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Redox Processes in the Blue Light Response of Guard Cell Protoplasts of *Commelina communis* L.

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ABSTRACT

Guard cell protoplasts from *Commelina communis* L. illuminated with red light responded to a blue light pulse by an H^+ extrusion which lasted for about 10 minutes. This proton extrusion was accompanied by an O_2 uptake with a $4H^+$ to O_2 ratio. The response to blue light was nil in darkness without a preillumination period of red light and increased with the duration of the red light illumination until about 40 minutes. However, acidification in response to a pulse of blue light was obtained in darkness when external NADH (1 millimolar) was added to the incubation medium, suggesting that redox equivalents necessary for the expression of the response to blue light in darkness may be supplied via red light. In accordance with this hypothesis, the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (10 micromolar) decreased the acidification in response to blue light more efficiently when it was added before red light illumination than before the blue light pulse. In the presence of hexacyanoferrate, the acidification in response to a blue light pulse was partly inhibited (53% of control), suggesting a competition for reducing power between ferricyanide reduction and the response to blue light.

Stomatal response to light depends on two photoreceptor systems: one covers the photosynthetic spectrum and corresponds to chloroplast pigments; the second is blue light specific (16, 26). This cryptochrome-like photoreceptor, which has not yet been identified, may be located at the plasma membrane (16). The specific response to blue light has been well characterized by means of double-beam experiments, *e.g.* pulses of blue light on a strong red light background (21). With whole plants, these experiments have made it possible to define the action spectrum of the blue light photoreceptor, which may correspond to a flavin-cytochrome complex (7). The blue light signal may be "memorized" during anoxia and needs O_2 to be expressed (23). The response to blue light saturates at low fluence and is synergistically enhanced by red light (6). Other characteristics have been obtained from studies with GCPs.[1] Under a strong red light background, blue light triggers a proton efflux from GCPs after a 20- to 30-s delay (21), accompanied by a plasma membrane hyperpolarization (1). Interestingly, the response to blue light is suppressed by protonophores or inhibitors of redox processes (16). The capability of guard cells to oxidize exogenous NADH (14) and to reduce tetranitroblue tetrazolium chloride more after irradiation in blue light (22) may indicate the intervention of a plasmalemma redox chain. Such a redox chain has been described in many plant tissues (11) and may participate in cellular osmoregulation (10).

The aims of this study were to investigate the role of red light in the enhancement of the response to blue light of GCP and to determine whether the response to blue light may proceed from the activation of a redox pump located at the plasmalemma with O_2 as the terminal electron acceptor. Some experiments were conducted with MCPs for comparison.

MATERIALS AND METHODS

Preparation of GCPs and MCPs

GCPs and MCPs were isolated enzymatically from abaxial epidermis of young fully expanded leaves of *Commelina communis* L., as previously described (4). Protoplasts were suspended in a basic medium (5 mM Mes and 5 mM Tris, 20 μM $CaCl_2$, 10 mM KCl, pH adjusted to 7 by addition of HCl). Osmotic pressure was adjusted with mannitol to 300 mosmol for GCPs and 500 mosmol for MCPs. Protoplast diameters were determined from microscopic observation under weak light ($n = 60$).

Measurements of O_2 Concentration and pH

Measurements of O_2 exchanges and pH were performed with a Clark-type O_2 electrode (DW2; Hansatech, Kings Lynn, U.K.) at 25°C. The plunger was modified to provide an access for a micro-pH electrode (Ingold 104023522). O_2 concentration and pH were continuously recorded (radiometer ion 85; Copenhagen, Denmark). The buffering capacity of the protoplast suspension was determined by addition of 10 nmol H^+ at the end of each experiment.

Protoplasts (0.6 to 1 10^6) were suspended in 1 ml medium weakly buffered by 0.5 mM Mes (pH = 6.7). The suspension was continuously stirred with a magnetic flea. After 5 min in darkness, red light (LH7, Hansatech; maximal transmittance at 665 nm, half-band width 30 nm, fluence 2000 μmol m^{-2} s^{-1} at the output) was switched on. A pulse of blue light was usually applied for 30 s, after 40 min in red light. Blue light (maximal transmittance at 450 nm, half-band 65 nm, fluence

[1] Abbreviations: GCP, guard cell protoplast; DES, diethylstilbestrol; FeCN, hexacyanoferrate; MCP, mesophyll cell protoplast; SW26, 2,2,2-trichloroethyl-3,4-dichlorocarbanilate; SOD, superoxide dismutase.

100 μmol m^{-2} s^{-1}) was obtained by filtering white light (LS2, Hansatech) through two blue filters (Corning 4-36 and Kodak 47).

The effects of the following chemicals were studied: catalase (650 and 1300 units) (Boehringer, Mannheim, Germany), DCMU (10 μM) dissolved in methanol:water (80:20), DES (100 μM) dissolved in ethanol, $K_3Fe(CN)_6$ (500 μM), KCN (1 mM), antimycin A (5 μM) dissolved in ethanol, NADH (100 μM or 1 mM) (Boehringer), SOD (50 units), sodium orthovanadate (150 μM), SW26 (a specific inhibitor of plasmalemma H^+-ATPase; 100 μM; ref. 2) dissolved in DMSO. SW26 was a generous gift from Dr. Calmon (Ecole Nationale Supérieure d'Agronomie de Toulouse, Toulouse, France). Each experiment was repeated at least twice.

Ferricyanide Oxidoreductase Activity

$K_3Fe(CN)_6$ and NADH were added to give 500 and 100 μM final concentrations, respectively. Rates of ferricyanide reduction and NADH oxidation were simultaneously determined at 420 nm (E_{420} = 1 mM^{-1} cm^{-1}) and at 340 nm (E_{340} = 6.2 mM^{-1} cm^{-1}) with a dual-beam spectrophotometer (Kontron uvikon 860). Turbidity of the sample was deduced by monitoring the optical absorption at 500 nm which was proportional to protoplast concentration. The sample (1 ml) was kept in darkness and continuously stirred with a magnetic glass flea; a low-speed magnetic stirrer was adapted to avoid protoplast damage. Stock solutions of $K_3Fe(CN)_6$ (50 mM) and NADH (10 mM) were prepared daily.

All chemicals were purchased from Sigma unless otherwise specified.

RESULTS AND DISCUSSION

H^+ Efflux and O_2 Consumption after a Blue Light Pulse

Typical variations in O_2 concentration and pH of the GCP suspension during a dual-beam experiment are presented in Figure 1. In darkness, GCPs strongly acidified the medium (253 ± 58 neq H^+ h^{-1} [10^6 GCP]$^{-1}$) and had a high O_2 uptake (364 ± 40 nmol O_2 h^{-1} [10^6 GCP]$^{-1}$). These two processes resulted from respiratory metabolism and were inhibited (80%) after addition of both salicylhydroxamic acid (2 mM) and KCN (1 mM). The respiration rate was in the range of previous ones obtained with GCPs (4). Under red light illumination, pH of the medium and O_2 concentration were almost constant because of a low net photosynthesis (−49 ± 44 nmol O_2 h^{-1} [10^6 GCP]$^{-1}$) compared with previous results (4). Alkalinization of the medium under red light, attributed by Shimazaki *et al.* (21) to CO_2 fixation, was observed when the plunger of the electrode was removed. After 40 min of red light, a blue light pulse (30 s) triggered a transitory acidification concomitantly with an increase in O_2 uptake. This acidification of the medium has been shown to be independent of CO_2 evolution (21) and characterized as an H^+ efflux from GCPs (1). The O_2 uptake was correlated with H^+ efflux (Fig. 1, inset). The stoichiometry was approximately $4H^+$:O_2 (4.1 ± 0.5; n = 23), and acidification and O_2 consumption were suppressed after grinding, heating at 80°C, or overdigestion of the GCPs. In contrast, MCPs had a high O_2 evolution rate under red light (2.9 ± 0.3 μmol O_2 h^{-1} [10^6 MCP]$^{-1}$) but did not respond to a blue light pulse.

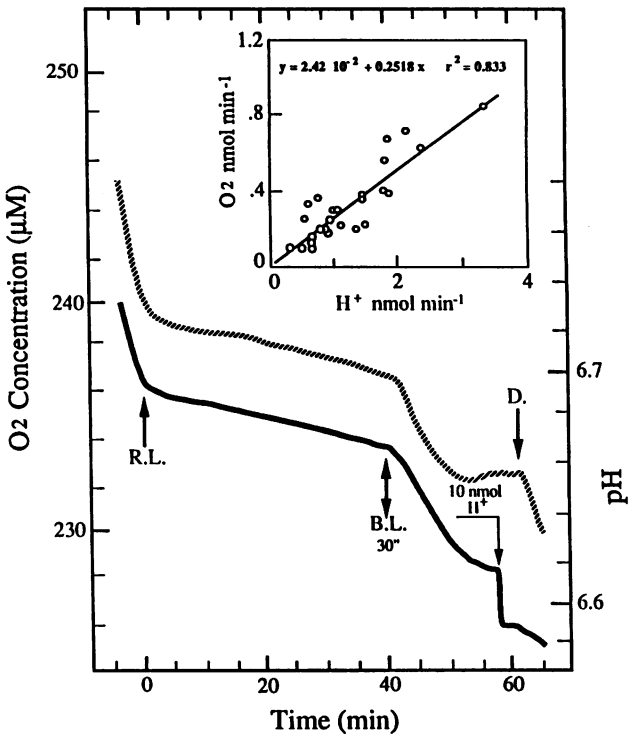

Figure 1. Typical effect of a blue light (B.L.) pulse (30 s, 100 μE m^{-2} s^{-1}) applied after 40 min under red light (R.L.) (2000 μE m^{-2} s^{-1}) on O_2 concentration and pH of a GCP suspension (10^6 GCP ml^{-1}). Dashed line, O_2; solid line, pH; D., darkness. Inset, Relation between the rate of H^+ efflux and O_2 consumption after a blue light pulse with different protoplast batches.

Addition of KCN under red light resulted in a pH decrease and an O_2 consumption comparable to those observed in darkness. When KCN was added 15 min before the blue light pulse, the acidification and O_2 uptake in response to blue light were inhibited up to 84% (Fig. 2). As reported by Shimazaki *et al.* (21), the response to blue light was poorly affected by 150 μM orthovanadate (78% of control). However, there is contradictory data concerning the effects of vanadate (3) which may be due to cell penetration problems and vanadate polymerization. After addition of DES (100 μM), a putative inhibitor of plasmalemma ATPase (12), or SW26 (100 μM), the response decreased to 37 and 0% of the control, respectively. However, microscopic observation revealed that DES and SW26 induced much destruction of GCPs, so that it was impossible to conclude that H^+-ATPase was involved in the response to blue light.

Effect of Red Light

Red light was necessary to obtain acidification in response to a blue light pulse (Fig. 2). The response to blue light was weak in darkness, increased after 15 min of red light, and saturated after approximately 40 min of illumination. The enhancement of the response by red light was not related to the simultaneous supply of blue and red light: GCPs preilluminated during 20 min responded well to a blue light pulse

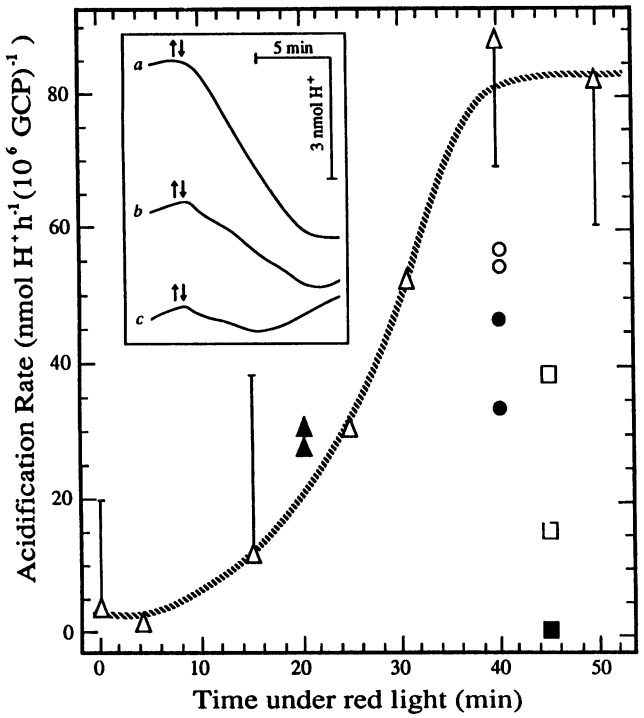

Figure 2. Change in the acidification rate in response to a blue light pulse as a function of red light preillumination; effects of KCN (1 mM) and DCMU (10 μM). △, Control experiments with different durations of red light preillumination; ▲, red light was switched off 5 min before the blue light pulse; ●, DCMU added before red light; ○, DCMU added after 30 min of red light; ■, KCN added before red light; □, KCN added after 35 min of red light. Lines associated with some of the control values are SEM. Inset: a, control experiment; b, DCMU added after 30 min of red light; c, DCMU added before red light. ↑, blue light on; ↓, blue light off.

applied 5 min later in darkness (Fig. 2). These results are in close agreement with those from wheat plantlets obtained by Karlsson (6) who concluded that the guard cell response to blue light may depend on the filling of an energy pool by red light. Assmann *et al.* (1) observed, using patch-clamp techniques, that the supply of ATP through the micropipette was necessary to obtain a response to blue light under a red light background. They concluded that the acidification in response to blue light resulted from the activation of an electrogenic plasma membrane ATPase. Similarly, Serrano *et al.* (19) showed that the red light-stimulated current needs the presence of ATP and Pi. Thus, it is difficult to conclude whether the response to blue light is directly dependent on an ATPase activity or whether blue light transduction requires a previous activation of a red light-stimulated ATPase. In a recent study (4), we showed that GCPs under red light were able to evolve O_2 at a higher rate (61 μmol O_2 mg^{-1} Chl h^{-1}) than they fixed CO_2 (40 μmol CO_2 mg^{-1} Chl h^{-1}). Thus, in contrast with MCPs, energy produced by photophosphorylation at the chloroplast level (25) is not totally dissipated for CO_2 reduction and may be used in the response to blue light. To investigate whether red light stimulates the response to blue light via linear electron transfer, the effect of the PSII inhibitor, DCMU, was studied (Fig. 2). Addition of DCMU restored O_2 consumption equivalent to dark respiration. To avoid anoxia, which totally inhibits the blue light response (data not shown), the O_2 electrode was reopened before the blue light pulse. This led to a CO_2 reequilibration resulting in slight alkalinization (Fig. 2, inset). When DCMU was added before illumination, the acidification rate following the blue light pulse was 46% of control. When DCMU was added after 30 min of red light, the initial rate of acidification triggered by a blue light pulse was 61% of control, a response similar to that observed after 30 min of red light. Duration of the acidification was also affected by DCMU (Fig. 2, inset). We hypothesize that, under red light, linear electron transfer provides NADPH which is stored as reduced compounds. The concentration of "stored reducing equivalents" provided by 40 min of red light would be approximately 28 μmol NADPH mg^{-1} Chl (calculated from the O_2 and CO_2 fluxes [4]). According to Hampp and Schnabl (5), the total amount of pyridine nucleotide in GCP of *Vicia faba* represents 0.6 nmol $(10^6$ GCP$)^{-1}$ or 0.3 μmol mg^{-1} Chl. This difference suggests that the high level of reducing equivalents produced by red light may be used to reduce an intermediate metabolite pool which could be stored until the blue light pulse. These metabolites could then be consumed after activation of the blue light photoreceptor. Moreover, ATP supply by cyclic photophosphorylation (20) which may operate in the presence of DCMU was unable to promote the blue light-induced acidification. We cannot explain the discrepancy between the high inhibition of the blue light response by DCMU added before illumination in this study and the limited effect observed by Schwartz and Zeiger with *C. communis* epidermis treated for 2 h (18).

Effects of External NADH and FeCN

To investigate the intervention of reducing power in the blue light response, the effect of externally added NADH was studied. The addition of 1 mM NADH under darkness restored the response of GCPs to blue light (Fig. 3, a and b), with an acidification rate equivalent to that triggered by a blue light pulse applied after 30 min of red light. In contrast, under red light, addition of NADH did not enhance the response to blue light. This is in agreement with the hypothesis that red light fills an energy pool necessary for the blue light response. However, we cannot exclude a direct effect of NADH on chloroplast via a shuttle of redox equivalents through the plasmalemma and the chloroplast membrane.

When FeCN (0.5 mM), an impermeant electron acceptor, was added under red light, acidification took place in the GCP suspension (Fig. 3c). The presence of FeCN diminished the response to a blue light pulse (Fig. 3c). Thus, FeCN was an inhibitor of the response to blue light, perhaps by accepting electrons, which should reduce oxygen (Fig. 4). The inhibition of the blue light response by FeCN, could be explained by regulation of the redox system by blue light. Obviously, this is not the case for MCPs, because they do not respond to blue light. Different plasmalemma redox systems have been described in plant cells (11) and redox pumps from MCPs and GCPs may be different in their regulation and activation.

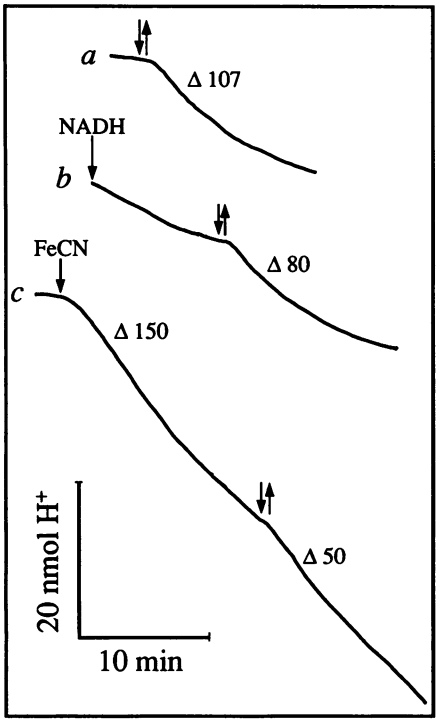

Figure 3. Effects of exogenous NADH (1 mM) in darkness and ferricyanide (500 μM) under red light on the response of GCPs to a 30-s pulse of blue light. a (control experiment), A pulse of blue light was applied after 40 min under red light; b, 1 mM NADH was added in darkness before the blue light pulse; c, 500 μM ferricyanide was added under red light before the blue light pulse; arrows, blue light pulse. Δ, Change in acidification rate in nmol H^+ h^{-1} $(10^6$ GCP$)^{-1}$.

NADH-Ferricyanide Oxydoreductase Activity of GCPs and MCPs

To study the GCP capability to transfer electrons across the plasmalemma, the reduction of FeCN with or without external NADH was followed. In darkness, GCPs reduced exogenous ferricyanide at a rate of 145 nmol h^{-1} $(10^6$ GCP$)^{-1}$. Under the same conditions, MCPs also reduced ferricyanide (317 nmol h^{-1} $(10^6$ MCP$)^{-1}$) (Fig. 5). On a surface area basis, ferricyanide reduction rate by GCPs was 50% higher than that of MCPs (mean diameters in darkness, GCP = 17.1 ± 0.4 μm and MCP = 31.0 ± 0.4 μm). Similar rates were reported by Neufeld and Bown (12) for *Asparagus* cells in darkness (93 nmol h^{-1} $(10^6$ cells$)^{-1}$). FeCN reduction was accompanied by acidification of the medium with a stoichiometry H^+/e^- 0.9 for GCPs and 0.95 for MCPs. NADH (or NADPH, 100 μM; data not shown) strongly stimulated FeCN reduction (Fig. 5) and produced a change in H^+/e^- ratio (3 for GCPs and 2.5 for MCPs). This change in the stoichiometry may result from the use of internal or external NADH (Fig. 5). KCN, antimycin A, and vanadate had no effect on ferricyanide reduction.

When GCPs were removed from the medium, FeCN was still reduced (30%). This residual activity could be attributed to the presence of remaining protoplasts and membrane fractions from burst protoplasts. To investigate whether peroxidase excreted by GCPs (14, 15) could be responsible for ferricyanide reduction, catalase and SOD were added to the medium, but they had no effect on the acidification rate (data not shown). Such little effect of SOD and catalase on ferricyanide reduction by GCPs does not totally discount the intervention of a peroxidase. However, Pantoja and Willmer (15) recently hypothesized that ferricyanide reduction by peroxidase may occur by a direct electron transfer from NADH to FeCN without involvement of oxygen-free radicals or H_2O_2.

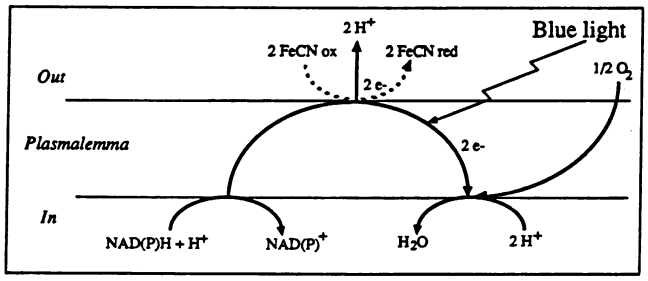

Figure 4. Model of the hypothetical competition between ferricyanide and O_2 as electron acceptors. Solid line, Transfer of electrons from NAD(P)H to O_2; dashed line, in the presence of FeCN, internal NAD(P)H could be used to reduce FeCN.

Origin of O_2 Overconsumption after a Blue Light Pulse

Different hypotheses could explain the enhancement of O_2 consumption following the blue light pulse: inhibition of

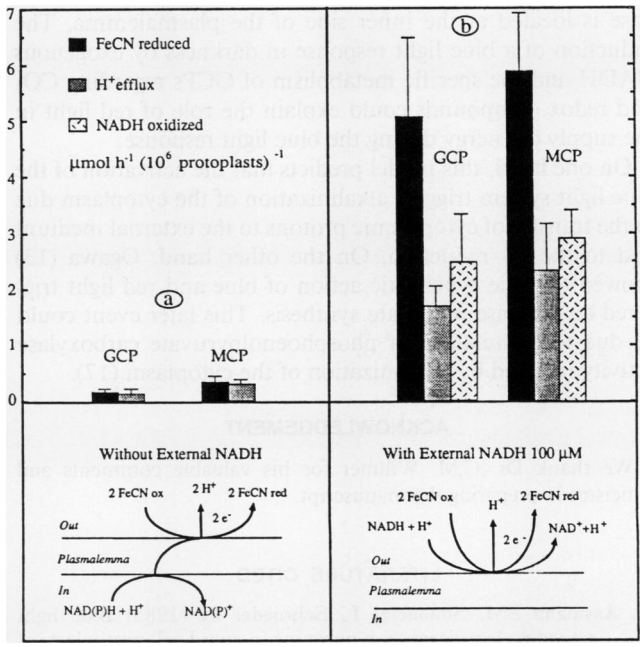

Figure 5. Reduction of ferricyanide (500 μM) in darkness by GCPs and MCPs; a, Without exogenous NADH; b, with exogenous NADH (100 μM). The hypothetical transfer of electrons is schematized at the bottom of the histograms. These models are consistent with the stoichiometry observed between ferricyanide reduction and H^+ efflux.

linear electron transfer, stimulation of mitochondrial uptake, or activation of a plasmalemma oxidase as suggested by Vani and Raghavendra (22). The first hypothesis concerning a decrease in water splitting can be dismissed because O_2 overconsumption following the blue light pulse was observed either in red light in the presence of DCMU or in darkness with exogenous NADH.

The inhibition of the blue light response by KCN might indicate a role of respiration in the O_2 overconsumption. Such stimulation of mitochondrial activity by blue light has been observed in different plant tissues (8). However, inhibition of mitochondrial ATP synthesis may dramatically alter the energy status of cytoplasm (9), resulting in a large decrease of chloroplast electron transfer. On the other hand, KCN may also affect chloroplast electron transfer via ribulose bisphosphate carboxylase inhibition (24) or inhibit a putative plasmalemma oxidase from guard cells (14).

The acidification triggered by FeCN in red light and the diminished response to a subsequent blue light pulse suggest that FeCN and O_2 may compete at the plasmalemma for a common redox pool. Such a plasmalemma oxidase may be directly regulated by blue light or via a flavoprotein.

Model of a Blue Light-Dependent Plasma Redox Chain

The model of a plasmalemma redox chain (Fig. 4) is close to the one proposed by Raghavendra (16) and is in accordance with the hyperpolarization observed in patch-clamp (1) and proton efflux (21) experiments. Moreover, the present study indicates the terminal electron acceptor and its localization within the plasmalemma. In comparison with different models of redox chain presented by Møller and Crane (11), the stoichiometrically observed $4H^+/O_2$ implies that the oxidase is located at the inner side of the plasmalemma. The induction of a blue light response in darkness by exogenous NADH and the specific metabolism of GCPs regarding CO_2 and redox compounds could explain the role of red light in the supply of energy during the blue light response.

On one hand, this model predicts that the activation of the blue light system triggers alkalinization of the cytoplasm due to the transfer of cytoplasmic protons to the external medium and to the O_2 reduction. On the other hand, Ogawa (13) showed that the synergistic action of blue and red light triggered an increase in malate synthesis. This later event could be due to stimulation of phosphoenolpyruvate carboxylase activity induced by alkalinization of the cytoplasm (17).

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