyzed from the perspective of electron carriers. Whereas PDH and OGDH in the TCA cycle use NAD^+ as the electron acceptor, the reverse reactions in the reductive TCA cycle are catalyzed by POR and OGOR using reduced Fd as the electron donor (Ikeda et al. 2010; Yamamoto et al. 2010). This difference is feasible because the carboxylation reactions catalyzed by POR and OGOR are energetically unfavorable and require strong reductants with a low redox potential. In addition, while succinate oxidation in the TCA cycle donates electrons to quinone, *Hydrogenobacter thermophilus* is reported to reduce fumarate using NADH as the reductant and not quinol (Miura et al. 2008). Considering that the redox potentials of NAD^+/NADH is significantly lower than those of fumarate/succinate and quinone/quinol,

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the use of NADH is advantageous to drive the fumarate/succinate conversion irreversibly in the direction of the reductive TCA cycle.

2.3.2 Nitrogen metabolism

Nitrogen forms a constituent of inorganic compounds with various redox states: +5 in nitrate (NO_3^-), +3 in nitrite (NO_2^-), +2 in nitric oxide (NO), +1 in nitrous oxide (N_2O), 0 for dinitrogen (N_2), −1 in hydroxylamine (NH_2OH), and −3 in ammonium (NH_4^+). Organisms utilize these compounds/intermediates as electron donors or acceptors in energy synthesis processes known as denitrification, anammox, and nitrification, as already reviewed elsewhere (Canfield et al. 2010; Stein and Klotz 2016; Kuypers et al. 2018).

Besides energy synthesis, reduction of inorganic nitrogen compounds occurs in cellular anabolism. Organisms can assimilate nitrogen only in the form of NH_4^+, thereby necessitating the reduction of the oxidized forms of nitrogen to NH_4^+ before assimilation. Assimilatory nitrate reductase (aNar) and nitrite reductase (aNir) catalyze the reduction of NO_3^- into NH_4^+ in their coupling reaction. NH_4^+ is incorporated into Glu by the coupling reaction of Gln synthetase and Glu synthase (GOGAT) (Kameya et al. 2006). While many bacterial aNar and GOGAT are NAD(P)H-dependent, cyanobacteria and chloroplast possess Fd-dependent aNar and GOGAT, which had been considered to be "plant-type" enzymes. However, recent studies discarded this paradigm by reporting Fd-dependent aNar and GOGAT in non-phototrophs, such as hydrogen-oxidizing bacteria and haloarchaea (Martinez-Espinosa et al. 2001; Kameya et al. 2007; Zafrilla et al. 2011; Pire et al. 2014; Kameya et al. 2017).

Nitrogen fixation is a process where N_2 is reduced to NH_4^+ by nitrogenase (EC 1.18.6.1) with the aid of ATP hydrolysis and electron supply from Fd (Buckel and Thauer 2018b). Because ammonification of N_2 is energetically unfavorable, the use of Fd with a low redox potential is the most suitable resort to drive the reaction, as for the carboxylation reactions discussed above.

2.3.3 Sulfur metabolism

As with nitrogen, sulfur forms various inorganic species at different redox states; for instance, −2 in thiosulfate ($S_2O_3^{2-}$), 0 in elemental sulfur, +4 in sulfite (SO_3^{2-}), and

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+6 in sulfate (SO_4^{2-}). Many microbes use sulfur species for electron transfer during energy synthesis and redox metabolism.

Oxidation of sulfur compounds is catalyzed by the coupling of several enzymes and systems, such as the sulfur oxidizing (Sox) enzyme system, heterodisulfide reductase (Hdr)-like system, sulfide:quinone oxidoreductase (EC 1.8.5.4), and sulfur oxygenase reductase (EC 1.13.11.55) (Friedrich et al. 2005; Wang et al. 2018). Electrons produced through these oxidizing reactions are transferred to quinone, which can be linked to the electron transport chain to produce energy.

Sulfate-reducing bacteria anaerobically oxidize organic compounds to synthesize energy and carbon skeletons, and the electrons produced through this oxidation are donated to the terminal electron acceptor, sulfate (Qian et al. 2019). Sulfate reduction is also ecologically important to maintain the activity of anaerobic methane-oxidizing archaea (ANME) because symbiotic sulfate-reducing bacteria accept and consume electrons produced by methane oxidation, thus allowing a continuum (McGlynn et al. 2015).

Sulfur is one of the primary elements of all organisms and is contained in many biomolecules, such as Cys, Met, glutathione, and proteins harboring Fe-S clusters. In general sulfur anabolism, inorganic sulfur compounds are incorporated to produce Cys, and the Cys serves as a sulfur donor for biosynthetic reactions of sulfur-containing metabolites. Sulfate and sulfite are reduced to sulfide before being incorporated into Cys, where NAD(P)H and electron carrier proteins (thioredoxin and glutaredoxin) serve as electron donors (Sekowska et al. 2000).

2.4 Respiratory electron transport pathway

2.4.1 Branched bacterial respiratory chain

In the presence of oxygen, aerobic and facultative anaerobic bacteria produce most of the energy required for cellular function, such as anabolic metabolism, ion homeostasis, or motility, through aerobic respiration like that of mitochondria in eukaryotic cells. Mitochondrial respiratory chain consists of four complexes, NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), a cytochrome bc_1 complex (complex III), and a cytochrome *c* oxidase (complex IV). Protons are pumped across the inner mitochondrial membrane during electron transfer through complexes I,

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III, and IV, producing a transmembrane chemiosmotic gradient. The generated proton motive force (PMS) drives ATP synthesis by F_0F_1-ATP synthase. This entire process of PMS generation and subsequent ATP synthesis is called oxidative phosphorylation. Many bacteria have more complex and flexible respiratory electron transfer pathways because they use a variety of electron donors and acceptors for oxidative phosphorylation (Anraku 1988).

Bacterial respiratory chains are composed of primary dehydrogenases that feed electrons to quinone/quinol pool from low-potential substrates, and terminal oxidoreductases that reduce the high-potential electron acceptors. Respiratory substrates, such as NADH, succinate, lactate, malate, and hydrogen, are used as electron donors. Among them, NADH, generated by the TCA cycle, is the most common substrate for aerobic respiration. *Escherichia coli* is known to have a multi-component NADH dehydrogenase corresponding to the mitochondrial complex I, which has proton-pumping activity, and a single-subunit type flavin-containing enzyme, which does not couple the redox reaction to proton translocation (Calhoun et al. 1993). Some bacteria such as *Vibrio cholerae* have a sodium-pumping NADH:ubiquinone oxidoreductase (Steuber et al. 2015). The electrochemical potential generated by sodium excretion is also used for oxidative phosphorylation.

Oxygen is used as the terminal electron acceptor for aerobic respiration. The enzymes that catalyze the four-electron reduction of oxygen to water at the end of the aerobic respiratory chain are called terminal oxidases. They can be divided into two groups, cytochrome *c* oxidases and quinol oxidases. The former receives electrons from quinol via the cytochrome bc_1 complex and *c*-type cytochromes, while the latter receives electrons directly from quinol (Garcia-Horsman et al. 1994). Most bacteria have more than one terminal oxidases with different features, that are used differentially depending on the growth conditions. The branched respiratory pathways of *Pseudomonas aeruginosa* and *Rhodobacter sphaeroides* are shown in Fig. 2.1 for reference.

2.4.2 Heme-copper oxidases for aerobic respiration

Cytochrome *c* oxidases and most quinol oxidases are membrane-bound multi-subunit enzymes belonging to the heme-copper oxidase superfamily. Although they have a variation in heme types and subunit composition, their catalytic subunits (subunit I)

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commonly contains 12 core transmembrane helices and are characterized by the presence of a six-coordinated low-spin heme and a binuclear catalytic center composed of a high-spin heme and a copper ion, Cu_B. The second subunit (subunit II) of most cytochrome *c* oxidases have a binuclear copper center, Cu_A, that receives electrons from cytochromes *c* and passes them to the catalytic subunit. The Cu_A site is absent in quinol oxidases.

The heme-copper oxidases, irrespective of cytochrome *c* oxidases and quinol oxidases, are proposed to be divided into three major types, A, B, and C, based on the constituents of their proton channels. The classification is also in good accordance with their phylogenetic relationship (Pereira et al. 2001; Sousa et al. 2012). Type A includes the mitochondrial complex IV and is distributed in many bacteria and some archaea. The type A oxidases have two proton conducting channels, named D- and K-channel, according to specific aspartate and lysine residues present in the channels, respectively. Type A can be divided into two subfamilies, A1 and A2, according to the conserved motif in the helix VI at the hydrophobic end of the D-channel. The motif sequences are –XGHPEV– and –YSHPXV– for the types A1 and A2, respectively (Pereira et al. 2001).

The aa_3 cytochrome *c* oxidases from *Paracoccus denitrificans* and *Rhodobacter sphaeroides*, and the bo_3 quinol oxidase from *E. coli* are type A enzymes. These oxidases have low affinity for oxygen and are utilized under high oxygen conditions. The proton pumping stoichiometry (H^+/e^- ratio) of the type A oxidases has been reported to be 0.75–1 (Pereira et al. 2008). The aa_3-like oxidases, which have C-terminal extension of subunit II containing one or two heme *c* binding motifs, have been found in many bacterial species such as *Thermus thermophilus*, *P. aeruginosa*, and *Shewanella oneidensis* (Lyons et al. 2012; Osamura et al. 2017; Le Laz et al. 2016). These enzymes are referred to as caa_3 or $ccaa_3$. The caa_3 oxidase of *P. aeruginosa* and the $ccaa_3$ oxidase of *S. oneidensis* have been reported to be expressed under starvation conditions (Kawakami et al. 2010; Le Laz et al. 2016).

Type B is mainly distributed in Crenarchaeota and thermophilic bacteria. A catalytic tyrosine residue in helix VI, which is covalently linked to a copper-binding histidine is conserved both in the type A and type B enzymes. The type B oxidases are thought to have only one proton conducting channel. The three-dimensional structure of the ba_3 oxidase of *T. thermophilus* indicated three possible proton-conducting pathways (Soulimane et al. 2000). One of these is an alternative of the K-channel of the type A

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enzymes, although some amino acid residues are substituted. The other two are not conserved in other type B enzymes and might not be functional (Sousa et al. 2012).

Type C is found only in bacteria, mainly in Proteobacteria. The cbb_3 cytochrome *c* oxidase is the only member of this type. The cbb_3 oxidases are encoded by the *ccoNOQP* (or *fixNOQP*) genes. CcoN is the core catalytic subunit corresponding to the subunit I of the other types. CcoO and CcoP are membrane-bound monoheme and diheme cytochromes *c*, respectively. CcoQ is known to affect the stability of the cbb_3 complex (Ekici et al. 2012; Buschmann et al. 2010). The position of the catalytic tyrosine residue is different from that of the type A and B enzymes and located in helix VII of CcoN. The cbb_3 oxidases do not have the Cu_A center. The electrons are thought to be received by the distal heme group of CcoP from the electron donor cytochromes *c*. The electrons are transferred to the catalytic center in CcoN via the proximal heme of CcoP and the heme group of CcoO. The cbb_3 oxidases have only one proton-conducting channel, which is an alternative of the K-channel, but its amino acid composition is different from those of the type A and B enzymes (Buschmann et al. 2010). The H^+/e^- ratios of the type B and C enzymes are reported to be 0.5–0.75 and 0.2–0.4, respectively, which is lower than those of the type A enzymes (Pereira et al. 2008). The lower values are thought to result from the lack of the D-channel. It is has been recently reported that the stoichiometry is close to unity among the three types if there is no proton leak under the optimal experimental conditions (Rauhamäki and Wikström 2014).

The cbb_3 oxidases have a very high affinity for oxygen with K_m values ranging from 6.5 to 40 nM and are usually utilized under low oxygen conditions (Arai et al. 2014; Mouncey and Kaplan 1998; Otten et al. 2001). Many *Pseudomonas* species have two tandemly located *ccoNOQP* gene clusters. In *P. aeruginosa*, one of the clusters is expressed constitutively and the other is induced under low oxygen conditions (Kawakami et al. 2010). *P. aeruginosa* also has two additional orphan *ccoNQ* gene clusters and can produce sixteen cbb_3 oxidase isoforms via combinations of four CcoN, two CcoO, and two CcoP isosubunits (Hirai et al. 2016). The isoforms derived from the orphan clusters confer resistance to nitrite and cyanide under low oxygen conditions.

2.4.3 Non-heme-copper oxidases for aerobic respiration

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The heme-copper oxidases are very sensitive to cyanide, however, many bacteria have cyanide-resistant respiration pathways that use non-heme-copper oxidases. Two major types of quinol oxidases, the cytochrome *bd* oxidase and the alternative oxidase (AOX), are known as the cyanide-resistant respiratory oxygen reductases. The *bd* oxidase is a two-subunit enzyme containing a low-spin heme b_{558} and a binuclear center consisting of a high-spin heme b_{595} and a heme *d*. The *bd* oxidase does not pump protons, but it generates proton gradient across the cell membrane by proton extrusion from quinol oxidation at the periplasmic side and proton uptake from the cytoplasm for oxygen reduction (Safarian et al. 2016).

The cyanide-insensitive oxidase (CIO) is known as a subfamily of the *bd* oxidase. CIO is homologous with, but phylogenetically distinct from the canonical *bd* oxidases. The conserved sequence of the hydrophilic loop (Q-loop), which contains the putative quinol oxidizing site, is shorter in CIOs than in the canonical *bd* oxidases (Cunningham et al. 1997). CIO contains the hemes b_{558}, b_{595}, and *d*, but shows very weak spectroscopic features of heme b_{595} and heme *d* (Miura et al. 2013; Mogi et al. 2009). The canonical *bd* oxidases have high affinity for oxygen and are generally utilized under low oxygen conditions such as in *E. coli* (Jünemann 1997). However, CIOs of *Campylobacter jejuni*, *Gluconacetobacter oxydans*, and *P. aeruginosa* have been reported to have a low affinity (Arai et al. 2014; Jackson et al. 2007; Miura et al. 2013).

AOX is a non-heme di-iron protein found in all higher plants and certain eukaryotes including some algae, fungi, and protists (Moore and Albury 2008). The crystal structure of AOX from a protozoan parasite *Trypanosoma brucei*, which causes human African sleeping sickness, has been reported (Shiba et al. 2013). The genes encoding AOX have also been identified in the genomes of some bacteria, especially those inhabiting marine environments, such as *Vibrio fischeri* and *R. denitrificans* (Dunn 2018; Swingley et al. 2007). AOX does not create proton gradient across the membrane and supports the oxidative phosphorylation only when coupled with proton-translocating NADH dehydrogenase.

AOX is responsible for the production of heat in thermogenic plants. It also contributes to stress resistance by maintaining redox and metabolic homeostasis in plant cells (Saha et al. 2016). When the parasite *T. brucei* lives in blood stream of its mammalian host, the glycolytic pathway is the major source of ATP, and the oxidative

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phosphorylation using cytochromes is not operative. Because AOX is required for re-oxidation of NADH accumulated during glycolysis in the blood stream form of the parasite, it is expected to be a drug target for the infectious disease (Shiba et al. 2013). The function of bacterial AOX was only reported in *V. fischeri*. The gene encoding AOX is regulated by an NO-sensing regulator, NsrR, and involved in reducing stress during exposure of the bacterium to NO (Dunn 2018). Our preliminary investigation has indicated that AOX is required for the growth of the photosynthetic bacterium *R. denitrificans* under low temperature conditions.

2.4.4 Respiratory protection by high-affinity oxidases

The *bd* quinol oxidase and the *cbb*$_3$ cytochrome *c* oxidase have a very high affinity for oxygen. These high-affinity oxidases are thought to have a role in protecting oxygen-sensitive enzymes or processes by scavenging oxygen. The protective role of the *bd* oxidase was initially proposed in a free-living nitrogen-fixing bacterium *Azotobacter vinelandii*, in which oxygen scavenging by the *bd* oxidase is thought to be required for the oxygen-sensitive nitrogenase activity (Poole and Hill 1997), although the role is still arguable (Oelze 2000). The *bd* oxidase has also been reported to be involved in conferring resistance to oxidative and nitrosative stresses in bacteria such as *E. coli* and *Mycobacterium tuberculosis* (Giuffrè et al. 2014).

Some chemolithoautotrophic bacteria such as *Hydrogenophilus thermoluteolus* and *Hydrogenovibrio marinus*, which fix carbon dioxide by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in the Calvin-Benson-Bassham cycle, have the *cbb*$_3$ oxidase as the only terminal oxidase for aerobic respiration (Arai et al. 2018; Arai and Ishii 2019). The oxygenase reaction of RubisCO is accompanied by wastage of energy and fixed carbon. Scavenging oxygen by the *cbb*$_3$ oxidase might minimize the futile oxygenase reaction and be advantageous for the autotrophic growth of these chemolithotrophs under aerobic conditions.

2.4.5 Anaerobic electron transport pathways

In the absence of oxygen, many bacterial species utilize alternative electron acceptors for the oxidative phosphorylation by anaerobic respiration. Many organic and inorganic compounds and metal ions, such as fumarate, dimethyl sulfoxide (DMSO),

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trimethylamine *N*-oxide (TMAO), nitrogen oxides, sulfate, elemental sulfur, ferric iron, and arsenate, are known to be utilized for anaerobic respiration. These compounds and ions are reduced by specific reductases that receive electrons from the respiratory chain.

E. coli uses nitrate or fumarate as the terminal electron acceptor for anaerobic respiration. Nitrate is catabolically reduced to ammonium via nitrite and the process is called ammonification. Because oxygen is a preferred electron acceptor for oxidative phosphorylation, the genes for anaerobic respiration enzymes are usually repressed under aerobic conditions. The induction of these genes under the anaerobic conditions is regulated by an oxygen-sensing global regulator FNR (fumarate and nitrate reductase regulator) (Gunsalus and Park 1994).

Some facultative anaerobic bacteria such as *P. aeruginosa* reduce nitrate to dinitrogen via nitrite, NO, and N_2O by the process called denitrification (Arai 2011). (Fig. 2.1a). The four steps of the denitrification pathway are each catalyzed by metalloenzymes, which receive electrons from quinol, cytochromes *c*, or copper proteins in the respiratory chain. Among the denitrification enzymes, NO reductase (NOR) is phylogenetically related to the terminal oxidases of the heme-copper oxidase superfamily, however, it lacks the proton-pumping activity. The catalytic binuclear center of NOR contains a non-heme iron, Fe_B, instead of Cu_B (Shiro 2012). In some pathogenic bacteria, NOR also has a function for detoxification of NO produced by the host immune defense system. Expression of the denitrification genes under the anaerobic conditions is regulated by an oxygen-sensing regulator ANR (anaerobic regulation of arginine deiminase and nitrate reduction), which is the analog of *E. coli* FNR, and a NO-sensing regulator DNR (dissimilatory nitrate respiration regulator) in *P. aeruginosa* (Arai et al. 1997; Kuroki et al. 2014).

Photosynthesis is another system to produce ATP using the proton gradient by a mechanism similar to oxidative phosphorylation. In contrast to the oxygenic photosynthesis of plants and cyanobacteria, photosynthetic bacteria, such as *R. sphaeroides*, perform anoxygenic photosynthesis under anaerobic conditions in the presence of light (Mackenzie et al. 2007). The process of ATP production in anoxygenic photosynthesis is called cyclic photophosphorylation. In this process, low-energy electrons in the reaction center bacteriochlorophyll are excited by light energy. The excited electrons are transferred through cyclic electron transport chain composed of

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quinones and cytochromes and return to the bacteriochlorophyll (Fig. 2.1b). PMS is generated during the electron transport and ATP is produced using PMS, akin to oxidative phosphorylation. Some photosynthetic bacteria, such as the *Roseobacter* clade, produce the anoxygenic photosynthetic apparatus even under aerobic conditions (Rathgeber et al. 2004). Because photosynthetic and respiratory pathways share a common electron transfer chain, regulation of electron flow in the diverse electron transport pathways is more important in these photosynthetic bacteria.

2.4.6 Regulation of the electron transport pathway

Aerobic respiration is a preferred ATP synthesis system and the anaerobic respiration and anoxygenic respiration genes are generally induced in the absence of oxygen. As described above, the oxygen-sensing regulator FNR and its homologs function as global regulators for the expression of the anaerobic respiratory enzymes in several facultative anaerobes. They are also involved in the expression of anoxygenic photosynthesis apparatus in photosynthetic bacteria.

The ambient oxygen tension and the redox status of the respiratory chain form important signals for the regulation of multiple terminal oxidases. The FNR-type regulators also regulate the expression of the high-affinity *bd* oxidase and cbb_3 oxidase in response to oxygen depletion. The *fnr* gene is often clustered with the *ccoNOQP* and *hemN* genes, which encode the cbb_3 oxidase and the oxygen-independent coproporphyrinogen III oxidase, respectively. The latter is used for heme biosynthesis under hypoxic or anoxic conditions.

The redox status of the respiratory chain is predicted to be sensed by two-component regulatory systems. In *E. coli*, the ArcBA two-component system regulates many genes for energy metabolism, including those for the *bd* oxidase, in combination with FNR, by monitoring the redox status of the quinone pool (Gunsalus and Park 1994). The PrrBA/RegBA/RoxSR-type two-component systems, which are predicted to sense the redox status of the respiratory chain, either by the redox status of the quinone pool or by the electron flow through terminal oxidases, are found in several Proteobacteria. PrrBA or RegBA regulate the expression of the genes responsible for photosynthesis, carbon dioxide fixation, nitrogen fixation, and many other functions in photosynthetic bacteria of the *Rhodobacter* species (Mackenzie et al. 2007). In *P. aeruginosa*, five

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terminal oxidase gene clusters are directly or indirectly regulated by RoxSR (Kawakami et al. 2010; Arai 2011). Because quinones are located at the pivotal point of the divergent electron transport chain of aerobic and anaerobic respiration and photosynthesis (Fig. 2.1), the redox status of the quinone pool serves as a critical leverage on the trafficking of electrons.

2.5 Electron bifurcation

In several biochemical reactions, an electron carrier with a higher energy level gets reduced by an electron carrier with a lower energy level. Such instances are mediated by electron bifurcation, unless membrane potential is used. In the biochemical field, quinone-based electron bifurcation and flavin-based electron bifurcation have been verified. In this section, a brief history, the current research status, and future prospects, of electron bifurcation has been discussed.

2.5.1 What is electron bifurcation?

In electron bifurcation process, a pair of electrons with the same energy level is essentially converted to two electrons with different energy status (Buckel and Thauer 2018a). That is, one electron is converted to one with higher energy level and the other electron is converted to one with lower energy level. Usually, the first electron leaves as one with the lower energy level, and the second electron comes out as one with the higher energy level. Therefore, by adopting electron bifurcation process, it becomes possible to reduce an electron carrier with higher energy level (lower redox potential) by an electron carrier with a lower energy (higher redox potential).

In view of the history of biochemistry, electron bifurcation process was firstly found in complex III (cytochrome bc_1 complex) (Mitchell 1976). Still, it was about 40 years later, when the existence of another type of electron bifurcation system, flavin-based electron bifurcation, was discovered (Li et al. 2008). Nowadays, metal-based electron bifurcation has also been suggested (Peters et al. 2018).

2.5.2 Quinone-based electron bifurcation

As mentioned above, electron bifurcation is operative in cytochrome bc_1 complex. In the complex, quinol is converted to semi-quinone by transferring one electron to Fe-S

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protein. This first electron is with low energy. Then, the generated semi-quinone state with higher energy level than the quinol state, is able to discharge an electron with high energy. Further, with the aid of large conformational changes in the cytochrome bc_1 complex, the semi-quinone makes a contact with cytochrome *b* and reduces it through electron transfer (Crofts et al. 2017). Here, it may be emphasized that quinone has three redox states; oxidized state (quinone state), one-electron-reduced state (semi-quinone state), and two-electrons-reduced state (quinol state), and is therefore capable of harboring two electrons simultaneously.

2.5.3 Flavin-based electron bifurcation

As mentioned above, flavin-based electron bifurcation was discovered around 40 years after the discovery of the quinone-based electron bifurcation system (Li et al. 2008). Since the first discovery of flavin-based electron bifurcation, many metabolic systems with such electron bifurcation have been reported (Buckel and Thauer 2018b). Similar to quinone, flavin also displays three states, which forms the theoretical basis for electron bifurcation. It should be considered that flavin can adopt neutral or anionic structures under the three redox states; oxidized, semi-quinone, and reduced; which might explain the limited clarification of mechanism for flavin-based electron bifurcation metabolic reactions.

A detailed structural analysis has been performed on the NADH-dependent reduced ferredoxin $NADP^+$ oxidoreductase (Nfn) from *Pyrococcus furiosus* (Lubner et al. 2017). Nfn is an enzyme that catalyzes the following reaction: $2NADPH + NAD^+ + 2Fd_{ox} \rightarrow 2NADP^+ + NADH + 2Fd_{red}$.

The bifurcating flavin in this enzyme has an electrochemical potential difference of +359 mV between HQ (hydroquinone state)/ ASQ (Anionic semi-quinone state) and -911mV ASQ / OX (oxidized state). For electron bifurcation, flavin is converted to a fully reduced state (hydroquinone state). The first electron, which comes out between HQ / ASQ, reduces NAD. The second electron, which is released between ASQ / OX, reduces ferredoxin. The electron generated between ASQ / OX does not reduce NAD. However, unlike cytochrome bc_1 complex, a large conformational change has not been detected. Although physical distance between flavin and [2Fe-2S]-type ferredoxin (14.1 Å) is minutely larger than the distance between flavin and [4Fe-4S]-type ferredoxin (7.5 Å),

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this is not enough to explain the high specificity of the second electron transfer. The reason can be explained by including the Marcus theory (Marcus and Sutin 1985) in addition to physical distance.

The Marcus theory states the energy gap law. In chemical reactions, it is reasonable to consider that lower the free energy of the final state, the higher would be the electron transfer rate. However, according to Marcus theory, there exists a reversal region, wherein after a threshold energy difference is crossed, the speed of electron transfer would decrease. This theory is now used to explain the mechanism by which the second electron reduces ferredoxin, and not NAD.

2.5.4 Metal-based electron bifurcation

Any redox cofactor that can receive at least two electrons and can then donate them sequentially can serve as a candidate for the essential catalytic part of electron bifurcation. Indeed, redox-factor architecture similar to that of Nfn is found in tungsten-containing formate dehydrogenase, [FeFe]-hydrogenase, and [NiFe]-hydrogenase. For this reason, these enzymes have been proposed to bear electron bifurcation capacity (Peters et al. 2018).

2.5.5 Future prospects of electron bifurcation

Since the discovery of flavin-based electron bifurcation, several findings are accumulating that precisely explain the theoretical basis of electron bifurcation through physics and structural information. Efforts ensue to develop electron bifurcation systems using biotechnological advances. This would require the construction of protein structures and/or peptide stretches, which would hold the bifurcating carriers and recruit electron acceptors. To this end, detailed understanding of the molecular machinery and biochemistry of the existing quinone-based, flavin-based, and metal-based bifurcation reactions is critical. Such studies would enable the control of metabolism on the basis of energetics.

Also, because electron bifurcation system has been identified from the microorganisms with old lineage (Schut and Adams 2009), biochemical analyses of these systems might provide avenues for generating insights on the emergence of life.

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2.6 Conclusions

We may appreciate that microorganisms take up external energy to convert it into bioenergy, and utilize the bioenergy to conduct various life activities such as proliferation, metabolism, and survival. The entire process of transforming external energy into bioenergy is governed by the flow of electrons. Therefore, electron transfer in microorganisms is a key facet to understand their metabolism and survival tactics.

Although there are unconventional instances such as an apparent uphill reaction during electron bifurcation, the flow of electrons is generally downhill. Since the electrons can be accepted by various molecules, microorganisms have evolved mechanisms to tightly regulate electron flow to ensure smooth functioning of all biological processes. In this light, microorganisms fine-tune the energy conservation systems so that electrons are received by the most appropriate redox systems. Yet, instances of erroneous transfer of electrons exist, which ultimately lead to the production of reactive oxygen species (ROS). The microorganisms utilize detoxification systems to ameliorate these conditions and use additional electrons for that.

Together, microorganisms orchestrate a highly complex system of handling electrons while sensing and responding to their external environment and cell-intrinsic cues.

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Figure legends

Fig. 2.1 Schematic representation of the branched respiratory electron transport pathway in *Pseudomonas aeruginosa* PAO1 (a) and *Rhodobacter sphaeroides* 2.4.1 (b). *P. aeruginosa* produces sixteen isoforms of the cbb_3 cytochrome *c* oxidase, which can be divided into four types. Two quinol oxidases of the heme-copper oxidase superfamily (Qox) and the cyanide-insensitive oxidase (CIO)-type (Qxt) are encoded in the genome of *R. sphaeroides*. Arrows indicate electron flow. The "up-hill" reverse electron flow that regenerates NADH under the anaerobic photosynthetic conditions is indicated by a dotted arrow. Q/QH_2, ubiquinone-ubiquinol pool; *hv*, light irradiation; LH1, LH2, light harvesting complexes; RC, photosynthetic reaction center.

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Index Word

aerobic respiration

alternative oxidase

cytochrome *c* oxidase

denitrification

Entner-Doudoroff pathway

heme-copper oxidase

hydrogen-oxidizing bacteria

Hydrogenobacter thermophilus

oxidative phosphorylation

pentose-phosphate pathway

photosynthetic bacteria

proton motive force

Pseudomonas aeruginosa

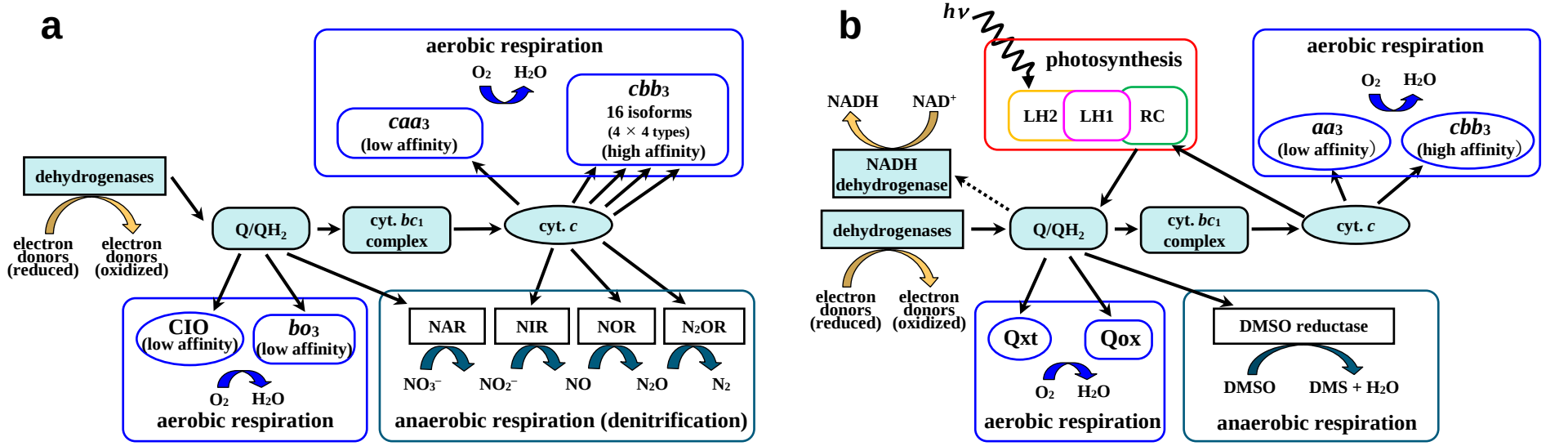
quinol oxidase

reductive TCA cycle

Roseobacter denitirificans

terminal oxidase

tricarboxylic acid cycle


a
aerobic respiration
O2 H2O
caa3
(low affinity)
cbb3
16 isoforms
(4 × 4 types)
(high affinity)
dehydrogenases
electron donors (reduced)
electron donors (oxidized)
Q/QH2
cyt. bc1 complex
cyt. c
CIO
(low affinity)
bo3
(low affinity)
O2 H2O
aerobic respiration
NAR
NIR
NOR
N2OR
NO3− NO2− NO N2O N2
anaerobic respiration (denitrification)
b
hν
photosynthesis
LH2 LH1 RC
NADH NAD+
NADH dehydrogenase
dehydrogenases
electron donors (reduced)
electron donors (oxidized)
Q/QH2
cyt. bc1 complex
cyt. c
aerobic respiration
O2 H2O
aa3
(low affinity)
cbb3
(high affinity)
Qxt
Qox
O2 H2O
aerobic respiration
DMSO reductase
DMSO DMS + H2O
anaerobic respiration