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The effect of cyanide on state transitions in *Chlamydomonas reinhardtii*

Pierre Gans [a,1], Francis-André Wollman [b,*]

[a] *Section de Bioénergétique Cellulaire, Département de Physiologie Végétale et Ecosystèmes, C.E.N. de Cadarache, Commissariat à l'Energie Atomique, 13108 Saint-Paul-Lez-Durance cedex, France*

[b] *Service de Photosynthèse, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France*

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Abstract

In *Chlamydomonas reinhardtii*, addition of KCN at low concentrations of 50–500 μM induced a transition to State II in vivo, as expected from its action as an inhibitor of mitochondrial electron transfer (Bulte, L., Gans, P., Rebeille, F. and Wollman, F.-A. (1990) Biochim. Biophys. Acta 1020, 72–80). However, raising the KCN concentration up to 20 mM induced a rise in the maximal fluorescence yield and resulted in an extensive dephosphorylation of the antenna proteins. The latter effect was also observed in vitro with isolated thylakoids. Thus, KCN at high concentrations locked the photosynthetic apparatus in State I by preventing phosphorylation of antenna proteins. Further investigations on the mode of action of KCN allowed us to rule out the possibility that KCN per se would hydrolyse phosphate bonds of phosphoproteins. Evidence is presented that the effect of KCN at high concentrations cannot be attributed to a stimulation of the activity of the LHC-phosphatase, but is rather due to an inhibition of the LHC-kinase. The mechanism by which KCN prevents activation of the kinase is discussed.

Keywords: State I–State II transition; Cyanide inhibition; LHC-kinase; (*C. reinhardtii*)

1. Introduction

State I to State II transitions have been described as a short-term chromatic adaptation in higher plants and algae (reviewed in [1]). The molecular basis of these transitions have been found in the reversible phosphorylation of several subunits of the light-harvesting complex antenna, LHC II, which may then migrate reversibly between the two photosystems, thus balancing the excitation energy distributed to each photosystem (for reviews see [2,3]). The kinase involved in the phosphorylation of LHC II is activated when the intersystem electron carriers are reduced, for instance when the number of charge separations per time unit in PS II exceeds that in PS I. The physiological significance of this reorganization of the photosynthetic apparatus has been mainly understood as an adaptation mechanism by which plants and algae restore optimal photosynthetic rates under conditions where the two photosystems receive unbalanced light excitation (bottom leaves in a canopy, inner side of a leaf). However, it has also been shown in *Chlamydomonas reinhardtii* that transition to State II takes place in darkness in conditions where the intracellular ATP supply is limited [4,5]. In particular, this is observed when mitochondrial ATP synthesis is prevented by oxidase inhibitors [6] or by anaerobiosis [7]. The molecular basis for the interaction between the mitochondria and the chloroplast originates from a deregulation of the glycolytic pathway. Starch is the only source of carbohydrates in *C. reinhardtii* and the initial part of glycolysis takes place in the chloroplast [8]. A decrease in intracellular ATP will induce a deregulation of phosphofructokinase activity, which is tightly controlled by ATP [9]. This leads to increased NADPH production in the chloroplast by glyceraldehyde phosphate dehydrogenase and in turn to a reduction of the PQ pool [6,10]. In the

Abbreviations: PQ, plastoquinone; PS I, Photosystem I; PS II, Photosystem II; SHAM, salicylhydroxamic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHC II, light-harvesting complex of Photosystem II ; AA, antimycin A; cyt. b_6f, cytochrome b_6f; PMSF, phenylmethylsulfonyl fluoride; WT, wild-type; F_{max}, maximal level of fluorescence in presence of DCMU; CP, chlorophyll protein; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, dinitrophenyl ether iodonitrothymol.

* Corresponding author. E-mail: wollman@citi2.fr. Fax: +33 1 40468331.

[1] Present address: Laboratoire de Résonance Magnétique Nucléaire, Institut de Biologie Structurale CEA/CNRS, 41 Avenue des Martyrs, 38027 Grenoble cedex, France. E-mail: pierre@rmn.ibs.fr.

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present study, we report on the paradoxical effect of cyanide on state transitions in *C. reinhardtii*. At concentrations below 5 mM, KCN promotes transition to State II, as expected from a genuine inhibitor of mitochondrial electron transfer. However, at a concentration of 20 mM, KCN places the photosynthetic apparatus in State I both in vivo and in vitro.

2. Material and methods

Chlamydomonas reinhardtii wild-type and mutant strains FUD6, deficient in b_6f complexes [11], and FUD7, deficient in PS II centers [12], were grown in TAP medium [13] unless when otherwise indicated. For each experiment, algae at mid-exponential phase of growth were harvested by low-speed centrifugation ($2500 \times g$ for 5 min) and resuspended in their growth medium at the desired cell concentration.

In vivo state transitions were performed in the dark as previously described [4]. For State 1, cells were placed in darkness and aerated by vigourous shaking for 10 min. For State II, cells were placed in the same conditions as for State I, then incubated with 1 mM SHAM and 1 μM Antimycin A for 10 min. In experiments with isolated thylakoid membranes, State I was attained by incubating the sample with ATP in the dark; State II was subsequently obtained by placing the same sample under continuous illumination for 15 min [7]. In all experiments, the pH of the 1 M KCN stock solution was adjusted to pH 8 prior to use.

We used two methods for fluorescence determination. Experiments in Fig. 1 and Fig. 4 were performed using a fluorescence apparatus constructed in house. Continuous illumination at 605 nm was provided by electroluminescent diodes HLMA/CLOO. Fluorescence was measured at right angles with photodiodes UV444B. The experiment in Fig. 2, which presents the kinetics of state transitions, was performed using the pulse modulation technique (basic system PAM 101 and saturation pulse unit 103, H. Walz, Effeltrich, Germany) [14]. The fluorescence level was measured with a weak modulated light (averaged intensity 0.8 μE $m^{-2}s^{-1}$) and the maximal fluorescence yield was determined with 500 ms pulses (light intensity 1500 μE $m^{-2}s^{-1}$) applied every minute.

Spectrophotometric measurements at 520 nm were performed with an apparatus similar to the one described in [15] and improved according to [16]. Actinic excitation was provided by Xenon flashes (3 μs half-time duration), filtered through a RG 680 filter. Algal cells were kept in darkness under strong aeration for 5–10 min before each measurement and placed in the presence of 7% Ficoll to avoid sedimentation.

For phosphorylation experiments in vivo, cells were harvested in the mid-exponential phase of growth ($3 \cdot 10^6$–$4 \cdot 10^6$ cells ml^{-1}) and resuspended at $2 \cdot 10^7$ cells ml^{-1} in minimum medium without phosphate. Cells were then incubated for 90 min in the presence of 2 μCi ml^{-1} [^{32}P]P_i, washed once with medium, and placed in the various states under investigation. Cells were then rapidly broken in a French press in the presence of 10 mM EDTA, 10 mM NaF, 0.3 M sucrose and 100 μM PMSF. Thylakoid membranes were isolated in the presence of NaF as previously described [7]. For phosphorylation experiments in vitro, thylakoid membranes at 400 μg Chla/ml were resuspended in 50 mM Tris, 0.3 M sucrose, 10 mM $MgCl_2$. 100 μl aliquots were then removed and incubated under various experimental conditions, with 2 μCi [^{32}P]ATP (final 100 μM ATP concentration). The reaction was terminated by addition of 10 vols. of chilled Tris buffer containing 50 mM EDTA. Gel electrophoresis and autoradiography were performed as in [7].

3. Results

We analyzed the effects of a broad range of cyanide concentrations on the fluorescence yield of *Chlamydomonas reinhardtii* in vivo. The fluorescence experiment described in Fig. 1 was performed with intact cells kept under strong aeration by vigourous shaking and incubated for 10 min in darkness with variable concentrations of KCN. In the absence of KCN this treatment places the cells in State I [4], where they display a high maximal fluorescence yield. This is due to an organization of the peripheral antenna favouring excitation transfer to Photosystem II. Curve (a) represents the relative fluorescence yield, measured at the F_{max} level reached in the presence of DCMU, as a function of cyanide concentration. Between 20 μM and 100 μM KCN, the F_{max} level continuously decreased. This fluorescence decrease corresponded

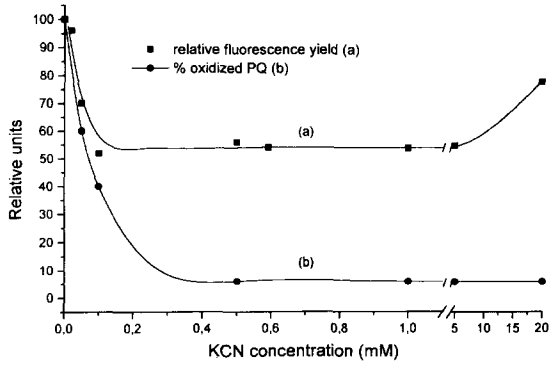


Fig. 1. Fluorescence changes upon incubation of *C. reinhardtii* intact cells with increasing KCN concentrations. Relative levels at F_{max} in WT cells, incubated in the presence of 10^{-5} M DCMU, with 100% corresponding to the sample without KCN (curve a); % of oxidized plastoquinones was estimated in the absence of DCMU, using the FUD6 mutant deficient in cyt. b_6f complexes, by measuring the area bound by the fluorescence induction curve and its F_{max} asymptote, with 100% corresponding to the area developed in the absence of KCN (curve b).

to a transition to State II, where an increased proportion of light excitation energy is diverted towards PS I. At concentrations from 100 μM to 5 mM KCN, the F_{max} reached a plateau, corresponding to a low fluorescence yield, typical of State II. This KCN-driven transition to State II most likely resulted from the action of KCN as an inhibitor of cytochrome oxidase in the mitochondria. As we previously reported [6,10], a block in mitochondrial electron transfer prevents ATP production in the dark. Under these conditions a deregulation of the glycolytic pathway generates reducing power in the chloroplast, which should lead to a reduction of the intersystem electron carriers in the thylakoid membranes. We therefore estimated the proportion of plastoquinones remaining oxidized upon incubation with increasing cyanide concentration. This was achieved in a mutant lacking cytochrome b_6f complexes, by measuring the area bound by the fluorescence induction curve and its F_{max} asymptote [17] at each KCN concentration. The results are shown in Fig. 1 curve b. The plastoquinone pool was increasingly reduced as the KCN concentration rose from 50 μM to 500 μM, in a concentration range close to those reported for the inhibition of the cytochrome oxidase pathway in WT *Chlamydomonas* cells [18].

Surprisingly, increasing the KCN concentration up to 20 mM induced a rise in the F_{max} level of the WT cells with no detectable reoxidation of the plastoquinone pool. This latter effect was confirmed by placing the algae, originally adapted to State I, in State II conditions by an incubation with 1 mM SHAM + 1 μM AA. A subsequent addition of 20 mM KCN restored an F_{max} level close to that of State I (Fig. 2). A similar effect of cyanide was observed when the cells were initially placed in State II by anaerobiosis (result not shown). It is noteworthy that the kinetics of the KCN-induced changes occurred with a half time of 5 min, i.e., on a time scale close to what has been previously reported for State II to State I transitions [19].

In order to determine whether the effect of high cyanide concentrations on fluorescence yields was related to a decreased phosphorylation of the antenna proteins — as it is the case for a genuine transition to State I in *C. reinhardtii* — we labelled the cells in vivo with ^{32}P and compared the phosphorylation patterns in the thylakoid membranes from cells placed in the same three conditions as in Fig. 2. The corresponding autoradiogram is shown on Fig. 3. As we reported previously [4] incubation of WT cells with SHAM + AA for 20 min in darkness (lane 2) induced an increase in polypeptide phosphorylation as compared to that in cells adapted to State I (lane 1). ^{32}P-labelling increased on five antenna proteins: three LHCII subunits, p13, p11 and p17, and the apoproteins of CP29 and CP26 corresponding respectively to p9 and p10 [20]. Subsequent incubation of these State II cells for another 20 min with 20 mM KCN (lane 3) induced an extensive dephosphorylation of the antenna proteins, thereby producing a phosphorylation pattern typical of State I.

This dephosphorylation effect of cyanide could arise from its action on the kinase/phosphatase system which controls state transitions [3]. However, it was also possible

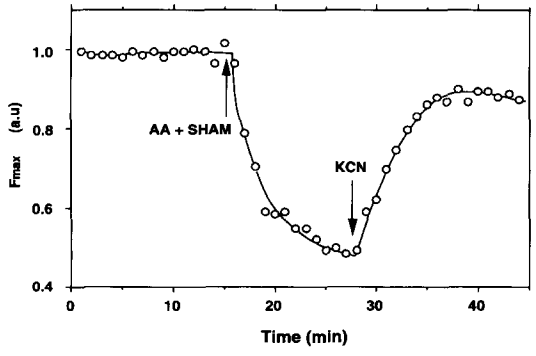


Fig. 2. Kinetics of the changes in fluorescence yield in WT cells of *C. reinhardtii* upon addition of 1 μM Antimycin A + 1 mM SHAM followed by addition of 20 mM KCN. Fluorescence levels were plotted relative to the Fmax level recorded before addition of these compounds.

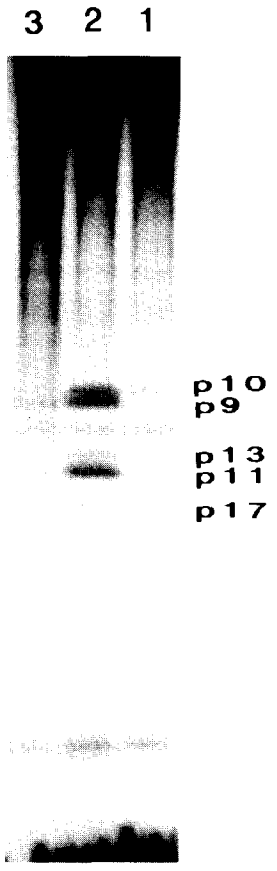


Fig. 3. Antennae phosphopolypeptides viewed after autoradiography of an electrophorogram loaded with SDS-solubilized thylakoids membrane proteins from ^{32}P-labelled cells. WT cells were placed (1) in State I by a strong aeration in the dark during 15 min.; (2) in State II by an incubation in the dark with 1 μM AA + 1 mM SHAM; (3) as in (2) with a subsequent incubation in presence of 20 mM KCN. p9 and p10 are apoproteins of CP29 and CP26, respectively; p11, p13, p17 are LHC II subunits.

that, at high concentrations, cyanide would cause a direct chemical hydrolysis of the phosphate bonds on the antenna proteins. Although monoalkyl phosphate esters are generally inert to alkali treatment [21], we could not rule out the possibility of a nucleophilic substitution of the phosphate group by cyanide. In this case we reasoned that NaF, a potent inhibitor of the LHC phosphatase [22], would not prevent a dephosphorylation of the LHC-complexes driven by 20 mM KCN. Although 10 mM NaF successfully inhibits the LHC-phosphatase in broken chloroplasts from higher plants [23], we had to use a much higher concentrations (100 mM NaF) with intact cells of *C. reinhardtii*. This is most likely because of the restricted diffusion of NaF through both the plasma membrane and the double chloroplast envelope. We have previously shown that the mechanism of state transitions are similar in the WT and PS II mutants from *C. reinhardtii* [7,19]. Therefore the experiments shown in Fig. 4A and 4B, were performed with intact cells from a PS II mutant which are most convenient for the study of the reversion from State II [7]. Due to the absence of PS II centers, the reduced plastoquinone pool from cells placed in State II in darkness, can be fully reoxidized by PS I upon illumination, thus generating State I. On the other hand, such mutants display a simple fluorescence pattern with no induction phase [24]. Their constant fluorescence emission originates from the free antenna proteins which are not associated with PS I. Traces 1 in Fig. 4 correspond to cells placed in State I by a strong aeration in darkness. The much lower fluorescence level in traces 2 reflects an increased connection of antenna proteins with PS I when cells were placed in State II by an incubation for 10 min in darkness with SHAM + AA. We then attempted to revert State II to State I in the absence (traces 3) and presence (traces 4) of 100 mM NaF. In the case of Fig. 4A the reversion to State I was achieved by merely illuminating for 10 min. the cells originally adapted in the dark to State II. The oxidation of the intersystem electron carriers, driven by PS I, deactivated the kinase and allowed the LHC-phosphatase to restore the full complement of non-phosphorylated free antenna proteins. The fluorescence level thus obtained was typical of State I (compare traces 1 and 3 in Fig. 4A). When 100 mM NaF was added to the cells in State II during the last 10 min of darkness, a subsequent illumination for 10 min did not cause the fluorescence yield to rise significantly above its level in State II (Fig. 4A, trace 4). This indicated that 100 mM NaF was able to block transitions from State II to State I in vivo. In the case of Fig. 4B, the reversion from State II to State I was achieved by adding 20 mM KCN to the sample adapted to State II in darkness (trace 3). Incubation with 100 mM NaF in State II prior to the addition of KCN prevented the restoration of this high fluorescence yield (compare traces 3 and 4 on Fig. 4B). These data ruled out the possibility that KCN acted by itself as a phosphatase since restoration of State I still depended on the activity of the endogeneous LHC-phosphatase.

KCN could then act on the kinase/phosphatase system either by stimulating LHC-phosphatase activity or by inhibiting LHC-kinase activity. To distinguish between these two hypotheses, we studied the effect of KCN on the changes in protein phosphorylation in vitro using purified thylakoid membranes from the wild-type strain (Fig. 5). In this case, the low phosphorylation state corresponding to State I was obtained by labelling the membranes with [γ-^{32}P]ATP in the dark for 15 min (Fig. 5, lane 1). A subsequent illumination for 15 min reduced the plastoquinone pool and activated the kinase leading to a State II situation with an increased phosphorylation on several antenna proteins (lane 2). We then added 5 mM (lane 3) or 20 mM KCN (lane 4) in the last 5 min of darkness before switching on the light for 15 min. Whereas 5 mM KCN did not prevent transition to State II, 20 mM KCN locked the membranes in their low phosphorylation state, typical of State I. This is reminiscent of the effect on phosphorylation that we observed in vivo (Fig. 3). We then looked to the effect of a subsequent 15 min incubation in the dark after the membranes were fully phosphorylated in the light (lane 5). The fact that the antenna proteins remained phosphorylated in these conditions indicated that the phosphatase was no longer active in our in vitro system. Consequently, antenna proteins remained equally phosphorylated whether the experiment was performed in the absence or presence of NaF (compare lanes 5 and 6). Once

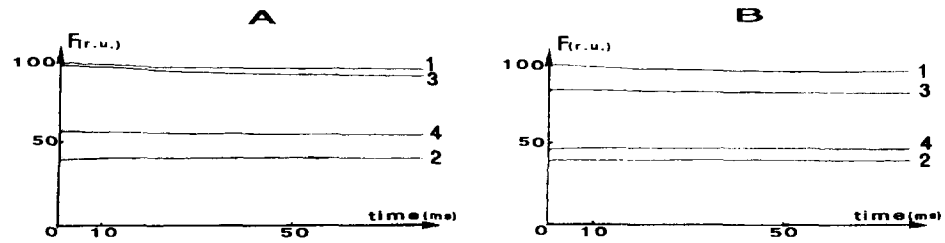


Fig. 4. Fluorescence yields associated with state transitions in cells from the FUD7 mutant lacking PS II centers. Experiments show the NaF sensitivity of a reversion from State II to State I by a 10 min. illumination (A) or by addition of 20 mM KCN (B). Curves 1: cells were placed in State I by a strong aeration in darkness. Curves 2: cells were placed in State II by an incubation with Antimycin A + SHAM in darkness. Curves 3: reversion from State II to State I by a 10 min. illumination (A) or by incubation in darkness with 20 mM KCN (B). Curves 4: same experimental conditions as for curves 3, except that 100 mM NaF was added in darkness, 10 min before setting the reversion conditions.

protein phosphorylation was induced in the light a further addition of 20 mM KCN for another 15 min had no dephosphorylating effect (lane 7). This in vitro system allowed us to observe an inhibition of antenna protein phosphorylation by 20 mM KCN in the absence of an active phosphatase. Thus, we conclude that KCN specifically inhibited the activity of the LHC-kinase and did not act through a stimulation of the LHC-phosphatase activity.

We have previously demonstrated that cytochrome b_6f complexes are involved in the activation of the kinase [25,26]. Therefore the possibility existed that high concentrations of KCN would block the activation of the kinase by altering the function of cyt. b_6f complexes. For instance, it has been shown that cyanide at high concentrations induces a removal of the methionine ligand in cytochrome *c* [27]. Electron transfer through the cyt. b_6f complexes is accompanied by a spectral change at 520 nm in the 1–10 ms time range after an actinic flash, known as phase b in the rise of the electrochromic shift [28]. This spectral change has been attributed to the so-called Q-cycle [29,30] or semiquinone cycle [31] and fully develops when reduced plastoquinones are available at the Qz site of the cyt. b_6f complex. We next examined these shifts in algal cells treated with 20 mM KCN. As shown in Fig. 6, the slow electrochromic phase (phase b) was not observed in aerated cells in the absence of KCN, which is consistent with the presence of an oxidized PQ pool [32]. Addition of KCN at 20 mM induced a large increase in phase b, which is indicative of a reduction of the PQ pool. This electrochromic rise was very similar to the one obtained with algae either treated with 0.5 mM KCN (results not shown) or placed under anaerobic conditions or incubated with SHAM + AA, two situations which are known to reduce the PQ pool in *C. reinhardtii*. [6–10]. Deconvolution of the decay signal into different exponential components [28] allowed us to confirm that the changes in the kinetics of the shift at 520 nm in the presence of KCN resulted from a genuine increase in phase b and not from a mere change in the decay rates of the spectroscopic signal (result not shown). This spectroscopic study indicated that PS I charge separation, electron transfer from cytochrome b_6f to PS I via plastocyanin, and the so-called Q cycle were not significantly altered by the 20 mM KCN treatment.

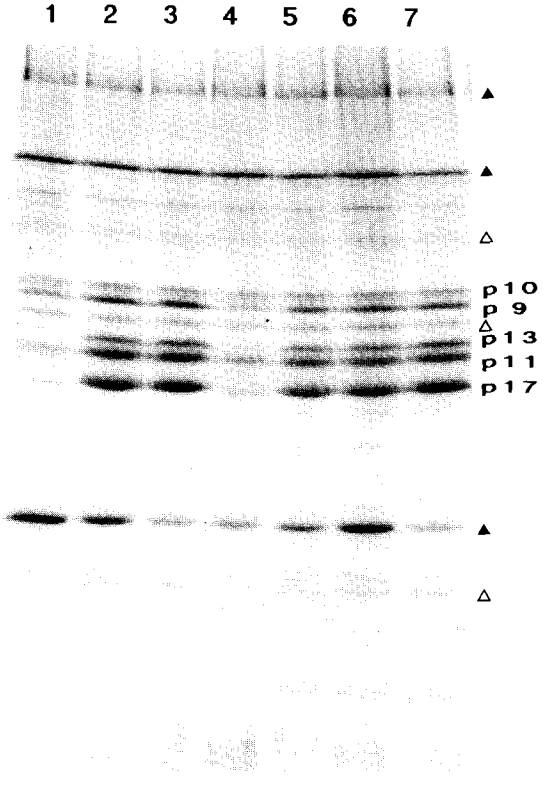


Fig. 5. Phosphorylation patterns in WT thylakoid membranes incubated with [γ-^{32}P]ATP in various conditions. Thylakoid membranes were kept in darkness for 15 min (lane 1), then illuminated for 15 min in the absence (lane 2) or presence of KCN at 5 mM (lane 3) or 20 mM (lane 4). Samples from lane 2 were subsequently incubated for another 15 min either in the dark, in the absence (lane 5) or presence (lane 6) of 10 mM NaF, or in the light with 20 mM KCN (lane 7). (△) PS II phosphoproteins, and (▲) unidentified phosphoproteins.

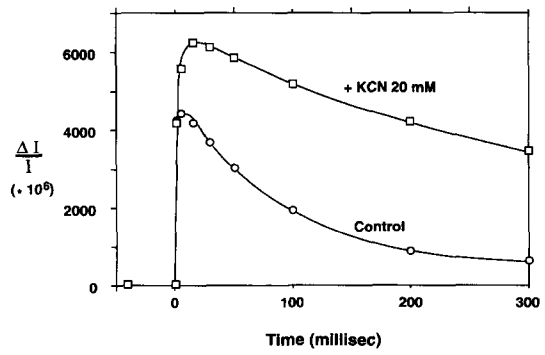


Fig. 6. Time-course of the 520 nm absorption change after a saturating flash in *C. reinhardtii*. To avoid anaerobiosis, the WT cell suspension was kept well aerated in darkness during the experiments. The 520 nm absorption change was recorded 10 min after addition of KCN. Chlorophyll concentration was in the range 20 to 30 μg Chl. ml^{-1}. (○) Dark-adapted cells, and (□) +20 mM KCN.

4. Discussion

The present study showed that cyanide has two contrasting effects regarding state transitions in *C. reinhardtii*. At low concentrations, below 5 mM, KCN acted as a regular inhibitor of the mitochondrial electron transfer chain [9]. As expected from the consecutive decrease in the intracellular ATP content [4], it induced a reduction of the plastoquinone pool and a transition to State II in TAP-grown cells. In a recent study with a mixothiazol-resistant mutant [33] Bennoun confirmed our previous observations [4] that this reduction of the plastoquinone pool arises from a metabolic interaction between the chloroplast and the mitochondria.

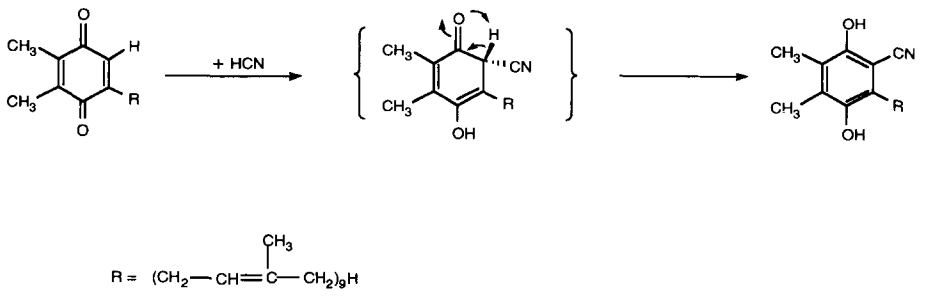
At higher concentrations, KCN locked the photosynthetic apparatus in State I. These experimental conditions still produced a large increase in phase b of the electrochromic shift and a decreased area bound by the fluorescence induction curve and its F_{max} asymptote. Therefore we could exclude that the lock in State I would arise from an artefactual CN^--induced reoxidation of the PQ pool. In addition these experiments showed that both the cyt. b_6f complexes and plastocyanin were active in the presence of 20 mM KCN.

As expected from a genuine transition to State I, addition of 20 mM KCN produced an inhibition of the phosphorylation of antenna proteins both in vitro and in vivo. We first considered a possible chemical hydrolysis of the phosphothreonine residues of the antenna proteins by CN anions. Two lines of evidence argue against this possibility. The rate of reversion from State II to State I upon addition of 20 mM KCN was not faster than during a genuine state transition. Moreover, the effect of 20 mM KCN on the fluorescence yield was NaF-sensitive, which points to its action on the kinase/phosphatase system. We ruled out an action on the LHC-phosphatase itself, since the inhibition of antenna protein phosphorylation by KCN was still observed with isolated thylakoids, in conditions where the LHC-phosphatase was inactivated. Thus, high concentrations of KCN most likely inhibit the activity of the kinase, either by an action on the enzyme itself or by blocking the process leading to kinase activation. Although several hypothesis have been discussed in the literature (see ref. [3]), neither the kinase nor the activating species have been rigorously identified so far. We show here that the cyt. b_6f complex, which is involved by some as yet unknown mechanism in kinase activation [25,26], remained active in the presence of 20 mM KCN. This is consistent with other reports showing that much higher concentrations, about 100 mM KCN, which inactivate plastocyanin, still have little effect on cyt. b_6f complexes [34].

From their chemical properties, cyanide anions can interfere in a number of ways with both the photosynthesis proteins and their cofactors. They could act at the active site of the kinase, for instance near a site of autophosphorylation, thereby preventing its activation due to a competition between two negatively charged anions. They can also complex various metal ions, in particular iron and copper. In this respect it should be mentioned that an extra copper has been identified in thylakoid membranes from spinach, besides the copper complement associated with plastocyanin [35]. The authors suggested that the extra-copper was associated with an antenna apoprotein, CP26. One should then consider the possibility that kinase activity would be copper-dependent through an interaction with CP26 and therefore cyanide-sensitive. Our previous observation that the BF4 mutant from *C. reinhardtii*, which lacks CP26 together with most of the LHC subunits, is also defective in PS II phosphorylation [36] would be consistent with such an hypothesis.

On the other hand, cyanide could inhibit the activation process by reacting directly with plastoquinones. This is consistent with the known properties of quinones [37], where reductive addition of cyanide on the plastoquinone lead to a cyano-plastoquinol (Scheme 1). This could be followed by an inhibition or a deactivation of the kinase either because of a loss of recognition of the substituted plastoquinone for the activation site or merely because of a change in the midpoint potential of cyanoplastoquinol versus plastoquinol: for instance the midpoint potential of 1,4-benzoquinone is about 400 mV lower than that of 2-cyano-1,4-benzoquinone [38], which makes the latter less oxidizable. We have verified that decyl plastoquinone, an synthetic homologue of the 9-plastoquinone, undergoes a reductive addition of cyanide at 20 mM NaCN (P. Gans, unpublished results). This hypothesis could also explain the intriguing observation that WT cells grown in minimal medium still undergo a reduction of the PQ pool upon addition of 20 mM KCN although mitochondrial respiration is poorly KCN-sensitive in these growth conditions (P. Gans, unpublished observation).

Although a number parameters controlling LHC-kinase and LHC-phosphatase activities has been assessed in a variety of studies (reviewed in [3] and [4]) the search for specific inhibitors of the LHCII-kinase met with limited success. For instance, EDTA may be used to block both kinase and phosphatase activities in vitro since the two enzymes are Mg^{2+}-dependent. *p*-Benzoquinone also inhibits both enzymatic processes in vivo in *C. reinhardtii*. [39]. Several inhibitors, including Qz-site inhibitors of the cyt. b_6f complex, have been used with some success to



Scheme 1. Proposed reductive addition of cyanide on plastoquinone 9, according to [37].

alter state transitions in vitro (reviewed in [3]) but similar attempts were not successful in vivo [26]. Thus, cyanide ions may prove to be a unique tool for selectively inhibiting the phosphorylation process controlled by the LHC-kinase. It may be used in the future as a possible probe for labelling the enzyme or some species participating in its activation.

Acknowledgements

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