

Sulfide dehydrogenase is identical with the SoxB protein of the thiosulfate-oxidizing enzyme system of *Paracoccus denitrificans* GB17

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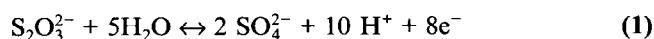
Abstract

Thiosulfate induced cells of *Paracoccus denitrificans* GB17 oxidize thiosulfate and sulfide to sulfate. A mutant carrying a Tn5-*mob* insertion in the *soxB* gene is unable to oxidize thiosulfate or sulfide suggesting a linkage of both activities. To test this assumption we have separated the components of the thiosulfate-oxidizing enzyme system of the wild-type by ion exchange chromatography. The SoxB protein coeluted with a highly active sulfide dehydrogenase. Analysis by polyacrylamide gel electrophoresis revealed one major protein of *M_r* 32k. Thus, the SoxB protein appeared to be identical with sulfide dehydrogenase.

Key words: Sulfide dehydrogenase; Thiosulfate oxidation; *Paracoccus denitrificans*

Introduction

Oxidation of reduced inorganic sulfur compounds is the most characteristic physiological trait of aerobic neutrophilic or acidophilic thiobacteria. The neutrophilic thiobacteria *Paracoccus denitrificans* and *Thiobacillus versutus* oxidize thiosulfate according to equation 1 [1,2].



The thiosulfate-oxidizing enzyme system of *T. versutus* has been purified and characterized. Four periplasmic proteins are required for oxidation of thiosulfate to sulfate: enzyme A (*M_r* 16,000), enzyme B (*M_r* 63,000), cytochrome *c*_{552.5} (*M_r* 56,000), and cytochrome *c*₅₅₁ (*M_r* 260,000) [3]. Although no catalytic function could be attributed to the components of *T. versutus* they were designated as enzyme A or enzyme B [2]. Enzyme A, however, was shown to bind thiosulfate with high affinity [3]. The current hypothesis indicates the oxidation of the sulfane sulfur of thiosulfate by *T. versutus* and the possible role of an enzyme-bound intermediate formally equivalent to dithionate ($\text{O}_3\text{S} - \text{SO}_3^-$) [3].

The first genetic analysis of the sulfur oxidizing enzyme system was initiated with *P. denitrificans* GB17. Mutants were isolated with the kanamycin-resistance (*Km^r*) encoding transposon Tn5-*mob*. One of these mutants was characterized in more detail. Strain TP19 did neither convert thiosulfate nor sulfide to sulfate [1]. A DNA fragment bearing the Tn5-*mob* insertion was cloned and used to identify the corresponding wild type

fragment by Southern hybridization. The resulting 13 kb fragment was cloned in plasmid pEG12. This fragment was shown to generate a strong signal in heterologous hybridization with genomic DNA of the closely related thiobacterium *T. versutus* [4]. Three lines of evidence were drawn from these observation: (i) the enzyme systems for sulfur oxidation of *T. versutus* and *P. denitrificans* appear to be highly related; (ii) both systems depend on the function of a common locus designated *soxB*; and (iii) thiosulfate and sulfide oxidation appear to be linked. To get further insight into the sulfur oxidation pathway we analyzed the components of thiosulfate and of sulfide oxidation of *P. denitrificans* GB17.

We here report on the detection of sulfide dehydrogenase, its purification, and the identity of sulfide dehydrogenase with the SoxB protein.

2. Materials and methods

2.1. Strains, media and culture conditions

Paracoccus denitrificans GB17 [5,6] and its Tn5-*mob* insertional derivative strain TP19 harboring *soxB::Tn5-mob* [1,4,7] was used.

Cells were cultivated aerobically at 30°C. Cells were pregrown in mineral medium with 0.2% (w/v) of glucose for 24 h. Main cultures were inoculated with 1% (v/v) of a preculture. After growth on glucose media were adjusted to pH 8.0, and 0.05% (w/v) sodium bicarbonate and 20 mM of sodium thiosulfate were added. Media of 200 ml were incubated in 1 liter baffled Erlenmeyer flasks with screw caps. Caps were tightened after addition of thiosulfate and bicarbonate. This resulted in a specific thiosulfate oxidizing activity equivalent to that of cells grown lithoautotrophically with thiosulfate. Cells treated in this manner for 5 h were referred to as 'thiosulfate induced'. Cells referred to as 'sulfide induced' were cultivated identically, however, with 1.0 mM disodium sulfide instead of thiosulfate.

To obtain cells for enzyme preparations *P. denitrificans* GB17 was cultivated in a fermenter (New Brunswick Scientific, NJ) with 60 liter of working volume which was stirred at 600 rotations per minute (rpm) and aerated with 20 liters of air per minute. After growth on glucose thiosulfate was added to a final concentration of 40 mM. The culture was then aerated with a mixture of 98% (v/v) of air supplemented with

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Abbreviations: SDH, sulfide dehydrogenase; cyt, cytochrome; Sox, sulfur oxidation.

2% (v/v) of carbon dioxide at a rate of 20 liter per minute. The pH was maintained at 8.0 by automatic titration with 5 N sodium hydroxide. After 6 h the cells were concentrated to 5 liter by cross flow filtration (Sartorius, Göttingen, Germany), subsequently collected by centrifugation at 4°C for 45 min at 5,000 rpm in a Sorvall GS3 rotor, washed once with 25 mM potassium phosphate buffer pH 7.5 and stored at 70°C.

2.2. Purification of sulfide dehydrogenase

Cell-free extracts were prepared from 10 g wet wt. and the components of the thiosulfate-oxidizing enzyme system of *P. denitrificans* were purified according to the protocol described for *T. versutus* [2]. All steps were performed at 4°C. Cell debris of cell-free extract was removed by centrifugation at 5,000 × g for 30 min and the supernatant was subjected to centrifugation at 142,000 × g for 45 min. The resulting soluble extract was treated with solid ammonium sulfate to a saturation of 65%. Precipitated protein was removed by centrifugation and the pellet was dialyzed against 5 liter of 25 mM potassium phosphate buffer, pH 7.5, for 16 h. The dialyzed extract (20.0 ml) was applied to a DEAE-Sepharose CL-6B column (2.6 × 16.5 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7.5, and washed with 210 ml of the equilibration buffer. Protein was eluted by a step gradient using phosphate buffer containing sodium chloride at concentrations of 0.1 M (340 ml), 0.2 M (270 ml), 0.3 M (110 ml), and 0.35 M (430 ml).

2.3. Enzyme assays

Enzyme activities were determined at 30°C. The sulfide or thiosulfate dependent oxygen uptake rate was determined with whole cells using an oxygen electrode (Rank Brothers, Bottisham, UK). The assay (2.0 ml) contained 100 μmol of Tris buffer, pH 8.0, about 0.5 mg of protein and 2 μmol of disodium sulfide or of thiosulfate to start the reaction.

The overall activities of the thiosulfate oxidizing enzyme system were determined as described for *T. versutus* [2]. The thiosulfate and sulfide dependent reduction of cytochrome c_{550} (horse heart type III) was recorded at 550 nm ($\epsilon_{550} = 27.8 \text{ cm}^2/\mu\text{mol}$) with a Shimadzu UV/vis 160 spectrophotometer. Enzyme activities of soluble extracts were assayed in 1.0 ml of a reaction mixture containing 50 μmol of Tris buffer, pH 8.0, 0.07 μmol of horse heart cytochrome c_{550} , about 0.2 mg of protein of soluble extracts, and 2 μmol of disodium thiosulfate or sulfide to start the reactions.

Activity of the SoxB protein was assayed from 0.2 ml of the DEAE-Sepharose CL-6B eluate in 1.0 ml with 50 μmol of Tris buffer, pH 8.0, 0.07 μmol of horse heart cytochrome c_{550} , 0.42 mg of protein of the 0.35 M NaCl (II) fraction (containing cytochrome $c_{552.5}$), 0.27 mg of protein of the 0.10 M NaCl fraction (containing the SoxA protein), and 2 μmol of disodium thiosulfate to start the reaction.

Activity of the SoxA protein was assayed from 0.2 ml of the DEAE-Sepharose CL-6B eluate in 1.0 ml with 50 μmol of Tris buffer, pH 8.0, 0.07 μmol of horse heart cytochrome c_{550} , 0.2 ml of fraction 100 (containing SDH), 0.18 mg of the 0.35 M NaCl (II) fraction (containing cytochrome $c_{552.5}$), and 2.0 μmol of disodium thiosulfate to start the reaction.

Activity of SDH was assayed from 0.2 ml of the DEAE-Sepharose CL-6B eluate in 1.0 ml with 50 μmol Tris buffer, pH 8.0, 0.07 μmol of horse heart cytochrome c_{550} , 0.42 mg of protein of the 0.35 M NaCl (II) fraction of the DEAE-Sepharose CL-6B eluate (containing cytochrome $c_{552.5}$), and 2 μmol of disodium sulfide to start the reaction.

One unit (U) of enzyme activity was defined as the reduction of one μmol of horse heart cytochrome c_{550} per min at 30°C. Protein was determined according to Bradford [8].

Protein analysis was done by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described [9].

2.4. Quantification of sulfate ions

Sulfate was quantified from an SDH assay mixture of 3.0 ml. The reaction was started by the addition of 18 nmol of disodium sulfide, and the reduction of cytochrome c_{550} was followed. After 28 min the reaction was completed and 2.0 ml were diluted with 2.0 ml of distilled water, microfiltrated and subjected to a DX 300 ion chromatograph equipped with IonPac AG9 and IonPac AS9 columns (Dionex, Idstein, Germany). The control assay was treated identically except that distilled water was added instead of sulfide.

2.5. Chemicals

All chemicals used were of analytical (p.a.) grade and obtained from

Merck, Darmstadt. Horse heart cytochrome c_{550} (type III), iodoacetamide and *N*-ethylmaleimide were from Fluka, Buchs, Switzerland. Molecular size marker proteins and DNase I were from Boehringer, Mannheim. DEAE-Sepharose CL-6B was from Pharmacia, Freiburg, Germany.

3. Results

3.1. Oxidation of sulfide by whole cells of *P. denitrificans* GB17

Thiosulfate induced wild-type cells oxidized sulfide at a rate of 0.78 μmol of oxygen/min · mg of protein and thiosulfate at a rate of 0.58 μmol of oxygen/min · mg of protein. Disodium sulfide induced cells oxidized sulfide or thiosulfate at a rate of 0.04 μmol of oxygen/min · mg of protein. Strain TP19 was unable to oxidize thiosulfate or sulfide to sulfate when incubated with thiosulfate [3]. The stoichiometry of sulfide oxidation was determined previously. Two moles of oxygen were required for the oxidation of either one mol of sulfide or thiosulfate (data not shown) which is in accordance to previous findings [10].

3.2. Oxidation of sulfide in soluble extracts

Soluble extracts exhibited a specific sulfide-dependent cytochrome c_{550} reducing activity of 0.129 U/mg of protein. With respect to sulfide oxidation this activity was about 5% of that obtained with whole cells. The thiosulfate-oxidizing activity was even lower (0.033 U/mg of protein; Table 2) and was similar to that of *T. versutus* extracts [11]. Both reactions were linear with time for well over 5 min. Soluble extracts of strain TP19 did not exhibit sulfide or thiosulfate-dependent cytochrome c_{550} reducing activity (data not shown).

3.3. Purification of the components of the thiosulfate-oxidizing enzyme system

The components of the sulfide- and of the thiosulfate-dependent cytochrome c_{550} reducing activities of

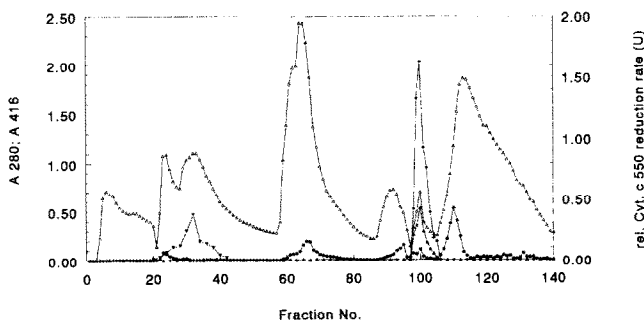


Fig. 1. Chromatography of the components of the sulfide- and thiosulfate-oxidizing enzyme system of *P. denitrificans* GB17 on DEAE-Sepharose CL-6B. The activity of the components of the enzyme systems was assayed as described in section 2.3. SoxA protein (▼); SoxB protein (▲); SDH (◆); cytochrome absorbance at 416 nm (●); absorbance at 280 nm (—).

Table 1

Assay composition of the sulfide- and thiosulfate-dependent cytochrome c_{550} reduction of *P. denitrificans* GB17

Substrate	Component ^a			Spec. cyt. c_{550} reduction rate (U/mg of protein) ^b
	SoxA	SoxB	SoxC	
$\text{Na}_2\text{S}_2\text{O}_3$	+	+	+	2.60
	–	+	+	0.0
	+	+	–	0.0
Na_2S	+	+	+	8.55
	–	+	+	8.46
	+	+	–	0.0
	–	+	–	0.0

^a The components were present in the assay in the following amounts: SoxA, 0.27 mg of protein of fraction 0.1 M NaCl; SoxB, 0.26 mg of protein of fraction 0.35 M NaCl (I); SoxC, 0.42 mg of protein of fraction 0.35 M NaCl (II).

^b Specific activity was related to protein of fraction 0.35 M NaCl (I).

P. denitrificans GB17 were purified from crude extracts by ammonium sulfate precipitation and separated by ion exchange chromatography on DEAE-Sephacrose CL-6B as described [2]. Also, the procedure to assay the components of the thiosulfate-oxidizing enzyme system of *P. denitrificans* GB17 was as described for that of *T. versutus* [2].

Thiosulfate-dependent cytochrome c_{550} reduction was obtained when three different fractions were reconstituted as eluted from the DEAE-Sephacrose CL-6B column with 0.1 M NaCl, 0.35 M NaCl fraction I and II. According to the previous designation of sulfur oxidation of *P. denitrificans* the components were designated SoxA, SoxB, and SoxC as they were eluted from the column (Table 2).

The SoxA component of the thiosulfate-dependent activity was eluted with 0.1 M NaCl in fraction 24 to 40 (Fig. 1). The SoxB component was eluted with 0.35 M NaCl and the combined fractions 97 to 105 were designated 0.35 M NaCl (I). In these fractions a soluble cytochrome was also present as determined from the absorbance at 416 nm (Fig. 1). The integrated SoxB activities resulted in a 79-fold purification and a yield of 81% (Table 2). The SoxC component was eluted in fraction 105 to 115 as determined from the absorbance at 416 nm and was designated 0.35 M NaCl (II) (Fig. 1). This frac-

tion contained cytochrome $c_{552.5}$ as determined from the fourth derivative of a dithionate-reduced minus ascorbate-oxidized difference spectrum (data not shown).

3.4 Purification of sulfide dehydrogenase

Sulfide-dependent cytochrome c_{550} reducing activity was obtained when two different fractions were reconstituted from the DEAE-Sephacrose CL-6B eluate. This activity required the 0.35 M NaCl (II) fraction containing the SoxC component, cytochrome $c_{552.5}$. The 0.1 M NaCl fraction containing the SoxA component was not required for activity (Table 2). Since the sulfide-dependent activity was strictly linked to the c -type cytochromes the enzyme was designated sulfide dehydrogenase (SDH). SDH was eluted with 0.35 M NaCl and the elution profile was identical to that of the SoxB protein as assayed in the reconstituted system for thiosulfate oxidation (Fig. 1). The integrated SDH activities resulted in a 67-fold purification and a yield of 69%. A summary of the purification procedure of SDH and of the SoxB protein is given in Table 2.

The requirement of both activities for cytochrome $c_{552.5}$, their coelution from the DEAE-Sephacrose CL-6B column, and the similar ratios in purification and yield was evidence that SDH and the SoxB protein of the thiosulfate-oxidizing activity were identical proteins.

The proteins of the 0.35 M NaCl (I) fraction of the DEAE-Sephacrose CL-6B eluate were analyzed by SDS-PAGE. Only one major protein band was observed with a M_r of 34 k (Fig. 2). This result was taken as further evidence that the SoxB protein of the thiosulfate-oxidizing system and SDH were identical.

3.5. Characterization of sulfide dehydrogenase

Fraction 100 of the DEAE-Sephacrose CL-6B eluate was used to characterize SDH. Sulfide was oxidized by SDH to sulfate as determined by ion exchange chromatography. An SDH assay described in section 2.4 converted 6 nmol of sulfide per ml to 5.42 nmol of sulfate per ml. Concomitantly, absorbance of cytochrome c_{550} reduction at 550 nm increased by 1.22, equivalent to 43.8 nmol of cytochrome c_{550} . Since 8 electrons are required for sulfide oxidation to sulfate this cytochrome reduction was equivalent to 5.48 nmol of sulfate formed per ml. Intermediates of sulfide oxidation could not be detected (data not shown).

Table 2

Summary of the purification procedure of sulfide dehydrogenase and the SoxB protein of *P. denitrificans* GB17

Step	Sulfide dehydrogenase				SoxB protein		
	Total protein (mg)	Total act. (U)	Spec. act. (U/mg)	Yield (%)	Total act. (U)	Spec. act. (U/mg)	(%)
Soluble extract	1260	162.5	0.129	100	41.6	0.033	100
0–65% $\text{NH}_4(\text{SO}_4)_2$	860	145.0	0.169	89	38.7	0.045	93
DEAE-Sephacrose CL-6B	13	112.3	8.640	69	33.8	2.600	81

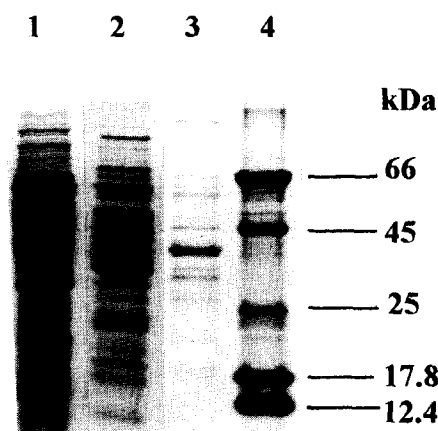


Fig. 2. SDS-PAGE of extracts of *P. denitrificans* GB17. Proteins (12 µg) from the different purification steps as summarized in Table 2 were analyzed according to [9]. Lane 1, soluble extract; lane 2, 0–65% ammonium sulfate fraction; lane 3, 0.35 M NaCl (I) fraction; lane 4, molecular size markers were bovine serum albumin (66k), ovalbumin (45k), chymotrypsinogen A (25k), myoglobin (17.8k), cytochrome *c* (12.4k).

The affinity constant of SDH for sulfide as determined from the linear double reciprocal plot was $0.94 \mu\text{M}$. Sulfide-dependent reduction was not observed with artificial electron acceptors like ferricyanide or methylene blue instead of cytochrome c_{550} (data not shown).

Iodacetamide or *N*-ethylmaleimide did not affect SDH activity while the thiosulfate-oxidizing activity was modestly inhibited by iodacetamide. Sulfite ions activated SDH slightly by 11% but inhibited the thiosulfate-oxidizing activity by 31%. Other reductants like mercaptoethanol had no effect on both enzyme activities. Although the dissociation constant of mercuric sulfide is very low, $10 \mu\text{M}$ mercuric (II) chloride inhibited SDH by 65% and inhibited the thiosulfate-oxidizing system completely (Table 3).

4. Discussion

A new enzyme activity of *P. denitrificans* designated sulfide dehydrogenase (SDH) was identified and this SDH was identical to the SoxB protein of the thiosulfate-oxidizing system. This finding was based on three bits of evidence. First, wild-type cells induced sulfide- and thiosulfate-oxidizing activity upon exposure to thiosulfate. The mutant strain TP19, harboring a *Tn5-mob* insertion in the *soxB* gene [7] was unable to form these activities. Second, SDH activity was demonstrated from soluble extracts and this activity was copurified with the SoxB protein of the thiosulfate-oxidizing enzyme system. Third, SDH required for activity the 0.35 M NaCl (II) fraction, containing cytochrome $c_{552.5}$. This fraction was also required for the thiosulfate-oxidizing activity in addition to the SoxA and the SoxB protein.

The *soxB* gene product of *P. denitrificans* and enzyme B of *Thiobacillus versutus* appeared to be identical [7]. Since the catalytic function of the SoxB protein was that of SDH, SDH is the product of the *soxB* gene. The M_r of SDH (34k) was similar to that reported for enzyme B of *T. versutus* (32 k) [2]. SDH did not oxidize thiosulfate. Since enzyme B of *T. versutus* was reported not to react with thiosulfate [3] we suggest a biochemical identity of the sulfur-oxidizing systems of *T. versutus* and *P. denitrificans* GB17.

The activity of the thiosulfate-oxidizing enzyme system of *T. versutus* was inhibited by sulfite at low concentrations of thiosulfate but activated at high thiosulfate concentrations. Enzyme A of *T. versutus* binds thiosulfate and this binding is inhibited by sulfite. However, the activation remained obscure [5]. Sulfite activated SDH of *P. denitrificans*. Thus, determination of SDH allowed to discriminate between the SoxA and the SDH activities and may explain the observation with the system of *T. versutus*.

Intermediates of sulfide oxidation were not detected from *P. denitrificans*. According to the deduced amino acid sequence of the *soxB* gene SDH contains one cysteine [7]. This cysteine may act as active site for sulfur oxidation since mercuric (II) ions inhibit SDH. As the first step of sulfide oxidation we propose covalent binding of sulfur to the cysteine of SDH (Eqn. 2). Sulfur may be further oxidized as shown in Eqns. 3 to 5. Final hydrolysis of the thionate moiety would regenerate SDH and form sulfate (equation 6). This model postulates two functions for SDH: oxidation of the enzyme-bound sulfur and hydrolysis of the finally formed thionate moiety.

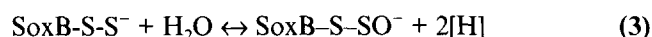
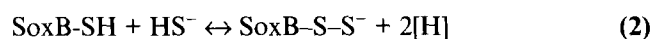


Table 3

Effect of additives on the purified reconstituted sulfide- and thiosulfate-dependent cytochrome c_{550} reducing activity of *P. denitrificans*

Additive	Concentration (mM)	SDH ^a (rel. act.)	Thiosulfate oxidation ^b (rel. act.)
None	–	100	100
HgCl ₂	0.01	35	0
Na ₂ SO ₃	0.10	111	69
β-Mercaptoethanol	2.0	97	95
Iodacetamide ^c	2.0	101	91
<i>N</i> -Ethylmaleimide ^c	2.0	100	97

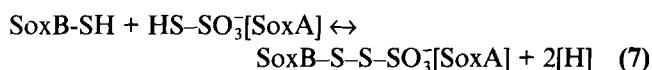
^aReference activity of SDH was $1.136 \mu\text{mol cyt } c_{550} \text{ reduced/min.}$

^bThe thiosulfate-dependent $\text{cyt } c_{550}$ reduction rate was $0.264 \mu\text{mol/min.}$

^cThe assay was preincubated for 2 min prior to the start with sulfide or thiosulfate.



The thiosulfate-oxidizing enzyme system of *P. denitrificans* requires the SoxA protein in addition to SDH. Since SDH appeared to be identical with enzyme B of *T. versutus* we suggest an identical function of the SoxA protein of *P. denitrificans* and of enzyme A of *T. versutus*. Therefore, we propose the formation of a covalent bond of the sulfane sulfur of thiosulfate to the cysteine of SDH enabled by the thiosulfate binding SoxA protein (Eqn. 7).



Since SDH releases sulfate as product the thionate moiety may be hydrolyzed by SDH according to Eqn. 8 or the SoxA protein may as well hydrolyze the thionate moiety of thiosulfate once the sulfane-sulfur was covalently bound to SDH. The thionate hydrolysis leads to the enzyme-bound sulfur as shown for the initial reaction of sulfide oxidation (Eqn. 2). This model is in accordance with the dual function of SDH in sulfide and in thiosulfate oxidation.

P. denitrificans is closely related with the phototrophic purple bacteria able to utilize reduced inorganic sulfur compounds for anaerobic phototrophic growth [6,12]. This raises the question if the sulfur-oxidizing enzyme system of the neutrophilic thiobacteria is similar to or originated from these phototrophic bacteria. Enzyme B of *T. versutus* contains a binuclear manganese cluster [13]. The identification of the SoxB protein as sulfide

dehydrogenase may help to elucidate the role of manganese in sulfide oxidation and may help to understand the role of manganese in oxygenic phototrophic water oxidation.

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