*Biochimica et Biophysica Acta*, 462 (1977) 414–421


BBA 47396

# PROTON EVOLUTION FROM PHOTOSYSTEM II
# STOICHIOMETRY AND MECHANISTIC CONSIDERATIONS

CHARLES F. FOWLER

*Martin Marietta Laboratories, 1450 South Rolling Road, Baltimore, Md. 21227 (U.S.A.)*

(Received April 12th, 1977)

## SUMMARY

1. In a sequence of flashes given to dark-adapted chloroplasts, the flash yield of proton release oscillates with a period of 4, which is similar but not identical to the oscillation of the $O_2$ flash yield.

2. Using the proton release associated with ferricyanide reduction as a calibration, we computed that two protons are released in the terminal $O_2$-liberating reaction; the other two protons are released in precursor conversion steps.

3. Analysis of the effect of preflashes on the oscillation pattern showed that the $S_1 \rightarrow S_2$ transition releases no proton, the $S_0 \rightarrow S_1$ transition somewhat less than one (0.75), and the $S_2 \rightarrow S_3$ transition somewhat more than one (1.25).

4. The precision of the data was sufficient to exclude the possibility that in the four-step water oxidation, proton release follows a simple 1, 0, 1, 2 pattern.

A possible model to interpret the observed flash yield patterns is discussed.

---

## INTRODUCTION

In a recent paper, we reported that the flash yield of proton release, in a sequence of flashes given to dark adapted chloroplasts, oscillates with a period of 4 [1]. Because of the similarity to the flash yield pattern of $O_2$ evolution (and because it was the simplest interpretation of the data), we leaned towards a "concerted" reaction where $4H^+$ were released concurrently with every $O_2$. We recognized at least two problems with this interpretation: (1) the measurements were made in relative units and the stoichiometry with $O_2$ evolution undetermined, and (2) several differences of a rather variable nature were encountered between the flash yield patterns of proton and $O_2$ release.

In more recent papers we presented (1) a procedure to "calibrate" the proton flash yields, [2] and (2) data that showed the occurrence of 2 distinct oscillatory components, during a sequence of flashes [3]. One of these – with a periodicity of two – was described in some detail and was shown to be associated with electron pairing and unpairing in the transport chain between the photosystems. The other oscillation exhibits a dominant periodicity of 4, is associated with $O_2$ evolution and is the subject of this paper.

## MATERIALS AND METHODS

Chloroplasts were isolated as previously described [1]. Flash-induced changes in pH were measured with a rapidly responding glass membrane cup electrode. Details of construction and use were given previously [1–3]. 0.25 ml of the required chloroplast suspensions were gently layered on to the glass membrane and followed by a short dark period to allow the system to stabilize. Suspensions were illuminated with 2 $\mu$s duration saturating xenon flashes spaced 1 s apart. Other details are described in the text.

## RESULTS

### *Interference of binary oscillation*

A difficulty in the study of flash-induced proton release associated with the $O_2$ evolution process is the interference caused by the binary oscillation of proton release coupled to electron transport between the photoacts. While it is relatively easy to obtain the binary oscillations by itself, e.g., in the presence of semicarbazide (Ref. 3 or see Fig. 1d), it proved more difficult to create conditions which show the quaternary oscillation entirely free of the binary one.

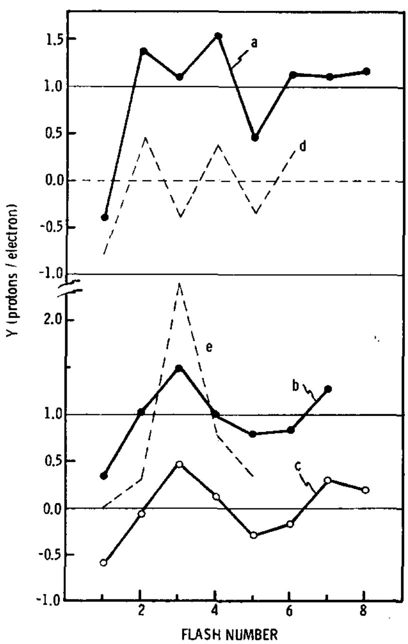


Fig. 1. Flash induced pH changes in spinach chloroplast plotted as a function of flash number. Chloroplasts were dark adapted 5 min prior to the initiation of each flash sequence. (a) Sample contained gramicidin D, $3 \cdot 10^{-6}$ M and ferricyanide (1.0 mM). (b) Chloroplasts were osmotically shocked prior to measurement. Sample contained only ferricyanide (1.0 mM). (c) Sample contained only $3 \cdot 10^{-6}$ M gramicidin D. (d) Binary proton exchange associated with electron pairing in the chain connecting the photosystems. (e) $O_2$ flash yield pattern obtained after 5 min dark adaptation.

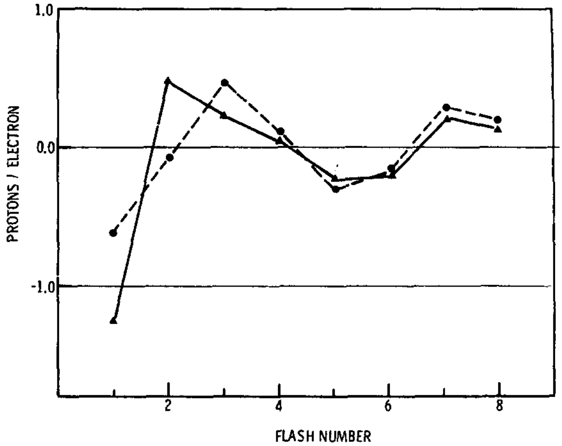


Fig. 2. Triangles : flash-induced pH changes in spinach chloroplasts following preillumination with far-red (720 nm interference filter) and 5 min dark. Sample contained no acceptor and $3 \cdot 10^{-6}$ M gramicidin D. Dots: data of Fig. 1C replotted for comparison.

The observation of the binary oscillation in pH depends upon the release of protons which follows the reduction of cytochrome *f* and/or plastocyanin by a protonated intermediate such as quinone. It occurs to the full extent in the presence of 1.0 mM ferricyanide, which in normal chloroplast preparations: (a) acts as an electron acceptor for System I but (b) slowly oxidizes all intermediates between the two photoacts (10–30 s darkness). Fig. 1 (a) shows the pattern of proton flash yields observed under these conditions. Presumably, it reveals both binary and quaternary oscillations to their full extent [3].

In osmotically shocked chloroplasts (as used in the experiment in Fig. 1b) the binary oscillation is lost, presumably because ferricyanide has direct access to System II [3]. We assume that the quaternary oscillation is unaffected.

With viologen as an acceptor and normal chloroplasts, the same phenomenon occurs as with ferricyanide but in this case dark oxidation of the intermediate pools (by $O_2$) requires several minutes. After this dark period, *P*-700 and its immediate electron donors are reduced, but these electrons are removed in the first 2–3 flashes, which makes the non-protonated electron acceptors available.

In the absence of an electron acceptor, the observation of binary oscillations depend mostly upon the prehistory of the sample. A far red preillumination fully oxidizes the intermediates between the photoacts [5]. In the subsequent dark period, the $O_2$ system deactivates and *P*-700, etc. are reduced. The flash sequence still reveals the binary oscillations but only during the first 2–3 flashes (Fig. 2, full line). On the other hand, if white light is used for the preillumination, no binary oscillation is seen (Figs. 1 (c) and 2, dashed). Presumably in this case, some protonated reducing equivalents remain in the pool; these can pair with those produced in the first few flashes so that the oscillation is fully damped.

*Description of quarternary oscillation*

Fig. 1 shows four proton flash yield patterns, the binary (d) the quarternary (1b and c) and the sum of both (a). Patterns b and c are very similar; adding either of these to pattern d results in sequence a. We therefore are confident that pattern c truly reflects the proton release associated with $O_2$ evolution.

For quantitative analysis, we assumed that in the presence of ferricyanide, the average value of the first 8 flash yields (Fig. 1a, b) was $1H^+$/flash [2, 3]. When plotting data, obtained in the absence of an electron acceptor (when the net proton release was zero), the average yield value of the sequence was shifted to 1.0, the average yield obtained in parallel experiments with ferricyanide (cf. Fig. 3b, right-hand ordinate).

A comparison of the proton yield pattern in Fig. 1b with a typical $O_2$ yield pattern shown in Fig. 1e [6, 7] reveals the considerable differences. For example, protons are evolved after flash one, but $O_2$ is not; the oscillation of the pH yield is much more shallow than the $O_2$ oscillation (e.g., the ratio $Y_3/Y_5$ is 1.8 for $H^+$ and 7 for $O_2$), as if the oscillations were superimposed on a monotonous background flash yield. If only the oscillatory part of the proton yield pattern is considered, we find that $Y_2$ is larger, $Y_3$ smaller, and $Y_4$ larger than the corresponding $O_2$ yields.

Evidently, our earlier speculation [1] viz, that all four protons are released in the final $O_2$ evolution step in a "concerted reaction", is inadequate since more than one, and possibly all four, charge accumulation steps appear to contribute to proton release. On the other hand, the fact that $Y_3$ and $Y_7$ are maximal indicates that the final step $S_3 \rightarrow S_0+O_2$ releases a substantial fraction of the protons. Data presented below will show that this fraction is 50 % ($2H^+$ per $O_2$).

*Contributions of precursor states to proton evolution*

To obtain more quantitative information concerning the contribution of the four steps, we performed the pre-flash experiments shown in Fig. 3a and b. Each point is the average of 10 measurements on independent samples. The chloroplast suspensions contained either ferricyanide or no acceptor respectively. Each sample was first illuminated with 25 flashes, spaced 1 s apart, to randomize the states and then dark adapted for 5 min. The appropriate pre-flash regime was then given and followed by another 5 min dark period prior to the measurement of the flash yield sequence.

Inspection of the data shows that the degree of modification of each individual flash yield induced by pre-flashes is not influenced by the presence or absence of the binary oscillation which is present in the experiment shown in fig. 3a, but absent in 3b. Evidently the binary oscillation is unaffected by the pre-flashes.

A cursory examination of Fig. 3 reveals that one pre-flash causes a decrease of yields 1, 4, and 5, and an increase of yields 2 and 3. The changes induced by three pre-flashes are more or less the opposite. Since these modifications of flash yield are sustained in the subsequent cycle of 4, they quite probably are due to changes in the S state distribution. Presumably, [6, 7] one pre-flash increases the initial ratio $[S_1]/[S_0]$ from 75/25 to approx. 100/0, while 3 pre-flashes decrease this ratio to approx. 50/50. Thus, the size of the first proton flash yield, $Y_1$, appears to correlate with the amount of $S_0$ present before the first flash, i.e., the step $S_0 \rightarrow S_1$ is associated with proton release, but the transition $S_1 \rightarrow S_2$ might not be. Similarly, $Y_2$ is enhanced by one preflash and decreased by three preflashes, indicating that the transition $S_2 \rightarrow S_3$ (large after one, small after three preflashes) is associated with substantial proton release.

For a more quantitative analysis of the data, we calculated the population of each S state during sequences of 8 flashes using the formulations developed in refs. 6

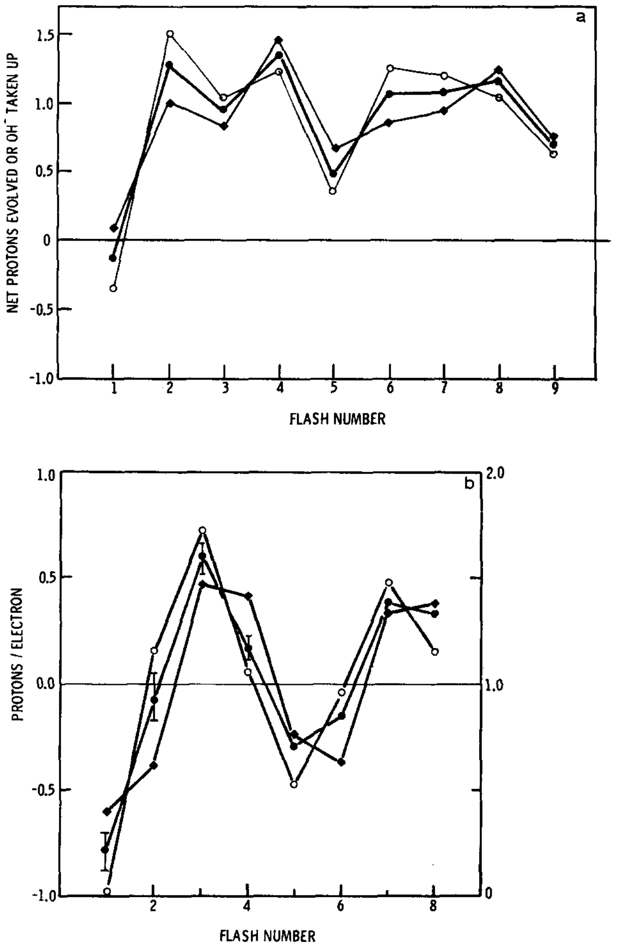


Fig. 3. Flash-induced pH changes obtained in spinach chloroplast following 3, 1 and no pre-flashes. A 5 min dark period preceded and succeeded each pre-flash regime. Sample 3A contained $3 \cdot 10^{-6}$ M gramicidin D and 1.0 mM ferricyanide and 3B contained gramicidin D and no acceptor. ●, No pre-flash ○ 1 pre-flash, ◆ 3 pre-flashes. The right hand ordinate was used for numerical computation.

and 7.

$$Y_{n+1}/1-\alpha = a[S_0]_n+b[S_1]_n+c[S_2]_n+d[S_3]_n$$

where the coefficients $a$, $b$, $c$, and $d$ denote the number of protons liberated in the four respective transitions (e.g., $S_0 \xrightarrow{h\nu} S_1 + a\ H^+$).

We assumed a homogenous value $\alpha = 0.1$ (representing the misses on each step). We further assumed that after 5 min darkness, following 0, 1 and 3 preflashes, states $S_3$ and $S_2$ disappeared and the ratio $[S_1]/[S_0]$ became 75/25, 97/3 and 50/50, respectively.

The initial S state distribution and the first four flash yields provide four equations from which the four coefficients can be calculated. The four flash yields

TABLE I

VALUES OF THE FOUR COEFFICIENTS, REFLECTING THE NUMBER OF PROTONS RELEASED IN THE FOUR S STATE CONVERSIONS

The values were calculated from the data in Fig. 3b (0, 1, 3 preflashes). Column 4 is the average of the first 3 columns. See text for further description.

| Coefficient | 0 | 3 pre-flashes | 1 pre-flash | Average |
|---|---|---|---|---|
| $a$ | 0.70 | 0.78 | 0.78 | 0.75 |
| $b$ | 0.10 | 0.00 | 0.00 | 0.03 |
| $c$ | 1.40 | 1.27 | 1.28 | 1.32 |
| $d$ | 2.00 | 2.00 | 1.80 | 1.93 |
| $\Sigma$ | 4.2 | 4.05 | 3.86 | 4.04 |

TABLE II

COMPARISON OF THE OBSERVED FLASH YIELDS IN FIG. 3B WITH THOSE COMPUTED FOR SEVERAL SETS OF COEFFICIENTS ($a$, $b$, $c$, $d$).

Yields taken from Fig. 3b were multiplied by 0.9 in order to take into account the 10 % miss factor. Other details can be found in the text. Observed values are the average of 10 independent experiments. The average deviation is 0.05.

| Flash No. | Observed | (0.67, 0.0, 1.33, 2.0) | (0.75, 0.0, 1.25, 2.0) | (1.00, 0.0, 1.00, 2.0) | (0.5, 0.0, 1.5, 2.0) |
|---|---|---|---|---|---|
| Zero preflash | | | | | |
| 1 | 0.19 | 0.15 | 0.17 | 0.23 | 0.11 |
| 2 | 0.84 | 0.82 | 0.78 | 0.63 | 0.92 |
| 3 | 1.45 | 1.50 | 1.48 | 1.40 | 1.56 |
| 4 | 1.05 | 1.09 | 1.13 | 1.23 | 1.04 |
| 5 | 0.64 | 0.44 | 0.47 | 0.64 | 0.40 |
| 6 | 0.76 | 0.66 | 0.64 | 0.56 | 0.70 |
| 7 | 1.25 | 1.22 | 1.19 | 1.11 | 1.27 |
| 1 preflash | | | | | |
| 1 | 0.00 | 0.02 | 0.02 | 0.03 | 0.02 |
| 2 | 1.04 | 1.07 | 1.01 | 0.81 | 0.41 |
| 3 | 1.55 | 1.67 | 1.66 | 1.62 | 1.70 |
| 4 | 0.94 | 0.92 | 0.97 | 1.13 | 0.83 |
| 5 | 0.48 | 0.26 | 0.31 | 0.35 | 0.22 |
| 6 | 0.86 | 0.76 | 0.73 | 0.61 | 0.84 |
| 7 | 1.33 | 1.40 | 1.37 | 1.29 | 1.44 |
| 3 preflashes | | | | | |
| 1 | 0.35 | 0.30 | 0.34 | 0.45 | 0.23 |
| 2 | 0.55 | 0.58 | 0.55 | 0.45 | 0.64 |
| 3 | 1.32 | 1.34 | 1.30 | 1.19 | 1.42 |
| 4 | 1.28 | 1.26 | 1.28 | 1.33 | 1.23 |
| 5 | 0.68 | 0.62 | 0.65 | 0.75 | 0.55 |
| 6 | 0.56 | 0.54 | 0.54 | 0.51 | 0.56 |
| 7 | 1.20 | 1.05 | 1.02 | 0.93 | 1.11 |

were normalized, so that a consistent answer should yield $a+b+c+d = 4$ for the three experiments in Fig. 3b. The resulting coefficients for the 3 pre-flash regimes are tabulated in Table I. The average for each coefficient is listed in the last column. The variation of the individual coefficients is small, which supports the supposition that the modification of the flash yields is due only to changes of the initial S-state distribution by preflashing.

We can confidently conclude from Table I that 2 protons are released during the terminal $O_2$ evolving step ($d = 2$). The lack of any proton release in the $S_1$ to $S_2$ transition is also evident ($b = 0$). The coefficients $a$ and $c$ are close to unity and one is tempted to assume that proton release occurs in a straightforward stoichiometric sequence 1, 0, 1, 2.

Upon closer inspection, however, we observe that $a$ and $c$ are consistently smaller and greater than one, respectively for all three pre-flashing regimes. To test this conclusion, we computed yields for 3 assumed sets of coefficients where $a$ and $c$ were varied between 0.5 and 1 and 1.5 and 1 respectively. On first sight, all three yield patterns seem to agree qualitatively with the observed data (Table II). However, a more careful inspection of the individual yields show that (1) more of the computed yields for the 1, 0, 1, 2 and the 0.5, 0. 1.5, 2 sets of coefficients fall outside one standard deviation than the 0.75, 0, 1.25, 2.0 set. (2) Yields for specific items most sensitive to variation of $a$ and $c$, particularly $Y_2$ show much larger deviations. One predicts an actual decrease in $Y_2$ following a single preflash for the 0.5, 0, 1.5, 2.0 case which is opposite to that observed. Also, the second flash yield for the 1, 0, 1, 2 case is much less than that observed for both the 0 and 1 pre-flash regimes. Therefore, we conclude that the most likely distribution falls near the 0.75, 0.0, 1.25, 2.0 set.

*Two paths to $O_2$ evolution*

To explain the deviation from the 1, 0, 1, 2 distribution, we assume that two pathways lead to $O_2$ evolution. A proposed model is illustrated in Fig. 4. One path releases a proton during the $S_0$ to $S_1$ transition and one during the $S_2$ to $S_3$ transition (1, 0, 1, 2). The other path releases no protons during the $S_0$ to $S_1$ transition, but releases two during the $S_2$ to $S_3$ transition (0, 0, 2, 2). The observed yields are linear combinations of the proton evolution in the two pathways. For instance, if $a = 0.75$, 75 % of $S_0$ go by the 1, 0, 1, 2 path and 25 % by the 0, 0, 2, 2 route, the net result being a (0.75, 0, 1.25, 2) distribution. The model shown in Fig. 4 accounts for the number of electrons (lower subscripts) as well as the number of protons (upper subscripts) which

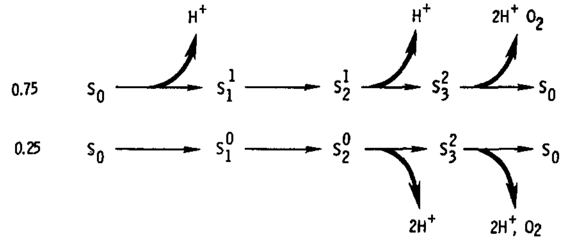


Fig. 4. A model for proton evolution associated with $O_2$ evolution. The upper subscript denotes loss of proton and lower, loss of electrons.

are released in each step. $S_1$ and $S_2$ can occur in one of two protonated states $S_1{}^0$ or $S_1{}^1$, and $S_2{}^0$ or $S_2{}^1$. However, neither $S_0$ nor $S_3$ can be distinguished on this basis.

No obvious explanation can be given at this time for the apparent existence of two pathways. We may point to resemblance between the 75/25 distribution of the two pathways in Fig. 4 and (a) the dark equilibrium ratio of $[S_1]/[S_0] = 75/25$ [6, 7] following white light illumination and (b) the dark equilibrium state of the pairing site $[B]/[B^-] = 70/30$ [3] in the chain connecting the photosystems. Whether these similarities are fortuitous or have a mechanistic implication remains to be seen.

One must be cautious in relating these results to a specific mechanism for $O_2$ evolution because of the inability to distinguish between $H^+$ release or $OH^-$ uptake. Based simply on the number of oxidizing equivalents accumulated, $S_3{}^2$ might represent a superoxide state which is oxidized to $O_2$ in the final step. In order to be consistent with the data, this would require the uptake of 2 $OH^-$ during the terminal step. However, an equally likely possibility is that $S_3$ exists at the peroxy level. The third oxidizing equivalent would combine with the 4th made in the light during the terminal step and together carry out the final oxidation to $O_2$ and $2H^+$.

The evolution of $2H^+$ in 3 steps leaves one charge unstabilized. Since $O_2$ evolution has an obligatory requirement for one of the ions, chloride, bromide, or iodide [8], but none for cations (other than $H^+$), the stabilization might require the uptake of an ion during transitions of the precursor states. According to results, this would most likely occur during the $S_1$ to $S_2$ transition in both pathways, and the $S_0$ to $S_1$ transition in one of the pathways.

Although we feel that these numbers represent our best estimate of the contribution each S-state transition makes to proton evolution, we do observe, infrequently, unexplained anomalous proton yield patterns, see for example Fig. 5 in ref. 1. They may simply represent an occasional erroneous measurement, but we cannot totally discount the possibility that they reflect a real but infrequent alteration in the $O_2$ evolving system.

## ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Energy Research and Development Administration Contract E(11-1)-3326. This material is also based upon research supported by the National Science Foundation under Grant No. PCM74-20736. Any opinions, findings, conclusions or recommendations expressed herein are those of the author and do not necessarily reflect the views of NSF. Appreciation is also expressed to Dr. Bessel Kok for helpful discussions during the preparation of this manuscript.

## REFERENCES

1 Fowler, C. F. and Kok, B. (1974) Biochim. Biophys Acta 357, 299–307
2 Fowler, C. F. and Kok, B. (1976) Biochim. Biophys Acta 423, 510–523
3 Fowler, C. F. (1977) Biochim. Biophys Acta 459, 351–363
4 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256
5 Marsho, T. V. and Kok, B. (1970) Biochim. Biophys. Acta 223, 240–250
6 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
7 Forbush, B. and Kok, B. (1971) Photochem. Photobiol. 17, 307–321
8 Hind, G., Nakatani, H. Y. and Izawa, S. (1969) Biochim. Biophys. Acta 172, 277–289