

NADPH-Dependent Thioredoxin Reductase and a New Thioredoxin from Wheat

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An albumin fraction extracted from wheat flour contains thioredoxin reductase ($M_r=65,000$) and a heat-stable thioredoxin ($M_r=15,000$) which are separated on DEAE cellulose and further purified by gel filtration. Wheat thioredoxin stimulates *E. coli* ribonucleotide reductase but not chloroplast fructose-bis-phosphatase. The enzyme is NADPH-dependent ($K_m=3.2 \times 10^{-6}$ M). In presence of the thioredoxin it slowly reduces other proteins like insulin or ribonuclease. Therefore it is most likely identical with a protein disulfide reductase (of unknown specificity) previously described in wheat. This new thioredoxin system is a counterpart of the ferredoxin-dependent system found in photosynthetic plant cells, suggesting different, specific mechanisms for regeneration of reduced thioredoxins in germinating seeds and green plants.

Introduction

Plant thioredoxins are an interesting group of proteins because they possess multiple species and an organelle specificity unknown from other organisms [1, 2]. An even greater difference is the existence of NADPH-dependent thioredoxin reductases (EC 1.6.4.5) in bacteria and animals [3–5] whereas a light-dependent, ferredoxin-coupled thioredoxin reductase was discovered in leaves [6]. There remains the question, however, whether the latter enzyme represents the only system for regeneration of reduced thioredoxins in plants. It could not function in germinating seeds and other non-photosynthetic plant cells in which DNA precursor biosynthesis proceeds by the apparently universal ribonucleotide reduction pathway [7, 21, 22] which is normally linked to NADPH and thioredoxin.

Previous experiments had failed to demonstrate in plant extracts a NADPH-thioredoxin reductase of the properties known for microbial and liver enzymes [1, 8]. We therefore asked whether the NADPH-requiring protein disulfide reductases (EC 1.6.4.4) described in pea and wheat seeds [9, 10] might be involved in thioredoxin metabolism. The catalytic function of these little-known enzymes appears to be similar to that of glutathione reductase and thioredoxin reductase, but their substrate specificity has not been investigated in detail. Identity of

a protein disulfide reductase in rat liver [11] with the thioredoxin system has recently been suggested [5]. In an attempt to characterize protein disulfide reductase from wheat we have now found that the grain proteins in fact contain both a new thioredoxin and an enzyme having the specificity of NADPH-thioredoxin reductase. In this paper we describe partial purification and some properties of the two proteins.

Materials and Methods

Reagents, coenzymes, and proteins of highest purity available were obtained from Merck, Boehringer Mannheim, and Serva. Crystalline insulin was rendered zinc-free by passage over a column of Chelex 100-chelating resin (Bio-Rad) prior to use. Thioredoxin and ribonucleotide reductase from *E. coli* B3 cells, and fructose-bis-phosphatase from spinach leaves were prepared by published procedures [12–14]. Two different brands of wheat flour (type 405) were used without significant change in the results.

Chromatographic materials were purchased from Whatman and Pharmacia. Protein was determined by the method of Lowry, photometric measurements were done with a Zeiss PMQ3 spectro-photometer.

The amperometric titration of SH groups in proteins [15, 16] was performed with a Metrohm potentiograph model E 436, equipped with a platinum and a Ag/AgCl electrode. The apparatus was calibrated with solutions of reduced glutathione and of serum albumin chemically reduced by NaBH_4 . Op-

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timum conditions for titration of SH with 1 mM AgNO_3 solutions were 0.15 M Tris- HNO_3 buffer pH 7.4 containing 0.01 M KCl, and a voltage of 0.070 V applied to the electrodes. A sensitivity of $> 0.05 \pm 0.01$ μmol SH in 2–4 ml sample volume could be reached on the instrument's 5 μA scale. Air-free, double-distilled water had to be used throughout. Enzyme assays were incubated in Thunberg tubes under nitrogen at 25 °C for 5–30 min periods and then immediately titrated.

Colorimetric assays of thioredoxin and thioredoxin reductase with 5,5'-dithiobis(2-nitrobenzoic acid) were done in 0.1 M phosphate buffer pH 7.0 following the method described for yeast thioredoxin system [17]. The most active fractions of either component were first sought in a column eluate (Fig. 2) and then mutually used for all other activity determinations.

Ribonucleoside diphosphate reductase (EC 1.17.4.1) was assayed exactly as described [8], using [^3H] cytidine diphosphate as substrate. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutathione reductase (EC 1.6.4.2), and fructose-bis-phosphatase (EC 3.1.3.11) were determined by standard procedures [18, 14].

All preparative work was done at 0–4 °C. Wheat flour (500 g) was stirred in 0.05 M Tris-HCl buffer, pH 7.65, containing 1 mM EDTA (2000 ml) for 3 h and insoluble material was removed by centrifugation. To the turbid supernatant (1625 ml) solid ammonium sulfate was added up to 30% saturation (167.4 g/l), maintaining the pH at 7.0 with concentrated ammonia. The precipitate was removed by centrifugation and the yellowish supernatant

(1720 ml) was brought to 65% $(\text{NH}_4)_2\text{SO}_4$ saturation (223.5 g/l). After 3 h the precipitate was collected by centrifugation (20 min at $9,000 \times g$), redissolved in 190 ml of 0.02 M ammonium acetate buffer, pH 8.6, containing 1 mM EDTA and then dialyzed for 14 h against the same buffer. The solution was centrifuged and applied to a column of DEAE-cellulose (DE 32, 6.5×2.8 cm). The column was washed for 4 h with the above acetate buffer and then eluted with a linear gradient of 0 to 0.3 M NaCl in the starting buffer. Fractions of 11.7 ml/30 min were collected and assayed, as indicated in Fig. 2.

The fractions containing thioredoxin (fraction I, 63 ml) were heated to 70 °C for 5 min. After rapid cooling insoluble proteins were removed by centrifugation, and the supernatant was dialyzed against a neutral, saturated solution of ammonium sulfate for 14 h. The precipitated proteins were redissolved in 11 ml Tris-HCl-EDTA buffer, 5 ml of the centrifuged solution were layered on top of a Sephadex G-50 column (84×2.8 cm) and chromatographed at a flow rate of 16.8 ml/h. 5.6 ml-fractions were collected and were concentrated by means of immiscible molecular separators (Millipore) for further experiments.

The DEAE-cellulose fractions containing thioredoxin reductase were concentrated by dialysis against ammonium sulfate solution. The proteins were redissolved in 12 ml Tris-HCl buffer, and portions of 6 ml were subjected to gel filtration on a Sephadex G-100 column (92.5×3.0 cm). Fractions of 7.9 ml/20 min were collected and assayed for enzyme activities.

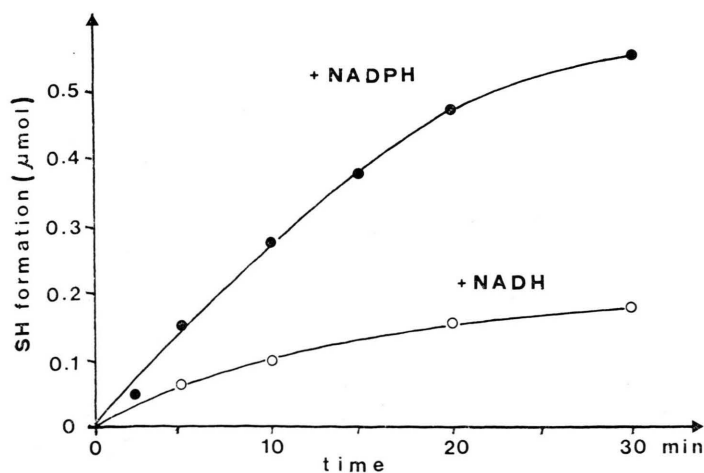


Fig. 1. Liberation of SH groups in wheat albumin during incubation with NADPH. 20 mg protein samples from a wheat flour extract (ammonium sulfate precipitate, redissolved and dialyzed in Tris-HCl buffer pH 7.4) were incubated with 0.6 mM NADPH (●) or NADH (○) and titrated with AgNO_3 by the amperometric technique. No reaction occurred in the absence of reduced coenzyme.

Results

The unknown substrate specificity of protein disulfide and thioredoxin reductases makes their differentiation in crude extracts difficult, if not impossible, by standard methods of SH determination. We were not able to detect NADPH-specific reductase activities in wheat embryo using the colorime-

tric assay with 5,5'-dithiobis(2-nitrobenzoate) because of a high level of unspecific reagent reduction. The more direct amperometric titration of SH groups with Ag^+ , which had been used for determination of protein disulfide reductase from wheat with wheat albumin as substrate [10], was therefore adopted. In our hands the albumin fraction extract-

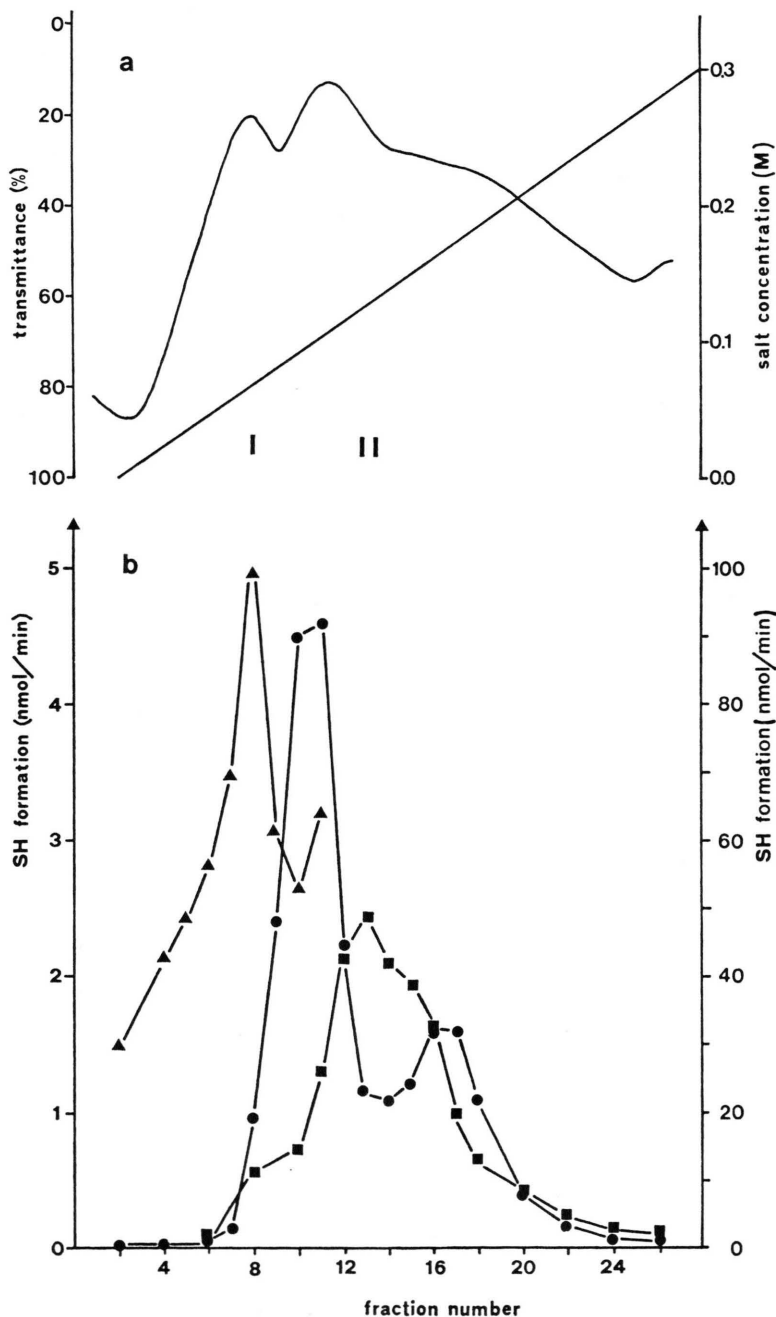


Fig. 2. Fractionation of wheat proteins (30–65% ammonium sulfate precipitate) on DEAE cellulose. a: Protein elution profile and NaCl gradient. b: Enzyme activities determined with the dithio-bis(nitrobenzoate) assay; NADPH, 0.1 mM. ● Glutathione reductase (50 μl samples, right scale). ▲ Thioredoxin (I, determined with 340 μg protein of fraction 13 as enzyme). ■ Thioredoxin reductase (II, 200 μl samples determined with 7.4 μg thioredoxin from the subsequent gel filtration step as substrate; left scale).

Table I. Liberation of SH groups during incubation of wheat protein fractions with NADPH.

Protein	Titrateable SH groups [$\mu\text{mol}/\text{mg}$ protein/30 min]
fraction I	0
fraction II	0.050
I+II	0.280
I, heat-treated, +II	0.275
I+II, heat-treated	0

NADPH, 0.60 mM; glucose-6-phosphate, 3 mM; pH 7.4; 25 °C. Fractions I and II were obtained from DEAE-cellulose (*cf.* Fig. 2), SH groups were determined by amperometric titration.

ed from commercial wheat flour showed already a strong, endogenous activity to liberate titrateable SH residues in the presence of NADPH and was thus not suitable as disulfide reductase substrate. However, this activity could be precipitated from the solution with ammonium sulfate, it was completely destroyed by incubation at 70 °C for 10 min, and it was dependent upon added NADPH but much less stimulated by NADH (Fig. 1), suggesting the combination of enzyme(s) plus substrate(s) in the same protein fraction. Glucose-6-phosphate was necessary for prolonged periods of NADPH consump-

tion, and the expected presence of glucose-6-phosphate dehydrogenase in the extract was indeed established. Because of the ready availability of the active material we decided to further characterize its specificity for disulfide reduction.

Chromatography on DEAE-cellulose removed a large amount of brown-colored substances and partially resolved two main protein fractions I and II (Fig. 2). They were almost inactive separately but SH-liberating activity was restored by combination of both proteins (Table I). Glucose-6-phosphate as NADPH-regenerating substrate could now be omitted from the assay mixtures, and separation from other SH-generating reactions was sufficient to allow NADPH-dependent colorimetric assays instead of discontinuous amperometric titration. Glutathione reductase was also present in the column eluate but did not interfere because its substrate, glutathione, was absent from the preparation at this stage.

The material eluting first from DEAE-cellulose (fraction I) can be identified as thioredoxin by the following criteria. (1) It is heat-stable at 70 °C like all other known thioredoxins. (2) Further purification on a column of Sephadex G-50 (Fig. 3) in-

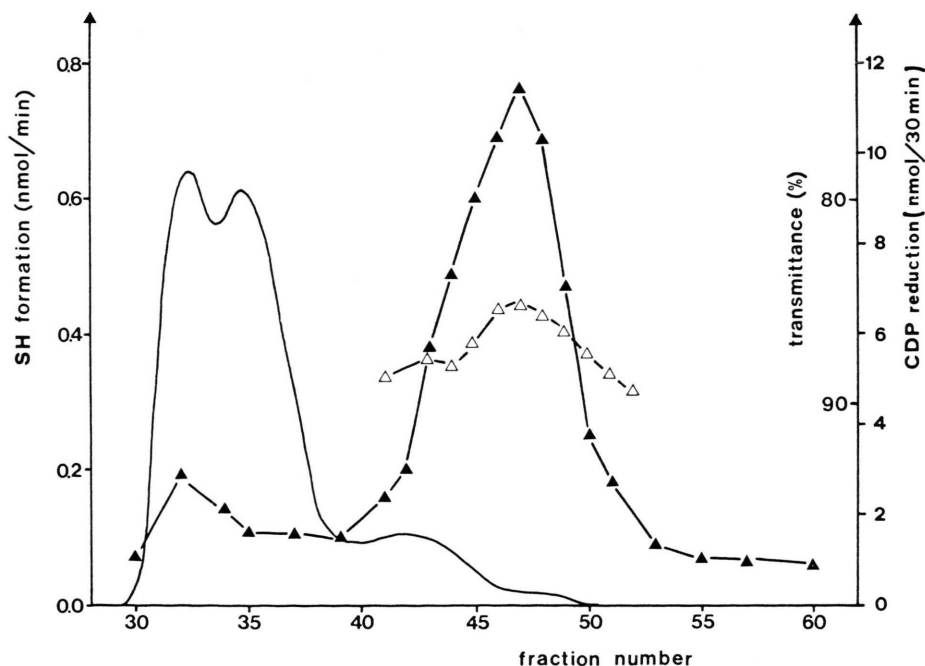


Fig. 3. Gel filtration of fraction I (thioredoxin) on Sephadex G-50. Drawn line: protein elution profile. ▲ Thioredoxin activity in the dithiois(nitrobenzoate) assay using 400 μl samples and 68 μg thioredoxin reductase from gel filtration step (Fig. 4); no reaction in the absence of enzyme. △ Stimulation of *E. coli* ribonucleotide reductase by 150 μl samples of thioredoxin under standard conditions [8] (right scale). The column was calibrated with cytochrome c ($M_r=13,500$), horse myoglobin (18,000) and chymotrypsinogen A (25,000) as mol weight markers.

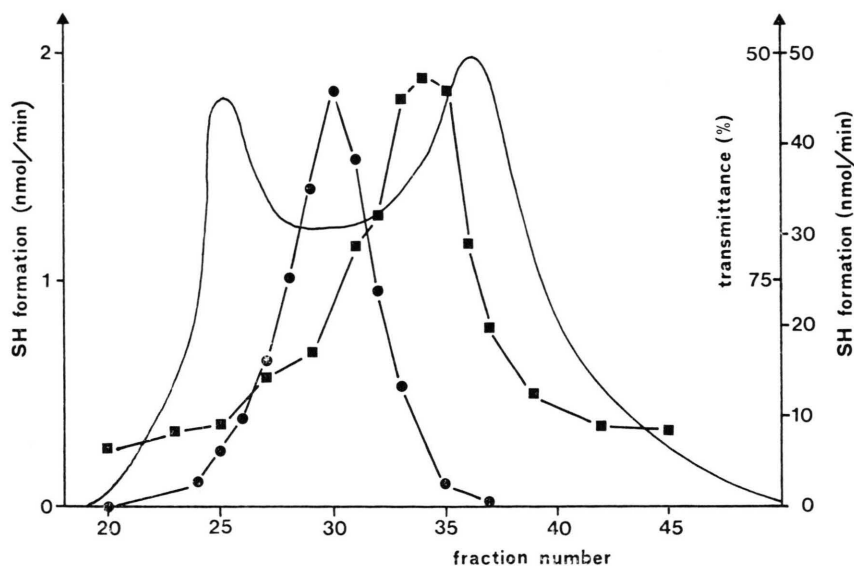


Fig. 4. Gel filtration of fraction II (thioredoxin reductase) on Sephadex G-100. Drawn line: protein elution profile. ● Glutathione reductase (50 μ l samples, right scale). ■ Thioredoxin reductase (200 μ l samples, 5.5 μ g purified thioredoxin as substrate; left scale). No enzyme activities were found in the absence of glutathione or thioredoxin. The column was calibrated with cytochrome c ($M_r=13,500$), chymotrypsinogen A (25,000), ovalbumin (45,000) and serum albumin (67,000) as mol weight markers.

licated a molecular weight of 15,000 which is in the size range of plant thioredoxins. (3) The protein stimulates CDP reduction in the presence of ribonucleotide reductase from *Escherichia coli* (Fig. 3). (4) It is enzymatically inactive by itself but is a good substrate for the homologous, NADPH-dependent protein fraction II (thioredoxin reductase) described below. In contrast, fraction I is *not* able to activate spinach fructose-bis-phosphatase like the thioredoxins of bacterial, algal, or chloroplast origin.

The enzyme nature of fraction II is implied by its rapid inactivation at 70 °C. Gel filtration of this fraction on Sephadex G-100 (Fig. 4) allowed safe differentiation, although not complete separation, of glutathione reductase and the thioredoxin (fraction I) reducing enzyme. A molecular weight of 65,000 was established for the latter, in close similarity with the thioredoxin reductases isolated from *E. coli* or Novikoff rat tumor [3, 4]. The enzyme can also be absorbed to 2', 5'-ADP-sepharose and eluted with 5 mM NADPH, in analogy to *E. coli* thioredoxin reductase [19]. At this stage it was entirely NADPH-dependent (Fig. 5) and showed virtually no activity in the presence of NADH. The apparent K_m value for NADPH is $3.2 \cdot 10^{-6}$ M, which may be compared with the value of $5 - 8 \cdot 10^{-6}$ M reported for *E. coli* thioredoxin reductase [12].

Purification of the two proteins is summarized in Table II; specific activities can only be calculated after they become separated.

The substrate specificity of enzyme fraction II clearly indicates that it is not a general protein

Table II. Purification of thioredoxin and NADPH-thioredoxin reductase from wheat flour.

Purification step	Total protein [mg]	Total activity [nmol/min]	Specific activity [nmol/min · mg]
crude extract	2259	14308	—
ammonium sulfate precipitate	790	1446	—
<i>thioredoxin</i>			
DEAE cellulose	67	583	8.7
fraction I			
heat treatment supernatant	54	545	10.1
Sephadex G-50	1.3	401	308.3
<i>thioredoxin reductase</i>			
DEAE cellulose	141	860	6.1
fraction II			
Sephadex G-100	86	800	9.3

Activities were determined with the dithiobis(nitrobenzoate) assay. In the first two steps protein samples were incubated with NADPH without further addition; in the following steps 76 μ g thioredoxin reductase (purest sample) were used for thioredoxin and 11 μ g thioredoxin (purest sample) for thioredoxin reductase determination, respectively.

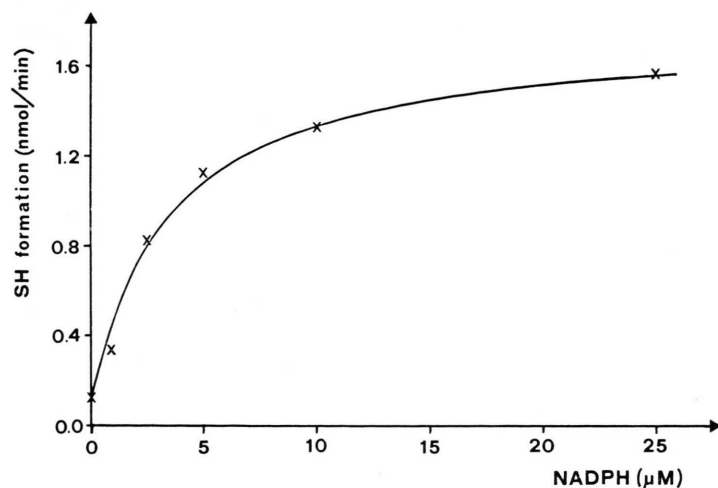


Fig. 5. NADPH dependence of thioredoxin reductase. 76 μg of enzyme from the gel filtration step and 11 μg thioredoxin were combined in the dithiobis(nitrobenzoate) assay. SH formation in presence of 25 μM NADH: 0.12 nmol/min.

disulfide reductase (Table III). Serum albumin, ribonuclease, or insulin showed no increase in titrable SH groups during incubation with the enzyme plus NADPH. However, when homologous thioredoxin (fraction I) was also present there occurred a slow reduction of ribonuclease and insulin (under precipitation of the insoluble B chain). Likewise, thioredoxin of *Escherichia coli* was slowly reduced by the wheat enzyme both under the amperometric and dithiobis-(nitrobenzoate) assay conditions. These reactions are in full agreement with the catalytic properties of other thioredoxin/thioredoxin reductase systems. For example, *E. coli* thioredoxin reduces insulin [12], and bovine thioredoxin reductase accepts heterologous thioredoxins as substrates besides the calf liver protein [5].

Table III. Specificity of wheat NADPH-thioredoxin reductase.

Substrate	Titrateable SH groups [$\mu\text{mol/h}$]
wheat thioredoxin (3.5 mg)	0.90
<i>E. coli</i> thioredoxin (1.7 mg)	0.10
serum albumin (10 mg)	0
insulin (10 mg)	0
insulin (10 mg) + wheat thioredoxin (3.5 mg)	1.16
ribonuclease (10 mg)	0
ribonuclease (10 mg) + wheat thioredoxin (3.5 mg)	1.05

NADPH, 0.80 mM; pH 7.4; 25 °C; thioredoxin reductase (fraction II), 3 mg protein. Wheat thioredoxin was the heat-treated fraction I, reduction of which is complete after 20 min; reduction of pure *E. coli* thioredoxin is 35% complete after 1 h and 44% after 2 h. Reduced insulin precipitates after 30 min.

Discussion

All available data suggest that the two proteins extracted from wheat flour constitute the wheat grain's thioredoxin and NADPH-thioredoxin reductase. Unambiguous definition of a protein as thioredoxin presents an unexpected difficulty in view of the multiple intracellular functions of thioredoxins recently observed [1, 20]. However, without exception the heat-stable proteins isolated from bacteria, yeast, green plants, and mammalian sources are substrates of *E. coli* ribonucleotide reductase which may thus be considered the most reliable enzymatic test for thioredoxin activity. A corresponding homologous assay could not be performed with the wheat protein because ribonucleoside diphosphate reductase of germinating wheat is only poorly characterized *in vitro* [21]. Another property of wheat fraction I compatible with a plant thioredoxin is its size: While microbial and animal thioredoxins all possess molecular weight of about 12,000, the species characterized in algae and in spinach leaves cover the range from 12,600 to 23,500 (in *Scenedesmus*) and 9,000 to 16,000 (spinach) [1, 2], indicating a greater diversity in the plant kingdom. The observed molecular weight of 15,000 also rules out that the activity of protein I is due to possible bacterial contamination of the starting material. The NADPH-dependent enzyme fraction II must then be thioredoxin reductase. Its molecular size and specificity are closely comparable to those of the known thioredoxin reductases. We assume that this enzyme system is identical with the described pro-

tein disulfide reductase activity of wheat [10] which had not been purified by column chromatography.

Thioredoxins and thioredoxin reductases are known as rather stable proteins, and their presence in a wheat flour extract is therefore not uncommon. While flour is obviously a good source for their purification, localization in the grain remains to be established. Besides thioredoxin reductase we also partially purified the glutathione reductase of wheat [23] which has a molecular weight of 90,000 according to its elution from a calibrated gel filtration column (Fig. 4), and a heat-stable fructose-bisphosphatase (mol. weight, 30,000) previously unknown from this source. In contrast to the light-dependent chloroplast enzyme [6] this phosphatase could *not* be activated by wheat or any other thioredoxin.

Our results are the first evidence for thioredoxin reductase of the NADPH-dependent type in plants. Such an enzyme is not present in spinach, nor in the green alga, *Scenedesmus obliquus* cultured under phototrophic or heterotrophic conditions [8]; these cells contain a ferredoxin-dependent reductase which was not as yet characterized in detail. It is thus conceivable that non-photosynthetic plant tissues and chloroplasts use two different mechanisms of enzymatic thioredoxin regeneration, in keeping with the much higher specificity of that reaction than of thioredoxin utilization [1]. The lack of crossreactivity between wheat flour thioredoxin and spinach chloroplast fructose-bis-phosphatase further points to a high degree of specialization of the two systems. Presence of the light-independent enzyme apparatus in dry seeds is particularly important for deoxyribonucleotide biosynthesis during germination which involves inducible ribonucleotide reductases [21, 22] whereas thioredoxin levels are known to change

little during cell proliferation. Other biochemical processes may also require reduced thioredoxin in the germinating seed.

The properties of this plant thioredoxin system cast doubt on the existence of unspecific protein disulfide reductase in general. An activity like that of the pea seed reductase towards enzyme proteins (e.g., alcohol dehydrogenase or hexokinase) as substrates *in vitro* [9] is entirely unphysiologic: Enzyme disulfide bridges have to be maintained in a specific, native state *in vivo* rather than being fully reduced; transhydrogenases and protein disulfide isomerases can catalyze rearrangements of disulfide bridges if necessary. In the earlier studies of wheat the substrate albumin fraction was claimed to be enzymatically inactive. This is in contrast with our own observations but may be explained with inactivation of thioredoxin reductases in the sample of wheat flour previously used. Nevertheless the protein extract must still have contained thioredoxin, which upon reduction by NADPH and an independent reductase preparation from wheat seedlings could easily have catalyzed rather unspecific disulfide reduction in other proteins, in analogy to known thioredoxin functions [5, 12]. The presence of an additional, unspecific protein disulfide reductase is then considered unlikely or unnecessary. This does not rule out that such an enzyme exists for special metabolic functions like storage protein mobilization in seeds [10]. Scrupulous fractionation of enzymes from different parts of the wheat seed, and specificity and inhibitor studies are under way to resolve that question.

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