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Minireview

The unfinished story of cytochrome *f*

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Abstract

Cytochrome *f* is a unique, integral membrane protein. The background to its discovery by Robert Hill (1899–1991) and Ronald Scarisbrick over 60 years ago and the influence of David Keilin (1887–1963) and Frederick Gowland Hopkins (1861–1947) are discussed. The development of methods for isolating cytochrome *f* is outlined, emphasizing the remarkable achievement of Hill and Scarisbrick at a time when few if any membrane proteins had been isolated, and the importance of the discovery of a natural proteolysis in *Brassica* spp., stimulated by organic solvents, by Eijiro Yakushiji and coworkers and by Masa-aki Takahashi and Kozi Asada in 1975. The significance of different types of instrumentation in the study of cytochrome *f* is discussed, drawing attention to the importance of the microspectroscope ocular for its discovery, to types of spectrophotometer developed especially by Britton Chance for spectrophotometric measurements on turbid suspensions of cells and plastids, and to the history of stopped-flow spectrophotometry. The stopped-flow instrument originated in the bucket-scale flow methods of Hartridge and Roughton (1923), and was later developed on the microscale by Chance. Finally, the problems that remain for understanding the behavior of cytochrome *f* in the thylakoid lumen are contrasted with the significance of *in vitro* studies that provide a paradigm for transient protein–protein interactions in the wider field of biology as a whole.

Discovery

Cytochrome *f* is a unique protein. In the Scop database of protein structures (http://scop.mrc-lmb.cam.ac.uk/scop/; Murzin et al. 1995) it forms both a family and a superfamily as the sole member. Its central position in the photosynthetic electron transport system ensures its importance in the economy of life. Its discovery and isolation were remarkable achievements by the standards of the 1930s, and the former would not be entirely trivial even today. It would be interesting to see how modern students of biochemistry would respond to an examination question on how to observe cytochrome *f* in leaves. This article is not a full history or review of cytochrome *f*, but is intended to trace the background to some key features in the development of our knowledge of it, and to indicate some of the lines of investigation that keep it a lively subject of research.

The word 'discovery' is used here in the sense of the first recognition of a distinctive component. Cytochrome *f* is inseparably linked to the name of Robert Hill (who became known almost universally as Robin), but the first person to observe its spectrum in leaf material was probably Eijiro Yakushiji, who in 1935 reported the presence of an absorption band at 550 nm in acetone powders of leaves of *Brassica* and spinach (Yakushiji 1935). He thought, however, that he was observing the spectrum of cytochrome *c*. The α-band of cytochrome *c* at 550 nm should be easy to distinguish from that of cytochrome *f* at 554.5 nm. Nevertheless, the true position of the band

Yakushiji observed was likely to have been 554.5 for two reasons. Firstly, in the same paper he describes the presence of 'cytochrome *c*' in extracts of various red, green and brown algae, giving absorption bands described in the text as at 550 and 520 nm, even though the spectrum in the figure clearly has the α-band at a significantly greater wavelength (this is likely to have been the soluble cytochrome now described as c_6, but see below under 'Nomenclature'). Secondly, Hill and Scarisbrick (1951) reported that leaf acetone powders show the α-band of cytochrome *f* in its normal position, at 555 nm. The importance of Yakushiji's early work probably lies in the stimulation of explicit work on cytochrome *f* in Japan at a much later date.

As an undergraduate Robin Hill studied chemistry and published a paper on the coordination state of the pink and blue complexes of cobalt (Hill and Howell 1924). He already had a deep love of plants, and, when he joined the fledgling Biochemistry Department at Cambridge in 1922, he wanted to study plant pigments, inspired by his knowledge of natural dyes and the book on Anthocyanins by Muriel Wheldale (later to become Mrs Huia Onslow, see Weatherall and Kamminga 1992; Wheldale 1916), who was a member of the Department. The professor, Gowland Hopkins, considered this a bad idea. Perhaps he thought it merely premature to work on plants, but, if so, Robin did not seem to agree. Instead, Hopkins 'directed' Robin to work on hemoglobin and its derivatives. Despite the fact that hemoglobin was being actively studied in the Physiology Department under Joseph Barcroft, especially by Anson and Mirsky (1925), Hopkins' decision turned out to have been a wise one, later fully appreciated by Robin. He has described the situation in the following words:

> When we all moved into the building in 1924 there were close links with other departments on the Downing Site. In particular there were the Department of Physiology with Joseph Barcroft for the respiratory function of the blood and the Molteno Institute where David Keilin was analysing the respiratory function of cytochrome. To these studies of extra-cellular respiration and intracellular respiration there came to be added, by Hopkins, biological oxidations in terms of molecules. The School of Biochemistry appeared on the site at that time like the brightest star Sirius which forms the triangle with its two partners Procyon and Betelgeuse and it directed the searchers after the knowledge as did the stars for the ancient mariners (Hill 1974).

Robin Hill met Keilin at the opening ceremony for the new building, at which Keilin seems to have been the only person to appreciate the display of brightly colored metalloporphyrins put on by Robin (see Figures 1–3 for photographs of Hill and Keilin). The discovery of cytochrome was a classic example of serendipity, and came as the result of Keilin's study of the fate of hemoglobin during the life cycle of the horse bot fly, *Gasterophilus*. In his first publication on cytochrome, Keilin (1925) fully acknowledged the earlier discovery of 'myohaematin' and 'histohaematin' by MacMunn (1886). MacMunn's work had been forgotten, largely as the result of its dismissal by Felix Hoppe-Seyler as observations on spectra derived from hemoglobin (Hoppe-Seyler 1890). Keilin felt justified in renaming MacMunn's pigments cytochrome, signifying cellular pigment, mainly because it had a wider distribution and importance than 'was ever anticipated even by MacMunn.' Somewhat ironically,

Figure 1. From left to right: Bill Rathmell, Marijana Plesničar, Robin Hill, Dana Sofrová and Derek Bendall on the steps of the building in which cytochrome *f* was discovered. Photograph taken in summer 1970.

Figure 2. From left to right: Hiroshi Tamiya (standing), Robin Hill, David Keilin and an unidentified visitor. Photograph taken at Keilin's home in 1962.

Figure 3. David Keilin in the old Quick Laboratory in Cambridge, 1916. The microspectroscope ocular, which Keilin later used, was designed to replace the normal eyepiece of the microscope. The Quick Laboratory preceded the Molteno Institute which was opened in 1921.

Keilin regarded the term cytochrome as temporary, 'pending the time when its composition shall have been properly determined.' We now have a wealth of information of all kinds, but seem to be little nearer a really satisfactory nomenclature.

Keilin's initial survey of the distribution of cytochrome included a variety of non-green plant tissues, but its direct observation in green leaves was not possible. Robin Hill had collaborated with Keilin on the isolation of cytochrome *c* from yeast and muscle and they obtained the first evidence that the porphyrin of cytochrome *c* is related to both protoporphyrin and hematoporphyrin (Hill and Keilin 1930). He was thus well placed to take on the task of studying cytochrome in plant tissues when he felt sufficiently well established to throw off the restraints initially imposed by Hopkins. The initial experiments were done with an Indian student, Kamala Bhagvat, again with non-green tissues. They were able to obtain the first particulate preparations from plants with succinate oxidase and cytochrome oxidase activities (Hill and Bhagvat 1939). Hill and Scarisbrick (1951) then tackled the question of cytochrome in leaves. When they examined acetone powders of leaves in the presence of a reducing agent they observed an absorption band at 555 nm, rather than at 550 nm as recorded by Yakushiji (1935). They were able to extract cytochrome *c* from leaves, with a 'normal' absorption peak at 550 nm, but showed that it was present at a much lower concentration than the component at 555 nm. The new component was present in acetone powders from leaves of all plant species examined, and also of isolated chloroplasts, but could not be detected in non-green tissues. It was therefore named cytochrome *f*, after Latin *frons*, for leaf. They also showed that the porphyrin of cytochrome *f* is similar to that previously found with cytochrome *c*. The name cytochrome *f* has stuck, because of its distinctive properties, and this has been fully justified by its X-ray crystal structure. The structure solved by Janet Smith, William (Bill) Cramer and colleagues at Purdue University (Martinez et al. 1994) showed that, unlike all other *c*-type cytochromes, the large, luminal fragment is a β-sheet protein with two domains, and the axial heme ligand is the N-terminus, which is otherwise unknown.

Hill and Scarisbrick (1951) attempted to observe cytochrome in intact leaves by examining leaves of yellow varieties, and the non-green portions of some variegated leaves. What they observed was an absorption band at 560–565 nm, presumably a cytochrome *b* component, but could not observe cytochrome *f*. The suspicion was that cytochrome *f* was photooxidized in the strong light necessary to make observations on leaves. The notion that in an illuminated leaf cytochrome *b* became photoreduced and cytochrome *f* photooxidized was a factor lying behind the later formulation of the 'Z'-scheme of photosynthetic electron transport (Hill and Bendall 1960; see also Walker 2002). Davenport (1952) was able to show the presence of cytochrome *f* in plastids from etiolated barley leaves without treatment with organic solvent.

Isolation

Cytochrome *f* is an integral membrane protein. Its solubilization was first reported by Hill (1943). At that time few if any other membrane proteins had been extracted into solution and no general methods were available. Purification of the cytochrome was first developed by Scarisbrick and then by Harold (Fearless) Davenport working with Hill (Hill and Scarisbrick 1951; Davenport and Hill 1952). The protein could be extracted from fresh leaves of elder (*Sambucus nigra*) with 50% ethanol in the presence of ammonia

and then partially purified by acetone precipitation and ammonium sulfate fractionation. Further purification was inhibited by the leaf polyphenols and a better source was found to be the curly leaved variety of parsley. The leaf was passed through an electric meat grinder together with the ethanol, entrapped in the crinkles of the leaf, so that the final concentration of 50% ethanol was achieved as rapidly as possible in the leaf juice. After acetone precipitation and ammonium sulfate fractionation the protein was fractionated again with calcium phosphate gel. Further purification, however, was difficult because of aggregation, as we now know.

Preparations from other species of leaf were seldom successful. Giorgio Forti and colleagues were able to dispense with the meat grinder and use a Waring Blendor instead by adding 1% Triton X-100 to the ethanol/ammonia mixture (Forti et al. 1965). John Gray eventually found that cytochrome *f* could be extracted from the leaves of most plants with butanone (Gray and Phillips 1982). It is curious that ethanol/ammonia extraction failed with fresh chloroplast preparations, which would have avoided the worst of the problems introduced by leaf polyphenols and organic acids. However, Davenport found that the difficulty was overcome by addition of ethyl acetate to the ethanol/ammonia mixture (Bendall et al. 1971). Singh and Wasserman (1971) succeeded with frozen spinach chloroplast fractions using cold butanol and Triton X-100. Nelson and Racker (1972) were also able to extract cytochrome *f* from spinach chloroplasts with digitonin followed by fractionation with ethanol and acetone. A monodisperse octameric preparation was obtained by purification in the presence of sodium cholate.

None of these procedures was entirely satisfactory, especially as they led to an oligomeric preparation unrelated to its state *in vivo*, until in 1975 Yakushiji and colleagues (Matsuzaki et al. 1975), and also Takahashi and Asada (1975), discovered that solubilization from leaves of cruciferous species led to a monomeric, truncated protein. This method was thoroughly exploited by Gray (1978), and it soon became apparent that in crucifers an endogenous protease cleaves the molecule at the start of the membrane anchor, liberating intact the thylakoid lumen domain. This large, soluble fragment contains the heme group and is fully active in electron transfer. It is interesting to note that although no other group of higher plants has been found to give rise to this spontaneous proteolysis, it has been observed when extracting cytochrome *f* from thylakoid membranes of the cyanobacterium, *Phormidium laminosum* (Wagner 1997).

In recent years isolation of cytochrome *f* from the plant or microbial culture has largely given way to heterologous gene expression in the periplasm of *Escherichia coli*. Gray (1980) obtained the first unambiguous evidence that cytochrome *f* is chloroplast encoded from study of interspecific hybrids of *Nicotiana tabacum* and *N. glutinosa*, and this led to determination of the nucleotide sequence for pea and wheat (Willey et al. 1984a, b). The spinach sequence was determined at about the same time by Reinhold Herrmann and colleagues (Alt et al. 1983). But isolation from the plant may still have a role, because of the low yields of the recombinant protein. The best yield obtained so far is 8 mg/l culture for the protein from *P. laminosum* (Schlarb et al. 1999; B.G. Schlarb-Ridley, personal communication), and higher-plant genes usually give much lower yields (Gong et al. 2000).

Nomenclature

Cytochrome *f* means cellular pigment from a leaf. Mention of leaf pigments naturally brings to mind chlorophylls and carotenoids, and one might not guess that the term cytochrome *f* refers to a tiny amount of pink protein that has been fished out of this sea of chlorophyll. Nevertheless, the name is good if one treats it as merely a convenient label, which draws attention to the unique character of the protein. Cytochrome *f* soon became known to be a *c*-type cytochrome (Hill and Keilin 1930), but it sits uncomfortably in any attempt at classification with other cytochromes containing heme *c*. In 1961 the Report of the Commission on Enzymes of the International Union of Biochemistry tried to bring it into line by giving it the name cytochrome c_6. Fortunately, the new label did not stick – it was seldom, if ever, used for the true cytochrome *f* – but it was too good a label to throw away and has now become firmly attached to another cytochrome component. Many algae contain a cytochrome which is superficially similar to cytochrome *f* in that it has a midpoint redox potential about 100 mV higher than that of mitochondrial cytochrome *c* and a red shifted α-band which is markedly asymmetric. However, it is invariably a soluble protein which, as Yakushiji (1935) showed, can easily be extracted from acetone powders of cells, contrary to what Hill and Scarisbrick (1951) found with cytochrome *f*. As the only well-known *c*-type photosynthetic

cytochrome of algae, it was thought to play the same role as the membrane-bound component of leaves and was often referred to as the algal cytochrome *f*. By the early 1970s the presence of a membrane-bound component had been reported in several species of green alga [*Chlamydomonas* (Gorman and Levine 1966a), *Chlorella* (Grimme and Boardman 1975) and *Scenedesmus* (Powls et al. 1969) and a cyanobacterium, *P. luridum* (Biggins 1967)]. The confusing use of the name cytochrome *f* for the soluble algal component was probably prolonged artificially by the discovery of a *Chlamydomonas* mutant (*ac*-206) by Gorman and Levine (1966b) that lacked both the soluble and insoluble components, suggesting that they were different forms of the same protein. A similar type of mutant was found for *Scenedesmus* by Powls et al. (1969).

In 1977, Wood reported that a membrane-bound *c*-type cytochrome could be extracted into aqueous solution from photosynthetic membranes of *Chlamydomonas*, *Euglena* and *Anacystis nidulans* (now named *Synechococcus* 6301) by following a recipe designed for higher-plant cytochrome *f* (Wood 1977). He argued conclusively that the membrane-bound cytochrome *f* was a component distinct from the soluble cytochrome hitherto called *f*, and probably invariably present. The soluble and membrane-bound components had markedly different molecular masses as well as distinct physical properties despite some similarities. Wood therefore recommended that the soluble component should be named cytochrome *c*-552 or *c*-553 according to the position of its α-band, using a system originally proposed by Scarisbrick (1947). This practice, clumsy as it is, was adhered to for some years until in the late 1980s and the 1990s the soluble component became known, apparently inappropriately, as cytochrome c_6. The change was possibly the result of a confused recommendation of the Nomenclature Committee of the International Union of Biochemistry in 1984 that the name cytochrome c_6 should be applied to the soluble algal component, 'more usually called "cytochrome *f*:lp".' The membrane-bound cytochrome *f* received no mention. It might have been better if the silence had been maintained in the 1992 report (still current), in which the only mention of cytochrome *f* is under cytochrome c_1, 'the related protein present in the *bc* complex of green plants is also called cytochrome *f*.' The use of the term cytochrome c_6 for the soluble component is confirmed, however, and is now firmly established in current practice. In some algae and cyanobacteria cytochrome c_6 takes over the role of plastocyanin under copper deficient conditions, and in a few organisms it is the only mediator of electron transfer between cytochrome *f* and Photosystem I. This functional equivalence is remarkable considering the unrelated structures of the two proteins.

The recent discovery of cytochrome c_6 in higher plants has given a further twist to this story of names (Gupta et al. 2002; Wastl et al. 2002). What is known about this protein strongly suggests homology with algal cytochrome c_6 and yet there are some crucial differences. The higher-plant protein has been shown, despite a claim to the contrary (Gupta et al. 2002), to be incapable of performing the same function as algal cytochrome c_6 or plant plastocyanin (Molina-Heredia et al. 2003; see Katoh 2003 for a discussion of early research on the role of plastocyanin). It has a substantially lower redox potential, and there is an intriguing insertion of 12 amino acid residues, including two cysteines, in the sequence (Wastl et al. 2002). The last point suggests that its true function may lie in the regulation of electron transport. Is the name c_6 still appropriate for such a protein or should it be clearly distinguished from the algal component? Should classification be based primarily on function or on structure?

Instrumentation

Progress in the study of cytochromes has naturally been dependent upon the available instrumentation, much of which has not always been readily obtainable commercially, especially for study of photosynthesis. The rediscovery of cytochrome by Keilin, however, depended on a simple spectroscopic device which was well known at the time, the microspectroscope ocular. The same is true of the discovery of cytochrome *f* by Hill and Scarisbrick. Keilin has described how his studies on hemoglobin of *Gasterophilus* would probably have followed a different, much less fruitful path, if he had had access to a good spectrophotometer in 1924 (Keilin 1966). The usefulness of the microspectroscope depends on the fact that it has a low-dispersion prism so that the absorption bands of cytochrome appear as sharp lines, and that spectra can readily be observed with turbid suspensions and even intact tissues. With a little experience one can obtain an immediate impression of the whole visible spectrum, which we still find occasionally useful in my laboratory to check the expression of cytochromes in *E. coli*. The eye tends to differentiate the absorption bands, exaggerating changes of intensity. The beauty

of the consequent appearance of the cytochrome *f* spectrum, in which the α-band appears as a pair of contiguous lines, one strong, the other weaker, is not quite matched by the spectrum traced out by a spectrophotometer. The latter shows a mere shoulder, which the unpractised eye might miss, on the short wavelength side of the main peak. It is of interest to note that this shoulder is a common feature of *c*-type cytochromes. In mitochondrial cytochrome *c* the subsidiary band is only distinguishable after sharpening at very low temperatures, whereas in cytochrome *f* from the cyanobacterium *P. laminosum* the shoulder is more clearly separated from the main peak because the latter has shifted 2 nm to the red compared with the plant or algal protein. This shift has been attributed to the presence of a tryptophan residue which is in contact with the heme ring (Ponamarev et al. 2000). Observations with the microspectroscope are as much craft as science, and spectra were normally recorded by hand drawings. Robin Hill built a spectrograph which allowed the spectrum to be photographed. An example is reproduced in the classic paper on cytochrome *f* by Davenport and Hill (1952), but the instrument received little further use as visual spectroscopy gave way to the spectrophotometers developed by Britton Chance.

The weakness of the microspectroscope lay in the difficulty, although not impossibility, of making quantitative measurements. It would be a little optimistic to claim that one could assign the positions of absorption bands to the nearest nm. Keilin routinely used the Hartridge reversion spectroscope, which improved the accuracy by a factor of 10, but few others followed his example. Britton Chance, through the instruments and experimental techniques that he introduced, has had an unrivalled influence on the study of cytochromes. In 1938 he set out to study the properties and kinetics of enzyme–substrate intermediates, and soon came under the influence of F.J.W. Roughton who, with Hamilton Hartridge, had developed the flow method for measuring the rates of rapid reactions. After completing a PhD in Philadelphia in 1940, Chance obtained a second PhD in Cambridge in 1942 under the supervision of Roughton. Doubtless his interest in cytochromes was stimulated by meeting Keilin during his stay in Cambridge.

Chance's spectrophotometers were original and sophisticated and gave an unsurpassed signal-noise ratio (Chance 1957). In addition they included a simple principle that made it possible to obtain spectra with turbid suspensions of organelles or cells, although seldom with whole tissues. This was to place a photomultiplier tube with a large end-window close to the cuvette so as to collect the maximum proportion of the incident light scattered by the sample. By 1954 two types of instrument had been built in the Johnson Research Foundation at the University of Pennsylvania in Philadelphia. The split-beam instrument, designed by C.C. Yang, had a rapidly oscillating mirror which directed the measuring beam alternately between sample and reference cuvettes (Yang and Legallais 1954). This gave sensitive measurements of difference spectra, even with samples frozen in liquid nitrogen, which Keilin and Edward Hartree had shown to be such a useful technique for improving spectral resolution (Keilin and Hartree 1949). The manual wavelength drive retained a degree of physical satisfaction for the operator. With this instrument it was possible to determine α-band spectra of cytochromes in untreated chloroplasts. Most commercial instruments ignore the principle on which this instrument was based. Cary Instruments, however, early produced a scattered light attachment for their model 14 spectrophotometer, which enabled Keith Boardman and Jan Anderson (Boardman and Anderson 1967) to undertake the first systematic study of cytochromes in untreated chloroplast preparations with a recording instrument. This study revealed the presence of a new component, cytochrome *b*-559, associated with Photosystem II, whereas cytochrome *f* was more closely associated with Photosystem I. Protocols were later devised for the quantitative analysis of the individual components (Bendall et al. 1971). Cytochrome *f* was present at about 1 nmol/mg chlorophyll, which indicated that it was present at a concentration equivalent to that of the photosystems.

The second instrument, designed by Chance himself, was a double-beam (dual wavelength) spectrophotometer in which measuring beams from two different monochromators were passed with rapid alternation through a single cuvette (Chance 1951). This was a kinetic instrument which made it possible to follow changes in absorbance with time against a reference wavelength, usually chosen immediately to one side of the absorbance peak, so as to minimize changes from light scattering effects. This became a popular instrument, marketed by Aminco, and has been much used for the study of photosynthetic cytochromes, especially by Bill Cramer and colleagues (Cramer and Whitmarsh 1977).

Chance's instruments were not the only ones to be developed around this time, although they were the most influential. Louis N.M. Duysens independently

designed an instrument for studying light-induced absorbance changes in bacterial and algal cells; the measuring beam from a single monochromator was split so as to provide a compensating beam which bypassed the sample cuvette (Duysens 1957). With this instrument he was able to observe photooxidation of cytochrome c_2 in *Rhodospirillum rubrum*, the first example of light-induced oxidation of a cytochrome in a photosynthetic organism (Duysens 1954b). A little later Duysens and Jan Amesz provided clear evidence for the two-light reaction scheme of Hill and Bendall (Hill and Bendall 1960) in terms of the light-induced redox changes of cytochrome *f* (Duysens et al. 1961; Duysens and Amesz 1962; Amesz et al. 1972). Duysens had already published observations on cytochrome *f* photooxidation in *Chlorella* in 1954 (Duysens 1954b) and in red algae in 1955 (Duysens 1955).

Henrik Lundegårdh, however, had also been busy in his private retirement laboratory at Penningby, near Stockholm, building spectrophotometers for observation of cytochromes in plant cells and tissues (see Larkum 2003). Early in 1954 he published observations on cytochrome *f* photooxidation in *Chlorella* (Lundegårdh 1954). There is a slight doubt now about whether he was observing the true cytochrome *f* or the soluble cytochrome c_6, and this question applies also to the early experiments of Duysens, especially with red algae, which have no plastocyanin. It would seem churlish, nevertheless, to doubt the real significance of either of these pioneering experiments.

Two further types of instrument have played essential roles in developing knowledge of the kinetics of the photosystems and of secondary electron-transfer processes, including those of the cytochromes, associated with them. Britton Chance played a crucial role in both developments. Electronic flashes had been used in the study of photosynthesis since the classic experiments of Emerson and Arnold (1932), which led to the concept of the photosynthetic unit. Gas-discharge flash lamps could be arranged to give extremely high energies in a single flash [10,000 J in the first experiments of Ronald Norrish and George Porter (Norrish and Porter 1949)], but for photosynthetic reaction centers, with their built-in antenna systems, the priority was for extremely brief, clean flashes. These were eventually achieved with lasers. The first laser experiments were carried out by Don DeVault and Chance in the 1960s (DeVault 1964; DeVault and Chance 1966), who were able to show that the rate of cytochrome photooxidation in *Chromatium* is independent of temperature below 100 K, indicating a role for quantum mechanical tunnelling in the mechanism. Shortly afterwards Horst Witt and colleagues introduced laser flashes for the study of the kinetics of responses in chloroplasts and green algae (Witt 1967). Witt had already made extensive use of flash methods, and had introduced the valuable method of averaging repetitive flashes, which improves the signal/noise ratio by a factor of $\sqrt{n}$, where n is the number of flashes.

Chance's other major contribution was the development of the stopped-flow method for analyzing the kinetics of 'dark' reactions, which, of course, include the oxidation of cytochrome *f* by plastocyanin or cytochrome c_6. The stopped-flow method, now widely used, is a direct descendant of the continuous flow method with rapid mixing invented by Hartridge and Roughton in 1923 for measuring the very rapid ligand binding reactions of hemoglobin. It is interesting to note that Roughton had been stimulated to work on hemoglobin in Cambridge by Gowland Hopkins, as had Robin Hill at about the same time. Their interests and backgrounds were different, however, Roughton being essentially a physiologist whereas Hill had been trained as a chemist. At the very start of his career Roughton entered into a collaboration with Hartridge, who was also a physiologist with a passion for instrument making. Hartridge designed a device with which solutions of dithionite and oxyhemoglobin could be mixed in less than 1 ms. By arranging for a steady flow along an observation tube they could follow the rate of dissociation of oxygen from hemoglobin by making observations at various points along the tube with Hartridge's reversion spectroscope (Hartridge and Roughton 1923). By this technique Hartridge and Roughton improved the time resolution for measuring chemical reactions by a factor of 50,000, but they were strongly dependent on the methods available for measurement of the progress of the reaction. In their early experiments the volume of reagent required for a single experiment was 3–4 l. The only biological material which could be studied was hemoglobin, fortunately available by the bucket. In an attempt to conserve reagents Hartridge and Roughton had tried out the stopped-flow method in which the flow was suddenly stopped and the course of the reaction followed in the stationary fluid. At that time, however, there were no photocells and no electronic equipment that would allow rapid changes to be followed (Roughton 1953).

By 1938 the situation had changed. Britton Chance's aim was to study intermediates in enzyme

catalyzed reactions, concentrating on catalase and peroxidase for which spectroscopically detectable intermediates had been defined by Keilin and Hartree. Encouraged by Roughton and G.A. Millikan, Chance designed the first effective stopped-flow spectrophotometer, which reduced the volume requirements of reagents by four or five orders of magnitude (Chance 1953). Following improvements by Quentin Gibson (Gibson and Milnes 1964; Gibson 1969) the instrument was taken up commercially, and has become an essential tool for the study of cytochrome *f*.

Life in the lumen

In its natural environment cytochrome *f* is a component of the cytochrome *bf* complex embedded in the thylakoid membrane. Its large heme-containing domain is exposed to the aqueous phase of the lumen. Most of what we know about the properties and structure of cytochrome *f* comes from studies of the purified protein *in vitro* (see below), and yet its reactivity *in vivo* must depend on the little known characteristics of the thylakoid lumen. A structureless solution may, indeed, be rather a poor model for the lumen. Electron micrographs depict it as a thin sheet of liquid, the width of which is roughly the same as the dimensions of a plastocyanin molecule. Surface effects must therefore be important in the diffusion of plastocyanin between cytochrome *f* and Photosystem I, taking place in two rather than three dimensions. If one imagines oneself as a tiny Lilliputian creature peering out from the surface of a plastocyanin molecule and looking for a cytochrome *f* molecule to give you an electron, one would observe not a featureless landscape but a frightening array of craggy mountains stretching almost from one side to the other and seriously obstructing the path one wished to take. These mountains consist of the luminal domains of the relatively stationary protein complexes of the membrane. One type of mountain contains the gently sloping surface of cytochrome *f*, which would more than span the narrow luminal space if standing upright. Perhaps more alarming would be the soluble protein molecules, similar in size to one's own, moving about at a similar speed, and occupying much of the space between the mountain peaks. Recent estimates place the total soluble protein concentration of the lumen at more than $20\,\mathrm{mg\,ml^{-1}}$ (Kieselbach et al. 1998), which is perhaps not insignificant from the point of view of macromolecular crowding (Ellis 2001). Returning to normality from our Lilliputian state we can appreciate that crowding tends to enhance macromolecular interactions, although the higher viscosity will slow diffusion. The more important effect might in fact be the enhancement of diffusion by restriction to two dimensions. There is evidence that under most conditions plastocyanin remains bound to the membrane surface even when the luminal volume is greatly increased by artificial swelling of the thylakoid (Bendall and Wood 1978; Lockau 1979). We may conclude that we still have much to learn about the factors which influence the interactions between thylakoid proteins *in vivo*.

A paradigm for weak interactions

The first stopped-flow experiments with cytochrome *f* were carried out in 1974 by Paul Wood, using an oligomeric preparation from parsley and a Durrum–Gibson apparatus (Wood 1974). The rate constant he observed for reaction with parsley plastocyanin was only five times smaller than has subsequently been observed with monomeric cytochrome *f*. Experiments with the monomeric form were much less troublesome. An extensive study of the reactivity of *Brassica* cytochrome *f* with plastocyanin was reported by Teruhiro Takabe and colleagues in 1980 (Niwa et al. 1980). They showed for the first time the marked effect of ionic strength, indicative of the strong electrostatic attraction between the higher plant proteins in solution.

Stopped-flow work in my laboratory was stimulated not only by Paul Wood's original experiments, but also by the realization in the mid-1980s that it was time to turn from the study of electron transport pathways and their components (the 'curly arrow biochemistry' of photosynthesis) to the study of the mechanisms of electron transfer. My interest in the latter owed much to the landmark reviews by DeVault (1980) and Marcus and Sutin (1985), and the opportunity for a change of direction came with the establishment of the interdepartmental Centre for Molecular Recognition in Cambridge. My thoughts turned first to reaction centers and it was John Gray who suggested studying the interaction between cytochrome *f* (declared to be his favorite protein) and plastocyanin, as experimentally the easier, although conceptually the more difficult problem because of the diffusional element. David Last and John Gray had already cloned the gene for pea plastocyanin and

established a transgenic expression system in tobacco (Last and Gray 1989, 1990), which provided the basis for the first experiments with mutant forms of plastocyanin (He et al. 1991). These were followed by experiments with mutant spinach plastocyanin (Modi et al. 1992; Kannt et al. 1996), using the *E. coli* expression system of Margareta Nordling and Lennart Lundberg (Nordling et al. 1990) and later of mutant turnip cytochrome *f* (Gong et al. 2000a, b). The first question asked was whether Tyr-83 or His-87 was the most likely route for electrons to enter plastocyanin. Because of an eightfold decrease in the rate constant with the plastocyanin mutant Y83L it was concluded that Tyr-83 was likely to be part of the main route of electron transfer (He et al. 1991). These experiments were carried out with cytochrome *f* purified from oil-seed rape, however, which gave rates four to five times slower with wild-type plastocyanin than those found later with turnip cytochrome *f* obtained commercially or expressed heterologously in *E. coli*. With the latter sources of cytochrome *f* the large effect of mutation of Tyr-83 could not be repeated (Bendall et al. 1999), and Marcellus Ubbink's NMR model of the complex between cytochrome *f* and spinach plastocyanin (Ubbink et al. 1998), in which there was van der Waals contact between the heme ligand, Tyr-1, of cytochrome *f* and the copper ligand, His-87, of plastocyanin, demonstrated convincingly that this is the main pathway for electron transfer. NMR experiments with soluble proteins have provided the first clues of a second binding site on the 'backside' of cytochrome *f* (Crowley et al. 2002), which might be the site for entry of electrons from the Rieske Fe–S protein.

Mutagenesis of both spinach plastocyanin and turnip cytochrome *f* has confirmed the importance for the solution reaction of the acidic patches on plastocyanin and the basic ridge of cytochrome *f*, as had been predicted by chemical modification (Takenaka and Takabe 1984) and cross-linking (Morand et al. 1989). These charged residues are well conserved in plants and algae, and it is curious, therefore, that loss of the basic ridge of cytochrome *f* in *Chlamydomonas* leads to little change of the kinetics of cytochrome *f in vivo* (Soriano et al. 1998). Although this lack of change may result from rate limitation by release of plastocyanin from Photosystem I, the conservation of the charge pattern remains unexplained. The constant charge pattern is not present, however, in cyanobacteria (Wagner et al. 1996; Schlarb-Ridley et al. 2002; Hart et al. 2003), which have a common ancestor with chloroplasts. The reaction with proteins from *P. laminosum* in solution is only slightly influenced by ionic strength, and a rate constant as high as $3 \times 10^7\,M^{-1}\,s^{-1}$ can be observed when the electrostatic effects have been largely screened out. This is an exceptionally high rate constant for the formation of any complex between soluble proteins.

Electron transfer reactions such as these have now taken on a much wider relevance, important as is photosynthesis. The high rates of electron transfer observed in photosynthetic systems are only possible when the interactions between diffusible proteins are transient with, say, $k_{off} > 10^3\,s^{-1}$. Such weak protein–protein interactions have been difficult to study and so largely neglected until recently, but they occur in many different biological systems. Some diverse examples of weak interactions can be found in the binding of CD2 to CD58 in T-cell activation (Wang 2002), the assembly of hepatitis B virus capsids from capsid protein homodimers (Ceres and Zlotnick 2002), and in the interactions of SH3 domains in some signalling pathways (Mayer 2001). Electron transfer systems provide experimental handles. Thus a well-studied reaction, such as that between cytochrome *f* and plastocyanin becomes a paradigm for weak protein–protein interactions which can provide insight into the structural basis of their transient nature.

Concluding remarks

The 1930s were the heroic period for photosynthesis research and the 1950s and 1960s saw its explosive development. Cytochrome *f* sprang to view towards the end of the heroic period, and its central position in photosynthetic electron transport was understood, if a little shakily, by the end of the 1960s. And yet it continues to surprise us. We still do not know its evolutionary origin and the full significance of its unique structure, and questions remain unanswered about its behavior in the thylakoid lumen. Its physico-chemical properties and reactivity, however, have an interest which takes us beyond the boundaries of photosynthesis into areas of biology which many of us may not have explored before.

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