COMMENTARY

New functional and biophysical insights into the mitochondrial Rieske iron-sulfur protein from genetic suppressor analysis in *C. elegans*

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ABSTRACT

Several intragenic mutations suppress the *C. elegans isp-1(qm150)* allele of the mitochondrial Rieske iron-sulfur protein (ISP), a catalytic subunit of Complex III of the respiratory chain. These mutations were located in a helical region of the “tether” span of ISP-1, distant from the primary mutation in the extrinsic head, and suppressed all pleiotropic phenotypes associated with the *qm150* allele. Analysis of these suppressors revealed control of electron transfer into Complex III through a “spring-loaded” mechanism involving a binding force for formation of enzyme-substrate complex, counter balanced by forces (a chemical “spring”) favoring helix formation in the tether. The primary P→S mutation results in inhibition of electron flow into the Q-cycle by decreasing the binding force, and the tether mutations relieve this inhibition by weakening the “spring.” In this commentary we discuss additional control features, and relate the primary inhibition to outcomes at the organismal level. In particular, the sensitivity to hyperoxia and the elevated reactive oxygen species (ROS) seen in *isp-1(qm150)*, likely reflect over-reduction of the quinone pool, which is upstream of the inhibited site; at high O₂, this would lead to increased ROS production through complex I. We speculate that alternative NADH:ubiquinone oxidoreductase activity in *C. elegans* from the worm apoptosis inducing factor (AIF) homolog (WAH-1) might also be involved, and that WAH-1 might have a “canary” function in detection of this adverse state (high O₂/reduced pool), and a role in protection of the organism by transformation to AIF-like products, and apoptotic recycling of defective cells.

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Introduction

The Rieske iron-sulfur protein (ISP) is a subunit of ubiquinol-cytochrome *c* oxidoreductase (the *bc*₁ complex) in prokaryotes and a subunit of complex III in mitochondria of eukaryotes^{1,2} (Fig. 1A). Within mitochondria, complex III is embedded in the inner membrane where it functions in the electron transport chain (ETC) and is required for ATP generation through oxidative phosphorylation. Complex III is a homodimer, in which each monomer has a catalytic core of 3 subunits: cytochrome (*cyt*) *b*, ISP, and *cyt* *c*₁. Many bacterial complexes have only this core, but mitochondrial complexes have up to 8 ancillary subunits.

All *bc*₁ complexes operate by the same Q-cycle mechanism (Fig. 1A and B, coupling the work from the oxidation of ubiquinol (QH₂, the reduced coenzyme Q)

by soluble *cyt c* to transfer of electrons across the inner mitochondrial membrane to reduce quinone (Q) at the Q_i site, and to generate the proton gradient that drives ATP synthesis. ISP itself consists of 3 domains: a trans-membrane α -helix that serves to anchor ISP into the membrane, and 2 extrinsic domains, the head that contains the 2Fe-2S cluster involved in electron transfer, and the tether that connects the head to the anchor through a chemical “spring,” acting through extension and relaxation of a helical span (Fig. 1C). The ISP head acts as a tethered substrate, moving between the Q_o site of the complex in *cyt b*, where the oxidized 2Fe2S cluster accepts electrons from ubiquinol (QH₂), and *cyt c*₁, where the heme *c*₁ accepts electrons from the reduced 2Fe2S cluster.

The worm ISP is encoded by the *isp-1* gene. Loss of function mutations in *isp-1* are associated with

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Figure 1. (For figure legend, see page 3.)

pleiotropic phenotypes including defective mitochondrial respiration, slow developmental rate, decreased fecundity, and small body size, slower pharyngeal pumping rate, decreased movement, activation of the mitochondrial unfolded protein response (UPR^{mt}), and increased lifespan.³ Our group became interested in studying ISP-1 based on the enhanced longevity of the *isp-1(qm150)* mutant. Although several mechanisms have been proposed, it was unclear precisely why this particular mutation, as well as other *C. elegans* strains with reduced mitochondrial function, live longer.^{4,5}

To gain additional insight into ISP-1-mediated mechanisms of lifespan control, we performed a genetic suppressor screen to identify mutations that could suppress the slow developmental rate of *isp-1(qm150)* animals. We chose to focus initially on developmental rate, because this phenotype was much easier to score (2 d analysis compared with measuring lifespan, which requires several weeks of observation) and thereby enabled ready identification of suppressing mutations.⁶ Fortunately, multiple mutant lines were identified from 3 saturated screens that showed partial suppression of both the delayed development and the lifespan extension, as well as several other phenotypes associated with the *isp-1(qm150)* allele.⁷

We mapped these suppressing mutations to the tether region of the ISP, all but one within the tether helical span or its H-bonding cap (the chemical “spring”) (Fig. 1C), which changes configuration in synchrony with mechanical movement of the ISP head to facilitate interaction with its redox partners^{7,8} (Fig. 2). On the basis of earlier work on the bacterial *bc₁* complex in *Rhodobacter* species, where similar mutations had been studied,⁹ we had suggested a “spring-loaded” mechanism,¹⁰ and invoked this to

explain these results. In addition, we reported a similar structure-function relationship between analogous mutations in the yeast ISP, Rip1, as well as an unexpected new phenotype, “sensitivity to hyperoxia,” in *C. elegans* with reduced ISP activity. Here we expand upon these findings and discuss implications for future studies of ISP proteins and their roles in maintaining cellular and whole animal physiology.

Why are intragenic suppressors of *isp-1(qm150)* largely restricted to the tether region?

As already noted the majority of the suppressors of *isp-1(qm150)* were intragenic and restricted to a small 6 amino acid “tether” region of ISP-1⁷. This region is composed of the amino acids DQRALA, where D__A__A are conserved in α -proteobacteria, yeast, worm, chicken, and human. We have used the term “tether” rather than the previously used “hinge” for the span,⁹ since the role of those 6 amino acids is clearly more dynamic.

Crystallographic studies provided the first topological information showing the ISP head in several different configurations in structures with different occupants of the Q_o site, and analysis of plausible rate constants using a simple distance-dependence showed that none of the different positions could account for observed electron transfer rates, indicating that ISP must undergo movement, in order to transfer an electron from the Q_o site to heme *c*₁^{8,11, 12} (Fig. 1C, Fig. 2). The movement involved a rotational displacement in which the structure of the extrinsic head was essentially unchanged, and the main action was in the tether. This showed an extended conformation when ISP interacted with an occupant of the Q_o site and a helical form when the site was empty, occupied with a different class of inhibitor, or engaged with

Figure 1. (see previous page) Overview of the spring-loaded mechanism in the context of the Q-cycle. (A) The respiratory chain, showing the central role of complex III (purple). On inhibition in complex III, the upstream pools will become reduced, triggering secondary effects in cellular metabolism (see text). (B) The Q-cycle mechanism operating in the catalytic core of a monomer: *cyt b* (cyan), *cyt c*₁ (pink) and ISP (yellow); only the transmembrane anchor (top) and the extrinsic mobile head (bottom) are visible, with the latter docked at the Q_o site. The Q-cycle mechanism is overlaid on the structure, rendered transparent so as to reveal the redox centers. Stigmatellin bound at the Q_o site in *cyt b*, is a proxy for SQ_o, Q, or QH₂ at different phases of turnover. The Q_o site reaction is initiated by formation of the ES-complex when QH₂ enters (green arrow) to H-bond ISP_{ox}, pulling the head into the position shown. In the first electron transfer (yellow arrows) the electron and H⁺ are transferred to reduce ISP_{ox} to ISPH, leaving SQ_o as a low-occupancy intermediate. On dissociation from ISPH, the neutral SQH[•] can lose a H⁺, transferred to the aqueous P-phase (red arrows) down a H⁺ channel, allowing SQ[•] to migrate close to heme *b_L* and reduce it (blue arrows) in the second electron transfer. Meanwhile, ISPH diffuses to find heme *c*₁ to accept the electron and release the H⁺ to the P-phase, and then reduce *cyt c*. In the low-potential chain, the electron moves via heme *b_H* (blue arrows) to the Q_i site to reduce the occupant, which can be Q, or a stable SQ_i, tightly bound after Q is reduced. Because it takes 2 electrons (and 2 H⁺ taken up from the N-phase, red arrows) to reduce Q to QH₂, the Q_o site now has to turnover a second time (the paired arrows) to generate the second electron in the low potential chain to reduce SQ_i, and complete the reaction. The bifurcation of electrons from QH₂ to separate chains is critical to the mechanism, allowing the chemical worked stored in SQ_o to drive charges across the membrane and generate the $\Delta\psi$ component of the proton gradient. (C) Cartoon of the spring-loaded mechanism (see Fig. 2 for details).

Figure 2. The structural basis for the spring-loaded mechanism. The extrinsic domain of ISP transfers electrons and protons from the Q_o site to heme c_1 . The yellow cartoons show ISP in different configurations (A–D), with the 2Fe2S cluster shown by space-filling atoms. The cyan surface is that of *cyt b* at the docking interface where ISP forms a complex with the Q_o site occupant. In this model, the site contains a ubiquinone species, which would change from (initially) QH_2 , then (transiently) SQ_o , then Q , the product (change not shown). After formation, Q diffuses out to the membrane phase, and is replaced by QH_2 for the next turnover. The heme of *cyt c*₁ is reduced through a propionate (peeking in on the right, shown without its protein scaffold), and oxidized by *cyt c* at the orthogonal ring edge (not shown) (see ref.⁸ for information on structures). Starting with the *ES*-complex (QH_2 .ISP_{ox} at the Q_o site, A), the first electron transfer generates a transient SQ_o .ISP_H intermediate which dissociates so that the reduced ISP (ISP_H) ((A)→(B)) can swing away to carry the electron and H^+ from QH_2 to heme c_1 ((B)→(C)→(D)). On transfer of the electron to heme c_1 , the H^+ is released to the P-phase (positive proton potential) (D). In the reverse direction ((D)→(C)→(B)→(A)), the oxidized ISP (ISP_{ox}) diffuses back to the Q_o site interface to complex with QH_2 to reform the *ES*-complex for the next turnover. The tether domain is in an extended configuration when ISP is docked in complex at the Q_o site (A). In the relaxed configuration (C), the extended tether has formed into a helical configuration. The change from relaxed to extended configuration involves the breaking of 8–10 weak H-bonds. The work required ($\Delta G^{\text{spring}} \sim -17$ kJ/mol) is provided by the binding reaction ($\Delta G^{\text{bind}} \sim -23$ kJ/mol). The remaining work determines the apparent binding free energy ($\Delta G^{\text{app}} = (\Delta G^{\text{bind}} - \Delta G^{\text{spring}}) \sim -6$ kJ/mol), giving $K_{\text{app}} = 10^{-\Delta G^{\text{app}} / 2.303RT} \sim 11$. When the complex dissociates, ΔG^{spring} is returned to the “spring” as the H-bonds reform. (See supplemental data online for a dynamic picture of the spring-loaded mechanism on action. The animation steps through the frames of Fig. 2, and is set up for stereo viewing by the crossed-eye method.)

heme c_1 . Apparently, the work available from binding was enough to “pull” the tether out to an extended configuration, and the tether relaxed to a helix when no binding force was involved, suggesting a “spring-loaded” mechanism.¹⁰ The forces involved could be quantified by analysis of data reflecting changes in binding energy upon mutation of the tether region.^{7,13} A further discussion of this can be found in the supplementary information of our report.⁷ We noted that the forces calculated for the spring-loaded model could be applied to

understand control of the reaction and gating of harmful bypass reactions.⁷

Why did evolution build the spring-loading into the reaction?

The most plausible answer is that spring-loading brings the reactions for binding with occupants of the Q_o site under dynamic control. Complexes important in mechanism are the enzyme-product (*EP*-) complex

of Q with ISPH, and the enzyme-substrate (*ES*-) complex of QH₂ with ISP_{ox}. Early experiments with bacterial complexes had shown that when the spring function was weakened by mutations, the binding affinity for the *EP*-complex was so high that electron transfer could be completely inhibited.^{10,13} In wildtype strains, this product-inhibition was controlled, - held in check when the pull from the spring weakened the binding, - so as to prevent inhibition, and allow rapid forward reaction. The same forces would be in play on formation of the *ES*-complex, but in that case, would have a more subtle effect. Formation of the intermediate product in normal forward chemistry leads to dissociation of ISPH to allow it to swing away on its tether to deliver its electron to heme c₁. Since at the moment of dissociation, the tether must be in its extended configuration, this release is driven by the pulling forces to reform the tether helix. It seems likely that this spring-aided retraction is exploited in gating of the reaction (Figs 1 and 2).

The consequences of the P→S mutation of *isp-1* (*qm150*) at the mechanistic level can be attributed directly to interference in formation of the *ES*-complex. Through this, the mutation slows electron transfer by inhibiting flux into the Q_o site reaction in its first step (Fig. 3A).

The product of the first step is the semiquinone intermediate, SQ_o, which can reduce O₂ to superoxide, a precursor of free-radical reactive oxygen species (ROS, implicated in aging). Normally, SQ_o is rapidly removed in the second step of the bifurcated reaction by transfer of the electron to heme b_L. The product, Q, equilibrates with the membrane phase, and the electron is transferred on to the Q_i site via heme b_H. All these reactions are at least 10-fold faster than the limiting first electron transfer, so SQ_o is kept at low occupancy, and mostly harmless (Fig. 3B). As a consequence of this rapid removal, by inhibiting the input flux, the primary mutation reduces the likelihood of harmful ROS production. The suppressor mutations relieve the primary inhibition by easing the tension of the spring, to allow more normal formation

of the *ES*-complex, but at the expense of more ROS production.

Although analysis of previous crystallography studies^{14,15} showed the importance of the tether span in the conformational changes of ISP, our study was the first in a multicellular organism, and showed that mutations of the tether region indeed have a significant impact, not only on the function of complex III, but also on the whole oxidative phosphorylation chain, and through translation of the impact to other complexes, to maintenance of the proton gradient, and to cellular function and physiology.

The *isp-1*(*qm150*) mutation results in sensitivity to hyperoxia that is also suppressed by intragenic tether region suppressors

During our study of tether suppressor mutants, we observed a previously unreported phenotype of *isp-1* (*qm150*) animals: sensitivity to hyperoxia (100% O₂). *C. elegans* carrying the *isp-1*(*qm150*) allele were unable to develop past the L2 stage in the presence of hyperoxia, while wild type N2 animals developed normally. As with the other *isp-1*(*qm150*) phenotypes, this defect was also partially suppressed by the tether region suppressors.

The mechanistic basis for sensitivity to hyperoxia in *isp-1*(*qm150*) animals is unclear. Cell culture studies have shown that exposure to 100% O₂ can mimic physiological oxidative stress and reactive oxygen species (ROS) generation,^{16,17} and *isp-1*(*qm150*) animals have been reported to produce a higher level of superoxide than wild type animals.¹⁸ Thus, one possibility is that both hyperoxia and mutation of *isp-1*(*qm150*) increase ROS levels independently such that, when combined, it results in ROS levels that exceed a threshold consistent with continued development. On the other hand, it seems unlikely from the arguments above that the *increase* in ROS comes from complex III, and much more likely that it is associated with complex I, or some other upstream site. In this context, it is worth noting that inhibition at complex III

Figure 3. Partial processes of the bifurcated reaction. (A) Formation of the *ES*-complex, and first electron transfer. (B) Second electron transfer to the low potential chain, and reduction of heme c₁ in the high-potential chain.

will generate “cross-over” effects¹⁹ such that upstream pools will become over-reduced, and downstream pools over-oxidized. Physiologically, this state is generated when back-pressure from the proton gradient inhibits the low potential chain, and is mimicked by inhibition of the Q_i site by antimycin, both conditions leading to generation of ROS. In contrast, the mutation in *isp-1(qm150)* inhibits flux into the Q_o site, reducing ROS. In both cases, over-reduction of the quinone pool will feed-back on complex I, and through that to the metabolic input reactions of the Krebs’s cycle.

Hirst and colleagues have suggested that the generation of ROS in complex I is through 1-electron reduction of O_2 to superoxide by the reduced flavin (FMN) of the NAD-binding domain.²⁰ Under conditions in which flux through complex I is inhibited by inhibitors or by reduction of the Q-pool, the reduced flavin can accumulate in the presence of O_2 so as to exacerbate this pathway. Because of the crossover effect to reduce the quinone pool, these conditions match those showing sensitivity to hyperoxia in *isp-1(qm150)*, accounting for the increased ROS production.

A second interesting twist to this story is the finding that the mitochondrial apoptosis inducing factor, AIF, is a fragment of the NADH-binding subunit of Ndi1, an alternative NADH-Q oxidoreductase in yeast and mammalian mitochondria, homologous to NDH-2.^{21,22} In *C. elegans*, a protein in the same family, the worm AIF homolog (WAH-1), has also been identified as a key player in apoptosis,²³ and is likely an NDH-2 homolog,²¹ with a flavoprotein (FAD) in its redox active form. In support of this, expression of yeast Ndi1 in *C. elegans* with impaired complex I function restored respiration and proton-pumping.²⁴ Although FAD in AIF can form a tight, air-stable charge transfer complex with NADH that is inaccessible to O_2 , in its redox active form it shows NADH-Q oxidoreductase activity higher than complex I²¹; when in communication with the Q-pool, the FAD will be kept oxidized as long as the pool is partly oxidized. It seems quite likely that when the pool goes reduced, so will the FAD, which might also be a source of ROS through Hirst’s mechanism.²⁰ Alternatively, accumulation of a ubiquinone SQ intermediate might generate ROS.

Putting these diverse observations together allows for some interesting speculation. Perhaps the NDH-2 class of enzymes serves a “canary” function in

detecting conditions threatening the cell’s viability. When appropriately triggered through conversion to the AIF form, this initiates a cascade of apoptotic processes to recycle the defective cells before further damage can occur. Triggering would have to detect the (rare) adverse condition in which the Q-pool is over-reduced in the presence of O_2 , and could come from a local effect of ROS, or by formation of the NADH. FADH complex as the mitochondrial NAD-pool becomes reduced, or perhaps both. We note that in vertebrates, these adverse conditions reflect the situation on oxygenation following ischemia in stroke or heart attack, known to lead to tissue damage.

Clearly, there are opportunities for further research in *C. elegans* to explore the above possibilities.

Conservation of ISP-1 structure and function between worms and yeast

It is perhaps not surprising to see a high level of conservation in the tether region of ISPs, given that changes in the composition of these few amino acids are associated with changes in the balance of forces that hold the ISP head domain in one position versus another position. In order to confirm the generality of the relationship between the *isp-1(qm150)* mutation and the tether region suppressors, we took advantage of the homology between worm and yeast ISPs and engineered a series of yeast strains expressing different alleles of the yeast ISP, Rip1. Although complete deletion of *RIP1* in yeast yields cells that are respiratory-deficient and unable to grow on non-fermentable carbon sources such as glycerol, *rip1Δ* cells are able to grow on medium containing glucose, which yeast can ferment to ethanol. Introduction of the analogous *isp-1(qm150)* mutation of proline to serine into Rip1 yields cells that display drastically diminished respiratory function. Strikingly, 2 different worm tether region suppressors (D87N and A90T) could suppress this respiratory defect. These findings demonstrated a remarkable conservation of the structure-function relationship across widely divergent phyla.

Although several mutations in the tether region identified by our group could partially rescue the *isp-1(qm150)* respiratory defect, the A149T (*sea4*) mutation was the most potent suppressor examined in both worm and yeast (A90T). Interestingly, Fisher et al.,²⁵⁻²⁷ previously reported mutations in the Rip1 tether region

that could partially rescue reduced complex III activity resulting from mutations in the mitochondrial-encoded complex III subunit, *cyt b*, that correspond to human alleles associated with cardiomyopathy²⁸ or exercise intolerance.²⁹ Notably, these *cyt b* mutations are located at the docking interface on *cyt b* for ISP, identified in structures. Building on their study, our work emphasized that: *i*) multiple suppressors of the respiratory deficient phenotypes were identified within the tether region of ISP, *ii*) most of the suppressor mutations were located in the alanine residues of the tether, and *iii*) mutation of yeast A90T (worm counterpart: A149T) was among the strongest suppressors of the yeast *cyt b*, yeast Rip1-P166S, and worm *isp-1(qm150)* respiratory defects. Similar complementary mutations in *cyt b* and the ISP tether were previously discussed in *Rhodobacter*.³⁰ These data illustrate that tether suppressor mutations can suppress not only the ISP P→S mutation, but also respiratory deficient mutations at the opposing interface in the *cyt b* subunit of complex III through which ISP interacts in binding to occupants of the Q_o site.

Collectively, these findings highlight the importance of these 6 conserved amino acids of the tether region in the interactions of *cyt b* with ISP, and suggest that potential pharmacological manipulation of the tether may be of therapeutic value for certain conditions or diseases associated with specific defects in complex III.

Conclusion

By identifying suppressor strains with mutations in the ISP at a distant site from the primary lesion of *isp-1(qm150)*, characterizing the reversal of a wide range of pleiotropic effects, and proposing a model to relate these phenotypes mechanistically to the immediate inhibition of electron transfer, we have provided for the first time a comprehensive molecular understanding of at least these important features of the aging process in terms of a unique reaction site in a central metabolic process. By highlighting the importance of the *bc₁* complex, we have focused attention on the control and gating of its Q-cycle reaction to guard against the generation of damaging free-radical species. The secondary pleiotropic phenotypes have provided clues as to how defects in the primary mechanism translate further out to cellular metabolism and physiology, thus identifying additional

targets for medical intervention. These latter effects will obviously require more detailed study in higher organisms.

Having a yeast model of the worm *isp-1(qm150)* mutation provides the opportunity for future experiments to leverage yeast genetic approaches (e.g., multi-copy suppressor library screening or spontaneous or mutagenesis-induced suppressor strategies) in order to identify novel modulators of the respiratory defect associated with this mutation. In addition, some types of biochemical studies are more easily performed with yeast, where large numbers of cells (>10¹⁰) can be obtained from a single overnight culture. Thus, we view this type of complementary multi-organism approach as quite valuable both for confirming the generality of highly conserved relationships, as well as providing opportunities to leverage the strengths of different model systems.

The synergies of the multi-organism approach can be extended to the bacterial system for detailed mechanistic studies. The photosynthetic *Rhodobacter* species have served as model systems for understanding the *bc₁* complex, because the ability to photoactivate the complex through the photochemical reaction center allows ready assay of kinetics through synchronized single turnover.³¹ In addition to following the electrons as they skip through the Q-cycle, the proton pumping activity can be directly measured through electrochromic changes of carotenoids to assay electrogenic flux, and through use of pH indicators. Since molecular engineering is simple in prokaryotes, and well-developed protocols are available, the structure-function interface and its architectural features can be explored. By expressing mutations in *Rhodobacter sphaeroides* that mimic those in the eukaryotic systems, we can more readily explore ramifications at the molecular level. These synergies can translate to real benefits in medicine, and a multi-faceted understanding of the aging process.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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