# SYNTHESIS OF A MIXED DISULFIDE OF EGG WHITE LYSOZYME AND GLUTATHIONE – A MODEL SUBSTRATE FOR ENZYMATIC REDUCTION OF PROTEIN MIXED DISULFIDES

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## 1. Introduction

A considerable proportion of the endogenous GSH has been demonstrated to be bound as mixed disulfides to proteins in cells [1–4]. The functional importance of such mixed disulfides is so far not well established, but one function which can be proposed is the modulation of the biological activity of specific proteins. Another possibility is that protein mixed disulfides can serve as a reservoir of GSH, which can regulate the concentration of mobile GSH. Such a system involving free and protein-bound GSH has the capacity to act as a redox buffer.

However, the study of the biological function of protein mixed disulfides has been limited by the lack of suitable experimental systems. Current research in our laboratory aims at the elucidation of the biochemical reactions which effect the release of GSH from protein mixed disulfides. The present paper describes the synthesis of a mixed disulfide of lysozyme and GSH, which is suitable as a model compound in such studies, and recent work [5] describes how this compound can be reduced by an enzyme present in rat liver cytosol.

## 2. Materials

### 2.1. *Commercial products*

The following products were obtained from the manufacturers indicated: egg white lysozyme (Serva); dithioerythritol and GSH (Sigma); [$^{35}$S] GSH (Schwarz); Sephadex G-50 Fine and G-75 Fine (Pharmacia); PM 10 membrane filters (Amicon).

### 2.2. *Synthesis of the mixed disulfide of lysozyme and GSH*

Lysozyme (1.4 g) and dithioerythritol (0.31 g) were dissolved in 10 ml 10 mM glycine–NaOH (pH 9.6) containing 8 M urea and the solution was incubated for 2 hr at 30°C. Dithioerythritol and its oxidized form were removed by gel filtration on a 250 ml column of Sephadex G-50 equilibrated with the urea-containing glycine buffer. Reduced lysozyme was collected in about 100 ml of effluent, which was immediately added drop-wise to a stirred solution of GSSG. The GSSG solution (10 ml) in 8 M urea had previously been adjusted to pH 9.6 and contained 2 g of GSSG synthesized by iodate oxidation of GSH (cf. [6]). The reaction was allowed to proceed for 15 hr, and the mixture was then acidified to pH 3 with 1% acetic acid and concentrated to 15 ml by ultrafiltration on a PM 10 membrane. The mixed disulfide of lysozyme and GSH was separated from low mol. wt compounds on a 300 ml Sephadex G-50 column equilibrated with 1% acetic acid.

Labeled mixed disulfide was synthesized on a smaller scale from [$^{35}$S] GSSG (prepared by aeration of an alkaline solution [$^{35}$S] GSH) and reduced lysozyme principally as described above.

### 2.3. *Enzymes*

Rat liver glutathione reductase (spec. act. >200 units/mg) was prepared according to Carlberg and Mannervik [7]. A thioltransferase (for nomenclature, see [8]) active with GSH and *S*-sulfocysteine or GSH and the mixed disulfide of lysozyme and GSH was purified by the same procedure [7] through step 4. The last step separated completely the thioltransferase from any

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glutathione reductase activity. The overall purification was about 300-fold.

## 3. Methods

The concentration of solutions of lysozyme was determined spectrophotometrically on the basis of $E^{1\%}_{280} = 26.35 \pm 0.18$ and a mol. wt of 14 400 [9]. It was assumed that the extinction coefficient of GSH-containing derivatives of lysozyme was not significantly different from that of lysozyme.

Quantitative determination of disulfide bonds in mixed disulfides of GSH and proteins was based on reduction with $NaBH_4$ followed by determination of acid-soluble sulfhydryl groups: solid $NaBH_4$ was added in a 2000-fold molar excess of the estimated maximal amount of disulfide bonds in a 1 ml sample, which had been adjusted to pH >9 with NaOH. After 15 min 5 ml 20% trichloroacetic acid was added, and the precipitated protein was then removed by centrifugation. The concentration of sulfhydryl groups in the centrifugate was determined by transferring an aliquot (0.1 ml) to a mixture of 0.7 ml 0.2 M sodium phosphate pH 7.6 and 0.2 ml 10 mM 5,5′-dithiobis(2-nitrobenzoate). In the calculations an extinction coefficient of 13 600 $M^{-1}$ $cm^{-1}$ was used for the reduced thiol reagent [10].

Determination of radioactivity was made by liquid scintillation counting in Bray's solution [11], and the specific radioactivity of [$^{35}$S] GSH was obtained from the ratio of radioactivity to sulfhydryl titer of GSH solutions. GSSG, which was present in [$^{35}$S] GSH solutions, was reduced enzymatically before determination of the sulfhydryl content.

## 4. Results

A mixed disulfide of lysozyme and GSH was obtained in 18% yield. Freshly prepared mixed disulfide was homogeneous in gel electrophoresis (fig.1) and its electrophoretic mobility showed that the derivative was more acidic than native lysozyme, as expected after incorporation of GSH. The amounts of GSSG and GSH remaining in the preparation were determined (after precipitation of the protein with trichloroacetic acid) with glutathione reductase and Ellman's method [10], respectively, and were found to be $\leq 0.0075$ and

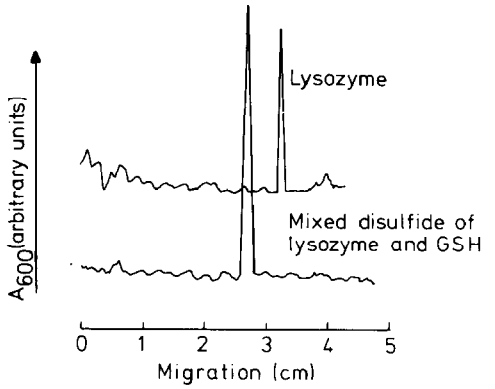


Fig.1. Polyacrylamide gel electrophoresis of lysozyme and its mixed disulfide with GSH. The proteins (about 40 μg) moved towards the cathode at pH 4.3 (system 7 of table 4 in [21]). The gels were analyzed in a gel scanning apparatus after staining with Coomassie Brilliant Blue G-250.

$\leq 0.005$ mol per mol of lysozyme mixed disulfide. The sulfhydryl titer of the protein was 0.007 mol per mol.

The disulfide content of the freshly prepared lysozyme derivative was found to be 7.54 mol per mol indicating that all 8 cysteinyl residues of the protein had been linked to GSH. In the radioactive derivative of lysozyme a content of 6.0 mol of [$^{35}$S] GSH per mol was determined. The difference between the two estimates of the GSH content is probably due to underestimation of the radioactivity caused by precipitation of protein and consequent low counting efficiency. Lack of radioactive material prevented new radioactivity determinations in the presence of solubilizing agents.

Aged preparations of the mixed disulfide of lysozyme and GSH contained several components, which were separable by disc gel electrophoresis. Gel filtration on Sephadex G-75 in the presence of reference proteins (lacking sulfhydryl groups) separated these components into three distinct fractions: one appearing in the void volume (presumably containing oligomeric and polymeric lysozyme-GSH derivatives), the remaining two appearing at effluent vols corresponding to mol. wts of 16 500 and 33 000, respectively. These components correspond to a monomeric lysozyme derivative containing 8 GSH residues (calculated mol wt 16 800) and a dimer linked by one disulfide group, which substitutes for one GSH residue in each of the monomer

moieties (calculated mol. wt 33 000). The elution profiles of the lysozyme-GSH derivatives were determined by use of the ability of these compounds to serve as substrate in a thioltransferase assay (see below). The disulfide content of the monomer was found to be 7.65 mol per mol in agreement with the value of the freshly prepared mixed disulfide.

Frozen solutions (in 1% acetic acid) of the monomeric form of the mixed disulfide of lysozyme and GSH could be stored at −30°C for some weeks without noticeable decomposition. After longer periods of time evidence for dismutation of the disulfide was obtained by demonstration of the presence of GSSG, which was assayed with glutathione reductase. At a concentration of 25 μM the lysozyme derivative formed a heavy precipitate within a few minutes when the pH value was raised to 7.6, but lower concentrations could be used in enzymatic assays.

The lysozyme derivative was tested as a substrate for yeast glutathione reductase. In a reaction medium (30°C) containing 0.15 M sodium phosphate (pH 7.6), 1 mM EDTA, and 0.1 mM NADPH, 5 μM lysozyme mixed disulfide gave no detectable activity (< 2 nmol reduced per min per nmol glutathione reductase). Neither could any activity be demonstrated with highly purified rat liver glutathione reductase [7].

On the other hand the lysozyme derivative was a good substrate for the thioltransferase [12] from rat liver which catalyzes the reaction between disulfides or thiosulfate esters and GSH [13,14]. The activity of highly purified thioltransferase with 5 μM lysozyme derivative was about the same as that obtained with 0.5 mM *S*-sulfocysteine. The progress curve of the reaction with the lysozyme derivative was biphasic, and demonstrated an initial rapid rate followed by a slower reaction velocity. This finding indicates that more and less reactive disulfide bonds are present in the protein. When the reaction was allowed to go to completion in the presence of glutathione reductase and excess of NADPH, the total number of disulfide bonds reduced per mol of lysozyme derivative was about 3. Thus only about half of the disulfide bonds are accessible for reduction by GSH and the thioltransferase.

The oligomeric forms, obtained by dismutation of the mixed disulfide, were also active as substrates for the thioltransferase. This was demonstrated after fractionation of the dismutation products on Sephadex G-75 (see above). A quantitative comparison of the ability of the different lysozyme components to serve as substrates has not been made so far.

## 5. Discussion

A mixed disulfide derivative of lysozyme has previously been prepared, in which the low molecular weight moiety was cysteine [15]. Neither this derivative nor the one described in the present paper has been detected in biological specimens, but of the two mixed disulfides the glutathione derivative of lysozyme can be assumed to have more properties in common with naturally occurring mixed disulfides of proteins, which are believed to have glutathione as the predominant thiol component. Other protein mixed disulfides involving GSH have been prepared from hemoglobin [16–18], serum albumin [4,19], and lens crystallins [20]. These derivatives are less suitable than the lysozyme compound for the study of general reaction pathways of interactions between protein disulfide groups and GSH, since they are not equally well characterized chemically, and in the case of hemoglobin, a high absorbancy limits spectrophotometric measurements. Recent work in our laboratory [5] demonstrates the usefulness of the mixed disulfide of lysozyme and GSH for the investigation of the biological reduction of protein mixed disulfides.

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