A Study on the Life Time of the S₃-State in the Filamentous Cyanobacterium Oscillatoria chalybea

G. H. Schmid, K. P. Bader, and R. Schulder

In the filamentous cyanobacterium Oscillatoria chalybea deactivation of the S-states starting from steady-state conditions in which S₀ = S₁ = S₂ = S₃ = S₄ = 25% reveals that S₃ deactivates to a finite level of approx. 10%. This level is reached under normal conditions between 10–15 seconds. This quasi metastable S₃ meets all requirements for S₄ in that one flash eliminates this redox conditions to give S₄ and therewith molecular oxygen. An analysis of the cyanobacterial S-state system in the S-state Kok model shows that the S-state population in the dark adapted sample contains no contribution from S₃, 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 S₄ is the most reduced state. Under these conditions S₃ seems to deactivate to S₄ and S₅ to S₆ and to a smaller extent to S₅. 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 S₃-state (and thereby on S₄), not only the deactivation kinetic of S₃ (and S₄) is accelerated (hence the life time of the S₃-state is shortened), but also the level of metastable S₃ 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 S₄. It looks as if in this condition S₃ deactivates not only to S₄, but also to an appreciable extent by two steps to S₅.

Another agent ABDAC (alkyl-benzyl-dimethyl-ammoniumchloride) seems to lengthen the lifetime of the S₃ and S₄ condition in this cyanobacterium by apparently acting on the membrane condition.

---

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

Reprint requests to Prof. Dr. Georg H. Schmid.

Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0959–5075/94/0100–0108 $ 01.30/0

---

This work has been digitized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.
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 • s⁻¹ • cm⁻² or 4.5 W • 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 to 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 illumination 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 Thiery [10], usable on IBM compatible computers. All specific algorithms for the modelling of oxygen are described by Thibault and Thiery [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 then 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.](image-url)
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. 2a) 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. 2a 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. 3a), i.e. from a more reduced state than \( S_0 \).

The metastable \( S_1 \) perfectly meets the criteria of a normal \( S_0 \). 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. 4a 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 probabilities.

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. 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 $S_1$ is after $10-15$ seconds practically zero, whereas that of $S_{-1}$ may reach $\sim 10\%$. 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 $S_3$, 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 $S_1$ strictly not deactivates to $S_{-1}$, a state, which is nonexistent 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 $S_{1} + S_{-1} + S_2$ 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>
<thead>
<tr>
<th>Dark time [s]</th>
<th>S-states (%)</th>
<th>Transition Probability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$S_0$</td>
<td>$S_1$</td>
</tr>
<tr>
<td>2</td>
<td>26.3</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>25.1</td>
<td>52.1</td>
</tr>
<tr>
<td>7</td>
<td>23.5</td>
<td>57.5</td>
</tr>
<tr>
<td>10</td>
<td>24.5</td>
<td>60.2</td>
</tr>
<tr>
<td>15</td>
<td>25.0</td>
<td>68</td>
</tr>
</tbody>
</table>

Flash sequence of 30 flashes spaced 300 msec apart.
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. 4 a) a situation is depicted with practically 6 per cent metastable S3, a level to which also S2 deactivates. In the presence of Ant-2-p not only the decay kinetics of S2 are faster but the level of S3 is substantially further decreased (Fig. 4 b). Deactivation of S2 under these conditions seems to go to S1 and to S3.

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 O2 evolved. The damping was explained to be due to three transition probabilities by which in a given biological system a defined state S; transits upon absorption of a light quantum to S+1 (success), stays in its state S (misses the transition) or advances by two steps to state S+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.***

<table>
<thead>
<tr>
<th>Dark time [s]</th>
<th>S-states (%)</th>
<th>Transition Probabilities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S−1</td>
<td>S0</td>
</tr>
<tr>
<td>2</td>
<td>14.9</td>
<td>32.5</td>
</tr>
<tr>
<td>5</td>
<td>11.8</td>
<td>34.5</td>
</tr>
<tr>
<td>7</td>
<td>13.2</td>
<td>38.9</td>
</tr>
</tbody>
</table>

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 \(^{18}\text{O}_2\)-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 \(\text{NH}_3\cdot\text{OH}\) or \(\text{H}_2\text{N}^+ - \text{NH}_2\) 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 \(S_2\)-state are still of particular interest. In \textit{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 \(S_4\) 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 \(S_4\) 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 \(\text{O}_2\)-evolution. The extent of the deactivation level of this state depends in \textit{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 \(S_3\) in the system (Table I, Fig. 4). The fact that in \textit{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**

This work was supported by the Deutsche Forschungsgemeinschaft within the scope of the Forschergruppe Pu 28/14-1.