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Minireview

The isolation of a functional cytochrome b_6f complex: from lucky encounter to rewarding experiences

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Abstract

The recognition that, in photosynthesis, the plastoquinol oxidizing cytochrome b_6f complex resembles the ubiquinol oxidizing cytochrome *bc*1 complex in respiration is one of the examples of exciting universalization in biological research. A peripheral observation towards the end of 1979 initiated an intensive investigation, which is still ongoing today: next to the ATP synthase the cytochrome b_6f complex could be selectively solubilized from the chloroplast membrane by the combined action of octyl glucoside and cholate. It was mere luck that the isolate was substantially active as an electrogenic, proton translocating plastoquinol–plastocyanin oxidoreductase, and that it also catalyzed oxidant-induced reduction of cytochrome b_6, a signature of the Q-cycle mechanism. The basic findings during the first characterization of the complex are summarized, and the excitement among the collaborating groups is remembered. More recent developments, including the impact of gene technology and the elucidation by the crystal structure, are additionally traced here.

Abbreviations: Cyt – cytochrome; Cyt b_6f – cytochrome b_6f complex; OG – octyl glucoside; PS I – Photosystem I; PS II – Photosystem II; SDG – sucrose density gradient

Introduction

Cytochrome (Cyt) *bc* complexes play a pivotal role in energy conserving electron transport chains as proton translocating quinol oxidoreductases, from Bacteria and Archaea to plants and animals (Berry et al. 2000; Schütz et al. 2000). The two most prominent representatives are the Cyt *bc*1 complex in mitochondria, also known as complex III, and the Cyt b_6f complex from chloroplasts. They function as ubiquinol-cytochrome *c* oxidoreductase in respiration, or as plastoquinol–plastocyanin oxidoreductase in oxygenic photosynthesis, the pathways that constitute the biological cycle of carbohydrate (Figure 1A). Although they are involved in the two counteracting halves of the cycle, the two quinol oxidoreductases are closely related, and appear to share a common ancestor. Furthermore, in versatile microbial systems respiration in the dark and photosynthesis in light may employ one and the same Cyt *bc* complex (Figures 1B and C). Our growing knowledge of the Cyt b_6f complex has been summarized repeatedly over the years, either in broad context (Hauska et al. 1983; Kallas 1994; Cramer et al. 1996; Hauska et al. 1996; Berry et al. 2000), or with more specific emphases (Hauska et al. 1988; Hope 1993; Wollman et al. 1999; Schütz et al. 2000). A further burst of information at the atomic level is expected from the presentations of crystal structures for the complexes from the cyanobacterium *Mastigogladus laminosus* (William A.Cramer's group), and from the green alga *Chlamydomonas rheinhardtii* (Jean-Luc Popot's group), which have been presented at two separate 2003 Gordon Conferences (see 'Note added in proof').

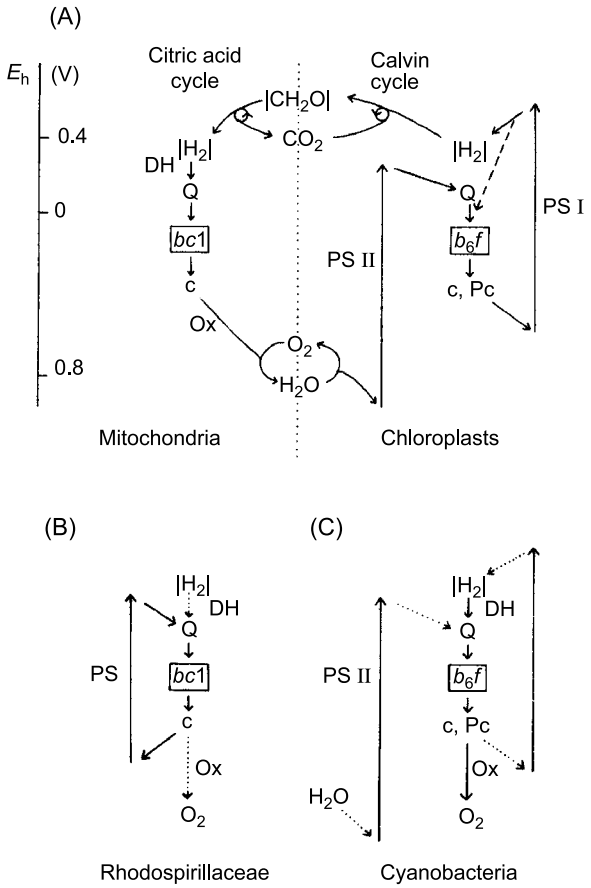

Figure 1. (A) Biological cycle of carbon, hydrogen and oxygen. (B) and (C) Photosynthetic and respiratory electron transport in purple bacteria and cyanobacteria, respectively. Abbreviations are: $|H_2|$ – hydrogen bound to NAD(P)+ or FAD, $|CH_2O|$ – carbohydrate, DH – dehydrogenase, Q – quinone, c – Cyt *c*, Pc – plastocyanin, Ox – oxidase, PS – photosystem. Figure taken from Hauska (1985).

This article is not intended as an updating review. Instead, it presents a recollection of our contributions to the notion that the Cyt b_6f complex of chloroplasts is closely related to the Cyt *bc*1 complex of mitochondria. I would like to convey the excitement we felt about this universalization. I also include personal remarks on the people with whom we were in close contact. Since a mind, naturally, is limited, I apologize for any inherent omission or bias. Whenever recent progress is referred to 'as known today,' the reader is referred to the comprehensive review on Cyt *bc* complexes by Berry et al. (2000), unless otherwise cited.

Prologue

'Did you ever look into the brownish band?' I asked Nathan Nelson in the fall of 1978, during one of his visits from Haifa (Israel) to Regensburg (Germany). 'It must have occurred to you during your numerous preparations of the ATP synthase (coupling factor complex = CF_1CF_0) with Uri Pick and Ef Racker. It's always there in the sucrose density gradients (SDG), slightly above the ATP synthase.' He had not yet looked, which is somewhat surprising in view of his infectious curiosity.

Nathan, during that haunting three-day visit to my lab, had just successfully reconstituted photophosporylation in lipid vesicles with Photosystem I (PS I) reaction centers, the quinoid mediator phenazinium methosulfate (PMS), and the ATP synthase (Pick and Racker 1979; see Nelson and Ben-Shem 2002). Over a bottle of champagne we missed the encounter with the brown band at that moment.

In those days, non-denaturing detergents, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and antibodies were important. It was the great time of membrane complexes and the assignment of chloroplast proteins – the era just before gene technology.

Nathan and I first met at the end of 1970 at Cornell University (Ithaca, New York), when I was about to finish a postdoc period with Dick McCarty and Efraim Racker, and he had started one with Racker, whose lab joined the expertise of 'mitochondriacs' and 'chloroplastologists.' It contributed substantially to one of the great universalizations in biochemistry – the merger of energy conservation in respiration and photosynthesis, under the roof of Peter Mitchell's chemiosmotic theory. Our common interest was the topography of the chloroplast membrane. Using antibodies and other means, we discovered that plastocyanin and Cyt *f* were both located on the lumenal surface, opposite the ATP synthase. This location resembled the site of cytochromes *c* in mitochondria (Racker et al. 1972), and a universal architecture for energy conserving biomembranes began to emerge. Nathan and I have stayed in contact ever since, with mutual visits over the years. He is probably my most important, certainly the most persistent, companion in science, and a true friend in life, despite the geographical distance between Israel and Germany. I owe him a lot. Figure 2 was taken in the spring of 1982, south of Tiberias, at Lake Galilee.

A brownish band? Why did not the bell ring immediately? Was there not already J.S.C. Wessel's observation of a 'pink' band on SDG with digitonin extracts from chloroplasts (Wessels 1966; Wessels and Voorn 1972)? Was there not Nathan's own description with Jossi Neumann of a Cyt fb_6 particle

Figure 2. With Nathan Nelson (left) in the spring of 1982, south of Tiberias, at Lake Galilee, when the author was visiting him at the Technion, Haifa, to prepare the cytochrome b_6f complex from the thermophilic cyanobacterium *Mastigogladus laminosus*. We never published the results, and the work was later independently resumed by Bill Cramer (see Zhang et al. 1999).

which shared features with the mitochondrial Cyt *bc*1 complex (Nelson and Neumann 1972)?

Later, after our excitement about reconstitution of photophosphorylation had settled down after publication (Hauska et al. 1980), I remembered and took difference spectra of the brownish band, together with Raj Sane, another of my influential companions who was visiting from India, also at the of end 1979 (see Hauska and Arnold 2000). And what a satisfying surprise! The brown band represented spectrally pure Cyt b_6f! Isolation by chance? A lucky encounter, after all! Thus the isolation procedure of the Cyt b_6f complex with alkylglycoside detergents actually was a spin-off from the isolation of the ATP synthase. And it was not the consistent extension of our work, although it may appear that we wanted to isolate the Cyt b_6f complex to replace PMS by the plastoquinol–plastocyanin oxidoreductase in the reconstitution of photophosphorylation.

Why did I not notify Nathan immediately? Well, I have to admit that I felt selfish about this lucky discovery, recognizing it as my chance. I suspect that he knows, and that may well be part of our friendship.

Eduard Hurt's PhD thesis work, 1979–1983

At the end of 1979, Eduard Hurt started his PhD work with me, just in time to pick up Raj's and my pilot experiment. During his master's thesis he had accomplished the functional reconstitution of PS I reaction centers into vesicles from chloroplast lipids, joining the project with Nathan. This work remained unpublished in favor of the brown band.

Eduard turned out to be the other lucky encounter in our Cyt b_6f story, a rare case of experimental efficiency. He combined an outstanding motivation to pick up new methods with a most admirable care for documentation – figures and tables from his notebooks could be taken for publication as they were. In addition, he was very communicative, establishing numerous experimental contacts, be it with the Melandris (Assunta and Bruno Andrea) in Bologna (Italy), Dick Malkin at Berkeley (California), Jeff Hind on Long Island (New York), or Yosepha Shahak at the Weizmann Institute (Israel). In 1984 he moved, as a postdoc, to Jeff Schatz at the Biocenter in Basel (Switzerland), studying protein import into mitochondria, and today he is established in Heidelberg (Germany), well recognized by his work on the nuclear import/export system for proteins and RNAs in yeast.

In 1979 it was already known that the chloroplast Cyt *f* and Cyt b_6 copurify (Nelson and Neumann 1972; Ke et al. 1975). Both were discovered long ago by Robin Hill (Hill and Scarisbrick 1951; Hill 1954; see Derek Bendall, this issue, for Cyt *f* discovery). In Hill's tradition at Cambridge – and most important for us – was the demonstration by Wood and Bendall (1976) that chloroplast homogenates and even Wessel's pink band had plastoquinol–plastocyanin oxidoreductase activity in the dark. Peter Rich took on that work in fruitful contact and competition with us (see Rich 1984). In that context, I should mention how proud I felt that I was the second choice of Professor Ulrich Heber in 1986, to address Robin Hill on the occasion of his honorary degree from the University of Würzburg. The first choice had been Achim Trebst, who unfortunately was unable to attend.

The pink band of Wessels and other Cyt b_6f isolates using digitonin additionally contained Cyt *b*-559 (Anderson and Boardman 1973; Cox 1979), which our isolate did not. Our main progress, however, mainly resided in introducing SDS-PAGE.

In Eduard's hands the brown band soon turned reddish (Figure 3), and a first characterization was quickly published: 'A cytochrome *f*/b_6 complex of five polypeptides with plastoquinol–plastocyanin oxidoreductase activity from spinach chloroplasts' (Hurt and Hauska 1981).

The isolation involved four steps: (1) removing peripheral proteins by sequential washings of the chloroplast membrane, in particular with the chao-

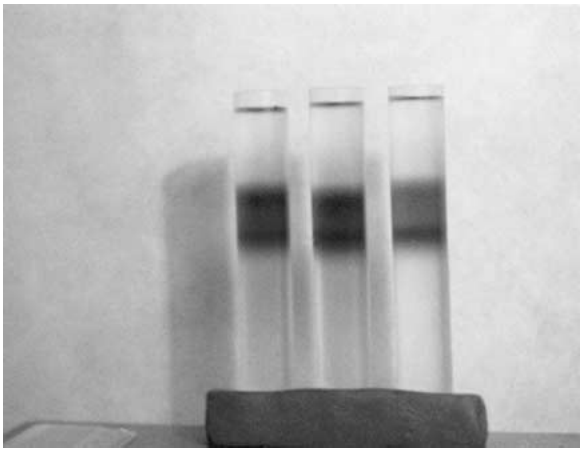

Figure 3. The 'reddish-brown band' (lower band) of the cytochrome b_6f complex on sucrose density gradients (Hurt 1983). For a color version of this figure see color section in the front of the issue.

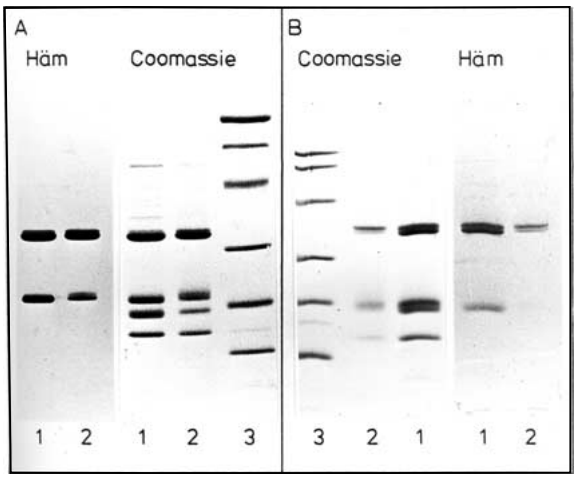

Figure 4. 'Five' polypeptides of the isolated cytochrome b_6f complex (Hurt 1983). (A) SDS-PAGE on a 12–18% gradient gel, and (B) in the presence of urea, stained for protein with Coomassie and for heme (Hurt and Hauska 1981); lanes 1 and 2 represent two different preparations of the complex; lane 3 the protein standard corresponding to 94, 68, 45, 30, 21 and 14.5 kDa.

tropic agent NaBr at 2 M, to strip off CF_1, the headpiece of the ATP synthase (Kamienietzky and Nelson 1975); (2) selective solubilization of the Cyt b_6f complex by a mixture of octyl glucoside (OG)/cholate leaving the chlorophyll proteins largely behind; (3) ammonium sulfate precipitation; and (4) centrifugation in SDG. Initially, Eduard used Triton X-100 in the SDG in place of OG/cholate for two reasons: OG was very costly, and separation of the bands in the presence of Triton was better than with OG/cholate. However, since Triton was inhibitory for the activity (see below), OG/cholate was later used throughout our research (Hurt and Hauska 1982a), or was replaced by cheaper *N*-methyl-*N*-nonaoylglucamide (MEGA-9)/cholate (Hauska 1986). Our isolation strategy has been modified several times over the years (see Berry et al. 2000), but in principle the basic procedure still holds today. Nowadays the detergent of choice may well be 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside (HECAMEG), which yields highly active preparations from the green alga *Chlamydomonas rheinhardtii* (Pierre et al. 1997), as well as from spinach (Dietrich and Kühlbrandt 1999).

The polypeptides can be seen in Figure 4 again, which is taken from Eduard's PhD thesis, as well as the following ones (Hurt 1983). As we now know, the polypeptides represent the three membrane-bound redox components generally functioning in Cyt *bc* complexes – a mono-heme Cyt *c*, a double heme Cyt *b*, and the high potential Rieske FeS protein – in the following way: the largest runs as a double band at 33/34 kDa, stains for heme, and represents the c-type Cyt *f* (Hurt and Hauska 1982a). The dual form is not found in every plant (Hauska 1986), and may reflect genetic microheterogeneity in spinach, or proteolytic truncation at the C-terminus. The truncation would be smaller than the one known for *Brassicaceae* (Gray 1992), which cleaves before the C-terminal transmembrane helix.

The second heme-active polypeptide running at 23 kDa in Laemmli gels (Figure 4) represents Cyt b_6, and carries two low spin hemes of different redox potential (Hurt and Hauska 1982a, but see 'Note added in proof'). Together with the fourth polypeptide of 17 kDa, it makes up the Cyt *b* complement (see below). The remaining 20 kDa band represents the Rieske FeS protein, discovered by John Rieske in connection with the respiratory chain in mitochondria (Rieske et al. 1964), and later also detected in choroplasts by Malkin and Aparicio (1975). The relative positions of Cyt b_6 and the Rieske FeS protein are shifted around in the presence of urea, which increases the relative mobility of the heme-carrying Cyt b_6 (Figure 4, right panel).

The complex eluted between catalase and ferritin from Sephadex G-200, at an M_r of 370 kDa, and was initially estimated as monomeric. It is predominantly dimeric, (Huang et al. 1994). Actually, a reinterpretation of our original data, which probably overestimated the Triton content, also favors a dimeric organization: the stoichiometry of the four protein

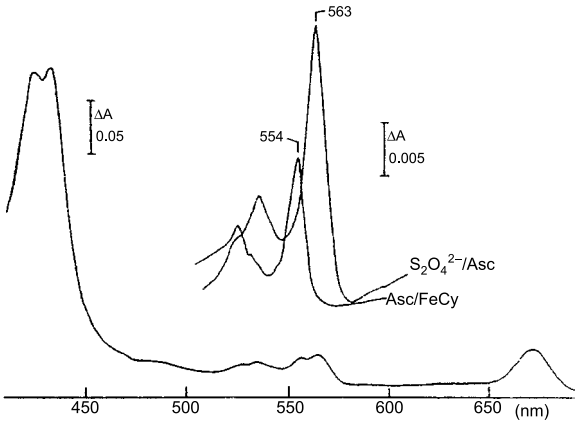

Figure 5. Spectra of the cytochrome b_6f complex. The redox difference spectra in the insert, Asc/FeCy (ascorbate minus ferricyanide) and $S_2O_4^{2-}$/Asc (dithionite minus ascorbate) reflects Cyt *f* and Cyt b_6, respectively.

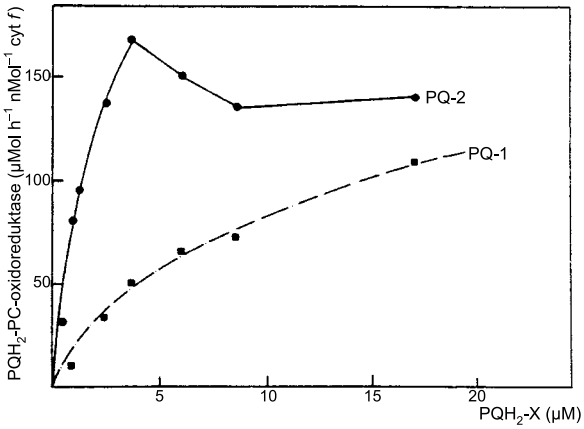

Figure 6. Dependence of specific activity on the concentrations of plastoquinols. PQ-2 and PQ-1 stand for plastoquinol with one and two isoprenoid units in the side chain (Hurt 1983).

subunits Cyt *f* (33 + 34 kDa), Cyt b_6 (23 kDa), Rieske FeS protein (20 kDa) and subunit IV (17.5 kDa) has been measured as 1:1:1:1 (Hauska and Hurt 1982a). This adds up to 94 kDa. With the Triton micelle size of some 90 kDa, and providing for the small subunits (Hauska and Hurt 1982a; also see below) and the pigments plus lipids, a dimer can well be accommodated within 370 kDa. In fact the crystal structure reveals a dimer which is clamped by the Rieske FeS protein (see 'Note added in proof').

The absolute spectrum plus redox differences of the reddish-brown band are seen once more in Figure 5. Cyt *f* and Cyt b_6 are present in a ratio close to 2:1, and Cyt *b*-559 is completely absent, as was shown by redox potentiometry (Hauska and Hurt 1982a), as well as immunologically by W blots later (G. Hauska, unpublished). One molecule of chlorophyll *a* per Cyt *f* is still present, which we regarded as residual contamination. However, this chlorophyll is actually firmly bound to Cyt b_6 in Cyt b_6f complexes (Pierre et al. 1997), and bound β-carotene has since been detected additionally (Zhang et al. 1999).

Plastoquinol–plastocyanin activity in our routine assay using plastoquinol-1 was around 10 μmol per nmole Cyt *f* per hour, which corresponds to a turnover of 3 s^{-1}.With plastoquinol-9 at decreased Triton and higher substrate concentrations this could be increased to about 14 s^{-1} (Hurt and Hauska 1981). Omission of Triton and the use of plastoquinol-2 (Hurt 1983) further increased the turnover to 47 s^{-1}, corresponding to 168 μmol per nmol Cyt *f* per hour, as shown in Figure 6. With HECAMEG for isolation, turnover numbers up to 500 s^{-1} have now been reached (Dietrich and Kühlbrandt 1999). Decylplastoquinol has been used as substrate, which can be conveniently prepared from commercially available decyl-plastoquinone (Rich 1978). As electron acceptor, Eduard replaced plastocyanin by Cyt *c*-552 from *Euglena*, which has a four times higher redox differential extinction coefficient. We obtained it from Erich Elstner (Munich), a close colleague from my time with Achim Trebst in Bochum (Germany). It is noteworthy that, in *Euglena*, plastocyanin is functionally replaced by Cyt *c*-552 (Elstner et al. 1968) and also resembles its topographical location, on the inner surface of the thylakoids (Wildner and Hauska 1974).

The inhibitor sensitivity of the isolated complex was in line with the sensitivity of electron transport in the chloroplast membrane (Trebst 1974), the quinone analogs 2,5-dibromo-3-methyl-6-isopropyl-p-benzpoquinone (DBMIB), and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), being efficient, while antimycin, a valuable inhibitor of the corresponding ubiquinol-Cyt *c* oxidoreductase in mitochondria, did not inhibit.

The impact of this first publication on the Cyt b_6f complex in 1981 was great, and, to our satisfaction, was recognized by Trends in Biochemical Sciences (Bennet 1981).

Eduard's further findings during his PhD work were: (1) At Berkeley, together with Dick Malkin the Rieske FeS subunit was isolated from the complex and characterized (Hurt et al. 1981); (2) Cyt *f* was isolated and characterized. Its redox potential was 340 mV, as expected (Hurt and Hauska 1982a);

(3) Cyt b_6 was isolated and studied in detail, in contact with Jeff Hind's lab at the Brookhaven National Laboratory on Long Island. The two hemes could be distinguished by spectroscopy at low temperature (Hurt and Hauska 1983), and in the complex had redox potentials of −40 and −172 mV at pH 6.5 (Hurt and Hauska 1982a), very similar to more recently determined values in chloroplasts (Kramer and Crofts 1994), but at variance with older measurements; (4) The presence of up to three additional, very hydrophobic (extractable with acidic acetone) and small (3–4 kDa) proteins was described (Hurt and Hauska 1982a), which have since been characterized in more detail (Wollman et al. 1999; Berry et al. 2000; see 'Note added in proof').

Most important, the isolated complex catalyzed 'oxidant induced reduction' of Cyt b_6, which is signatory for the so-called Q-cycle mechanism of quinol oxidoreductases in electron transport chains (Mitchell 1976; see Tony Crofts, this issue). In essence, the mechanism envisages two quinone interaction sites, one for quinol oxidation and one for quinone reduction. These are located on opposite membrane surfaces, and are connected via the two hemes of Cyt *b*. The cycle is initiated with the oxidation of quinol by the Rieske FeS center to the semiquinone, which reduces Cyt *b* in a concerted reaction. For further details, see Brandt and Trumpower (1994) and Berry et al. (2000).

One of our experiments, which was brought to my mind again by Bacon Ke (Ke 2001, Figure 12D in Chapter 35, p. 655), is shown in Figure 7. As can clearly be seen, the transient reduction of Cyt b_6, after adding limited amounts of ferricyanide, depended on the presence of the Rieske FeS protein. Corresponding spectral changes were also obtained by illuminating a mixture which coupled the isolated Cyt b_6f complex to PS I reaction center in the presence of plastocyanin (Hurt and Hauska 1982b). Furthermore, extending the studies of oxidant-induced reduction, evidence for a bound plastoquinone molecule was provided by differential extraction with organic solvents (Hurt and Hauska 1982c); this finding has been questioned later, however, (Willms et al. 1988). With Walter Oettmeier at Bochum, binding of plastoquinone was studied with a photoaffinity label (Oettmeier et al. 1982). The Rieske FeS protein, Cyt b_6 and subunit IV were labeled. The crystal structure contains one plastoquinone molecule, bound close to the site of quinol reduction (see 'Note added in proof').

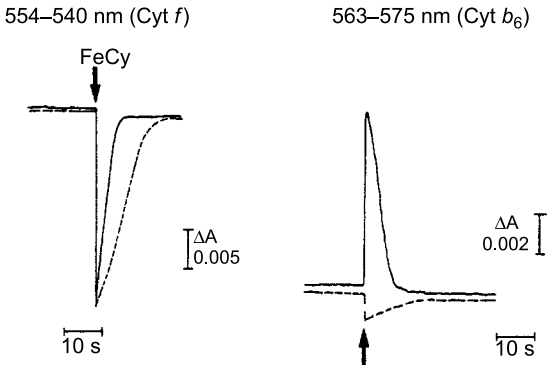

Figure 7. Ferricyanide-induced oxidation of Cyt *f* and reduction of Cyt b_6 (Hurt and Hauska 1982b). The reaction mixture buffered at pH 8.0 contained in 0.5 ml: 1.4 μM Cyt *f* in form of the Cyt b_6f complex, 2.8 μM plastocyanin and 0.5 mM plastoquinol-1; the reaction was started by addition of ferricyanide (FeCy) to 0.25 mM; the absorption changes at 554–540 nm and 563–575 nm correspond to Cyt *f* and Cyt b_6, respectively; the dashed traces were obtained with a complex deficient in the Rieske FeS protein (Hurt et al. 1981).

Another highlight was the functional reconstitution of the Cyt b_6f complex into liposomes, which was done with Yosepha Shahak from the Weizmann Institute in Israel. Figure 8 shows her celebrating the success with us (Hurt et al. 1982). The complex was studied in 'inside-out' orientation by adding external plastocyanin. Its plastoquinol–plastocyanin oxidoreductase activity was stimulated by the uncoupling ionophore combination valinomycin/nigericin. Accompanying electrogenic proton translocation out from the vesicles was documented by measuring external acidification, as well as by membrane-potential-indicating carbocyanine dyes.

Yosepha and I first met in the summer of 1974, on the occasion of the 3rd Photosynthesis Congress at the Weizmann Institute, when she was still a student with Noun Shavit at Beer Sheva, before she joined the group of Mordhay Avron at Rehovot (Israel). She was my guide during a most impressive tour into the Negev, and so the other still standing personal acquaintance with Israel started. With Avron she had characterized a b_6f mutant from *Lemna* (Shahak et al. 1976; see Gal et al. 1987), but our common interest in the Cyt b_6f complex arose in the summer of 1981, when she was collaborating with Jeff Hind at the Brookhaven National Laboratory on Long Island and I was visiting. I learned to appreciate her as a sporty and determined mind, shaped by experiences in the army, and as a skilled and persistent experimentalist. Later, together with Etana Padan and Michal Bronstein at the Hebrew University in Jerusalem, we shifted to the mi-

Figure 8. Yosepha Shahak with Eduard Hurt (left) and the author (right), on 15 September 1982, celebrating the successful reconstitution experiment at the 'Italian,' on campus of the University of Regensburg.

crobial sulfide-quinone reductases (SQR), induced in stressed cyanobacteria of the Negev for instance, and which are linked to quinol-oxidation by *bc* complexes (Bronstein et al. 2000).

With Wolfgang Lockau – another companion from my time with Trebst at Bochum in the 1970s, now at the Humboldt University in Berlin – the isolation procedure was extended to the Cyt b_6f complex of the cyanobacterium *Anabaena variabilis* (Krinner et al. 1982). With Nadia Gabellini, a most charming and efficient collaborator, coming from the Melandris' group at Bologna, we also applied it to isolate the Cyt *bc*1 complex from a carotinoid deficient strain of purple bacterium, which we regarded as *Rhodopseudomonas sphaeroides* GA (Gabellini et al. 1982), known as *Rhodobacter sphaeroides* GA today. This was a regrettable mistake, straightened out later by Davidson and Daldal (1987), who identified the organism as a strain of *Rhodobacter capsulatus*. Both isolates were studied in parallel to the Cyt b_6f complex from spinach (Hurt and Hauska 1983; Gabellini and Hauska 1983a, b), including functional reconstitution into liposomes (Hurt et al. 1983). It is noteworthy that the Cyt *bc*1 complex from *Rhodobacter capsulatus* consisted of only three polypeptides (Cyt *c*1, Cyt *b* and the Rieske FeS center), and was functionally intact. Thus these three proteins are sufficient for the proton translocating quinol oxidation via the Q-cycle mechanism. The more complicated composition of the mitochondrial counterpart is obviously of secondary importance. Indeed, a role in protein import is indicated (Neupert 1997).

Already in 1983, before Eduard graduated, the new results with the Cyt b_6f complex were compared to the properties of the Cyt *bc*1 complexes from mitochondria and bacteria in a first, comprehensive review (Hauska et al. 1983).

Further developments

I travelled and lectured a lot at that time. Figures 9A and B exemplify the many contacts I was able to make. Details of what I could tell can be found in Chapter 35 of Bacon Ke's book on photosynthesis (Ke 2001).

As early as 1981, I brought Eduard's preparation to Les Dutton's group at the Johnson Foundation in Philadelphia (PA), for another day of exciting experimental experience. Roger Prince designed and Katsumi Matsuura carried out the measurements, impatiently observed over their shoulders by Les, me and Andrea Melandri, who also happened to be visiting. The Cyt b_6f complex from spinach was coupled to reaction centers from *Rhodobacter sphaeroides*, and cytochrome changes were studied with flash trains. The observed reduction of Cyt b_6 titrated with an ambient redox potential of +280 mV, which corresponds to the value expected for the Rieske FeS center, exemplifying once again the oxidant-induced reduction of Cyt b_6 (Prince et al. 1982). Wolfgang Nitschke later determined the redox potential of the Rieske FeS center in the isolated complex more accurately by EPR. His value of +320 mV was somewhat higher (Nitschke et al. 1992).

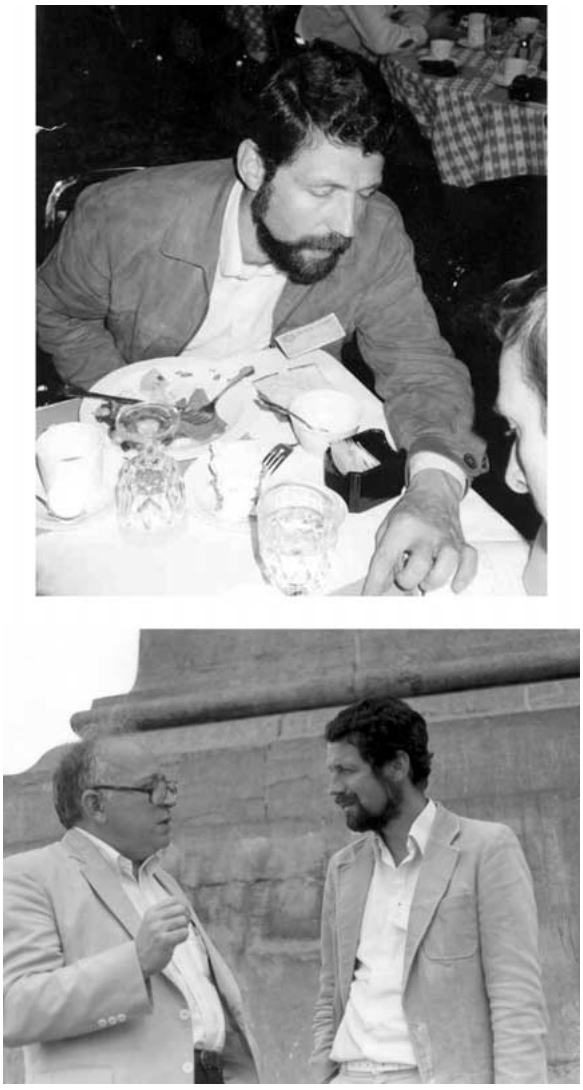

Figure 9. Top: The author with Bruce Diner (only partially seen) at the Gordon Conference on Photosynthesis, at Ventura (California), February 1983. *Bottom*: The author with David Krogmann (left) at Waterloo, on the occasion of the 6th International Congress on Photosynthesis, Brussels, 1–6 August 1983.

In March 1985 I was invited to Paris by Pierre and Anne Joliot, and I think that this visit encouraged the groups of Francis-André Wollman and Jean-Luc Popot at the Collège de France to go for the characterization of the Cyt b_6f complex from *Chlamydomonas rheinhardtii*, a very successful venture (Wollmann and Lemaire 1988; Pierre et al. 1995; Wollman et al. 1999), culminating in the crystal structure in 2003 (see 'Note added in proof'). The Joliots had in addition developed a sensitive spectrophotometer which allowed the observation of cytochrome changes in chloroplasts by single flashes (Joliot and Joliot 1984). This technical improvement attracted Tony Crofts (see Kramer and Crofts 1994) to spend a sabbatical in Europe. On this occasion, Tony spent three months in Regensburg with his young family, which was most stimulating for us.

Tony and I had been in contact since the Gordon Conference on Photosynthesis in 1970. After I had moved to Bochum, Roger Prince, his PhD student visited from Bristol, and with the Melandris from Bologna, we formed the triple party 'BBB' (= Bochum–Bologna–Bristol), 150% as strong as the French idol BB. We discovered that Cyt *c*2 in purple bacteria was located in the periplasmic space (Prince et al. 1975). The picture of universal topography in energy conserving biomembranes (see above) has been significantly substantiated by this finding.

With advice from Tony, Wolfgang Nitschke obtained evidence for heme-to-heme electron transfer in cytochrome b_6 during the turnover of the Cyt b_6f complex (Nitschke et al. 1988). He had taken over the work on the Cyt b_6f complex from Eduard very efficiently. Coming from physics he introduced EPR to the subject, concentrating on the Rieske FeS center (Nitschke et al. 1989), but also studying the cytochromes (Nitschke and Hauska 1987). After graduating in 1987 he joined Bill Rutherford at Saclay (France) and continued the work in contact with Piere Joliot and our group, especially with Astrid Riedel, another expert on EPR (Riedel et al. 1991; Nitschke et al. 1992). Wolfgang established himself in France and is now residing in Marseille (Schoepp et al. 1999; Brugna et al. 2000). More recently, a joint paper on the evolution of the Cyt *bc* complexes was published (Schütz et al. 2000).

Very valuable, too, was our contact with Gebhardt von Jagow and his coworkers Uli Brandt, Thomas Link and Herrmann Schägger, first at Martin Klingenberg's Institute in Munich and later in Frankfurt. Together, from 1985 to 1990, we served the German Bioenergetics group within the Gesellschaft für Biologische Chemie. Mutual stimulation, from methods (Engel et al. 1983; Schägger et al. 1988) to mechanistic considerations (Brandt and Trumpower 1994) was great, which was later also experienced in Frankfurt by Bill Cramer (Huang et al. 1994).

Since quinol oxidation is the rate-limiting step in the electron transport chains, it naturally lends itself to redox regulation (see Allen 2003). Indeed, the Cyt b_6f complex functions as a redox sensor

of the plastoquinone pool, in addition to its central role in energy transduction. The redistribution of quanta between PS I and II upon changes in light quality, the so-called 'state transitions' (see Myers 1971), occur via movement of the outer chlorophyll antennae LHCII, which is caused by phosphorylation/dephosphorylation (Bennet 1977; see historical perspective by Allen 2002). An excess of plastoquinol is 'sensed' by the Cyt b_6f complex at the quinol oxidation site, and it causes activation of an attached LHCII kinase. Phosphorylated LHCII moves from PS II to I, funelling more quanta into PS I, which leads to reoxidation of plastoquinol and deactivation of the kinase (Vener et al. 1997; Zito et al. 1999; Hamel et al. 2000). We were also able to participate in these studies initially, in contact with Francis-André Wollman in Paris (Wollman and Lemaire 1988), and with the late Alma Gal in Itzak Ohad's group, again at the Hebrew University in Jerusalem (Gal et al. 1987, 1990). Most intriguing is the recent finding that this redox sensing is linked to the conformational movement of the redox-center-carrying domain in Rieske FeS protein (Schoepp et al. 1999; Brugna et al. 2000; see also Berry et al. 2000).

In 1993, I lectured at the European Molecular Biology Laboratory in Heidelberg, bringing our Cyt b_6f preparation to Germaine Mosser and Werner Kühlbrandt for 2D-crystallization (Mosser et al. 1994). While Germaine went back to France, turning to Popot's preparation from *Chlamydomonas* (1997), Werner improved the isolation procedure from spinach (Dietrich and Kühlbrandt 1999). High resolution structures had not been published for the whole Cyt b_6f complex, neither by X-ray nor by electron crystallography until September 2003 (see 'Note added in proof'). Such structures from X-ray diffraction have, however, been available for several mitochondrial Cyt *bc*1 complexes, and for the soluble parts of Cyt *f* and the Rieske FeS protein of the Cyt b_6f complex (see Berry et al. 2002). At the time of writing there is a rumor, however, that both Bill Cramer and Jean-Luc Popot will report on high resolution structures of the whole complex at the next Gordon Conference on photosynthesis (Don Bryant and Francis-André Wollman, personal communications; see Cramer, this issue). Great!

The impact of gene technology

Already in 1982, Nathan in Haifa and I had started independently to prepare antibodies against the individual subunits of the Cyt b_6f complex. I had learned to deal with the rabbits from Richard Berzborn, who originated from Wilhelm Menke's group at Köln, and shared many years with me, first at Cornell with Racker, later at Bochum with Trebst. Immunizing and bleeding the rabbits myself, I used to spend a lot of time around their cages until I developed an unbearable allergy. Thus, I quit after obtaining two rabbits with reasonable titers of antibodies against the Rieske FeS protein, while Nathan was successful at acquiring the rest – Cyt *f*, Cyt b_6 and subunit IV. The two rabbits, numbered 8 and 9, 'visited' numerous laboratories and became very precious in donating their Rieske antisera to several Cyt b_6f projects. They were guests at my home during Easter breaks, where they were called 'Pünktchen und Anton' by my little daughter Konstanze who loved to play with them in our garden. It almost wrecked the family when they got lost in the bushes for two days, once.

Nathan and I joined forces once again in Reinhold Herrmann's lab in Munich, leading to a most fruitful extension of the Cyt b_6f studies. Reinhold had just moved from Düsseldorf where he had developed his interest in the plastid genome (plastome), advised by Wilfried Stubbe, and meanwhile had grown into a leading plant molecular biologist. Very valuable for the progress was the participation of Peter Westhoff, who was starting his career with Reinhold, and is now well established, back in Düsseldorf. After the genes for Cyt *f*, Cyt b_6 and subunit IV had been located on the plastome, and the gene for the Rieske FeS protein in the nucleus (Alt et al. 1983), they could be sequenced (Alt and Herrmann 1984; Heinemeier et al. 1984; Steppuhn et al. 1987). This opened the field to a study of their expression, as well as the transport and assembly of their products (Zak et al. 1997; Wollman et al. 1999).

Because chloroplast Cyt b_6 and mitochondrial Cyt *b* both bind two low spin hemes, the significance of conserved histidines in the relatively short Cyt b_6 with respect to mitochondrial Cyt *b* was recognized as soon as the sequence became available (Saraste 1984; Widger et al. 1984). Together with the hydropathy pattern, the following structural predictions were made: (1) Cyt b_6 plus subunit IV of the Cyt b_6f complex add up to Cyt *b* in the Cyt *bc*1 complex, with nine originally predicted transmembrane helices; Cyt b_6 represents the first N-terminal five helices and subunit IV corresponds to the C-terminal helices 6–8 in Cyt *b*; (2) the four His-residues conserved in the Cyt b_6 part are located in two pairs on helix 2 and 5, and

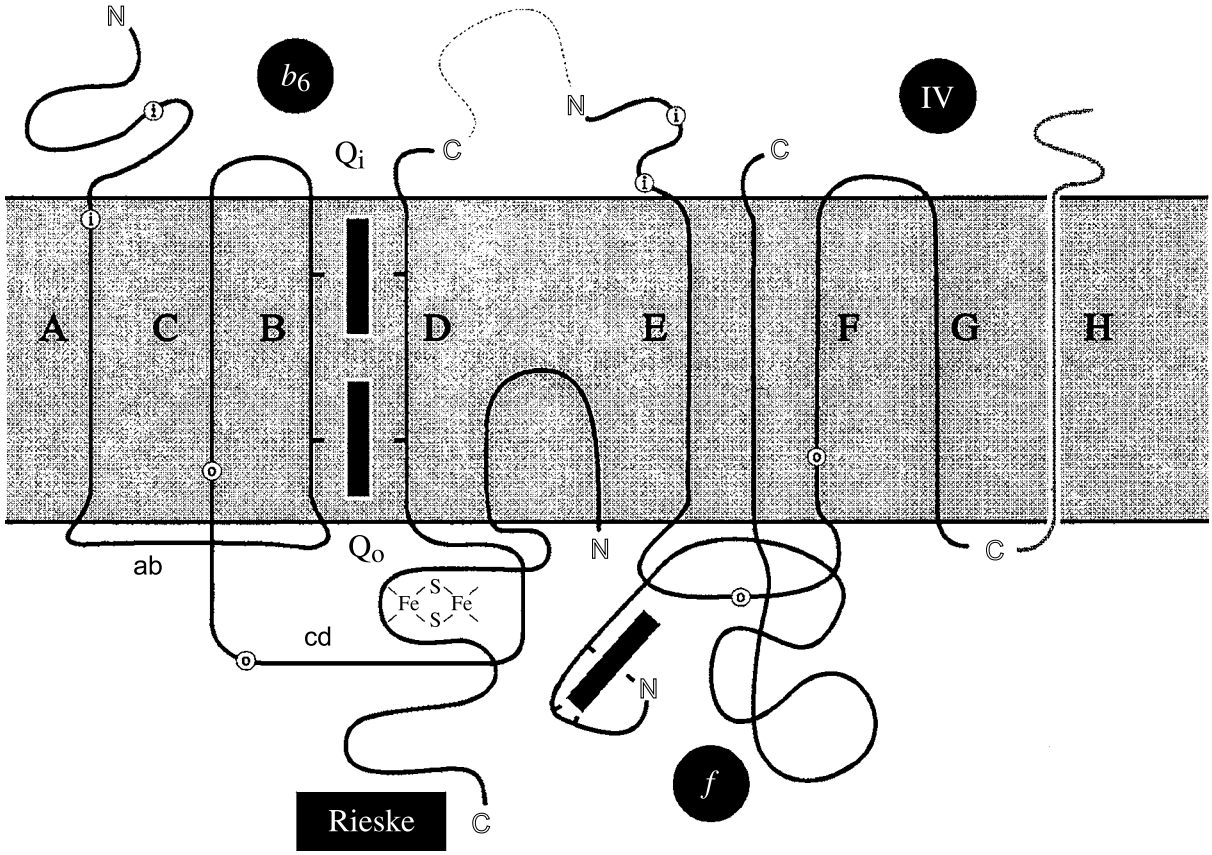

Figure 10. Folding model for the major polypeptides of the cytochrome $b_6 f$ complex in the chloroplast membrane. Figure taken from Hauska et al. (1996). The top of the figure represents the stromal surface of the membrane; N- and C-termini are indicated by N and C; Q_i and Q_o stand for quinone reduction and quinol oxidation site, respectively; the eight putative transmembrane helices are lettered A–H, the two surface helices are indicated by 'ab' and 'cd'; the connection between b_6 and subunit IV, as well as the missing C-terminal part with helix H, which form a cytochrome *b* complement of *bc*1 complexes are also indicated. The encircled 'o' and 'i' show positions of inhibitor sensitivity for the quinol oxidation site Q_o and the quinone reduction site Q_i, respectively.

hold the two hemes in transmembrane orientation, in connection with the two quinone interaction sites on opposite surfaces of the membrane. What an intriguing agreement with the proposal of the Q cycle! The model for Cyt *b* folding was later revised, mainly on the basis of point mutations affecting the differential effects of inhibitors for the two quinone binding sites on the opposite membrane surfaces (Colson 1993; Gennis et al. 1993). The fourth helix was taken out from the membrane and was placed on the surface, which holds the quinol oxidizing site. The resulting fold is shown in Figure 10, which is taken from our review (Hauska et al. 1996) and also depicts Cyt *f* and the Rieske FeS protein. The model is largely in accordance with the crystal structures (see Berry et al. 2000 and 'Note added in proof').

Similarly, Nadia Gabellini, who had moved from our lab to Walter Sebald at the Gesellschaft für Biotechnologische Forschung in Braunschweig, characterized (Gabellini et al. 1985) and subsequently sequenced (Gabellini and Sebald 1986) the Cyt *fbc* operon, in parallel with Fevzi Daldal's group (Davidson and Daldal 1987; Gennis et al. 1993). From Braunschweig, Nadia went to Dieter Oesterhelt, at Martinsried (Munich) to continue studying the bacterial Cyt *bc*1 complex. In renewed collaboration with B. Andrea Melandri at Bologna, she accomplished something that we had in mind before – the reconstitution of photophosphorylation solely from natural components (Gabellini et al. 1989). From Munich, after almost a decade in Germany, she returned to Italy, where she is now lecturing and working in neurobiology at the University of Padova.

Several other bacterial Cyt *bc* complexes have been characterized meanwhile, and they are either more like the mitochondrial Cyt *bc*1 type, with a continu-

ous Cyt *b* polypeptide of some 40 kDa, or like the plastidal Cyt b_6f type, with the Cyt *b* complement split into Cyt b_6 and subunit IV. Cyt *bc*1 complexes occur in Gram-negative bacteria (proteobacteria), and are characteristically encoded by an *fbc* operon; the b_6f complexes are found in Gram-positive species (firmicutes), including the cyanobacteria. They usually contain two transcription units, one coding for the Rieske FeS protein plus Cyt *f*, and one for Cyt b_6 plus subunit IV (see Brandt and Trumpower 1993; Hauska et al. 1996; Berry et al. 2000). A particularly interesting case from a phylogenetic view point occurs in *Chlorobium limicola*, a green sulfur bacterium, since its Cyt *b* complement combines features from the Cyt *bc*1 and Cyt b_6f types (Schütz et al. 1994). The gene for Cyt *b* is continuous, but other characteristics resemble Cyt b_6 and subunit IV. Eduard Hurt had already begun studying this Cyt *bc* complex (Hurt and Hauska 1984), which was continued by Michael Schütz in our lab, in yet another fruitful collaboration with Nathan Nelson, who thrived at the Roche Institute of Molecular Biology, Nutley, New Jersey, at that time. Nowadays, due to genomics, many more of the sequences for Cyt *bc* complexes, including those from Archaea, are available for phylogenetic and other considerations (Berry et al. 2000; Schütz et al. 2000; Schmidt and Shaw 2001). The 'respiration early' hypothesis is one of the key conclusions, maintain ing that anaerobic respiration preceded chlorophyll-based photosynthesis (Castresena and Saraste 1995; Castresana and Moreira 1999).

Concluding remarks

Genomics and the availability of structures at atomic resolution mean that our notions about quinol oxidizing Cyt *bc* complexes, like biological research in general, face a tremendous expansion of information which has become difficult to oversee. An admirable attempt to cover the state of the art is provided by Berry et al. (2000). Another one, concentrating on the Cyt b_6f complex with a historical perspective, was presented by Ke (2001).

We ourselves are still concerned with the Cyt b_6f complex. On the one hand, we are investigating the occurrence of iso-genes for the Rieske FeS protein in nitrogen-fixing cyanobacteria (see Schneider et al. 2002), and their possible role in redox regulation for heterocyst formation (Arnold 2001), and on the other hand, we are studying the involvement of the Rieske FeS protein in programmed cell death of the mother cells in the green alga *Volvox* (M. Lichtinger, H. Küpper, G. Hauska and R. Schmitt, in preparation).

The encounter with the band on SDG, be it pink or brown, has certainly turned into a rewarding experience for us. What if J.S.C. Wessels had applied SDS-PAGE in 1966 already? Well, who knows?

Appendix A shows a list of persons involved/ discussed in this paper.

Note added in proof

After this review had been submitted for publication, the crystal structures of the cyanobacterial and the algal Cyt b_6f complex were made accessible to the author by courtesy of Bill Cramer and Jean-Luc Popot, respectively (Kurisu et al. 2003; Stroebel et al. 2003). In essence, they resemble the structure of the Cyt *bc*1 complex, as discussed above (see Berry et al. 2000; Ke 2001), but also brought a surprise: the Cyt b_6f complexes contain an additional heme! It is of the high-spin form, without axial ligands – which is why it has been overlooked in the redox spectra so far – and it is covalently bound by one vinyl group to Cys 35 in Cyt b_6, at the Q_i site, between a bound plastoquinone molecule and the high-potential heme *b*. This finding explains why Cyt b_6, in contrast to Cyt *b* in Cyt *bc*1 complexes, remains active in heme staining after denaturation (Hurt and Hauska 1981; Kuras et al. 1997), and why it contains extra mass (Whitelegge et al. 2002). It may also explain why electron transport through the Cyt b_6f complex is insensitive to antimycin. In addition, it is consistent with the pathway for heme insertion into Cyt b_6 (Kuras et al. 1997). The extra heme may be involved in cyclic electron flow around PS I. Furthermore, the cyanobacterial as well as the algal complex both contain the four small hydrophobic subunits, PetG, PetL, PetM and PetN, which form a transmembrane helix bundle, distal to the symmetry center of the dimeric structure.

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I am grateful to the collaborators who have contributed invaluably to this rewarding experience over the years. In addition to those I have made stand out in the foregoing text, these were my PhD students in Regensburg, Wolfgang Nitschke, Michael Schütz and Matthias Arnold, and my associates Wolfgang Lockau, Astrid Riedel and Iris Maldener. Furthermore I would like to recall my teachers, the late Otto

Hoffmann-Ostenhof who introduced me to biochemistry at the University of Vienna and supervised my PhD in 1965–1967, and the late Efraim Racker. I believe that it was at the end of 1967 when he returned to Vienna for the first time after his emigration to lecture at the place where he had studied. He accepted me as a postdoc for 1968–1970 at Cornell, and I thank him for the sporting way he taught me mitochondriology, and for funding my collaboration with Dick McCarty on photophosphorylation. Dick, my friend and mentor, had a profound influence, introducing me to Mitchell's chemiosmotic theory, together with André Jagendorf, Rod Clayton and Peter Hinkle, all at Cornell at that time. Then it was Achim Trebst in Bochum who took over supervision, sharing an enjoyable period of photosynthetic experimentation from 1971 to 1976, which additionally shaped me. The colleagues at this time I would like to mention were Richard Berzborn, Herrmann Bothe, Erich Elstner, Susanne Reimer, Walter Oettmeier, Rolf Thauer and Günter Wildner. Finally, the contact with Gebhardt von Jagow and his group with Uli Brandt, Thomas Link, Herrmann Schägger, first in Munich, later in Frankfurt, was very influential. I am extremely thankful to Nathan Nelson and Francis-André Wollman for reading this story and for giving me their approval. Finally, the courtesy of W.A. Cramer and Jean-Luc Popot for providing access to their beautiful data before publication is acknowledged with pleasure. This paper was edited by Govindjee, who also took the photos in Figure 9.

Appendix A

Matthias Arnold, Richard Berzborn, Uli Brandt, Bill Cramer, Tony Crofts, Fevzy Daldal, Bruce Diner, Erich Elstner, Alma Gal, Nadia Gabellini, Reinhold Herrmann, Geoffrey Hind, Eduard Hurt, Anne and Pierre Joliot, Bacon Ke, David Krogman, Werner Kühlbrandt, Thomas Link, Wolfgang Lockau, Dick Malkin, Katsumi Matsuura, Assunta and B. Andrea Melandri, Germaine Mosser, Nathan Nelson, Wolfgang Nitschke, Walter Oettmeier, Itzak Ohad, Jean-Luc Popot, Roger Prince, Peter Rich, Astrid Riedel, Raj Sane, Herrmann Schägger, Michael Schütz, Walter Sebald, Yosepha Shahak, Achim Trebst, Gebhardt von Jagow, J.S.C. Wessels, Peter Westhoff, Günter Wildner, Francis-André Wollman and Paul Wood.

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