ACTIVATION OF ALA SYNTHETASE BY REDUCED THIOREDOXIN IN *RHODOPSEUDOMONAS SPHEROIDES* Y

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1. Introduction

Rhodopseudomonas (Rps.) spheroides, a non-sulphur purple bacterium, which belongs to the group Rhodospirillaceae, can grow anaerobically in light and aerobically in both light and dark. Light intensity and molecular oxygen are the most prominent environmental factors that influence the synthesis of bacteriochlorophyll-membrane structures in growing photosynthetic bacteria [1].

There is strong evidence that the activity of 5-amino levulinic acid (ALA) synthetase, the first enzyme in tetrapyrrole biosynthesis, is directly affected [2]. ALA synthetase (EC 2.3.1.37) may occur in multiple forms [3–5] with high activity (a forms) or with low activity (b forms). Another interesting feature of tetrapyrrole synthesis in *Rps. spheroides* is the important part played by sulphur-containing compounds of low molecular weight in this activation [5] which lead to the postulation [2] that the ALA synthetase activity is controlled by the cellular concentration of trisulphides, whose formation would be regulated by an enzyme-dependent sulphydryl–disulphide couple linked to the electron transport chain.

Our results [6,7] showed that the *Rps. spheroides* Y pure enzymes with high activity are active when reduced, extremely sensitive to oxidation, contain, respectively, 2 or 8 titrable SH groups/mol, and that one form is absent from the depigmented bacteria cultivated in dark aerobically. A new regulatory system of chloroplasts has been described [8] whereby enzymes are activated in the light by reduction and are deactivated in the dark by oxidation. This mechanism depends on two newly identified soluble chloroplast proteins: one of these new proteins, ferredoxin-thioredoxin reductase, catalyses the reduction of the second protein, thioredoxin, in a reaction that utilizes photoreduced ferredoxin. The thioredoxin reduced in this manner is used for the activation of soluble regulatory enzymes of chloroplasts:

$$\text{Enzyme}_{\text{inactive}} \xrightarrow[\text{thioredoxin}]{\text{reduced}} \text{Enzyme}_{\text{active}}$$

The thioredoxin needed for enzyme activation can be reduced experimentally in the dark with the non-physiological sulfhydryl reagent dithiothreitol [8–11]. With dithiothreitol (DTT), neither ferredoxin nor ferredoxin-thioredoxin reductase is needed for enzyme activation by thioredoxin [8,11,12].

This study was undertaken to investigate whether:

(i) Thioredoxin exists in *Rps. spheroides* Y (as in *Rhodospirillum* (*Rhs.*) rubrum [10]);
(ii) ALA synthetase could be activated either by DTT or by DTT-reduced thioredoxin.

2. Materials and methods

Rps. spheroides Y was grown photosynthetically in 'L + 25 μM' medium [13] modified as follows: 0.02 M potassium phosphate buffer (pH 7); 3.5 g/l ammonium succinate; 1 g/l sodium acetate, 3 H_2O; 1 g/l sodium glutamate, 1 H_2O; 20 ml/l Hutner's base (pH 7) [14]. Bacteria were grown at 30°C with continuous bubbling of CO_2–N_2 (5 : 95 by vol.) and mag-

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netic stirring in 10 l bottles thermostated in water baths. Illumination of 5000 lux was provided by incandescent bulbs. The cells were harvested during late exponential phase, resuspended and washed once with 0.05 M phosphate buffer (pH 7.4) and stored at −20°C until used.

Purification of ALA synthetase was carried out as in [7,15] with the following modifications: the supernatant of the first centrifugation was centrifuged 3 h at 100 000 × g; the first precipitation by 25% $(NH_4)_2SO_4$ was omitted; all buffers contained 5% glycerol and 0.1 mM pyridoxal 5′-phosphate (PLP) except in the last Sephadex G-200 chromatography (10% glycerol + 0.1 mM PLP) and during the DEAE–Sephadex A-25 chromatography where pyridoxal phosphate was replaced by 0.01 M 2-mercaptoethanol. Homogeneity was controlled by thin-layer gel electrofocusing in polyacrylamide gel (LKB multiphor PAG plate technic).

ALA synthetase activity was assayed by determining the amount of ALA formed at 37°C after 15 min in 1 ml final vol. containing: 400 μM Tris (pH 7.8); 0.2 μM PLP; 600 nmol succinyl coenzyme A [16]; 100 μM glycine. The reaction was terminated by addition of 0.2 ml 50% trichloroacetic acid. To 1 ml of this reaction mixture (I) were added 0.25 ml 2 M sodium acetate and 0.05 ml acetylacetone, kept for 10 min at 100°C. After cooling, 0.6 ml Ehrlich–Hg reagent [17] was added and A_{553} measured after 15 min ($\epsilon_{mM} = 53$). High concentrations of DTT interfere with the reaction of Ehrlich's reagent. DTT at $\leqslant 1$ mM in the trichloroacetic acid mixture (I) had no effect on the color. However most of the experiments were made using blanks in which DTT was added after trichloroacetic acid addition.

Cell free extracts containing thioredoxin were prepared according to [10]: 20 g frozen *Rps. spheroide* cells were thawed at 4°C in 40 ml 25 mM Tris buffer (pH 8), 1.4 mM 2-mercaptoethanol in the presence of DNase; sonicated 6 min at 0°C, centrifuged 10 min at 75 000 × g and adjusted to pH 4.5 with 2 N formic acid. The precipitate was centrifuged off (5 min 48 200 × g), the supernatant fraction adjusted to pH 7 with 1 N ammonium hydroxide and dialysed overnight at 4°C against the preparative solution. This treatment removed fructose 1,6-bisphosphatase (FDPase) (EC 3.1.3.11).

FDPase was prepared according to [18] and used after Sephadex G-100 chromatography (spec. act. 3.1 units/mg), or purified to homogeneity according to [19] (spec. act. 100 units/mg).

Protein was estimated by the Lowry method with BSA as standard [20].

3. Results and discussion

The occurrence of thioredoxin in *Rps. spheroides* Y was demonstrated by two different methods (table 1). First, thioredoxin was assayed by its capacity to activate spinach FDPase [10]; secondly, it was estimated in a coupled reaction involving thioredoxin and NADPH thioredoxin reductase by measuring the reduction of

Table 1
Occurrence of thioredoxin in *Rps. spheroides*
a. Activation of spinach FDPase

	Thioredoxin (units/mg protein)
Rhs. rubrum [10]	1.1
Rps. spheroides	0.43 ± 0.04

One unit is defined [10] as the thioredoxin-mediated release of 1 μmol inorganic phosphate by 8 μg FDPase in 0.5 ml final vol. containing the indicated dialysed (pH 4.5) supernatant fraction and the following in μmol: Tris–HCl buffer (pH 7.9) 50; $MgCl_2$ 0.5; DTT 2.5; fructose 1,6-bisphosphate (FDP) 3. The reaction was started by adding FDPase continued for 30 min at 25°C and stopped by adding 0.5 ml of 10% trichloroacetic acid. P_i was estimated as in [18]. Blanks were without FDP or FDPase. The value obtained for *Rps. spheroides* is the mean of 3 different experiments, one of which was made with pure FDPase (detailed in section 2)

b. Reduction of DTNB – details in text

	Thioredoxin (units/mg protein)
Mean of 3 expt	0.0045 ± 0.0004

One arbitrary unit which is by no means to be compared to the unit defined above corresponds to the amount of enzyme required to produce ΔA_{412} in the conditions of the assay. Assay, 500 μl, contained 40 μmol Tris (pH 8.0), 10 μM EDTA, 0.2 mg BSA, 0.05 μM NADPH, 0.08 μM DTNB (0.05% methanol), 0.48 mg thioredoxin extract (containing NADPH-thioredoxin reductase). In the blank cuvette NADPH was replaced by water. The ΔA_{412} (37°C) was recorded on a thermostated Beckman Acta M VII spectrophotometer, for 15 min.

DTNB (5,5′-dithiobis-2-nitrobenzoic acid) in the presence of NADPH [21] (scheme 1):

$$\text{Thioredoxin } (S)_2 + \text{NADPH} + H^+ \xrightarrow[\text{reductase}]{\text{NADPH-thioredoxin}} \text{Thioredoxin } (SH)_2 + NADP^+ \quad (1)$$

This reaction demonstrates the two components: thioredoxin and NADPH thioredoxin reductase, in the extracts. Specificity with respect to thioredoxin has been demonstrated for *Escherichia coli* [22]. On the other hand the equivalence of chloroplast assimilation regulatory protein b (ARPb) required for the activation of spinach FDPase by DTT and *E. coli* thioredoxin was established [8]. The observation that *E. coli* thioredoxin is active in assays specific for ARPb allows one to take the capacity to activate homogenous preparation of FDPase as a proof of the existence of thioredoxin in the organism tested [10,23].

Having demonstrated the existence of thioredoxin in *Rps. spheroides* we studied the activation of ALA synthetase by DTT and DTT-reduced thioredoxin.

Table 2 shows the activation of purified ALA synthetase by DTT. The reaction of DTT with protein disulfides may be compared with that of the dithiol-protein thioredoxin [24] and its effect tested as a model for the action of thioredoxin. ALA synthetase is well known to be rather unstable. The stability was so much improved by the use of glycerol and PLP in the buffers that the activation became small or null. We then first inactivated the enzyme by Sephadex G-15 or G-25 chromatography to remove cofactors and stabilizing agents.

The effect of DTT on the protein can be followed by the measure of its oxidation. This is shown in fig.1.

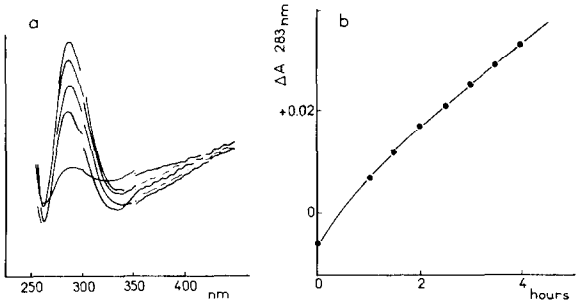

Fig.1. (a) Repetitively scanned difference spectra recorded every 30 min on Beckman Acta M VII spectrophotometer (24°C) showing the oxidation of DTT due to ALA synthetase concomitant reduction. 4 cuvettes were used, 2 mm path length, 0.5 ml vol. Cuvette 1 containing 0.2 ml enzyme, 0.2 ml 2 M Tris (pH 7.8) joined side by side to cuvette 2 containing 0.1 ml 12 mM DTT, constituted the reference. Sample was cuvette 3: 0.2 ml enzyme, 0.2 ml 2 M Tris (pH 7.8), 0.1 ml 12.5 mM DTT joined to cuvette 4: 0.2 ml 2 M Tris (pH 7.8). (b) Corresponding ΔA_{283} are plotted as function of time.

Table 2
Activation of ALA synthetase by DTT

Activity before treatment			Conditions of preincubation	Activation	
FI	FII			FI	FII
2007	516	day 2	5 min 37°C	+ 30%	+ 12%
1491	380	day 4	30 min 37°C	+ 49%	+ 52%
			2 h 24°C	2.8-fold	1.6-fold

ALA synthetase was chromatographed on Pharmacia columns PD 10 (Sephadex G-25). Elution was performed with 0.03 M phosphate buffer (pH 7.4) which will separate the protein from any stabilizing factor such as 2-mercaptoethanol, PLP or glycerol, and the eluate kept at 4°C. FI and FII are the two forms with high activity obtained after DEAE–Sephadex A-25 chromatography [4,15]. Activation was performed by preincubation with 1.25 mM DTT for the time and temperature indicated. Substrates were then added and the reaction allowed to take place for 15 min at 37°C. Samples without DTT were preincubated in the same conditions. 1 unit = 1 nmol ALA formed/h. The initial specific activity was on day 1; 4702 units/mg protein for FI; 1081 units/mg protein for FII

The ultraviolet spectrum of the oxidized DTT has a maximum at 283 nm, which will obviously mask any spectral change in the protein at that wavelength. The oxidation of DTT against time at 24°C is shown in fig.1. As found for regulatory enzymes of CO_2 assimilation in higher plants the rate of activation was quite slow relative to the rate of catalysis. Time and temperature of preincubation are of importance and a systematic study is in progress.

From table 3 it can be seen that extracts containing reduced thioredoxin activated ALA synthetase and markedly increased the activation due to DTT alone, even though the conditions of preincubation were not optimal. By analogy with the other enzymes [8,11] it would appear that the DTT is needed to reduce thioredoxin which in turn activates ALA synthetase.

These results show thioredoxin and NADPH thioredoxin reductase to be present in the facultative phototroph *Rps. spheroides* Y. It provides evidence that reduced thioredoxin stimulates the activity of a key regulatory enzyme in bacteriochlorophyll synthesis: ALA synthetase. Like other thioredoxin-linked activation reactions the ALA synthetase reaction consists of two phases which differ in rate:

1. An activation phase in which reduced thioredoxin changes the enzyme from an inactive to an active form;
2. The catalytic phase.

As is the case for other enzymes, activation is slow relative to the rate of catalysis.

Table 3
Activation of ALA synthetase by DTT-reduced thioredoxin

Activity before treatment	Activation by	
FI	DTT 1.25 mM	+ 85%
765 units	DTT 1.25 mM + 50 μl thioredoxin	+ 112%
FII	DTT 2.5 mM	+ 46%
327 units	DTT 2.5 mM + 50 μl thioredoxin	+ 49%
	DTT 2.5 mM + 0.1 ml thioredoxin	+ 73%

The enzyme was preincubated 5 min at 37°C with or without the indicated compounds prior to measuring catalysis activity (detailed in section 2). 1 unit = 1 nmol ALA formed/h

The possibility that thioredoxin could be reduced photochemically remains to be determined.

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