fluorescence quenching both in the dark and under steady-state illumination, and (*b*) that changes in the maximum quantum yield of fluorescence can be quantitatively related to the fraction of open PS II reaction centers (Falkowski et al., 1988; Genty et al., 1989).

In conclusion, we agree with Trissl et al. and Holzwarth that the interpretation of the standard fluorescence induction curve is complex. However, their criticisms regarding fluorescence induction measurements in relation to both the quantum efficiency and the transfer of reducing equivalents to the acceptor side of PSII are unwarranted. We concur that the interpretation of the conventional fluorescence induction measurements within the context of the absorption cross section of PSII or the probability of transfer of excitation between reaction centers is poorly supported on theoretical grounds. We point out, however, that measurements of variable fluorescence induced by single turnover flashes can be conveniently used to derive the desired parameters, and that such measurements are consistent with both picosecond fluorescence lifetime analyses and with conceptual models of exciton trapping and charge separation in PSII.

REFERENCES

Falkowski, P. G., Z. Kolber, and Y. Fujita. 1988. Effect of redox state on the dynamics of Photosystem II during steady-state photosynthesis in eucaryotic algae. *Biochim. Biophys. Acta.* 933:432–443.

Falkowski, P. G., Y. Fujita, A. Ley, and D. Mauzerall. 1986a. Evidence for cyclic electron flow around photosystem II in *Chlorella pyrenoidosa*. *Plant Physiol.* 81:310–312.

Falkowski, P. G., K. Wyman, A. C. Ley, and D. Mauzerall. 1986b. Relationship of steady state photosynthesis to fluorescence in eucaryotic algae. *Biochim. Biophys. Acta.* 849:183–192.

Genty, B., J.-M. Briantis, and N. R. Baker. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta.* 894:183–192.

Greene, R. M., R. J. Geider, Z. Kolber, and P. G. Falkowski. 1992. Iron-induced changes in light harvesting and photochemical energy conversion processes in eucaryotic marine algae. *Plant Physiol.* 100:565–575.

Haehnel, W., J. A. Nairn, P. Reisenberg, and K. Sauer. 1982. Picosecond fluorescence kinetics and energy transfer in chloroplasts and algae. *Biochim. Biophys. Acta.* 680:161–173.

Holzwarth, A. R. 1986. Fluorescence lifetimes in photosynthetic systems. *Photochem. Photobiol.* 43:707–725.

Holzwarth, A. R. 1993. Is it time to throw away your apparatus for chlorophyll induction? *Biophys. J.* 64:1280–1281.

Kolber, Z., J. Zehr, and P. G. Falkowski. 1988. Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in Photosystem II. *Plant Physiol.* 88:923–929.

Leibl, W., J. Breton, J. Deprez., and H.-W. Trissl. 1989. Photoelectric study on the kinetics of trapping and charge stabilization in oriented PSII membranes. *Photosyn. Res.* 22:257–275.

Ley, A. C., and D. Mauzerall. 1986. The extent of energy transfer among photosystem II reaction centers in *Chlorella*. *Biochim. Biophys. Acta.* 850:234–248.

Malkin, S., and B. Kok. 1966. Fluorescence induction studies in isolated chloroplasts I. Number of components involved in the reaction and quantum yields. *Biochim. Biophys. Acta.* 126:413–432.

Mauzerall, D. 1972. Light-induced changes in *Chlorella*, and the primary photoreaction for the production of oxygen. *Proc. Nat. Acad. Sci. USA.* 69:1358–1362.

Mauzerall, D., and N. L. Greenbaum. 1989. The absolute size of a photosynthetic unit. *Biochim. Biophys. Acta.* 974:119–140.

Paillotin, G. 1976. Movement of excitations in the photosynthetic domains of photosystem II. *J. Theor. Biol.* 58:237–252.

Roelofs, T. A., C.-H. Lee, and A. R. Holzwarth 1992. Global target analysis of picosecond chlorophyll kinetics from pea chloroplasts. A new approach to the characterization of the primary processes in photosystem II α and β-units. *Biophys. J.* 61:1147–1163.

Schatz, G. H., H. Brock, and A. R. Holzwarth. 1988. Kinetic and energetic model for the primary processes in photosystem II. *Biophys. J.* 54:397–405.

Trissl, H.-W., Y. Gao, and K. Wulf. 1993. Theoretical fluorescence induction curves derived form coupled differential equations describing the primary photochemistry of photosystem II by an excition-radical pair equilibrium. *Biophys. J.* 64:974–988.

Response to Falkowski et al.

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The comments of Falkowski et al. in response to our article on theoretical fluorescence induction curves Trissl et al. 1993 require confirmation in some parts but also some criticism.

It is unfortunately true that our article contained some errors in the numerical values of F_v, F_m, F_a, and Φ_p. For parameter set No. 3 the rate constant for losses of the radical pair, k_r, was not quantified. An erratum has been printed (*Biophys. J.* 65:982–983, 1993). Despite this, all conclusions listed at the end of our article remain valid, in particular the statement of the absolute value of the complementary area depending on the rate constants selected for the exciton radical pair equilibrium model. Regrettably, this point is not discussed in the article of Falkowski et al.

Falkowski and his colleagues argue on whether the relations $(F_v/F_m)/\Phi_p$ and $(F_a/F_v) \cdot \Phi_p$ are constant or not. Recently, Dr. Jérôme Lavergne and I have succeeded in deriving analytical formulae for fluorescence induction curves derived from the exciton-radical pair equilibrium model. These equations allow effortless tests of any proposed relationship with high numerical accuracy, and they do not suffer from time-consuming computational calculations. With the availability of the analytical solution it is easy to prove that $(F_v/F_m)/\Phi_p = f(k_i)$, i.e., is a function of the rate constants, and that $(F_a/F_v) \cdot \Phi_p = 1$, i.e., is independent of the rate constants. The term $(F_a/F_v) \cdot \Phi_p$ expresses the number of electrons transferred. It thus turns out that the rela-

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tionship originally derived by Malkin and Kok (1966) still holds in the framework of the exciton-radical pair equilibrium model.

Finally, I would like to point out that the interpretation of fluorescence induction curves with blocked electron transfer between Q_A and Q_B has been subject to critique for different reasons for a long time (Doschek and Kok, 1972; Hemelrijk and van Gorkom, 1992; France et al., 1992). A critical review of the widely used commercial PAM-fluorimeter has been published recently (Büchel and Wilhelm, 1993). It is also worth mentioning that even experimental data on the shape of fluorescence induction curves obtained with the pump-probe technique are contradictory (Hemelrijk and van Gorkom, 1992; France et al., 1992), and thus appear not to be a "better" alternative to conventional methods. In my view a complete interpretation of experimental fluorescence induction curves is still an open subject that will progress by comparison with model predictions. Experimental observations like those offered by Falkowski et al. do neither prove nor disprove the self-consistency of any conclusions drawn from a purely theoretical treatment of the problem.

REFERENCES

1. Trissl, H.-W., Y. Gao, and K. Wulf. 1993. Theoretical fluorescence induction curves derived from coupled differential equations describing the primary photochemistry of photosystem II by an exciton/radical pair equilibrium. *Biophys. J.* 64:984–998.
2. Malkin, S., and B. Kok. 1966. Fluorescence induction studies in isolated chloroplasts. I. Number of components involved in the reaction and quantum yields. *Biochim. Biophys. Acta.* 126:413–432.
3. Doschek, W.W., and B. Kok. 1972. Photon trapping in photosystem II of photosynthesis. The fluorescence rise curve in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. *Biophys. J.* 12:832–838.
4. Hemelrijk, P.W., and H.J. van Gorkom. 1992. No double hit involved in fluorescence induction of photosystem II of spinach chloroplasts. *In* Research in Photosynthesis. Vo. II. N. Murata, editor. Kluwer Academic Publishers, The Netherlands. 33–36.
5. France, L.L., N.E. Geacintov, J. Breton, and L. Valkunas. 1992. The dependence of the degrees of sigmoidicities of fluorescence induction curves in spinach chloroplasts on the duration of actinic pulses in pump-probe experiments. *Biochim. Biophys. Acta.* 1101:105–119.
6. Büchel, C., and C. Wilhelm. 1993. In vivo analysis of slow chlorophyll fluorescence induction kinetics in algae: progress, problems and perspectives. *Photochem. Photobiol.* 58:137–148.

Methods for Calibration of Fluorescent Calcium Indicators in Skeletal Muscle Fibers

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In a recent paper in this journal Baylor and co-workers concluded that the free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in frog skeletal muscle fibers at rest is at least 100 nM and may be as large as 300 nM (Harkins et al., 1993; see also Kurebayashi et al., 1993). This $[Ca^{2+}]_i$ is higher than that frequently reported for muscle fibers, and hence, it raises the question about the accuracy of various methods used to measure $[Ca^{2+}]_i$ (Morgan, 1993; Ross, 1993).

In the paper by Kurebayashi et al. (1993), $[Ca^{2+}]_i$ was measured with the fluorescent indicator fura red, whereas Harkins et al. (1993) used another fluorescent Ca^{2+} indicator, fluo-3. The methods of calibration of these two indicators were similar in that the fraction of indicator in the Ca^{2+}-bound form at rest (f_r) was established by a combination of in situ and in vitro measurements. The value of f_r was then combined with estimates of the apparent dissociation constant (K_D) to get $[Ca^{2+}]_i$ at rest; estimates of K_D were obtained either in vitro or from rate constants obtained from $[Ca^{2+}]_i$ transients due to stimulation. The estimates of f_r and K_D depended on the calibration technique and showed a large spread both with fura red and fluo-3. However, the authors conclude that the $[Ca^{2+}]_i$ at rest is at least 100 nM and can be as large as 300 nM (Harkins et al., 1993).

The methods described by Baylor and co-workers are attractive in that a complete calibration can be performed without causing damage to the cell. The disadvantage is that the techniques are technically rather cumbersome, and to some degree, they depend on in vitro measurements in uncertain solutions. For instance, Harkins et al. (1993) show that when F_{max}/F_{min} of fluo-3 (the fluorescence intensity of the Ca^{2+}-bound and Ca^{2+}-free form, respectively) is large, a term in the calibration equation (their Eq. 4) is of little importance. In simple ionic solutions in vitro they found F_{max}/F_{min} to be 200. To mimic the intracellular environment, they added protein to their calibration solutions, and this markedly reduced the ratio; hence, they assumed an intracellular F_{max}/F_{min} of 100, which required a small (5%) reduction to their estimate of $[Ca^{2+}]_i$. In a recent study (Westerblad and Allen, 1993; see below), we measured R_{max}/R_{min} (the equivalent expression for a ratiometric dye) for another fluorescent Ca^{2+} indicator, indo-1, both in simple ionic solutions in vitro and in situ, and the resulting ratios were 68 and 11, respectively. If a similar intracellular change occurs with fluo-3, then the error in estimated resting $[Ca^{2+}]_i$ by Harkins et al. (1993) becomes rather large, and their value of resting $[Ca^{2+}]_i$ should be reduced by a considerable amount.

We have recently used another method for intracellular calibration of indo-1 (Westerblad and Allen, 1993). Intact,

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