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GLUTATHIONE-BOUND CELLULOSES: PREPARATION WITH THE LINKING REAGENT *s*-TRIAZINE TRICHLORIDE AND USE IN CHROMATOGRAPHY

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Glutathione has been covalently bound to cellulose via the linking reagent *s*-triazine trichloride (sTT) in three ways: the tripeptide through its sulfur and either reduced or oxidized glutathione through its amino nitrogen. The use of glutathione-bound cellulose in chromatography was studied with bovine serum albumin (BSA) and glutathione reductase. The enzyme was eluted with 1 M NaCl.

INTRODUCTION

A number of methods have been described in which thiols or disulfides including glutathione were covalently bound to solid supports (1–8). Our interest in glutathione-requiring proteins led us to search for a system in which glutathione could be bound through either its sulfur or amino nitrogen. In addition, a nonporous carrier was desired in order to alleviate any problems that might arise due to entrapment of substances within the gel pores. Cellulose activated by *s*-triazine trichloride (sTT) has been described and used for binding amino acids and proteins (9). This procedure has been utilized in preparing three cellulose derivatives of bound glutathione: one in which oxidized glutathione is bound through the amino group, and two others in which the tripeptide is bound through either the amino or thiol group.

MATERIALS

Chemicals were purchased from the following companies: *s*-triazine trichloride, Aldrich Chemical Company, Milwaukee, Wisconsin; reduced glutathione, oxidized glutathione, nicotinamide adenine dinucleotide phosphate (reduced form) and bovine serum albumin (BSA), Sigma

Chemical Company, St. Louis, Missouri; glutathione reductase, Boehringer–Mannheim Biochemicals, New York; dioxane and naphthalene (scintillation grade), Matheson, Coleman and Bell, Los Angeles, California; and 2,5-diphenyloxazole (PPO), Beckman Industries, Fullerton, California. ^{3}H-glutathione (250 mCi/mmol) was purchased from New England Nuclear Corporation, Boston, Massachusetts. The following celluloses were used: cellophane dialysis tubing ($1\frac{1}{8}$ in.), H. A. Thomas, Philadelphia, Pennsylvania; cotton, Parke Davis, Los Angeles; and fibrous cellulose (column Chromedia-Grade CF11), Whatman–Reeve Angel, Clifton, New Jersey. All other reagents were reagent grade.

METHODS

Activation of cellulose by sTT was carried out according to the procedure of Smith and Lenhoff (9). In the case of cellophane, the dialysis tubing was cut open and flattened between two pieces of Teflon mesh to provide for maximum exposure to the activating solution. Cotton and fibrous cellulose were transferred to the various solutions after filtration on coarse-grade sintered glass. Precautions were taken with fibrous cellulose particles because they tended to clump in the dioxane–xylene solvent; this difficulty was overcome by using 105–210 μm particles. The activated species will be referred to as sTT-cellophane, sTT-cotton, or sTT-cellulose. The various activated cellulosic species were either used immediately or stored in acetone for no more than 36 h prior to use.

Two control celluloses were prepared. Control A, used for experiments with ^{3}H-GSH, consisted of a cellulosic species that had been taken through the activation process in the absence of sTT. In the case of cellophane, control A was turbid and brittle as opposed to the transparent, flexible quality exhibited by sTT-cellophane.

Control B was sTT-cellulose that was allowed to remain in 0.1 M phosphate, pH 7.7, for 5–20 h, and then washed as described below. In the resultant triazine-bound cellulose, all reactive chlorines were replaced by hydroxyl groups (9,10). In an experiment designed to measure the binding of ^{3}H-GSH to sTT-cellulose, approximately 2% of the counts could be accounted for by reacting with this control. Control B contributed slightly to the ninhydrin assay, probably a reflection of the lability of the triazine nucleus when hydroxyl groups are present (10). Control B cellulose was also used in chromatographic studies to assess the effect of the triazine nucleus on the protein under study.

Prior to reacting with glutathione, the activated cellulosic species was hydrated with cold buffer. Attachment was carried out at room temperature.

The reaction was stopped by separating the cellulose from the reaction solution and washing extensively with 1 M NaCl to remove noncovalently bound material. After further washing with water, the derivatives were stored in water at 4°C.

Bound radioactive glutathione was measured in a Beckman Liquid Scintillation Counter (model LS-250). The cocktail consisted of 12.5% naphthalene + 0.75% 2,5-diphenyloxazole (PPO) in dioxane. An efficiency of 45% was used to convert CPMs to DPMs. This efficiency is based upon known standards and does not take into account quenching of radiation by the solid supports. When radioactive binding data are expressed as specific DPMs, they refer to control A. As pointed out by Smith and Lenhoff (9), exact quantification of the amount of radioactive peptide bound to cellulose is difficult because of the uncertain degree of quenching exerted by the solid support on the radioactive emissions. Nevertheless, it is possible to compare the amounts of bound radioactivity of a given substance under different sets of conditions, for example, pH (Fig. 2).

The α-amino groups were measured with ninhydrin (11) and the thiol groups with 5,5′-dithio bis(2-nitrobenzoic acid), hereafter abbreviated as DTNB (12,13). When assaying cellulose-bound glutathione, the particles were continuously agitated or rotated. The rate of reaction with DTNB was slower when applied to particle-bound thiol groups than when applied to thiols in solution. A 30-min reaction time was sufficient, but generally a full hour was allowed for the DTNB assay. The use of DTNB to quantify bound thiol groups has been described by Cuatrecasas (4).

Protein was measured by the procedure of Lowry et al. (14). Glutathione reductase (EC 1.6.4.2.) was measured by the method of Racker (15) using 0.05 M phosphate (Na^+), pH 7.4.

Chromatography was carried out at 4°C with polyethylene columns. Flow rates were one drop every 10–20 sec, and the volumes collected ranged from 0.6 to 1.5 ml per fraction. The amount of cellulose used in the columns is expressed as a dry weight. Such determinations were based on a calibration curve relating the dry weight of cellulose sample to its hydrated volume.

RESULTS

Reaction of Na_2S or GHS with Activated Cellulose

The binding of Na_2S and glutathione to the sTT-activated cellulose was investigated. Na_2S was chosen as a model compound because of the ultimate goal of attaching glutathione to the activated cellulose through sulfur. In addition, the reaction between sTT and Na_2S is accompanied by spectral changes that make this reaction a convenient one to follow.

sTT exhibits a λ_{max} at 242 nm ($E = 2 \times 10^3$) in aqueous solution (16). Cellophane activated with sTT exhibited a λ_{max} below 250 nm (Fig. 1a). Due to the intensive reaction with sTT, absorbance values below 250 nm could not be read accurately, but the spectrum turned out to be a good diagnostic aid to assess the extent of activation. When sTT was incubated with 0.1 M Na_2S at pH 9 for 0.5 h the λ_{max} occurred at 280 nm (Fig. 1b). The shift of the λ_{max} to higher wavelengths appears to be a property of triazine derivatives in which sulfur is bound to the carbon of the triazine ring (17), and the peak of 280 nm is in agreement with the spectral data reported by Hirt et al. (18) on trithiocyanuric acid. Cellophane activated with sTT also reacted with ^{3}H-GSH, although there was a high extent of nonspecific binding (about 30%) due perhaps to the physical alterations shown to exist in control A cellophane; see the Methods section.

To determine the optimal pH and incubation time for covalently binding ^{3}H-GSH, sTT-cotton was used to increase the surface area for binding sites and to eliminate the nonspecific binding of ^{3}-H-GSH seen with

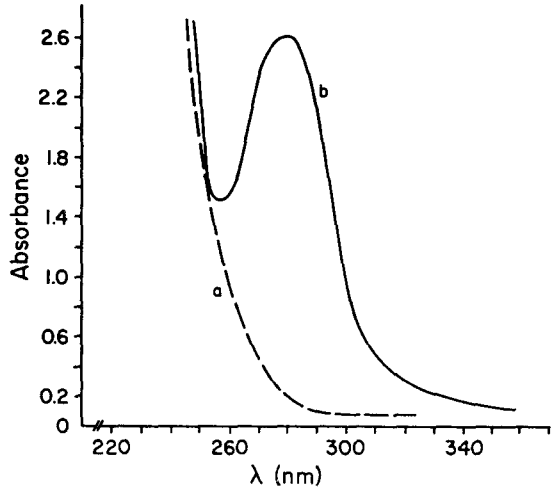


FIG. 1. Spectra of sTT-cellophane before and after reaction with Na_2S. The sTT-cellophane was prepared according to the procedure outlined in the Methods section and was stored for 1 day in acetone. Conditions of the reaction of sTT-cellophane with Na_2S: [Na_2S], 0.1 M; pH, 9; room temperature; 0.5 h. Solvent for absorption spectra: 0.05 M acetate (Na^+), pH 5.5. (a) sTT-cellophane; (b) reaction product of sTT-cellophane plus Na_2S. The light beam passed through one thickness of the cellophane (0.0016 mm). Untreated cellophane did not exhibit significant absorption between 230 nm and 320 nm.

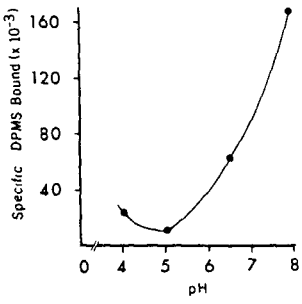


FIG. 2. pH dependence of ^{3}H-GSH binding reaction with sTT-cotton. The sTT-cotton was prepared as described in the Methods section. Conditions for ^{3}H-GSH binding: [^{3}H-GSH] = 8.0×10^{-7} M; weight of cotton, 50 mg; buffers, pH 4, 5 = 0.1 M acetate (Na^+) and pH 6.5, 7.7; 0.1 M phosphate (Na^+); 3 h at room temperature.

cellophane. Figure 2 shows that such binding was favored by an increase in pH. The binding of ^{3}H-GSH was substantially complete in 5 h. Essentially the same results were observed when sTT-cellulose was used.

Preparation and Analysis of Glutathione Bound to sTT-Cellulose

Glutathione Bound Through Sulfur. One hundred–milligram quantities of activated fibrous cellulose (see Methods) were hydrated briefly with 0.1 M phosphate, pH 7.7, and then added to 5 ml of 0.01 M GSH in the same buffer. When the reactants were mixed, the pH dropped immediately to 7.3 and remained there throughout the reaction. After 2–3 h, the reaction mixture was filtered through coarse-grade sintered glass; the derivative was washed three times with 1 M NaCl while on the filter, stirred for 15 min in 1 M NaCl, and filtered again. The process was repeated with water. Table 1 shows that there are insignificant quantities of thiol groups present in this derivative either before or after DTT treatment, whereas large amounts of amino groups were observed. Hence, this derivative probably has

TABLE 1. **Functional Group Analysis of GSH(S)-sTT-Cellulose**[a]

Group[b]	nmol/mg conjugate	
	Prep 1	Prep 2[c]
Amino	11	22
Sulfhydryl before DTT	undetectable	undetectable
Sulfhydryl after DTT	0.17	0.87

[a] Conditions for binding reaction: Room temperature, 0.1 M phosphate (Na^+), pH 7.3, 2 h (Prep 1), or 3 h (Prep 2).

[b] Amino and sulfhydryl group analyses are described in Methods. Dithiothreitrol reduction is described in the text.

[c] A control was also analyzed at the same time. It consisted of control A (see Methods) which was then reacted with GSH under the conditions used for Prep 2 (see text). When analyzed for amino and thiol groups, values of 1.3 nmol/mg and less than 0.07 nmol/mg, respectively, were found.

glutathione bound to the triazine nucleus through its sulfur, and will subsequently be referred to as GSH(S)-sTT-cellulose.

Oxidized Glutathione Bound Through Nitrogen. Activated fibrous cellulose (100 mg) was hydrated briefly with cold 0.1 M phosphate, pH 7.7, and then added to 5 ml of 0.01 M GSSG in the same buffer. The pH immediately dropped to 7.3 but did not change from this value during the reaction. After 10–20 h with constant stirring, the reaction was terminated as described above. The final product, GSSG(N)-sTT-cellulose, was stored in H_2O at 4°C until further use.

This derivative contained 4–7 nmol α-amino groups per milligram of dried conjugate (six preparations). Thiol group analysis following dithiothreitol (DTT) reduction [used for the subsequent preparation of GSH(N)-sTT-cellulose] showed that the concentration of bound thiol groups was greater than the concentration of bound α-amino groups in the original GSSG(N)-sTT-cellulose. The increase amounted to 12–54% in five preparations. Such results could be obtained only if (a) GSSG(N)-sTT-cellulose contained a cross-linked species and (b) the C(triazine)–N(GSSG) bond was stable to the conditions of the ninhydrin assay.

Reduced Glutathione Bound Through Nitrogen. GSSG(N)-sTT-cellulose was reduced with 10^{-2} M DTT in 0.1 M phosphate, pH 7.7, for 30 min. The DTT was removed by filtration and extensive washing with 1 M NaCl and H_2O. Control celluloses were treated in an identical way in order to check that DTT was removed by the wash procedure. Alternatively, the filtrate can be tested for the presence of thiol groups with the DTNB reagent.

The glutathione celluloses were stored in water at 4°C. After 6–8 weeks, about 70% of the original peptide was found bound to GSH(S)-sTT-cellulose and GSSG(N)-sTT-cellulose when they were measured for bound α-amino groups (11).

Chromatographic Studies. Glutathione celluloses were examined for their chromatographic properties with two purified proteins, bovine serum albumin (BSA) and yeast GSSG reductase (EC 1.6.4.2.), each possessing a pI between 4 and 5. BSA was chosen because it exhibits no biochemical specificity for GSH or GSSG and might thus serve as a control. The GSSG reductase does bind GSSG, and its behavior in a different chromatographic system involving immobilized GSSG has been described (6).

Bovine Serum Albumin. Bovine serum albumin (0.58 mg in 0.5 ml of 0.05 M phosphate, pH 6.5) was placed on a column (1.5 cm × 0.9 cm) of control B cellulose, GSH(S)-sTT-cellulose or GSSG(N)-sTT-cellulose (Fig. 3). All elutions were carried out at 4°C with 0.05 M phosphate (Na^+) pH 6.9 (control B and GSH(S)-sTT-cellulose) or pH 7.4 GSSG(N)-sTT-cellulose. The total recoveries were 96% from control B cellulose, 88% from GSH(S)-sTT-cellulose, and 98% from GSSG(N)-sTT-cellulose.

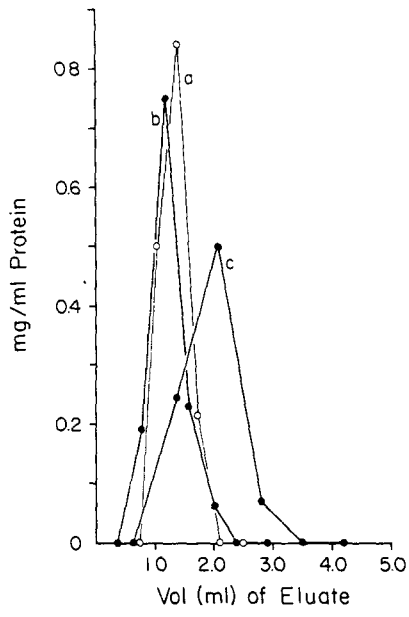


FIG. 3. Elution profile of bovine serum albumin. (a) Control B cellulose: 91 mg; $d = 9$ mm. (b) GSH(S)-sTT-cellulose: 104 mg containing 1.5 μmol GSH; $d = 9$ mm. (c) GSSG(N)-sTT-cellulose: 144 mg containing 0.62 μmol GSSG; $d = 9$ mm. 0.58 mg of BSA was applied to each column. The weight of cellulose was determined as described in the Methods section.

Glutathione Reductase. A dialyzed (0.05 M phosphate, pH 7.4) preparation of glutathione reductase containing 3.6 units of activity was placed on a column of control B cellulose or GSSG(N)-sTT-cellulose. The elution profile for each column is shown in Fig. 4. Using control B cellulose, 93% of the units applied were recovered by eluting with 0.05 M phosphate (Na^+), pH 7.4, at 4°C, whereas none was eluted from the GSSG(N)-sTT-cellulose. Application of 1 M NaCl resulted in the recovery of greater than 70% of the enzyme from the GSSG(N)-sTT-cellulose column. Recoveries of greater than 85% have also been obtained. No enzyme elution was observed in the presence of 3.3×10^{-3} M GSSG or 10^{-3} M DTT.

When GSSG reductase was applied to GSSG(N)-sTT-cellulose in the presence of 3.3×10^{-3} M GSSG, enzyme activity was retarded just as it was when GSSG was omitted during application. It was then eluted with 1 M NaCl.

GSSG reductase was also applied to a column of GSH(N)-sTT-cellulose. Figure 5 shows that the enzyme adsorbed to this column. As with GSSG(N)-sTT-cellulose, elution was achieved with 1 M NaCl.

The tightness of binding of GSSG reductase to GSSG(N)-sTT-cellulose appears to be influenced by the amount of GSSG bound to the matrix. In general, with columns consisting of 4 nmol/mg conjugate or less, successful elution of the enzyme was obtained. The use of columns containining a higher degree of substitution frequently resulted in no elution with 1 M

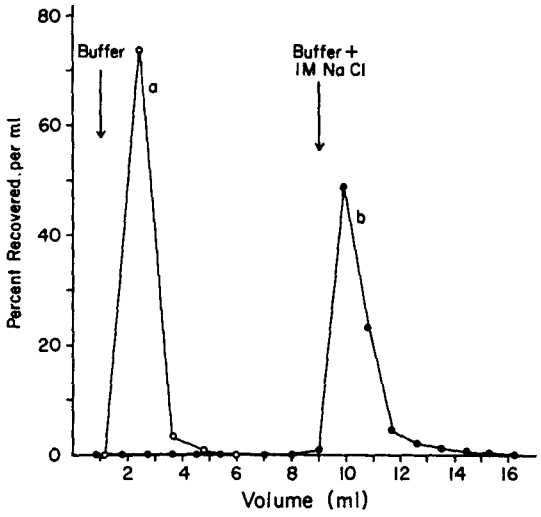


FIG. 4. Elution profile of glutathione reductase. (a) Control B cellulose: 139 mg, $d = 9$ mm. (b) GSSG(N)-sTT-cellulose: 139 mg containing 0.23 μmol GSSG, $d =$ 9 mm. Buffer: 0.05 M phosphate (Na^+), pH 7.4. GSSG reductase was stable in the presence of 1 M NaCl.

NaCl. Obtaining celluloses with 4 nmol/mg conjugate or less was accomplished by carrying out the GSSG attachment reaction for no more than 5 h.

DISCUSSION

Three types of glutathione-bound celluloses have been prepared utilizing the linking reagent *s*-triazine trichloride. They are the tripeptide

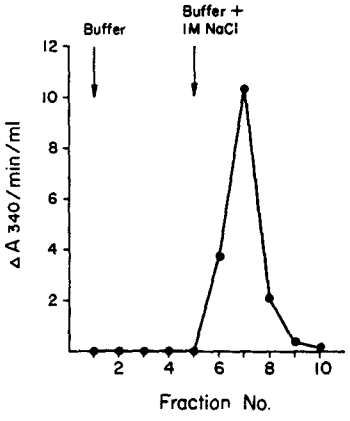


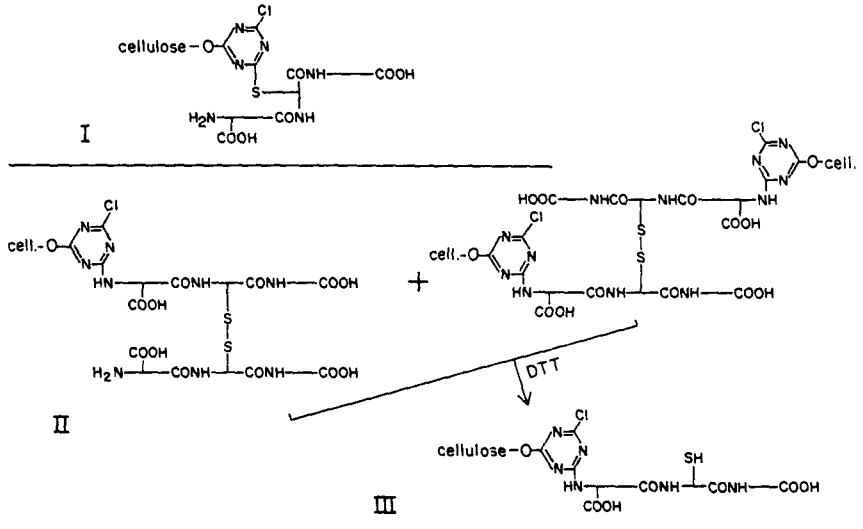
FIG. 5. Elution profile of glutathione reductase on GSH(N)-sTT-cellulose. Glutathione reductase (3.1 units) was applied to a column (9 mm in diameter) of GSH(N)-sTT-cellulose (see text) containing 0.85 μmol of bound GSH. Buffer: 0.05 M phosphate (Na^+), pH 7.4; 1.1 ml/fraction.

glutathione bound through its sulfur or free amino group, and oxidized glutathione bound through its free amino group.

Another system in which GSH has been bound through its sulfur to a nonporous support has been described (7). In this case, the product was a result of reaction between GSH and a polymer-bound thiol reagent, *N*-phenylenemaleimide. The analogous derivative described in the present paper was obtained using the less specific reagent, *s*-triazine trichloride, and carefully controlling the conditions. Thus, by carrying out the binding reaction between sTT-cellulose and GSH at pH 7.3 for 2–3 h, attachment through sulfur was assured (see Table 1). The use of a higher pH (see Fig. 2) would result in a higher content of bound GSH, but the use of a high pH was avoided because GSH is autooxidizable at an alkaline pH. A separate measurement showed that at pH 7.3, 90% of the glutathione remained reduced for 3 h.

The quantities of GSH and GSSG bound to cellulose are less than those reported for bound thiols and disulfides in other systems (1–7). The lowered degree of glutathione binding may be a consequence of the nonporosity of the cellulose matrix.

The nature of the linkage between glutathione and the triazine nucleus was investigated by the use of amino and thiol group analyses. The structures that are consistent with the data obtained from these measurements (see the Results section) are diagrammed below.



I. GSH(S)-sTT-cellulose
II. GSSG(N)-sTT-cellulose
III. GSH(N)-sTT-cellulose
Only the functional groups of GSH and GSSG are shown

It is improbable that *reactive* chlorines remain on the triazine ring after the various glutathione celluloses have been prepared. [The chlorine shown in the structure is unreactive (10,19).] Control B cellulose represents the control used for the preparation of glutathione cellulose. If reactive chlorines had withstood the treatment, the control derivatives should have bound substantial amounts of protein. That this was not the case is shown by its inability to retard either BSA or GSSG reductase under conditions in which GSSG(N)-sTT-cellulose did retard the enzyme. These observations are consistent with the knowledge of both the susceptibility of sTT to hydrolysis in aqueous solution (9,10) and the unreactivity of the third chlorine (10). Also, the ability to elute GSSG reductase with NaCl (Fig. 4) shows that the protein was not bound covalently to the triazine nucleus.

The exclusion of bovine serum albumin by control B cellulose shows that the presence of the triazine nucleus itself did not cause retention of the albumin molecule. Although triazine is aromatic and should provide a hydrophobic environment, hydroxyl groups decrease aromaticity of the triazine ring due to the ketone–enol equilibrium that favors the ketone form (10). Because of the uncertainty of the extent of hydrolysis it is difficult to assess to what extent the triazine nucleus in control B cellulose would contribute to favorable hydrophobic interactions with BSA. Such interactions have been shown to operate during the chromatography of BSA on CNBr-activated agarose to which 4-phenylbutylamine was bound (20).

That BSA did not bind to either GSH(S)-sTT-cellulose or GSSG(N)-sTT-cellulose indicates that neither of the linked peptides caused non-specific binding of this protein. Yet, at the pH values of the experiments, the carboxyl groups were negatively charged, and the behavior of BSA on these columns may be due to charge repulsion.

As with BSA, the exclusion of GSSG reductase by control B cellulose shows that subsequent retention of enzyme activity by GSSG(N)-sTT-cellulose is not due to a property of the triazine nucleus. It may be argued that the retention of reductase activity by GSSG(N)-sTT-cellulose and its elution with 1 M NaCl are reflections of the ion-exchange properties of the column because at pH 7.4 the carboxyl groups of GSSG will be carrying a negative charge. The pI of the enzyme, however, is 4.8 (Boehringer–Mannheim Corp., personal communication). Thus, at pH 7.4 repulsion of enzyme would be expected if binding occurred only through non-specific electrostatic interactions.

Failure to elute GSSG reductase with GSSG at a concentration greater than its K_m (21) might suggest that the interaction between GSSG(N)-sTT-cellulose and the enzyme is not biospecific. Recent evidence (to be reported), however, shows that the binding of GSSG reductase to GSSG(N)-sTT-cellulose does exhibit biospecificity.

The inability to elute GSSG reductase with free GSSG, in agreement with the results of Harding (6), may be explained in at least two ways. The rate of binding of the enzyme to GSSG(N)-sTT-cellulose may be greater than its rate of dissociation (22). Since no NADPH was present during application and subsequent elution, such binding must occur with the oxidized form of the enzyme. That GSSG does bind to the oxidized form of the enzyme has been shown (23). It is also possible that the affinity of GSSG reductase for GSSG bound to sTT-cellulose is greater than its affinity for free GSSG. Consistent with this hypothesis is the observation that retardation of enzyme activity also occurred when GSSG was present during application of the reductase to the column.

The tight rebinding of GSSG reductase to GSH(N)-sTT-cellulose may explain the inability to elute GSSG reductase with dithiothreitol (DTT). DTT treatment of GSSG(N)-sTT-cellulose leads to the formation of GSH(N)-sTT-cellulose, and Fig. 5 shows that GSSG reductase also binds to this derivative. Such data are consistent with the observations that GSH binds to the soluble enzyme (21,24).

In conclusion, the binding of GSSG reductase to GSSG(N)-sTT-cellulose has been demonstrated and shown not to be a result of nonspecific electrostatic interactions alone. Elution with 1 M NaCl, but not with free GSSG, is consistent with results obtained by other workers (6,22) and may be explained by the higher affinity of GSSG reductase for the bound GSSG relative to its affinity for soluble GSSG.

ACKNOWLEDGMENT

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