4. A. A. Abduvakhabov, Sh. K. Kasymov, E. V. Rozengart, I. N. Soboleva, Kh. A. Aslanov, and A. S. Sadykov, *Dokl. Akad. Nauk SSR* **220**, 961 (1975).
5. Sh. M. Gafurova, A. A. Abduvakhabov, and Kh. A. Aslanov, *Uzb. Khim. Zh.*, No. 2, 56 (1979).
6. Sh. M. Gafurova, A. M. Saitkudov, Kh. A. Aslanov, A. A. Abduvakhabov, A. I. Ishbaev, and A. M. Maksudov, *Uzb. Khim. Zh.*, No. 3, 36 (1978).
7. G. L. Ellerman, K. D. Courtney, V. Anders, and K. M. Featherstone, *Biochem. Pharmacol.* **7**, 88 (1961).

ISOLATION, PURIFICATION, AND PROPERTIES OF THIOREDOXIN FROM *Ankistrodesmus braunii*

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Two forms of thioredoxin possessing the capacity for reactivating glutamine synthetase have been isolated from the cells of a green alga. Thioredoxins I and II are heat-stable proteins with molecular masses of 12 and 24 kDa, respectively. Analysis of the amino acid compositions of (I) and (II) have shown that they each contain two cysteine residues participating in the reduction of the oxidized form of glutamine synthetase.

Thioredoxins were initially found in extracts of *E. coli* as electron donors for the reduction of ribonucleotides to deoxyribonucleotides [1]. Thioredoxins are heat-stable low-molecular-mass proteins consisting of a single polypeptide chain containing two functionally active SH groups in the reduced state. NADPH-dependent thioredoxin reductase participates in the reduction of the disulfide form of thioredoxin in vivo. In plants a soluble low-molecular-mass protein — ferredoxin-dependent thioredoxin reductase — participates in the reduction of the disulfide groups of thioredoxin. In vitro, thioredoxin can be reduced nonenzymatically — by dithiothreitol [2, 3]. The thioredoxins of bacteria, yeasts, animals, and plants differ appreciably with respect to amino acid composition and have molecular masses of from 11 to 24 kDa [4, 5].

There are no reports in the literature on the properties and structures of the thioredoxins of algae or of their influence on the activity of the isoforms of glutamine synthetase (GS). The task of our investigations was to isolate, purify, and study some properties of the thioredoxin from the cells of *A. braunii*.

Electrophoretically homogeneous preparations of thioredoxins I and II were obtained with the aid of ion-exchange chromatography (Figs. 1 and 2), gel filtration, and ammonium-sulfate fractionation from the cells of the alga *A. braunii*. The UV spectra of preparations (I) and (II) showed their protein nature with an absorption maximum at 280 nm.

We have determined the molecular masses of (I) and (II). A graph of the dependence of the electrophoretic mobility on the molecular mass of the thioredoxins is shown in Fig. 3. The results obtained indicate that thioredoxins I and II each consists of a single polypeptide chain and they have molecular masses of 14 and 24 kDa, respectively.

The amino acid compositions of thioredoxins I and II have also been determined. As can be seen from Table 1, they differ in amino acid composition but have the same number of histidine residues which undergo reversible dithiol-disulfide transformations.

We have shown previously [8] that on the gel filtration of extracts of homogeneous preparation of GS there is a loss of activity of the enzyme. As can be seen from Table 2, the addition of 1 mM dithiothreitol to the enzyme solution partially restores the activity of the two forms of glutamine synthetase. The subsequent addition of 0.5 μg/ml of thioredoxin to the same enzyme solution leads to a rapid restoration of the activity of the enzyme to the initial level.

In contrast to dithiothreitol, monothiols — cysteine, mercaptoethanol, and reduced glutathione, which are stabilizers of the activity of GS [8] — do not possess the capacity for reactivating GS either in the presence or in the absence of thioredoxin.

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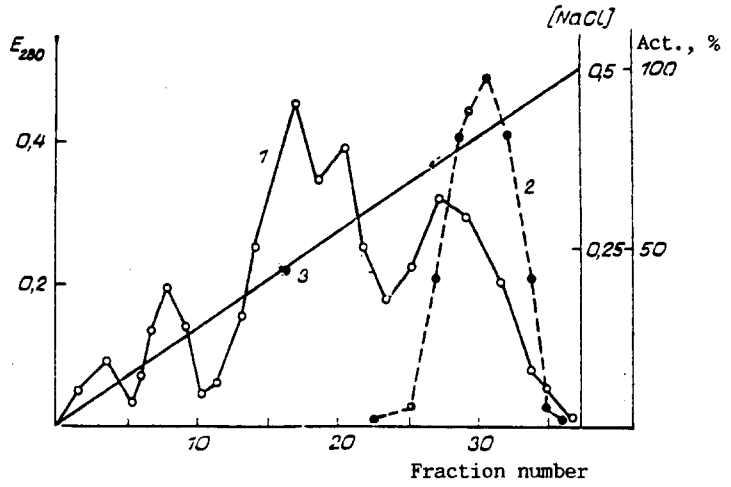

Fig. 1. Chromatography of thioredoxin on DEAE-Sephacel in a concentration gradient of NaCl: 1) protein (A-280); 2) activity of thioredoxins I and II; 3) concentration gradient of NaCl.

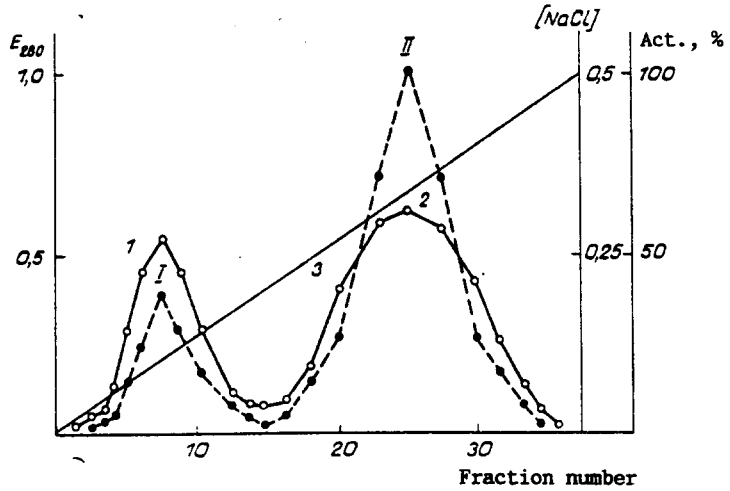

Fig. 2. Separation of thioredoxins I and II on CM-cellulose with rising concentrations of NaCl in acetate buffer: 1) Protein (A-280); 2) thioredoxin activity; 3) concentration gradient of acetate buffer. Act., % is the degree of activation of glutamine synthetase by the fractions of thioreduction I and II.

TABLE 1. Amino Acid Compositions of Thioredoxins I and II from *A. braunii*

Amino acid	Number of residues in the molecule		Amino acid	Number of residues in the molecule	
	I	II		I	II
Lysine	12	20	Glycine	10	18
Histidine	1	2	Alanine	11	20
Arginine	1	3	Cysteine	2	2
Aspartic acid	16	25	Valine	1	3
Threonine	6	8	Methionine	1	2
Serine	4	6	Isoleucine	8	15
Glutamic acid	8	10	Leucine	14	30
Proline	5	11	Tyrosine	2	5
Phenylalanine	5	9	Tryptophan	1	3

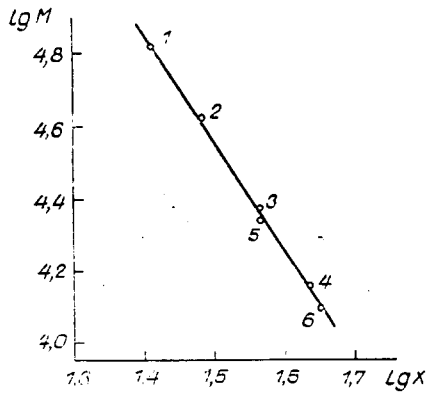

Fig. 3. Determination of the molecular masses of thioredoxins I and II by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate: 1) bovine albumin (65,000); 2) ovalbumin (44,000); 3) thioredoxin II (24,000); 4) thioredoxin I (14,000); 5) trypsin (24,000); 6) cytochrome (13,000).

TABLE 2. Influence of Thiol Reagents and Thioredoxins I and II on the Inactivated Form of the *A. braunii* GS

Thiol reagent	Concentration	Activation, %	
		GS_1	GS_2
Thioredoxin I + DTT	0,5 μg + 1 mM	80	90
Oxidized thioredoxin I	0,5 μg	0,0	0,0
Oxidized thioredoxin II	0,5 μg	0,0	0,0
Thioredoxin II + DTT	0,5 μg + 1 mM	100	100
Dithiothreitol	1 mM	50	63
Cysteine	1 mM	0,0	0,0
2-Mercaptoethanol	1 mM	0,0	0,0

Thus, our investigations have shown that the *A. braunii* glutamine synthetase possesses the redox type of regulation, and in the absence of a reducing agent the enzyme passes into an inactive state.

Reduced thioredoxin (TR) can be used as specific endogenous reducing agent for GS in algal cells (reaction 1), and dithiothreitol as a nonspecific reducing agent for GS (reaction 2). The dithiol groups of these compounds are donors for the reduction of the disulfide bonds in the protein molecule of the oxidized form of GS:

$$\text{GS}-(\text{S})_2+\text{TR}-(\text{SH})_2 \rightarrow \text{GS}-(\text{SH})_2+\text{TR}-(\text{S})_2, \quad (1)$$

$$\text{GS}-(\text{S})_2+\text{DTT}-(\text{SH})_2 \rightarrow \text{GS}-(\text{SH})_2+\text{DTT}-(\text{S})_2. \quad (2)$$

EXPERIMENTAL

Determination of the Functional Activity of Thioredoxin. As a test for detecting thioredoxin during its isolation and purification we used the oxidized form of glutamine synthetase. The activity of the glutamine synthetase was determined in a transferase reaction as described previously [9]. As the unit of glutamine synthetase activity we took the amount of enzyme catalyzing the synthesis of 1 μmole of glutamylhydroxamate. The number of activity units was calculated per 1 ml of enzyme solution.

Preparation of the Oxidized form (inactivated form) of GS. An extract of algal cells (after centrifugation at 18,000 × g) was passed through Sephadex G-50 that had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.025 M $MgSO_4$ and 0.05 M glutamate. The fractions eluted from the column in the void volume were collected and used in the subsequent experiments (these fractions contained the oxidized or inactivated form of GS). The addition to these fractions of 0.5 ml of the eluate obtained in various stages of the purification of thioredoxin led to the reactivation of the oxidized form of GS if the eluates contained thioredoxin. The relative concentration of thioredoxin in each fraction was judged from the degree of reactivation of the GS. Fractions that did not restore the activity of the enzyme were used as controls.

Purification of Thioredoxin. The whole procedure was carried out at room temperature. To obtain extracts containing thioredoxin, the algal cells were isolated from the medium with the aid of a separator and were washed with 0.05 M Tris-HCl buffer, pH 8.0. The cell paste so obtained (200 g) was suspended in 200 ml of the same buffer. Quartz microbeads No. 14 were added to the suspension in a ratio of paste, buffer, and beads of 1:1:1.25. The cells were disrupted in a planetary mill of the L-17 type for 30 min. The fragments of disrupted cells and beads were separated by centrifugation at 80,000 × g, and the supernatant was heated at 100°C for 5 min and was then cooled. The residue of disrupted cells was resuspended in 200 m of 0.05 Tris-HCl buffer and the suspension was heated at 100°C for 5 min.

After cooling and centrifugation, the supernatants were combined and were acidified with 6 N HCl to pH 2. After 30 min, the precipitate was separated off by centrifugation at 18,000 × g, ammonium sulfate to a concentration of 40% was added to the supernatant liquid, and the resulting precipitate was separated off by centrifugation. Then ammonium sulfate to 80% saturation was added to the supernatant liquid. After 30 min, the precipitate was collected by centrifugation and was dissolved in the initial buffer. This solution of thioredoxin was dialyzed against 600 volumes of 1 mM Tris-HCl buffer, pH 8, for 12 h. The dialyzed solution of thioredoxin was deposited on a column of DEAE-Servacel (1.5 × 5 cm).

Linear gradient elution was performed with Tris-HCl buffer, pH 8, with increasing ionic strengths of NaCl (up to 0.5 M) (Fig. 1). The active fractions were combined and dialyzed against 400 volumes of 0.01 M acetate buffer for 12 h and were then deposited on a column of CM-cellulose equilibrated with 0.02 M acetate buffer. Linear gradient elution of the thioredoxin was carried out with an acetate buffer having increasing ionic strengths (up to 0.3 M). As can be seen from Fig. 2, at this stage of purification two peaks of thioredoxin activity were detected, which we have designated as thioredoxins I and II. Electrophoretograms of these fractions revealed small amounts of protein impurities.

For the further purification of the thioredoxins I and II, chromatography was performed on a column (2.0 × 10 cm) of TSK-gel HW-50 equilibrated with 0.05 M Tris-HCl buffer. The amino acid analysis of the thioredoxins was carried out on a 4101 LKB amino acid analyzer (Sweden).

Samples were hydrolyzed with 6 N HCl in evacuated glass ampuls at 105-115°C for 72 h. After the evaporation of the HCl, the samples were redissolved in 0.2 N citrate buffer, pH 2.2.

The molecular masses of thioredoxins I and II were determined by analytical electrophoresis in 10% polyacrylamide gel in the presence of 1% of sodium dodecyl sulfate [10].

LITERATURE CITED

1. P. Gonzales, A. Baldeten, and P. Reichard, *J. Biol. Chem.* **245**, 2263 (1970).
2. B. Buchanan, *Annu. Rev. Plant Physiol.* **31**, 341 (1986).
3. L. Anderson, *Encyclopedia of Plant Physiology*, Springer New Series, New York, Vol. 6 (1979), p. 271.
4. W. Wagner, H. Follmann, and A. Schmidt, *Z. Naturforsch.* **33**, 517 (1978).
5. C. I. Schmidt and V. Christen, *Z. Naturforsch.* **34**, 1272 (1979).
6. R. Wolosiuk, B. Buchanan, and N. Crawford, *FEBS Lett.* **81**, 253 (1977).
7. A. S. Rasulov and Z. S. Shakirov, *The Industrial Cultivation of Microorganisms* [in Russian], Moscow (1985), p. 125.
8. A. S. Rasulov and Z. S. Shakirov, *The Biosynthesis of Enzymes by Microorganisms* [in Russian], Pushchino (1986), p. 70.
9. A. S. Rasulov, Z. S. Shakirov, Z. G. Evstigneeva, and V. L. Kretovich, *Biokhimiya* **51**, 413 (1986).
10. B. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).