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Review

The chloroplast cytochrome *bf* complex: a critical focus on function

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Key words: Chloroplast; Cytochrome *bf* complex; Cytochrome structure; Protonmotive cycle; Electron donor

Contents

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Dedicated to the memory of Peter Mitchell, whose influence on Bioenergetics was so profound.
Abbreviations: AQS, anthraquinone sulphonate; chl, chlorophyll; cyt, cytochrome; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCCD, dicyclocarbodiimide; DNP-INT, dinitrophenylether of iodonitrothymol; EPR, electron paramagnetic resonance; ECU, European currency unit; Fd, ferredoxin; FeCN, ferricyanide; FeS(R), Rieske iron-sulphur centre; H, high-potential cyt *b*-563; HQNO, heptylhydroxyquinoline *N*-oxide; L, low-potential cyt *b*-563; MOA, *E*-β-methoxyacrylate; $NADPH_2$, reduced nicotinamide adenine dinucleotide phosphate; NQNO, nonylhydroxyquinoline-*N*-oxide; *n*-sites, sites on chloroplast cytochrome *bf* complex nearer the stroma (electrically *n*egative) side, postulated to bind plastoquinone and plastosemiquinone (also called Q_i or Q_r sites); $PC^{(+)}$, plastocyanin (oxidized); PQ, plastoquinone; PQH_2, plastoquinol; *p*-sites, sites on chloroplast cytochrome *bf* complex nearer the lumen (electrically *p*ositive) side, postulated to bind plastoquinol (also called Q_o or Q_z sites); PS I, Photosystem I; PS II, Photosystem II; PMF, proton motive force; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SQ, semiquinone; UHDBT, undecylhydoxydioxobenzothiozol; UQ, ubiquinone.

I. Introduction

The chloroplast thylakoids contain three main complexes involved in electron transfers. These are Photosystem I, Photosystem II and the cytochrome *bf* complex. The reactions of the chloroplast that involve light, as opposed to the carbon-fixation reactions, use these three complexes in different combinations with the ATP-synthase complexes to achieve non-cyclic and cyclic photophosphorylation.

In non-cyclic electron flow in vivo, electrons from water are driven by PS II into bound or mobile plastoquinone molecules, while in an independent light reaction, electrons from the reaction centre of PS I are driven into ferredoxin molecules, later forming $NADPH_2$. The latter is used, together with ATP formed in ATP-synthetase complexes from proton electrochemical energy, by the carbon-fixing mechanism. In cyclic flow, which may be the main function of strictly stromal PS I (as opposed to marginal or fret PS I: see Fig. 1), the cytochrome *bf* complex is again part of the pathway of electrons, PS I-Fd-quinone-*bf*-PC-PS I. The coupling of redox energy to the formation of an electrochemical potential difference for protons, across

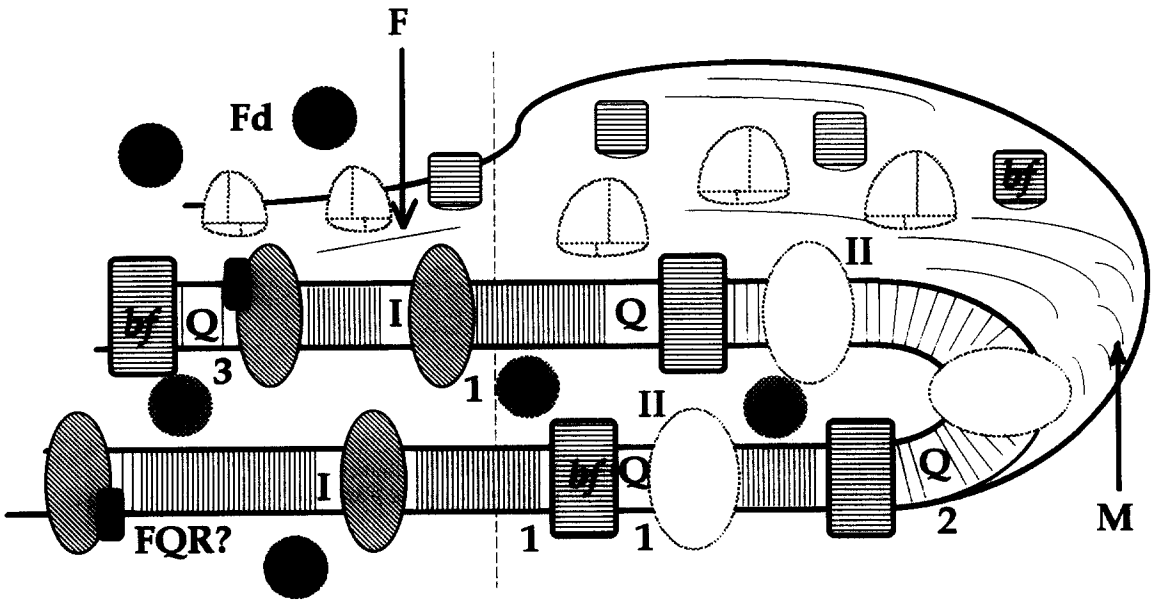


Fig. 1. A generalized diagram of the chloroplast thylakoid membrane showing the relation between the cytochrome *bf* complex and PS II, PS I. The dashed vertical line approximately divides stromal membrane on the left from a granal thylakoid on the right. Plastocyanin ('PC') is in the lumenal space; ferredoxin ('Fd') is in the stroma; PS II ('II') is confined to the granum; PS I ('I') is confined to the stroma and margins of the granum. According to developing views, discussed in this review, the kinetic relationship between cyt *bf* and PS II, and probably between PC and cyt *bf*, is non-homogeneous due to the varying distances that have to be traversed by diffusion. Thus in the group labelled '1', non-cyclic electron flow is facile, through closely related complexes. In group '2', both plastoquinone ('Q') and PC are some distance from their donors and acceptors. In group '3' in the stromal region, cyclic electron flow may be catalyzed by ferredoxin/quinone reductase ('FQR') associated with PS I. No attempt has been made to display the proper relative sizes of the complexes and their packing densities. The fret regions ('F') are those joining the grana with the stromal membrane, but nearer the former; the margins of the granal membranes are labelled 'M'.

the thylakoid membranes, is a vital function of the *bf* complex under most conditions.

The past few years have seen progress in envisaging the three-dimensional structure of the complex, and in understanding the details of its functioning. There is a clearer idea of its distribution amongst the regions of the chloroplast granal and stromal membranes, of the make-up of the subunits, their individual functions, and of the conditions under which the *bf* complex works to translocate protons across the thylakoid membranes, arguably its most important function. In addition, the *bf* complex mediates transitions between State I and State II, thus constituting a vital link in the mechanism controlling the balance of excitation energy between PS I and PS II, and hence helping to maximize photochemical efficiency.

The cytochrome *bf* complex occupies conceptually a central position between the two photosystems, mediating and controlling the overall electron-transfer rate from primary donor to terminal acceptor, and balancing the input of electrons (from PS II in non-cyclic, or from Fd in cyclic flow) with the output of electrons via PS I. Enzymically the *bf* complex is a plastoquinol/plastocyanin oxidoreductase. That is to say, plastoquinol produced by PS II or Fd is oxidized by the *bf* complex, electrons becoming available to reduce plastocyanin oxidized by PS I. Fig. 1 summarizes these concepts.

There have been several important reviews of the cytochrome *bf* complex in the last several years, to which due acknowledgment is made by the present author [1–3]. In addition, older reviews [4–6] should not be neglected, as much fundamental information may be gleaned.

Drawing particularly on studies published in the last 3–4 years, the present review has a different emphasis from most of those mentioned, namely a physico-chemical one, in which quantitative details of function are given emphasis. Thus, a very detailed examination of the behaviour of the redox components of, and associated with, the *bf* complex, of proton uptake and deposition, and of the slow electrochromic signal enables the conclusion that a protonmotive cycle with many of the attributes of a Q cycle is the normal mode of operation of the *bf* complex under a wide range of conditions.

We have to consider seriously the probability that the *bf* complex is a dimer in vivo, and possibly in vitro, depending on the isolation procedure. Recent evidence [7–9] is persuasive that plastoquinol formed at PS II has a restricted diffusion path to cytochrome *bf* complexes, and suggests a percolation model wherein quinone reduction and quinol oxidation are kinetically heterogeneous. These findings fall short of the necessity of envisaging 'supercomplexes' between *bf* complexes on the one hand, and PS I or PS II complexes on the other, as may be the case for *Rhodobacter* reaction centres and cyt bc_1 complexes [10].

Homology in the general sense of functional as well as secondary-structural similarity exists between the chloroplast complex and the cyt bc_1 complexes from mitochondria and photosynthetic bacteria. Because of this, conclusions from studies of the other complexes are at least suggestive for the properties of the *bf* complex and may turn out to be applicable to it. Thus, literature on these related complexes has been drawn upon in places in this review, but not at all comprehensively covered.

II. Location of the *bf* complex

II-A. Distribution in the chloroplast membranes

The cyt *bf* complex is found throughout the granal and stromal lamellae of chloroplasts [11,12]. It has been visualized by means of gold labelling of electron-micrograph sections as well as by immunological techniques. In electron-microscopy visualizations, the complex has been reported to appear as a dimer, as it has in the mitochondrial cyt bc_1 complex. While a general distribution throughout the lamellae is accepted, some variation in the content on a per chl basis has been noted in vesicles derived from granal disks, fret and margin regions [13,14].

Of great significance is the recent finding [15] that the complexes may be subject to redistribution between the granal and stromal regions, much as is found for LHCs. This aspect is covered fully by Anderson [3] and it is not proposed here to refer in any detail to the function of the *bf* complex in controlling its own or the LHC redistribution; the importance of these phenomena is that they are part of changes (so-called State changes) that help control the balance of excitation energy reaching the two populations of photosystems.

II-B. Stoichiometry of the bf complex

P700 has a concentration on a per-chlorophyll basis of about 1.6–2.0 mmol(mol chl)$^{-1}$ in some common higher plant chloroplasts [16–19]. This concentration is relatively constant in a range of growth illuminances. For a full discussion of photosystem stoichiometry see Ref. 20. Then, relative to P700, the *bf* complex varies in concentration depending on (amongst other things) illuminance during the plant growth [18,19,21]. Estimated from cyt *f* content, *bf* complex stoichiometry was found to be 0.7–1.2 per P700, when growth illuminance was 300–1500 $\mu E\,m^{-2}\,s^{-1}$ [18,19]. In simulations of electron-transfer events [22–24], a 1 : 1 ratio is reasonable, and frequently appropriate for glasshouse peas or spinach.

III. Structure of the complex and its subunits

III-A. Structurally a dimer?

Isolation and chromatography with SDS-PAGE shows the *bf* complex of chloroplasts to consist of four subunits, though five polypeptides frequently appear [25–27]. Here we define a *bf* complex as an association of one copy each of four (possibly five – see Section III-C.5) subunits, three of which contain prosthetic groups; these three groups are implicated in electron-transferring steps in the complex, while the remaining one probably has a function in binding electron donors and/or in stabilizing the other three.

Does it exist as a dimer? The cyt *bf* complex when isolated in the standard way [26], after which it clearly acts as a quinol/plastocyanin oxidoreductase (see Section III-E), probably consists of a mixture of monomer and dimer. A partial separation of the two forms has been achieved [28] but activity measurements were not reported. Cross-linked homodimers of the cytochrome *b* subunit and Subunit IV were correlated with retarded dimer-to-monomer conversion during centrifugation in a sucrose density gradient.

III-B. Functionally a dimer?

Does the *bf* complex function as a dimer? The above information does not say whether its function in any obvious way depends on a dimeric association. Unfortunately, the Atlantic ocean stands between two particular, differing conclusions about the complex functioning as a dimer. The main experimental approach has been to determine what ratio of strongly-bound inhibitor of *bf* complex activity to *bf* (monomer) is necessary for inhibition of function. Usually the function observed is the rate of electron transfer from water or exogenous quinol to terminal acceptor methyl viologen, via *bf* complexes and Photosystem I.

Thus, on the one hand, Graan and Ort [29] concluded that the number of DBMIB-binding sites was only half, approximately, of the *bf* complexes. The possibility was raised that full inhibition of a functionally dimeric structure could occur if DBMIB were bound to only one of the pair of complexes comprising the dimer. Indeed, the analysis requires that only one DBMIB binding site be available per dimer.

Rich et al. [30] on the other hand, while obtaining rather similar data, concluded that their results best matched a monomeric *bf* complex, with half-maximum inhibition occurring when DBMIB was bound to half the total *bf* complexes. These two studies:

(i) used different theoretical treatments,

(ii) used different material, grown under different conditions and

(iii) assayed chlorophyll with differing extinction coefficients,

but had a common problem, that none of the data was precise enough for the conclusions reached. Using figures entirely from the experiments in Ref. 29 the mean ratio of [DBMIB binding sites]/[*bf*] was actually 0.65 $\pm$ 0.25 (SE), which does not place the ratio at 0.5 with complete confidence. The point has been made [30] that the *bf* complexes should be turning over rapidly for DBMIB to remain bound to them. Oxidized DBMIB is the preferred bound form. Different results might be obtained according to differing turnover rates in such experiments.

Several continents separate conclusions about the same matter, arrived at as a result of radiation inactivation studies [31,32]. Here, one might prefer the conclusion in [31], that the *bf* complex is functionally a monomer. The second study [32] contains some inconsistent results (recognized by the authors), and it is questionable whether target theory applied to single electron-transfer enzymes can be extended to the multi-enzyme arrangement of PS II + *bf* + PS I, complexes which are connected electronically by multiple diffusible components. For example, inactivation of one of several plastocyanins would not inactivate the chain in the same way as would inactivation of a single, essential intermediate. For today, we assume the *bf* complex is able to function monomerically, but that it may exist physically as a dimer both in thylakoids and in isolation. Such dimeric association may lead to

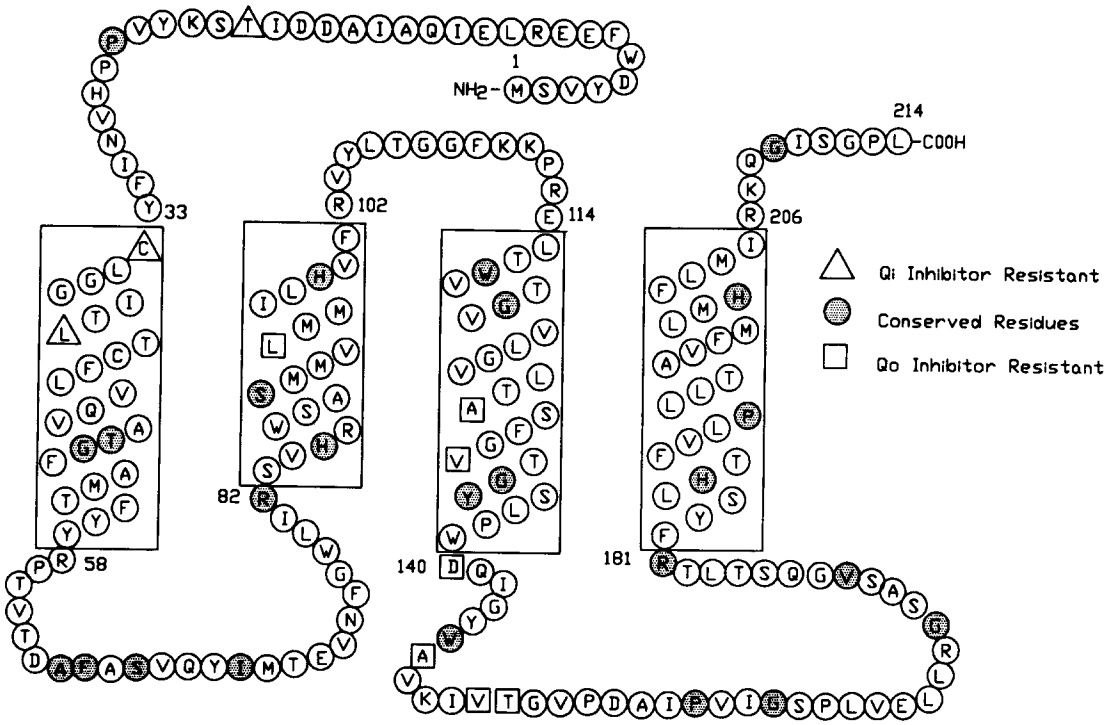
TABLE I

Known subunits of the cytochrome bf complex of spinach chloroplasts and their properties

Subunit	Molecular weight (kDa)	Membrane helices	Likely function
I. cyt *b*	24	4	Transverse electron transfer
II. cyt *f*	31	1	Accepts e^- from FeS(R); donates to PC
III. Rieske	20	2	Accepts first electron from PQH_2
IV.	19	3	Binds quinone
V.	4	?	?

Fig. 2. Above: the components of the cytochrome *b* polypeptide from spinach with its four currently envisaged helices and connecting residues; below: the 19-kDa subunit, Subunit IV, from spinach *bf* complex. Residues enclosed in triangles refer to point mutations that confer resistance to *n*-site (Q_i) inhibitors, those in a square, to *p*-site (Q_o) inhibitor resistance and those in darkened circles are conserved throughout all tested *bf* or bc_1 complexes. Reproduced from Ref. 34 with the kind permission of Drs. W. Widger and W. Cramer, and Academic Press.

"n" Side



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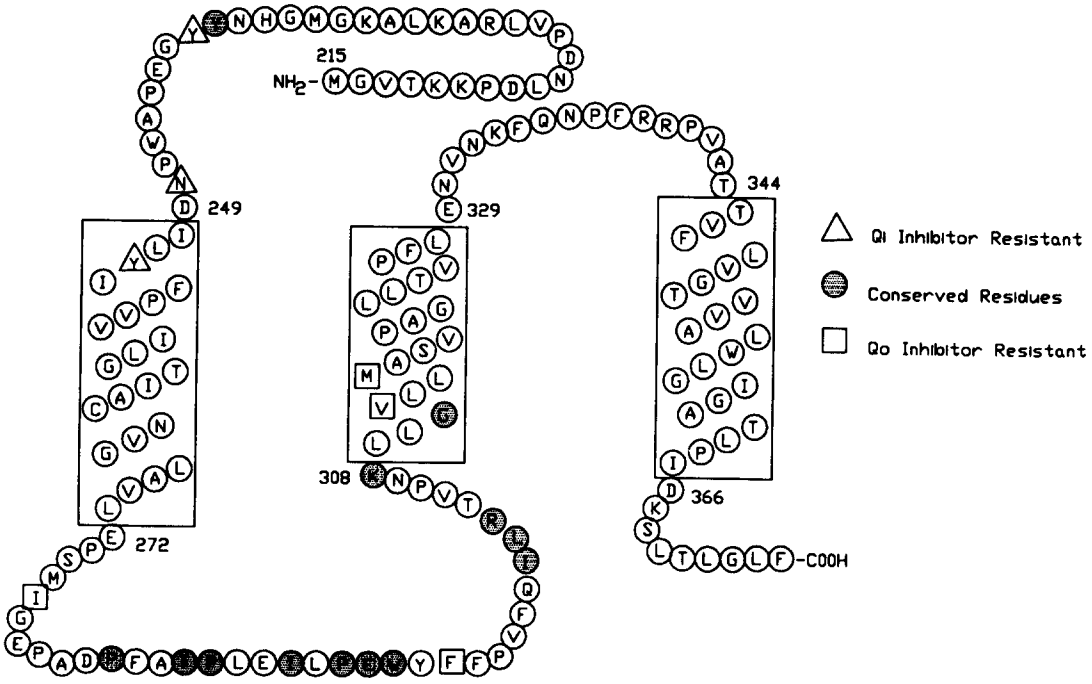

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unique function under some conditions (see Section IV-D below).

III-C. The subunits

Table I summarizes the molecular weight and assumed function of the known subunits. Complete data on the amino-acid sequences of four subunits now exist for many plants, including spinach, and are assembled and discussed in Refs. 33,34; note that the amino-acid numbering differs in these accounts. Until X-ray diffraction data from protein crystals are available, the progression from amino-acid sequence to confident knowledge of three-dimensional structure is not possible. Nevertheless, general guiding principles have helped to envisage a probable structure of the native subunits as they exist in relation to their host membrane. Thus, observing predominantly hydrophobic amino acids in a linear segment of, say, 15–20 leads to the idea of a membrane-spanning segment, probably alpha-helical, a motif that may be repeated several times within the membrane, the segments being joined by variable lengths of extra-membrane residues.

The other guiding principle has been to assume confidence in a suggested structure according to whether homology exists between corresponding amino acids of the *bf* complex compared with other similar oxido-reductases such as the bacterial and mitochondrial bc_1 complexes. When a high degree of homology is noted, that region of the amino-acid chain is assumed to have significance for a function such as liganding the prosthetic group, a property common to all the complexes.

III-C.1. The cyt b-563 subunit

Fig. 2 shows how the above ideas were applied to the subunit containing the two cyt *b*-563 haem centres. Fig. 3 shows the region where cyt *b*-563 haems are likely to be liganded to the amino-acid chain via homologous histidine residues. Since there are only the four totally conserved His throughout the entire range of these complexes, and since the haems are known from spectroscopic studies to be liganded to two His each [35], this feature of the *b* subunit can be said to be well-based. Originally proposed for the chloroplast complex in 1984 [36], and later revised [37,38], this

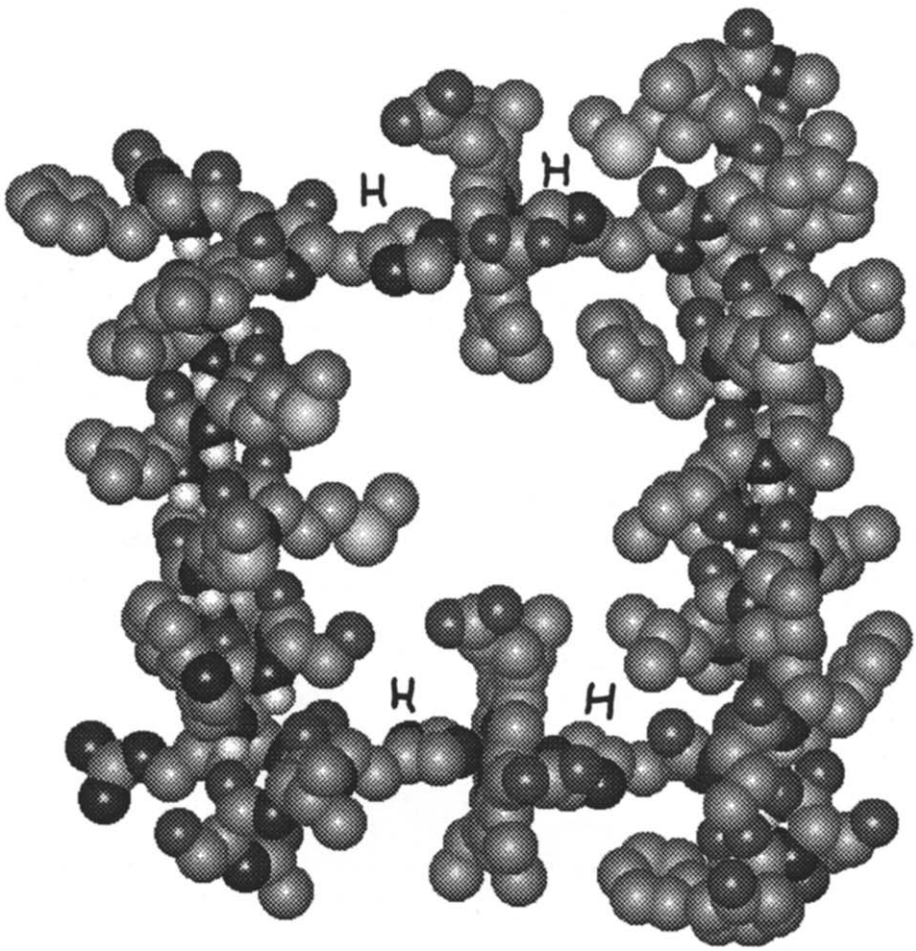


Fig. 3. Proposed relationship between helices II and IV in the *b* subunit of the cyt *bf* complex. The histidine residues assumed to form ligands with the Fe of the haems are indicated. The molecular structure was drawn with the aid of *Hyperchem* software (AutoDesk).

important concept remains current. The number of membrane-spanning helices has recently been reduced from 5 to 4 because of the necessity for both the N- and C-termini to be accessible to trypsin [37]. The previously spanning fifth segment, in any case, was in doubt for that location [33] because of a high complement of polar amino-acid side-chains, which led only to a weak indication of a membrane helical segment on a hydropathy plot. Finally, bacterial mutant studies suggested sites of inhibition by myxothiazol at residues facing both sides of the membrane, whereas myxothiazol is regarded as specific for *p*-sites (see Sections III-D and IV-B below). A possible arrangement of six helices to form the environment of the *p*- and *n*-sites in *R. sphaeroides* has been derived by the Crofts group [39]. This scheme comprises the four helices of the *b*-subunit and two which would be in Subunit IV (below) in the *bf* complex.

III-C.2. The cyt f subunit

This subunit, which has 285 residues in spinach [34], is regarded as crossing the supporting membrane once only in an α-helical stretch between residues 251 and 270. Conserved residues VCnnCH (20–25), because they are homologous for many organisms, are said to contain the Cys, and proximal His ligands for the haem of cyt *f*. The latter is located on the lumenal side of the membrane. The good news comes, not from Ghent but from W. Lafayette, that this subunit has been crystallized [40] so that a high-resolution structure will be soon available.

III-C.3. The Rieske subunit

In spinach, this subunit has 180 amino-acid residues and contains two possible membrane-spanning sectors on each side of a hairpin bend [34]. Like the cyt *f* subunit, the prosthetic group, the Fe_2S_2 centre, is in the lumen-located portion of the structure. The probable liganding residues for the centre in bc_1 complexes from yeast [41] and *Rhodobacter* [42] have been identified by means of the following strategy. Firstly, there exist two sets of six amino acids (CTHLGC, and CPCHGS) that are conserved in all Rieske centres of the bc_1/bf type. Again, there are different numberings in these references and in Refs. 33,34. Single-site mutations in the underlined residues caused inability to grow or to assemble the subunit to include the Fe_2S_2 centre and these residues were therefore proposed as the ligands, the two His to one of the Fe, the two Cys to the other.

III-C.4. Subunit IV

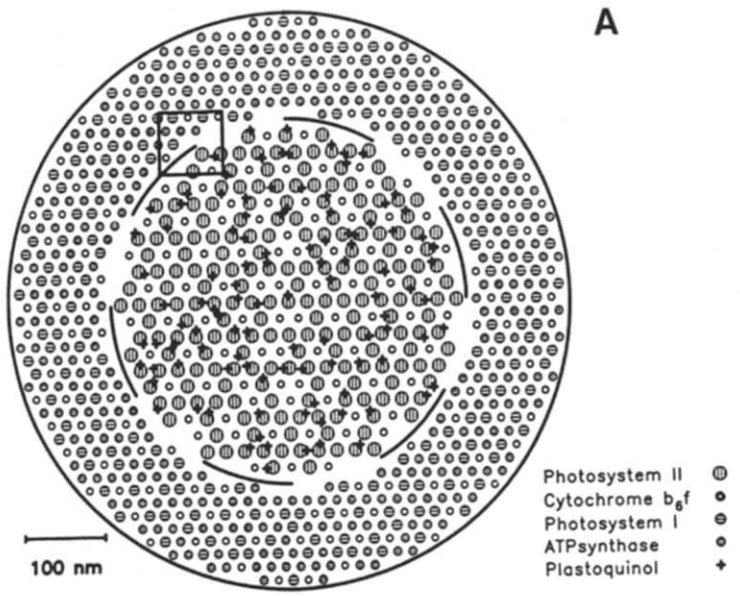
This subunit, which in spinach has a mass of 19 kDa, has partial homology with a string of amino acids, leading from the C-terminal, found in the cyt *b* subunit of the mitochondrial bc_1 complex [33]. It might therefore be the equivalent of three of the alpha-helical coils in that mitochondrial subunit, a subunit that is heavier and has eight membrane-spanning segments. The chloroplast Subunit IV has been found, by means of photoaffinity labelling of isolated *bf* complex with the quinone derivative 'azido-Q', to be implicated in quinone binding [43]. There was little label to be found on the cyt *b* subunit. Subunit IV has further been concluded to be essential for the catalytic activity of the *bf* complex [44].

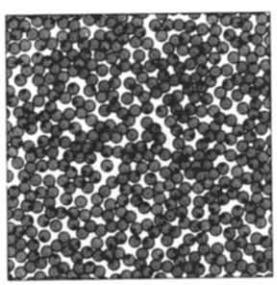
III-C.5. Another subunit?

A 4-kDa polypeptide from maize was found to be co-purified with the other *bf* subunits [27] but it is too early to grace this with the title of Subunit V, despite the actual finding being repeated with *Chlamydomonas* (R. Malkin, personal communication).

III-D. Envisaging the complete structure

It has proved more difficult to settle on the way the membrane-spanning segments of one subunit associate with each other and with other subunits. Here, molecular genetics techniques have provided some advances. Since inhibitors such as DBMIB, stigmatellin (*bf* complex) and myxothiazol (bc_1 complex) interfere with quinol oxidation (see further Section IV-B below), they are assumed to bind to a closely related region on the subunit responsible for oxidizing quinol, and/or to nearby subunits. A separate set of inhibitors is happily available for bc_1 complexes in connection with quinone reduction, namely antimycin A, funiculosin, the quinoline *N*-oxides; absence of corresponding clean inhibitors for the chloroplast system has led to dismay, but as compensation we do have light as a plaything. Single-site mutations that endow the bc_1 complexes in yeast and mouse mitochondria, and in *R. capsulata* with resistance to the above inhibitors have been assembled [34]. These amino acids are therefore assumed to lie near or at quinol oxidation sites. Thus the amino acids Phe 132, Gly 140, Ile 150, Tyr 151 are in the cyt *b* subunit, the last two at least just outside the membrane, near helix three; Asp 276, Leu 295 would be in Subunit IV; in some way that is not yet obvious, these residues are a part of, or nearby, *p*-sites in the *bf* complex. By analogy with the bc_1 results for AMA-resistant mutations, the amino acids around about *n*-sites might be Glu 40, well within helix 1 of the *b* subunit, and Lys 248 just outside putative helix 1 of Subunit IV. In Fig. 2 the residues that have been mutated are enclosed in a square for *p*-site, or in a triangle for *n*-site affinity changes (resistance to inhibitor). The residue numbers agree with those listed above, but the actual amino acids at these positions are for spinach, and differ from the corresponding ones listed above for


A
100 nm
Photosystem II
Cytochrome b_6f
Photosystem I
ATPsynthase
Plastoquinol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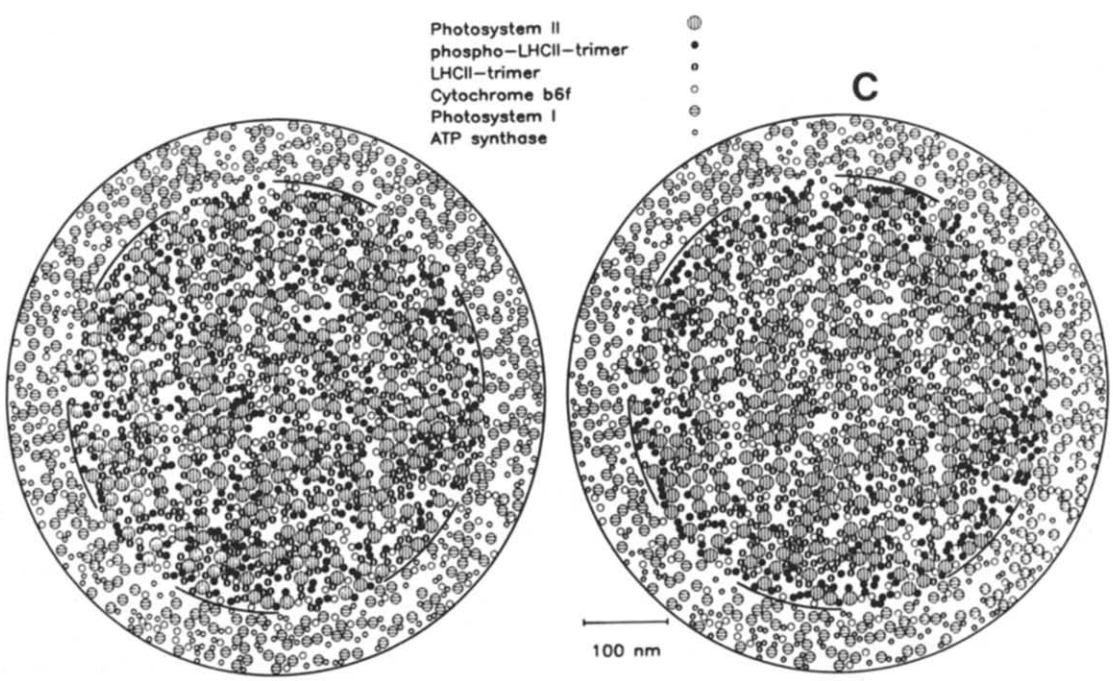
Photosystem II
phospho–LHCII–trimer
LHCII–trimer
Cytochrome b6f
Photosystem I
ATP synthase
C
100 nm

the yeast, mouse or *R. capsulata* complexes in which the mutational work was done.

III-E. Isolated preparations

Cytochrome *bf* preparations have been shown to require associated lipid for their functioning as PQH_2/PC oxidoreductase [45]; the exact mix of endogenous lipid and extractant detergent present in isolated *bf* preparations is not known. The significance of varying amounts of PQ co-isolated with the complexes [45,46] is still unclear.

Turnover numbers of 25–35 s^{-1} by isolated complexes given favourable quinol forms and excess PC^+ have been measured [26]. Using ferricyanide as oxidant this has been increased to ~ 80 s^{-1} [47], so the ability to turn over as fast as in chloroplasts (300 s^{-1}) is conceivable under optimal conditions. The Hurt and Hauska preparation [26,48] is proving a useful vehicle with which to explore function.

IV. External electron donors and acceptors

IV-A. Quinol diffusion

The transport of quinol to its site of oxidation has been assumed to occur by diffusion within the thylakoid lipid bilayer. Modelling of this process [23] was combined with measurements of $P700^+$ re-reduction kinetics to test the hypothesis that quinol diffusion is rapid enough to account for the appearance of an electron at $P700^+$, under a variety of conditions. Fig. 4a shows the packing arrangement of the protein macromolecular complexes and quinone assumed by considering published data on the concentration and size of the complexes. The conclusion was reached that diffusion was not limiting the overall electron-transfer process from PS II to $P700^+$. Depending on whether quinol oxidation was by a collisional process (second-order between [PQH_2] and [*bf*]) or occurred after binding between the reactants (first-order rate-coefficient), the diffusion coefficient of PQH_2 (D) was minimally $2 \cdot 10^{-12}$ m^2 s^{-1} or $3 \cdot 10^{-11}$ m^2 s^{-1} respectively. On other grounds (see next section) the collisional mechanism is favoured, leading to the lower figure for D. The lower figure has experimental support, because D measured for PQ in phospholipid vesicles [49,50] averaged $2 \cdot 10^{-11}$ and it was about 10× lower when 16–26% of the membrane area was occupied by protein, forcing a longer diffusion path [50]. If D is $2 \cdot 10^{-12} m^2$ s^{-1} the root mean square distance diffused in 1 ms is 89 nm; in 200 μs, it is 40 nm. Conversely, if a distance of 100 nm has to be traversed to support a turnover rate of 300 s^{-1}, D must be as high as $8 \cdot 10^{-12}$ m^2 s^{-1}.

Recent reports [7–9] draw attention to the restricted diffusion paths for plastoquinol as it attempts to find an oxidation site on cytochrome *bf* complexes nearby. The reduction of PQ during saturating light, estimated by three different methods, was found to be heterogeneous, with some of the pool being quickly reduced by PS II, namely about 60% of the total, with a half-time of about 40 ms. The remainder, the slow pool, had a half-time of nearly 1 s. In [9] the reduction kinetics of cyt *b*-563(H) were analyzed following saturating flash- or DC light-induced PQH_2 production by PS II; isolated thylakoids were in oxidizing conditions (+FeCN). About two thirds of the *bf* complexes were in rapid (< 10 ms) communication with the quinol, the remainder being reduced only after about 100 ms. The conclusion was drawn that diffusion of PQH_2 should be seen as a 'percolation' between densely packed protein complexes (see Fig. 4b for their planar density) and would be too slow to effect electron transport from PS II in the grana to stromal regions. Of course it has never been asked to fulfil that precise duty, only to reach cytochrome *bf* complexes. Clearly, however, that task will be the slower, the further these are located from PS II, putting the onus on PC to account for high rates of non-cyclic electron transport.

Realizing that the packing density of protein complexes had been underestimated in the earlier modelling of quinone diffusion [23], Drepper et al. [51] have used the model shown in Fig. 4c on which to base Monte Carlo simulations of the diffusion, particularly, of phosphorylated LHC II. In passing, they state that, even with the restricted percolation model, plastoquinol with a D of 10^{-12} m^2 s^{-1} will distribute laterally over the granal area within about 100 ms. This figure agrees with that for the reduction of the 'slow' portion of cyt *b*-563(H) [9], but the situation needs more sophisticated analysis, since the percolation model predicts a continuum of characteristic times for the reduction of *bf* complexes by PQH_2.

Fig. 4. Three different representations of the packing of protein complexes in the lipid of chloroplast thylakoid membranes, to illustrate the evolution of models intended to give insight into the diffusion of plastoquinone, etc. (A) Plan view of a model of a chloroplast thylakoid membrane from Ref. 23; the inner area is the appressed granal region, the outer, the unappressed membrane. The boxed area was expanded in the original to show random diffusion of plastoquinol. (B) from [10] showing increased packing density based on freeze-fracture studies; the larger circles represent PS II, the smaller, PQ. (C) from [51], also using more realistic particle densities, with in addition appropriate particle sizes (e.g., diameter in nm: PS II with bound LHC, 16; cyt *bf*, 8.0; PS I, 12). Reproduced with the kind permission of the authors, and publishers of Trends Biochem. Sci. and Biophys. J.

Note that in Fig. 4b and c, packing density is such that some PQ have virtually no access to a PS II, suggesting to Lavergne and Joliot [10] a possible explanation of 'disconnected' PS IIs [52]. Further to this, but in passing, it has recently been reported [53] that a period of light flashing was able to increase the apparent number of connected PS II, since the oxygen per flash increased to match the total number of DCMU-binding sites. Does 'light activation' of PS II alter the communication between PS II and PQ?

A puzzle also seems to persist with regard to the interaction of the presumed-diffusing PQ/PQH_2 with the lipids of thylakoid membranes. Earlier proton NMR and other studies, discussed in Ref. 54, revealed little change in order parameters in the lipid hydrocarbon chains in the presence of various Ubiquinone-related quinones. More elaborate (solid-state deuterium) NMR studies have reinforced this view [55]. Millner and Barber [54] discussed evidence in relation to an earlier proposal [56] that the isoprenoid side-chain of physiological quinones could lie in a plane parallel to the membrane surface, between the lipid monolayers, not parallel to their acyl chains. Freedom of the PQ to diffuse would only be limited by the (dense) packing of membrane-spanning proteins, reducing the maximum D proposed, of 10^{-10} m^2 s^{-1} (see above). Fig. 5 attempts to illustrate this. Evidence consistent with this attractive idea was not, however, forthcoming from neutron diffraction studies made of UQ_{10} in phospholipid multilayers [57]. Indeed the whereabouts of the UQ could not be established, except that it was not intercalated between the lipid acyl chains, nor was it in the centre of the lipid bilayers.

Less attention has been paid to the behaviour of the quinone ring, which is not nearly so hydrophobic as the hydrocarbon tail; it should tend to locate nearer the membrane surfaces. Conclusions from relatively old NMR observations with artificial phospholipid bilayers [58] were that (a) quinol ring methoxy groups were closer to the membrane surface than those in a quinone ring, and (b) a sufficient ring flip-flop rate (across the coupling membrane) was available to support the Q-

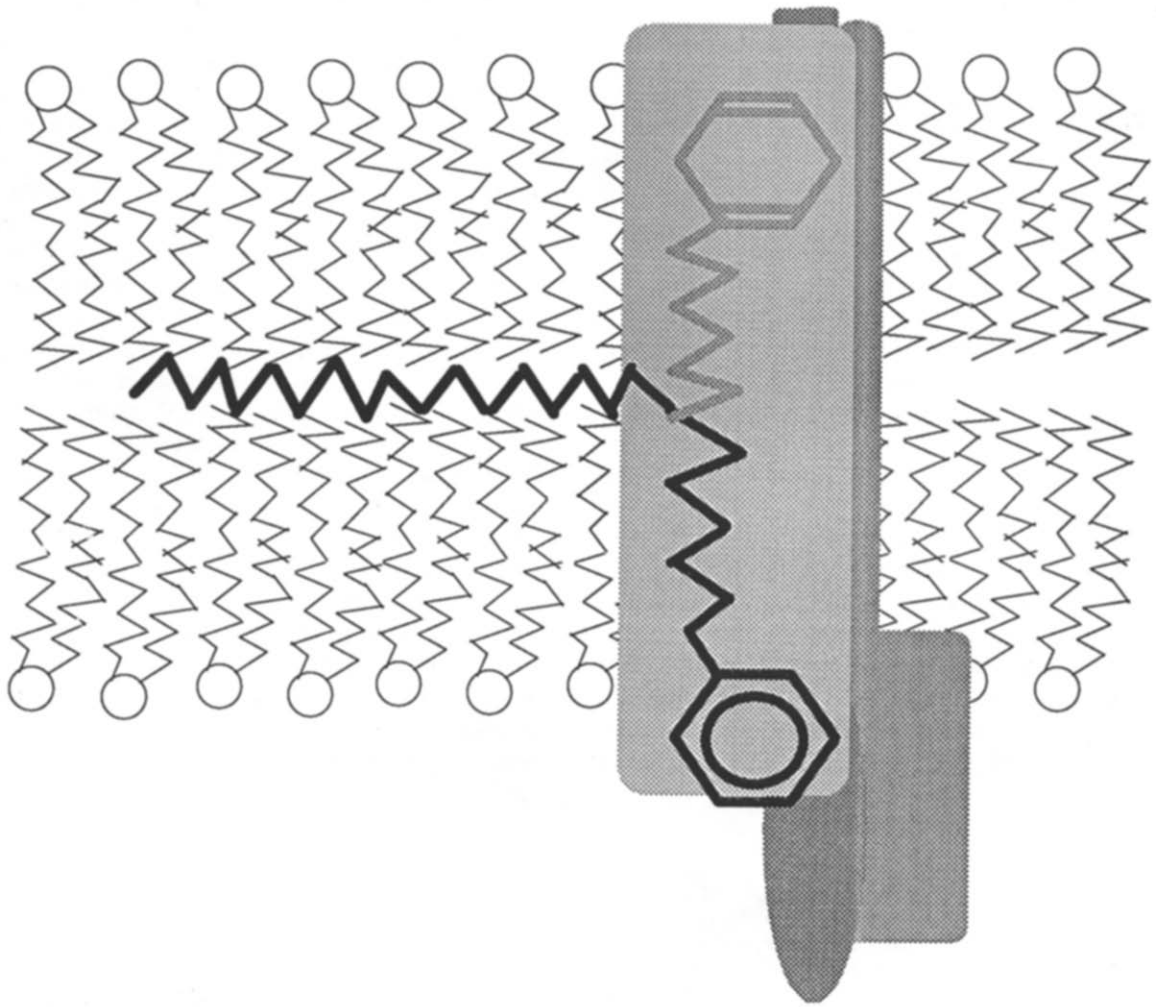

Fig. 5. Suggested relationship between the isoprenoid tail and ring of plastoquinone(ol), and the thylakoid membrane and subunits of the cytochrome *bf* complex, respectively. The ring is shown alternatively binding as quinol to a *p*-site or as quinone to an *n*-site.

TABLE II

Reduction potentials (mV), peak wavelength, λ_{peak} (reduced minus oxidized, α-band) and extinction coefficient $\Delta\epsilon$ (reduced minus oxidized, mM^{-1} cm^{-1} at λ_{peak}) of the two b-563 cytochromes

Preparation	Cyt *b*-563(H)			Cyt *b*-563(L)		
	$E_{m.7}$	λ_{peak}	$\Delta\epsilon$	$E_{m.7}$	λ_{peak}	$\Delta\epsilon$
Isol. *bf* compl.	-30 ± 20	–	–	-150 ± 15 [97]	–	–
	−25	–	–	−120 [1]	–	–
	−45	564	16.8 [a]	−140	563.5	25.2 [a] [132]
Thylakoids	−40	–	–	−40 [93]	–	–
	−15 [c]	–	–	−110 [c] [111]	–	–
Chlorella	–	564.0	18.4 [b]	–	563.4	23.6 [b] [100,120]

[a] Assuming the average $\Delta\epsilon$ was 21; $\Delta A(H)/\Delta A(L)$ was 0.67.
[b] Assuming the average $\Delta\epsilon$ was 21; $\Delta A(H)/\Delta A(L)$ was 0.78.
[c] Because redox changes due to the measuring beam were minimized, these values are to be preferred.

cycle concept. Ulrich et al. [59] used a thicker bilayer, namely dipalmitoylphosphatidylcholine (DPPC, 18-C), which was taken as more comparable to natural membranes than the dimyristoylphosphatidylcholine (DMPC, 14-C) used previously [58] and contested the ability of UQ_{10} to undergo such flip-flop processes. UQ_{10} did not appear to perturb the transition temperature of the bilayer lipids in DPPC and 50% of the membrane-located UQ_{10} was not reducible by borohydride over 30 min. Ferricyanide enclosed in the vesicles was virtually unreduced by external borohydride via UQ_{10}, although this was possible through the medium of membrane-dissolved UQ_2 or UQ_3; dithionite was able to effect some reduction. These results suggested a location for much of the UQ_{10} in the centre of the bilayer from which it was effectively unable to reach the membrane surface. Nevertheless, 20% of the UQ_{10} methoxy groups were detected by NMR in more polar environment frequented by UQ_2, which leaves the picture a little inconclusive.

Measurements of the partition coefficient of many quinones and quinols [60] revealed a very marked preference of quinone for the hydrophobic phase compared with the quinol; nevertheless, part of the mechanism of the *bf* complex involves transverse movement of quinol from the stroma to the lumenal side of thylakoids (see below); this movement may be in a space surrounded by subunits of the cytochrome *bf* complex but still in an environment expected to be rather hydrophobic.

IV-B. The site of quinol oxidation

The chief evidence that there is a specific site for quinol oxidation comes from studies with inhibitors of electron transport. The substances DBMIB, UHDBT, stigmatellin, and DNP-INT all inhibit the passage of electrons from quinol to a terminal acceptor. As well, they tend to prevent cyt *b*-563 reduction, proton deposition at *p*-sites, the slow electrochromic signal and cyt *f* re-reduction (examples in [61]). All these processes depend on effective oxidation of quinol. The effectiveness of these inhibitors, at least one of which is a plastoquinone analogue, must depend on a type of competitive binding to the site of quinol oxidation, or to a nearby site from where it exerts 'conformational interaction' [62]. In the bc_1 complex, where stigmatellin, the MOA-based inhibitors and myxothiazol form separate groups (see [63]), the common action is to prevent quinol oxidation and $FeS(R)^+$ reduction. However, it has been shown using mutants of the bc_1 complex of *R. capsulatus* [64] that resistance to stigmatellin is not accompanied by impaired *p*-site Q/QH_2 binding ability or kinetic competence (oxidoreductase function of the complex). By contrast, mutants conferring myothiazol or mucidin resistance were impaired in *p*-site catalysis. When inhibitors are added to chloroplasts, chromatophores or isolated complexes, they have specific effects on the physical properties of the Rieske centre which is close to where they are supposed to bind:

(i) DBMIB [65] and stigmatellin [66] alter the reduction potential of FeS(R) (Table III),

TABLE III

Reduction potentials (mV) of the Rieske centre and cytochrome f

Preparation	FeS(R)	Cyt *f*
Thylakoids [a]	+310 [65]	+358 [1] +355 [133]
+DBMIB	+180 [65]	–
Thylakoids, kinetic meas.	+370 [47] +380 [24]	– –
Isol. *bf* [a]	+320 [66]	+350 [132]
+STIG.	+460 [66]	+325 [132]

[a] Redox titration of EPR g_y line at 15 K.

(ii) DBMIB [67] specifically markedly alters the low-temperature EPR spectrum of the reduced FeS centre, stigmatellin less so.

(iii) Quinones and quinols alter the low-temperature EPR spectrum of reduced FeS(R) but more subtly [68,69].

We noted above the evidence for recognizing Subunit IV involvement in quinone binding, but the electron-donating portions of the quinols must also be within a reasonable distance of the acceptors FeS(R) and cyt *b*-563(L) for prompt transfer. This suggests that when the PQH_2 docks it interacts with no less than three of the *bf* subunits.

The location in the Rieske centre of probable binding sites has been clarified by reference to the common or homologous amino acids in the Rieske centres in other organisms [33]; in this work, it is argued that homology means a common relation between structure and function. The argument is persuasive: though there is only 20% overall homology between the chloroplast and other Rieske proteins, there exists a small homologous group of amino acids suggested to bind quinols. The detailed nature of the interaction is close to being envisaged; it should include the formation of hydrogen bonds [60,69]. Quinols can form up to five hydrogen bonds with a binding site. Two may arise from each of the the hydroxyl groups; less recognized is a possible hydrogen bond due to the benzene ring [70]. This might suggest a stronger binding of quinol than of quinone, but no doubt other factors contribute. Specific schemes whereby quinone and quinol bind to interact with both the Fe_2S_2 and cyt *b* regions have been advanced [69]. Both of the His shown to ligand to the Fe_2S_2 (III-C.3 above), and at least one of the His ligands to the cyt *b* haems are proposed to be H-bond donors to the O = of quinones or the -O-H of quinols, giving rise to characteristic EPR line shapes and *g*-values. In addition, this study concluded that the *p*-site could be occupied by two quinones, so that double turnover of the complex was possible without exchange between the *p*-site and the quinone pool.

IV-C. Quinone reduction at n-sites

As has been repeatedly observed, the extent of cyt *b*-563 reduction after a single flash, in reducing conditions where there is enough quinol to reduce each *bf*, does not exceed about 0.3 per *bf* [71,61,72,22,24] and up to 0.5, with frequent multiple flashes [73]. On the second flash given to *Chlorella* cells with added ferricyanide ('oxidizing conditions') about 0.7 of a cyt *b*-563 per complex was reduced [74]. After a pulse of DC illumination [75] also about 0.7 of a cyt *b*-563 was reduced. Production of quinol by light appears to result in the reduction of less than one molecule of cyt *b*-563 of the two in a single complex. Because of this, it might be inferred that electrons do not stop at cyt *b*-563(H) but are able to be shared with a quinone; direct evidence has been absent. The expected reduced species, $PQ^{-\cdot}$, was not detected until recently, though the corresponding radical in mitochondria or photosynthetic bacterial bc_1 complexes has a longer provenance. A flash-induced EPR signal centred on $g = 2.005$ has been observed, which was by several criteria associated with *bf* complex turnover [76] and attributed to quinone radicals; this has provided a missing link in the chain of facts that indicate the reality of a protonmotive cycle of the Q-cycle variety (more below).

IV-D. The rate of cyt b-563 oxidation and its implications

Cyt *b*-563(H)$^-$ might be oxidized in several ways: (i) as just discussed, its electron is probably normally shared with a quinone at adjacent *n*-sites, (ii) further oxidation may occur if the quinone radical either dismutates by unbinding and finding a similar partner, or (iii), as in several literature references [77,73,76], by dismutating with a quinone radical on a nearby *bf* complex. This last process would explain two very intriguing facts:

(i) The oxidation of *b*-563 is much faster in reducing conditions ($t_{\frac{1}{2}} = 20$–30 ms [71,61,22,24] compared with oxidizing conditions (200–800 ms [74,72]).

(ii) The line-width of the flash-induced EPR signal we observed in chloroplasts [76] was too small to represent a normal quinone radical but was consistent with electron-sharing with another, close quinone. The line-width observed in experiments with bc_1 complexes is normal for an isolated quinone radical (approx. 0.8 mT, see Fig. 2 in Ref. 78) but these radicals were induced not by normal turnover of the complex but by redox poising; the narrow line-width in the chloroplast experiment was observed during turnover at room temperature.

With respect to (i), if the concentration of free quinone were to control the oxidation rate, in a second-order reaction such as envisaged for the *p*-sites [22,23] and proposed for *n*-sites in PS bacterial complex [79], this situation should be reversed, because the concentration of quinone should be minimal in reducing conditions, and maximal in oxidizing conditions. To explain this apparent contradiction, it might be proposed that the quinone radical that is formed at *p*-sites travels across the membrane in a SQ-cycle type of mechanism, from *p*-site to *n*-site, where it oxidizes *b*-563$^-$. There is nothing against this, indeed possibly something for it [80], but a more attractive explanation beckons.

As to (ii), what if the close-by quinone were on the *n*-site of the second *bf* (No. 2) of a dimeric pair? In oxidizing conditions, the probability that this quinone

has an electron is low. The radical on *bf* No. 1 is relatively stable, only dismutating at a low rate as it unbinds, etc. In reducing conditions, *bf* No. 2 has a quinone radical more frequently, because the quinol/*bf* ratio is perhaps 6, and therefore more *bf* s are in the state $R.b^- \leftrightarrow R.bQ_n^{-\cdot}$. In other words, the whole situation is more comfortably explained if the *bf* complexes are associated as dimers, as the biochemical evidence urges. Fig. 11 in Ref. 76 shows the necessary spatial relationship between the quinone rings to account for the EPR observations.

IV-E. Plastocyanin diffusion

The transport of plastocyanin to its site of reduction at possibly distant *bf* complexes, from PS I where it has been oxidized, and in the reverse direction, has been assumed to occur by diffusion in the lumenal space of thylakoids. Of the mobile electron-carriers, there is probably more conceptual difficulty with plastocyanin than with PQH_2 or Fd. Firstly, the lumenal space is narrow, with many protruding proteins. Secondly, PC is a 10-kDa protein, with a maximum diffusion coefficient in aqueous solution, if spherical, of $1-2 \cdot 10^{-10}$ m^2 s^{-1}. Thirdly, the sites of its oxidation and reduction may be separated by hundreds of nm (Fig 1; separations of 20–200 nm are referred to in Ref. 81). There are 2–3 PC per P700 [16,81]. Most P700 have an associated bound PC in the dark [81], because $P700^+$ is very promply reduced ($t_{\frac{1}{2}} = 11$ μs [81] or 4 μs [82]) after a laser flash.

Since most PS I is located in stromal membranes, for each granal *bf* to be oxidized, a PC^+ must travel a comparatively large average distance. Haehnel et al. [81] observed a greater concentration of PC in granal regions than in stromal, after illuminating chloroplasts, compared with dark conditions. This was interpreted as favouring the long-distance lateral transport of electrons from *bf* complexes (grana) to PS I (stroma). This situation needs further analysis, taking into account the redox state of the *bf*s and PC, which might determine, in steady-state light, the direction of any lateral concentration gradient of PC. Delosme [82] has reported two differing oxidation rates for cyt *f* which were thought to correspond to different diffusion distances necessary for PC^+ between P700 and *bf* complexes. The different oxidation rates were possibly related to chloroplast ATP levels and the transition of the oxidation rate towards 'fast' caused by the protonophore FCCP brought to mind State transitions.

IV-F. The site of cyt f oxidation

Oxidized plastocyanin is the normal acceptor of electrons from the *bf* complex. It has overall a negative charge of 9 per molecule [83] and should be isoelectric at pH 4.5, a pH that could well be approached during light-driven lumen acidification. Electrostatic interaction (leading to favourable conditions for electron transfer) of PC^+ with basic amino acids Arg 88 and 154 of the cyt *f* subunit, as proposed in [84], would therefore depend on lumenal pH. Is this influence part of electron-transport control in coupled chloroplasts? The general area identified in that consideration may nevertheless be correct, since it has been reported [85] that a mutation from Tyr 83 to Phe 83 or Leu 83 caused much weakened binding of PC to cyt *f*.

IV-G. Exogenous quinols

It is common to use exogenous quinols such as duroquinol to explore foreshortened, non-cyclic electron flow, that from quinol to PS I acceptor without PS II (this should be distinguished from cyclic flow though it is not always done). It was shown [86] that DQH_2 was able to donate electrons directly to *bf*; the result has been confirmed [30] and further, it was shown that chloroplasts lacked a transhydrogenase able to mediate between DQH_2 and PQ. By contrast we [22,24] assumed that DQH_2 and similar quinols could reduce PQ which then acted as direct reductant of the *bf* complex. The disagreement may be more apparent than real, since the turnover rates under the two experimental conditions were widely different, continuous light or rapidly repeated single-turnover flashes on the one hand, and spaced-out single-turnover flashes on the other. Thus, is it reasonable to believe that DQH_2, etc., can keep the PQ pool reduced during the infrequent turnovers. Otherwise, we have to postulate that quinols as dissimilar as decyl-dimethyl benzoquinol and duroquinol (tetramethyl benzo-) are similar reductants, though they are very **dissimilar** when presented to isolated complexes.

IV-H. Where is FQR?

Little progress has been made in confirming the location of the site at which Fd donates electrons which eventually reduce PQ, during cyclic flow (Fig. 1). Some text-books show the *bf* complex as this site regardless of the negative evidence. In fact the existence of a Ferredoxin Quinone oxidoReductase is putative. The inference that FQR will be found on or sometime associated with PS I and not with the *bf* complex [87] is consistent with present views [3] of cyclic electron flow as the main function of PS I and *bf* complex located far away from grana membranes. Another inhibitor class for Fd-catalyzed cyclic photophosphorylation, the dihalogen-naphthoquinones, inhibited at submicromolar concentrations, and were thus analogous to antimycin A [88]. They were identified as acting on the *bf* complex itself, but further analysis of their involvement is necessary. These inhibitors were

effective only on Fd-mediated cyclic phosphorylation, and not when the latter was induced by using duroquinol as electron donor to the *p*-sites of *bf* complexes. Since these complexes are common to the two situations, it is thought that Fd-cyclic must involve a separate oxidase for Fd; hence the postulate of FQR, and its further recruitment as the site of action of AMA and the dihalogen-naphthoquinones. The situation has been confused for many years by the undoubted action of AMA at *n*-sites in the bc_1 complexes; the analogy is proven to be limited.

V. Events internal to the *bf* complex

V-A. The high-potential path – the relation between the Rieske centre and cyt f

A small hiatus exists in the description of electron-transfer events between quinol and cytochrome *f*. Though light-induced turnover of the FeS(R) centre was established in early EPR studies [89,90] the time-scale was long and to this day the kinetics of the oxidation or reduction of the centre remain unknown. It is generally considered that the Rieske centre is an intermediate between quinol and cyt *f*. There is no reason to doubt this. But while published reduction potentials place FeS(R) 30–50 mV more reducing than cyt *f* (see Table III), kinetic evidence suggests that FeS(R) is in fact more redox positive. For example, when the *bf* complex was oxidized by PC^+ after a single-turnover, saturating flash, about 0.4 of the cyt *f* was promptly oxidized [89,24]. A kinetic analysis of this type of data ([24] and see below) suggested that FeS(R) was 30 mV more redox positive than cyt *f*. Despite that, the kinetic coefficients were large, such that FeS(R) and cyt *f* were in rapid redox equilibrium.

The same conclusion had been reached by Rich et al. [47], also using thylakoids, namely that the operational value of the mid-point potential for FeS(R) had to be 370 mV rather than 280–320, to match the observations of quasi-equilibrium concentrations.

Figures obtained through redox titrations (Table III) refer to a long time-scale, using various lipophilic mediators, in the absence of the normal reductants of FeS(R). As seen above, when inhibitors are bound near the FeS(R) centre, the reduction potential (of not only FeS(R)) is profoundly affected. It seems reasonable to suppose that the normal substrates, quinols, also have an effect, as once proposed by Whitmarsh et al. [91]. Use of the isolated *bf* preparation can be expected to clarify this.

V-B. The low-potential path – the relation between cyt b-563(L) and cyt b-563(H)

Reduced cyt *b*-563(L) can probably only be oxidized by cyt *b*-563(H), though it has been suggested [92] that *b*-563(L) might reduce oxidized FeS(R) under certain conditions. This has not been observed. Most accounts put L ≅ 100 mV more reducing than H (Table II), contributing, together with an effective separation distance of < 1 nm between the haem edges, to a fast L → H transfer ($\approx 10^5\ s^{-1}$ [19]) and an equilibrium well towards the high-potential haem. Conclusions in [93] that these two haems are not in electronic communication were not watertight, in that full reduction of *b*-563 (H) by $NADPH_2 + Fd^-$ may not have been achieved in those experiments; indeed the involvement of cyt *b*-559 (low-potential, non-ascorbate reducible) could be inferred from the spectra presented. The argument hinged on assuming the prior reduction of the H haem, because there was under these conditions flash-induced, 'extra' reduction of cyt *b* in the presence of NQNO (discussed further in VI-A below), which, if the H haem were truly reduced, would have to be attributed to cyt *b*(L). The two electrons thus in the complex should have been removed promply by reduction of Q at *n*-sites, unless there was no Q, or indeed there was no electron path between the haems.

VI. Protonmotive cycles

VI-A. Signs of cycling

VI-A.1. The slow electrochromic signal

The slow phase of the absorbancy change at 515–520 nm, the 'slow electrochromic bandshift', which arises after a flash of light, is almost universally atributed to cytochrome *bf* complex activity. Thus the signal is inhibited by DBMIB, which virtually shuts down the complex. It is reduced in size by HQNO or NQNO [71,94,61], substances which were proposed [95,96,22] to alter the charge distribution between species $R.b^-$ and $R.b.Q_n^-$ favouring the $R.b^-$ form, through a change in relative reduction potential. Note, however, that redox titration of the *bf* complex or of chloroplasts [30] has failed to confirm earlier reports [97] of an N(H)QNO effect on the mid-point potentials of the *b*-563 haems. In any case, because of their failure to prevent proton uptake in proton-motive cycling [96] the *n*-quinoline oxide substances are now thought not to be simply inhibitory in the sense of tight binding to prevent electron transfer. This is discussed further below, in the context of the increased degree of cyt *b*-563 reduction caused by NQNO.

Whether inhibitory or acting to modify reduction potential, the explanation of its effect on the slow phase is that charge on species $R.b.Q_n^-$ is situated nearer the stroma than that on $R.b^-$, which is about halfway across the membrane [98,99]. The charge causing the EC slow signal is thus stalled at about the half-way point. In agreement with that concept, H(N)QNO reduces the slow phase to about half of control values (by 40–60%) [100,94,61,30].

The slow phase is detectable in oxidizing as well as reducing conditions [74,94], but is more obvious in the latter when observing the average in a series of spaced-out turnovers. The reason for this is that the number of *bf* complexes able to turn over in oxidizing conditions is less, because usually only about 0.5 PQH_2 per *bf* is generated by PS II, whereas under reducing conditions the PQ pool is reduced, and all *bf* complexes are able to oxidize a quinol [101]. Both the relative sizes, and the kinetic coefficients describing the onset of the slow phase are consistent with the different prevailing quinol concentrations, as simulations show (unpublished). The maximum size of the slow phase signal under reducing (+DCMU) conditions is usually just less than the fast phase, which is due to PS I charge separation. This is also consistent with its suggested mode of generation (charge translocation across the *bf* complex) because in uninhibited complexes one equivalent electronic charge is translocated across the thylakoid, in the steady-state, per flash. The slow phase can be modelled using Q-cycle properties, but other models may also be as consistent (see Section VI-C).

In an different interpretation, which may be more relevant to phosphorylating conditions, Vredenberg and co-workers (e.g., recently [102]) have delineated a 'slow reaction 2' said to contain a component due to proton movements relative to sequestered/free proton domains as well as specifically to *bf* complex reactions. The quantitative importance of the proton domains in the interpretation of the slow EC phase has not been made clear, and it is felt justified to present the interpretation of the previous paragraphs as the majority view, if not that for all time. It is certainly true that the decay of the 518-nm signal is frequently not simple, depending on non-linear conductance through the $CF_0.CF_1$ complex.

VI-A.2. Proton uptake

In nearly all models of cyt *bf* complex activity, in the steady-state quinol is generated at *n*-sites from PQ, electrons from cyt *b*-563(H) and stromal protons:

$$PQ_n + 2e^- + 2H_n^+ \rightarrow PQH_2(n).$$

This proton uptake, combined with that following reduction of PQ_B, contributes to the overall proton-to-electron ratio, the electrons being those passed to the high potential acceptors and PS I. If a ratio in excess of one is observed when using ferricyanide as electron acceptor (in excess of two for the acceptor methyl viologen since it auto-oxidizes with uptake of one proton from the stroma side, per electron), protonmotive cycling of the *bf* complex is inferred. Early reports of ratios greater than one (ferricyanide) were sometimes equivocal (see comments in Ref. 103), delaying general acceptance of the reality of protonmotive cycling in thylakoids. The first valid indication of the 'extra' proton uptake was probably that by Velthuys [104] under conditions where pre-illumination had produced a partly reduced PQ pool; proton uptake was then detected after flashes in the added presence of DCMU to inhibit PS II. Proton uptake over and above that due to reduction of PQ_B or to auto-oxidation of the electron acceptor methyl viologen was clearly demonstrated by Hangarter et al. [105] under oxidizing and reducing conditions. Under reducing conditions, *bf* proton uptake has been kinetically distinguished from that due to methyl viologen auto-oxidation [96], and shown to be NQNO-insensitive. It was flash number-dependent at pH 7.4, but less so at other pH values. Under 'super-oxidizing' conditions, with a background of FR illumination, cyt *bf* complex activity has been shown also to include proton uptake [106].

VI-A.3. Cytochrome b-563 reduction

Similarly, most models of protonmotive cycling by the *bf* complex include some measure of cyt *b* reduction. Is the cycle always able to be completed (in the absence of inhibitors) even if an electron reaches cyt *b*? It has been suggested that cyt *b*-563(L)$^-$ might reduce oxidized FeS(R) or cyt *f* under very oxidizing conditions [92], but evidence that a normal protonmotive cycle occurs under these conditions [106] is against this. If the turnover rate for a particular complex is low, it is possible for the 'one-electron state' to decay, as discussed above (Section IV-D). Thus, in dim light there could be an indirect oxidation of cyt *b*, but the electron sink may ultimately be oxygen or alternatively quinone as in the regular cycle.

VI-B. Q cycle is the product most recommended by doctors

The Q cycle has undergone several successful metamorphoses to account for new evidence, since it was proposed for mitochondria by Mitchell [107]. For this history, refer to Refs. 5,6. In a perceptive review [108], several sticking points were aired, which the Q-cycle model was thought not to dissolve. These included some quite relevant to chloroplasts:

(i) The necessity for quinone radicals at *n*-sites to be more tightly bound, or in other words for the couple $PQ/PQ^{-\cdot}$ to have a much more positive reduction potential, than those generated at *p*-sites; the latter couple is assumed to be about -100 mV to accomplish reduction of cyt *b*-563(L). Reduction of Q_n by cyt *b*-563(H) would be difficult if this difference in binding property were absent.

(ii) The lack of evidence (at that time) that quinols bind to the FeS(R) protein (above), and the suggestive evidence from photoaffinity labelling that it does not. The label in question was, however, not as suitable as the azido-Q used with *bf* complexes [43].

(iii) The apparently faster reduction of cyt *b*(-562) than of oxidized cyt c_1. Jones and Whitmarsh [109] have referred to the same apparent difficulty in chloroplast experiments with respect to cyt *b*-563 and cyt *f*.

Of (i), little more can be said at present, without new information about stability constants of the quinone radicals at the two sites. The slowness of cyt *b* oxidation, following two flashes which led to the reduction of fully one cyt *b* per complex was taken to show that electron sharing was difficult and the reduction potential of the couple $PQ/PQ^{-\cdot}$ at *n*-sites was set at -100 mV [110]. However, as seen later, kinetic modelling is more consistent with a reduction potential of about 0 mV.

Point (ii) seems not a problem, since there is now a solid basis for implicating the hydrogen-bonding amino acids liganding the Rieske centre (Section IV-B); the apparent inconsistency between the photoaffinity binding information, and the implications from inhibitor site mutation experiments remains.

Point (iii) implies that the reduction of *b* is not 'oxidant-induced', which is a cornerstone of the Q cycle (and others). Some plausible qualitative arguments to explain the apparent anomaly and retain oxidant-induced *b* reduction were given in the literature as early as 1979 [73] and later [47,22,111]; the reverse order of reduction, *b* before *f*, would necessitate a change in the accepted reduction potential of the couple $PQH_2/PQ^{-\cdot}$ or of *b*-563(L)/*b*-563(L)$^{-\cdot}$ for the former readily to reduce the latter. Attempts have also been made to explain the observations quantitatively, in two approaches. In [47], electron transfers through the high-potential components and towards cyt *b*-563 were simulated with the preferred model wherein PQH_2 first reduces the Rieske centre then cyt *b*-563, but only the initial rate of *b*-563 reduction was returned. This rate and that for cyt *f* re-reduction were well matched by the experimental data.

In Refs. 22,24, as well as modelling the high-potential path, cyt *b*-563(H) was considered as kinetically the middle component of three, the others being $PQ_p^{-\cdot}$ and PQ_n. Cyt *b*-563(L) was omitted because all the indications are that electrons spend little time on it on the way through. The data was satisfactorily described if the production of the PQ radical at *p*-sites, followed by its rapid reduction of cyt *b*-563, is simultaneous with the reduction of cyt *f* (via a rapid path through FeS(R)). In other words, the oxidation of PQH_2 is the concerted loss of two electrons (and two protons, the order is not addressed here). Even if it were possible to prevent the electron on cyt *b* being shared with the quinone at *n*-sites, and still observe a faster cyt *b* reduction than cyt *f* re-reduction (a comparable observation has been made with the cyt bc_1 complex when Q-reduction sites (*n*-sites) were blocked: see [6]), the above model would not necessarily fail because the apparent reduction rate of cyt *f* depends on the kinetics of electron-transfer events on both sides of it, namely $FeS(R) \rightarrow f^+$ and $f \rightarrow PC^+$.

Related to the above is the effect of NQNO on cyt *b*, cyt *f* reduction and on the slow EC signal, for which new data have been obtained [61,75]. Our model, of 'concerted quinol oxidation', predicts that the half-time for reduction of cyt *b* will be greater with NQNO (figure 4 in ref. [22]), if it steers the equilibrium towards more electrons on cyt *b*-563(H), even though the inherent rate-coefficients for QH_2 oxidation are unchanged. This half-time was not observed to change with up to 1 μM NQNO in [61], but was clearly greater in earlier reports of the same authors [71], and in our experiments [22]: $t_{\frac{1}{2}} > 3$ ms, 1 μM NQNO, but ~ 2 ms without NQNO; better deconvolution of the spectral changes from 0–5 ms to give more detailed cyt *b* reduction kinetics might resolve this disagreement.

Other effects of NQNO [61] are impossible to explain by the hypothesis that it affects only *n*-sites. Cyt *f* re-reduction rate, onset rate of the slow EC signal and the extent of cyt *b*-563 reduction were all affected similarly over the same range of concentration (0.01–1.0 μM) of NQNO. The most economical hypothesis is that NQNO binds to a single site, suggested to be the *n*-site [61], at which it influences both quinol oxidation and quinol reduction. The slowing of both cyt *f* re-reduction and of the onset of the slow phase EC signal are characteristic of a decrease in the second-order rate-coefficient k_{QR} used in modelling the Q cycle [22,24]. The best explanation of the increased extent of cyt *b* reduction is the kinetic reasoning offered by Rich et al. [30], that the rate-coefficient for cyt *b* oxidation by PQ_n (k_{HQ}) is diminished by NQNO; a factor of nine was proposed. The diminished rate-coefficient would explain also the slower oxidation rate for cyt *b* in the presence of NQNO [112,72]. A different set of coefficients must apply to the oxidation of the one-electron-reduced *bf* by $PQ_n^{-\cdot}$, since proton uptake consequent on that reaction is unaltered (above). The lack of inhibition of electron throughput in DC light with less than about 1 μM NQNO is unfortunately not explained.

The substance MOA-Stilbene has recently been reported to act like NQNO with respect to changes in cyt *b* reduction and oxidation and diminution of the slow electrochromic signal, but with the difference that it does not appear to inhibit at PS II [75]. In this study, a maximum reduction level of cyt *b*-563 of 0.7 molecules per *bf* complex after a DC light pulse was understandable only if, for example, the one-electron reduction state of the complex had access to another site (compare IV-C above).

Some further odd observations exist that are not easy to fit to the 'simple' Q cycle:

(i) Proton release into the lumen space from PQH_2

oxidation (reducing conditions, +DCMU, $+DQH_2$) was biphasic [113], about half of the deposition being fast, the remainder quite slow by comparison. The rate of the slow portion could be accellerated up to the fast phase rate by the addition of, at most, 100 nM valinomycin or nonactin. This observation suggests a scheme where one of the protons is translocated against an electric potential difference, the other, not. This remains unexplained.

(ii) The anomalous oxidation rate of cyt *b*-563(H) in reducing conditions (Section IV-D).

(iii) The slow phase of the electrochromic signal decays very slowly [94,24], more slowly than the fast phase(s) due to charge separation at reaction centres. Its decay can also be accellerated by protonophores such as nonactin. The onset of the slow phase should have the same time-course as those species carrying or storing charge across (in) the thylakoid. These include cyt *b*-563(H)$^-$, $Q_n^{-\cdot}$, and OH^- arising from proton uptake at the stromal side. Of these the last is subject to decay as ions are conducted passively across the membrane; it should have a decay similar to that of the fast electrochromic signal. The first species is relatively stable in oxidizing conditions (see Section IV-D), but the stability of the slow EC phase extends to reducing conditions, where cyt *b*-563 oxidizes quickly. At the least, it seems that protonophores can reach and discharge all the charged species, which is not obvious a priori. The slow phase is almost universally inferred from a subtraction of a 518-nm signal, +DBMIB, from that of −DBMIB. There could be an error in estimating the slow phase decay rate if the decay of the fast EC signal (+DBMIB) is not representative of its decay in the absence of DBMIB. But since stigmatellin produces the same slow phase, this error seems unlikely.

VI-C. The SQ cycle

The semi-quinone cycle, originally proposed for the mitochondrial bc_1 complex [114], describes an alternative means of oxidation of the semi-quinone radical [1] produced when QH_2 is oxidized at *p*-sites. Instead of reducing the low-potential cyt *b*, the SQ traverses the membrane from the lumenal side towards the stroma side where it shares its remaining electron with cyt *b*-563(H). Earlier experiments [116] where a slow EC signal was observed under redox-clamped conditions designed to reduce both the *b* cytochromes constituted one of the few pointers towards the SQ cycle for chloroplasts. One of the problems with this type of experiment is to be sure that the haems are indeed reduced between light flashes; even a slightly actinic measuring beam will tend to oxidize them. Opposing results have been reported [117], the slow EC signal being titrated out at redox potentials where the *b* cytochromes would have been reduced; reasons were later proposed for the disagreement [118], based on the differing redox mediators that were used. Rich and Moss [119] in considering the above and their (unpublished) experience with other mediators concluded that the slow phase can be titrated out at low reduction potentials if using an appropriate lipophilic mediator.

Joliot and Joliot [100,120] used intact *Chlorella* cells in which both the *b*-563 haems were in the reduced state in the dark due to an interaction with reducing species of the respiratory chain. The subsequent flash-induced oxidation of cyt *b* was associated with a slow electrochromic signal; this was interpreted as indicating that a quinone radical produced at *p*-sites was able to traverse the membrane to oxidize cyt *b*-563(H) with subsequent electrogenic proton uptake. Ref. 121 is also notable for its description of a reducible component 'G', on the *bf* complex stroma side; G was not detectable in chloroplast preparations and has not been further characterized. Because of a difference in the reduction rates of the two cyt *b*s, it was possible to distinguish their separate oxidized-minus-reduced spectra (Fig. 11 in [120], and see Table II). In the 1986 interpretation [100], the PQ radical oxidized cyt *b*-563(L), with proton uptake occurring via a proton channel from the stroma to the region of the *p*-sites (see Section VI-D below). Moss and Rich [121] reduced thylakoid *b*-563 haems with AQS plus benzyl viologen. While reporting essentially similar results to those in [100], they have argued for the transmembrane movement of PQH, not the radical anion, to oxidize cyt *b*-563(H). In that event, the cycle would not be coupled to proton translocation across the thylakoid. It may be that the SQ cycle occurs under such conditions, but that a Q cycle operates in more oxidizing conditions. In racing parlance, this is betting two bob (ECU) each way.

Kinetic modelling of redox changes in chloroplast *b* and *f* cytochromes and PC (VI-E below) was unable to differentiate the SQ-model from the conventional Q-cycle version [24], but the distinction might be made by careful measurement of the H^+/e^- ratio together with that of the slow electrochromic signal, in the appropriate conditions.

VI-D. Proton pumps

The proton movement alluded to above, in cells with reduced *b* haems [100], where protons were supposed to move through the thylakoid from *n*- to *p*-side to reduce $PQ_p^{-\cdot}$ together with an electron from cyt *b*-563(L), is a form of redox proton pump, since the

[1] A primitive SQ-cycle was proposed by Kröger [115] in which semiquinone radicals traversed the membrane but did not specifically interact with a bc_1 complex.

protons are not carried by PQ. A proton pump is usually defined, and distinguished from the proton movements described in the conventional Q cycle, as the translocation of protons as a result of redox changes in prosthetic group(s) at a distance from the proton events [122]. The use of the proton pump hypothesis, it is fair to say, has been influenced by the solid evidence indicating such a proton-translocating mechanism in the cytochrome *c* oxidase system [122]. The facts of quinone chemistry might incline one to favour Q or SQ cycles, but surprises must not be ruled out. For example, DCCD, the classic inhibitor of proton movement through the F_0 proton channel when bound to a Glu carboxyl [123], has been found to bind, with mild specificity, to spinach chloroplast *bf* complex [124]; DCCD inhibited proton ejection from vesicles loaded with *bf* complex without effect on electron throughput (definitely against the rules). In Ref. 125, somewhat similar inhibition was found but the effective [DCCD] was for some reason 300–400 mol per mol cyt *f*, 10-times that used in Ref. 124. The binding site could have been Asp 155 or Glu 166 in the *b* subunit [125]. Reference to Fig. 2 will show that, in the current model, these residues are in a sequence that joins hydrophobic helices 3 and 4. Whether this area forms or guards a proton channel in spite of being close to the lumen phase is an intriguing question.

VI-E. Factors controlling the protonmotive cycle

Several factors have been seen as important in the operation (or not) on turnover rate of the *bf* complex. Thus the potential difference across the thylakoid was supposed, if great enough, to prevent the charge transit from *p*-side to *n*-side signalled by the slow EC signal. This latter was observed apparently to decline or disappear in repeated flashes [126,127,94], but more recent experiments [101] have thrown doubt on the interpretation of the data. It appears that the decay of the electrochromic signal, at an increasing rate as its total size increased, may have obscured the slow phase, and that the charge movements giving rise to it normally are still occurring during a regime of repeated flashes.

High light intensity was found to affect the observed proton-to-electron ratio found from steady-state proton release rates using ferricyanide [128]. In these experiments, the ratio decreased from about 3:1 to 2:1 over a range of increasing light intensity, leading to the hypothesis that a high prevailing proton motive force in strong light inhibited the charge transfer across the membrane, and hence the protonmotive activity. Rich [101], having noted a DCMU-insensitive proton release in very strong light in such experiments, has suggested that the electron-transport rate in very high light (more than needed to saturate oxygen evolution)

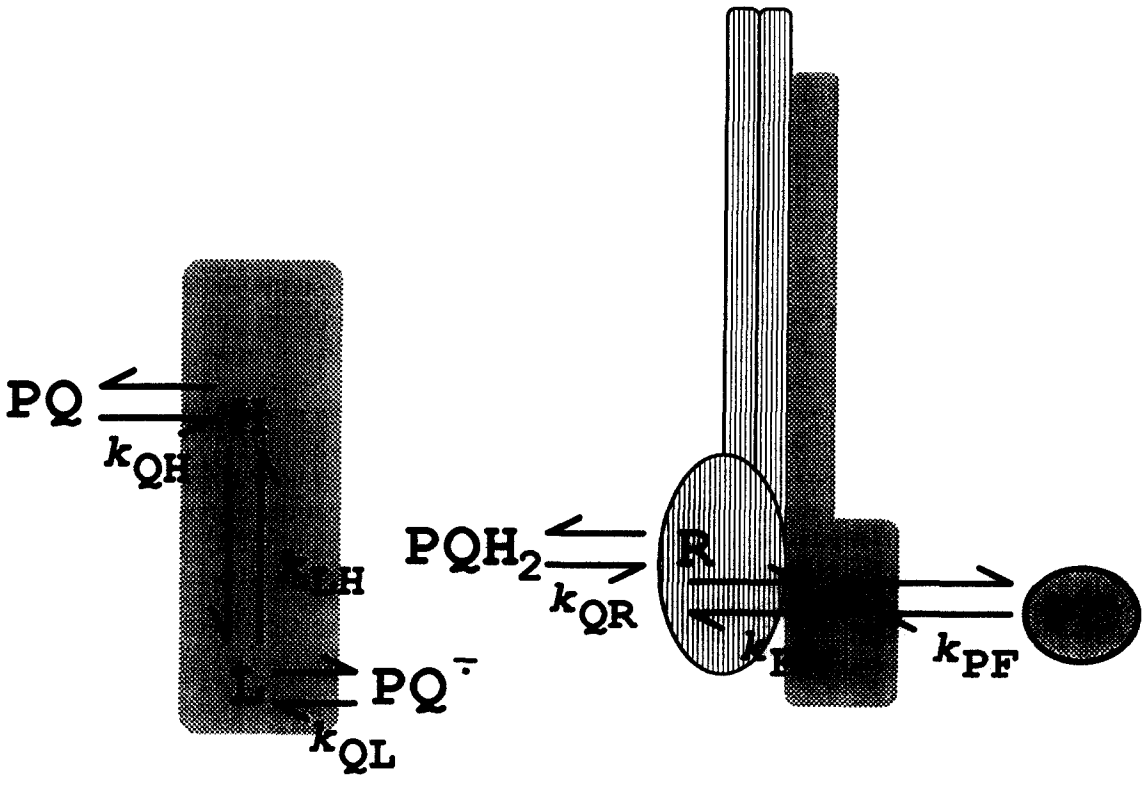

Fig. 6. Diagram showing the 12 kinetic rate-coefficients necessary for a full description of the electron-transfer events between the cytochrome *bf* complex, plastoquinol, plastoquinone radical anion, plastoquinone and plastocyanin, together with internal electron transfers. Reverse coefficients are not named. The cyt *b* subunit is on the left.

could have been overestimated. This would have led to an **under**-estimation of the proton-to-electron ratio, which may well be 3:1 over the physiological range of light intensity.

Though the cherished beliefs of yesteryear have been quite eroded, the proton motive force may still indirectly influence the turnover rate of the complex, though not, as we see, acting as a throttle on the low-potential path only. High values of PMF are linked with a lowered lumenal pH, which may affect the reaction:

$$PQH_2 + R^+.b.Q_n \xrightarrow{k_{QR}} PQ + R.(b.Q_n)^- + 2H_p^+$$

through mass action. Alternatively, a weakly basic group (pK 6.5) near the Q binding region may need to be ionized to catalyze quinol oxidation. The following sequence has been suggested [5] ('+' outside the external brackets signifies the charge on this group):

$$PQH_2 + (R.b.Q_n)^+ \rightarrow PQH^- + (R.b.Q_n)^+ + H_p^+$$

$$PQH^- + (R^+.b.Q_n)^+ \xrightarrow{k_{QR}} PQ + (R.(b.Q_n)^-)^+ + H_p^+$$

This scheme has the advantage of giving the required pH-dependency, in which activity increases up to ~pH 6.5, but not beyond, despite the pK for ionization of PQH_2 being 10.8. As well, a species suitable for reduction of the Rieske centre is produced, it being thought that the couple is not sufficiently reducing. Despite these reasonable suggestions, based on known electrochemistry, the precise mode of quinol oxidation is still hypothetical.

VII. Modelling electron- and proton-transfer steps

Modelling of various degrees of complexity has been attempted [129,47,22,92,23,24] with the aim of testing hypotheses about the operation of the cytochrome *bf* complex.

In a complicated system like this complex plus its electron donors and acceptors, the observable kinetic coefficients may not be easily related to the rate-coefficients of individual electron-transfer steps. Also, if these are reversible reactions, the complication goes further. In Fig. 6 are shown the probable rate-coefficients which should be known for a full prediction of

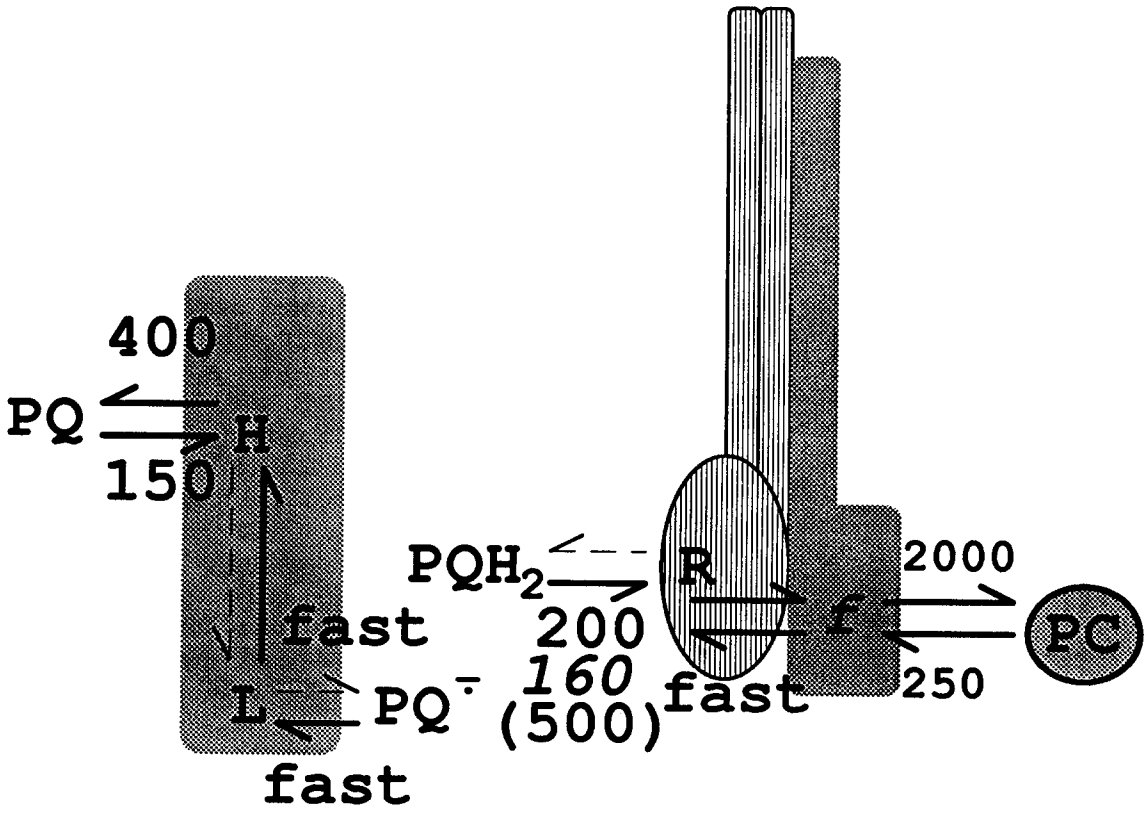

Fig. 7. Some estimates of a few of the rate coefficients of Fig. 6; from [24] except for italicized k_{QR} from [23] which refers to oxidizing conditions, and the bracketted figure for k_{QR} from [129] which refers to a rate-coefficient that optimized the agreement between data for $P700^+$ reduction in far red light, following a flash; values half or twice as large gave a worse agreement. The other estimates were arrived at [24] by applying the Inverse Method to data on averaged cyt *b*-563, cyt *f*, PC turnover following repeated single-turnover flashes together with data for the slow electrochromic signal and proton deposition, all in reducing conditions with propyl benzoquinol as reductant of the *bf* complex. Some rate-coefficients that refer to second-order reactions are reduced coefficients, with units s^{-1}, because the rate equations were for relative concentrations of *bf* complex species, e.g., '$[f^+.R.H]$' divided by [P700], the latter being one per *bf* complex.

the performance of the system. The number exceeds by far the number of data sets obtainable by current techniques. Inevitably, simplifications are necessary to simulate or model the system. These take the form of (i) assuming some steps are essentially irreversible, (ii) assuming some steps are so fast relative to all others that certain intermediates can be omitted from the scheme and (iii) ignoring some side reactions on the grounds that the yield is small.

Our own approach [22,24] has included all three simplifications. Some are justifiable on reasonable grounds; thus we have argued [22] that the relative reduction potentials and separation distance between cyt *b*-563(L) and cyt *b*-563(H) lead to a very fast transfer between the two (Section V-B above), which is virtually irreversible. In agreement with that, attempts to deconvolute a signal indicating reduction of Cyt *b*-563(L) have failed [130] and it is thus omitted from some simulations. With reference to (iii), some forms of the system, such as $(f^+.R^+.b.Q_n)^{0/-}$ may be quite low in yield because, in this instance, to produce it, a second oxidation of a *bf* complex is required during the life of the singly oxidized species.

Thus, we have set up a minimal set of reactions, with differential equations to describe the rates of variation of the concentration of all relevant species in terms of the rate-coefficients of the reactions; given the initial concentrations of the species, suitable software programs will return the species concentrations as functions of time. The simulations have mostly used the device of instantaneously generating the species $P700^+$ or PC^+ as by a brief, saturating light flash. Time-courses obtained from such simulations can be compared with experimental data. Possibly, several model variables such as $[f.R.b^-]$ and $[f^+.R.b^-]$ would have been summed to compare with the measured reduced cyt *b*-563. While trial-and-error changes to the model rate-coefficicents could optimize them with respect to the observations [24], the procedure is lengthy and such subjectivity is to be avoided. The Inverse Method [131] was employed to solve both problems, but has to be used with care to avoid solutions where the error between the model data and the observations has merely reached a local minimum and not the global minimum. The Inverse Method is a means of objectively optimizing the rate-coefficients by systematically varying them while comparing the model data with the corresponding experimental data until some specified, minimum error integral is reached.

The result of this process has been to suggest, with varying degrees of precision, some new rate-coefficients for chloroplast thylakoids. These are shown in Fig. 7. It is difficult to describe their precision, except that the error integral was more sensitive to changes in some parameters than others [24]. Having settled on a satisfactory model (in this instance the Q cycle), the experimental conditions can be widened to test further the model. Extension to isolated systems of complexes in suspension or in vesicles can be anticipated. Improvements to the simulations might in some cases include consideration of binding/unbinding rates and equilibria, for example in PC/P700, cyt *f*/PC and PQH_2/FeS(R) interactions.

VIII. Conclusions

The overall operation of the cyt *bf* complex, in its electron-transfer role, is to act as a transformer of two-electron redox energy to one-electron. It is specialized to oxidize plastoquinol which has received electrons, two at a time, from either PS II or from Fd via a ferredoxin/quinone oxidoreductase as yet unidentified. The processing involves withdrawal of one electron into the high-potential side of the complex, and simultaneously the other into the low-potential side. Accompanying the electron loss is the release of two protons into the lumenal space. This mode of operation is the norm for non-cyclic and cyclic electron flow, and extends to all redox conditions of the *bf* so far examined. The electrons arriving in the low-potential arm of the complex are claimed by PQ or $PQ^{-\cdot}$, with the uptake of protons and generation of PQH_2. The rate and mode of formation of this quinol depend on turnover rate, and on the redox state of the Q pool and of the *bf* complex.

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