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Minireview

Membrane-anchored cytochrome *c* as an electron carrier in photosynthesis and respiration: past, present and future of an unexpected discovery

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Abstract

In the mid 1980s, it was observed that photosynthesis could still occur in the absence of the diffusible electron carrier cytochrome c_2 in the purple non-sulfur facultative phototrophic bacterium *Rhodobacter capsulatus*. This serendipic finding led to the discovery of a novel class of membrane-anchored electron carrier cytochromes and their associated electron transfer pathways. Studies of cytochrome c_y of *R. capsulatus* (and its homologues in other species) have modified the previous dogma of electron transfer between photosynthetic and respiratory membrane protein complexes with a new paradigm, in which these proteins and their electron carriers can form 'hard-wired' structural super-complexes. Here, we reminisce on the early days of this discovery, its impacts on our understanding of cellular energy transduction pathways and the physiological roles played by the electron carrier cytochromes *c*, and discuss the current knowledge and emerging future challenges of this field.

Abbreviations: bc_1 complex – ubiquinone; cyt – cytochrome; cyt *c* – oxidoreductase; ET – electron transfer; Ps – photosynthesis; RC – reaction center; res – respiration

Introduction

Prior to the mid-1980s, models of photosynthetic (Ps) and respiratory (Res) electron transfer (ET) always involved a soluble, freely diffusible protein, often a *c*-type cytochrome (cyt), either called cyt *c*, c_{550} or c_2 (Prince et al. 1975) depending on the species, or a plastocyanin that functions as an obligate electron carrier between the photochemical reaction centers (RC) and the cyt *bc*/*bf* oxidoreductases (bc_1 complex) (Prince et al. 1982; Crofts and Wraight 1985; Dutton 1986). A salient feature of these models was that membrane proteins that engaged in these ET reactions did not form structural super-complexes, but were solitary entities freely diffusing in lipid bilayers. Thus, they were not 'hard-wired' to each other, but loosely 'soft-wired' with connections provided by mobile, water-soluble and lipid-soluble electron carriers like cyts *c* and quinones, respectively (Figure 1). At that time, this view was well supported by biophysical and biochemical studies, and no exceptions were known. Consistent with this dogma, available mutants of photosynthetic bacteria lacking cyt c_2, like MT113 of *Rhodobacter capsulatus* (Zannoni et al. 1980), were deficient in light-induced ET reactions between the RC and the bc_1 complex, and unable to grow by Ps. In such mutants, Res ET between the bc_1 complex and the cyt *c* oxidase (C_{ox}) was also severed (Hudig et al. 1986), although Res growth could still occur via an alternative pathway using a hydroquinone oxi-

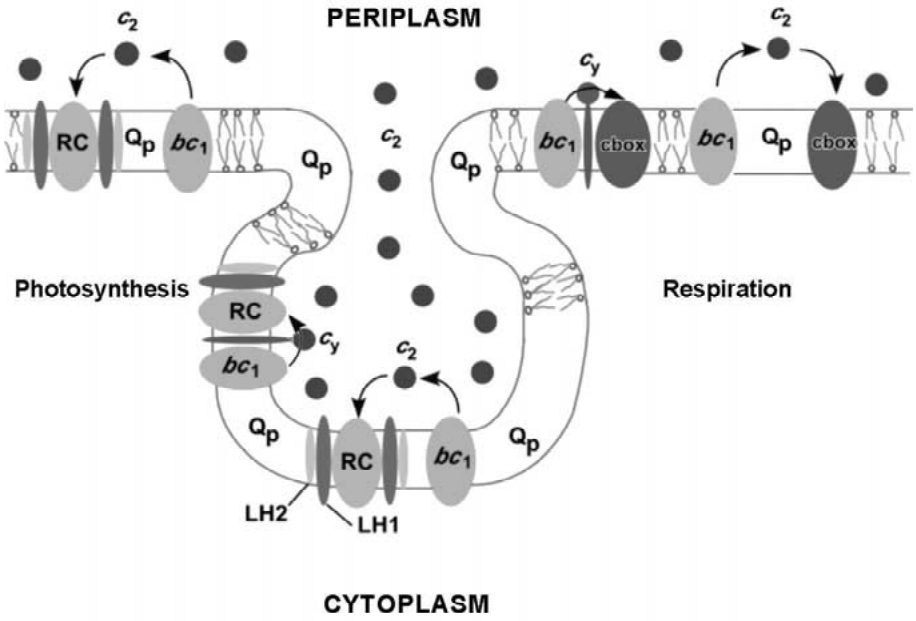

Figure 1. Diagrammatic representation of the mosaic structure of cytoplasmic membranes from *Rhodobacter* species exhibiting both 'soft-wired' solitary and 'hard-wired' super-complex protein structures that mediate electron transfer during photosynthesis and respiration. RC, LH1, LH2, bc_1, cbox, c_2, c_y, and Q_p refer to the reaction center, light-harvesting complexes 1 and 2, cytochrome bc_1 complex, cbb_3-type cyt *c* oxidase, cyt c_2, cyt c_y and the membrane quinone pool, respectively.

dase (Q_{ox}) as a terminal acceptor (Baccarini-Melandri et al. 1978). Ironically, it had previously been pointed out that Ps in *Rhodobacter sphaeroides* involved not only cyt c_2 but also the membrane-bound cyt c_1 (Wood 1980). However, our grasp of the implications of this finding had to await the availability of a *bona fide* mutant lacking only cyt c_2.

Cold Spring Harbor years: discovery of cyt c_2-independent photosynthesis

In the early 1980s, one of us (F.D.), then at Cold Spring Harbor Laboratory (CSHL), joined the photosynthesis-focus group supported by the Exxon-CSHL collaboration. Daldal became acquainted with the impressive field of cyts *c* while searching for a model protein with which to initiate protein structure-function analyses, and was inspired by the close proximity of Marc Zoller, a former student from Michael Smith's laboratory where site-directed mutagenesis techniques had just been developed. Since the early 1960s cyts *c* from various species had been studied by leading bioenergeticists as important physiological electron carrier proteins (Boyer et al. 1977). These proteins had been extensively characterized by Martin D. Kamen (1982) and his colleagues, several high-resolution 3 D structures had been solved, and remarkable evolutionary insights had already emerged (Dickerson et al. 1976). This wealth of information immediately convinced Daldal that cyts *c* were a valuable model system for protein structure-function studies. However at that time, their molecular genetics was largely unknown. Only the iso-1-cyt *c* from *Saccharomyces cerevisiae* had been cloned and sequenced (Smith et al. 1979), and mutants lacking it were available (Sherman et al. 1974). Indeed, the yeast protein was already under study by M. Smith and his colleagues (Pielak et al. 1985). Daldal resolved to initiate a similar effort within the photosynthesis program at CSHL, developing molecular genetics of a cyt *c* from a phototroph. As a novice to the field of photosynthesis but guided by Barry L. Marrs' earlier ingenious genetic studies (see Marrs 2002), insightful suggestions and congenial support (Figure 2), Daldal chose *R. capsulatus* as the most suitable bacterium for such approaches.

The initial idea that a mutant lacking only cyt c_2 was needed came from the studies of Edgar Davidson (Figure 2), then a postdoctoral fellow in Marrs' group, in collaboration with one of us (R.C.P.) who had then joined Exxon Research and Engineering Co.

Figure 2. A few of the players who have participated in the various episodes of the cyt c_y story described here. *Top (left to right)*: Barry L. Marrs, Edgar Davidson, Davide Zannoni, Roger C. Prince and Fevzi Daldal. *Bottom (left to right)*: Francis E. Jenney, Hannu Myllykallio, Paul Mathis, Sevnur Mandaci and Yavuz Ozturk.

Our research (Davidson et al. 1987) demonstrated that mutant MT113 lacked not only cyt c_2 but also the bc_1 complex, including its cyt c_1 subunit. Thus, the absence of Ps in MT113 could not be attributed solely to the absence of only cyt c_2. Indeed, later work established that MT113 was missing a chaperone required for the maturation of all *c*-type cyts, and it was the very first bacterial mutant defective in cyt *c* biogenesis (Lang et al. 1996). Unfortunately at that time, our view of cyt *c* biogenesis was very naïve as we thought that MT113 lacked only a component then referred to as the cyt *c* synthetase or cyt *c* heme lyase.

Attracted by the highly desirable features of cyt c_2 such as the small size, known amino acid sequence, established biochemical characterization methods, and an important physiological role, we undertook, together with Joy Applebaum, a molecular analysis of *R. capsulatus* cyt c_2 (Daldal et al. 1986). The screening of a chromosomal gene library with synthetic, degenerate oligonucleotide probes derived from the amino acid sequence of cyt c_2 led us immediately to its structural gene, *cycA*, which was sequenced and inactivated by the insertion of an antibiotic resistance cassette. Using the Gene Transfer Agent-mediated allele exchange technique (interposon mutagenesis; Scolnik and Marrs 1987) we constructed a mutant, MT-G4/S4 that lacked only cyt c_2. To everyone's surprise, MT-G4/S4 (unlike MT113) was Ps^+ like its wild-type parent. This showed for the first time that Ps growth in the absence of cyt c_2 was possible. Detailed analyses of MT-G4/S4 demonstrated that the component required for Ps growth was a membrane-associated *c*-type cyt (Prince et al. 1986). We thought that it might be cyt c_1 that was then the only known light activated, membrane-associated cyt *c*. However, the possibility that cyt c_1 could donate electrons directly to the RC, although intriguing, was received with skepticism by the photosynthesis community. Alternative possibilities, such as the presence of other diffusible proteins that could substitute for cyt c_2 as an electron carrier in MT-G4/S4, were quickly eliminated. Using a double mutant lacking both the cyt c_2 and the bc_1 complex we further found that in their absence no electron donation to the RC could be detected (Prince and Daldal 1987). In addition, using multiple mutants lacking both the cyt c_2 and the alternate Res pathway dependent on Q_{ox}, we showed that cyt c_2-independent ET pathways also operated during Res growth of *R. capsulatus* (Daldal 1988). Later on, other groups obtained indications for similar ET pathways in other species (Laudenbach et al. 1990; Bott et al. 1991; Turba et al 1995; Oh-Oka et al. 1998).

Despite this progress, the molecular nature of the critical component(s) of the cyt c_2-independent ET

pathways remained obscure, precluding its general acceptance. In retrospect, this was perhaps due to our erroneous implication of cyt c_1 in this pathway, the reticence of the senior scientists in the field who had studied cyt c_2 in great detail as the unique mobile electron carrier in Ps and Res ET, and to conflicting data emerging from other groups. Some *in vitro* reconstitution experiments using the RC and the bc_1 complex suggested that cyt c_2 was absolutely required for Ps ET to the RC (Gabellini et al. 1989), yet others indicated that cyt c_1 could indeed mediate it, albeit at a slower time scale (Venturoli et al. 1990). Among the competing groups, perhaps the most thorough work was that of Tim J. Donohue and his colleagues. They repeated our initial experiments using the closely related *R. sphaeroides*, and found that in this species, unlike in *R. capsulatus*, cyt c_2 was absolutely required for Ps growth (Donohue et al. 1988). In addition, further confusion arose from their remarkable finding that *R. sphaeroides* mutants lacking cyt c_2 could regain Ps growth ability via suppressor mutations that overproduced another soluble *c*-type cyt, named cyt isoc_2 (Roth and Donohue 1990), apparently involved in formaldehyde oxidation (Barber et al. 1996). These findings raised the possibility that our *R. capsulatus* mutant MT-G4/S4 lacking cyt c_2 might also give rise to similar suppressors. These discrepancies between two *Rhodobacter* species thought to be closely related stimulated us to systematically exchange the RC, the bc_1 complex and the cyt c_2 between *R. capsulatus* and *R. sphaeroides* (Davidson et al. 1989). Although these experiments established unequivocally that the molecular basis of the cyt c_2-independent pathway was unrelated to any of the exchanged components, the 'culprit' remained unidentified (Daldal 1990; Zannoni and Daldal 1993). Unfortunately, a more incisive effort towards uncovering this novel component was delayed because of intense competition in the field of bc_1 complex research and also the move of F.D. from the CSHL to the University of Pennsylvania. However, during this time period, an insightful finding came from the work of J. Bas Jackson and his colleagues (Jones et al. 1990). They repeated our work with the double mutant lacking both the cyt c_2 and the bc_1 complex (Daldal and Prince 1987) that we had given them for their study on the ET pathway to nitrous oxide in *R. capsulatus* (Richardson et al. 1989). In contrast to our findings, they detected in this mutant photo-oxidation of a membrane-associated cyt *c* distinct from cyt c_1, which they designated cyt c_x, and proposed it to be the electron donor to the RC (Jones et al. 1990). Resolution of this initially perplexing controversy awaited the discovery of the structural gene of cyt c_y, and the demonstration that its steady-state amount depended on the composition of the growth medium used.

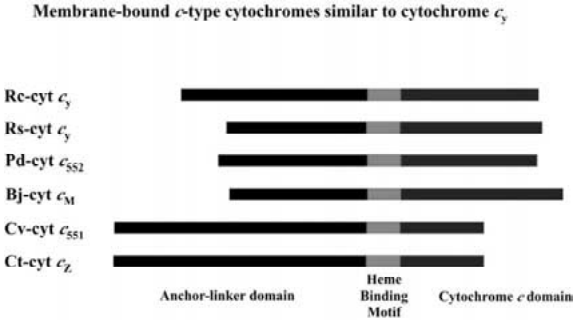

Figure 3. Schematic alignment of a few membrane-bound *c*-type cyt c_y homologues from various species. The membrane-bound cyt c_y and its homologues have two distinct domains on either side of the heme binding motif: an amino terminal anchor-linker domain which varies in length depending on the species and a carboxyl terminal cyt *c* domain that is highly homologous to mitochodrial cyts *c*. Rc-cyt c_y: *Rhodobacter capsulatus* cyt c_y; Rs-cyt c_y: *Rhodobacter sphaeroides* cyt c_y; Pd-cyt c_{552}: *Paracoccus denitrificans* cyt c_{552}; Bj-cyt c_M: *Bradyrhizobium japonicum* cyt c_M, Cv-cyt c_{551}: *Chlorobium vibrioforme* cyt c_{551}; Ct-cyt c_z: *Chlorobium tepidum* cyt c_z.

University of Pennsylvania years: discovery of cyt c_y as molecular basis of cyt c_2-independent photosynthesis

In retrospect, we were extremely lucky that our first graduate student at the University of Pennsylvania, Francis E. Jenney (Figure 2), became deeply intrigued by the cyt c_2 riddles. As part of his graduate work, he initiated two independent approaches to uncover the component(s) responsible for the cyt c_2-independent pathway. In the first approach, he sought a Ps-*minus* mutant derivative of MT-G4/S4 that could be complemented for Ps^+ growth only by cyt c_2, hypothesizing that such a mutant would lack the missing component(s). In the second approach, encouraged by our earlier work (Davidson et al. 1989), he attempted heterologous complementation between *Rhodobacter* species. Using a *R. capsulatus* chromosomal library from a strain lacking the structural gene for cyt c_2, he complemented for Ps^+ growth a mutant of *R. sphaeroides* lacking cyt c_2 (Caffrey et al. 1992).

The first approach yielded a unique mutant that produced only cyt c_1 due to the absence of a chap-

erone that was required for all c-type cyts that are not carboxyl-terminally anchored to the membrane (such as cyt c_1), and this rekindled our interest on the process of cyt *c* biogenesis (Lang et al. 1996). The second approach led us directly to the structural gene, *cycY*, of a membrane associated cyt *c*, that we called cyt c_y (Jenney and Daldal 1993). With an unusual bi-partite structure, cyt c_y defined a novel subclass of cyts *c* (Figure 3). It had a membrane-anchor extended by a large flexible stretch of amino acids (amino terminal 'anchor-linker' domain) followed by a *c*-type cyt sequence (carboxyl terminal 'cyt *c*' domain) homologous to cyts *c*, especially from plant mitochondria.

Finding *cycY* ended all controversies and shifted attention from the question of whether cyt c_2-independent ET pathways existed to how such pathways operated. In collaboration with Davide Zannoni (Figure 2), Jenney undertook detailed studies to define the properties of cyt c_y and the ET reactions it catalyzed during both Ps (Jenney et al. 1994) and Res growth (Hochkoeppler et al. 1995), both in *R. capsulatus* and *R. sphaeroides* (Jenney et al. 1996). These works clearly demonstrated that in wild-type *R. capsulatus*, unlike *R. sphaeroides,* two independent electron carriers with distinct properties operate simultaneously. The membrane-integral cyt c_y communicates with only a subset of RCs and exhibits ET rates comparable to those seen with the diffusible cyt c_2, but only when it coexists with the bc_1 complex. Moreover, in the absence of the bc_1 complex, cyt c_y was only present in the membranes when cells were grown in minimal, but not in enriched, medium (Jenney et al. 1994). Thus, the enigmatic difference between our work (Daldal and Prince 1987) and that of Jones et al. (1990) was apparently due to different growth media. The picture emanating from these findings nicely completed the then prevailing dogma, in the sense that photosynthetic and respiratory membranes were mosaics of proteins that either remained as solitary entities 'soft-wired' to each others via diffusible electron carriers, or formed 'hard-wired' structural super-complexes (Figure 1).

This research also neatly explained a nagging loose end in the thesis work of R.C.P., when he was a student in the laboratory of Tony R. Crofts in Bristol. The Crofts laboratory was part of a NATO-sponsored collaboration with Gunter Hauska in Bochum, and the B. Andrea Melandri and the late Assunta Baccarini-Melandri (1940–1981) (husband and wife) team in Bologna to produce antibodies to the cyt c_2 of *R. sphaeroides* and *R. capsulatus*, with the aim of localizing the protein in the cell. These experiments were unequivocal in *R. sphaeroides*, where the antibody completely inhibited *c*-type cyt oxidation in chromatophores made permeable by the addition of cholate. But similar experiments with *R. capsulatus* indicated that antibodies inhibited only approximately half the cyt *c* oxidation (Prince et al. 1975). In hindsight, this was the first clear observation of cyt c_y oxidation in *R. capsulatus*, although it was overlooked at the time.

The stage was now neatly set for detailed analyses of cyt c_y as a redox protein, and its ET pathways, a task that was tackled by Hannu Myllykallio (Figure 2), our second graduate student at the University of Pennsylvania. He first established that cyt c_y was associated with the membrane via its amino terminal signal sequence-like anchor which remained unprocessed (Myllykallio et al. 1997). Myllykallio then showed that this anchor could also attach other cyts, like cyt c_2, to the membrane. These cyts are not naturally membrane-bound, emphasizing that it was the anchor-linker domains of cyt c_y-like electron carriers that were unique for their function (Figure 3). On the other hand, it initially appeared impossible to obtain a periplasmic form of cyt c_y by simply eliminating its membrane anchor. However, very recently, Yavuz Ozturk (Figure 2), a visiting graduate student from Turkey, found that at least two different mutational events were necessary to support the Ps growth of *R. capsulatus* via a periplasmic form of cyt c_y, which he dubbed cyt S-c_y (Y. Ozturk and F. Daldal, in preparation). Next, using epitope-tagging technology, Myllykallio purified cyt c_y from the membrane and defined its redox properties to establish that, as expected, they were similar to other electron carrier cyts *c*. He also initiated a remarkable collaboration with Paul Mathis (Figure 2) to examine the electron donor properties of cyt c_y at a much faster time scale (μs) than we had analyzed previously. These experiments revealed that cyt c_y could donate electrons about as fast as cyt c_2 to the RC (1 and 5 μs half times), and also exhibited a much slower phase (about 50 μs half time) that depended on the presence of the bc_1 complex (Myllykallio et al. 1998). Perhaps most remarkably, cyt c_y was more efficient than cyt c_2 during multiple turnover ET reactions, because upon photo-oxidation c_y re-reduction by the bc_1 complex was much faster than cyt c_2 (about 50 *versus* 400 μs, respectively) (Myllykallio et al. 1998). At last, we could understand why cyt c_y, which connected only a small fraction (about 15–20%) of available RCs to the bc_1 complex,

could still maintain Ps growth with continuous, saturating light intensity during which multiple turnovers occurred efficiently. Clearly, Nature has found two distinct solutions to allow efficient ET between membrane proteins either using a component like cyt c_2 that is diffusion limited but produced in large amounts to form a pool, or a membrane anchored component like cyt c_y, that is topologically constrained and produced in smaller amount but could turn over much faster. In this way, not only could both the solitary complexes and the super-complexes communicate efficiently with each other, but also their ratio could be controlled physiologically according to different cellular needs (Myllykallio et al. 2000).

The next remarkable impact on cyt c_y studies came, by serendipity again, from the work of Zeilstra-Ryalls et al. (1995) who were at that time studying the role of *fnrL* in oxygen mediated Ps gene regulation in *R. sphaeroides.* They found an ORF upstream of *fnrL* that is homologous to *R. capsulatus cycY* and graciously gave us this clone. Myllykallio immediately pursued this lead to establish that the ORF was indeed the cyt c_y orthologue in *R. sphaeroides*, and demonstrated together with D. Zannoni that it was an efficient electron carrier in Res ET although not involved in Ps ET (Myllykallio et al. 1999). Comparison with the *R. capsulatus* cyt c_y indicated that the *R. sphaeroides* cyt c_y had a shorter anchor-linker domain, suggesting a possible reason for its lack of function in Ps ET. Chimeric constructs in which the anchor-linker and the cyt *c* domains were exchanged between the two *Rhodobacter* species demonstrated that the cyt *c* domain of *R. sphaeroides* cyt c_y was unable to support Ps ET in *R. capsulatus*, although it supported Res ET. However, such constructs could not be used to determine whether this was due to the length, or perhaps the sequence, of the different linkers. Finally, our work together with Sevnur Mandaci (Figure 2), a visiting scientist to our group, confirmed that in *R. sphaeroides,* cyt c_y could convey electrons *in vitro* to both the cbb_3- and the aa_3-type cyt *c* oxidases (Daldal et al. 2001).

Future studies

The coexistence of efficient hard-wired and soft-wired Ps and Res ET pathways raises important issues that need to be addressed to complete our understanding of cellular energy metabolism. Perhaps one of the most pressing questions is to understand how various protein complexes are organized in the membrane to allow efficient interactions between cyt c_y-like electron carriers and their physiological partners, the RCs which are surrounded by the light-harvesting complexes, the bc_1 complexes and the different types of cyt *c* oxidases. Are all of the RCs always surrounded by complete rings of light-harvesting complexes (Cogdell et al. 1999), and if so, how can cyt c_y reach both the RC located inside, and the bc_1 complex located outside of these rings? Could cyt c_y be part of these rings, or does it reach over them? In different species, could the length or the amino acid sequence of the anchor-linker domain of cyt c_y be important to allow, or to impede, such interactions? What are the important amino acid residues of the cyt *c* domain that mediate productive ET to the cyt *c* oxidases but not to the RCs and *vice versa*? On the other hand, as already seen with some mutants (Jungas et al. 1999), could it be that only solitary RCs have rings of light-harvesting complexes, and are therefore constrained to interact only with the cyt c_2, while those that are connected via cyt c_y are surrounded by crescent-like light-harvesting complexes (i.e., not completely closed rings) leaving room for both the cyt c_y and the bc_1 complexes to be located near the RCs? If so, are the bc_1 complexes that function in Ps ET mainly located in different membrane regions, like intra cytoplasmic invaginations for example, than those involved in Res ET? And what are the physiological advantages of a higher order of organization of membrane protein complexes for Ps or Res ET pathways? Considering that super-complexes have now been found even in mitochondria (Schagger and Pfeiffer 2000), what are the signals and the signal transduction pathways that regulate this complexity?

Undoubtedly, continued studies of the structure, function, regulation and biogenesis of membrane-associated cytochromes will further our understanding of cellular energy transduction, which is among the most essential of biological processes in living cells.

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