Pea Chloroplast Glutathione Reductase: Purification and Characterization

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ABSTRACT

Glutathione reductase (EC 1.6.4.2) was purified from intact pea (*Pisum sativum*) chloroplasts by a method which includes affinity chromatography on ADP-agarose. Fractions from the affinity column which had glutathione reductase activity consisted of polypeptides of 60 and 32 kilodaltons. Separation of the proteins by electrophoresis on native gels showed that glutathione reductase activity was associated with 60 kilodalton polypeptides and not with the 32 kilodalton polypeptides. Antibodies to spinach whole leaf glutathione reductase (60 kilodaltons) cross-react with the chloroplast 60 kilodalton glutathione reductase but not the 32 kilodalton polypeptides. In the absence of dithiothreitol the 60 kilodalton polypeptides showed a shift in apparent molecular weight on sodium dodecyl sulfate gels to 72 kilodaltons. Dithiothreitol did not alter the activity of the chloroplast enzyme. Chloroplast glutathione reductase is relatively insensitive to NADPH.

Glutathione is a widely distributed thiol-containing tripeptide. Although mutants of microorganisms lacking detectable glutathione grow normally (7) it is probably essential in eukaryotic organisms. This is in agreement with the wide range of functions, both enzyme and nonenzyme dependent, in which glutathione participates (15, 19). For almost all of these functions glutathione must be in the reduced state. The enzyme glutathione reductase (EC 1.6.4.2) uses the reducing power of NADPH to regenerate reduced glutathione (GSH) from oxidized glutathione (GSSG).

GSH and glutathione reductase have been reported to be present in the chloroplast, mitochondria and cytoplasm of plant cells (22, 28). In chloroplasts, removal of photosynthetically derived H_2O_2 was proposed as a function for GSH and glutathione reductase (6) in conjunction with ascorbate and ascorbate peroxidase (11). This detoxification pathway could be important in the chloroplasts of plants which experience water deficits. In water stressed plants daytime stomatal closure limits the availability of CO_2 thereby limiting NADP as an electron acceptor from photosystem I (5). Under these conditions electron flux to O_2 may increase, resulting in increased production of superoxide and H_2O_2. Maintenance of high levels of glutathione reductase in water stressed plants is consistent with the involvement of GSH in O_2 product detoxification (8).

Glutathione reductase has been purified from several plant sources (12, 26) including spinach leaves (10) and fractions enriched in pea chloroplasts (13). Glutathione reductase purified from spinach leaves had an apparent mol wt of 72 kD (10). The native form of the enzyme was reported to be a dimer of identical subunits (10) similar to glutathione reductase from other sources (12). The glutathione reductase purified from spinach leaf extracts had a specific activity of 246 units/mg protein (10). In

contrast, the enzyme purified from pea chloroplasts was reported to have a native mol wt of 156 kD and consisted of 2 each of 2 subunits of 40 and 42 kD (13). This enzyme preparation had a specific activity of 26 units/mg protein and pretreatment of the isolated enzyme with NADPH did not inhibit enzymic activity (13). In this paper we have reinvestigated glutathione reductase from chloroplasts. Our results demonstrate that the chloroplast glutathione reductase monomer is 60 kD and is relatively insensitive to incubation with NADPH.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L cv Progress No. 9) were purchased from Burpee, dithiothreitol from Boehringer Mannheim, SDS from Pierce, and protein standards from BRL. All other reagents were obtained from Sigma Chemical Co. unless noted.

Growth of Peas. Peas were grown in flats of vermiculite in controlled environment chambers (Conviron) with a constant temperature of 23°C and constant illumination. Material for isolation of chloroplasts was harvested between 15 and 25 d after planting.

Isolation of Chloroplasts. Chloroplasts were isolated on Percoll gradients according to the method of Bartlett *et al.* (2) except that GSH and ascorbate were not included in the isolation media. Chloroplast intactness was estimated by phase-contrast microscopy. Chl was determined by the method of Arnon (1). All steps were performed at 4°C unless otherwise indicated.

Purification of Glutathione Reductase. The following procedure, modified from Halliwell and Foyer (10) was used to isolate glutathione reductase. Percoll purified intact chloroplasts were lysed by diluting to less than 1 mg Chl/ml in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The membranes were then pelleted by centrifugation at 12,000g for 15 min at 4°C. The light greenish stromal extract was applied to a column of DEAE-52

Table I. *Assessment of Cytoplasmic Contamination of Plastids Purified on Percoll Gradients*

Sample	Specific Activity	
	PEP carboxylase	Glutathione reductase
	units/mg protein	
Crude homogenate[a]	0.055 ± 0.002	0.152 ± 0.009
Percoll purified plastids[b]	0.00	0.228 ± 0.011

[a] Crude homogenates were prepared by homogenizing pea leaves in a mortar and pestle with 25 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, 0.8 mM phenyl methyl sulfonyl fluoride, 1 mM benzamidine, and 5 mM ε-NH_2 caproic acid (0.5 g fresh wt tissue/ml solution). The homogenate was filtered through two layers of Miracloth and centrifuged at 10,000g for 10 min. Fifty μl of the supernatant were used per assay. Three homogenates were prepared. [b] Plastids purified on Percoll gradients (from 2 separate isolations) were lysed by diluting 1:5 with 25 mM Hepes-KOH (pH 8.0) and membranes were removed by microfuging.
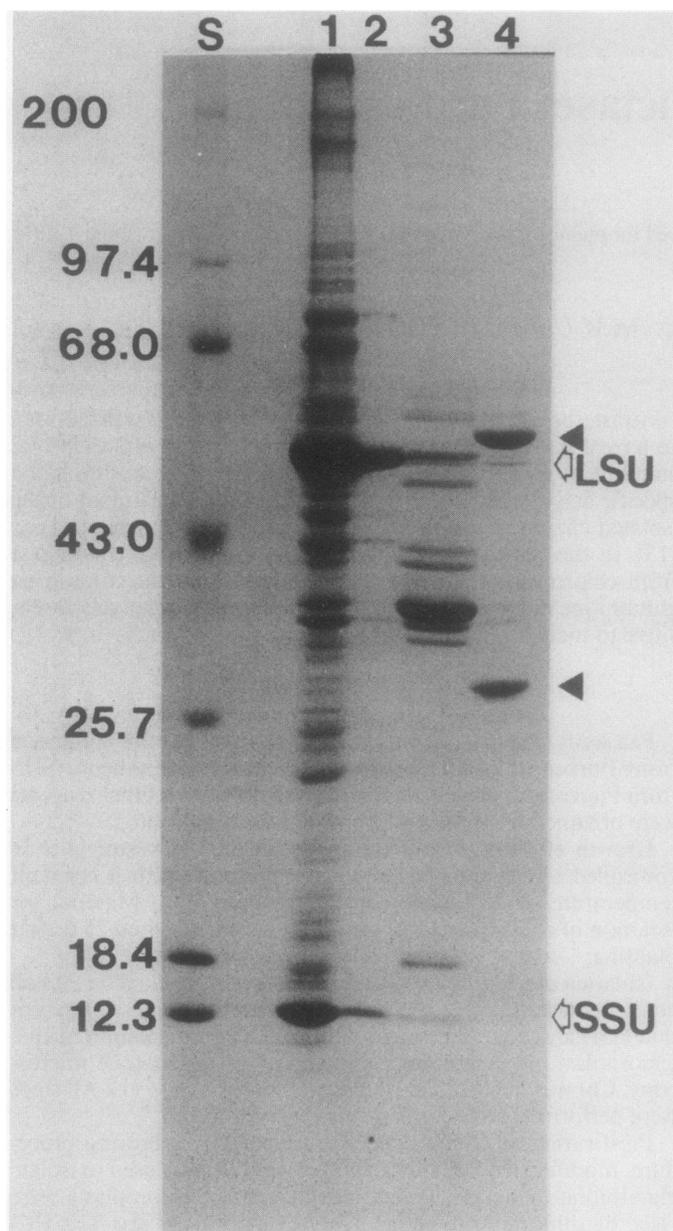
FIG. 1. SDS-polyacrylamide gel of proteins from fractions at each step of the purification of glutathione reductase from isolated chloroplasts. Lane 1, Crude stromal extract; lane 2, DEAE-52 pool; lane 3, concentrated 0.4 M KH₂PO₄ wash from affinity column; and lane 4, concentrated glutathione reductase from affinity column, 0.80 unit. Closed arrows indicate major proteins in the glutathione reductase affinity fraction. Open arrows indicate positions of the large (LSU) and small (SSU) subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase. Standards (lane S) are myosin heavy chain (200,000), phosphorylase B (97,400), BSA (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400) and lysozyme (12,300).

(Whatman) (bed volume: 2.5 × 22.5 cm) equilibrated in 50 mM Tris-HCl (pH 8.0). The enzyme was eluted after washing with 2 column volumes of buffer by applying a gradient of 0 to 150 mM KCl. Fractions containing glutathione reductase activity were pooled and loaded onto a 2′,5′-ADP agarose column (bed volume 1.75 × 6.0 cm). The column was then washed with 1 column volume of lysis buffer followed by 1 column volume of 0.4 M KH₂PO₄ (pH 7.5) and finally one column volume of lysis buffer (3). Glutathione reductase was eluted from the column by

applying a 2 ml pulse of 10 mM NADP.

Fractions containing glutathione reductase were pooled and concentrated to less than 500 μl (40–140 units/ml) by means of a Pro-Di-Con vacuum dialysis concentrator (Bio-Molecular Dynamics).

Enzyme Assays. Glutathione reductase activity was assayed in 0.1 M Tris HCl (pH 8.0) containing 0.1 mM EDTA and 0.5 mM GSSG in a final volume of 1 ml. Reactions were initiated by adding NADPH to a final concentration of 0.2 mM and the progress of the reaction monitored by the decrease in absorbance of NADPH at 340 nm. One unit of enzyme is that amount of protein which will oxidize 1 μmol of NADPH min⁻¹ at 25°C.

Phosphoenol pyruvate carboxylase activity was measured by the method of Wong and Davies (27). The decrease in A at 340 nm was followed in a 1 ml reaction mixture containing 50 mM Tris (pH 8.0), 5 mM DTT, 10 mM NaHCO₃, 5 mM PEP, 0.2 mM NADH, and 2 units malate dehydrogenase.

Protein Determination. Protein was estimated with the Bio-Rad Protein Assay (Bradford method) using BSA as the standard.

Electrophoresis. *(a) SDS-PAGE.* PAGE was performed on gradients of 7.5 to 15% acrylamide according to the method of Laemmli (14). Unless otherwise indicated, gels were stained with Coomassie brilliant blue. Electrophoresis was carried out at room temperature at 6 W/gel constant power.

(b) Native Gels. Gels of 10% acrylamide, 0.27% bisacrylamide, and in 375 mM Tris-HCl (pH 8.0) were prepared. Sample buffer was the same as that of Laemmli (14) except that SDS was omitted. Electrophoresis was performed at 8 to 10 W/gel at 4°C for 6 h.

(c) Purification of Proteins from Native Gels using DEAE-Membranes. Proteins were isolated from native gels by electroelution onto a strip of DEAE-membrane (NA-45, Schleicher & Schuell). After electrophoresis, a slice from the gel was stained with Coomassie blue. Staining was done with 0.6 g of Coomassie blue in 450 ml of 50% TCA (w/v) for 15 to 20 min. The gel slice was destained in 7% acetic acid, 11% methanol (v/v). If staining and destaining were done rapidly (total time less than 30 min) the gel slice did not shrink appreciably.

The gel slice was wrapped in Saran wrap and aligned with the remaining gel and a cut was made below the desired protein band. NA-45 membrane, prepared according to the directions of Schleicher & Schuell, was placed between the two pieces of gel. An additional strip of NA-45 was placed above the desired protein to prevent cross-contamination during electroelution. The gel plates were clamped together and the half of the gel from which the stained slice was taken was filled with a plug made of 10% acrylamide, 0.1% bisacrylamide (w/v). The gel was then electrophoresed 3 to 4 h at 4°C at 8 W.

After electrophoresis, the DEAE-membrane was carefully placed in a 1.5 ml microfuge tube and 100 to 300 μl of NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl (pH 8.0)) was added. The sample was then centrifuged, the supernatant removed, the membrane washed once more with NET buffer, and the washes pooled.

Preparation of Antisera. Commercially obtained whole leaf spinach glutathione reductase (500 units) was further purified by SDS-PAGE in the absence of reducing agent. After electrophoresis the yellow band was excised and the gel slice soaked in sample buffer containing 10 mM DTT for 30 min. The gel slice was placed on a second gel and the protein reelectrophoresed. The yellow band was again excised and the protein eluted into a dialysis bag by electrophoresis against one-tenth strength running buffer. Overall recovery was 70% on the basis of enzymic activity.

The purified spinach glutathione reductase was used to generate antibodies by intrascapular injection of 2 New Zealand white rabbits. Enzyme (50 units) was emulsified with complete adjuvant for the first injection. A second injection of 50 units in

Table II. *Purification of Glutathione Reductase from Pea Chloroplasts*

Sample	Total Units	Total Protein	Specific Activity	Recovery	Purification
		mg	units/mg protein	%	-fold
Crude stromal extract	47.3	200	0.236		
DEAE pool	46.0	66.5	0.692	97	2.9
Affinity pool	15.6	0.240	65	33	275
Electroeluted 60 kD protein	ND[a]	ND	146	ND	619

[a] ND = not determined.
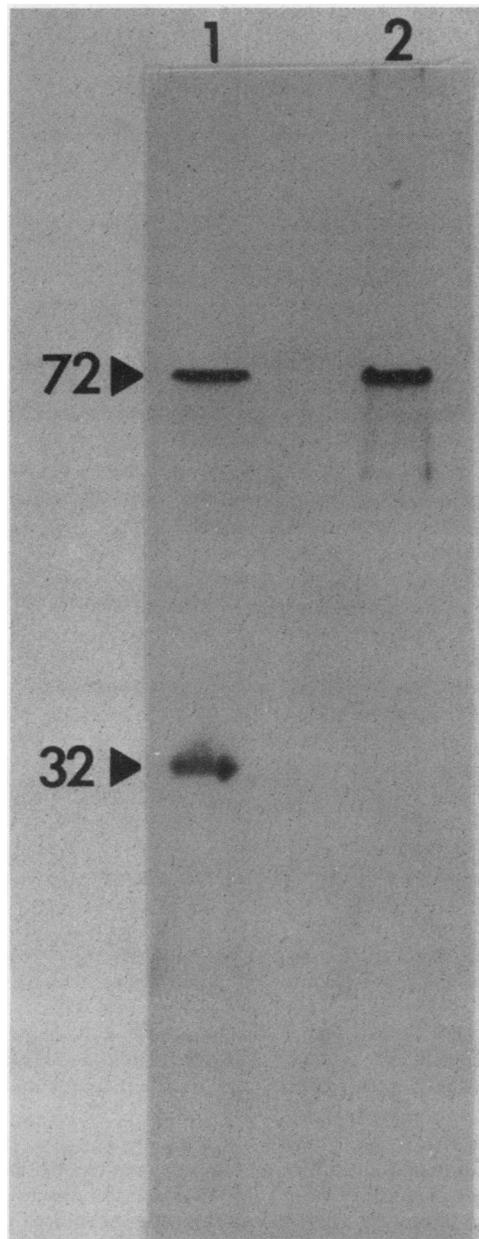
FIG. 2. SDS-polyacrylamide gel of chloroplast glutathione reductase isolated from native gels (lane 2) compared with affinity purified chloroplast glutathione reductase (lane 1). Lane 1 contains 0.41 unit of affinity purified chloroplast glutathione reductase, lane 2 contains 0.41 unit of glutathione reductase isolated after separation on a native gel. The gel was stained with silver.

incomplete adjuvant was given 2 weeks later. Subsequent injections were given at 4, 5, and 6 weeks of 25 units each. Bleeding by heart puncture was initiated on the 7th week. The IgG containing fraction of the antiserum was purified by the method of Chua *et al.* (4).

Protein Blots. Protein was transfered from polyacrylamide gels to nitrocellulose paper (30 V, 12 h) according to the procedure of Towbin *et al.* (25). Visualization of antiglutathione reductase IgG was achieved using alkaline phosphatase conjugated goat antirabbit IgG. Color development was done using Nitro blue tetrazolium and BCIP (Promega Biotec).

RESULTS AND DISCUSSION

Isolation of Glutathione Reductase from Chloroplasts. Chloroplasts were purified on Percoll gradients (90–95% intact) (20). The amount of cytoplasmic contamination in the Percoll purified plastid fraction was determined by measuring the activity of the cytoplasmic enzyme PEP carboxylase (Table I). This analysis showed that cytoplasmic contamination is negligible after purification of chloroplasts on Percoll gradients. The isolated chloroplasts were lysed and membranes were separated from soluble proteins by centrifugation. Each fraction was assayed for glutathione reductase. Over 90% of the enzyme activity was found in the soluble protein fraction consistent with previous reports (6, 13).

A two-step procedure was used to isolate glutathione reductase from the chloroplast soluble protein fraction. This involved chromatography on DE-52 cellulose followed by separation on a $2',5'$-ADP agarose affinity column. A similar procedure was used previously to isolate glutathione reductase from whole leaf extracts of spinach (10). We made two modifications of the previously described method. First, the column order was reversed so that crude extracts were chromatographed on DEAE-52 prior to the $2',5'$-ADP agarose column. This change was introduced because glutathione reductase can be separated from other NADP-dependent enzymes by DEAE chromatography (8). This allowed greater amounts of glutathione reductase to be loaded onto the affinity column. The second modification in the isolation procedure was the introduction of a 0.4 M KH_2PO_4 wash of the $2',5'$-ADP agarose column prior to elution of glutathione reductase with NADP (3). We have noted that the concentration of KH_2PO_4 used to wash the column should be optimized for the particular plant investigated. Glutathione reductase was not removed from the affinity column by the 0.4 M KH_2PO_4 wash in contrast to a number of other proteins which were eluted by this procedure (Fig. 1, lane 3). Proteins in samples from each step in the glutathione reductase preparation are shown in Figure 1. Table II shows the data for the purification of pea chloroplast glutathione reductase. After the affinity chromatography step the specific activity of glutathione reductase was 65 units/mg protein.

Characterization of Affinity Purified Glutathione Reductase. After affinity chromatography the sample consists of 2 major proteins which can be visualized on SDS-PAGE along with several minor proteins (Fig. 1, lane 4). The major polypeptides
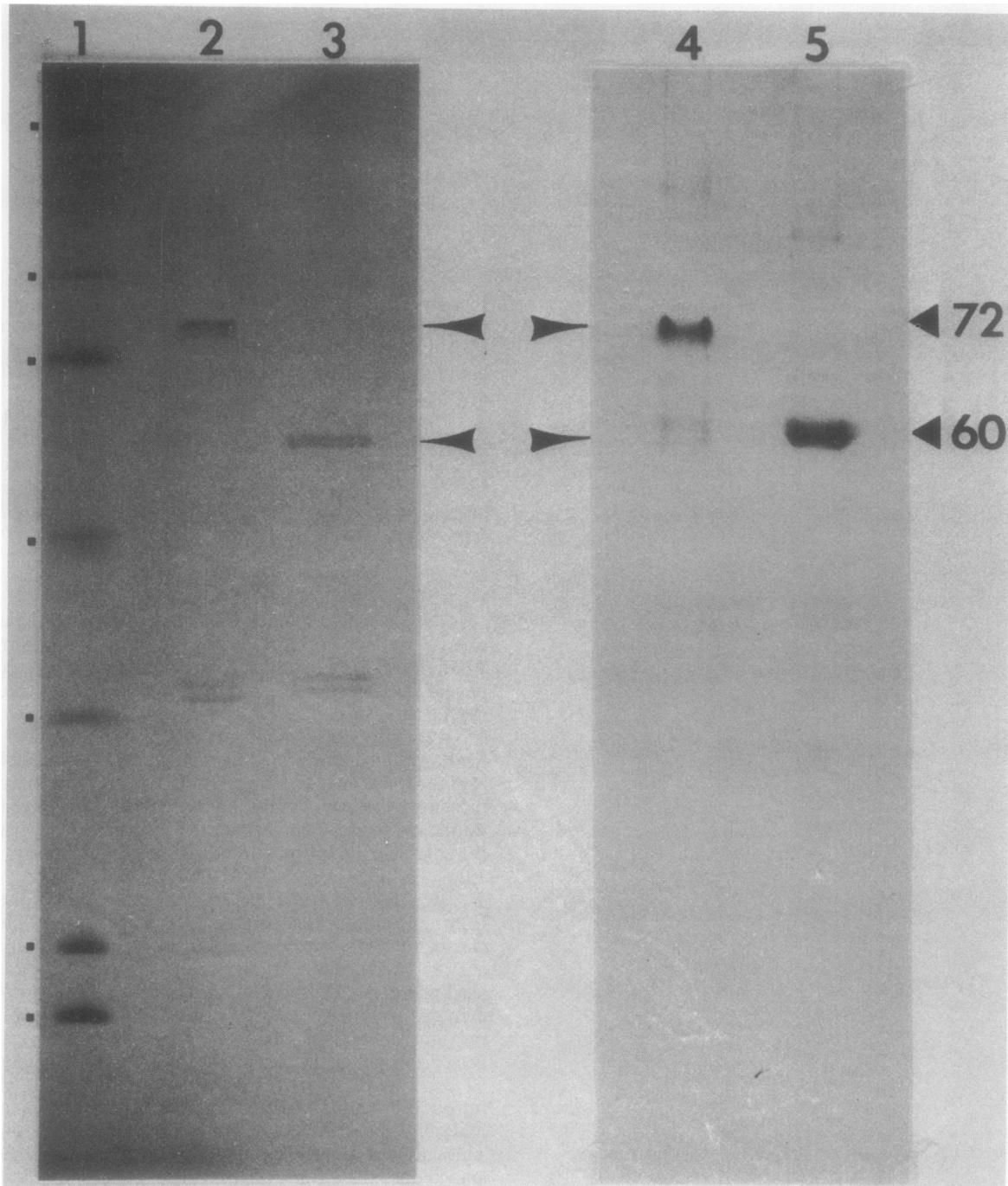
FIG. 3. SDS-polyacrylamide gel of glutathione reductase purified from pea chloroplasts (lanes 2 and 3) and spinach leaf glutathione reductase (lanes 4 and 5). DTT was omitted from the sample buffer in lanes 2 and 4 but was included in lanes 3 and 5. Mol wt standards (lane 1) are the same as those in Figure 1. Approximately 0.68 unit of glutathione reductase was loaded in lanes 2 and 3. Lanes 4 and 5 contain approximately 1 unit each. Arrows mark the position of 60 and 72 kD protein bands.

had apparent mol wt of 60 and 32 kD. To assign enzymic activity to these polypeptides, a sample was electrophoresed on a native gel and the major peptides electroeluted as described in "Materials and Methods." The 32 kD protein purified from the native gel had no glutathione reductase activity. In contrast the purified 60 kD protein exhibited glutathione reductase activity. An equal amount of enzyme activity from the affinity purification step (Fig. 2, lane 1) and the 60 kD protein isolated from the native gel (Fig. 2, lane 2) were run on an SDS-gel. This experiment showed that most of the glutathione reductase activity in the affinity purified fraction is associated with 60 kD polypeptides.

Affinity purified glutathione reductase was electrophoresed in the presence or absence of DTT (Fig. 3). In the absence of DTT glutathione reductase migrated at an apparent mol wt of 72 kD, whereas in the presence of DTT its apparent mol wt shifted to 60 kD. For comparison, spinach glutathione reductase from whole cell extracts is also shown electrophoresed in the absence and presence of DTT (Fig. 3). The lower mol wt polypeptides in the chloroplast sample are not affected by this treatment. DTT also had no effect on glutathione reductase activity.

We next tested whether the DTT-induced shift in glutathione reductase mobility was reversible. Figure 4 shows this experiment
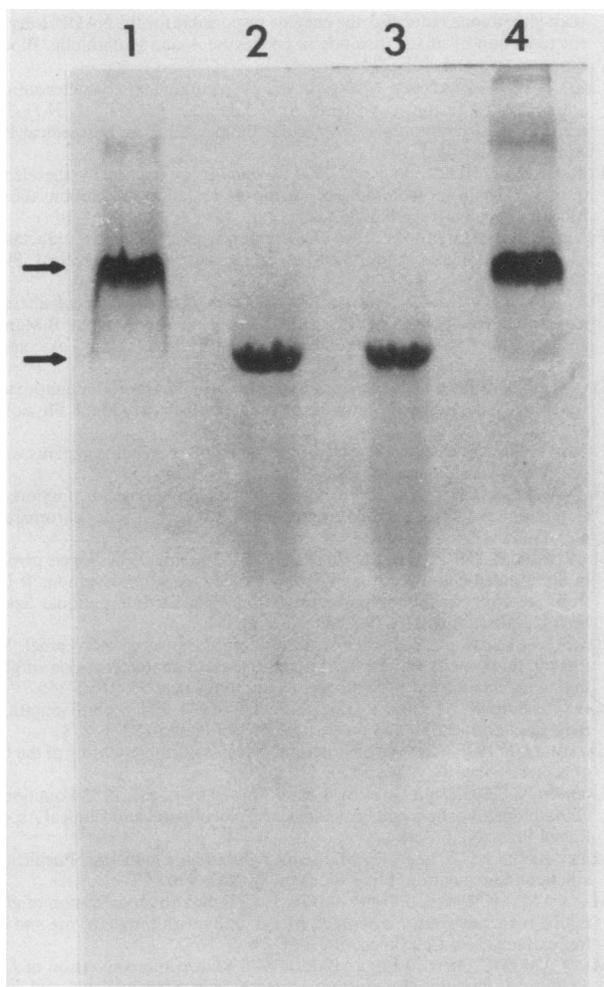
FIG. 4. SDS-polyacrylamide gel of spinach glutathione reductase under reducing and oxidizing conditions. Lane 1, enzyme without added reductant; lane 2, enzyme reduced with 17 mM DTT 30 min prior to loading; lane 3, enzyme reduced with 17 mM DTT 60 min prior to loading; lane 4, enzyme reduced with 17 mM DTT for 30 min, then diamide added to 34 mM final concentration for 30 min prior to loading. Approximately 1.2 units of enzyme were loaded per lane. Gel was stained with silver.

Table III. *Molecular Weight and Kinetic Constants of Pea Chloroplast Glutathione Reductase*

Kinetic parameters were determined from Hanes-Woolf plots; 2.0 μg protein was used per reaction point.

M_r Subunit	K_m			V_{max}		
	NADPH	NADH	GSSG	NADPH	NADH	GSSG
		μM			μmol/min	
60,000	3	17	62	0.263	0.0046	0.295

with spinach glutathione reductase. Glutathione reductase without reductant is shown in lane 1. In lanes 2 and 3 the reductase was reduced for 30 and 60 min prior to electrophoresis. In lane 4 the reductase was reduced 30 min with DTT and then oxidized 30 min with a 2-fold molar excess of diamide. This experiment suggests that the basis for the DTT-induced change in apparent mol wt of glutathione reductase involves a thiol-disulfide interchange.

One explanation for this finding then would be a reductant dependent dissociation from dimers to monomers. It is known that glutathione reductases from mammalian sources possess a
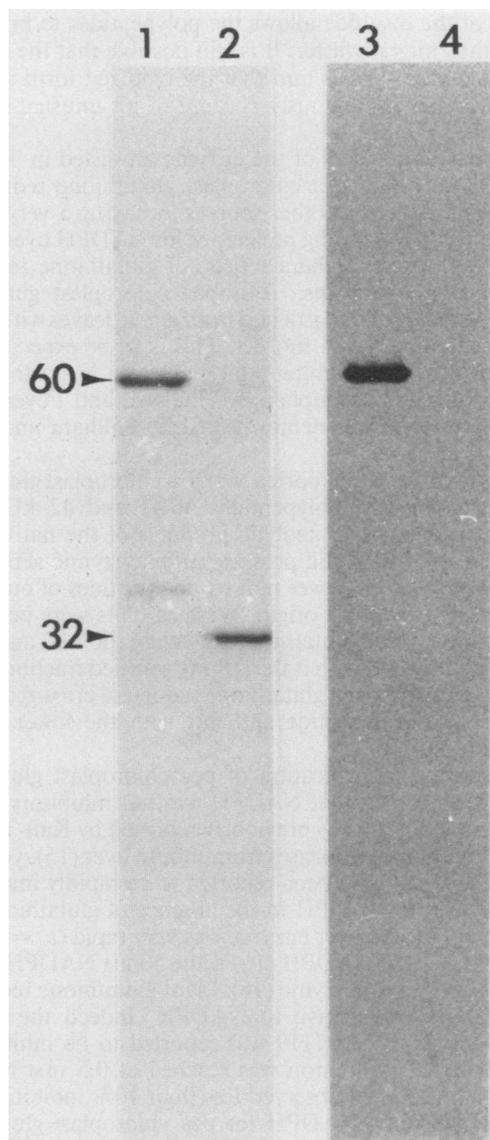
FIG. 5. SDS-polyacrylamide gel and immunoblot of the fractions enriched in 60 kD glutathione reductase subunits (lanes 1 and 3) and 32 kD polypeptides which copurify with glutathione reductase on ADP-agarose (lanes 2 and 4). The proteins in lanes 1 and 2 were immunoblotted and reacted with antibodies against spinach glutathione reductase (lanes 3 and 4).

disulfide between the 2 subunits (at Cys 90 for the human erythrocyte species) (24). Electrophoresis of bovine intestinal mucosa glutathione reductase in the absence and presence of reductant yields polypeptides with apparent mol wt of 100 and 50 kD, respectively. The change in mol wt for the spinach and pea chloroplast enzymes, however, is too small to be accounted for by such a mechanism. Indeed if bovine intestinal mucosa glutathione reductase is electrophoresed alongside the spinach enzyme it is seen that the change in mobility is greater for the bovine form, with the oxidized form running well above the oxidized spinach enzyme and the reduced form well below the reduced spinach enzyme (data not shown). A possible explanation for the change in mobility therefore is the breaking of a disulfide bridge within one subunit for the spinach enzyme as opposed to breaking a disulfide bridge between subunits, as happens in this case of mammalian glutathione reductases. If this is the case then the increase in mobility upon reduction is puzzling but perhaps can be explained if one assumes that

reduction of the disulfide allows the polypeptides to bind more SDS and thus migrate faster. It is still possible that the disulfide is between the 2 subunits and that the oxidized form would in this case migrate anomalously because of an unusual shape of the subunits.

The kinetic parameters of the enzyme are listed in Table III. This table shows that pea chloroplast glutathione reductase is similar to enzymes from other sources in having a very low K_m value for NADPH. A strong preference for NADPH over NADH was observed, which is characteristic of glutathione reductases from all higher organisms. Thus pea chloroplast glutathione reductase is similar to that isolated from whole leaves with respect to kinetic parameters and mol wt. This is to be expected if the majority of the cellular glutathione reductase is located within the chloroplast, as was implied by Halliwell and Foyer (10) in spinach leaves and later demonstrated by Gillham and Dodge (9) in peas.

Kalt-Torres *et al.* (13) reported that pea chloroplast glutathione reductase consisted of polypeptides of 41 and 42 kD. These polypeptides could be proteolytic products of the native gluta-thione reductase which still possess partial enzymic activity. To test whether any of the lower mol wt polypeptides of our prepa-ration were of proteolytic origin, Western blots were performed on samples of purified glutathione reductase (60 kD) and the 32 kD polypeptide found with it the affinity purified fraction. Figure 5 shows that anti-spinach glutathione reductase cross-reacts only with the 60 kD polypeptide and not with the lower mol wt polypeptides.

In the course of purification of pea chloroplast glutathione reductase it was noted that NADPH was not inhibitory even at mM concentrations, as was previously reported by Kalt-Torres *et al.* (13). Glutathione reductases from mouse liver (16), yeast (21) and *E. coli* (17, 18) have been reported to be rapidly inactivated by incubation with NADPH in the absence of glutathione. The inactivation of mouse liver enzyme was very rapid ($t_{1/2} = 14$ min) by as little as 0.3 mM NADPH (16) while 50 μM NADPH rapidly inactivated the *E. coli* enzyme (16). Plant glutathione reductases appear relatively insensitive to NADPH. Indeed the enzyme purified from *Anabaena* 7119 was reported to be inhibited by NADPH but 60% inhibition was reached at 0.5 mM NADPH only after 9 h (23). We observed less than 10% inhibition after 90 min with 2.5 mM NADPH for pea chloroplast glutathione reductase. Since the physiological function of the inhibition by NADPH is not understood, it is not possible to draw conclusions at present on the insensitivity of plant reductases to reduced pyridine nucleotide. However, as Kalt-Torres *et al.* (13) have noted, high concentrations of NADPH are present in the illu-minated chloroplast and the enzyme for this reason may be less sensitive to NADPH.

LITERATURE CITED

1. ARNON I 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BARTLETT S, AR GROSSMAN, N-H CHUA 1982 In vitro synthesis and uptake of cytoplasmically synthesized chloroplast proteins. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomed-ical Press, Amsterdam, pp 1081–1091
3. CARLBERG I, B MANNERVIK 1977 Purification by affinity chromatography of

4. yeast glutathione reductase, the enzyme responsible for the NADPH-depend-ent reduction of mixed disulfide of coenzyme A and glutathione. Biochim Biophys Acta 484: 268–274
4. CHUA N-H, SG BARTLETT, M WEISS 1982 Preparation and characterization of antibodies to chloroplast proteins. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam pp 1063–1080
5. EGNEUS H, U HEBER, M KIRK 1975 Reduction of oxygen by the electron transport chain of chloroplasts during assimilation of carbon dioxide. Biochim Biophys Acta 408: 252–268
6. FOYER CH, B HALLIWELL 1976 The presence of glutathione reductase in spinach chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21–25
7. FUCHS JA, B HALIER, CK TUGGLE 1983 Mutants of *Escherichia coli* altered in glutathione metabolism. *In* A Larsson, S Orrenius, A Holmgren, B Manner-vik, eds, Functions of Glutathione, Biochemical, Physiological, Toxicological and Clinical Aspects. Raven Press, New York, pp 385–393
8. GAMBLE P, JJ BURKE 1984 Effects of water stress on the chloroplast antioxidant system. I. Alterations in glutathione reductase activity. Plant Physiol 76: 615–621
9. GILLHAM DJ, AD DODGE 1986 Hydrogen-peroxide-scavenging systems within pea chloroplasts. Planta 167: 246–251
10. HALLIWELL B, CH FOYER 1978 Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatogra-phy. Planta 139: 9–17
11. HALLIWELL B, CH FOYER, SA CHARLES 1981 The fate of hydrogen peroxide in illuminated chloroplasts. *In* G Akoyunoglou, ed, Photosynthesis II Elec-tron Transport and Photophosphorylation. Balaban International Science Series, Philadelphia, pp 279–283
12. IDA S, Y MORITA 1971 Studies on respiratory enzymes in rice kernel. VIII. Enzymatic properties and physical and chemical characterization of gluta-thione reductase from rice embryos. Agric Biol Chem 35: 1550–1557
13. KALT-TORRES W, JJ BURKE, JM ANDERSON 1984 Chloroplast glutathione reductase: purification and properties. Physiol Plant 61: 271–278
14. LAEMMLI UK 1970 Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature 277: 680–685
15. LARSSON A, S ORRENIUS, A HOMGREN, B MANNERVIK, eds, 1983 Functions of Glutathione, Biochemical, Physiological, Toxicological and Clinical Aspects. Raven Press, New York
16. LOPEZ-BAREA J, C-Y LEE 1979 Mouse liver glutathione reductase. Purification, kinetics and regulation. Eur J Biochem 98: 487–499
17. MATA AM, MC PINTO, J LOPEZ-BAREA 1985 Redox interconversion of gluta-thione reductase from *Escherichia coli*. A study with pure enzyme and cell-free extracts. Mol Cell Biochem 67: 65–76
18. MATA AM, MC PINTO, J LOPEZ-BAREA 1985 Redox interconversion of *Esch-erichia coli* glutathione reductase. A study with permeabilized and intact cells. Mol Cell Biochem 68: 121–130
19. MEISTER A, ME ANDERSON 1983 Glutathione. Annu Rev Biochem 52: 711–760
20. MORGENTHALER JJ, MPF MARSDEN, CA PRICE 1975 Factors affecting the separation of photosynthetically competent chloroplasts in gradients of silica sols. Arch Biochem Biophys 168: 289–301
21. PINTO MC, AM MATA, J LOPEZ-BAREA 1985 The redox interconversion mechanism of *Saccharomyces cerevisiae* glutathione reductase. Eur J Biochem 15: 275–281
22. SCHAEDLE M, JA BASSHAM 1977 Chloroplast glutathione reductase. Plant Physiol 59: 1011–1012
23. SERRANO A, J RIVAS, M LOSADA 1984 Purification and properties of glutathi-one reductase from the cyanobacterium *Anabaena* Sp. strain 7119. J Bacte-riol 158: 317–324
24. THREME R, EF PAI, RH SCHIRMER, GE SCHULZ 1981 Three-dimensional structure of glutathione reductase at 2 Å resolution. J Mol Biol 152: 763–782
25. TOWBIN HT, T STAEHELIN, J GORDON 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some ap-plications. Proc Natl Acad Sci USA 46: 83–91
26. WIRTH E, E LATZKO 1978 Partial purification and properties of spinach leaf glutathione reductase. Z Pflanzenphysiol 89: 69–75
27. WONG KF, DD DAVIES 1973 Regulation of phosphoenol pyruvate carboxylase of *Zea mays* by metabolites. Biochem J 131: 451–458
28. YOUNG LOT, EE CONN 1956 The reduction and oxidation of glutathione by plant mitochondria. Plant Physiol 31: 205–211