Role of Bicarbonate in Plastoquinone Reduction Using Site-selected and Site-directed Mutants

by

Govindjee

1. Introduction, Objectives and Significance

In plants and cyanobacteria, two photosystems (I and II) operate in series to extract electrons from water molecules in order to produce the reducing power needed to fix CO_2 to carbohydrates [1]. Photosystem II (PSII) transfers electrons from water to plastoquinone (PQ), reducing it to plastoquinol (PQH_2), whereas PSI transfers electrons from PQH_2, via several intermediates, to $C0_2$. A molecular understanding of PSII, the water-plastoquinone oxido-reductase, has been the focus of our laboratory (see our reviews [1-10]). PSII is unique in having a reaction center chlorophyll a (P680) that has a high enough redox potential to oxidize H_2O. As shown in our laboratory, PSII requires CO_2/HCO_3^- for the reduction of PQ to PQH_2 [3,5,11,12]. The reduction of plastoquinone (PQ) to plastoquinol (PQH_2) follows in two steps and involves two bound plastoquinones Q_A and Q_B, located, respectively, on D2 and D1 proteins of the reaction center [5,13]. These steps, omitting the primary donor P680 and the primary acceptor pheophytin, are:

(1) $Q_A\, Q_B \xrightarrow{h\nu} Q_A^-\, Q_B \xleftrightarrow[(H^+)]{K_1} Q_A\, Q_B^-\, (H^+)$

(2) $Q_A\, Q_B^-\, (H^+) \xrightarrow{h\nu} Q_A^-\, Q_B^-\, (H^+) \xleftrightarrow[(H^+)]{K_2} Q_A\, Q_B\, H_2 \xleftrightarrow{PQ} Q_A\, Q_B + PQH_2$

(1) and (2) stand for odd and even-numbered flashes and K_1 and K_2 are equilibrium constants. Herbicides inhibit electron flow and kill photosynthetic organisms by displacing Q_B [14,15]. Our results show [16,17] that a major effect of bicarbonate-depletion is in the inhibition of electron flow from Q_A^- to Q_B^- (step (2), above) and this, we suggest, arises from the inhibition of the protonation

steps since the electron flow from Q_A^- to Q_B is nearly normal. Furthermore, $C0_2/HCO_3^-$ binding is affected by the binding of herbicides and vice versa [18]. A hypothesis has emerged in which the function of CO_2/HCO_3^- is to aid in the protonation steps and, thus, in the electron flow on the electron acceptor side of PSII [11,16,17]. Furthermore, $HC0_3^-$ may bind on the one hand to the non-haem iron [19,20] located between Q_A and Q_B, and on the other hand, through hydrogen bonding to other amino acids such as arginine (see e.g. [11]) and lysine (B. Diner and coworkers, personal communication) residues on the D1/D2 protein complex.

Our objective in this proposal is to understand, using a multidisciplinary (molecular biology, biophysics and plant physiology) approach, the molecular basis of the unique role of CO_2/HCO_3^- on the electron acceptor side of PSII. In particular, biophysical measurements on the site-directed D1 and D2 mutants, altered in single amino acids in the region of Q_A, Q_B and herbicide binding, will help us identify the binding sites and unravel the function of CO_2/HCO_3^- binding in PSII.

In view of the known interactions of bicarbonate, herbicides and Q_B, the significance of our research extends beyond understanding the details of the molecular mechanism of plastoquinone reduction in PSII. In the long run, it may also provide clues for the design of efficient herbicide-resistant plants.

2. Summary of Relevant Previous Work

The current hypothesis, discussed above, invokes liganding of bicarbonate to Fe^{II} and

hydrogen bonding to certain amino acids (e.g., arginine, lysine, etc.). This is in agreement with the picture obtained from the crystallographic structure of the Fe^{III} - (bi)carbonate protein known as human lactoferrin [21]. Last year, during my tenure as associate member of the Center of Advanced Studies (UIUC) and my sabbatical at Arizona State University at Tempe, I was able to obtain preliminary data showing differential sensitivity of several herbicide-resistant D1 mutants of Synechocystis 6714 to bicarbonate-depletion [22]. The $Ser^{D1\text{-}264}\rightarrow$ Ala mutant was most sensitive, whereas the double mutant $Phe^{D1\text{-}211}\rightarrow$ $Ser/Ala^{D1\text{-}251}\rightarrow$ Val was most resistant to bicarbonate depletion. In Chlamydomonas reinhardtii, the $Ser^{D1\text{-}264}\rightarrow$ Ala mutant was again the most sensitive and $Leu^{D1\text{-}275}\rightarrow$ Phe was the most resistant mutant [23] to bicarbonate depletion. Since Fe sits between Q_A (on D2) and Q_B (on D1), by analogy to bacterial reaction center [19], my graduate student J. Cao constructed several site-directed D2 mutants at two arginine residues (Arg-233 and Arg-251) in the region between the helix IV and V during a semester stay in W.F.J. Vermaas' laboratory at ASU. In addition, I constructed an another D2 mutant ($Arg^{D2\text{-}139}\rightarrow$ Proline) and obtained two mutants that are altered in the Q_A binding region: one of them has an additional arginine next to arginine 251, and in another one tryptophan 253 is mutated to glycine. Preliminary results on $Arg^{D2\text{-}233}\rightarrow$ Gln and $Arg^{D2\text{-}251}\rightarrow$ Ser mutants show that they are ten times more sensitive than the wild type to bicarbonate-depletion (J. Cao, W.F.J. Vermaas and Govindjee, submitted for publication, 1991). Independent work of B. Diner and co-workers (personal communication) has suggested the importance of $Lys^{D2\text{-}265}\rightarrow$ in the bicarbonate effect. The involvement of several amino acids falls in line with our hypothesis based on the human lactoferrin crystallographic structure.

3. Research Plans including Methodology and Roles of Personnel

The current hypothesis, that we are about to model on a computer, predicts that (bi)carbonate is liganded to iron between D1 and D2 and is hydrogen-bonded to several amino acids in the Q_A and Q_B niche on the D2 and D1 proteins. Our proposed research plan falls in two categories: (a) Construction of new site-directed D1 mutants; and (b) Biophysical analyses.

A. Construction of New Site-directed D1 Mutants in the Transformable Cyanobacterium Synechocystis sp. PCC 6803.

First of all, both my graduate student J. Cao and I have extensive experience constructing and characterizing site-directed mutants in Synechocystis 6803. Second, we plan to construct site-directed D1 mutants in this organism in collaboration with the laboratory of Prof A. R. Crofts. Crofts' laboratory has the necessary D1 deletion strain lacking two of the three copies of the psbA gene (that encodes for D1) as well as vectors containing the third remaining copy for site-directed mutagenesis and transformation of the deletion strain. In addition, R. Sayre (Ohio State University) has been generous in giving both Crofts and I strains and plasmids to aid in molecular engineering of the D1 of Chlamydomonas reiihardtii. Although Chlamydomonas mutation work may take some time to start, we plan to begin Synechocystis mutation work right away. The mutations that are specifically intended for the current proposal are in the region of amino acids 264 to 275 because Ser264Ala mutant is very sensitive and Leu275Phe mutant is very resistant to bicarbonate depletion [22,23]. We specifically plan to first target Arg-269 that is assumed to sit at the interface of the helix V and the loop between helix IV and V of the D1 protein. This choice is based on our hypothesis [11] that Arg-269 and His-272 pair aids in the protonation of Q_B^- and Q_B^{2-} (H^+) where

CO_2/HCO_3^- is involved. Other amino acids in the D1 264-268 region may also be involved in the proton relay mechanism according to Crofts (personal communication) and mutants in this region will be constructed in Synechocystis 6803.

One half-time research assistant is needed for 12 months to construct and charcterize the new Synechocystis 6803 D1 mutants, and to continue the maintenance, growth and test of all the existing fifteen D1 and D2 mutants that are differentially sensitive to bicarbonate depletion.

B. Biophysical Assays of the Mutants and the Construction of a Molecular Model for the Role of Bicarbonate in PSII.

Experiments needed to test the role of bicarbonate in the electron and proton flow on the electron acceptor side of PSII will involve parallel biophysical measurements on the existing fifteen D1 and D2 and the new D1 mutants (described under 3.A) that are altered in single amino acids in the plastoquinone and herbicide binding region. The techniques and the methodologies for all the experiments to be performed, are available either in our laboratory [16-18,24-27] or through collaborative arrangements with others. Experiments to be done include: (1) Overall electron flow [24,25]; (2) proton uptake and release (T.G. Ebrey's lab); (3) electron flow from Q_A^- to Q_B (or to Q_B^-) [16,17,26]; and (4) herbicide binding [18; Cao, Vermaas and Govindjee, unpublished]. All of the above experiments will be first done with thylakoid membranes from the mutants and the wild type cells by methods developed in our laboratory [26,27]. Whenever appropriate, intact cells [22,23,26] or photosystem II particles [27,28] will be used, the former to relate the effect to in vivo conditions, and the latter to eliminate any effects of PSI.

One half-time research assistant is needed for 12 months to perform the many biophysical assays on all the mutants. The construction of the molecular model for the role of bicarbonate in PSII will be done by the P.I. using, as base, the Q_A- Q_B model created by H. H. Robinson, C. Yerkes and A. R. Crofts (manuscript, courtesy of Crofts).

4. References

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5. Tasks to be Assigned to Graduate Students and Qualifications Required

Graduate Student #1 for Section 3A (site-directed mutagenesis): Tasks will include growth and maintenance of wild type and mutant strains in liquid or solid agar culture; and cyanobacterial DNA isolation and characterization. Most of the time will be spent in constructing site-directed mutants that requires prepartion of single stranded M13mp18/mp19 templates, DNA sequencing, cloning and subcloning using pUC vectors; transfection and transformation of competent E. coli cells; and transformation of cyanobacterial cells. Student for this job should be a graduate student in Plant Physiology, Molecular Biology, Biophysics or Microbiology; he/she should have had some prior training in Molecular Biology/Microbiology.

Graduate student #2 for section 3B (biophysical assays): Tasks will include biophysical instrumentation, kinetic analysis, measurements of electron flow, proton uptake and release by difference absorption spectrophotometry of pH-sensitive dyes, measurements of Q_A^- decay by a double-flash fluorometer, EPR spectroscopy for Q_A^{-Fe} signals, and for O_2 evolution (method developed in Prof. H. Schwartz's lab.), and other necessary analyses. Student for this job should be a graduate student in Biophysics or Biophysical Chemistry. He/she should have had some prior training in instrumentation and the use of computers.

6. Budget Justification

$6,000 supply money is needed for the site-directed mutagenesis, including oligonucleotide synthesis, enzymes for restriction analysis, cloning and sequencing, radiochemicals, antibiotics, inhibitors and glassware. Since the Department of Physiology and Biophysics will provide $1,500

for this purpose, only $4,500 is requested from the URB.

Salary money is needed for two half-time research assistants for 12 months for this project. Both tasks are labor intensive and require long hard hours. Department of Physiology and Biophysics has consented to provide $10,881 for one half-time research assistant as matching funds for this project. Thus, I have requested $10,800 from the URB for the second research assistant. Both assitants are necessary for the project.

7. Other Support from the University

The Department of Physiology and Biophysics will provide me with funds for the maintenance of our ultracentrifuge and $1,500 for supplies, as noted above. In addition, the department will provide a computer (worth $2,000) for word processing and a half-time research assistant, as noted earlier (see attached letter from Prof. John Zehr).

$500 has been provided by the Vice Chancellor for my shoe-string grant proposal on this topic for travel to complete a paper in a laboratory in the Netherlands.

8. Why Research Board?

My research had been supported without any break since 1961 till 1986 by the National Science Foundation. Due to angioplasty and other related problems at that time, I was unable to apply for renewal of this grant. During the last 4 years, I was one of the 12 Co-P.I.s of a McKnight research grant that provided me with two graduate students and the necessary supply money. My portion of this grant will be exhausted on July 21, 1991, and there is no provison for additional funds. In the meantime I had applied to DOE and GRGO-USDA (1989, 1990,

respectively), but was turned down. I believe it is because they were not convinced that I can move out of purely biophysical/physiological studies to a study that also includes molecular biology. I have used my sabbatical to train myself in the use of molecular biology, and have used site-selected and site-directed mutants. Our preliminary observations have provided exciting insight into the binding realm of $C0_2/HC0_3^-$ on D1 and D2 proteins. However, I believe a critical mass of data and a couple of high quality publications using molecular biology are needed before I can succeed with the National Agencies like NSF, etc. The support requested from URB will definitely help me in attracting funds from federal agencies. And, I plan to actively seek such funds.

9. Plans for Future Support.

Several grant applications will be submitted during 1991, the first one in June, 1991 to the NSF.

We have already submitted a research training grant (Regulation and molecular mechanism of photosynthesis: research training and education in a multidisciplinary environment) to the NSF ($1,418,500 for 5 years, 11 Co-P.I.'s including myself; C. Wraight, Director).