FEBS Letters 586 (2012) 603–616

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Back-reactions, short-circuits, leaks and other energy wasteful reactions in biological electron transfer: Redox tuning to survive life in O_2

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ARTICLE INFO

Article history:
Received 10 October 2011
Revised 15 December 2011
Accepted 24 December 2011
Available online 13 January 2012

Edited by Miguel Teixeira and Ricardo Louro

Keywords:
Electron transfer
Photosystem
Evolution
Cytochrome b_{c1} and b_6f
Bioenergetics
Reactive oxygen species (ROS)
Oxidative stress
Protective reactions

ABSTRACT

The energy-converting redox enzymes perform productive reactions efficiently despite the involvement of high energy intermediates in their catalytic cycles. This is achieved by kinetic control: with forward reactions being faster than competing, energy-wasteful reactions. This requires appropriate cofactor spacing, driving forces and reorganizational energies. These features evolved in ancestral enzymes in a low O_2 environment. When O_2 appeared, energy-converting enzymes had to deal with its troublesome chemistry. Various protective mechanisms duly evolved that are not directly related to the enzymes' principal redox roles. These protective mechanisms involve fine-tuning of reduction potentials, switching of pathways and the use of short circuits, back-reactions and side-paths, all of which compromise efficiency. This energetic loss is worth it since it minimises damage from reactive derivatives of O_2 and thus gives the organism a better chance of survival. We examine photosynthetic reaction centres, bc_1 and b_6f complexes from this view point. In particular, the evolution of the heterodimeric PSI from its homodimeric ancestors is explained as providing a protective back-reaction pathway. This "sacrifice-of-efficiency-for-protection" concept should be generally applicable to bioenergetic enzymes in aerobic environments.

1. Introduction

Biological energy conversion mainly operates through membrane-spanning enzymes that build up a transmembrane electrochemical potential using the driving force provided by exergonic reactions [1]. As with all enzymes, those involved in energy conversion act as catalysts. Thus, even though the reaction pathways may involve a series of intermediates, completion of the enzyme cycle brings the enzyme back to its ground state. As energy-converting devices, their reaction pathways involve high-energy intermediates, which react exergonically providing the work required to drive the accumulation of the electrochemical potential (see Fig. 1).

These two basic characteristics pinpoint one of the main challenges encountered by these enzymes: how to favour the energy-productive processes over competing reactions in which the high energy intermediates decay without going through the useful energy converting step(s). In other words, to be efficient they must avoid back-reactions, short-circuits, by-passes, side-reactions, futile cycles and leaks (Fig. 1).

Owing to the thermodynamic reversibility of the forward, productive reactions, intermediates may simply decay by back-reacting to the ground state of the enzyme via the thermally-activated repopulation of higher energy states (Fig. 1). The transiently-stored energy would thus be released without any work being extracted from the overall process.

Productive energy conversion can be lost by processes other than simple back-reactions (Fig. 1). Such processes could involve charge recombination from a high energy state directly to the ground state without retracing the steps of forward electron transfer. Equally they could involve the formation of a lower energy form of the same component, such as a change in the protonation state or a secondary change in the redox state, reactions common in quinone chemistry. These may be termed "short-circuits" or "by-passes" depending on rates, routes, distances and semantics. In other cases, the lower energy intermediates may involve components different from those involved in the productive pathway. Such routes can be called "side pathways", in some case these could fall into the category of "futile cycles". The range of routes that lead to energy loss is broad and this is reflected by the loosely fitting terminology (Fig. 1).

An additional route of energy loss is worth mentioning specifically: the leak. In this category the main focus of attention is the 1-electron reduction of O_2 by reducing components of the electron

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0014-5793/$36.00
doi:10.1016/j.febslet.2011.12.039

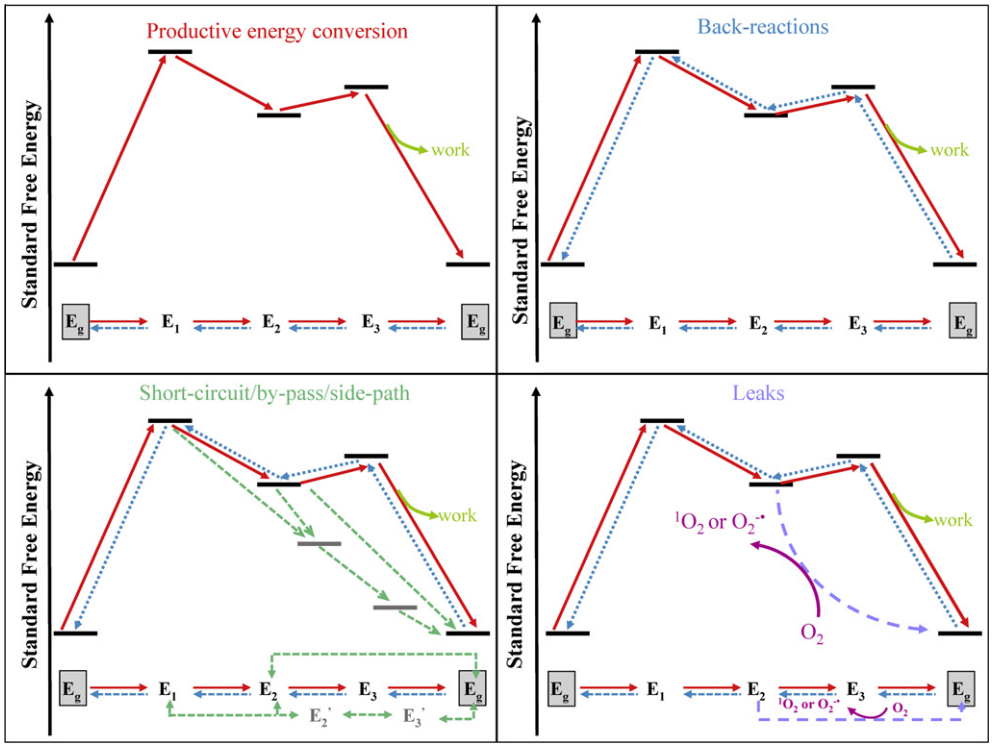

Fig. 1. Scheme illustrating the energy landscape of an energy converter (and an opportunity to define some terminology). A succession of different intermediates states denoted E_{1-3} where g stands for ground state are shown and their energy level is marked as a thick black bar. The intermediate higher energy states may represent excited states, charge pairs, or simply reduced or oxidised species. The main productive pathway is shown as solid red arrows: the energy input promotes the formation of the E_1 state at the expense of E_g. The decay of E_1 to E_g (via E_2 and E_3) is downhill in energy and is coupled to the desired energy conversion reaction, designated "work" in the scheme (i.e. oxidation or reduction of a substrate and/or the pumping of a proton or protons). The reversibility of these reactions is shown by the presence of broken blue arrows. In normal function these back reactions are disfavoured compared to the forward reactions. Under certain circumstances the energy can be wasted when the back reactions become significant. Energy can also be lost by short-circuits, by-passes and side pathways depicted in a simplified form by the broken green arrows in the lower left panel. The definition of a short circuit versus a by-pass or side pathway etc is somewhat semantic and may be decided by the rate and routes of the reactions occurring. Short circuits would be fast and direct, as illustrated by the direct conversion of E_2 into E_g. By-passes would involving additional intermediates, as illustrated by a conversion of E_2 into E_g via E_2' and E_3'. These additional intermediates may be relaxed or modified forms of the cofactors involved in productive reaction. Side-pathways can be defined as the involvement of other electron transfer components within the complex and these can also be considered as a type of by-pass, and in some cases these can be classed as "futile cycles". Energy may also be lost by electron transfer leaks. Perhaps the most relevant case is shown in violet and purple in the lower right panel, where reducing electrons reduce O_2 to form $O_2^{-\bullet}$. Another case which is particular relevant to photosynthetic systems is the energy leak that occurs when triplet O_2 is converted to the ultra-reactive singlet O_2 due to interaction with triplet state of chlorophyll.

transfer pathways to produce superoxide radical, $O_2^{-\bullet}$ (Fig. 1). Superoxide is one of the so-called "reactive oxygen species", it is easily reduced to form even more reactive species (peroxide and thence $OH^{\bullet}$) [2] that damage biological material and it is implicated in ageing and disease (see e.g. [3]). Superoxide is also thought to play an important role in signalling pathways within the cell [4].

The structures of all the major actors in the photosynthetic and respiratory electron transfer chains are now known at levels of resolution sufficient to identify the redox cofactors involved in the intracomplex electron transfer and to estimate the distances between them. This provides a robust framework for the theoretical determination of the electron transfer rates between a given donor and acceptor pair [5,6]. In addition, the functional characterization of some of these enzymes under various (usually inhibited) conditions has allowed the study of the non-productive pathways (short-circuits/by-passes/side-pathways etc). Thus a rather detailed picture has emerged, within which can be discerned the basic principles that determine the competition between productive and non-productive reactions and thus their respective yields. Taking Photosystem I, Photosystem II and the cytochrome bc_1 and b_6f complexes as examples, in this review we shall highlight the mechanisms that tune the yields of the competing pathways and discuss the possible role of short-circuits etc as protective routes under adverse conditions.

2. Photosynthetic reaction centres: an overview

All known photosynthetic reaction centres share the common structural feature of pseudo-C_2 symmetry both at the level of the protein backbone and of the arrangement of the redox cofactors (Fig. 2) (see e.g. [7–11]). This reflects their evolution from a common ancestral reaction centre which was made up of a homodimer of core protein subunits [12]. Despite this basic structural similarity, two classes of reaction centres are defined based on their terminal electron acceptors. Type II reaction centres use light to drive the reduction of quinone, while Type I reaction centres use light to drive the reduction of ferredoxin (or flavodoxin).

In Type II reaction centres, there are two bound quinones, Q_A and Q_B, which act in series as electron acceptors. Q_A acts as a 1-electron carrier, while Q_B undergoes two sequential reductions and associated protonations to form the quinol, Q_BH_2 [13]. The quinol then exchanges with an oxidized quinone from the membrane. This function requires that the semiquinone, $Q_B^{-\bullet}$, formed on the first photochemical turnover, remains stable until a second electron arrives upon the subsequent photochemical turnover. In Type I reaction centres, the terminal electron acceptors are iron-sulfur clusters but two bound quinones are also present. In this case, the quinones are both 1-electron carriers, relaying electrons from photoreduced chlorophyll to iron-sulfur clusters (see [14] for a review).

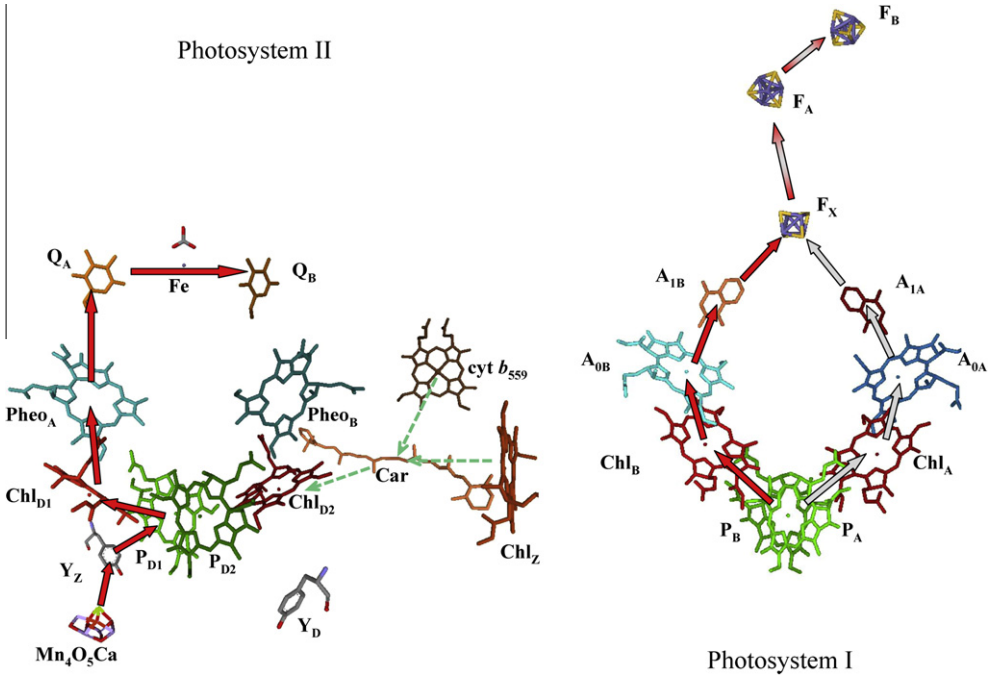

Fig. 2. Scheme illustrating the structure of Photosystems I and II. The left panel shows the structural arrangement of the cofactors involved in electron transfer in Photosystem II (redrawn from PDB: 3ARC [93]). Note that the two redox active tyrosines (Y_Z and Y_D) are symmetrically arranged but the perspective makes them look otherwise. In PSII the red arrows indicate the charge separation pathway and the broken green arrows shows the side-pathway (see [46] for a recent review). Cofactors are labelled with abbreviations P, chlorophylls on which the cation is localised, Chl, chlorophyll; Pheo, pheophytin: Q quinone, Car, carotene, cyt, cytochrome. The D1 and D2 subscripts relate some of the components to the protein to which they are mainly associated. Other subscripts are for distinguishing specific components. The non-heme iron Fe is also shown with its non-protein ligand, bicarbonate/carbonate. The right panel shows the structural arrangement of the cofactors involved in electron transfer in Photosystem I (redrawn from PDB: 1JB0 [8]). The red and grey arrows represent charge separation that can occurs on either side of the near pseudo homodimer with a near equal probability [138]. P, Chl and A_0 are three chlorophylls A1 is phylloquinone. The subscripts B and A designate that the cofactors have their main ligation coming from the PsaB and PsaA proteins. The iron sulfur centre Fx connects the B and A proteins, while FA and FB are cofactors in a third subunit psaC.

Thus, whereas Type I reaction centres work as purely monoelectronic devices that are not coupled to protons, Type II reaction centres couple the monoelectronic photochemical charge separation to the two-electron (and two-proton) reduction of a quinone to a quinol. As will be discussed below, this essential functional difference results in a strong mechanistic constraint that has shaped the evolution from Type I to Type II reaction centres.

The energy wasteful reactions are a particular problem for photosynthetic reaction centres because the photochemistry gives such high energy intermediates. This problem is mainly dealt with through kinetic control: i.e. the forward reactions are faster than the back reactions. This is achieved by having cofactors appropriately spaced within the protein to allow rapid vectorial electron transfer across the membrane, separating the positive and negative charges from each other. Small energy losses occur on the forward reactions thus making back-reactions thermodynamically unfavourable. In contrast, the direct recombination reactions of the radical pairs are strongly exergonic. In this case however the standard free energy gap is so big that the reactions fall in the "Marcus inverted region" and are thus relatively slow [15]. In addition, as the distance increases between the two charges of the radical pair, direct charge recombination electron transfer routes become slower: short-circuits decrease [16].

When highly reducing intermediates are formed they will have a tendency to react with O_2 if it is present. This is a particular problem for PSI where even the terminal acceptors are more reducing (-520 mV) than the O_2/ O_2^- couple (-330 mV under 10^5 Pa of O_2 and -160 mV for O_2 in aqueous solution, with $[O_2] = 1$ M [2]). When very oxidizing species are formed, and this is a particular problem of Photosystem II, the adventitious oxidation of cofactors (and perhaps proteins and lipid) can occur and may propagate out from the reaction centre. Both of these can be considered as "leaks".

When high energy radical pairs involving chlorophyll recombine, they can form chlorophyll triplet states that can react with O_2 to form singlet oxygen, a reactive oxygen species that is much more damaging than superoxide. This problem is common to all reaction centres in aerobic environments. This can be considered a short-circuit (chlorophyll triplet formation), resulting in a leak (triplet-sensitized $^1O_2$ formation, a leak of energy not of an electron), in some cases preceded by a back-reaction (e.g. $P^+Q_A^-$ to $P^{\cdot+}Phe^-$). In this case the energy loss to the leak does not drive the short-circuit, as the triplet state would decay, albeit more slowly, without reacting with oxygen. The consequences of $^1O_2$ formation are presumably so negative that a range of strategies are employed to prevent this route from occurring.

3. Type I reaction centers

Photosystem I is a plastocyanin/ferredoxin photooxidoreductase (in some species and conditions the donor may be cytochrome c_6 and the acceptor flavodoxin) and is present in plants, algae and cyanobacteria. It is thought to have evolved from an ancestral homodimeric reaction centre that resembled those in the present day *Heliobacteria* and *Chlorobiacae,* both of which grow in anaerobic conditions [17]. These homodimeric reaction centres are composed of two identical subunits, each bearing a redox chain capable of light-driven charge separation [18,19].

Even in the absence of a crystallographic structure, the main structural features of the *Heliobacteria* and *Chlorobiacae* reaction centres can be deduced given the similarities to PSI. The two electron transfer branches diverge from a (bacterio)chlorophyll pair,

which is close to one side of the membrane, and converge at the level of the F_X iron sulfur cluster on the other side of the membrane [12,20–22]. In a homodimer, with a truly symmetric structure, both pathways are expected to function symmetrically.

3.1. Heterodimeric PSI: adaptive redox tuning to deal with life in O_2

Existing PSI has evolved to have greater asymmetry, with duplication of the core reaction centre gene and separate evolution of the two resulting genes giving rise to a heterodimeric reaction centre [17,23]. The two near-symmetrical electron transfer branches of PSI, which have around 60% identity between the A and B subunits, thus show differences at the amino-acid side chain level resulting in several functional differences (see Figs. 2 and 3).

Under normal circumstances, when PSI undergoes photochemistry, reduced F_A/F_B is rapidly oxidized by ferredoxin or flavodoxin. Prior to the activation of the CO_2-fixation enzymes however, the amount of oxidized ferredoxin is limited and it builds up in its reduced form. The back-reaction $F_{A/B}$ with P_{700}^+ is 40 ms, but under normal circumstance reduced donors are available and electron donation prevents the back-reaction. $F_{A/B}^-$ will thus accumulate, when fully reduced, further light excitations will result in formation of F_X^- and the two A_1^- acceptors, all three of which are rather close in energy and these will back-react with P_{700}^+ when present

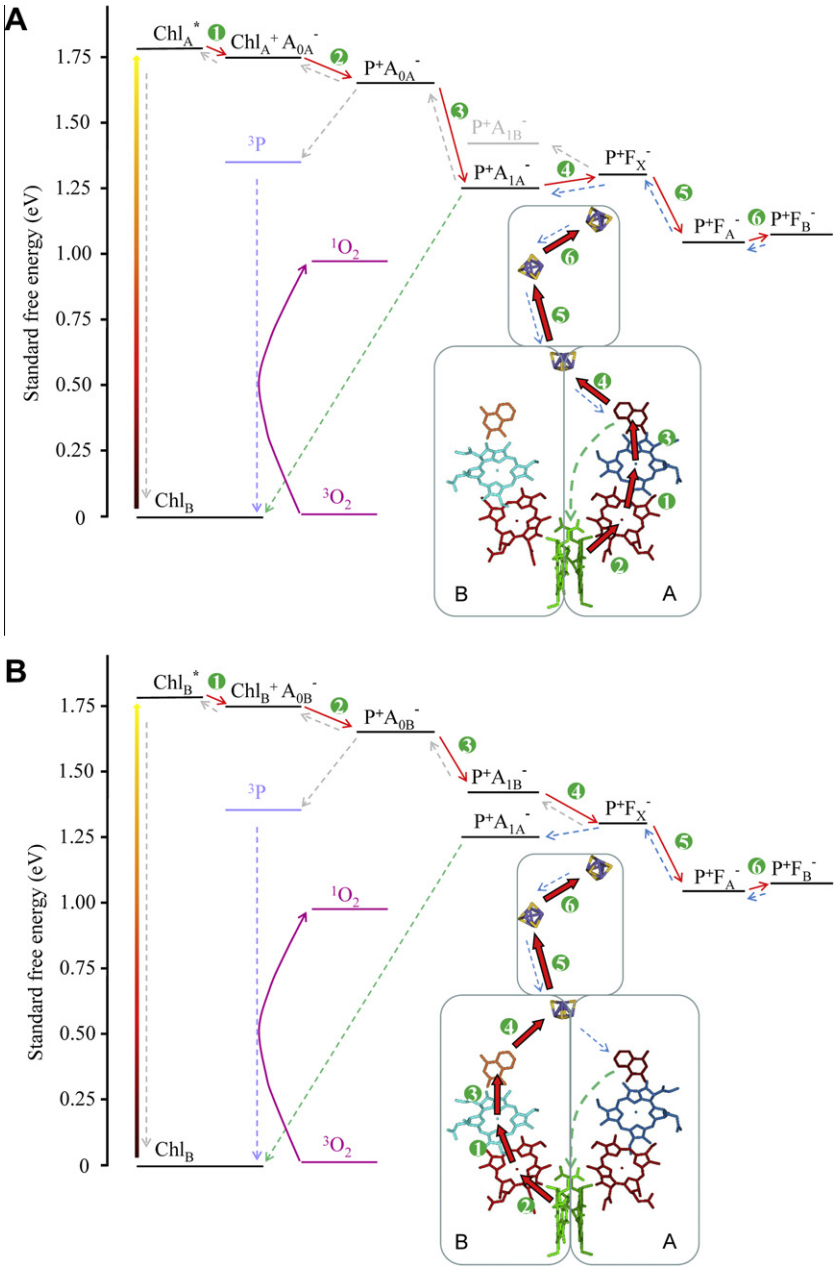

Fig. 3. Scheme illustrating the dominance of the A-branch as a back-reaction pathway in Photosystem I. Both panels show the standard free energy levels of the radical pairs formed by charge separation in Photosystem I (estimate from [14,24]) and a structural scheme of the same reactions [8]. The order of the forward reactions is indicated by numbered red arrows. Broken arrows show back-reactions with grey designating the disfavoured reactions. Panel A shows charge separation initiated on the A branch and the dominant back-reactions are shown, illustrating the idea that charge recombination occurs mainly between A_{1A}^- and P^+ thereby minimizing the formation of ^3P triplet state and thence $^1O_2$. Panel B shows charge separation on the B branch and here too the dominant back-reactions pathway is suggested to be the non-triplet generating A branch as explained in the text. Note that charge separation is considered to occur between the Chl and A_0 pigments followed by rapid donation from the P chlorophylls in accordance with [45,138].

[14,24,25]. These back-reactions take place with rates ranging from milliseconds to hundreds of microseconds. Again, unless the plastocyanin pool is fully oxidized, electron donation to P_{700}^{+} should be fast enough to trap the acceptors in the reduced form. Thus when the soluble electron acceptors are limited, electrons linger on the terminal electron acceptors and upon further turnovers the preceding acceptors can become reduced. Leaks and back reactions are then predicted to occur.

When the PSI electron acceptors are reduced, their very low potentials mean that O_2 can easily be reduced to $O_2^{\cdot-}$. This leak occurs not only at the stromal surface but also perhaps within the membrane, presumably from the A_1 semiquinones when the iron sulfur centres are reduced (see [26]). At the onset of illumination, before the enzymes for CO_2 fixation are activated, nearly all the electrons coming through the electron transfer chain end up forming $O_2^{\cdot-}$. In recent years $O_2^{\cdot-}$ has been recognised as a signalling molecule (in addition to its established reputation as a potentially damaging reactive oxygen species) and so this leak is not necessarily a bad thing. But it is something that needs to be regulated and exactly how that is done is still not clear in detail. It does seem likely that the regulation of electron donation into and out of the reaction centre in both linear and cyclic electron transfer, are all important and that redox tuning may also play a role.

One feature of PSI that may reflect redox tuning to limit O_2 reduction is the fact that the more stable, highest potential acceptor is not the F_B, the exposed terminal acceptor that interacts with ferredoxin, but rather the F_A centre which is buried inside the protein (see [27]). The more sequestered location of the reduced F_A could slow its reaction with O_2. Interestingly in the green sulphur bacterial reaction centre this F_B-type acceptor appears to be the more stable of the two iron-sulfur centres [21]. This would make sense since it lives in an anaerobic environment and thus does not need to protect itself against O_2.

The back-reactions occurring when the electron acceptor side of PSI is blocked can result in chlorophyll triplet formation and hence singlet O_2 formation. This might be one of reasons underlying the photosensitivity of PSI in mutants lacking PGR5 [28], a protein shown to promote cyclic electron flow and proposed to be involved in the formation of supercomplexes comprising all the players required for an efficient cyclic electron flow around PSI [29,30]. Indeed, such redox cycling supercomplexes, which function without the diffusion of soluble electron carriers such as ferredoxin or plastocyanin, are expected to be less susceptible to the electron acceptor-side limitations described above. Below we propose that back-reaction pathways leading to the chlorophyll triplet formation are specifically minimized by redox tuning.

Asymmetry in PSI also exists at the level of the phylloquinones (compare Fig. 3A and B). The forward electron transfer rates for the two phyllosemiquinones, $A_{1A}^{\cdot-}$ and $A_{1B}^{\cdot-}$, to F_X differ by an order of magnitude (200 and 20 ns respectively) [31–34] due to differences in the reduction potential of the two phyllosemiquinones (estimated to be −671 and −844 mV respectively [35], although functional studies point to the difference being smaller [36,37]). The origins of these very low potentials and the difference in the two potentials are discussed in detail elsewhere [25], briefly it results from a combination of electrostatic effects notably those from the F_X and $F_A/_B$ and from the protein environment with the asymmetry arising from differential effects of the protein backbone and asymmetry in specific ionisable amino acids [35].

The mechanistic significance of the kinetic and redox asymmetry associated with the quinones is unexplained. A rate of 200 ns compared to 20 ns is not expected to have any functional significance since these rates are both much faster than the competing back-reaction $P^{+}A_1^{\cdot-}$ recombination occurs in 200 μs). However, one can predict that the back-reaction pathway from F_X will be dominated by the A-side route since A_{1A} is slightly downhill from F_X, while the F_X to A_{1B} step is uphill. It seems possible that this characteristic of PSI could be beneficial under high-light conditions.

It is known that chlorophyll triplets can form in the PSI reaction centre when illuminated under reducing conditions [38] or when the secondary acceptors are removed [39]. Under physiological conditions it seems possible that PSI would encounter conditions in which electron donors and acceptors would be insufficient to prevent charge recombination within PSI, under supersaturating light intensities for example, and this may be expected to result in triplet formation. Fig. 3 illustrates how the A-side, with its high potential phylloquinone, would be favoured for the back-reaction from $F_X^{\cdot-}$, compared to the uphill transfer from $F_X^{\cdot-}$ to A_{1B}.

On the B-side, the energy gap between $P^{+}A_{1B}^{\cdot-}$ and $P^{+}A_{0B}^{\cdot-}$ is smaller than the equivalent reaction on the A-side. This means the population of $P^{+}A_{0B}^{\cdot-}$ from $P^{+}A_{1B}^{\cdot-}$ will be rapid and hence triplet formation will be favoured. On the A-side however, the equivalent back-reaction will be slower and the triplet formation will be disfavoured provided that the $P^{+}A_{1A}^{\cdot-}$ recombination reaction occurs directly to ground state with a rate that out-competes the back-reaction to $P^{+}A_{0A}^{\cdot-}$. Therefore, if the back-reaction occurs mainly down the A branch, as suggested above, then triplet generation overall will be minimized (see Fig. 3). The lowered triplet yield could constitute a significant advantage. A corollary of this idea is that native PSI should show less triplet formation per back reaction than seen in the anaerobic, homodimeric reaction centres of *Heliobacteria* and *Chlorobiacae*. In these bacteria, however, triplet formation would be less of a problem as these they grow in strictly anoxic conditions and thus singlet oxygen will not be formed.

When Ishikita et al. [35] calculated the potentials for the quinones, they found that a key amino acid responsible for the asymmetric potentials was Asp575-PsaB. This group is closer to A_{1A} than A_{1B} and it was suggested that it undergoes a deprotonation in response to the formation of the semiquinone, thereby stabilizing the semiquinone. Given the very rapid forward electron transfer in PSI however, the protonation may not have time to influence the forward reactions, and indeed site-directed mutations of this particular residue had little effect on the reoxidation rates of the phyllosemiquinones [40]. However under conditions where charges accumulate and back-reactions occur, it seems possible that this protonation does occur. This could switch A_{1A} to an even higher potential and thus the protective mechanism suggested above would be even more effective. Intriguingly Asp575-PsaB is changed to a Asn in the unusual gene variant present in nitrogen fixing cyanobacteria and expressed in heterocysts [41]. It seems clear the high potential A_{1A} and the tuning switch that we suggested above would not be needed in PSI functioning in the anaerobic conditions encountered during nitrogen fixation. If there is a change to two-sided low-potential PSI in anaerobic conditions, we must suppose that there is an advantage in doing this. A comparison of this anaerobic PSI with normal PSI might show differences in efficiency as well as susceptibility to O_2 related damage.

Current views of the evolution of Type II reaction centres propose that the Type II reaction centres diverged from the Type I reaction centres while both were homodimeric. This is particularly compelling since the majority of features (the cofactors and their environments) that differentiate Type II RCs from Type I RCs are symmetrical over both sides of the reaction centre. These differences were therefore almost certainly present in an ancestral homodimeric Type II reaction centre [42]. The heterodimericity of PSI, should the word exist, most likely evolved relatively recently compared to the separation of Type I and Type II reaction centres. Indeed the two sides of PSI, PsaA and PsaB, have not diverged greatly from each other (60% identity). In the preceding text, the asymmetry existing in PSI was rationalised in terms of protection and regulation in the context of reactions with oxygen.

We therefore suggest that heterodimerisation in PSI occurred after O_2 appeared in the environment, i.e. after the evolution of water splitting PSII and probably in the same membrane as the nascent water oxidizing reactions. Based on other arguments, a similar conclusion has been arrived at independently (John Golbeck personal communication). The tuning of electron transfer needed to deal with leaks and damaging back-reactions seems to be a requirement associated with life in the presence of oxygen.

4. Type II reaction centres

4.1. No charge separation in the B-branch of Type II reaction centres: a requirement for an efficient two-electron gate

In Type II reaction centres the quinone, Q_B is the last electron acceptor in the chain and $Q_B^{\cdot-}$ must be stable until another photochemical turnover provides the second electron required to complete its 2-electron reduction. At this stage there is no forward reaction which can compete with the back-reaction and so kinetic control is not an option to prevent the back-reaction. The simplest way to prevent the energy loss is to slow-down the backward rate. This is done by making the back-reaction, or at least one step in the back-reaction pathway, strongly uphill in energy.

The shortest route for electrons to get to $P^{\cdot+}$ from $Q_B^{\cdot-}$ is via the $Pheo_B$, the pheophytin on the "non-functional" B-side of the reaction centre. The distance between Q_B and $Pheo_B$ is similar to that between Q_A to $Pheo_A$ (~9 Å) [10] but the energy gap between Q_B and $Pheo_B$ is thought to be very large in all Type II centres (the potential of $Pheo_B$ has not been determined but is considered to be more negative than that of $Pheo_A$ and the potential of Q_B is around 100 mV higher than Q_A see [43,44]). Thus no $P^{+}Q_B^{\cdot-}$ back-reaction takes place by this route. This is a major factor contributing to the long lifetime of $Q_B^{\cdot-}$. In the evolution of the Type II reaction centres, the switching-off of the B branch presumably occurred by a mutation or mutations that lowered of the potential of $Pheo_B$, simultaneously switching-off the charge separation on the B-side and blocking $Q_B^{\cdot-}$ from back-reacting through $Pheo_B$. Interestingly, it has been shown in Photosystem I that raising the potential of A_{0B}, which is analogous to $Pheo_B$ in Photosystem II, lowers the yield of the B-branch without affecting the overall quantum yield of charge separation, suggesting that the proposed evolutionary tinkering does not impact the overall charge separation efficiency [45].

The evolution of a large energy gap between Q_B and Ph_B contributed to a more efficient reaction centre by elimination of this direct back-reaction route. Several other features of the current Type II reaction centres can be seen as greatly increasing the efficiency of reaction centre as a quinol-producing device, compared to the ancestral homodimeric quinol-producing reaction centre. These have been dealt with in detail elsewhere [42,46]. Basically a homodimeric quinone-reducing reaction centre would suffer inefficiencies associated with $Pheo^{\cdot-}$ encountering semiquinone (which would be awaiting the second electron) or an empty site (due to quinol/quinone exchange and incomplete occupancy). The heterodimer evolved (i) a specialised Q_A, which is always bound and only does rapid, 1-electron chemistry and (ii) a specialised Q_B site that stabilises a semiquinone adjacent to the non-functional PheoB.

4.2. Back-reactions in Type II reaction centres: the purple bacterial reaction centres

For $Q_B^{\cdot-}$ the first step in the back reaction is electron transfer back to Q_A, in this case however, these two components are not far apart in energy, the two semiquinones equilibrate ($K = 20$). It is on the next back-reaction step that a big energy gap exists: the $Q_A^{\cdot-}$ to Pheo step requires several hundred meV [47,48]. Across the Type II reaction centres this energy gap varies, having marked effects on back-reaction rates and this has clear mechanistic significance.

There are several examples that illustrate the extent to which the lifetime of the radical pair involving $Q_A^{\cdot-}$ depends on the free energy difference associated with the electron transfer from $Q_A^{\cdot-}$ to the nearby $Pheo_A$. In *Rhodobacter sphaeroides*, the lifetime of the radical pair changes as a function of the energy gap between Q_A and Pheo and this has been studied by substituting different quinones for Q_A and by imposing an external field [47,49,50]. When the energy gap is smaller than around 350 meV then repopulation of the $P^{\cdot+}Pheo^{\cdot-}$ state dominates; when the energy gap is larger than that, the direct tunnelling recombination reaction dominates [47,49,50].

Variations in this energy gap and hence the back-reaction kinetics are seen in different species of purple bacteria. In *R. sphaeroides* or *Rhodobacter capsulatus*, where Q_A is a ubiquinone (UQ), the lifetime of $Q_A^{\cdot-}$ is significantly longer than in *Rhodobacter viridis*, a bacteriochlorophyll b-containing species in which Q_A is a menaquinone (MQ) [49,51,52]. This is explained by the smaller energy barrier for the repopulation of $Pheo^{\cdot-}$ from $Q_A^{\cdot-}$ in *R. viridis* due to the higher potential of the Pheo (and perhaps a contribution from the slightly lower potential of MQ as Q_A). The smaller energy gap here is mainly a consequence of *R. viridis's* use of longer wavelength light for photosynthesis. The energy available from 960 nm light (1.292 eV) is significantly less than 870 nm light (1.425 eV) used by *R. sphaeroides* and yet the reaction centre bacteriochlorophyll *b* gives rise to a $P^{\cdot+}$ cation that has approximately the same oxidizing power as that from *R. sphaeroides* (+450 mV). This means that it has around 130 meV less reducing power on the acceptor side and this is mainly seen as a diminution in the Pheo to Q_A energy gap. The rapid recombination via repopulation of the $P^{\cdot+}Pheo^{\cdot-}$ is expected to give rise to a high yield of the triplet ^3P (see Fig. 4).

R. sphaeroides does not suffer from this energetic squeeze and so is able to have an energy gap between $Q_A^{\cdot-}$ and $Pheo_A$ that is more than enough to prevent the back-reaction by that route. Instead the $P^{\cdot+}Q_A^{\cdot-}$ recombination reaction takes place via a slow reaction that involves a tunnelling process. We shall see in the following section that PSII is like *R. viridis* insofar as it has insufficient energy in the absorbed photon to allow it to maintain an energy gap between Ph and Q_A that is big enough to render the Pheo to Q_A step irreversible. In PSII the energy squeeze is not caused by a lower energy photon (indeed it uses the highest energy photon of all the photosynthetic reaction centres) but by the high energy requirement at the oxidizing side of the reaction centre. PSII needs all the energy it can get to take electrons out of water with a reasonable over-potential.

4.3. Two back reaction pathways in PSII: redox switching

PSII contains two charge recombination pathways for $P^{\cdot+}Q_A^{\cdot-}$, one of which is comparable to that in *R. viridis* and the other more similar to that in *R. sphaeroides* [48,53]. As described in the purple bacterial reaction centre, the size of the energy gap between Q_A and the Pheo determines the back-reaction rate and thus the recombination route. Here however this has more important mechanistic implications and remarkably PSII is able to modulate the size of the energy gap and hence the yields of these pathways in order to mitigate damage and optimize function.

As described above for *R. viridis*, the indirect pathway in PSII takes place with the formation of the $P^{\cdot+}Pheo^{\cdot-}$ radical pair, which then decays to the ^3P triplet state ([54,55] and see [56]), for a review of the bacterial case see [57]). Unlike *R. viridis*, however, PSII is far from anaerobic, indeed it makes the O_2 and its ^3P triplet state

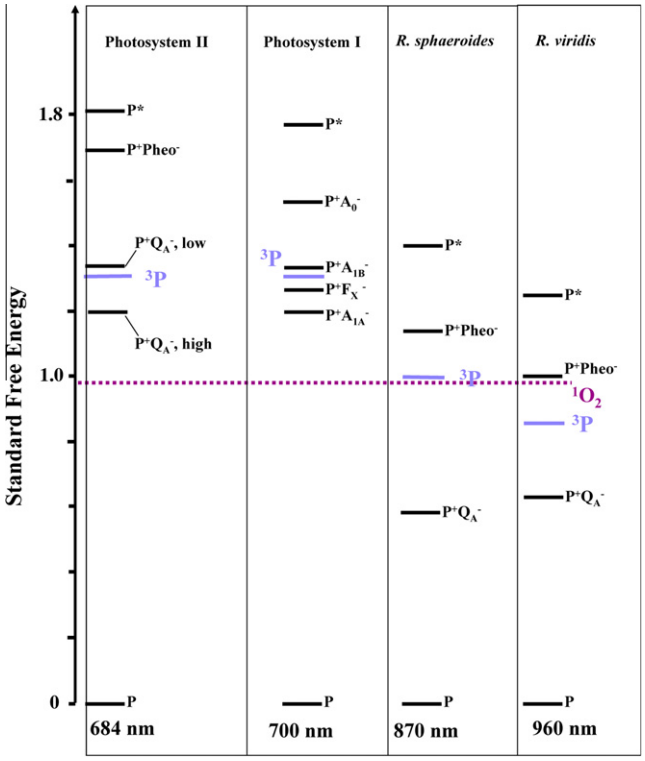

Fig. 4. A scheme showing estimates of the standard free energy levels of relevant excited states and radical pairs in photosynthetic reaction centres. The chlorophyll triplet (^{3}P) formed by charge recombination is shown in each case (in blue) as well as the energy needed for converting triplet oxygen into the highly reactive singlet form (in broken red line). For each type of reaction centre the energy available in the first excited singlet state (P*) corresponds to the photon absorbed and that is indicated at the foot of each column. Note that for *the R sphaeroides* and *R. viridis* the term P* represent the special pair of bacteriochlorophylls, In PSI and PSII this is more complicated. For PSI as shown in Fig 3, the excited state would be located on the component designated Chl_A in panel A (or Chl_B in panel B). For PSII the excitation is not thought to be localised on a single pigment at room temperature, instead is distributed over several pigments: mainly on the component Chl_{D1}, but also partly on P_{D1} and P_{D2} and also to a smaller extent on Chl_{D2} and even the Pheos [43,46].

lies about 1.3 eV above the ground state, which is more than enough energy to promote the triplet to singlet oxygen transition (0.98 eV [58]). Thus the chlorophyll triplet state is likely to encounter $^3O_2$, transfer excitation to it and generate singlet oxygen $^1O_2$, a highly reactive and damaging species [55,59].

Most purple bacterial reactions centres, even those in which the direct route is optimised and which live in low O_2 environments, still have a system for quenching ^{3}P when it is formed. In this case it is only formed by $P^{\cdot+}Pheo^{\cdot-}$ recombination occurring when $Q_A^{\cdot-}$ is already present, i.e. in the light when the acceptor-side is limited (i.e. when over-reduced). Even though there is not much driving force (~100 meV) in ^{3}P for the formation of singlet O_2, it seems it is worth protecting against this eventuality. These reaction centres have carotenoid in van der Waals contact with the bacteriochlorophylls of the reaction centre and these are able to quench ^{3}P before it can react with oxygen. In PSII however, the core reaction centre chlorophylls are so oxidising that carotenoids cannot approach them without being oxidised adventitiously [60]. Given this limitation and its O_2-rich environment, it is thus crucial for PSII to limit chlorophyll triplet formation. From an engineering point of view one mechanism by which this can be achieved is to increase the energy gap between Ph_A and Q_A, thereby minimising the thermally activated, indirect route via $P^{\cdot+}Pheo^{\cdot-}$ and favouring the direct recombination route instead. This can be done either by raising the free energy level of $P^{\cdot+}Pheo^{\cdot-}$ or by decreasing the energy level of $P^{\cdot+}Q_A^{\cdot-}$.

Each of these strategies may compromise the overall efficiency of the energy converter. Indeed, since Photosystem II is a "shallow photochemical trap" [61,62] (another consequence of being energy squeezed), raising the free energy level of the primary radical pair impacts the quantum yield [63–65]. Similarly, decreasing the free energy level of $Q_A^{\cdot-}$ (relative to Q_B and or the quinone pool) will increase its steady state concentration, thereby impacting the overall energetic efficiency of the system [53,66]). There is thus a trade-off between the need to limit potentially harmful but, under standard conditions, rare (chlorophyll triplet generating) back-reactions and the optimization of the energy conversion yield. The latter seems to have been favoured since the relative yield of the indirect, back-reaction pathway in Photosystem II, under functional conditions is rather high and accounts for ~70% of the charge recombination process [67]. Under normal functional conditions electrons are plentiful from water splitting and these potentially damaging reactions occur infrequently. When however conditions are encountered in which back-reactions are more frequent, then this damaging route can be essentially switched off [53].

While this switching process involves kinetic control, the switch itself is through a conformational change and this can be viewed as kinetic gating. Photosystem II is fully assembled in the membrane as a photosynthetic reaction centre without its active site, the Mn_4O_5Ca cluster. The cluster is assembled by a process known as photoactivation. Prior to and during photoactivation, electrons are much less readily available to stabilize the oxidizing equivalent resulting from photochemistry, thus charge recombination would be expected. However when the cluster is absent, the reduction potential of Q_A is higher by 150 mV than in the functional enzyme [69]. Consequently, the free energy gap between $P^{\cdot+}Q_A^{\cdot-}$ and $P^{\cdot+}Pheo^{\cdot-}$ is large and the direct charge recombination pathway is favoured, while the indirect pathway, and its troublesome ^{3}P intermediate, is avoided [53] (see Fig. 4).

It is known that the simple absence of Ca^{2+}, rather than the whole cluster, is responsible for the switching effect [68,69]. It is possible that this occurs under physiological conditions, for example in the presence of high local proton concentrations [68,69] and in certain S states [70], and this would result in the same kind of redox switching. This would protect PSII should this occur but it also could be a regulatory mechanism in high light conditions.

It is assumed that the structural modifications resulting from the absence of the Mn_4Ca cluster, or indeed just the Ca^{2+}, propagate over to the other side of the protein (almost 40 Å away) and induces a down-shift of the midpoint of the $Q_A/Q_A^{\cdot-}$ couple [53,69,71]. The nature of this change is not clear however it has been suggested to be related to the presence of an H-bond from threonine (217 of D_2) to the carbonyl on Q_A that is proximal to the non-heme iron [72]. The presence of this H-bond has been calculated to produce just such an up-shift in potential. The simple rotation of the OH group of the threonine could make or break this bond. It is not clear how the binding status of the Ca^{2+} 40 Å away would influence this rotation. Alternatively, the ionization of amino acids in the region of Q_A, perhaps the bicarbonate/carbonate that ligands the non-heme Fe, could also be responsible for this redox shift [46]. A well resolved crystal structure of the Mn_4Ca-depleted PSII may help understand this effect and how it is propagated across the protein.

The relationship between the redox potential of Q_A (and hence the $P^{\cdot+}Q_A^{\cdot-}$ $P^{\cdot+}Ph^{\cdot-}$ energy gap) and the generation of $^1O_2$ has been established experimentally using spin trap EPR methods. This was done in a site-directed mutant that lowered the potential of Q_A and it duly gave rise to more $^1O_2$ [73]. Another demonstration was done using herbicides. The binding of herbicides in the Q_B site results in changes in the potential of Q_A and this again affects the

Q_A to Pheo energy gap [74]. The yield of singlet oxygen increased when the binding of phenolic herbicides decreased the size of the energy gap due to the increased yield of the indirect pathway via the $Pheo^{\cdot-}$ giving rise to ^3P formation [59]. These herbicides not only block electron transfer but they also redox tune Q_A, favouring the back-reaction up to a high energy intermediate ($P^{\cdot+}Pheo^{\cdot-}$), this reacts by a short-circuit (charge recombination) to form a reactive state (^3P) that reacts with O_2 (a leak) and this kills the plant [75] .

In the Q_B site, phenolic herbicides seem to H-bond strongly to the imidazole that ligands the non-heme iron, this effect may be relayed to the H-bonded Q_A on the other side of the imidazole-Fe-imidazole motif leading to a weaker H-bond to the Q_A and thus generating the lower potential [76]. In line with this, the affinity of the Q_B pocket for phenolic herbicides depends on the redox state of $Q_A^{\cdot-}$ [77]. This herbicide-induced modulation of the Q_A potential is additive to the Ca-induced effect, so clearly the chemical origin of the redox effects are different [74]. These observations also could indicate that the native occupant of the Q_B site, PQ (its presence and absence and each of its different redox states), could influence the reduction potential of Q_A and thus tune the forward and back reactions. This has yet to be studied.

Based on the effect of Ca^{2+} on the potential of Q_A [78] and the fact that Ca^{2+} binding changes during the S-state cycle [70], it has been suggested that the potential of Q_A could be tuned to suit specific properties of the S states [46]. For example a short-lived $S_3TyrZ^{\cdot}$ state may have less chance of back-reacting if the Q_A potential were increased in S_3.

4.4. Back reactions in PSII: modulating the potential of pheophytin

The standard free energy level of the $Pheo_A^{\cdot-}$ state can be modified depending on the strength of the H-bond to the $C_{13}{}^1$=O of $Pheo_A$ from the amino acid side chain at D_1-Gln130. In nature this residue can be Gln or Glu but a range of site-directed mutants have been made and studied. The lifetime of $Q_A^{\cdot-}$ decreased as the H-bond was strengthened (as the Ph potential became more positive) and the corresponding increase in the lifetime of $Q_A^{\cdot-}$ occurred when the H-bond was weakened (as the potential of Ph became more negative) [48,65]. As expected these studies showed the correlation between the size of the standard free energy gap between $Pheo_A^{\cdot-}$ and $Q_A^{\cdot-}$ and the lifetime of the semiquinone.

The impact of modulating the free energy level of the $Pheo_A$ is not limited to the back electron transfer rate from $Q_A^{\cdot-}$, indeed the forward reactions are also affected. Site-directed mutants at the position D_1-130 position showed the rate and quantum yield of the primary charge separation were dependent on the H-bond strength, with increased rates and yields when the H-bond strength was decreased [63,64,79]. Lowering the potential of Pheo leads to lower yields of charge separation as well as slower back reactions.

In nature only the high potential form of Pheo (with the strong H-bond from D_1-Glu130) is present in plants and algae. By contrast, in cyanobacteria either Glu or Gln is found at position 130 in D_1. All known cyanobacteria possess several genes coding for the D_1 subunit, which together with D_2 constitutes the core of the reaction center. These multiple D_1 genes are differentially expressed depending on the environmental conditions [80,81]. In all known cyanobacterial sequences, the only conserved difference between the two expressed isoforms is at position D_1-130, being Glu in high light D_1 but Gln in the low light form. It is tempting to suppose that the reason for this differential gene expression is to decrease the yield of potentially damaging reactions when Photosystem II is exposed to over-saturating light. And indeed, the D_1 isoform expressed in high light does confer photo-resistance, compared to "low-light" isoforms and species [82–84]. Again, this tuning results in a kinetic control but this is obtained by substituting one isoform of the D_1 subunit by another, something that might be considered a special form of large-scale gating. However, the mechanisms underlying this photo-protection effect are not clearly understood.

The Glu at position D_1-130, which is present in all the high-light isoforms should result in a stronger H-bond to $Pheo_{D1}$ and thus have the following effects: (i) the potential of the PheoD1 is expected to become more positive; (ii) the quantum yield of charge separation is expected to increase because of the greater driving force for $P^{\cdot+}Pheo^{\cdot-}$ formation from $^*P$ and (iii) the yield of the indirect, triplet-generating, charge-recombination pathway should increase because the energy gap between $P^{\cdot-}Q_A^-$ and $P^{\cdot+}Pheo^{\cdot-}$ is smaller. These are not obvious ploys for coping with too much light, indeed we might expect them to have exactly the opposite effect and to make matters worse. How can this be rationalized?

One possibility is that the decreased photo-sensitivity of the high-light Photosystem II isoforms stems from the combination of multiple functional effects arising from the range of amino acid changes. The recently documented changes on the electron transfer rates on the donor-side of Photosystem II might be one of these [84,85]. But this is highly unsatisfactory as an explanation: why should the Glu be selected in *every* high-light strain, if it did not play a positive role? Here are some suggestions.

The photosensitivity of PSII mutants bearing either a Leu, Gln or Glu at position D_1-130 has shown that photoinhibition is more pronounced when the energy level of the $P^{\cdot+}Pheo_A^{\cdot-}$ state is high. This observation should help solve the paradox.

Vass and Cser [86] invoked the Marcus theory and pointed out that changes in the potential of $Pheo_A$ not only affect the thermally activated repopulation of the $P^{\cdot+}Pheo^{\cdot-}$ but also the electron transfer rate between $P^{\cdot+}Q_A^{\cdot-}$ and $P^{\cdot+}Pheo^{\cdot-}$. They proposed that increasing the midpoint potential of $Pheo_A$ would indeed favour the thermally activated repopulation of $P^{\cdot+}Pheo^{\cdot-}$ from $P^{\cdot+}Q_A^{\cdot-}$ but in addition it would favour radical pair recombination from the singlet over the triplet route. This can be rationalized in terms of Marcus theory: the very large driving force for singlet recombination to the ground state (above 1.6 eV) would put the reactions into the inverted region (i.e. a decrease in driving forces accelerates the reaction), while recombination to the triplet, with a driving force around 0.2–0.3 eV, behaves conventionally (i.e. a decrease in driving force slows the reaction). Thus a positive shift in the potential of Ph (i.e. with the stronger H-bond from D1Glu-130) would decrease the driving force for $P^{\cdot+}Pheo^{\cdot-}$ recombination, accelerating singlet recombination to the ground state but slowing triplet formation. This then is positive protective effect. While this is plausible, it is not wholly satisfying since the rate effects are not expected to be large and the increased decay of $P^{\cdot+}Q_A^{\cdot-}$ by the dangerous, indirect back-reaction route has to be compensated for before any protective benefit can be gained by this mechanism. Below we suggest some alternatives.

The modulation of the energy gap as a method of minimizing the formation of the PSII triplet, as originally formulated by Johnson et al [53], was seen as a protection strategy for preventing $P^{\cdot+}Pheo^{\cdot-}$ formation when populated from $P^{\cdot+}Q_A^{\cdot-}$, i.e. to prevent electrons from returning to Pheo from $Q_A^{\cdot-}$ (or from $Q_B^{\cdot-}$ via $Q_A^{\cdot-}$). This is a particular risk when the enzyme is unable to provide electrons: i.e. when the water splitting function is absent (before photoassembly of the MnCa cluster) or disabled (after photodamage or when Ca^{2+} is lost) or when the light intensities is so low that $S_2Q_B^{\cdot-}$ or $S_3Q_B^{\cdot-}$ charge recombination can take place [53,55]. Under high light intensities, however, the situation is quite different.

Under high light water splitting works and the acceptor side rapidly becomes unable to keep up with the electron input. Under these conditions, the $Q_A^{\cdot-}$ will accumulate, and despite the "shallow trap" lowering the quantum yield of charge separation, the

$P^{+}Pheo^{-}Q_A^{-}$ state will still be formed [61,62]. Recombination of this state will produce triplet [61]. Now, returning to the change in the potential of Ph associated with the H-bonding Glu/Gln at D1-130, we suggest that a smaller driving force from $P^{+}Pheo^{-}$ state (with stronger Glu 130 H-bond) may simply diminish the triplet yield and favour singlet recombination. For this to occur then, like Vass and Cser [86], we resort to the Marcus theory. In this case however, recombination is from $P^{+}Pheo^{-}Q_A^{-}$ (rather than $P^{+}Pheo^{-}Q_A$. Because of the electrostatic effect of the charge on Q_A^{-} on Ph^{-}, this radical pair would be at an even higher energy, even further into the Marcus inverted region for singlet recombination to the ground state and thus its rate would be more susceptible to a small changes in the driving force. The Glu 113 H-bonded Ph would thus be an advantage in the high light strains. The different feature of this model compared to that to Vass and Cser [86] is that here the energy gap between $P^{+}Q_A^{-}$ and $P^{+}Pheo^{-}$ is irrelevant to the protective mechanism in high light, the dominant problem is not repopulation of $P^{+}Pheo^{-}$ from $P^{+}Q_A^{-}$, but simply the decay route of $P^{+}Pheo^{-}$ (formed by the forward reaction from *P).

There is another possible explanation which is not necessarily exclusive. It is known that strong light under reducing conditions results in the second reduction of Q_A forming the Q_AH_2 state [87,88]. This results in an increased yield of charge separation, because the electrostatic influence of Q_A^{-} on the energy level of $P^{+}Pheo^{-}$ is removed, the energy gap between this state and *P increases and so does the quantum yield of charge separation: in short the shallow trap effect is reversed [61]. As a result of the increased quantum yield of $P^{+}Pheo^{-}$ formation, the yield of the ^3P is greatly increased. Futhermore, the proposed Q_A^{-} electron transfer quenching of ^3P will also be lost, allowing the triplet lifetime to increase by more than 2 orders of magnitude [61,89]. This would give rise to severe oxidative damage if O_2 were present. This has been suggested to occur under some photoinhibitory conditions [87,90]. The increase in the potential of the Pheo due to the presence of H-bonded D1Glu-130 would decrease the driving force for this second reducing step, $Ph^{-}Q_A^{-} + 2H^{+} \rightarrow PhQ_AH_2$, and might be expected to slow it down. This would constitute a significant advantage for selecting of Glu-130 in high light conditions.

4.5. Side path, futile cycle and oxidative leaks in PSII

The very high potentials involved in water photolysis can result in the adventitious oxidation of nearby organic species in and around the PSII reaction centre. The longest-lived of the chlorophyll cations formed during primary charge separation, P_{680}^{+} is thought to have a potential around 1.2 V [48,64]. As mentioned above, this is oxidizing enough to take electrons from carotenoids. To work as quenchers of chlorophyll triplets, carotenoids need to be very close, van der Waals contact is typical. Thus carotenoids cannot protect the core chlorophylls in PSII and indeed the crystal structure verified this prediction [91–93]. The nearest carotenoid is at a distance of 11 Å to Chl_{D2}, the nearest core chlorophyll and 21 Å to P_{D1} the location of the cation and even further to Chl_{D1}, the main location of the triplet [94,95]. All of these distances are too far for triplet quenching but not too far to prevent slow electron transfer from the β-carotene.

Electron donation from this β-carotene to P^{+} (probably via Chl_{D2}) occurs in a few ms [96]. This side-pathway donation is slow compared to the main forward reactions for P^{+} reduction (e.g. electron donation from TyrZ can occur in tens of ns). Nevertheless it is predicted to occur with a very low quantum yield under normal conditions and with an increased quantum yield whenever P^{+} lifetimes are longer (i.e. when electron donation from water is inhibited or absent). The carotenoid is a 20 Å-long cofactor and it gets within a short distance of Cyt b_{559} and makes van der Waals contact with $ChlZ_{D2}$. Given its low potential relative to the $ChlZ_{D2}$, Cyt b_{559} if reduced, donates electrons rapidly to the β-carotene cation. The oxidized Cyt b559 is relatively stable but can be reduced slowly by plastoquinol [97]. This completes a futile cycle. Not much is known about the reduction of the Cyt b_{559} except that it is slow and is blocked by the same herbicides that bind to the Q_B site. Given the long distance, electron transfer is expected to take around a second to occur from the Q_B site to the heme of Cyt b_{559}. It seems possible that faster electron donation may occur under other circumstances through a less well-defined quinone site that is closer to the Cyt b_{559} (a "Q_C site") and that is also herbicide sensitive [98,99]. A third functional quinone in isolated reaction centres [100] was detected by crystallography bound in a channel close to the heme of Cyt b_{559} [92], although electron transfer from here to the heme could be rapid, it seems somewhat unlikely that this corresponds to the earlier defined Q_C site [46].

This inefficient Cyt b_{559}-mediated cycle has been proposed to protect against oxidative damage caused by P_{680}^{+} [101] or by $Car^{\cdot+}$ (see citations in [34]). In marine plankton a much more efficient futile cycle exists under high light [102]. This may represent a souped-up version of the Cyt b_{559} cycle described here. In order to work so much more efficiently, some kind of redox switch seems to be required. It was suggested recently [46] that this could involve a perturbation of the relative redox potentials of the core chlorophylls so that the chlorophyll cation may be distributed onto Chl_{D2}, i.e. closer to the carotenoid. This could occur by for example the electrostatic influence of accumulated oxidising species, say $Tyr_Z^{\cdot}$ or at least its proton [46].

When the cytochrome b_{559} is already oxidized, the carotenoid cation can be reduced by chlorophyll, most likely $ChlZ_{D2}$, which is calculated to have a uniquely low potential [103]. This branch of the futile cycle may serve as a trap for the cation and as a fluorescence quencher that will protect against over excitation of PSII.

It is possible that other pigments further from the reaction centre may undergo oxidation due to further oxidation of side-path components. Reports exist in the literature of multiple chlorophylls and carotenoids undergoing slow bleachings with prolonged illumination [104]. Such oxidations, should they occur under physiologically relevant conditions, may be considered as oxidative leaks. The oxidation of carotenoids in the antenna will remove their protective (triplet quenching) influence from the nearby chlorophylls and this will start a chain reaction of light-driven, triplet-mediated $^1O_2$ damage [75]. These oxidative leaks (and several other oxidative leaks occurring under other circumstances) are clearly to be avoided and the futile cycle and the up-shift in the Q_A potential when water splitting is non-functional, as described above, are both useful for that.

5. The cytochrome bc_1/b_6f

In the Q cycle of cytochromes bc_1/b_6f, a reversible oxidation of quinol in the catalytic Q_o site delivers one electron into the high potential c-chain and the other into the low potential b-chain, giving rise to a charge-separated state in the enzyme [105]. This reaction relies on i) the energetic coupling of the two reduction/oxidation reactions, one involving the FeS center of the c-chain, the other heme b_L of the b-chain, and ii) on the split between the midpoint potentials of the quinol/semiquinone and semiquinone/quinone redox couples illustrated in Fig. 5, estimated to be at least ~800 mV [106,107]. The electrons are then transferred from the FeS center to the heme c_1/f in the c-chain and from the heme b_L, via the heme b_H to the second catalytic quinone site, the Q_i, site in the b-chain. While in mitochondria and purple bacteria the

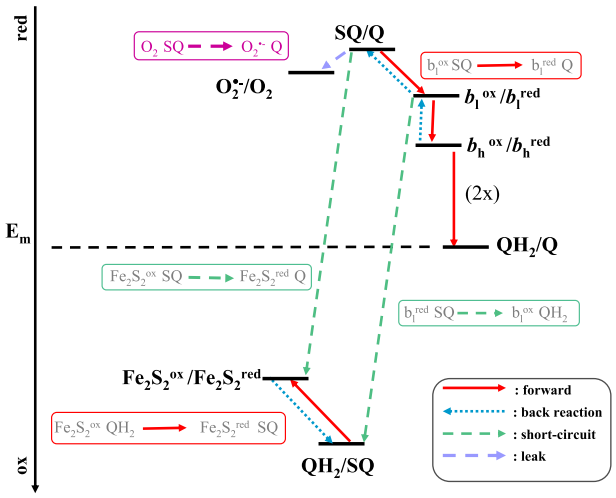

Fig. 5. Scheme illustrating the various electron transfer reactions occurring in the bc_1/b_6f complexes discussed in the text. Consistent with Fig. 1 the forward reactions are shown in red, solid arrows. The backward reactions are shown in dotted blue lines, the short-circuits in dashed green arrows and the leak toward oxygen as the dashed violet arrow. The reactions discussed in the text are specifically indicated. As a convention the arrows start from the redox couple that provides the electron donor and points toward the redox couple that provides the electron acceptor.

enzyme works as a quinol:cytochrome oxidoreductase, there are examples of bacteria which rely for their growth on reverse electron flow from cytochrome c to quinone [108].

Despite a great deal of research, the mechanism of the Q_o site catalysis and the way the two chains are connected are still not fully understood and remain the subject of intense debate (for recent references see for example [109–112]. The connection between the two chains has an additional level of complexity arising from the fact that the enzyme has a homodimeric structure with each monomer containing one *c*- and one *b*-chain that together form an H–shaped electron transfer system.

From a kinetic point of view, the direction of electron flow through the two coupled chains would be expected to depend exclusively on the rates of all partial reactions, including the Q/QH_2 exchange rate to and from the catalytic sites, provided that all the reactions within the chains are fully reversible. The equilibrium of one reaction in the coupled chains influences the equilibrium of all other reactions. A kinetic model based on this is sufficient to explain the re-equilibration reactions occurring following a flash-induced change in redox level of quinone pool and the cytochrome *c* pool [113].

However, rapid reversibility (within the catalytic timescale) of all partial reactions, including the initial charge separation at the Q_o site [114], raises a mechanistic problem in understanding how the productive (reversible) two-electron oxidation of quinol is kinetically separated from wasteful short-circuits (when single electrons directly flow from the low to the high potential chain), or leaks (when electrons are transferred to oxygen) (discussed in [114–117]), as shown in Fig. 5.

If one considers all possible types of short-circuits reactions that could potentially occur in this two-chain system (two- or three-step one-electron reaction sequences as discussed in detail in Refs. [114,115,118]), there are just two general solutions that ensure that all short-circuits occur with kinetics slower than the catalytic timescale. The first possibility is that the Q_o site allows the sequential oxidation of quinol when the two electron acceptors (i.e. the FeS and heme b_L) are both oxidized. This requires double gating of reactions at the Q_o site so that formation of semiquinone at the Q_o site, SQ_o, takes place only in a strictly defined set of redox conditions [114,115]. Since the reactions are reversible the same must apply to quinone reduction at the Q_o site, which would require both FeS and heme b_L to be present in the reduced form at the same time.

The second possibility is that the site favours a concerted, two-electron oxidation of the quinol that does not involve a semiquinone intermediate [114,115,119], or at least keeps its concentration extremely low. Again this would apply to the reverse reaction, quinone reduction.

Deciding between these two possibilities, as well as developing a precise understanding of the molecular mechanisms occurring await further studies. One interesting line of investigation has opened up recently with the reports of methods for the trapping of the semiquinone formed at the Q_o site [106,107].

The short-circuit reactions are traditionally referred to as "unwanted" because they dissipate energy and thus lower the energetic efficiency of the system. Recent studies however, indicate that their occurrence on a much longer time-scale (seconds) may have physiological relevance [120–122]. This relates to the redox conditions in which the cofactors remain in the reduced state for long periods, for example, when the Q_i site is unable to accept electrons from the reduced *b* hemes. As discussed below, under these circumstances short-circuits might compete effectively with electrons leaking onto O_2 and thus would diminish $O_2^{\cdot-}$ formation [120,121]. Furthermore, they might even allow quinol oxidation at Q_o site to remain functional at a residual, yet physiologically competent, level [122].

5.1. Competition between short-circuits and leaks of electrons

Superoxide, $O_2^{\cdot-}$ is formed by the cytochrome bc_1 complex under certain circumstances. If, as seems likely, the reaction of SQ_o with oxygen is directly responsible for the generation of $O_2^{\cdot-}$ [106,107,123], then the probability of this reaction will increase when the reduced forms of the heme b_L or FeS remain present long enough to prevent complete oxidation of the quinol. A similar argument applies for the reverse reaction, where complete reduction of the quinone will not be achieved if either heme b_L or FeS is not reduced upon arrival of the quinone in the Q_o site. But SQ_o may also engage in competitive reactions that retain electrons within enzyme rather than reducing oxygen. As described in detail in [120,121], several of those reactions result in short-circuits (see Fig. 5).

Because semiquinone in the Q_o site can be formed in two ways, either by the withdrawal of electron from QH_2 by FeS (the semiforward reaction) or the reduction of Q by heme b_L (the semireverse reaction), both of these reaction can potentially generate $O_2^{\cdot-}$. Experimental analysis of $O_2^{\cdot-}$ production in various mutants of *Rb. capsulatus* cytochrome bc_1, combined with modelling, suggested that the dominant reaction responsible for $O_2^{\cdot-}$ formation is electron transfer from heme b_L to quinone [121]. This leads to a build-up of steady state levels of SQ_o, which can react with O_2 [121]. According to this model, when SQ_o is formed at a time when the head domain of the mobile FeS is away from Q_o site, the FeS cluster cannot immediately react to neutralize SQ_o. Thus the lifetime of SQ_o is relatively long and the probability that SQ_o will reduce oxygen is therefore greater. If however the FeS cluster is close to the Q_o site, when in its oxidized form it simply picks up the electron from the SQ (resulting in a short-circuit), while if it is in its reduced form it will donate an electron to SQ (completing full quinone reduction, i.e. the reverse reaction). In either case SQ is rapidly removed by internal reactions occurring within the Q_o site before it can react with O_2.

This type of kinetic competition between short-circuits and leaks also probably occurs in the mitochondrial system, where the electron transfer from heme b_L to Q has also been proposed to be responsible for generation of reactive oxygen species by

cytochrome bc_1 [124,125]. Such competition may conceivably work as a protective mechanism with which the living cells would minimize the deleterious formation of $O_2^{\cdot-}$ through the use of competing energy-wasting but leak-proof and safer short-circuits [120]. But on the other hand, under some conditions the $O_2^{\cdot-}$ production could occur as part of a signalling pathway, reflecting the redox state of the electron transfer chain so the leak itself, when it occurs, could be physiologically relevant [126] .

5.2. Short circuits in the broken Q-cycle

We have discussed above the possibility of the back-reaction between reduced b_L and quinone and how this can lead either to the reduction of the oxidized FeS by the resulting SQ_o or to leaks to oxygen. As shown in Fig. 5, another possible short-circuit involving the reduced b_L is the electron transfer from b_L to SQ_o. Such a situation might occur when the oxidized FeS oxidizes the quinol yielding SQ_o while b_L is reduced.

Owing to the large separation between the potentials of the quinol/semiquinone and semiquinone/quinone couples (at least ~800 mV [106,107]), the unstable SQ_o is both an efficient electron donor and acceptor and it is thus capable of accepting an electron from the reduced b_L yielding a quinol.

The occurrence of this reaction was demonstrated recently in the b_6f complex when the Q_i site was inhibited [118] or disabled, and it was found that the reduction of SQ_o at the expense of b_L was very slow (~250 ms [122]). Despite its sluggishness, the very existence of this short-circuit is important because it represents an "emergency exit" pathway which bypasses the Q-cycle. This reaction thus allows the quinol-plastocyanin oxidoreductase activity to occur and thus the entire photosynthetic chain to function, even with a non-functional Q cycle.

This short circuit mechanism may be relevant to wild-type cytochrome *bc* and b_6f complexes under steady-state conditions. As the proton motive force builds up in the light, the driving force for the electrogenic electron transfer from b_L to b_H decreases, thereby increasing the steady state level of reduced b_L and thus the relative yield of the short-circuit pathway.

The slow rate of electron transfer from reduced b_L to SQ_o under these conditions is not understood. Notably it is much slower than electron transfer from SQ_o to oxidized b_L (~250 ms for the former *vs* 3 ms for the latter [122]). The slower electron transfer rate might be simply due to the standard free energy change for the electron transfer from b_L to SQ_o being much larger than that of SQ_o to b_L, putting the latter reaction into the "inverted region" of the Marcus curve [15]. According to the current available estimates for the ΔG_0's of these reactions, however, their reorganization energies would have to be unusually low for this to occur. Alternatively, and more likely, the rate-limiting step may not be electron transfer, but limited by a change in the binding of the quinol or of SQ in the site, indeed the appropriate configuration of the site for the formation of SQ_o may depend on the redox state of b_L (see [116,117] for a discussion of such possibilities).

5.3. Cytochrome bc_1 as a functional dimer and its possible role in diminishing ROS

The recent demonstration of all electron transfer paths in the *bc* dimer provided important functional principles. In addition to the well-known electron transfers through the c- and b-chains of each monomer, a functional electron transfer bridge connecting the two monomers formed between the two hemes b_L in a core of dimers was demonstrated [127,128]. With this bridge, all cofactor paths within the dimer assemble into an H-shaped electron transfer system linking the two Q_o sites on one side of the membrane with the two Q_i sites on the other side of the membrane. This system distributes electrons between these four catalytic sites within a timescale of the catalytic turnover (milliseconds) and acting like a molecular-scale "bus bar" with four terminals [127]. As a result, any connection between the catalytic sites on opposite sides of the membrane allows the enzyme to be catalytically competent. It remains to be seen whether and how this "bus bar" design contributes to regulation of electron flow in respiratory and photosynthetic systems. It does seem clear that the built in redundancy will allow enzymological function even if operation or mutation disables specific component branches.

An important question concerns the relative ratio of intra- versus inter- monomer pathways in the functioning dimer. Considering electrochemical properties of the cofactors and distances between them, it is expected that under the conditions of unperturbed electron flow in all parts of the dimer (i.e. when all four terminals are "fully open") the intra-monomer electron transfer would dominate [129]. However, as the equilibrium levels change and electron flow in parts of the dimer is suppressed (i.e. when any of the energetic and/or structural conditions lead to the bus bar terminals become "partly or entirely closed", for example where $\Delta\mu_H$ builds up), then the contribution of the inter-monomer electron transfer is expected to increase.

Another important question concerns a possible role of electron exchange between all four hemes b within a dimer to diminish leaks of electrons and generation of superoxide [114,127,130]. A general concept behind those possibilities assumes that the connection between hemes unites them with all four catalytic quinone oxidation/reduction sites in such a way that multiple unpaired electrons produced during the Q cycle can by collected and neutralized [114,127]. This means that the cross-dimer electron transfer may help in sweeping the b-chain of reduced heme b [114]. As discussed in paragraph 5.1, an increased level of reduced hemes b is associated with the increased probability of SQ and for $O_2^{\cdot-}$ formation. Thus, any means to diminish the level of reduced hemes b is expected to diminish the risk of $O_2^{\cdot-}$ formation.

Those two general questions set now the stage for further studies to clarify and define intra- and inter- monomer electron transfer in this system and its possible physiological role in regulating electron flow and guarding against unwanted ROS.

6. Overview and conclusions

The energy-converting enzymes evolved from ancestral enzymes that functioned in conditions of low O_2 concentration or anaerobicity. These ancestral proteins did not have to deal with the inevitable side-reactions (leaks) that occur when O_2 is present. The different redox tuning adaptations that we have discussed above can be rationalised in the context of avoiding side-reactions with O_2. The key physical values that seem to have had such an influence on this area of bioenergetics are −160 mV (the Em of $O_2/O_2^{\cdot-}$ but note this will have varied depending on the concentration of O_2 in the atmosphere) and 1 eV, the energy difference between O_2 triplet and its highly reactive singlet state.

Redox reactions occurring with intermediates with potentials in the region of −160 mV and lower face the possibility of electrons leaking out to O_2 and forming superoxide. This is dealt with in diverse ways, some of which, but not all, have been presented here: (i) the regulation of electron transfer to avoid the build up of reducing intermediates, e.g. cyclic electron flow around PSI, regulation of PSII; (ii) kinetic control, involving fast electron transfer steps through the most reducing states, e.g. F_X and A_1 in PSI or b_L to b_H electron transfer in bc_1/b_6f; (iii) redox tuning by raising the potential of some intermediates, e.g. stabilising the Q_i and Q_A Q_B semiquinones, or switching from low potential menaquinone to

high potential ubiquinone and plastoquinone [131], (iv) changes in mechanisms/structures, e.g. the appearance of an additional heme in the quinone reduction site of the b_6f complex with respect to its bc_1 counterpart [132–135]. Another adaptation was of course to remove O_2^- and peroxide with specific scavenging enzymes but this was clearly a second line of defence.

When chlorophyll-containing species found themselves in the presence of O_2 then there was a big problem: the excited singlet and more importantly the longer-lived triplet state had more than enough energy (1.3 eV) to drive the conversion of triplet O_2 to its highly reactive singlet form (1 eV). Redox tuning does not help here, this is an energy question and red light is as low in energy as possible for efficient oxygenic photosynthesis. The main fix was to wheel out the carotenoids as quenchers of chlorophyll triplet states and of singlet O_2. However in the reaction centres, chlorophyll triplet could be formed by short circuits such as charge recombination. Here again carotenoid quenching has been employed where possible but this is clearly insufficient and many mechanisms exist where by these short-circuits are minimised. These include the following: (i) big energy gaps, when energetically possible, to prevent back reactions, (ii) redox switching to control the energy gap, e.g. the high and low potential of Q_A in PSII which is related to donor side function; (iii) switching to a lower potential Ph in PSII in high light isoforms of D1, (iv) a bigger energy gap for the specific back-reaction side of the reaction centre (the A-side) in PSI, (v) also perhaps the switching on and off of a futile cycle in PSII (see [46]).

In PSII, given the extreme oxidising chemistry associated with water oxidation and generation of O_2, there are other protective mechanisms that appear to be there to prevent over-oxidation of the cofactors and groups in their environment (see [46]. These too may have secondary reactions associated with O_2 and its derivatives.

The basic requirements for electron transfer in biology have been established and a few basic rules have been defined [5,16]. What is required is proximity between cofactors and appropriate overall driving forces. Once these are in place, the system has little or no need for fine-tuning in order to promote productive electron transfer. Indeed, it has been shown that big variations in the energy levels of intermediates have little effect on the final (quantum) yield of the forward reactions (see e.g. [136,137]). This provides great robustness to the system. Here however we have presented several cases (and there are others), where the fine-tuning of energy levels does occur. Among these there are examples where redox tuning is used to obtain totally different outcomes, while the same cofactor distances are maintained. These situations are specifically associated with circumstances in which the desired energy-useful outcome of catalysis becomes less important than saving the system from damaging reactions, particularly with O_2, that put the viability of the organism in danger.

Some of the key features of the bioenergetic redox enzymes can thus be understood from an evolutionary viewpoint, where enzymes that were already optimised for productive energy conversion in an anaerobic or low O_2 environment had to compromise efficiency in order to survive the arrival of O_2. Here we have dealt with only a limited number of enzymes and a limited number of their features. It seems likely that similar thinking could provide further insights not only into these complex enzymes but also into other bioenergetic redox enzymes.

Acknowledgements

We would like to thank Wolfgang Nitschke for stimulating discussion. The authors would like to thank the proprietors of the Pré Verre, Paris, for the food and environment which fostered the project of this review. AWR is the recipient of the Wolfson Merit award of the Royal Society. A.O. acknowledges The Wellcome Trust International Senior Research Fellowship (WT076488).

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