A Thioredoxin Activated Glutamine Synthetase in *Chlorella*

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Z. Naturforsch. **36 c**, 396–399 (1981); received February 27, 1981

Glutamine, Synthetase, Thioredoxin, *Chlorella*

Glutamine synthetase was partly purified from Chlorella pyrenoidosa. This enzyme activity was stimulated by dithioerythritol and 2′-3′-dimercaptopropanol (BAL). Full activation was obtained by thioredoxins isolated from the cyanobacterium *Synechococcus* 6301, from spinach and by heated *Chlorella* extract. The apparent K_m for the purified thioredoxin B from *Synechococcus* 6301 was determined to be 0.022 μM (22×10^{-9} M), suggesting a specific role for thioredoxins in regulating glutamine synthetase activity in this alga.

Introduction

Glutamine synthetase has been detected in organisms ranging from bacteria to higher plants [1, 2]. This enzyme is at the branching point between assimilatory nitrate reduction and incorporation of the reduced nitrogen into protein. The activity of this enzyme controls the level of free ammonium which in turn could control the rate of assimilatory nitrate reduction [3]. A light-dependent regulation of glutamine synthetase activity has been found in higher plants [4], green algae [5], and cyanobacteria [6], suggesting that a signal is involved related to or similar to systems describing the light-dependent activation of enzymes by thioredoxin and the ferredoxin-thioredoxin reductase [7]. The studies in this paper were initiated by the observation that *Chlorella* growing on methionine sulfone as a sulfur source had a high specific acivity of glutamine synthetase (unpublished). It is known that the reduction of methionine sulfoxide is catalyzed by thioredoxin [8]. The idea was to link higher glutamine synthetase activities during growth on methionine sulfone to higher thioredoxin levels. Therefore the glutamine synthetase from Chlorella was analyzed for a possible activation by thioredoxin. The data of this paper will demonstrate that a partly purified glutamine synthetase from Chlorella is activated by thioredoxins.

Materials and Methods

a) Organism

Chlorella pyrenoidosa strain 211-8 b of the algal collection of the university of Göttingen was cultured as previously described [9].

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0341-0382/81/0500-0396 $ 01.00/0

b) Purification of glutamine synthetase

Chlorella cells were broken in a french-press at 12000 PSI in a buffer system containing 0.1 M Tris-HCl pH 8.0 and 10 mM mercaptoethanol. The crude extract was frozen over night, thawed and cleared by centrifugation. To the supernatant Polymin P was added (10 μl/ml of a tenfold diluted Polymin P solution) and the precipitated material was collected by centrifugation. The pellet was resuspended in the original buffer and ammoniumsulfate was added to 40% saturation. The solution was cleared again by centrifugation and the supernatant was discharged. The pellet was taken up in 0.02 M Tris-HCl pH 8, homogenized and cleared afterwards by centrifugation. The supernatant containing glutamine synthetase activity was placed on a small Sephadex G-50 column (1.8 × 23 cm) to remove traces of thioredoxin. Active fractions were pooled and stored frozen until use; this enzyme fraction was free of thioredoxin.

c) Purification of thioredoxins

Thioredoxin A and B from *Synechococcus* 6301 were obtained as described recently [10] and spinach thioredoxin was prepared as described earlier [11]. Heated Chlorella extract was obtained by boiling crude extract for 5 min; the supernatant after centrifugation was used.

d) Glutamine synthetase measurements

This activity was determined using the transferase assay according to Rhodes *et al.* [12]. The reaction was always started by the addition of arsenate.

e) Chemicals

Arsenate was obtained from Baker (Gross Gerau, Bundesrepublik Deutschland); Polymin P was generous gift of BASF (Ludwigshafen, Bundesrepublik Deutschland); all other chemicals were obtained from Merck (Darmstadt, Bundesrepublik Deutschland).

Results

Glutamine synthetase from *Chlorella*, partly purified and separated from low-molecular weight components, is activated by dithiols such as 2′-3′-dimercaptopropanol or dithioerythritol. The data of Table I demonstrate, that preincubation of the glutamine synthetase fraction with thioredoxin and dithioerythritol doubled the enzyme activity within about 5 min compared to a control without thiol or thioredoxin. A closer analysis of different thiols in the presence and absence of thioredoxin B from *Synechococcus* 6301 is given in Table II. It is evident that the monothiol mercaptoethanol is not activating this system; however, dithioerythritol and 2′-3′-dimercaptopropanol clearly activate this glutamine synthetase, also in the absence of thioredoxin. If, however, thioredoxin is added, the rate of the glutamine synthetase activity is stimulated further, demonstrating a specific activation of this activity by thioredoxin. This can be deduced also from the observation, that the monothiol mercaptoethanol is inactive in this system and that this thiol will not reduce thioredoxin for further activation of the glutamine synthetase activity. The thioredoxin/DTE system has been used to determine the apparent K_m-data for the purified thioredoxins A and B from the cyanobacterium *Synechococcus* 6301. These data are shown in Fig. 1 and 2. The apparent K_m for thioredoxin B is determined to 0.26 µg thioredoxin B/ml, which can be converted to an apparent K_m of 22×10^{-9} M for thioredoxin B having a molecular weight of 11800. The apparent K_m for the thioredoxin A fraction is 44 µg/ml. This *Chlorella* glutamine synthetase is activated also by spinach thioredoxin (data not shown) and by heated *Chlorella* extract (Table II).

Table I. Effect of preincubation on the glutamine synthetase activity.

Preincubation time [min]	Additions	µmol Glutamine metabolized	%
0	thioredoxin	0.81	100
0	thioredoxin + dithioerythritol	1.69	165
1	thioredoxin + dithioerythritol	1.92	188
2	thioredoxin + dithioerythritol	2.01	197
3	thioredoxin + dithioerythritol	2.12	208
5	thioredoxin + dithioerythritol	2.22	218

Conditions: Glutamine synthetase activity was determined as described in materials and methods. The assay was started by adding 60 µg of glutamine synthetase to the reaction mix without the arsenate fraction. After the time needed for preincubation the reaction was started by addition of arsenate. The assay was run for 10 min at 37 °C; the reaction was stopped by addition of the ferric chloride reagent. 0.56 µg purified thioredoxin B from *Synechococcus* was used and the thiol concentration was kept at 1 mM.

Table II. Activation of glutamine synthetase by various sulfur compounds.

Additions	glutamine metabolized
none	0.81
0.056 µg thioredoxin B	0.80
0.56 µg thioredoxin B	0.83
mercaptoethanol	0.91
mercaptoethanol + 0.056 µg thioredoxin B	0.89
mercaptoethanol + 0.56 µg thioredoxin B	0.93
dithioerythritol	1.23
dithioerythritol + 0.056 µg thioredoxin B	1.51
dithioerythritol + 0.56 µg thioredoxin B	2.25
dithioerythritol + 0.2 ml heated *Chlorella* extract	2.18
2′-3′-dimercaptopropanol	1.48
2′-3′-dimercaptopropanol + 0.056 µg thioredoxin B	1.58
2′-3′-dimercaptopropanol + 0.56 µg thioredoxin B	1.60
0.56 µg thioredoxin B − glutamine synthetase	0.01
0.2 ml heated *Chlorella* extract − glutamine synthetase	0.02

Conditions: Glutamine synthetase activity was determined as described in materials and methods. The assay was started by adding 60 µg of glutamine synthetase to the reaction mix without the arsenate fraction. After 5 min of preincubation in the presence of the thiol compounds listed the reaction was started by addition of arsenate. After 10 min at 37 °C the reaction was stopped by addition of the ferric chloride reagent. Thiol concentration: 1 mM. 0.2 ml of heated *Chlorella* extract is equivalent to 0.27 mg of chlorophyll.

Discussion

Thioredoxins are low molecular weight proteins originally found as an electron donor for the reduc-

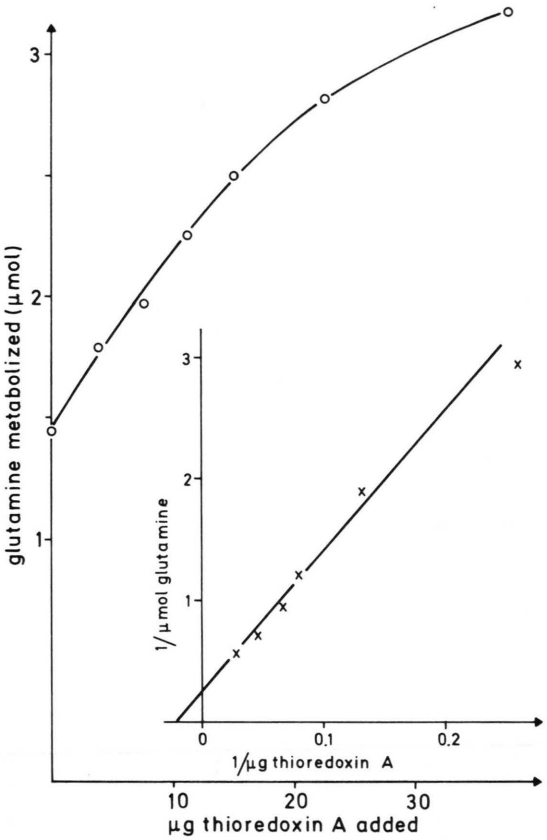

Fig. 1. Activation of glutamine synthetase by thioredoxin A from *Synechococcus* 6301. Conditions as in Table II, however thioredoxin A from *Synechococcus* was added as indicated. In the insert of this figure a Lineweaver-Burk plot for the activation is shown.

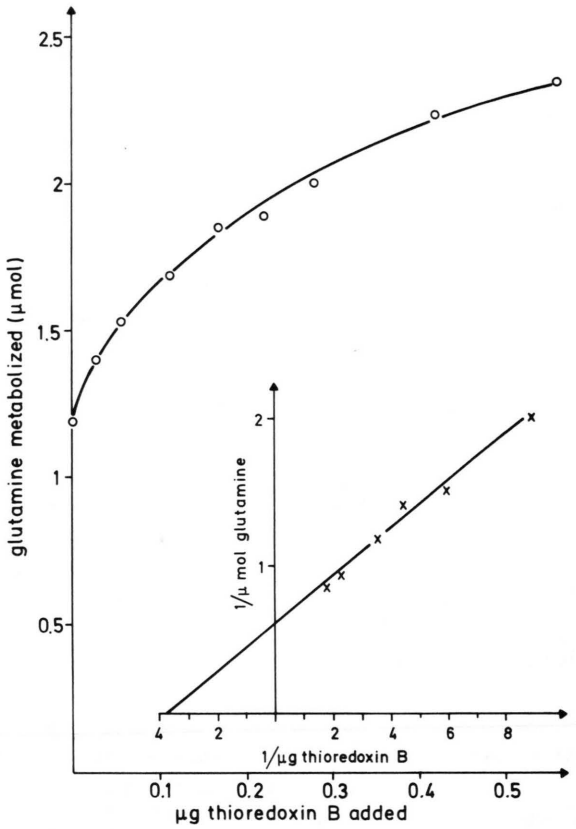

Fig. 2. Activation of glutamine synthetase by thioredoxin B from *Synechococcus* 6301. Conditions as in Table II, however thioredoxin B from *Synechococcus* 6301 was added as indicated. In the insert of this figure a Lineweaver-Burk plot for the activation is shown.

tion of ribonucleotides to deoxyribonucleotides [13]. Recent developements have shown a light-dependent reduction of thioredoxins by ferredoxin and a ferredoxin-thioredoxin reductase, and it was demonstrated that the activation of certain enzymes of the reductive pentose phosphate cycle could be achieved by light or by reduced thioredoxins [14, 15]. Evidence from bacteria and blue-green algae suggested the involvement of thioredoxin in assimilatory sulfate reduction [12, 16, 17]. These activations of different reactions suggested a regulatory role for thioredoxins in the synchronized activation of key pathways needing light (reducing power) as CO_2-fixation and assimilatory sulfate reduction and possibly nitrogen fixation [11, 18]. Another line for a possible involvement of thioredoxins in assimilatory nitrate reduction developed from observations that glutamine synthetase from higher plants [4], green algae [5], and cyanobacteria [6] is activated by light and inactivated partly in darkness. These light modulations suggested that a mechanism similar to the ferredoxin/thioredoxin system could be operative mediating this activation by reduced thioredoxins [14].

The data of this report clearly demonstrate that a thioredoxin-free glutamine synthetase from *Chlorella* can be activated partly by dithiols such as dithioerythritol (DTE), 2′-3′-dimercaptopropanol (BAL), or lipoic acid; whereas monothiols such as mercaptoethanol, GSH, or cysteine do not activate glutamine synthetase in the presence or absence of thioredoxin. Full activation, however, is obtained if reduced thioredoxins are added, and about 5 min are needed for this activation. Spinach, *Chlorella*, or *Synecho-*

coccus thioredoxins are active with this *Chlorella* glutamine synthetase using either DTE or lipoic acid (data not shown) for the reduction of these thioredoxins.

For *Synechococcus* thioredoxin B the apparent K_m obtained in this system was determined to be 22 nM (22×10^{-9} M). This apparent K_m can be compared directly with the K_m-values obtained for this thioredoxin in the PAPS-sulfotransferase from *Synechococcus* (10.7 μM), the fructose-1,6-bisphosphatase from the same organism (1.7 μM), or the APS-sulfotransferase from Chroococcidiopsis (5.4 μM) because the identical thioredoxin preparation has been used for these K_m-determinations [10] and the same holds for the K_m-values for the thioredoxin A fraction from *Synechococcus*. Thus, the sensitivity of this glutamine synthetase towards thioredoxins is about hundredfold below K_m-values reported for other thioredoxin-dependent systems, which have apparent K_m-data of one to ten mM [19]. It should be pointed out here that the glutamine synthetase was isolated from *Chlorella* cells growing in continuous light and that we did not find a stimulation of glutamine synthetase activity in freshly broken cells either by thioredoxins or by a thiol. This would be in agreement with the observation, that glutamine synthetase in Chlorella is activated by light [5]; therefore the enzyme is in the activated conformation in light grown cells. During the purification procedure the glutamine synthetase activity is slowly inactivated and this inactivated glutmine synthetase was used in the studies reported here. It has to be analyzed if the inactivation during the purification procedure is identical to the inactivation observed during darkness [5].

The regulation of glutamine synthetase in bacteria is achieved by an adenylation of this enzyme [1], however, such an adenylation has not been found so far in photosynthetic organisms. The suggestion is made here that organisms having photosystem I (and II) could have developed the thioredoxin-ferredoxin system with a regulation of glutamine synthetase by thioredoxin, whereas non-photosynthetic organisms could regulate the activity of this enzyme by the adenylation mechanism.

The regulation of the glutamine synthetase activity could control directly assimilatory nitrate reduction, since it has been shown that addition of ammonium ions (or inactive glutamine synthetase which leads to an increased ammonium pool) stops nitrate uptake and inactivates nitrate reductase [3, 20].

Acknowledgement

This study was supported by a grant from the Deutsche Forschungsgemeinschaft.

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