Hypothesis

ON THE ROLE OF CYTOCHROME b_{559} IN OXYGEN EVOLUTION IN PHOTOSYNTHESIS

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1. Introduction

The role of cytochrome b_{559} in photosynthesis has remained enigmatic despite considerable efforts to elucidate its function [1]. Some experiments show that cytochrome b_{559} is closely associated with the oxidizing side of photosystem II, others indicate redox activity between the two photosystems, while still others suggest participation in a cycle around photosystem II. Part of the enigma of cytochrome b_{559} is that its redox changes measured under normal physiological conditions are too small and too slow to be consistent with its expected role in the electron transfer chain between photosystem II and photosystem I. In order to observe appreciable light-induced changes of cytochrome b_{559} it has generally been necessary to modify the chloroplasts in ways which alter the redox properties of the cytochrome or to use non-physiological conditions, such as low temperature, so that the physiological significance of many of the experimental approaches has been open to question.

It was first proposed that cytochrome b_{559} functioned in the electron transport chain between the two photosystems on the basis of the reversible photooxidation by photosystem I and photoreduction by photosystem II which could be observed in the presence of low concentrations of CCCP [2] but those light-induced redox changes did not occur in the absence of CCCP. It was later shown that this characteristic redox behavior could also be induced by the addition of FCCP, antimycin A [3], PMS [4] or acid to pH 5.0 [5]. However, all of these reagents caused a decrease in the midpoint potential of the normally high potential cytochrome b_{559}. The photosystem I oxidation of cytochrome b_{559} which occurred in the presence of those reagents could be blocked by DBMIB which also indicated a low potential form of the cytochrome with a midpoint potential of 80–100 mV [6]. However, this low potential form of cytochrome b_{559} was generally thought to be associated with denatured chloroplasts while a high potential form with a midpoint potential near 370 mV was considered to be characteristic of normal chloroplasts.

At the same time, cytochrome b_{559} appeared to be in close association with the oxidizing side of photosystem II. This was shown most clearly by the photooxidation of cytochrome b_{559} by photosystem II at −196°C [7]. However, other lines of evidence also indicated an association between cytochrome b_{559} and the oxygen evolving apparatus. Reagents or treatments which inhibit oxygen evolution by blocking electron transport between water and photosystem II and which also cause manganese to be released from its active, bound state in the thylakoid membranes [8] invariably cause a marked decrease in the midpoint potential of cytochrome b_{559} [9]. In the case of heptane extracted chloroplasts in which oxygen evolution was absent and cytochrome b_{559} was in a low-potential form, oxygen evolution could

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; PMS, *N*-methylphenazonium methosulfate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; 1799, α,α′-bis (hexafluoroacetonyl) acetone; ADRY, acceleration of the deactivation reactions of the water splitting enzyme; DABS, diazonium benzene sulfonate

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be restored by reconstituting the extracted chloroplasts with plastoquinone A and β-carotene and the low potential cytochrome b_{559} was restored to a higher potential form (or forms) [10]. The effects of low concentrations of CCCP and FCCP to modify cytochrome b_{559} are not shared by most other uncouplers [2,11] but essentially the same effects were found with the uncouplers S-13 and 1799 [11] which, along with CCCP and FCCP, are known as ADRY reagents [12] (i.e., reagents which accelerate the decay of the S_2 and S_3 states involved in oxygen evolution). Thus, it appears that the ADRY effect and the decrease in the midpoint potential of cytochrome b_{559} are somehow related. It has also been shown that the photooxidation of cytochrome b_{559} by photosystem II at −50°C is greater in states S_2 and S_3 than in S_0 or S_1 [13]. I do not propose that cytochrome b_{559} is an electron donor to photosystem II under normal conditions but rather that these various types of results suggest a close physical association between cytochrome b_{559} and the oxygen evolving apparatus.

Much of the confusion and uncertainty about the function of cytochrome b_{559} derives from the unstable nature of its redox properties. It has been common in the literature to speak of the high potential and low potential forms of cytochrome b_{559} as if they were well-defined chemical species with specific midpoint potentials and to operationally define the high potential and low potential forms on the basis of whether or not they are reduced by hydroquinone [14]. However, early redox titration experiments on Tris-washed chloroplasts showed that the redox potential of cytochrome b_{559} was shifted to lower values but as a heterogeneous population which assumed a wide range of midpoint potentials [9]. It is important to bear in mind that while cytochrome b_{559} is normally present as a high potential form it can assume a broad spectrum of lower midpoint potentials. The midpoint potential of cytochrome b_{559} appears to have a normal lower limit of 40–80 mV which is obtained after incubation of chloroplasts in Triton [15,16] (still lower values can be obtained after lipase digestion [17] or extraction with acetone [16] but these treatments involve more extensive denaturation, probably of the lipoprotein character of the cytochrome [18]).

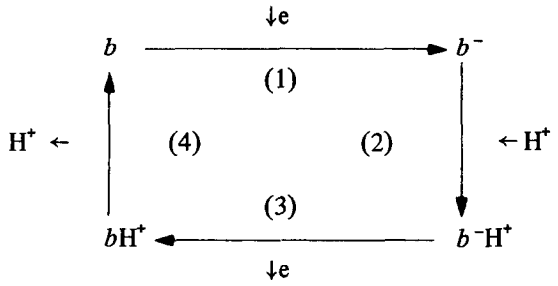
Even what is generally considered to be the high potential form of cytochrome b_{559} has been reported to have midpoint potentials over a range of values between 325 mV [19] and 450 mV [9]. While the value of 450 mV in [9] was assumed to be somewhat too high for unknown technical reasons, it was clear from the absorption spectra of chloroplasts measured at different redox potentials that the midpoint potential of cytochrome b_{559} in normal dark-adapted chloroplasts was appreciably more positive than that of cytochrome *f*. More recently, the midpoint potential of cytochrome b_{559} in dark-adapted spinach chloroplasts at pH 7.8 was determined [20] to be 395 mV, thus confirming that the midpoint potential is more positive than that of cytochrome *f*. However, a redox titration at pH 5.0, where cytochrome b_{559} is known to assume a lower potential form which can be photooxidized by photosystem I [21], gave a value of 335 mV. This change of midpoint potential which was reversible with pH, occurred primarily between pH 6.0 and 5.0. These results indicate that a relatively small shift of midpoint potential (which will move cytochrome b_{559} from the oxidizing side of cytochrome *f* to the reducing side) is sufficient to activate or facilitate the photosystem I mediated oxidation of cytochrome b_{559}. Furthermore, essentially the same results, as were produced by a pH shift to 5.0 in darkness, could be induced at pH 6.0 by preillumination with moderate intensities of red light or even at pH 7.8 by high intensities of preillumination [21]. These latter observations, made under nearly normal physiological conditions, strengthen the view that cytochrome b_{559} may function in the electron transport chain between photosystem II and photosystem I. It appears from such results that the midpoint potential of the high potential cytochrome b_{559} is adjustable and possibly under control by the photosynthetic apparatus in a feedback system that regulates electron flow between the two photosystems.

2. The scheme

My purpose here is to present a scheme for the function of cytochrome b_{559} which accounts both for its role as an electron carrier between the two photosystems and for its close association with the oxidizing side of photosystem II. The scheme proposes

a proton-linked function for cytochrome b_{559} in the water oxidation reactions of the oxygen evolving apparatus. I will assume that cytochrome b_{559} exists as a high potential or low potential form depending on whether or not it is protonated. The scheme in which the cytochrome cycles between a high potential, protonated form, and a low potential, nonprotonated form, is essentially the same as that presented in [22] for mitochondrial cytochromes where their general ligand L is assumed to be a proton. Here, however, it is proposed that the energy availabe between the low potential and high potential forms of the cytochrome can be used to extract a proton from water. The proton-linked function presented here for cytochrome b_{559} is different from that suggested in [23] where it was assumed that the protonated form was the low potential form of the cytochrome.

Using b and b^- to represent the oxidized and reduced forms of cytochrome b_{559}, I propose the following reaction scheme:



Reaction (1) represents the reduction of the low potential, nonprotonated form by photosystem II probably via plastoquinone, possibly in a type of Q cycle as proposed in [24]. Reaction 2 represents the binding of a proton released from water by b^- which results in the formation of the high potential form of the cytochrome. The reduced high potential form b^-H^+ in turn reduces cytochrome f (or possibly plastocyanin) with little loss of redox energy (reaction (3)). The proton associated with the oxidized form bH^+ is assumed to be bound relatively loosely and to be released at the inner side of the thylakoid membrane (reaction (4)). According to this scheme cytochrome b_{559} has both an electron carrier function on the reducing side of photosystem II and a proton-linked function on the oxidizing side which is integrally connected to its redox activity.

The binding constants of the proton for the reduced and oxidized forms may differ by a number of orders of magnitude with the stronger binding (higher pK) invariably occurring with the reduced form [22]. This stronger binding of the proton to the reduced form causes the standard reduction potential of the protonated cytochrome half cell (i.e., the reverse of reaction (3)) to be more positive than that of the nonprotonated cytochrome half cell (reaction (1)):

$$\epsilon_{M(HP)} = \epsilon_{M(LP)} + 0.059\ (pK_{red} - pK_{ox})$$

Thus, the midpoint potential of the protonated cytochrome b_{559} will depend on the pK values of the reduced and oxidized forms, probably with the greater dependence being on the reduced form since it has the stronger proton binding constant.

Experiments on the photooxidation of cytochrome b_{559} by photosystem II at −196°C showing that only half of the high potential cytochrome b_{559} was oxidized by a saturating irradiation established that there are two high potential cytochrome b_{559} molecules per photosystem II reaction center [25]. That factor of Q was confirmed in [26] where it was further shown that all of the high potential cytochrome b_{559} is accessible to photooxidation by photosystem II at −196°C. Recent redox titration experiments [27] indicate that the ratio of low to high potential cytochrome b_{559} in normal dark-adapted chloroplasts is ~1 : 2. Furthermore, in the dark-adapted state, the high potential cytochrome b_{559} is fully reduced and the low potential form fully oxidized (the scheme also predicts that the stable states should be b^-H^+ and b). This stoichiometry in the dark of one oxidized low potential cytochrome b_{559} and two reduced high potential cytochrome b_{559} molecules per photosystem II reaction center may be significant for the functioning of this cytochrome. The fact that very little redox change of cytochrome b_{559} is normally observed at the onset of irradiation could be due to a compensation of the absorbance changes due to the simultaneous photoreduction and photooxidation of the low and high potential forms.

The oxidizing side of photosystem II appears to be buried in hydrophobic regions of the thylakoid membranes. Treatments such as Tris washing or incubation

with chaotropic reagents or with detergents which disrupt membrane structure make the oxidizing side of photosystem II accessible to water soluble, artificial electron donors. These treatments also cause a marked decrease in the midpoint potential of cytochrome b_{559}. It was suggested [9] that the high potential cytochrome b_{559} requires the structural integrity of thylakoid membranes and that disruption of that integrity causes cytochrome b_{559} to be modified to lower potential forms. According to the scheme presented here, variations in the midpoint potential of the high potential cytochrome b_{559} can be attributed to changes in the pK of the reduced form. One would predict higher midpoint potentials and higher pK values of the protonated form if it resides in more hydrophobic regions of the membrane. The midpoint potential of certain heme complexes was reported [28] to be 300 mV more positive in a nonpolar solvent (400 mV in benzene) than in aqueous solution (100 mV). Simple changes of dielectric constant can have large effects on proton binding constants. The pK of benzoic acid, for example, changes from 4.20 in water with a dielectric constant of 78.3 to 10.25 in ethanol with a dielectric constant of 24.3 [29]. Thus, changes of membrane environment which alter the pK of bound, proton-linked, electron carriers can be expected to have profound effects on the redox properties of those carriers. Purified cytochrome b_{559} has been shown to be a relatively large (mol. wt 111 000) lipoprotein of somewhat over 50% lipid [18]. Although the heme appears to be reasonably accessible to water soluble redox reagents [30], it is reasonable to expect that other parts of the molecule will be buried in inaccessible hydrophobic regions of the membrane.

It is implicit in the scheme that the reduction of b to b^- occurs with the proton binding site of the cytochrome in an environment where the effective pH is comparable to or greater than the pK of that binding site. In that case the energy difference between the low and high potential forms of cytochrome b_{559} will be used to bind the proton and therefore stabilize the S state which released the proton. The reaction sequence which assumes that a change in the redox state of the heme will affect the pK of a group some distance removed from the heme, is analogous to a membrane Bohr effect [31] with an e/H^+ ratio of unity.

There is no requirement that the proton binding site of the oxidized protonated form be sequestered in a highly alkaline, hydrophobic region of the membrane. In fact, the reversible protonation–deprotonation would be facilitated if, in effect, the membrane opened up to release the proton from bH^+ and reclosed to allow the tight binding of the proton to b^-. Such turnover of structural changes coupled to the turnover of redox changes would represent interconversions between redox energy and conformational (or binding) energy.

I assume from the work in [20] that, under steady-state electron flow, the midpoint potential of the protonated cytochrome b_{559} is in the range 330–350 mV which can be readily oxidized by cytochrome f. Presumably, the dynamic properties of the membrane are such that the hydrophobic regions are a little less hydrophobic during electron flow so that the midpoint potential of the cytochrome may be somewhat less positive than the 380–400 mV which obtains in the dark-adapted state.

It was clear in the experiments on heptane-extracted chloroplasts [10] that, while β-carotene was sufficient to restore the primary photochemical activity of photosystem II, plastoquinone was needed in addition to restore oxygen evolution and a high potential form of cytochrome b_{559}. The plastoquinone-dependent restoration of a high potential form of cytochrome b_{559} to hexane-extracted chloroplasts was also shown in [34,35]. A requirement for plastoquinone on the water splitting side of photosystem II has also been reported [36,37]. I would suggest that oxygen evolution requires a certain minimum midpoint potential for the protonated form of cytochrome b_{559} and that plastoquinone contributes to the hydrophobic nature of the environment around the proton binding site which results in a more positive midpoint potential for the protonated form of the cytochrome.

It is not necessary to assume that all four of the protons released in the oxidation of water are bound by cytochrome b_{559}. If only one (or two) of the four were bound, i.e., if only one (or two) of the S states were stabilized then only one-quarter (or one-half) of the electron flow between photosystem II and photosystem I would pass through cytochrome b_{559}, the rest going to cytochrome f and plastocyanin by presumably more direct pathways. Thus, the scheme

predicts parallel pathways of electron transport between the two photosystems with only part of the flow passing through cytochrome b_{559}. It was estimated [38] that only 20% of the electron flow passed through cytochrome b_{559}. Various experimental observations have indicated that cytochrome b_{559} plays a relatively minor role as an electron transport component between the two photosystems. However, if the hypothesis presented here has some validity, then even the partial flow through cytochrome b_{559} may be important to the overall process since for each electron transferred, up to 0.3 eV of redox energy created on the reducing side of photosystem II would be available on the oxidizing side to aid in the extraction of a proton from water.

The extent to which such redox energy is required for oxygen evolution is not clear. Treatments which transform the high potential cytochrome b_{559} to very low potential forms inhibit the oxygen evolving apparatus but oxygen evolution survives less drastic modifications of the cytochrome. It is on this basis that I suggest that oxygen evolution may require a certain minimum midpoint potential for the high potential form of cytochrome b_{559}. If the energy difference between the low and high potential forms of cytochrome b_{559} can be used to facilitate oxygen evolution, that role should be most apparent at low light intensities where the need to stabilize the S states is greatest.

The scheme suggests that the action of ADRY reagents might be related to the proton-linked function of cytochrome b_{559}. It was pointed out [39] that ADRY reagents are all uncouplers which have an acidic NH or OH group with a pK between 5.5 and 6.0. If an ADRY reagent supplied protons to cytochrome b_{559}, then the S states normally stabilized would not be stabilized. The observation that ADRY reagents accelerate the decay of states S_2 and S_3 equally [40] could even be extrapolated to suggest that both states are normally stabilized by cytochrome b_{559}. The presence of ADRY reagents could lower the effective pH of the environment around the proton binding site and thus account for the lowering of the midpoint potential of cytochrome b_{559} which accompanies the addition of such reagents. While this proposed mechanism for the action of ADRY reagents is speculative, it does suggest that relevant experimental data might be obtained from flash profiles of proton release measured in uncoupled chloroplasts in the absence and presence of ADRY reagents. It was concluded [41] from such measurements made in the absence of an ADRY reagent that, to a first approximation, the numbers of protons released to the medium on the formation of S_1, S_2, S_3 and S_4 were 1, 0, 1, and 2, respectively. In the present context those results could be taken to indicate that the proton relased on the formation of S_2 was bound by cytochrome b_{559} and held in the membrane for later release at the $S_3 \rightarrow S_4$ transition. On the other hand, the results [42] obtained in the presence of an ADRY reagent showed that roughly equal pH changes occurred inside the thylakoids on each of the first four flashes. It may be that these different sets of results are due to factors other than the absence or presence of an ADRY reagent but that could be determined by direct experiment.

The scheme also predicts that oxygen evolution should be sensitive to chemical oxidants and reductants which react with the high and low potential forms of cytochrome b_{559}. It is not clear, however, that the scheme is consistent with the flash yields of oxygen evolution and the transformations the S states induced by repetitive flash illumination. It was shown with chloroplasts [43] that the maximum yield of oxygen evolution occurred on the fourth flash in the presence of ascorbate while the yield was maximal on the third flash in the control or in the presence of ferricyanide. These results which indicate ascorbate induces a dark transformation of S_1 to S_0 do not indicate that the redox state of cytochrome b_{559} affects the flash yield data to any major extent. However, working with *Chlorella* cells under anaerobic conditions much more dramatic changes in the flash yield data due to varying redox conditions were observed [44]: increasing the ratio of hydroquinone to benzoquinone caused a complete damping of the repetitive flash yield pattern and an inhibition of the steady-state yield. We (J. Farineau and W. L. B., unpublished) examined oxygen evolution from uncoupled chloroplasts with benzoquinone as the electron acceptor in continuous light with a Clark electrode. In the presence of 1 μM FCCP, oxygen evolution was markedly inhibited by the addition of 1 mM potassium ferricyanide but no inhibition occurred in the absence of FCCP. It is not clear that any of these effects of redox conditions on oxygen

evolution is due to changes in the redox state of cytochrome b_{559} but further experiments designed specifically to examine the role of cytochrome b_{559} might be more conclusive.

I have attempted to develop a scheme which accounts for the functioning of cytochrome b_{559} on both the reducing and oxidizing sides of photosystem II and which brings some order to a confusing array of data. To the extent that these efforts are successful, one must be concerned that the order has been imposed from without rather than revealed from within. Such self-fulfilling speculations can be both convincing and misleading and must be subjected to rigorous standards of experimental verification. At most, it is hoped that the scheme presented here will serve as a useful working model which will stimulate penetrating experimental explorations into this refractory region of the photosynthetic apparatus.

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