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Dynamic association of flavin cofactors to regulate flavoprotein function

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Abstract

Flavoproteins are key players in numerous redox pathways in cells. Flavin cofactors FMN and FAD confer the required chemical reactivity to flavoenzymes. In most cases, the interaction between the proteins and the flavins is noncovalent, yet stronger in comparison to other redox-active cofactors, such as NADH and NADPH. The association is considered static, but this view has started to change with the recent discovery of the dynamic association of flavins and flavoenzymes. Six cases from different organisms and various metabolic pathways are discussed here. The available mechanistic details span the range from rudimentary, as in the case of the ER-resident oxidoreductase Ero1, to comprehensive, as for the bacterial respiratory complex I. The same holds true in regard to the assumed functional role of the dynamic association presented here. More work is needed to clarify the structural and functional determinants of the known examples. Identification of new cases will help to appreciate the generality of the new principle of intracellular flavoenzyme regulation.

KEYWORDS

FAD, Flavin cofactors, flavoproteins, FMN, protein biogenesis, subunits

1 | INTRODUCTION

Flavoenzymes are oxidoreductases involved in key metabolic pathways in prokaryotic and eukaryotic organisms alike. Their chemical aptitude relies on the associated cofactors, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD are biosynthesized from the precursor molecule riboflavin. Riboflavin is phosphorylated by the riboflavin kinase (RFK) to become FMN. Subsequently, the FAD synthetase (FADS) adenylates FMN to produce FAD. Most prokaryotes combine both these activities in a single bifunctional protein. Many organisms, including mammalians, are not capable of synthesizing riboflavin and thus depend on its supply with food. To underline the nutritional essentiality of riboflavin in humans, it is called vitamin B2.

Chemically speaking, flavoenzymes are capable of dehydrogenation, oxidation and electron transfer. Less

Abbreviations: DD, death domain; DR, death receptor; ER, endoplasmatic reticulum; ETF, electron transfer protein; FAD, flavin adenine dinucleotide; FAO, fatty acid β-oxidation; FMN, flavin mononucleotide; I/R, ischemia/reperfusion; PDI, protein disulfide isomerase; RC, respiratory complex; RET, reverse electron transport; RFK, riboflavin kinase; ROS, reactive oxygen species; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; VLCAD, very long chain acyl-CoA dehydrogenase.

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than 10% are involved in nonredox processes, such as light sensing, circadian timing, and DNA repair.[1] The repertoire of biotransformations accomplished by flavin-containing enzymes is truly impressive.[2] Especially noteworthy are the reactions involving oxygen.[3] One group, the flavoprotein oxidases, transforms their substrates simultaneously reducing oxygen to hydrogen peroxide. Another group, the flavoprotein monooxygenases, incorporates one atom of molecular oxygen into their substrates while the second oxygen atom is reduced to water. The capacity to reduce oxygen is one of the reasons why flavoenzymes contribute significantly to the generation of reactive oxygen species (ROS) in cells. The NADPH oxidase family is a classical example, but also other ROS sources turned out to be flavoproteins. Mitochondrial respiratory complexes I and II, ketoglutarate and pyruvate dehydrogenases, and peroxisomal D-amino acid oxidase are only a few examples thereof. Furthermore, the recent elucidation of the network of proteins involved in the ferroptotic cell death revealed that a number of key players are flavoenzymes.[4] Ferroptosis is an iron-dependent peroxidic damage of unsaturated phoshpolipids in the cellular membranes. It can be exploited to target drug-resistant tumor variants. Because of their participation in the oxygen metabolism, it is important to understand the flavoenzyme function in pathophysiological and clinical context.

The human flavoproteome encompasses around 100 enzymes. More than half of them, when mutated, lead to devastating diseases.[5] Apart of symptomatic support, there have been few therapeutic options to help patients with these conditions up to now. Riboflavin supplementation shows sporadic effect, however, it could not be sufficiently rationalized in mechanistic terms yet.[6] These difficulties additionally emphasize the need for a fresh look at the intracellular workings of flavoenzymes. One possible direction is suggested by the accumulating evidence, which indicates a regulated association of FMN and FAD with their respective enzymes.

2 | COFACTOR BINDING TO FLAVOPROTEINS

Flavoenzymes prevail in redox bioprocesses because of the remarkable flexibility of the flavin cofactors in their role as electron donors and acceptors. For FMN and FAD, both one-electron and two-electron reversible transfer is possible. This capacity distinguishes flavins from obligatory one-electron systems, such as iron ions, as well as from the two-electron modules, such as NAD(P)H. Due to the ability to donate one electron, flavins can react with molecular oxygen, which is in contrast to oxygen-inert NAD(P)H. The chemical reactivity of the flavins is determined by the isoalloxazine composed of xylene, pyrazine, and pyrimidine rings. The pyrazine ring is where most of the reactivity resides. Its N(5) and C(4a) atoms are targets for nucleophilic addition. The pyrimidine ring is rather hydrophilic and can engage in hydrogen bond interactions with the protein. In contrast, the hydrophobic xylene remains usually unbonded.

Most flavoproteins bind FMN and FAD non-covalently. In around one tenth of the flavoproteins, the cofactors associate covalently, though. In most of these latter cases, the covalent bond is formed via the 8α-methylene of the xylene ring. Protein participates through histidine, cysteine, or tyrosine resulting in N-, S-, or O-linkage, respectively. In addition, there are cases of a direct bond between cysteine and isoalloxazine at C(6) atom. In rare instances, double linkages between proteins and FAD were found. Examples of covalently linked flavoproteins in humans are the mitochondrial succinate dehydrogenase, monoamine oxidases A and B, sarcosine dehydrogenase and dimethylglycine dehydrogenase. The role of the covalent associations with the cofactor remains unclear.

Classical view holds that flavin cofactors are tightly bound to their respective enzymes, even if in a noncovalent association. This is in contrast to other cofactors, first and foremost, NAD(P)H which dissociate and must rebind during each enzymatic cycle. The continuous association of flavin cofactor is believed to be a safeguard against the danger of autooxidation of flavins when they are free and not protected by the protein environment.[7] The binding of FMN and FAD is dominated by the enthalpic contribution to the free energy change.[8,9] Large negative enthalpy results from the hydrogen bond networks. On the other side, the entropic contribution can be negative or positive in rigid or flexible apoproteins, respectively.[10]

There have been few systematic studies to compare the biophysical parameters of the flavin cofactor binding among different flavoproteins under standardized experimental conditions. Despite the lack of comparative data, it seems plausible to expect variability of the cofactor-apoprotein association. Structural evidence supports this assumption. Nature has adapted a number of different folds to host FMN and FAD, such as the TIM barrel, flavodoxin-like fold and Rossmann fold to name a few.[1] Among different folds, the shape of the flavin pocket varies, which excludes the existence of a unique pharmacophore for the flavin cofactor binding.[11] Recent examples of the dynamic association of FMN and FAD with some flavoproteins further support the existence of different modes of the protein-cofactor interactions. Some of these examples will be presented and discussed below.

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3 | MEMBRANE NADPH OXIDASE

The activity of the NADPH oxidase (NOX2, also known as gp91) is low in resting phagocytes. This change upon bacterial encounter. Several mechanism are responsible for the NOX2 activation. Most noticeably, the assembly of the functional complex from the cytosolic and membrane subunits takes place during activation. Thereby, the cytosolic p40, p47, and p67 associate with the membrane-resident p22 and gp91. Phosphorylation of the cytosolic subunits causes their conformational change which contributes to the activation-driven assembly. In addition, a direct effect from membrane lipids has been proposed. Since long, saturated and unsaturated fatty acids have been known to stimulate the oxidative burst after being added to the leucocyte membrane preparations.[12,13]

When isolated from human neutrophils, NADPH oxidase lacks FAD and is inactive. The activity can be reconstituted by adding FAD in vitro. Noteworthy, the ratio heme-to-flavin in the reconstituted enzyme was 2:1, which indicates its incomplete loading with FAD.[14] The in vitro activated NADPH oxidase was estimated to bind FAD with a Kd value 0.94 nM.[15] The authors suggested that this strong association with the cofactor is active state-dependent and that the resting enzyme is only loosely bound to FAD. The low Kd might be necessary in order to avoid accidental generation of reactive oxygen species.[15] Although biologically appealing, this notion awaits rigorous experimental scrutiny.

A novel mechanism of the NADPH oxidase activation was discovered while performing a yeast two-hybrid screen for potential interactors with the death domain (DD) of the human TNF receptor 1 (TNFR1) (Figure 1).[16] Immune cells usually secrete high amounts of TNF during infection. Upon TNF binding to TNFR1, the adaptor protein TRADD associated with the DD of the cytosolic tail of the receptor initiating downstream signaling. It turned out that TRADD recruits also the riboflavin kinase (RFK), the enzyme phosphorylating riboflavin into FMN. RFK, in turn, associated with NOX1 and NOX2. Both enzymes need FAD as cofactor. Although the presence of the FAD synthase (FADS) in the complex was not demonstrated explicitly, a co-recruitment of FADS seems to be plausible. In support, increased amounts of FAD were detected in TNF-stimulated macrophages.[16] Taken together, these findings offered a mechanistic explanation of the observation that TNF is able to activate the NADPH oxidase for increased ROS production.[17] They also raised the question regarding the existence of similar regulatory loops that would locally link riboflavin metabolism with activation of other flavoenzymes.

Indeed, a similar case of RFK involvement in the induced generation of ROS was described soon afterwards. Death receptor (DR)4 and DR5 belong to the Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor family. Stimulation of DRs activates NOX1 to produce ROS. It was found that the DR4/DR5 signaling complex contains, among other components, TRADD and RFK.[18] Depletion of RFK affected the recruitment of NOX1 to DR4/DR5 and the generation of ROS. Contrary to the situation during TNFR1 stimulation, the adaptor protein TRADD did not affect the

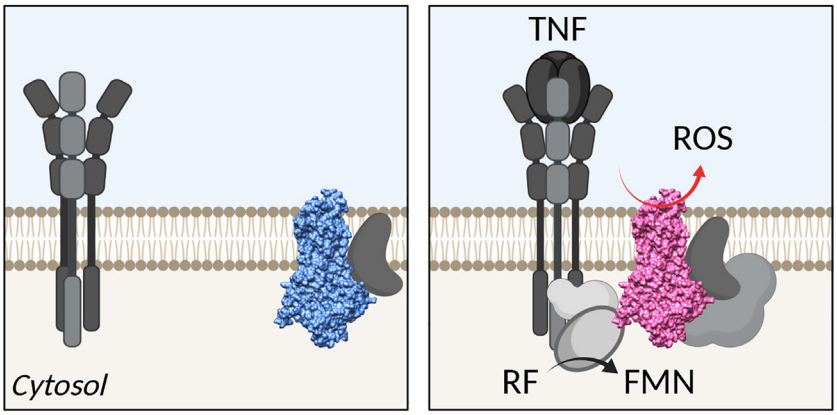

FIGURE 1 Activation of NADPH oxidase to trigger the oxidative burst. *Left*: inactive NOX2 (blue), the flavoenzyme subunit of the NADPH oxidase complex, is bound to p22, but does not associate with the trimeric TNF receptor 1 under normal condition. Other members of the complex, p40, p47, and p67, remain soluble in the cytosol (not shown). *Right*: upon pathogen encounter, TNF is secreted and engages its receptor. The TNF receptor initiates a downstream cascade by recruiting a number of signaling proteins, including the adaptor protein TRADD. TRADD recruits the riboflavin kinase which, in turn, binds NOX2. Enhanced conversion of riboflavin (RF) to FMN takes places, which contributes to the local activation of the flavoenzyme (pink). At the same time, soluble members of the complex dock ensuring a full-power generation of reactive oxygen species (ROS) to combat the invading microbes

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recruitment of RFK to the signaling complex. The molecular determinants of this difference remain unclear.

4 | ENDOPLASMATIC RETICULUM OXIDOREDUCTASE ERO1

Ero1 is an FAD-containing sulfhydryl oxidase that resides in the endoplasmatic reticulum (ER). During the oxidation reaction, hydrogen peroxide is produced. The Ero1 substrates are protein disulfide isomerases (PDI). Through the oxidative regeneration of PDI, Ero1 is centrally involved in the biogenesis of secreted and membrane proteins in the ER. The generation of each disulfide bond in a maturing polypeptide is accompanied be the production of one molecule of hydrogen peroxide. This sums up to a significant fraction of the hydrogen peroxide generated in a cell.

A tight regulation of oxidative protein folding is needed to prevent futile consumption of reducing equivalents, the accumulation of ROS and the ROS-initiated damage of cellular structures. In yeasts, it was observed that free FAD can influence the activity of Ero1 (Figure 2).[19] High levels of free FAD stimulated whereas low levels attenuated the Ero1-driven oxidative folding. The molecular basis of the effect remains unclear. The authors suggested the existence of a second, weaker FAD binding site in Ero1. The weak affinity binding site could function as an FAD sensor with the capacity to modulate the strong affinity binding site. This notion is interesting in view of the available structural data.[20,21] In Ero1, FAD is buried within the fold and the isoalloxazine ring is shielded by the cofactor's dinucleotide moiety. A channel for oxygen to reach the active site is not evident. Moreover, the active site disulfide is obscured by other elements as well. Thus, extensive structural changes seem to be required to enable an electron relay between PDI-engaging cysteines (shuttle cysteines) and the FAD-proximal disulfide (active site cysteines). Whether the postulated second FAD binding site is involved in the allosteric regulation of the required rearrangement of the Ero1 remains to be analyzed.

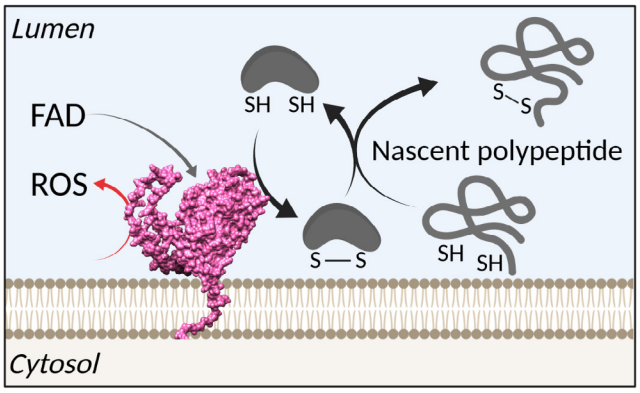

FIGURE 2 Enhancement of Ero1 activity. Nascent polypeptides undergo oxidative folding in the lumen of the endoplasmatic reticulum. Protein disulfide isomerases (PDI) oxidize sulfhydryls of nascent polypeptides to disulfide bonds. The regeneration of PDI for the next round of substrate oxidation is carried out by the flavoprotein Ero1 (pink). Ero1 itself passes electrons further to molecular oxygen generating reactive oxygen species (ROS). It was reported that free FAD enhances the PDI-oxidizing capacity of Ero1. An additional FAD binding site was suggested as explanation, although it has not been identified yet

The presence of free FAD in the endoplasmatic reticulum is required for its suggested role in the Ero1 activity regulation. However, the FAD synthase which converts FMN to FAD is localized in the cytosol. At the same time, it has been shown that yeasts are capable to import FAD into the ER lumen.[19] This transport system was shown to be fast and energy-independent. Better characterization of FAD transport into the ER would offer additional insights regarding the regulation of Ero1.

5 | BACTERIAL RESPIRATORY COMPLEX I

NADH:ubiquinone oxidoreductase (complex I) is a key component of the respiratory chain in the mitochondria of eukaryotic cells and in the membranes of many bacterial species. It transfers two electrons from NADH to ubiquinone and uses the energy of this exergonic process to translocate protons against the electrochemical potential across membranes. Featuring 14 core subunits, the bacterial complex I can be considered as a minimal model of the respective mitochondrial complex. It has been thoroughly characterized structurally and functionally.[22]

A hydrid ion from NADH is first transferred to a non-covalently bound FMN on the peripheral hydrophilic arm of the complex I. The electrons are then passed further down, along the path of several iron–sulphur (Fe–S) clusters, to the ubiquinone binding site. Also the reverse process can take place in the complex, namely, the reduction of NAD+ from the ubiquinol.[23]

Since many years, it was known that the addition of NADH to the complex I—before adding an electron acceptor to the reaction—affects the reduction of the acceptor.[24] Recently, this observation could be finally explained.[25] It turns out that the reduced bacterial complex I loses its affinity to FMN, such that FMN dissociates leaving the enzyme inactive (Figure 3). A Fe-S center in the vicinity of the FMN binding site, the N1a cluster, seems to be responsible for the effect. Due to its high

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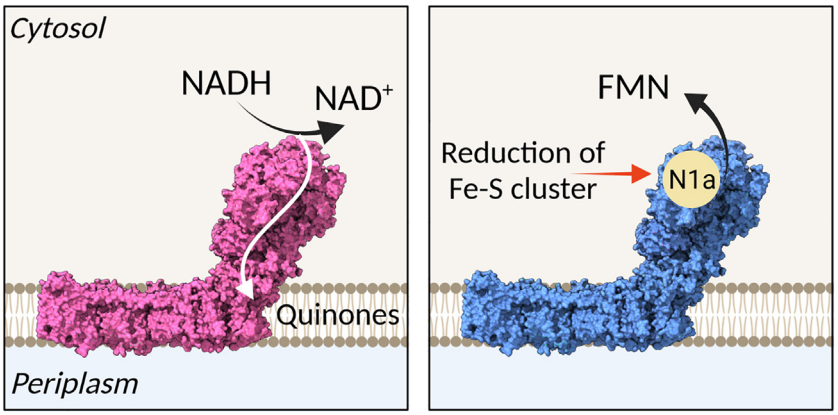

FIGURE 3 Inactivation of bacterial respiratory complex I. *Left*: respiratory complex I (pink) transfers electron from NADH to membrane quinones. The energy of the transfer is used to translocate protons against the electrochemical potential across the membrane. *Right*: reduction of the FMN-proximal Fe-S cluster N1a affects the binding of the cofactor. FMN is ejected, which disrupts the electron transport pathway inactivating the respiratory complex (blue)

redox potential, the N1a cluster in *E. coli* is much easier reducible in comparison to N1a from other organisms. Reduction of the cluster by NADH introduces a negative charge around the FMN binding pocket. This can result in structural alterations leading to the decreased affinity to the cofactor, especially because of the electrostatic repulsion of the orthophosphate of FMN.[25]

The process is reversible and FMN can rebind without the need for dedicated assembly factors. The dynamic nature of the effect poses the question of the possible physiological role of the dissociation, for example, in prevention of ROS generation by the complex I. During the stalling of electron transfer along the respiratory chain, electron acceptors in the membrane accumulate in their reduced state. This, in turn, facilitates reverse electron transport (RET) and the FMN-dependent generation of superoxide radicals. FMN dissociation from the reduced complex I under these conditions would mitigate ROS generation. Indeed, this scenario could be confirmed experimentally.[25]

6 | MITOCHONDRIAL RESPIRATORY COMPLEX I

When analyzed in vitro, the mammalian respiratory complex I exhibited a similar reduction-dependent dissociation of FMN.[26,27] However, the process was much slower and took more than an hour to complete. Furthermore, strongly alkaline pH was needed thus questioning the physiological relevance of this in vitro observation. Excitingly, the dissociation of FMN from the complex I was observed in a relevant pathophysiological context of brain ischemia/reperfusion (I/R) injury.[28–30] Consequences of ischemic stroke are often debilitating and contribute to a significant fraction of disability cases world-wide. Cellular damage of neurons during stroke is closely linked with the ROS generation in mitochondria. Succinate accumulation leads to the reverse electron transport and flavin-dependent reduction of oxygen.[29] Reperfusion recovers the activity of complex I, however, its thiols become oxidized, which affects the enzymatic activity.[28] The above studies in the murine brain I/R model demonstrated a complex dynamics of the FMN loss and reassociation. Thus, it is challenging to unequivocally define the role of the process mechanistically. Administration of the FMN precursor riboflavin before the injury attenuated the severity of biochemical and clinical manifestations.[30] This latter results argues for a net negative effect of the cofactor dissociation and allows to consider the loss of FMN as a molecular damage during brain ischemia/reperfusion.

The situation is more static in cases of genetically caused defects of FMN binding to the mitochondrial complex I. Dysfunction of complex I accounts for around one third of early-onset mitochondrial disorders.[31] The most prevalent clinical presentations include Leigh syndrome, leukoencephalopathy, lactic acidosis and hypertrophic cardiomyopathy among other symptoms. A number of disease-causing mutations have been identified in the FMN binding subunit NDUFV1 (OMIM #161015, #618225). Some of the mutations, predicted or known to be pathogenic, have been characterized at the molecular and cellular level in a yeast model recently.[32] The study revealed different groups of mutations in regard to the effect on the FMN association. Two mutations resulted in an FMN-free complex I and this defect could not be repaired by increasing riboflavin amount in cell growth medium. The finding suggested that the mutations directed NDUFV1 to an off-pathway during the complex I biogenesis. This situation seems to be in contrast to the reversible association of FMN discussed above.

It is commonly assumed that the turnover rates of individual subunits in stable protein complexes have converged to enable the efficient biogenesis and stability of the respective molecular machineries. Thus, no surprise that a number of disease-causing mutations in one of the

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45 subunits of the complex I often affect the stability of the entire complex. The more unexpected was the finding that the FMN-binding subunit NDUFV1 displays much higher turnover rate.[33] Also other proximal subunits in the soluble arm, the so-called N-module, displayed similar acceleration of their turnover (Figure 4). The authors showed that the superoxide production through the FMN site was responsible for the loss of FMN and contributed to the exchange of the N-module. The mitochondrial ClpXP protease was involved in the removal and degradation of the N-module subunits. The exchange restituted the module with the proper amounts of flavin, thus representing actually a complex I-salvage pathway—the exchange of the entire complex would be much more costly for a cell. In a strict sense, the mechanism does not represent a regulatory reversible binding of the cofactor. Nevertheless, it was included here because it is an example of an apparent turnover which ensures the proper function of a flavoprotein.

7 | ACYL-COA DEHYDROGENASE 9

Mitochondrial complex I is the largest among the respiratory chain complexes and consists of 45 different subunits. Its assembly in a crowded intracellular environment is conceivably a complicated process. Nevertheless, there has been recently significant progress in understanding how the subunits assemble into a functional unit and how additional factors assist thereby.[34] At least two assembly factors are flavoproteins, namely, FOXRED1 and ACAD9. ACAD9, the acyl-CoA dehydrogenase 9, was initially proposed as an enzyme of the mitochondrial fatty acid β-oxidation (FAO) pathway.[35] Later, it was shown to be active in the complex I assembly in concert with two other factors, NDUFAF1 and ECSIT.[36] The essential role of ACAD9 in this process is underscored by assigning it, together with NDUFAF1 and ECSIT, to the core components of the complex I assembly machinery. ACAD9 is most closely related to the very long chain acyl-CoA dehydrogenase (VLCAD), however, VLCAD is not required for complex I assembly and instead functions as an enzyme in the first step of the mitochondrial FAO. In contrast, the role of ACAD9 in FAO is debated and FAO-incompetent catalytically inactive ACAD9 can still support complex I biogenesis.[37] Although indirectly, the assembly sufficiency of the FAO-deficient variant was the first indication that the flavination status of the protein is relevant to its role during assembly. This was explicitly demonstrated in a recent study combining functional and structural approaches.[38] The authors discovered that the interaction of ACAD9 with ECSIT causes the loss of FAD from the catalytic site of ACAD9 (Figure 5). Since the interaction interface is far from the FAD binding site, an allosteric mechanism ought to be in place. The loss of FAD upon interaction with ECSIT was confirmed and substantiated by molecular modeling and small-angle X-ray scattering analyses by another group.[39] Furthermore, the authors identified the binding site of ECSIT on ACAD9, which turned out to overlap with the ACAD9-ETF binding interface

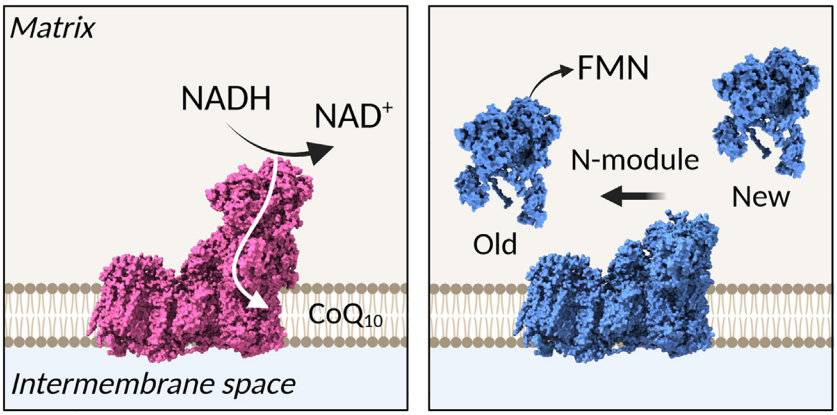

FIGURE 4 Salvage pathway of mitochondrial respiratory complex I. *Left*: mammalian mitochondrial respiratory complex I (pink) contains 45 subunits and transfers electron from NADH to ubiquinones, such as coenzyme Q_{10} (CoQ_{10}), in the mitochondrial inner membrane. FMN-associated NDUFV1 in the N-module of the complex is a core subunit which directly encounters NADH and is responsible for the first step of electron transportation. Superoxide can be generated due to one-electron reactivity of FMN. *Right*: inactive complex I (blue). To repair the local damage caused by reactive oxygen species, NDUFV1 and the entire N-module are exchanged more rapidly than the rest of the complex. Mitochondrial protease ClpXP hydrolyzes the old subunits. New NDUFV1 must be loaded with FMN to become functional

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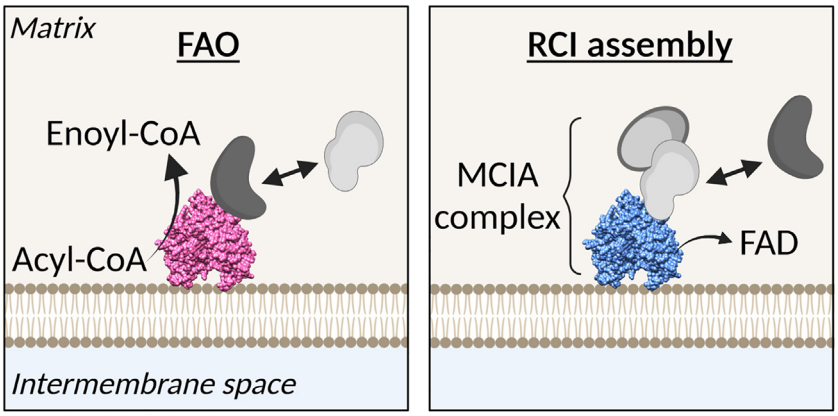

FIGURE 5 Two mutually exclusive functions of ACAD9. *Left*: during the mitochondrial matrix fatty acid ß-oxidation (FAO) ACAD9 acts as an FAD-dependent dehydrogenase (pink). Hereby, ACAD9 catalyzes the formation of a double bond between the C-2 and C-3 atoms in fatty acids. Electrons from reduction are passed to another flavoprotein, ETF (dark gray). ETF competes for the same binding site with the ECSIT protein (light gray). *Right*: binding of ECSIT ejects FAD from ACAD9 and thus renders ACAD9 enzymatically inactive. Cofactor-free ACAD9 (blue), ECSIT and NDUFAF1 form a stable complex which is a part of the mitochondrial complex I assembly (MCIA) machinery

required for the ACAD9 participation in FAO. The overlap demonstrates that the two functions of ACAD9 are mutually exclusive.

Because the FAD-free ACAD9 is excluded from FAO, it is tempting to speculate that the switch has a regulatory role in shifting the cellular metabolism from FAO to oxidative phosphorylation. This assumption needs to be tested experimentally, though, because the role of ACAD9 in FAO remains still questionable as mentioned above. When tested in vivo, the dehydrogenase activity of ACAD9 could be unambiguously established only in VLCAD-deficient cells.[37] The low ACAD9 activity could be a mere evolutionary rudiment not proving the proper involvement of the flavoprotein in fatty acid catabolism.

8 | ELECTRON TRANSFER FLAVOPROTEIN

It is possible that an active deflavination of flavoenzymes is employed to switch between different functions more often than previously anticipated. In addition to ACAD9, another case of this mechanism has been described recently. A recent proteomics study found that the electron transfer protein (ETF) loses its FAD cofactor upon interaction with LYRM5.[40] ETF is a heterodimer composed of ETFA and ETFB subunits. It accepts electrons from different mitochondrial dehydrogenases, such as acyl-CoA dehydrogenases, glutaryl-CoA dehydrogenase and sarcosine dehydrogenase. The electrons are subsequently transferred from ETF to ETF-ubiquinone oxidoreductase (ETF dehydrogenase). The association of ETF and LYRM5 was identified in a systematic analysis of the interactomes of poorly characterized mitochondrial proteins and was confirmed in vitro.[40] When mixed together, recombinant LYRM5 led to a linear reduction of ETF activity with a proportional loss of FAD from the complex (Figure 6). The authors suggested a number of possible scenarios regarding the biological relevance of the observation that all await experimental clarification. It cannot be excluded that ETF, similarly to ACAD9, possesses an additional, nonenzymatic function. Intriguingly, LYRM5 is known to associate with the mitochondrial respiratory complex I. It remains to be investigated whether the parallels between ACAD9-ECSIT and ETF-LYRM5 in regard to RCI are accidental or indicate functional similarities.

9 | DISCUSSION

It is basic biochemistry knowledge that the activity of a flavoprotein depends on its association with a flavin cofactor. As early as in the 30's of the last century, Hugo Theorell and Otto Warburg showed that the binding of FMN and FAD, respectively, is required to make their cognate proteins enzymatically active. So what are the news almost 100 years later when talking about the activation and deactivation of flavoproteins with respect to the cofactor binding? This time it is about the activity in the cells, not in a reagent glass. Things are more complicated in a crowded intracellular environment, where up to 300 mg/ml protein concentration can be reached, a thousand times more than what is usually set up for an in vitro experiment. In addition to proteins, other types of biomolecules are present in high concentrations as well. The crowding imposes additional biophysical and chemical constrains that flavoproteins must handle to

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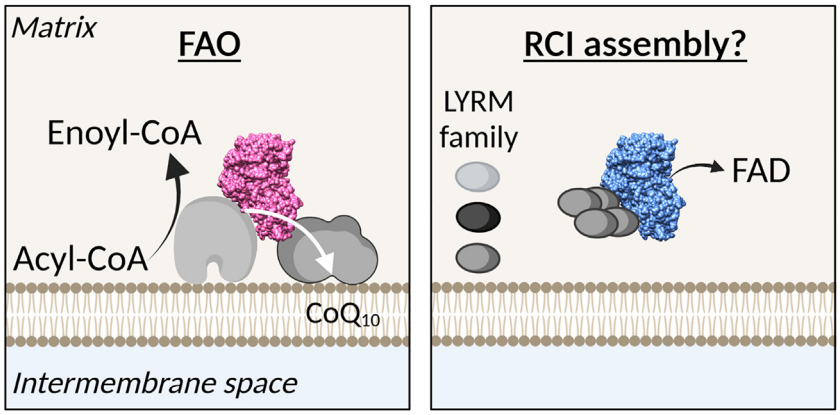

FIGURE 6 Deflavination of electron transfer protein ETF. *Left*: during fatty acid ß-oxidation (FAO), the heterodimeric flavoprotein ETF (pink) transfers electrons from the acyl-CoA-dehydrogenases to the ETF dehydrogenase that subsequently reduces ubiquinone (coenzyme Q_{10}, CoQ_{10}) in the inner mitochondrial membrane. *Right*: several LYR motif-containing (LYRM) proteins are involved in the respiratory complex I (RCI) assembly. The LYRM5 tetramer was found to interact with ETF causing the dissociation of FAD from and loss of the enzymatic activity of ETF (blue). Whether the LYRM5-ETF complex is involved in the assembly of RCI is not clear yet

successfully execute their enzymatic duties. In the biophysical regard, especially the kinetic aspects become critical when in a cell. For example, although thermodynamically unproblematic in principle, a given enzyme might not be able to reach its stable conformation intracellularly failing to find a cofactor to associate with. Such immature enzyme would be prone to misfolding and aggregation in the crowded cytosol. There are challenges also from the chemical point of view. Flavoenzymes are dangerous to bystander molecules as discussed above. Their capacity to reduce molecular oxygen is highly needed under specific circumstances, yet must be tightly controlled not to damage other proteins, lipids and other constituents present nearby.

In vivo, the local and timely regulation represents at least a partial solution to the task of controlling the structurally vulnerable and chemically perilous flavoenzymes. The cases presented in the review are meant to support this notion. An example of the local control is the recruitment of the riboflavin kinase (RFK) to the membrane-docked NADPH oxidase complex upon stimulation of the TNF receptor. Because NOX2 uses FAD instead of the immediate product of RFK, it remains to be confirmed that the FAD synthetase (FADS) is co-recruited to activate the NADPH oxidase locally. The action of RFK and FADS in the same complex is conceivable, the notion supported by the fact that most prokaryotes encode both activities in one polypeptide chain. FADS associates with its product FAD very tightly,[41] which suggests that the delivery of FAD to client proteins, such as NOX2, is strictly controlled. Structural rearrangements could be required for the FAD release. Whether local interactors facilitate the transfer of the cofactor from FADS to a client protein in a biologically relevant time-scale remains an open question.

An example of a timely deactivation of flavoproteins is the release of FMN from the mitochondrial respiratory complex I during I/R injury.[28–30] Depending of the oxygenation levels and the duration of hypoxia, flavin release can have opposite effects for tissue damage.[42] FMN dissociation from RCI decreases the production of ROS during the early stages of reoxygenation. From the other side, free reduced flavin is known to react with oxygen non-enzymatically, thus generating ROS. It was proposed that the protective or damaging nature of FMN release might depend on the oxygen availability and the length of ischemia.

The reversible association of FMN and FAD with flavoproteins cannot be considered separately from the cofactor availability and household in the cells. Apart of a handful of exceptions, cells do not store flavins and instead efficiently excrete the surplus of riboflavin supplies. This becomes relevant when the supplies are cut back.[43] Under deficiency, cells might require to undergo an extensive metabolic reorganization.[44] It is unclear how the affinity of individual flavoproteins to their respective cofactors determines the redistribution of the remaining flavin pool under starvation. More work in two areas of cellular flavin biochemistry needs to be done. First, there is still only rudimentary knowledge regarding the mechanisms of FMN and FAD loading during the synthesis and maturation of flavoproteins. Some flavoproteins will not need much and being capable of binding a cofactor upon encountering it.[8] Those with deeper or shielded binding sites might need specific assistance during the cofactor loading. Whether the mode of the cofactor loading correlates with its regulated dissociation will be an interesting question to address. Secondly, the distribution of flavins in subcellular compartments awaits clarification. The recent

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discovery of riboflavin transporters in the plasma membrane significantly advanced the understanding of the cellular and organismal flavin household.[45–47] However, its many intracellular details remain poorly understood. Especially the quantitative information of the flavin metabolism in and transfer of flavins between the organelles would be helpful for elucidating the biogenesis and regulation of flavoenzymes.

10 | CONCLUSIONS

Over the recent years, the flavoenzyme field has bestowed with a number of exciting discoveries and developments. One of them is the increasing appreciation of the dynamic nature of the FMN and FAD association with flavoproteins in the cell. From one side, it is about long-known observations, such as the enhanced association of NADPH oxidase with its cofactor when active or the loss of activity of the bacterial respiratory complex due to preceding reduction. Other examples are more recent, such as the functional switch of ACAD9, which accompanies the expulsion of FAD from the dehydrogenase. In the cases presented here, it is tempting to assume functional roles, and thus an evolutionary meaning. Yet, despite the progress in mechanistic elucidation of these phenomena, many details are still missing. The full appreciation of the dynamic association of flavin cofactors to regulate flavoprotein function needs additional work to be done. Almost certainly, more examples will be described in the future. The field that started with the Otto Warburd's "old yellow enzyme" almost 100 years ago, keeps surprising and inspiring us.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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