Glutathione Disulfide Inactivates, Destabilizes, and Enhances Proteolytic Susceptibility of Fructose-1,6-bisphosphate Aldolase*

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Disulfides (glutathione disulfide, cystine, cystamine) caused a first-order inactivation of rabbit-muscle fructose-1,6-bisphosphate aldolase at pH values of 7.4 and above. Inactivation by glutathione disulfide was partially reversed by reducing agents, but the enzyme became irreversibly inactivated with time. The disulfide-inactivated aldolase had a lower transition temperature and enthalpy of denaturation than the native enzyme. In addition, the disulfide-inactivated enzyme was extensively degraded by proteinases, whereas the native enzyme was resistant. Mixed disulfides were formed; a maximum ratio of 4–5 mol of glutathione/mol of the aldolase tetramer was found. The number of titratable —SH groups on aldolase decreased by 16 (out of 32 total) on the control enzyme) after inactivation by glutathione disulfide, indicating that other oxidation reactions in addition to those resulting in mixed disulfides occurred. The substrate, fructose 1,6-bisphosphate, prevented inactivation of aldolase by glutathione disulfide, the formation of glutathione-enzyme mixed disulfides, thermodynamic destabilization of the enzyme, and a decrease of —SH groups on the enzyme. These data indicate that covalent modification of aldolase by biological disulfides is important in modulating enzyme stability and vulnerability to proteinases as well as enzyme activity and that the substrate protects against modification by disulfides.

Modulation of enzyme activity by covalent modification is well documented, and the importance of this phenomenon in biological regulation is established. There is increasing interest in the role of covalent modification as an initial event in the degradation of enzymes and consequently in the regulation of enzyme concentration in cells. For example, Levine et al. (1), Levine (2), Bernlohr and Switzer (3) have evidence that oxidative reactions occur as initial events in the degradation of specific bacterial enzymes; Ciechanover et al. (4) proposed a covalent modification of proteins in a reaction with ubiquitin as a first step in degradation, and Francis and Ballard (5) proposed that reactions of cystine with proteins initiate the degradation of specific liver proteins. The latter modification involves the enzymatically catalyzed formation of a cysteine-protein mixed disulfide. There is evidence now that glutathione-protein mixed disulfides also form in cells and that the thiol/disulfide exchange reactions may be important in regulating several enzyme activities (6–9). In fact, biological disulfides have been referred to as "the third messenger" (9) and were suggested to have wide-ranging effects on metabolism. Fluctuations of thiol/disulfide ratios in vivo reflect changes in the redox state of cells, and several observations indicate that the rate of proteolysis in cells increases with an increase in the degree of oxidation. For example, in perfused cardiac tissue and diaphragm when the tissue was in a more reduced state, the overall rate of protein degradation was decreased (10, 11). Thus, there are indications that the state of oxidation of proteins in cells plays a determinative role in the degradation of intracellular proteins and that the molecular basis for some of these observations involves the formation of mixed disulfides.

To gain insight into how disulfides might influence the rate of proteolysis of cytosolic proteins, the effects of glutathione disulfide and other biological disulfides on fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) were examined. Aldolase is a good model for a relatively long-lived cytosol enzyme of mammalian cells. Its half-life in rabbit heart and soleus muscle or rat muscle has been estimated at 1–2 days (12, 13). Although the native enzyme is readily inactivated in vitro by severe proteases (cathepsins B, D, and L, papain, chymotrypsin, subtilisin, carboxypeptidase A, Meprin), only the C-terminal region (up to 20 amino acids) of the native form is readily vulnerable to proteolysis; this limited proteolysis causes a specific loss of activity with fructose 1,6-bisphosphate, while activity with fructose 1-phosphate is maintained (14–17). The bulk of the native enzyme and the partially degraded enzyme are equally resistant to thermal and urea denaturation and to purified proteases and mixtures of proteases (18). The goals of the present work were to determine how biological disulfides affect aldolase activity, structure, and proteolytic susceptibility. In addition, we asked whether glutathione disulfide actually formed mixed disulfides with the protein or whether it brought about other oxidative reactions with —SH groups in the aldolase molecule. We found that disulfides decrease aldolase activity towards fructose 1,6-bisphosphate and fructose 1-phosphate, destabilize and increase the susceptibility of the protein to proteinases, and cause formation of some mixed disulfides. Fructose 1,6-bisphosphate protects the enzyme from reactions with disulfides and all the subsequent changes.

EXPERIMENTAL PROCEDURES

Materials — Rabbit-muscle aldolase (specific activity, 17 µmol of fructose 1,6-bisphosphate cleaved min⁻¹ mg⁻¹) was purchased from Sigma. Human-liver cathepsins D and B, prepared by the method of

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Barrett (42) was the gift of Dr. Alan J. Barrett (Strangeways Laboratory, Cambridge, United Kingdom). Meprin, a metalloendoproteinase from mouse renal tissue, was purified by Dr. John D. Shannon. a-Chymotrypsin, crystallized three times from bovine pancreas (type II), was purchased from Sigma. Glutathione (specific activity of 934 mCi/mmol) was purchased from New England Nuclear. All other chemicals were obtained from Sigma, except where noted.

**Preparation and Assay of Aldolase**—Minor protein contaminants of aldolase preparations were removed by cellulose phosphate chromatography, and the protein concentration and activity were determined as previously described (18). Anti sera to rabbit-muscle aldolase were prepared in cooters, and immunoreactivity of preparations was tested on Ouchterlony plates as previously described (18).

**Titration of —SH Groups**—The number of reduced —SH groups in native or oxidized aldolase was determined using DTNB prepared on a molar absorptivity for 2-nitro-5-thiobenzoate of 13,600 M⁻¹ cm⁻¹ at 412 nm (20). The number of —SH groups was determined both under denaturing conditions (9.25 M glycine HC1 buffer, pH 3.5, and a final concentration of 1% sodium dodecyl sulfate) and non denaturing conditions (0.05 M Tris-Cl buffer, pH 7.5).

**Measurements of Glutathione-Protein Mixed Disulfides**—Radiola beled glutathione disulfide was prepared from L-[glycine-2,3H]glutathione (reduced) by oxidation with H₂O₂. Briefly, a glutathione solution (4 mg/ml) was adjusted to pH 5.5 with 0.1 M Ba(OH)₃. A slight excess of H₂O₂ was added until the glutathione solution no longer reacted with DTNB. The solution was neutralized with H₂SO₄, and the resulting BaSO₄ precipitate was removed by filtration. The filtrate was concentrated by lyophilization and subjected to Sephadex G-16 column (1.5 × 25 cm) chromatography. The eluate was monitored at A₂₅₀, and the peak corresponding to authentic glutathione disulfide was collected and lyophilized. Samples of the product were solubilized in 50% pyridine and subjected to thin-layer chromatography in butanol:pyridine:acetic acid:water (45:30:9:6). The product identified as one spot with fluorescamine (Pierce Chemical Co.) had an RF value consistent with that of Aldolase.

**Interaction of Biological Disulfides with Aldolase**

Aldolase was inactivated by glutathione disulfide in a first-order manner, and inactivation was completely prevented by the substrate fructose 1,6-biphosphate (Fig. 1). Fructose 1-phosphate (4 mM) also prevented inactivation, while dihydroxyacetone 3-phosphate (4 mM), a product of the aldolase-catalyzed reaction, had no effect on the rate of inactivation of the enzyme by disulfides (data not shown). Aldolase inactivation by glutathione disulfide was partially reversed by dithioerythritol but became partially irreversible with time.

The rate of inactivation of aldolase by disulfides was dependent on pH and disulfide concentration (Table I). For any 1 day's experiments, the estimated half-lives were reproducible (less than 3% variation); however, more variation was observed from day to day. For example, the half-life for the inactivation of aldolase with 12 mM glutathione disulfide at pH 8.0 ranged from 15 to 30 min on 10 different days, an average of 23 ± 2.3 (mean ± S.E.). Cystamine and cysteine consistently inactivated aldolase more rapidly than glutathione disulfide under comparable conditions. Graphical analysis of the data indicated that there was a linear relationship between disulfide concentration and the rate constant for inactivation (ksub) of aldolase. Ammonium sulfate, at concentrations of 5 mM or greater, inhibited the rate of inactivation by glutathione disulfide. Other sulfate salts (Na₂SO₄, MgSO₄) had the same protective effect as (NH₄)₂SO₄; while NH₄Cl and NH₄HCO₃ did not protect aldolase from disulfide inactivation (results not shown). These results indicate that the sulfate is the active ion in the protective effect.

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### RESULTS

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### Interaction of Biological Disulfides with Aldolase

The abbreviation used is: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).
Glutathione disulfide was released from the protein by glutathione reductase, and the released thiol was assayed with DTNB (20). The latter was determined under denaturing conditions (triangles) or nondenaturing conditions (inverted triangles) as described under "Experimental Procedures." Open and closed symbols indicate the presence or absence of substrate, respectively, in the original incubation mixture.

In order to determine whether the loss of sulphydryl groups was due to the formation of glutathione-enzyme mixed disulfides, rather than sulfdide bridging within the aldolase molecule or some other form of sulphydryl oxidation, two experiments were performed. First, aldolase was reacted with glutathione disulfide until the enzyme was 94% inactivated, the modified enzyme was separated from small molecules on a Sephadex G-25 column, and the glutathione was released from the protein with glutathione reductase, and the released thiol was assayed with DTNB (20).

Approximately 1 nmol of GSH/nmol of monomer could be released from aldolase (Fig. 3). At 50% inactivation, 0.5 nmol of GSH monomer was released (data not shown). Samples incubated with glutathione disulfide in the presence of substrate demonstrated little if any release of glutathione. Second, aldolase was in-

TABLE I
Interaction of Biological Disulfides with Aldolase

| Disulfide Type | Concentration (mM) | pH (NH₄)₂SO₄ | Half-life for inactivation of aldolase (kₐ) |
|---------------|-------------------|-------------|---------------------------------|
| Glutathione disulfide | 2 | 8.0 | None | 91 (0.007) |
| | 4 | 8.0 | | 72 (0.009) |
| | 8 | 8.0 | | 30 (0.023) |
| | 10 | 8.0 | | 25 (0.027) |
| | 12 | 8.0 | | 17 (0.040) |
| | 12 | 10 | 7.4 | 70 (0.009) |
| | 12 | 8.0 | | 19 (0.036) |
| | 12 | 8.5 | | 7 (0.099) |
| Cystine | 2 | 6.0 | None | 172 (0.004) |
| | 2 | 7.0 | | 49 (0.014) |
| | 2 | 2.0 | | 30 (0.023) |
| | 2 | 4.0 | | 14 (0.049) |
| Cystamine | 1.0 | 7.4 | None | 30 (0.023) |
| | 2.5 | 7.4 | | 15 (0.046) |
| | 5.0 | 7.4 | | 11 (0.063) |
| | 7.5 | 7.4 | | 7 (0.099) |
| | 10 | 8.0 | | 24 (0.026) |
| | 1.0 | 8.0 | | 12 (0.057) |
| | 2.0 | 8.0 | | 10 (0.069) |
| Glutathione disulfide | 12 | 8.0 | 0 | 20 (0.034) |
| | 12 | 8.0 | 5 | 42 (0.016) |
| | 12 | 8.0 | 10 | 49 (0.014) |
| | 12 | 8.0 | 20 | 64 (0.010) |
| | 12 | 8.0 | 50 | 75 (0.009) |
| | 12 | 8.0 | 100 | 96 (0.007) |
cubated with radiolabeled glutathione disulfide, and the amount of GSH bound per mol of aldolase monomer in the presence or absence of substrate was determined (Table II). Both of these studies indicated that mixed disulfides (approximately 1 mol of GSH/mol of aldolase subunit) were formed in the absence but not in the presence of substrate.

Differential scanning calorimetry revealed that glutathione-inactivated aldolase had less conformational stability than native aldolase (Fig. 4). The transition temperature for denaturation was decreased from 58 °C for the native enzyme to 49 °C for the disulfide-inactivated enzyme, and the enthalpy for denaturation was decreased from 4.3 to 2.0 mcal/mg. The substrate, fructose 1,6-bisphosphate, had no effect on thermodynamic parameters of native aldolase but completely protected the enzyme from the destabilizing effects of glutathione disulfide.

The loss of stability of aldolase caused by disulfides did not result from the dissociation of the tetrameric structure (Fig. 5). In addition, the disulfide-inactivated enzyme gave a single line of identity with native enzyme on Ouchterlony plates with antibody prepared against native rabbit-muscle aldolase (results not shown). There was no evidence that intersubunit disulfide bonds formed in the presence of disulfides because only monomers of aldolase (Mr = 40,000) were observed after sodium dodecyl-polyacrylamide gel electrophoresis (with no 2-mercaptoethanol added to samples) (data not shown). Some aggregation of disulfide-oxidized aldolase occurred as evidenced by the presence of protein at the bottom of sucrose density gradients (Fig. 5); aggregated forms of aldolase were not observed with native aldolase or aldolase inactivated by limited proteolysis (18).

Chymotrypsin inactivated native aldolase at pH 7.4 by limited proteolysis, as shown previously (39), but extensively degraded aldolase inactivated by glutathione disulfide (Fig. 6). Similar results were observed with the lysosomal proteinases cathepsin D at pH 4.8 and cathepsin B at pH 6.0, and with Meprin, a metalloendopeptidase (19), at pH 9.5 (results not shown). The substrate, fructose 1,6-bisphosphate, completely protected aldolase from both limited and extensive proteolysis by chymotrypsin, cathepsins D and B, and Meprin at their respective pH optima.

In order to determine whether the formation of mixed disulfides (between biological disulfides and protein) was essential for the observed change in aldolase proteolytic susceptibility, aldolase was treated with sodium periodate. Periodate is an oxidizing agent that, under the conditions used, catalyzes exclusively the formation of disulfide bridges between adjacent sulfhydryl residues in proteins (27). Periodate caused a first-order inactivation of aldolase (t1/2 = 9 min) against the substrate fructose 1,6-bisphosphate, and the inactivated enzyme was degraded by proteinases as extensively as the enzyme inactivated by biological disulfides.

**TABLE II**

| Incubation of          | Aldolase activity | GSH/aldolase monomer % nmol/nmol |
|------------------------|-------------------|----------------------------------|
| Aldolase + [3H]GSSG    | 6                 | 0.95                             |
| Aldolase + [3H]GSSG +  | 100               | 0.08                             |
| Fru-1,6-P2             |                   |                                  |

**FIG. 4.** Thermally induced transitions of native aldolase and aldolase incubated with glutathione disulfide in the presence or absence of fructose 1,6-bisphosphate. Aldolase was incubated with glutathione disulfide for 2 h (90% inactivation observed) or under the same conditions except in the presence of 1 mM fructose 1,6-bisphosphate (no inactivation). Glutathione and substrate were removed by Sephadex G-75 gel chromatography in Tris-HCl buffer, pH 7.4, containing 0.15 M KCl. Samples (approximately 1 mg/ml) were subjected to differential scanning calorimetry. Transition temperatures for denaturation were 58 °C for both native aldolase (curve 1) and aldolase incubated with glutathione disulfide plus substrate (curve 2) and 49 °C for glutathione-inactivated aldolase (curve 3). Esthers for denaturation were 4.3 mcal/mg for curves 1 and 2 and 2.0 mcal/mg for curve 3.

**FIG. 5.** Sedimentation of glutathione-inactivated aldolase in a sucrose density gradient. Aldolase which had been inactivated 92% by glutathione disulfide was sedimented in a 5-30% linear sucrose gradient for 12 h. Molecular weight standards were catalase (232,000), ovalbumin (45,000), and native aldolase (160,000).

**DISCUSSION**

The present work demonstrates that glutathione disulfide covalently modifies rabbit-muscle aldolase leading to inactivation against fructose 1,6-bisphosphate and fructose 1-phosphate, thermodynamic destabilization, and enhanced proteolytic susceptibility. The observed physicochemical changes in aldolase are associated with the formation of four to five mixed disulfides and the oxidation of approximately 16 sulfhydryl groups/tetramer under the conditions described. Our explanation of these results is that thiol/disulfide interchanges of two types occur:
Structure conformational changes in the enzyme that result in thermodynamic changes and an increased susceptibility to protease. Formation of specific glutathione-protein mixed disulfides does not appear to be critical for inactivation or enhanced susceptibility of aldolase to proteinases because 1) the linear relationship between glutathione disulfide or cystamine concentration and the $k_d$ for inactivation of the enzyme suggests there is no specific saturatable binding site for disulfides associated with inactivation, and 2) formation of intramolecular disulfide bridges alone (in the presence of periodate), without mixed disulfide formation, results in conformational changes that increase the vulnerability of the enzyme to proteinases. Portions of the tertiary and the quaternary structure of aldolase appear to be unchanged after oxidation of sulphydryl groups because the oxidized enzyme is able to cross-react with antibodies to native aldolase and it behaves primarily as a tetramer in sedimentation gradients. The fact that the enzyme becomes irreversibly inactivated by glutathione disulfide with time might reflect the formation of unstable conformations or more complex rearrangements than bridges between vicinal thiols.

There are conflicting reports about the number and role of sulphydryl groups in aldolase, and this is due partly to the fact that aldolase sulphydryl groups differ widely in reactivity toward different sulphydryl reagents (32). There are 32 sulphydryl groups/tetramer (21), and they are all accountable as free thiols in the native enzyme (33). Chlorodinitrobenzene, p-hydroxymercuribenzoate, N-ethylmaleimide, and $\beta$-carboxyethyl sulphydryl disulfide all have been reported to react with 10–12 sulphydryl groups/tetramer (32); these reagents all inactivated the enzyme, and in most instances, fructose 1,6-bisphosphate protected four to six sulphydryl groups and prevented inactivation. With regard to inactivation and protection by substrate, the biological disulfides used in the present work (glutathione disulfide, cystine, and cystamine) are similar to the sulphydryl reagents used previously. Our results are consistent with those of Steinman and Richards (33) who studied interactions of aliphatic disulfide monosulfoxides with aldolase and concluded that there are four accessible and four buried sulphydryl groups in the aldolase subunit. The present work does differ from that of Steinman and Richards in that they found that cystamine had no deleterious effect on catalytic properties of aldolase, whereas we found this compound to be a potent inactivator. Nonetheless, our results confirm that cysteinyl residues in aldolase are important for conformational integrity.

The protective effect of sulfate ions against inactivation by disulfides is more than likely due to the interaction of this ion with aldolase phosphate-binding sites (34). Phosphate is a competitive inhibitor of aldolase and also protects aldolase against oxidation by periodate (27) and against inactivation by cathepsin L and other proteinases (35). The stabilization that phosphate and sulfate ions afford to aldolase appears to be a more general affect of these ions on proteins in that many enzymes are protected against oxidation (27) and proteolytic inactivation (36) by these ions.

Inactivation of aldolase per se does not necessarily lead to increased instability and vulnerability to proteinases (18); e.g. cathepsin-inactivated aldolase is as stable as native aldolase thermodynamically and with regard to extensive proteolysis. But when inactivation is coupled with destabilization of the enzyme, as it is after sulphydryl oxidation of aldolase, there is enhanced susceptibility to proteolysis. Oxidized cell proteins are more vulnerable to degradation in erythrocytes (37) and in bacteria (1–3, 5) and it is possible that enhanced degradation in more oxidized states in muscle is also related to an
increase in oxidized proteins (10, 11).

The concentrations of glutathione disulfide used in these in vitro studies are much higher than physiological concentrations (38), and the half-lives obtained in vitro are much shorter than would be expected in vivo. Aldolase is a relatively stable enzyme in muscle tissue (the most recent estimates of its half-life in vivo range from 1 to 2 days), whereas in vitro half-lives described herein were expressed in minutes. In addition, the in vitro reactions between disulfides and aldolase were not enzyme catalyzed, whereas it is probable that thiol-exchange enzymes (39) catalyze the oxidation reactions in vivo. The rate of an enzyme-catalyzed reaction between aldolase in a reduced state and oxidizing agents may affect the thermodynamic properties of these muscle enzymes as well as their kinetic properties. The reducing environment within cells and the further protection of aldolase by its substrate, fructose 1,6-bisphosphat.e, against oxidizing agents can distinguish between the native structure and disulfide-modified aldolase (41). Thus, an increase in the percentage of sulfhydryl groups in muscle tissues. We have found that perfused livers preferentially endocytose glutathione-inactivated aldolase compared to native aldolase or cathepsin D-inactivated aldolase, indicating that cellular membranes can distinguish between the native structure and disulfide-modified aldolase (41). Thus, an increase in the percentage of sulfhydryl groups that are oxidized may result in unstable protein conformations, increased membrane association, and increased degradation of the protein to amino acids and small peptides.

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