A Membrane Bound Cysteine Oxydase from the Cyanobacterium *Synechococcus* 6301

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Membrane fractions of the cyanobacterium *Synechococcus* 6301 obtained by french press treatment following sonication catalyzed an oxygen-dependent oxydation of cysteine to cystine. For 1 O_2 consumed four cysteine were oxydized. Oxygen uptake was completely inhibited by 1 mM KCN. Only D- and L-cysteine were active and partial activity was observed with DL-homocysteine and cysteamine. No activity was found with glutathione, mercaptoethanol, thioglycerol, dithioerythritol, or N-acetyl-L-cysteine. Cysteines with a blocked acid group such as O-methyl-L-cysteine and O-ethyl-L-cysteine were oxydized rapidly by *Synechococcus* membrane fractions. Rates of about 200 µmol of cysteine oxydized per mg chlorophyll and hour were measured. This cysteine respiration is discussed in relation to dark inactivation of enzymes.

Introduction

ATP and NADPH are generated in the light as the energy source for CO_2-reduction in the reductive pentose-phosphate cycle. Light, however, is also needed to regulate certain key enzymes of different metabolic steps including CO_2-fixation [1, 2], sulfate reduction [3, 4], and nitrate reduction [5−7, 20]. Evidence has been presented that at least some of the light activation properties can be explained by coupling the electron transport chain to a thioredoxin reductase forming reduced thiol groups of thioredoxins, which in turn activate (or inactivate) key enzymes as discussed above.

The activation step by thioredoxins has been analyzed in detail using different organisms [1, 8, 9] including cyanobacteria [4, 10−13]. No information, however, has been obtained for the mechanism of the reoxydation of thiols commonly referred to as dark inactivation.

Evidence is presented in this paper that *Synechococcus* 6301 contains a membrane-bound cysteine oxydase, which catalyzes the oxydation of cysteine to cystine with molecular oxygen.

Materials and Methods

Organism

Synechococcus 6301 was obtained from the collection of algal cultures from Prof. Dr. Stanier (Pasteur Institute, Paris) and grown as axenic culture as described previously [3]. Thiol compounds to be analyzed were added to sulfate grown cultures after 3 days of growth by sterile filtration to a final concentration of 3×10^{-4} M [14] and cells were harvested 24 h later.

Preparation of membrane fractions

Cells were harvested by centrifugation and to 1 g of wet cells 5 ml of Tris-HCl buffer pH 8.0 (containing 10 mM $MgCl_2$ and 10 mM mercaptoethanol) was added. Cells were broken at 12.000 PSI; the membrane fraction was collected by centrifugation and the pellet fraction was washed once with 0.05 M Imidazol-buffer pH 8.0, afterwards taken up in the same buffer and treated 3 times for 10 s by sonication (Model Branson sonic power). The membrane fraction obtained in this way was used for the experiments.

Chlorophyll determination

Chlorophyll was determined according to the procedure of Arnon [15].

Measurement of thiol oxydation

Thiol oxydation was analyzed following the disappearance at thiol groups with the Ellman reagent (DTNB). For these measurements a Zeiss filter photometer was used at 436 nM; the ε for DTNB at this wavelength was calculated to 10.9 (mM). A control without addition was run in parallel to correct for autoxidation of thiol groups not catalyzed by the membrane fraction.

Measurement of oxygen uptake

Oxygen uptake was followed with a Hansatech clark type electrode coupled to a W + W recorder model 312 (Bachofer, Reutlingen).

Chemicals

Thiol compounds including DTNB were obtained from Sigma (München) whereas all other chemicals not mentioned were purchased from Merck (Darmstadt).

Results

Membrane fractions of *Synechococcus* catalyzed the oxydation of cysteine are shown in Fig. 1. This oxydation was followed by the disappearance of free thiol groups of cysteine using a control without addition of membrane fractions as a control for thiol autoxydation. The cell with the membrane
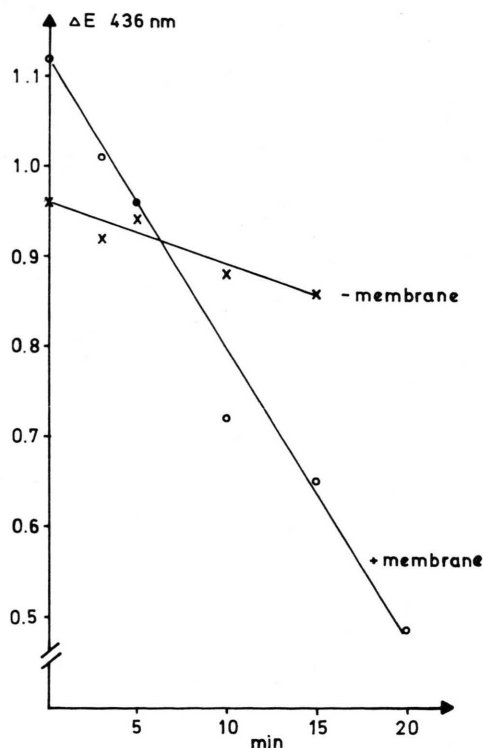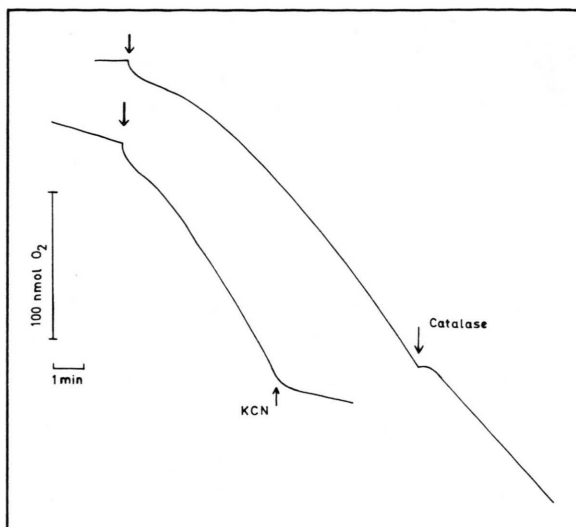
Fig. 2. Cysteine-induced oxygen uptake by *Synechococcus* membranes. Conditions as in Table I, however the temperature was held at 33 °C.

Fig. 1. Membrane-catalyzed oxydation of cysteine in relation to outoxydation of cysteine. Conditions: Incubation conditions as in Table 1; however the temperature was set to 37 °C. A_t the time intervals indicated 50 µl aliquots were withdrawn and pipetted into a cuvette containing 950 µl water and 1 µmol of DTNB-reagent. The optical density read at 436 nm was plotted against the time.

fraction starts at a higher absorbance since the membrane fraction adds to the absorbance at 436 nm, which was used for the DTNB measurements. It is evident from these data that a membrane-catalyzed cysteine oxydation can be followed in relation to the incubation time. The electron acceptor for this cysteine-oxydation is molecular oxygen (O_2) which is evident from the traces of Fig. 2. It shows a trace of the oxygraph; the reaction was measured first without addition of membrane fractions to measure cysteine autoxidation and the addition of the membrane fraction is marked with an arrow. Addition of 1 mM KCN stopped the oxygen consumption, whereas addition of 100 units of catalyse did not affect the rate. The ratio of oxygen uptake and cysteine oxydation was followed by measuring these two rates side by side using the same buffer, same temperature, same cysteine concentration and the identical membrane fraction. The data obtained are summarized in Table I.

A ratio of one oxygen to four cysteines oxydized was found, which suggests a direct reduction of oxygen to water. The intermediate formation of H_2O_2 was not detected, however the membrane fraction contained also catalase activity, although with rates evidently not high enough to allow a reaction sequence with H_2O_2 as an intermediate, which would be in accord with the observation of Fig. 2 that addition of catalase did not alter the

Table I. Comparison of oxygen uptake, L-cysteine oxidation and catalase activity. Conditions: Oxygen uptake was determined with a clark electrode using the following conditions (in µmol): Imidazol-buffer pH 8:200; L-cysteine: 3 and 0.023 mg membrane bound chlorophyll in a total volume of 2 ml at 37 °C. The reaction was started by adding the pellet fraction. Cysteine oxydation was followed using the identical conditions, however following the disappearance of thiol groups with the DTMB-method. Catalase activity was determined with the clark-electrode by replacing L-cysteine with H_2O_2 following oxygen production with the membrane fraction.

| Source measured	Rate [µmol/mg Chl × min]	O_2-uptake [%]
Oxygen uptake	1.08	100
L-cysteine oxidized	4.39	406
Catalase activity	1.6	148

Table II. Substrate specificity of the membrane fraction towards different thiols. Conditions: Each vessel contained in µmol: Imidazol-buffer pH 8.0: 300; thiol compound: 3; (with the exception of DTE: 1.5); membrane fraction containing 21.6 µg chlorophyll in a total volume of 3.0 ml. Incubation at 37 °C.

Thiol added	nmol thiol groups oxidized in 10 min	L-cysteine [%]
L-cysteine	429	100
D-cysteine	493	115
Glutathione	10	2
Cysteamine	187	44
DL-homocysteine	192	45
Dithioerythritol	0	0
Mercaptoethanol	10	2
Mercaptopropionic acid	25	6
Thioglycerol	15	4
N-acetyl-L-cysteine	10	2
O-methyl-L-cysteine	1816	424
O-ethyl-L-cysteine	2155	503

Table III. Cysteine oxidation capacity of membrane fractions from *Synechococcus* 6301 grown on different sulfur sources. Conditions. Each vessel contained in µmol: Imidazol-buffer pH 8.0: 300; D-cysteine, L-cysteine or O-methylcysteine: 3; and membrane fractions with 20 to 50 µg of chlorophyll in a total volume of 3 ml. Incubation at 37 °C. Aliquots were taken at 2 to 3 min intervals to measure the disappearance of thiol groups. The rates were calculated over the first ten to 15 min. The temperature in all cases was 37 °C.

Sulfur source	Thiol oxidation rate: µmol/mg Chl×h		
	L-cysteine	D-cysteine	O-methyl-cysteine
Sulfate	173	210	1034
L-cysteine	211	181	951
D-cysteine	235	218	971
L-methionine	261	150	854
D-methionine	233	163	1015
O-methylcysteine	1328	1314	3162

oxygen uptake kinetics. This cysteine oxidation can be formulated as follows:

$$4 \text{ cysteine} + O_2 \xrightarrow{\text{membrane fraction}} 2 H_2O + 2 \text{ cystine}.$$
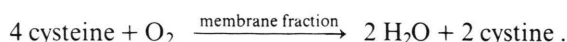
The thiol specificity of the *Synechococcus* membrane fraction for this reaction sequence was analyzed using the DTNB-method with different cysteine analogues and some artificial thiol. These data are summarized in Table II.

D- and L-cysteine were oxidized in a rapid manner by this membrane fraction, and about 40% of the activity was determined using either DL-homocysteine or cysteamine. All other thiols tested including glutathione, N-acetyl-L-cysteine, DTE, or mercaptoethanol were not oxidized above their autoxydation by *Synechococcus* membrane fractions. Synthetic cysteine derivatives with a blocked acid group (O-methyl-L-cysteine and O-ethyl-L-cysteine) were oxidized, however, with rates about five times higher when compared with L-cysteine.

Comparable rates of cysteine oxidation were measured using membrane fractions of cells which had been grown for 24 h before harvest with addition of different cysteines and methionines. These data are summarized in Table III. The rates are in the range of 200 µmol cysteine oxidized per mg chlorophyll and hour with the exception of O-methylcysteine, however, this thiol is already toxic for *Synechococcus*.

Discussion

Light activation and dark inactivation of enzymes has been documented for higher plants [1, 16], green algae [5, 20] and cyanobacteria [4, 17–19]. The activation mechanism has been traced to thioredoxins [1, 8, 9]. Thioredoxins are low-molecular weight proteins containing at least one disulfide group which can be reduced and oxidized in a catalytic manner [9]. These thioredoxins are reduced either by a ferredoxin-dependent ferredoxin-thioredoxin reductase [2, 10], or a $NADPH_2$-dependent thioredoxin-reductase [21, 22]; thus either ferredoxin or $NADPH_2$ oxydation is coupled to the reduction of thiol groups. By this mechanism the light perception of the electron transport chain is transferred via thiol groups to generate active enzymes.
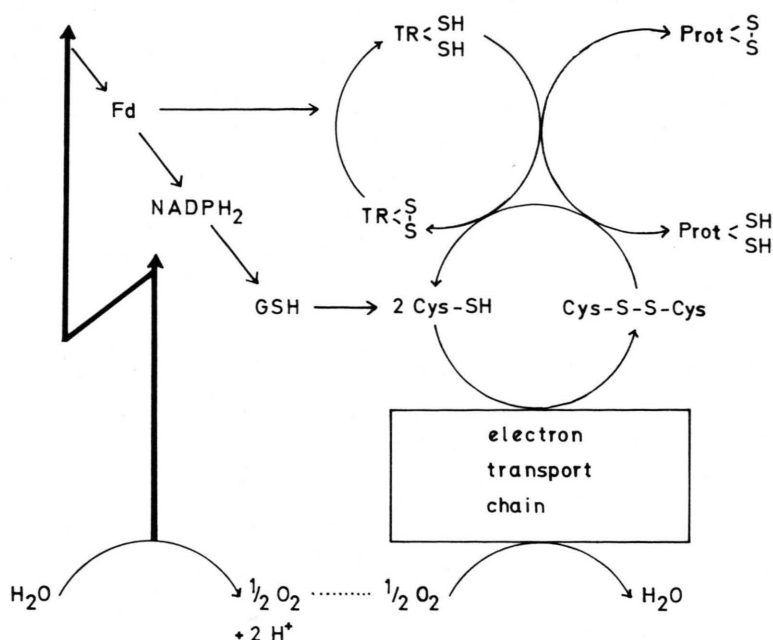
No information is available up to now for the light-independent oxydation of thiols, which seems to be necessary to inactivate these enzymes active during the light period. Evidently electrons have to be transferred to oxygen, since no inactivation was found under anaerobic conditions at least for a higher plant leaf [23].

Evidence from the cyanobacterium *Synechococcus* 6301 of this paper clearly demonstrates that a cysteine oxydase is present in a membrane bound form, which will transfer the electrons to molecular oxygen with the formation of water. If H_2O_2 is an intermediate in this reaction is not known; however catalase activity was present in these membrane fractions, although the rates measured seem to be not high enough to destroy all H_2O_2 which should be formed if it is an intermediate. Secondly addition of 100 units of catalase did not alter the oxygen uptake rate, which again favours the idea of a direct reduction of O_2 to water without H_2O_2 as intermediate. The inhibition of KCN points to inhibition of cytochromes, thus the easiest explanation would be the feeding of electrons from cysteine into the electron transport respiratory chain with coupling to oxygen by the terminal $a-a_3$ complex [24, 25], which then would possibly even be used for ATP-formation. Such a system would be a thiol respiration.

If living cells have a system for enzyme activation, as shown for the thioredoxin system, the cells should have an inactivation system as well, since it should be needed for an efficient regulation. Our view of the thioredoxin system interacting with the cysteine oxydation system is summarized in Fig. 3. Thiols are reduced by the electron transport chain and are inactivated by the oxydation of cysteine; the system thus provides a balance between the reducing power on the one side and the oxydation capacity at the other side. This reaction sequence (probably shortcut by a direct reduction of cystine with reduced thioredoxin) seems to be a Mehler-type reaction where water is split to oxygen and this oxygen is used to produce water again, however mediated by a thiol oxydation. This would be a new type of light-induced oxygen consumption.

For the balance of the reaction sequence discussed above, the cysteine concentration is of critical influence for the cell, since high cysteine concentrations would increase the oxydation rate and this would affect the activation state of thiol-dependent enzymes. This might be the reason that high cysteine concentrations (1 mM) are toxic for algae [14].

This membrane catalyzed cysteine oxydation is not a unique property of *Synechococcus* 6301, since we found it also with rates measured and expressed

according to Table III using L-cysteine in *Anabaena variabilis* (107); *Anabaena cylindrica* (92) (both strains from C. P. Walk); *Anabaena* PCC 7120 (347); *Spirulina* PCC 6312 (433); *Nostoc* PCC 6310 (409) and *Pseudoanabaena* PCC 7408 (72).

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