A Study on the Life Time of the S_3-State in the Filamentous Cyanobacterium *Oscillatoria chalybea*

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In the filamentous cyanobacterium *Oscillatoria chalybea* deactivation of the S-states starting from steady-state conditions in which $S_0 = S_1 = S_2 = S_3 = 25\%$ reveals that S_3 deactivates to a finite level of approx. 10%. This level is reached under normal conditions between 10–15 seconds. This *quasi* metastable S_3 meets all requirements for S_3 in that one flash eliminates this redox conditions to give S_4 and therewith molecular oxygen. An analysis of the cyanobacterial S-state system in the 5-state Kok model shows that the S-state population in the dark adapted sample contains no contribution from S_{-1} or a more reduced condition which under normal conditions is the case for *Chlorella* or higher plant chloroplasts. Hence under standard conditions, the *Oscillatoria* condition is a pure Kok-4-condition in which S_0 is the most reduced state. Under these conditions S_2 seems to deactivate to S_1, and S_3 to S_2 and to a smaller extent to S_0. In the presence of the ADRY-reagent Ant-2-p (2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene) introduced by Renger (Biochim. Biophys. Acta **256,** 428, 1972), which is supposed to specifically act on the S_3-state (and thereby on S_2), not only the deactivation kinetic of S_3 (and S_2) is accelerated (hence the life time of the S_3-state is shortened), but also the level of metastable S_3 becomes practically zero. An analysis of the deactivation pattern shows that the agent changes the mode of deactivation of the entire system. Thus, it is seen that after deactivation of a sample in presence of this agent the dark population of S-states contains the more reduced redox condition S_{-1}. It looks as if in this condition S_2 deactivates not only to S_1 but also to an appreciable extent by two steps to S_{-1}. Another agent ABDAC (alkyl-benzyl-dimethyl-ammoniumchloride) seems to lengthen the lifetime of the S_2 and S_3 condition in this cyanobacterium by apparently acting on the membrane condition.

Introduction

According to Kok the water-splitting complex of photosystem II exists in a certain number of redox conditions or “S-states” with the water splitting reaction simply requiring the successive transition from S_0 to S_4. In earlier publications we had shown that S-states in the filamentous cyanobacterium *Oscillatoria chalybea* were particularly long living when compared under identical external conditions (*e.g.* temperature) to those of *Chlorella* or higher plant chloroplasts [1–3]. In particular the S_3-state appeared *quasi* metastable. Thus, if photosynthetic oxygen evolution is measured as the consequence of short saturating light flashes, even after a prolonged dark adaptation of many minutes, a substantial amperometric signal is observed under the first flash [1]. Hence, the term metastable seemed to be justified. In a later publication Seibert and Lavorel have measured for S_3-deactivation 105 s [4] and Boussac *et al.* find for PS II-enriched membrane preparations half-decay times for S_2 and S_3 of 35 and 40 s respectively [5]. A more recent publication by Styring and Rutherford finally shows that in photosystem II-enriched membranes from spinach in the presence of an external electron acceptor the half-decay time for S_2 and S_3 is 3–3.5 min. and 3.5–4 min. respectively [6]. The experiment shows that the life time of S_2 and S_3 depends on the redox condition of the acceptor side of photosystem II. In particular, if the acceptor side is oxidized, a stabilization of S_3 and S_2 is observed. Hence, long living S_3 is no problem anymore as it was when the observation with *Oscillatoria* was made at the time [1]. The following is a study on deactivation characteristics of the S-state system with particular emphasis on the properties of the S_3-state and to conditions affecting the life-time of this state in PS II of the filamentous cyanobacterium *Oscillatoria chalybea.*

Abbreviations: Ant-2-p, (2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene); ABDAC, (Alkyl-benzyl-dimethyl-ammoniumchloride).

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Material and Methods

Plant material

Oscillatoria chalybea was grown in petri-dishes, in which a clay plate was just immersed, in a medium containing nitrate as the sole nitrogen source. Growing conditions were 25 °C and a light/dark cycle of 14 hours light/10 hours dark and illumination intensity of 4500 ergs $\cdot s^{-1} \cdot cm^{-2}$ or 4.5 $W \cdot m^{-2}$ essentially as described earlier [1].

Thylakoid preparations from cells of *Oscillatoria chalybea* were made as described by Bader *et al.* [2].

Mass spectrometric experiments were performed as previously described by Bader *et al.* 1987 [2] and 1992 [7]. The stable isotope ratio mass spectrometer "Delta" from Finnigan MAT (Bremen, Germany) is a magnetic sector field instrument and has been substantially modified for our experiments. These modifications have been described by Bader *et al.*, 1987 [2]. Calibration of the set-up and calculation of the isotope distribution was carried out by two procedures: 1st, the average of at least 10 determinations of the signals at $m/e = 32$, $m/e = 34$ and $m/e = 36$ for "normal" air was correlated with the well-known natural atomic abundance of 99.7587% oxygen-16 and 0.2039% oxygen-18, and 2nd, various concentrations of exogenously added hydrogen peroxide yield definite signals in the detection system upon decomposition by addition of catalase.

Corrections for the isotope dilution can be made according of the equation given by Peltier and Thibault [8]. The mass spectrometric set-up that we use is a closed system with an unidirectional gas flow towards the ion source. Signals for $^{16}O_2$, $^{16}O^{18}O$ and $^{18}O_2$ were simultaneously detected in Faraday cups and recorded on a SE 130-03 BBC Metrawatt 3-Channel recorder. Flash illimination was performed *via* a Stroboscope 1539 A of General Radio which yields flashes of 5 μs duration.

$H_2^{18}O$ was obtained from CEA-Oris, Bureau des Isotopes Stables, Gif-sur-Yvette, France.

Oxygen measurements were carried out by polarography with the "Three Electrode System" described by Schmid and Thibault [9]. Measurements were carried out at a polarization voltage of −680 mV. The electrode system was interfaced with an Atari Mega ST 4 computer. Flashes were provided as for the mass spectrometric assays by the Stroboscope 1539 A of General Radio with flash durations of 5 μs. Usually, a sequence of 30 flashes was given, spaced 300 ms apart.

Mathematic analyses

Experimental data were fitted with the *Kok* application of the *Voyon* general modelling software from Thiéry [10], usable on IBM compatible computers. All specific algorithms for the modelling of oxygen are described by Thibault and Thiéry [11] and Thibault [12] and have been used in this context in earlier publications [1, 13].

Results

We talk of S_3 in terms of the redox state of the water splitting system which requires one more light quantum to split water and to evolve oxygen. This is demonstrated by mass spectrometry for thylakoid preparations of *Oscillatoria chalybea* at the low temperature of approx. 0 °C at which the S_3 state is populated by 2 preflashes. The assay contains until there only normal water-($H_2^{16}O$-)containing buffer in an atmosphere of normal oxygen, *i.e.* $^{16}O_2$. Addition of $H_2^{18}O$ and an analyzing flash given within 15 seconds yields ^{18}O-labelled oxygen evolution (Fig. 1). In the earlier publication we had stated that S_3 at room temperature was in part metastable [1, 2]. This was to be understood in the sense that the deactivation of S_3 out of the steady state condition

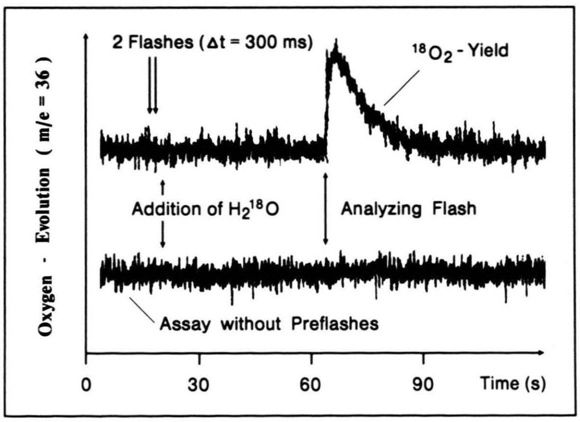

Fig. 1. O_2-flash yield measured by mass spectrometry at 0 °C in a thylakoid particle preparation of *Oscillatoria chalybea*. S_3 was populated by 2 flashes in an assay with *Oscillatoria* thylacoids corresponding to 40 μg Chlorophyll in Tricine 0.15 M/KCl 0.3 M. Within 15 sec. $H_2^{18}O$ was given and an analyzing flash fired 45 s after the preflashes.

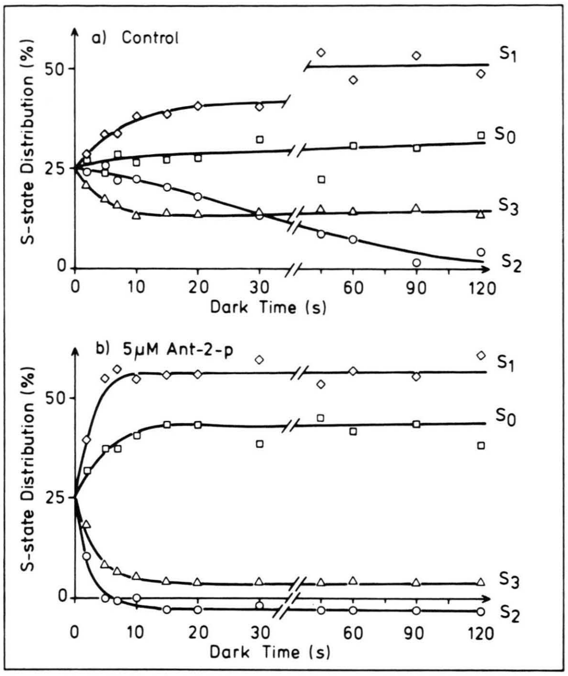

Fig. 2. Pattern of deactivation of S-states in thylakoids of *Oscillatoria chalybea*. The variation of the S-state population is given as a function of the dark time separating two flash sequences. The S-state distribution is calculated in the 4-state Kok model. a) thylakoids without additions (control); b) thylakoids in the presence of 5 µM Ant-2-p (2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene)).

where $S_0 = S_1 = S_2 = S_3 = 25\%$ does not go down to 0% (Fig. 2 a) even after prolonged dark adaptation, thus leaving even after 20–40 minutes of dark adaptation approx. 10% S_3 in the system [1, 2]. Fig. 2 a shows that deactivation time proper meaning the half-decay times of the deactivation of S_2 and S_3 are well comparable to those known in other plant material. It should be noted here that from mathematical fitting experiments it can be concluded that an *Oscillatoria* sequence obtained after dark adaptation represents a purer 4-state Kok-condition than the well known *Chlorella* sequences (see Refs [1] and [2]). Thus, it is clearly seen that the deactivation pattern, expressed in the 5-state Kok model, *i.e.* the dark population of S-states, contains absolutely no contribution from an S_{-1} state (Fig. 3 a), *i.e.* from a more reduced state than S_0.

The metastable S_3 perfectly meets the criteria of a normal S_3. If in a dark adapted sample the S-state population is (as described in Table II of Ref. [1]) assumed to be approx. 41% S_0, 50% S_1, 1% S_2 and 7.5% S_3, one flash (disregarding misses) should practically eliminate all S_3 in the system. In this example approx. 50 sec. after the preflash more than 90% of the states are S_1, 7.5% S_2 and S_3 should be less than 1%. In Fig. 4 a the deactivation kinetics of a preparation with a similar S-state distribution after dark adaptation, as the one just described, is shown after one preflash. The miss parameter which in *Oscillatoria* never is considerably below 25% explains that one preflash fails to eliminate absolutely all S_3. Table I gives the S-state distribution in *Oscillatoria* for various dark times after one preflash together with the relevant transition probablilities.

Ant-2-p (2-(3-Chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene) is an ADRY-reagent introduced by Renger [14] which is supposed to specifically act on S_2/S_3 by accelerating the deactivation kinetics, hence diminishing the life time [15]. The mechanism of action of this ADRY-reagent is thought to be that of an electron donor to Tyr 161. Clearly, addition of this agent specifically affects the deactivation of S_2/S_3 and brings both metastable states within 5 seconds practically down to zero

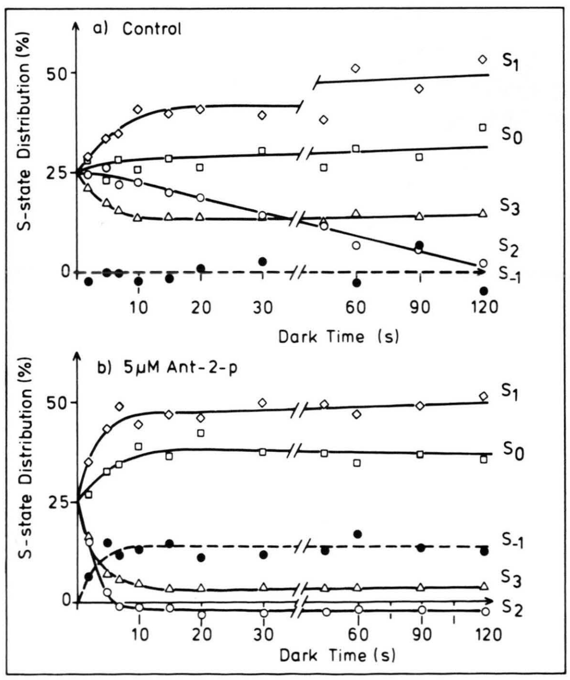

Fig. 3. Deactivation pattern of S-states in thylakoids of *Oscillatoria chalybea* expressed in the 5-state Kok model. a) control without additions; b) thylakoids in the presence of 5 µM Ant-2-p. Same experiment as Fig. 2.

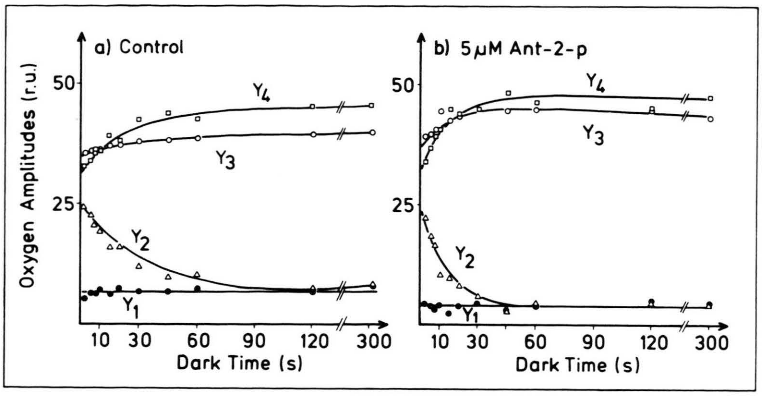

Fig. 4. Deactivation of S-states as a function of dark time separating one preilluminating flash from the flash sequence. The figure gives the amperometically measured oxygen-amplitudes (Y_1, Y_2, Y_3, Y_4) in relative units.

(Fig. 2b). The shortening of the life time of S_2 is particularly important (Fig. 2b). If the deactivation is calculated in the 5-state Kok model (Fig. 3) it is seen that Ant-2-p changes the mode of deactivation (Fig. 3b). Whereas in the control condition, that is in the absence of this agent, a dark adapted sample contains absolutely no S_{-1}, meaning that the most reduced state is S_0, it is seen that in the presence of Ant-2-p the system finds itself in a condition in which the per cent contribution of S_3 and S_2 is after 10–15 seconds practically zero, whereas that of S_{-1} may reach ~10%. Hence, the agent changes the mode of deactivation and the redox condition of the system.

Studies of the dark transitions in a normal system *e.g.* higher plant chloroplasts or chlorella have shown that S_2 deactivates in variable proportions to S_1 and S_{-1} whereas S_3 deactivates in a relative constant ratio of 2:1 to S_2 and S_0 [12]. Fig. 2a and 3a show that under control conditions in *Oscillatoria* S_2 strictly not deactivates to S_{-1}, a state, which is nonexistant in the system, whereas in the presence of Ant-2-p S_2 obviously deactivates to a considerable extent to S_{-1}. This can be verified by the fact that $\frac{S_1 + S_{-1} + S_2}{2}$ is constant demonstrating that S_{-1} is somehow derived from S_2 (Table II). The metastability of S_3 shown in Fig. 2 and 3 refers to the fact that the deactivation of S_3 leads to a defined low concentration of this state after which a further decay does not take place anymore. Hence, it looks as if the life time of this state depended on its concentration. The highest concentration of this state, found in a fully dark adapted sample, never exceeded 12–13% of the dark state population, and Fig. 2a describes this situation. This state can be almost eliminated by one single flash, if the redox situation of the S-state system is such, that the concentration of the S_2-state does not go below a certain level (Table I). This interpretation puts definite constraints on the deactivation sequence $S_3 \rightarrow S_2$. In this defined redox range S_3 deactivates in the *Oscillatoria* system with normal half-times. Anti-2-p obviously changes the redox condition of the system in such a way that the deactivation kinetic of S_3 (and S_2) is accelerated and the mode of deactivation

Table I. Variation of the S-state population as a function of the dark time separating a preillumination flash from a flash sequence.

Dark time	S-states (%)				Transition Probability (%)		
[s]	S_0	S_1	S_2	S_3	Miss (α)	Success (β)	Double hit (γ)
2	26.3	45	26	2.7	31	62	7
5	25.1	52.1	18.5	4.3	31	61	8
7	23.5	57.5	14.2	4.7	34	58	8
10	24.5	60.2	9.7	5.7	33	58	9
15	25.0	68	0.7	6.1	36	56	9

Flash sequence of 30 flashes spaced 300 msec apart.

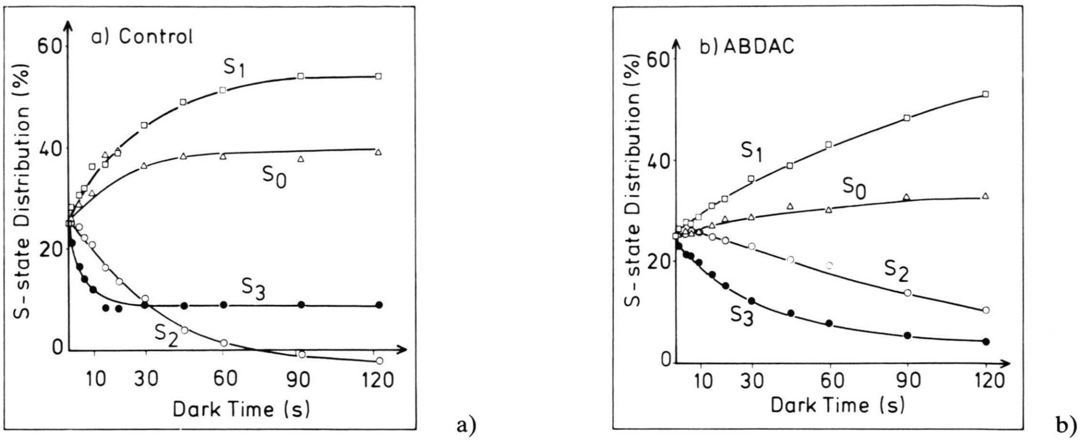

Fig. 5. Deactivation of S-states in thylakoids of *Oscillatoria chalybea* calculated in the 4-state Kok model. a) Control without additions; b) in the presence of 10^{-5} M ABDAC (Alkyl-benzyl-dimethyl-ammonium chloride).

changed, which permits the system to go practically down to the zero level (Fig. 2 and 3). The experiment in Fig. 4 demonstrated this. In this experiment a flash sequence is fired at increasing dark times after one preilluminating flash. In the control condition without Ant-2-p (Fig. 4a) a situation is depicted with practically 6 per cent metastable S_3, a level to which also S_2 deactivates. In the presence of Ant-2-p not only the decay kinetics of S_2 are faster but the level of S_3 is substantially further decreased (Fig. 4b). Deactivation of S_2 under these conditions seems to go to S_1 and to S_{-1}.

An opposite effect on the life time of the S-state system of *Oscillatoria* is observed in the presence of alkyl-benzyl-dimethyl ammonium chloride (ABDAC) (Fig. 5). The chemical clearly leads to a prolongation of all S-state life times which could be seen as a reduction of the overall reactivity of the redox states. The agent does not seem to act as a redox component but rather as an agent that modifies the membrane condition.

Discussion

When photosyntetic oxygen evolution is measured as the consequence of short saturating light flashes, the pattern of a damped oscillation with periodicity of four is observed [16, 17]. Kok explained the phenomenon by saying that the water splitting system occurred in four redox states [9], which have simply to be gone successively through before water is split and O_2 evolved. The damping was explained to be due to three transition probabilities by which in a given biological system a defined state S_i transits upon absorption of a light quantum to S_{i+1} (success), stays in its state S_i (misses the transition) or advances by two steps to state S_{i+2} (double hit). The relative distribution of transi-

Table II. Deactivation of S-states in the presence of the ADRY-reagent Ant-2-p in the filamentous cyanobacterium *Oscillatoria chalybea*.

Dark time [s]	S-states (%) S_{-1}	S_0	S_1	S_2	S_3	$\frac{S_{-1}+S_1+S_2}{2}$	Transition Probabilities (%) Miss (α)	Success (β)	Double hit (γ)
2	6.4	2.8	35.1	15.2	16.5	28.35	18.4	74.4	7.2
5	14.9	32.5	43.2	2.5	6.9	30.3	18.2	75.7	6.5
7	11.8	34.5	48.9	−1.0	5.8	29.9	20.8	72.9	6.8
10	13.2	38.9	44.3	−1.1	4.8	28.2	18.8	74.7	7.0

The S-state population was measured in dependence on the dark time between two flash sequences.

tions between these probabilities was supposed to be an inherent property of the respective system (*e.g.* spinach, chlorella or else) and independent from and hence constant for any given state S_i. This model known as the Kok model is up to now the most used model. Other models taking into account that the transition probabilities appear in certain conditions not constant for any S-state transition have insufficiencies which the Kok model does not have and complicate the description of phenomena substantially in comparison to the Kok model. The Kok model cannot only be used in the sense of the water splitting and oxygen evolving reaction but also in the sense of deactivation of S-States when oxygen evolution is measured as consequence of a train of flashes separated from another flash sequence by different dark times. Deactivation of S-states is supposed to be subject to the same transition probabilities as the forward reaction. Not much has been finally added in 25 years to this model which successfully describes most observations.

The most investigated states in this system are S_2 and S_3. The question whether water is stepwise oxidized in the course of this reaction sequence is still open. Mass spectrometric analysis have shown that in a condition in which the S_3 condition is populated (by two flashes for example) addition of $H_2{}^{18}O$ and a subsequent flash yielded $^{18}O_2$-evolution, suggesting that up to the S_3 condition no bound and no "partially oxidized" water should exist [2, 18, 19] a result which is not only difficult to interpret from thermodynamic point of view but also in view of results in which nitrogen compounds like NH_2OH or H_2N-NH_2 are oxidized by the S-state system [19, 20]. Here it can clearly be shown that these compounds interact preferentially with the S_2-state which from the point of view of reactivity seems to be the most reactive state, interacting finally with a variety of components [19–22], whereas S_3 appears to be relatively inert. This leads to the feeling that what is observed in the mass spectrometric analyses represents the "end" of an equilibrium situation. Fast binding reactions might occur *via* redox equilibria. In this situation the properties of the S_3-state are still of particular interest. In *Oscillatoria* this state is metastable in the sense that a certain portion of this state, when the system is dark adapted from the steady state illuminated condition, does not fully deactivate. It may happen that after a prolonged dark adaptation of several minutes the S-state population contains up to 10 per cent S_3 which does not deactivate further. The deactivation itself to this final level takes place within a normal delay when compared to the time range observed for S_3 deactivation in different systems under different redox conditions [1, 4–6]. It looks as if deactivation of this state depends on the redox condition of the S-state system as a whole in the membrane context, thus depending practically on its own concentration. This particular S_3-condition can be perfectly promoted by one more flash to S_4, thus leading to O_2-evolution. The extent of the deactivation level of this state depends in *Oscillatoria* clearly on the redox conditions of the enzyme system which can be seen from the fact that the deactivation level is intimately linked to the actual concentration of S_2 in the system (Table I, Fig. 4). The fact that in *Oscillatoria* S_3 deactivates under usual conditions to a constant finite level shows not only what has been discussed earlier with respect to the reactivity of the state [19, 20] but also that in a certain number of centers either a redox condition and/or an intermediate of water oxidation is conserved. This in turn seems to depend on the redox condition of the S-state/enzyme system as a whole because in the presence of the ADRY-reagent Ant-2-p, a condition under which electron attraction by photosystem I is diminished, this metastable condition is not maintained and deactivates. The agent apparently changes the enzyme condition (redox conformation) in such a way that the mode of deactivation is changed, yielding under this condition a dark population of S-states in which the reduced condition S_{-1} accumulates in the dark (Fig. 3).

Acknowledgment

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