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CHARACTERIZATION AND PARTIAL PURIFICATION OF A CYTOPLASMIC GLUTATHIONE : DISULFIDE OXIDOREDUCTASE (THIOLTRANSFERASE) FROM ADENOHYPOPHYSIS

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A glutathione-dependent thioltransferase (thiol : disulfide oxidoreductase) has been partially purified (70-fold) from anterior pituitary cytosol, and characterized. Purification was effected by differential centrifugation, precipitation between 30 and 60% $(NH_4)_2SO_4$, and sequential chromatography on Sepharose 6B, DEAE-cellulose, and CM-cellulose. Enzyme activity, monitored by the disappearance of NADPH, was associated with a protein of molecular weight 170000 both by gel filtration and by polyacrylamide gel electrophoresis in SDS. There was apparent charge heterogeneity after the gel filtration step, and only the major DEAE-cellulose peak was further purified on CM-cellulose. When SDS-polyacrylamide gel electrophoresis was carried out in the presence of mercaptoethanol, the two predominant bands seen in its absence were converted to five major bands, all of different apparent molecular weights from the originals. Isoelectric focusing yielded two major peaks of enzyme activity, at p*I* 7.0 and p*I* 4.5–5.0. These peaks were shown to be interconvertible upon reelectrofocusing. Both low- and high-molecular weight disulfides could be reduced. The pH optimum was sharp, at pH 8.2. The K_m values for glutathione and cystine (the standard assay disulfide) were 0.57 and 0.062 mM, respectively, each in the presence of saturating concentrations of the other substrate. *N*-Ethylmaleimide at 0.1 and 1.0 mM inhibited enzyme activity non-competitively, suggesting a non-catalytic role of enzyme thiol(s) for maintenance of optimal activity.

Introduction

The interconversion of thiols and disulfides, frequently involving the participation of glutathione, may influence a large variety of cellular processes. In addition to maintenance of cellular redox status, such conversions may influence peptide and protein synthesis [1] and structure and function [2–13]. The reacting residues may be on different molecules, with formation or scission of oligomers or mixed disulfides [14–17], on different chains within a single molecule [1], or on the same peptide chain [2–13].

Functions affected may include receptor binding [5,6,12,13,17], and the expression of the biological and immunological activity of enzymes [2,4], neurotransmitters [5–7] and peptide hormones [4,8–13]. Disulfide-bonded dimeric growth hormone (GH) [8,9] and prolactin [10,11] appear to retain immunologic activity similar to that of monomer; however, recent data suggest that for GH dimers, this activity is only 11% that of monomer [18]. Larger disulfide forms of GH and prolactin [8–13,17] are probably even less immunoreactive with monomer antiserum, as judged by the scanty data available [18].

Our interest in thiol : disulfide interchange arose as a result of observations indicating that large, disulfide-bonded forms of GH and prolactin exist in secretory granules [17]. A number of tissues are capable of mediating thiol : disulfide exchange

Abbreviations: GH, growth hormone; SDS, sodium dodecyl sulfate.

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enzymatically. In addition to its known presence in liver, kidney, and pancreas, we have demonstrated such activity in the pituitary [19]. This adenohypophysial GSH: protein disulfide oxidoreductase (thioltransferase) is capable of reducing monomeric or oligomeric GH and prolactin disulfides, and membrane disulfides of pituitary secretory granules as well [20].

In this paper, details are given for an approximate 70-fold purification of this thioltransferase. Also, properties of the enzyme are described which differentiate it from similar enzymes from other sources.

Materials and Methods

Materials

NADPH, reduced glutathione and cystine were purchased from Sigma Chemical Co., DEAE-cellulose, CM-cellulose, and acrylamide gel reagents were obtained from Bio-Rad, while Sepharose 4B and 6B were products of Pharmacia. All materials and equipment for isoelectric focusing were from LKB. Frozen porcine pituitaries were obtained from Pel-Freez.

Methods

Enzyme assay. Thioltransferase activity was routinely determined spectrophotometrically at 340 nm using a Gilford recording spectrophotometer. The enzyme has an absolute requirement for GSH and NADPH, and added glutathione reductase does not accelerate the reaction rate. Cystine was normally used as the disulfide substrate. The routine reaction was started by the addition of enzyme to a solution containing 0.05 M Tris-HCl, pH 8.0/1 mM GSH/1 mM cystine/0.1 mM NADPH in a final 1-ml volume. Blank reactions were incubated without GSH. Units of activity were defined as net nmol NADPH oxidized/min at 25°C. Protein concentration was estimated by the method of Lowry et al. [21].

Subcellular fractionation and $(NH_4)_2SO_4$ precipitation. Tissue (80–120 g) was homogenized (30%, w/v) in 0.3 M sucrose/0.05 M Tris-HCl, pH 7.4, and centrifuged for 10 min at $3\,000 \times g$. The pellet was washed with half the original volume of buffer, recentrifuged as above, and the combined supernatants were centrifuged at $45\,000 \times g$ for 20 min. This procedure conformed to the early steps of our procedure for preparing purified secretory granules [22]. Over 95% of the thioltransferase activity found in the adenohypophysial homogenate was located in this $45\,000 \times g$ supernatant fraction. Only 2.9% of the total homogenate activity sedimented when this supernatant was spun in the ultracentrifuge at $105\,000 \times g$ for 60 min. Of this amount, only 2.9% was found associated with the secretory granule fraction following sucrose gradient centrifugation of the cytosol at $96\,000 \times g$ for 120 min. When $(NH_4)_2SO_4$ was added to the $45\,000 \times g$ supernatant to a final concentration of 30%, about one-fifth of the total protein but virtually none of the thioltransferase precipitated. After this precipitate was discarded, enzyme activity was nearly quantitatively precipitated when the $(NH_4)_2SO_4$ concentration was raised to 60% (19.7 g/100 ml original volume).

Sepharose 6B chromatography. The precipitate was dissolved in a 25 ml 0.01 M sodium phosphate buffer, pH 8.0, and applied to a 2.5×90 cm column of Sepharose 6B equilibrated with the same buffer. Ascending flow was used for development. All column chromatography was carried out at 4°C; fractions were collected with an LKB fraction collector (2 070) equipped with an ultraviolet monitor. The flow rate was maintained at 20 ml/h, and 150 drops were collected per fraction. Those fractions containing thioltransferase activity (K_d^* 0.44–0.60) were combined and concentrated 5-fold by ultrafiltration (Amicon, PM-30 membrane).

DEAE-cellulose chromatography. The pH of the ultrafiltered Sepharose 6B enzyme was adjusted to pH 7.2, and this sample was then charged onto a 1.5×25 cm column of DEAE-cellulose equilibrated with 0.01 M sodium phosphate at pH 7.2. After washing with 150 ml of the same buffer, then with 250 ml 0.01 M sodium phosphate, pH 7.2/0.055 M NaCl, we began a linear gradient using equal volumes (250 ml) of 0.01 M sodium phosphate, pH 7.2/0.055 M NaCl and 0.01 M sodium phosphate, pH 7.2/0.15 M NaCl. When 350 ml of this gradient buffer had been washed onto the column, 150 ml of buffer containing 0.3 M NaCl were added to an additional chamber of the gradient maker (Buchler). The final elution step was addition of 100 ml of buffer/0.5 M

* K_d, partition coefficient calculated as $(V_e - V_0)/(V_t - V_0)$ where V_e is the elution volume, V_0 is the void volume and V_t is the column bed volume.

NaCl. The Na^+ gradient was monitored by flame photometry. Over 92% of the protein applied to the column was recovered. Several protein peaks from this column had thioltransferase activity; these were separately pooled, concentrated and desalted using an Amicon model 52 stirred cell and a PM-30 membrane.

CM-cellulose chromatography. The high specific activity protein fraction from the DEAE-cellulose column (elution position 60–75 mM Na, accounting for 40% of total enzyme activity applied) was adjusted to pH 5.8, clarified by centrifugation, and applied to a 1.2 × 11 cm column of carboxymethylcellulose equilibrated with 0.01 mM sodium acetate/1 mM EDTA buffer, pH 5.8. The flow rate was maintained at 15 ml/h and 60 drop fractions (4.2-ml) were collected. After washing the column with 40 ml of the above buffer, a linear gradient was begun with 250 ml buffer and 250 ml buffer/0.15 M NaCl. After the linear gradient step, little if any protein was eluted by the addition of buffer with 0.25 M NaCl or when the pH of this buffer was raised to 7. Fractions eluting in the range of 0.075–0.10 M NaCl contained more than 75% of the thioltransferase activity. These were combined, diafiltered with 100 ml 0.01 M sodium phosphate, pH 8.0 and then ultrafiltered to 10 ml and stored until use.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out using an LKB multiphor unit. Preparation of 8% gels, electrophoresis for 4.5 h at 195 mA, staining with 0.25% Coomassie blue for 2 h, and destaining in ethanol/acetic acid/water (3 : 1 : 10, v/v) were carried out according to the manufacturer's instructions.

Isoelectric focusing. The methods utilized for column isoelectric focusing using 1% Ampholine in a 0–50% (w/v) sucrose gradient were those of the supplier, LKB. Enzyme was solubilized in the light gradient solution if the anode was at the bottom of the column, and in the dense solution if the cathode was at the bottom. After electrofocusing for a minimum of 22 h, fractions were collected (2 ml each for 110 ml columns, 4 ml for 440 ml columns) using an Isco fraction collector equipped with a 280 nm monitor. Samples were neutralized and assayed for enzyme activity, peak fractions were combined, and sucrose and Ampholine were removed by diafiltration with 5 mM sodium phosphate buffer, pH 8.0.

Results

Our initial experiments were designed to determine the subcellular localization of pituitary thioltransferase. In contrast to most thiol: protein disulfide interchange enzymes from other sources, results shown in Fig. 1 demonstrate that the cytosol contains the bulk of the thioltransferase. Isolation of subfractions by differential and density gradient centrifugation showed nearly all the activity in the high speed supernatant. In order to determine if our homogenization conditions artifactually liberated enzyme from an organelle to which it was normally bound, we varied both the speed and the number of passes made with the Teflon-glass homogenizer; changing the speed from 300 to 1 400 rev./min, and the number of passes from 2 to our usual 8, had no effect on the subcellular distribution of enzyme activity as subsequently determined following differential centrifugation. Further, a comparison of

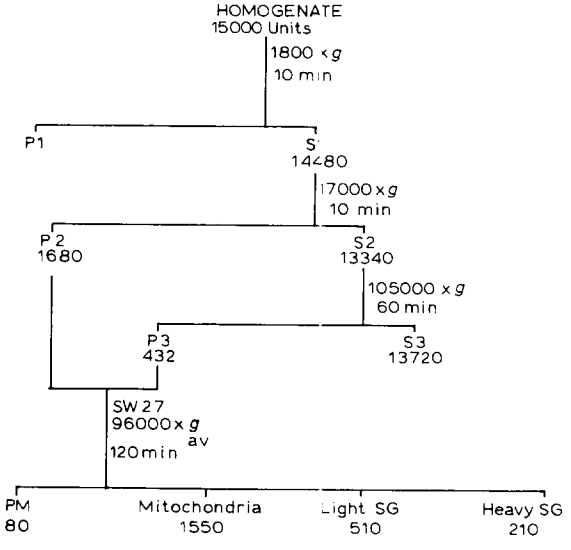

Fig. 1. Subcellular distribution of pituitary thioltransferase activity. Fresh porcine adenohypophysis (60 g) was homogenized, and fractionated by differential centrifugation as shown in the figure. The combined precipitate fractions (P2 and P3) were subjected to sucrose density gradient centrifugation, and thioltransferase activity was determined in the plasme membrane, (PM), mitochondrial and secretory granule (SG) fractions. Results were identical to those obtained routinely using frozen tissue in which enzyme activity was recovered in the supernatant fraction after two 3 000 × *g* spins and a 20 min 45 000 × *g* centrifugation.

TABLE I

PARTIAL PURIFICATION OF THIOLTRANSFERASE

All methods are as described in Materials and Methods. Values for the peak fractions are given in parenthesis.

	Specific activity (nmol/min per mg)	Purification	Recovery (%)
Homogenate	0.56	1.0	100
Cytosol	2.19	3.9	97
$(NH_4)_2SO_4$	5.23	9.3	95
Sepharose 6B	6.88 (9.11)	12.3	68.6
DEAE-cellulose	24.5 (30.5)	43.8 (54.5)	28.1
CM-cellulose	37.5 (68.0)	67.0 (121.4)	21.9

enzyme distribution in fresh and frozen tissue yielded essentially identical results.

$(NH_4)_2SO_4$ and chromatographic purification steps

Since a variety of pyridine-linked GSH-dependent enzyme activities might be present in crude preparations, partial purification was undertaken, as described in Methods. A summary of the results of a typical purification is given in Table I. The pituitary enzyme, unlike some others, was not inhibited by $(NH_4)_2SO_4$, and little loss in activity occurred but close to a 2.5-fold purification was achieved when soluble enzyme was subjected to 30–60% $(NH_4)_2SO_4$ fractionation. The next step in purification, chromatography on Sepharose 6B, is illustrated in Fig. 2. A broad enzyme peak was obtained; in the example shown, fraction numbers 50–60 were combined, corresponding to a K_d of 0.44–0.60, consistent with a molecular weight of approx. 180 000 for a globular protein. This step resulted in the removal of red and yellow chromophores from the sample, and also abolished materials present in the $(NH_4)_2SO_4$ fraction which interfered with the blank reaction (minus GSH); furthermore, no glutathione reductase activity remained in the chromatographed preparation, and no absorbance characteristic of FAD was seen upon spectrophotomeric examination (data not shown). Therefore, despite the limited increase in specific activity associated with this step (5.23 units/mg to 6.88 units/mg), it has been retained as an extremely useful procedure.

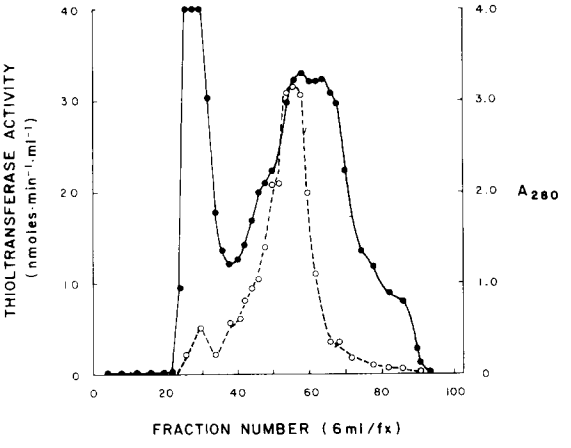

Fig. 2. Sepharose 6B chromatography of thioltransferase. All methods and conditions were as described in Materials and Methods. In this example, 25 ml $(NH_4)_2SO_4$-fractionated enzyme (2 500 total units, 750 mg protein) were applied to the column (2.5 × 90 cm, 0.01 sodium phosphate, pH 8.0). Absorbance at 280 nm is depicted by closed circles (●———●) and enzyme activity by open circles (○———○).

Ion-exchange chromatography was then utilized for further purification. A typical DEAE-cellulose fractionation, shown in Fig. 3, resulted in more than a 3.5-fold purification over the activity observed in the Sepharose 6B fraction. As shown, the major peak of activity eluted upon the addition of 0.055 M NaCl. However, only 42% of the enzyme activity was recovered in this peak. Several other protein peaks were eluted with increasing salt concentration; the specific activity in these regions was low, but contributed significantly to the overall recovery from the columns, which was over 90%. These data suggest that different forms of the enzyme occur with substantial differences in charge; this question is addressed in greater detail in the isoelectric focusing studies described below.

The major peak of thioltransferase activity from DEAE-cellulose was purified further by subjecting it to CM-cellulose chromatography at pH 5.8. Although a low level of enzyme activity was again observed in several peaks, as shown in Fig. 4, 78% of the activity was recovered in a major peak eluting at 0.065–0.085 M NaCl. The specific activity of the combined fraction was 37.5 units/mg; the activity in the peak tube was 68 units/mg.

Although not shown in Table I, the CM-cellulose fraction was filtered through an Amicon, PM-30

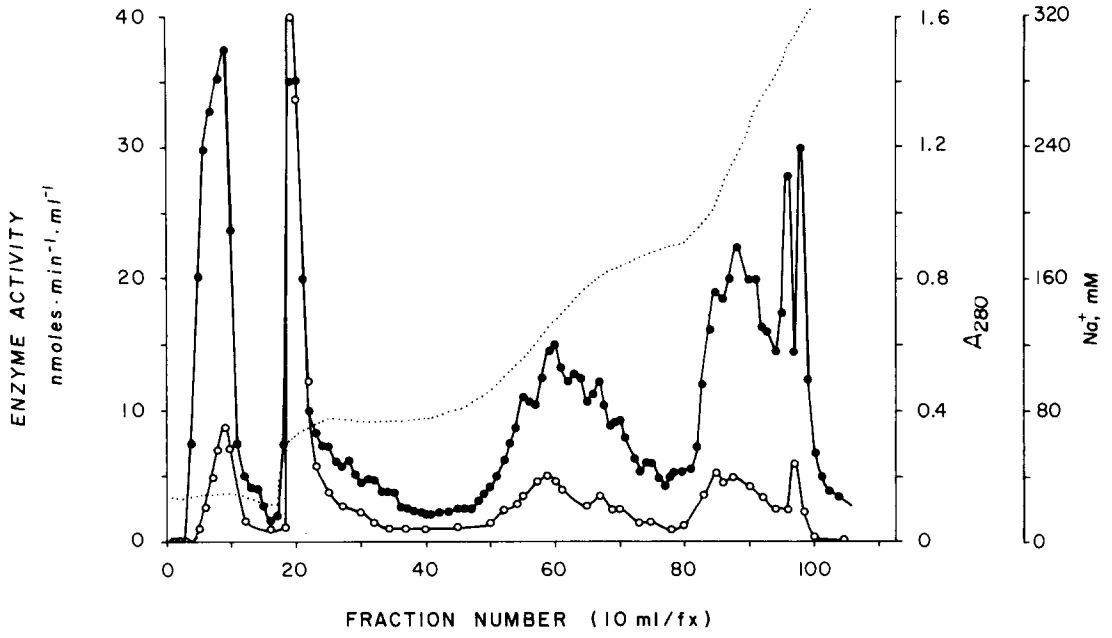

Fig. 3. DEAE-cellulose chromatography of thioltransferase. 50 ml (500 mg) of Sepharose 6B fractionated and ultrafiltered thioltransferase (5.57 units/mg) was applied to a 1.5 X 25 cm DEAE-cellulose column equilibrated with 0.01 M sodium phosphate, pH 7.2 buffer. A discontinuous salt gradient was begun after washing the column with 100 ml buffer. Details of all methods and gradient conditions are given in Materials and Methods. Absorbance at 280 nm is represented by closed circles (●———●), enzyme activity by open circles (○———○), and the Na^+ gradient by the continuous dotted line (· · · · · ·).

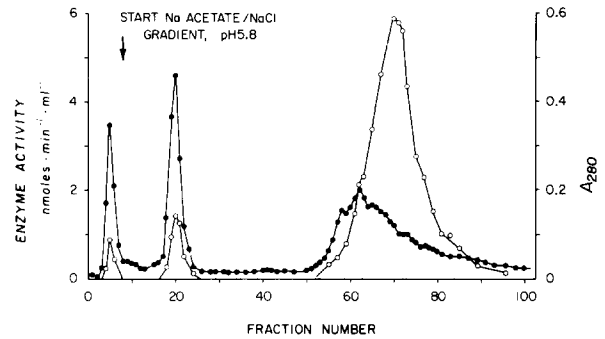

Fig. 4. CM-cellulose chromatography of thioltransferase. The high specific activity enzyme fraction from DEAE-cellulose chromatography (eluting with 0.055 M NaCl) was diafiltered, pH adjusted, and applied to a 1.8 X 11 cm CM-cellulose column equilibrated with 0.01 M NaOAc buffer, pH 5.8. A linear gradient of equal volumes (250 ml) of buffer alone and with 0.15 M NaCl was begun at fraction 8. Of the 375 enzyme units applied to the column, over 75% of the activity was recovered in the peak eluting at approx. 0.075 M NaCl. Fractions collected beyond fraction 100 are not shown since no further absorbance at 280 nm (●———●) or enzyme activity (○———○) was observed as the salt concentration and pH were increased. See text for further details on conditions and methods.

membrane and placed on a Sepharose 4B column (1.5 X 90 cm) previously equilibrated with 0.01 M phosphate buffer, pH 8.0/1 mM EDTA. The enzyme eluted as a single peak (K_d 0.607) but since no increase in specific activity was observed, this step was not retained in the purification procedure.

Polyacrylamide gel electrophoresis

The partial purification thus far described was monitored by SDS-polyacrylamide gel electrophoresis and is shown in Fig. 5. The possible involvement of disulfide/sulfhydryl equilibria in the protein conformation is demonstrated by comparing the gel patterns obtained when the protein was prepared for electrophoresis in the presence or absence of mercaptoethanol. It is also apparent from the figure that multiple bands are observable even in the CM-cellulose purified fraction. In this case, the predominant species with mercaptoethanol had an apparent molecular weight of 51 000 although bands at 90 000, 60 000, 40 000 and 28 000 were also visible. At this time, it is not known which bands may be subunits of active enzyme and which may be contaminants.

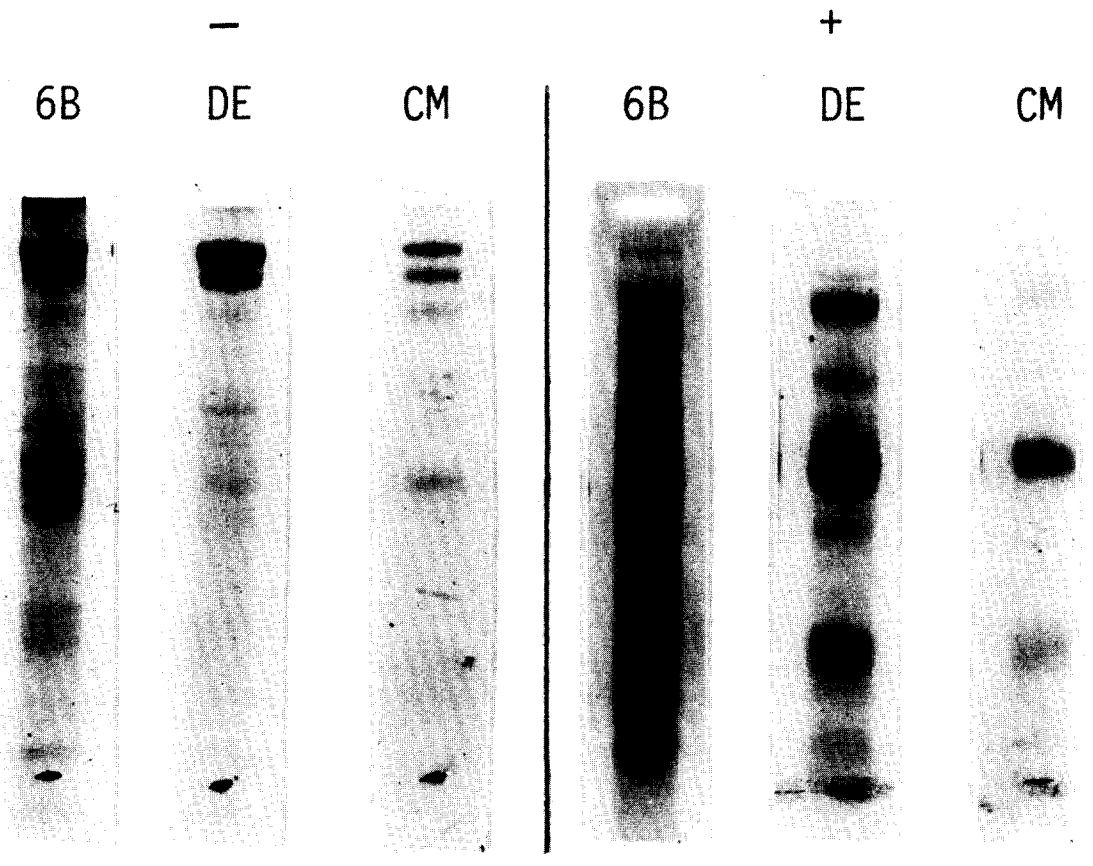

Fig. 5. Polyacrylamide gel electrophoresis of partially purified thioltransferase fractions in the presence or absence of mercaptoethanol. All methods and conditions for polyacrylamide gel electrophoresis, staining, and destaining using an LKB 2117 Multiphor are given in Materials and Methods. The specific activity of the enzyme fractions was 6.92 units/mg, 23.5 units/mg and 41.5 units/mg for fractions 6B (Sepharose 6B), DE (DEAE-cellulose), and CM (CM-cellulose), respectively, before overnight dialysis at 4°C against 0.01 M sodium phosphate, pH 7.1/0.1% SDS. The three gels on the left representing fractions solubilized at 100°C for 5 min in 1.5% SDS/0.01 M sodium phosphate, pH 7.1, are compared to those on the right which were solubilized in SDS buffer containing 0.15 M mercaptoethanol. For each sample, from 10–25 μg protein was applied. Protein standards (Pharmacia) were run simultaneously for molecular weight determinations. The India ink markers near the bottoms of the gels represent the migration of the bromphenol blue dye.

Isoelectric focusing of thioltransferase

Sepharose 6B enzyme was subjected to isoelectric focusing in the broad pH 3.5–10 range. As shown in Fig. 6, enzyme activity was observed both in the acid range (p*I* 4–6) and in a p*I* 7.0 peak. The scatter of points in the acid range was due to precipitation. Upon neutralization of these acid protein fractions, the turbidity disappeared and a more accurate specific activity of 3.75 units/mg was obtained. This was low compared to the specific activity of the p*I* 7.0 fraction (20.0 units/mg) but represented 31% of the total recovered activity (p*I* 7.0 = 44%).

The initial electrofocusing results seemed compatible with two major hypotheses; either the enzyme had two (or more) forms with large differences in net charge (greater than 1 pH unit), or the acidic p*I* form might represent irreversible denaturation of enzyme protein due to the acidic environment. Since over 75% of the enzyme activity was recovered after electrofocusing, it seemed unlikely that irreversible denaturation played an important role. In order to distinguish further between these possibilities, and determine if there were an equilibrium between the observed forms, we decided to re-electrofocus the

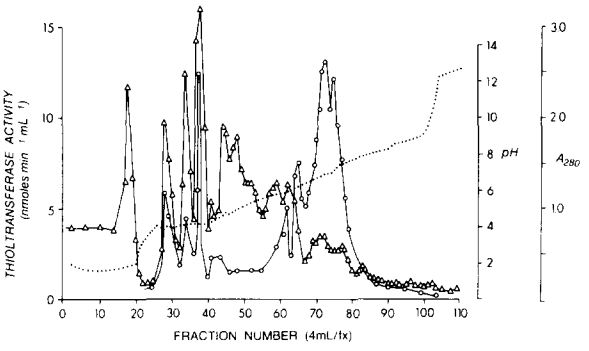

Fig. 6. Isoelectric focusing of Sepharose 6B thioltransferase. Details of the isoelectric focusing method and conditions are given in Materials and Methods. In this example, the anode was at the bottom of a 440 ml column, 1% Ampholine, pH 3.5–10 was used, and 175 mg enzyme (6.76 units/mg) were solubilized in the light Ampholine gradient solution. Focusing was begun with a maximum power limit of 15 W. After the first hour this was increased to 25 W and maintained until a maximum voltage of 1 800 V was reached. The voltage was held constant for the remainder of the 70-h run. Precipitation in the acidic region was observable after 30 min. o———o, thioltransferase activity; ·····, pH 3.5–10; Δ———Δ, A_{280}.

obtained peaks. If there were an equilibrium between the forms, then such re-electrofocusing would again result in an enzyme distribution with peaks of activity located in more than one p*I* region. This was the case, as is shown in Fig. 7. When the p*I* 4–6 peak was re-electrofocused for 41.5 h in a pH 3.5–10 gradient (Fig. 7, panel A), enzyme activity was again observed in both the acidic and neutral regions. As observed in the initial run, the specific activity in the acid region was markedly lower than that found at p*I* 7.0 (7.1 units/mg compared to 33.1 units/mg). The third protein peak, observed in the extreme alkaline portion of the gradient, is an artifact. This material represents precipitated acidic p*I* protein which had adhered to the walls of the column during the run. When the column was drained, this material remained stuck to the inner column wall until the alkaline portion of the gradient drained down to the level where it was adhering; at that time, the precipitate was solubilized and eluted at a position which falsely suggested an alkaline p*I*. In order to circumvent this problem when the initial p*I* 7.0 fraction was re-electrofocused, we placed the anode at the top of the electrofocusing column. The results of this experiment, shown in panel B of Fig. 7, demonstrate that both the acidic and the p*I* 7.0 fractions are in equilibrium, since re-electrofocusing of the p*I* 7.0 fraction produced peaks of enzyme activity at both p*I* values. This dramatic shift in p*I* values could be attributed to the exposure to the solution of additional charged moieties by an alteration in the redox state of key enzyme disulfides. Indirect evidence from separate

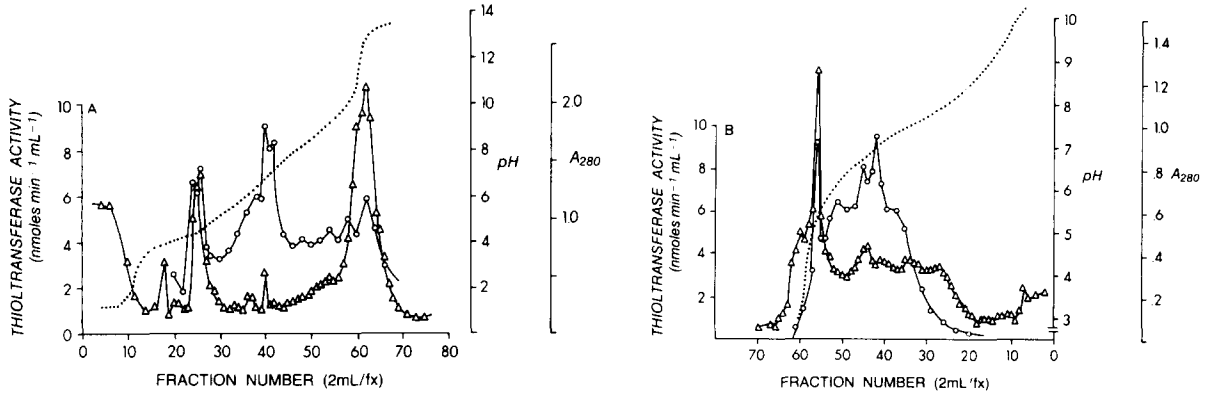

Fig. 7. Re-electrofocusing of thioltransferase fractions. Sepharose 6B enzyme was electrofocused as in Fig. 6, and an acidic p*I* fraction and a p*I* 7.0 fraction were isolated and re-electrofocused on 110 ml columns at 1 000 V constant voltage as described in the text. (A) The p*I* 4 fraction (80 mg) was re-electrofocused using 1% Ampholine (pH 3.5–10) for 41.5 h with the anode at the bottom. Precipitation was apparent in the acidic region after 30 min. (B) The p*I* 7.0 fraction (24 mg) was re-electrofocused using 1% Ampholine (pH 5–9) for 22 h. The cathode was placed at the bottom so acid precipitates would be at the top of the gradient and not solubilized by basic conditions on drainage. For ease of graphic comparison the data were plotted so the pH gradient was in the same order as in Fig. 7A. o———o, thioltransferase activity; ·····, pH; Δ———Δ, A_{280}.

experiments agree with this possibility. It was found that by lowering the pH of an enzyme preparation to 5.6, we could precipitate approx. 50% of the thioltransferase activity and that neutralization of the precipitate resulted in solubilization. When the same experiment was carried out in the presence of mercaptoethanol (0.4 M), however, only 18% of the starting enzyme activity precipitated. Inclusion of cystine in the solution did not effect the precipitation phenomenon. At present, we do not know whether the enzyme fraction which precipitates under acidic conditions in the test tube is equivalent to the p*I* 4–6 fraction from the electrofocusing experiments.

Properties of pituitary thioltransferase

The pH optimum of 8.2 for the enzyme is shown in Fig. 8. The study was carried out using either citrate phosphate, Tris-HCl or sodium bicarbonate buffers at concentrations of 50 mM. The enzyme was insensitive to the specific buffer ions used, since overlapping pH values with different buffers resulted in no significant change in activity. Histidine buffers were avoided due to the known nonenzymatic NADPH/thiol interaction in the presence of imidazole buffer [23]. Alterations in pH had no effect on the stability of substrates since only initial rates were utilized and exposure to extreme pH conditions was limited.

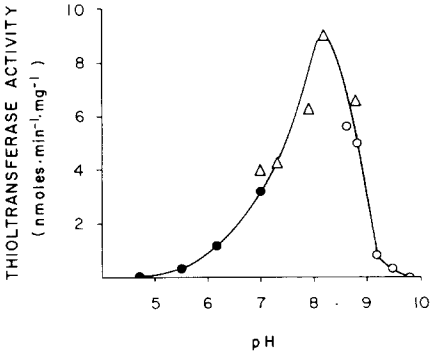

Fig. 8. pH dependence of thioltransferase activity. All methods and conditions were as described in the text using 50 mM buffers: sodium citrate phosphate (●———●) from pH 5 to 7, Tris-HCl (△———△) from pH 7 to 9, and sodium bicarbonate (○———○) from pH 8.7 to 9.75. Sepharose 6B enzyme (250 μg) was assayed in triplicate at each pH, and the average values are given.

In addition to insensitivity to phosphate, chloride and bicarbonate ions as shown in Fig. 8, NaCl concentrations up to 5 M were separately found to have no effect on activity. Also, as pointed out before, $(NH_4)_2SO_4$ did not inhibit the enzyme. On the other hand, as indicated in Table II, the presence of EDTA in the reaction resulted in a marked increase in activity, and the presence of zinc or copper was inhibitory. Table II also shows that zinc was the more potent inhibitor, and that *o*-phenanthroline, which has a higher affinity for zinc than EDTA, was more effective at reversing the zinc inhibition. EDTA, in turn, was a more potent enzyme activator than EGTA, but equipotent with *o*-phenanthroline, in the absence of added zinc. Calcium and magnesium at 1–5 mM did not significantly influence enzyme activity (data not shown). It should be noted that blank reactions (minus enzyme) were unaffected by EDTA, EGTA, *o*-phenanthroline or cations.

The substrate requirements for this enzyme are much more restricted for the thiol than the disulfide.

TABLE II

EFFECTS OF CHELATORS AND CATIONS ON PITUITARY GSH: DISULFIDE OXIDOREDUCTASE

The enzyme preprations usedin these experiments were pooled fractions from Sepharose 6B chromatography.

Experiment	Addition		Relative activity
1.	–		1.00
	EDTA	1 mM	2.03
	EGTA	1 mM	1.58
		10 mM	1.81
	o-phenanthroline	5 mM	2.03
	$ZnCl_2$	10 μM	0.18
	$ZnCl_2$ plus EDTA	10 μM	
		5 mM	0.39
	$ZnCl_2$ plus *o*-phenanthroline	5 mM	1.65
2.	–		1.00
	EDTA	1 mM	2.14
	$ZnCl_2$	5 μM	0.55
		10 μM	0.20
		20 μM	0.04
	$CuSO_4$	100 μM	0.77
		1 mM	0.35
	$CuSO_4$ plus EDTA	1 mM	
		1 mM	1.95

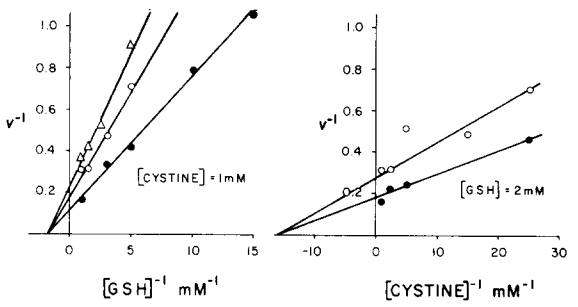

Fig. 9. *N*-Ethylmaleimide inhibition of thioltransferase activity: effect on kinetics with GSH and cystine as substrates. Sepharose 6B enzyme (1.1 mg) was incubated in a final volume of 0.75 ml with 0 (●——●), 0.1 (○——○) or 1 mM (△——△) *N*-ethylmaleimide for 10 min at 37°C, excess *N*-ethylmaleimide was removed by dialysis. K_m values for GSH (0.062 mM) and cystine (0.57 mM) were determined for the control and partially inhibited enzymes at saturating concentrations of the second substrate. v = nmol/min per mg protein.

There appears to be an absolute requirement for reduced glutathione and NADPH; other sulfhydryls (cysteine, CoASH, thioglycolate, mercaptoethanol and dithiothreitol) and NADH are ineffective. In contrast, the disulfide donor requirement is broad: cystine, homocystine, glutathione, growth hormone, prolactin and pituitary secretory granule membranes are all substrates [19,20]. As shown in Fig. 9, the apparent K_m values for reduced glutathione and cystine were approx. 0.57 and 0.06 mM, respectively, when the second substrate was held constant at saturating concentrations.

The involvement of sulfhydryl groups in activity was measured by inhibition studies using *N*-ethylmaleimide. Enzyme (1.1 mg) was incubated at 37°C for 10 min (pH 7.2) with varying concentrations of *N*-ethylmaleimide (0, 0.01, 0.1 and 1.0 mM) in a final volume of 0.75 ml. After excess *N*-ethylmaleimide was removed by dialysis, activity of the fractions was determined. Inhibition was not seen at 0.01 mM, but was 55 and 64% at the two highest ratios of *N*-ethylmaleimide/protein. K_m determinations on the partially inhibited enzyme are shown in Fig. 9, and suggest that *N*-ethylmaleimide is noncompetitive with respect to both GSH and cystine. Thus, the *N*-ethylmaleimide-reactive sulfhydryl group(s) critical for activity may not be at the active site per se but may be required to maintain the active conformation of the protein.

Discussion

These studies demonstrate the presence of a cytosolic thiol: disulfide oxidoreductase in the anterior pituitary, and report methods suitable for its partial purification and characterization. Its cytosolic localization, clearly established by the differential and density gradient centrifugation studies, shown in Fig. 1 and described in the text, demonstrate its separateness from a variety of other disulfide interchange enzymes which are membrane-associated [24–28]. The other major cytosolic thioltransferase activities which have been described are the hepatic enzyme described by Mannervik and his associates [14–16] and that described by Tietze [29]. The enzyme reported by the Swedish group is a glycoprotein of 11 000 molecular weight [15]. One of the implications of a cytosolic location is the rather wide access to potential substrates this affords: soluble compounds, as well as components of subcellular organelle-limiting membranes would be expected to be exposed to cytosolic enzyme in vivo. In contrast, such a location would not be expected of an enzyme playing a major role in degradative processes, since these are generally felt to be largely located within lysosomes. Since the enzyme we describe here can act on secretory granule membrane disulfides [20], it may have some relation to the secretory process.

The purification scheme outlined in this paper is straightforward and rather conventional. $(NH_4)_2SO_4$ precipitation and gel filtration chromatography, the initial steps, yielded a product with modestly enhanced specific activity, but separated from a variety of contaminating materials. The ion-exchange chromatographic steps demonstrated that significant charge heterogeneity prevailed following Sepharose 6B, but that little heterogeneity remained following the DEAE-cellulose step. Although the fraction chosen for additional purification after the DEAE-cellulose step represented only about 40% of the protein charged onto the column, total column recovery exceeded 90%; additional work will be required to establish the nature and extent of the other differences between the various DEAE-cellulose forms of the enzyme. This step represented the site of maximal loss of material, all the other procedures being associated with approx. 80% yields or better. An additional question which will require further

study is whether the DEAE-cellulose fractions bear a predictable correlative relationship with the fractions obtained on isoelectric focusing. At present, we have no data on this point.

Despite substantial purification based on several different physicochemical characteristics through five purification steps, polyacrylamide gel electrophoresis still showed heterogeneity of the enzyme preparation after CM-cellulose chromatography. At this stage, the preparation had been purified about 70-fold with an overall recovery of approx. 22%. The most prevalent stained protein species when specimens were run without mercaptoethanol had an apparent molecular weight of approx. 170 000; this result, agreeing with the non-denaturing Sepharose 6B estimate of size, suggested that the visualized protein did in fact represent the enzyme. Another possibility is that the enzyme is bound to a higher molecular weight material by $(NH_4)_2SO_4$-resistant mechanisms, or that the enzyme itself is not visualized on the gels. Judged in comparison to purification ratios achieved with many proteins, the present ratio of only 70-fold or so seems quite small; this may be interpreted as indicating rather inadequate purification, or alternatively, that the enzyme really represents a very substantial fraction of the cytosol protein. Until the enzyme is purified to homogeneity, this issue may not be fully resolvable; however, the available evidence suggests that the enzyme is an important component of the stained gel patterns. First, mercaptoethanol caused a major change in the electrophoretic pattern, indicating disulfide-bonded subunit structure, in all our enzyme preparations, from the crudest to the most purified. Second, mercaptoethanol but not cystine protected against acid precipitation of the enzyme. Third, acid exposure caused a major reversible conformational change altering the number of surface charges exposed to ampholyte during isoelectric focusing. Taken together, these data suggest but do not conclusively prove that the gel patterns in fact represent enzyme subunits.

The studies of pH dependence of enzyme activity shown in Fig. 8 provide additional evidence for differences between this enzyme and a number of others which have been isolated from various tissues, primarily liver. Ionic sensitivity, apparent molecular weight, substrate specificity, isoelectric point and subcellular location all demonstrate the separateness of this enzyme from others previously described [15,24,28,30].

The experiments summarized in Table II demonstrate the sensitivity of the enzyme to chelators and certain divalent cations. The relative potencies of both the activating chelators and the inhibitory cations suggest that the presumed metal binding site on the enzyme has a higher affinity for zinc than for copper, and does not bind calcium or magnesium.

Electrophoresis after exposure to mercaptoethanol, demonstrating major changes in the apparent molecular weights of the stained protein bands, suggests that the enzyme may possess a complex subunit structure held together by disulfides. There is precedent for thiol: disulfide oxidoreductases possessing their own disulfides, which may in fact play a role in the catalytic process [31]. Direct evidence that enzyme thiols may be required for expression of enzymatic activity was obtained from studies with *N*-ethylmaleimide (Fig. 9). These experiments indicated that presumably saturating concentrations of *N*-ethylmaleimide resulted in roughly 60% inhibition of enzyme activity. The kinetics indicated that this inhibition was non-competitive, however, and this observation, together with the fact that complete inhibition was not obtained, may indicate that the thiols titrated by *N*-ethylmaleimide are important for maintenance of molecular conformation optimal for catalysis rather than being involved in the catalytic site directly.

In summary, we have partially purified from pituitary cytosol a glutathione-dependent thiol: disulfide oxidoreductase. The enzyme appears to have a molecular weight of about 175 000 and a complex disulfide-linked subunit structure. Its pH optimum is about 8.2, and one or more enzyme thiols are required for optimal activity, though not located in the active site. Acid conditions inhibit activity and cause precipitation of the enzyme at pH 4.5–5.0, where a precipitated form is located in isoelectric focusing columns. This form is at least partly interchangeable with a p*I* 7.0 form. The relationships between the isoelectric focusing forms, the several forms eluting from DEAE-cellulose columns, and the acid precipitable form, remain to be established. The concentration of the enzyme in the pituitary will not be firmly established until we have purified it to homogeneity, but present data suggest the possibility

that this GSH: disulfide oxidoreductase could constitute as much as 0.5–1.0% of the cytosol protein.

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