

Specifically Targeted Modification of Human Aldose Reductase by Physiological Disulfides*

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Aldose reductase is inactivated by physiological disulfides such as GSSG and cystine. To study the mechanism of disulfide-induced enzyme inactivation, we examined the rate and extent of enzyme inactivation using wild-type human aldose reductase and mutants containing cysteine-to-serine substitutions at positions 80 (C80S), 298 (C298S), and 303 (C303S). The wild-type, C80S, and C303S enzymes lost >80% activity following incubation with GSSG, whereas the C298S mutant was not affected. Loss of activity correlated with enzyme thiolation. The binary enzyme-NADP⁺ complex was less susceptible to enzyme thiolation than the apoenzyme. These results suggest that thiolation of human aldose reductase occurs predominantly at Cys-298. Energy minimization of a hypothetical enzyme complex modified by glutathione at Cys-298 revealed that the glycol carboxylate of glutathione may participate in a charged interaction with His-110 in a manner strikingly similar to that involving the carboxylate group of the potent aldose reductase inhibitor Zopolrestat. In contrast to what was observed with GSSG and cystine, cystamine inactivated the wild-type enzyme as well as all three cysteine mutants. This suggests that cystamine-induced inactivation of aldose reductase does not involve modification of cysteines exclusively at position 80, 298, or 303.

Aldose reductase (alditol:NADP oxidoreductase, EC 1.1.1.21) (ALR2)¹ catalyzes with a broad catalytic efficiency the NADPH-dependent reduction of aldo-sugars and a variety of aromatic and aliphatic aldehydes to their corresponding alcohols. This enzyme is the first in a pathway that results in the transformation of glucose to fructose using sorbitol as a metabolic intermediate. This so-called "polyol pathway" is not a "high flux" metabolic route except in hyperglycemic conditions such

as diabetes mellitus and galactosemia, where elevated concentrations of glucose and galactose, respectively, result in enhanced accumulation of their corresponding polyols in various tissues such as the eye lens (1, 2). Since these polyols do not readily permeate cell membranes, their intracellular accumulation is thought to create an osmotic imbalance, resulting ultimately in sugar cataract formation (3–5). Intensive effort has been mounted to identify inhibitors of aldose reductase for use as therapeutic tools against diabetic complications such as cataract and retinopathy (6–9).

Aldose reductase is subject to modifications leading to enzyme forms with an altered sensitivity to various inhibitors. Thus, the so-called "activated" ALR2 generated through apparently different processes such as isomerization (10), glycosylation (11), and thiol-dependent oxidation (12–14), besides displaying differences in substrate specificity, has a greatly reduced sensitivity to different aldose reductase inhibitors. Indeed, others recently reported the purification of human ALR2 with kinetic properties consistent with those described for an oxidized form of the enzyme (15). The potential involvement of cysteine residues in catalysis and inhibition has been widely investigated (16–21). Many studies indicate that Cys-298, an accessible residue located close to the active site, is a possible modulator of ALR2 susceptibility to inhibition. Carboxymethylation of ALR2 generates an enzyme form with differentially altered susceptibility to inhibition by Tolrestat (*N*-[[5-(trifluoromethyl)-6-methoxy-1-naphthalenyl]thioxomethyl]-*N*-methylglycine) and Sorbinil ((*S*)-6-fluorospiro[chroman-4,4'-imidazolidine]-2',5'-dione), suggesting the existence of two distinct inhibitor-binding sites on ALR2 (14). Besides carboxymethylation, Cys-298 appears to be the target residue in the menadione-induced inactivation of human placental ALR2 (20) as well as in the dithiodiethanol-induced activation of the enzyme (22). Among significant differences in structural and kinetic properties (23–26), the enzyme form characterized by a mixed disulfide linkage between 2-mercaptoethanol and the cysteine residue appears completely insensitive to Sorbinil.

Cystine and glutathione disulfide are both capable of inducing reversible inactivation of bovine lens ALR2 *in vitro* (27). The enzyme form modified by GSSG, which displayed as much as 40% of the native enzyme activity and is insensitive to Sorbinil, has been demonstrated to occur *in situ* in cultured bovine lenses subjected to oxidative stress by hyperbaric oxygen treatment (28). Thus, the possibility that ALR2 may undergo *in vivo* reversible thiolation must be considered in the design of new ALR2 inhibitors. In this study, we demonstrate that ALR2 is susceptible to glutathione disulfide- and cystine-

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¹ The abbreviations used are: ALR2, aldose reductase; DTT, dithiothreitol; r.m.s., root mean square.

dependent oxidation and that Cys-298 is the target residue for such post-translational modifications.

EXPERIMENTAL PROCEDURES

Materials—NADPH, NADP⁺, DL-glyceraldehyde, DTT, glutathione, glutathione disulfide, cystamine, and isoelectric focusing standards were purchased from Sigma. L-Cystine was from Carlo Erba. Matrex Orange A, Centricon 10 microconcentrators, and YM-3 ultrafiltration membranes were from Amicon, Inc. All electrophoresis reagents were obtained from Bio-Rad. All inorganic chemicals were of reagent grade and were from BDH. Glutamylcysteinyl[2,3H]glycine (³H[GSH]) was purchased from DuPont NEN. Ampholine PAG plates (pH 4.0–6.5) for isoelectric focusing were obtained from Pharmacia Biotech Inc.

Purification of Recombinant Human Aldose Reductases—Wild-type and mutant aldose reductases were expressed and isolated as described previously (19) with the exception that the enzymes were additionally purified by column chromatography over Affi-Gel blue (Bio-Rad). Apparent homogeneity of the purified enzyme preparations was confirmed by the appearance of a single protein band following 12% SDS-polyacrylamide gel electrophoresis and silver staining.

Enzyme Assays—Aldose reductase activity was determined at 37 °C as described previously (13) using 4.7 mM DL-glyceraldehyde as substrate in 0.25 M sodium phosphate buffer (pH 6.8) containing 0.38 M ammonium sulfate and 0.11 mM NADPH. One unit of enzyme activity is the amount of the enzyme that catalyzes the oxidation of 1 μmol of NADPH/min. These assay conditions were found to be suitable for determination of the activity of both unmodified wild-type and glutathione-modified enzyme forms. A slight decrease (up to 20%) in activation by ammonium sulfate was observed for glutathione-modified ALR2.

Enzyme Modification—To assess the susceptibility of ALR2 to thiol-dependent modification, incubations with different reactive thiols were performed in 10 mM sodium phosphate buffer (pH 7.0) (S-buffer). Unless stated otherwise, enzyme preparations used in the modification experiments (~15 μM) were dialyzed first against 100 volumes of S-buffer supplemented with 0.1 mM NADP⁺ and 2 mM DTT, followed by dialysis against 100 volumes of S-buffer supplemented with 2 mM DTT. Incubations to measure the stoichiometry of [³H]GSSG incorporation into wild-type and mutant aldose reductases were carried out at least three times. Values representing moles of glutathione incorporated per mole of enzyme are reported as the mean ± S.E. of at least three independent determinations.

Preparation of Tritium-labeled GSSG—Tritium-labeled GSSG (³H[GSSG]) was prepared by thiol exchange reaction between 1.3 mM GSSG and [³H]GSH as described previously (27). The final specific radioactivity of [³H]GSSG was 129,000 dpm/nmol. A Beckman LS500CE scintillation counter was used for radioactivity measurements using Opti Phase Hi Safe II scintillation fluid (Pharmacia Biotech Inc.) with a counting efficiency of 50% as determined by the tritium standard quench curve of the instrument.

Evaluation of NADP⁺-bound ALR2—Quantitation of NADP⁺-bound ALR2 was performed by circular dichroism analysis of the enzyme preparation in 2 mM DTT (25). The relative amount of ALR2-NADP⁺ complex was determined from the elongation at 330 nm before and after the addition of saturating amounts of NADP⁺. Circular dichroism spectra were obtained using a Jasco 40AS spectropolarimeter with a cylindrical 10-mm path length cuvette kept at 10 °C. A spectral bandwidth of 2 nm was used.

Isoelectric Focusing—Isoelectric focusing was carried out at 4 °C on a Biophoresis horizontal electrophoresis cell (Bio-Rad) using Ampholine PAG plates (pH 4.0–6.5). Gels were prefocused for 20 min at 15 watts. Samples were then applied ~2 cm from the cathode, and focusing was allowed to proceed for 90 min. After focusing, gels were immediately fixed in 10% trichloroacetic acid, 0.135 M sulfosalicylic acid for 30 min and then rinsed for 5 min with 25% ethanol, 8% acetic acid. Gels were stained for 15 min with 1.16 g/liter Coomassie Blue R-250 in 25% ethanol, 8% acetic acid and then destained with 25% ethanol, 8% acetic acid. Isoelectric point values reported for different enzyme forms represent the mean ± S.E. of at least three independent determinations.

Structural Modeling—Energy minimization, solvent accessibility, and r.m.s. deviation calculations were made using the XPLOR package of programs (29). In all cases, the starting model was the 1.8-Å structure of the human aldose reductase-NADPH-Zopolrestat ternary complex (Protein Data Bank code 1MAR; Ref. 30) after stripping the inhibitor molecule, Zopolrestat (3,4-dihydro-4-oxo-3-[[5-(trifluoromethyl)-2-benzothiazolyl]methyl]-1-phthalazineacetic acid), and all ordered waters from the structure. Solvent accessibility was then calculated for

the S-γ atoms of the cysteines at positions 80, 298, and 303 using a probe radius of 1.4 Å.

Modifications of Cys-298 and Cys-303 were made manually, placing the glutathione adduct in what appeared to be regions that would cause minimal steric conflict with protein atoms. Since Cys-80 is buried in the interior of the structure, it was not possible to model the glutathione adduct to this site without introducing numerous steric collisions between the glutathione and protein atoms. The glutathione was therefore positioned arbitrarily prior to minimization.

Each modified structure was subjected to energy minimization without harmonic restraints until convergence (350 steps). The areas considered most likely to be perturbed in the resulting structures were then compared with the unmodified starting structure to determine the possible consequences of each specific glutathione modification. These comparisons were done by performing a rigid-body minimization between the starting coordinates and the energy-minimized coordinates using all atoms within 10 Å of the particular cysteine S-γ. r.m.s. deviations were then calculated on this oriented subset of atoms.

Other Biochemical Methods—Protein concentration was determined according to the method of Bradford (31) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (32), and gels were stained with silver nitrate according to the method of Wray *et al.* (33). The following standards were used for calibration: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa).

RESULTS AND DISCUSSION

Characterization of GSSG-modified Aldose Reductase—When incubated in the presence of the physiological disulfides GSSG and cystine, human ALR2 shows a progressive loss of enzyme activity (Fig. 1A). The effectiveness of DTT in restoring the enzyme activity when added either to the inactivated enzyme after removal of the modifying disulfides by dialysis on YM-3 ultrafiltration membranes (Fig. 1A, *inset*) or directly to the ALR2/disulfide inactivating mixture (data not shown) is consistent with an oxidative modification of one or more cysteine residues. Isoelectric focusing analysis of ALR2 before and after treatment with GSSG revealed a decline in the pI from 6.1 ± 0.05 for the reduced native enzyme to 5.9 ± 0.05 for the GSSG-treated material (Fig. 2, *lanes c* and *d*). The observed anionic shift in pI as a result of GSSG treatment is compatible with the incorporation of a carboxylate group as it occurs with the insertion of an S-glutathionyl residue on the protein (34).

Similar to what has been observed for bovine ALR2 (28), affinity chromatography on Matrex Orange A can be used to separate native human ALR2 and the enzyme form modified by GSSG. The native enzyme is retained by the affinity chromatographic support, whereas the modified form appears in the column flow-through fraction (Fig. 3A). Indeed, when [³H]GSSG was used as modifying agent, radioactivity was associated only with those fractions corresponding to modified ALR2. Specific activity measurements on this material were consistent with incorporation of 1 molar eq of glutathione residue/mol of enzyme.

Human ALR2 contains seven cysteine residues, three of which (Cys-80, Cys-298, and Cys-303) are located close to the catalytic pocket (35, 36) and represent possible targets for thiol-induced modification of ALR2. To evaluate whether one or more of these residues are involved in GSSG- or cystine-mediated inactivation, we compared the susceptibility of wild-type ALR2 with mutants containing serine substitutions at Cys-80 (C80S), Cys-298 (C298S), and Cys-303 (C303S). Some kinetic properties of these cysteine mutants were reported previously. All three mutants express robust activity using DL-glyceraldehyde as substrate, with catalytic efficiencies of ~53 and 54% that of the wild-type enzyme for the C80S and C303S mutants, respectively (19). The x-ray structure of the C298S mutant (catalytic efficiency of 6.5–10% compared with the wild-type enzyme) complexed to NADPH (36) is highly similar to that of

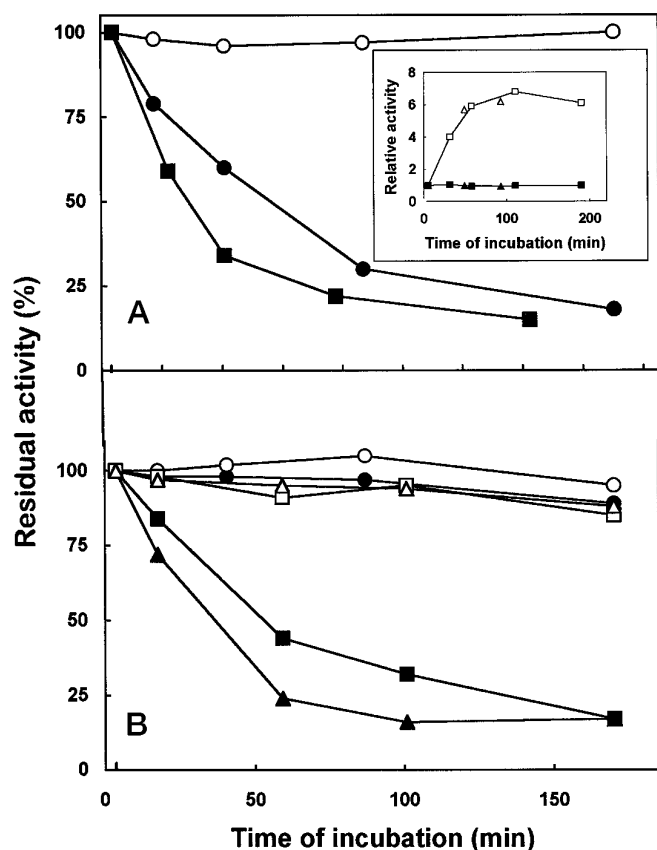


FIG. 1. Inactivation of wild-type and mutant human ALR2 by physiological disulfides. A, purified ALR2 (60 $\mu\text{g/ml}$, corresponding to 0.240 units/ml) was incubated at 25 $^{\circ}\text{C}$ in S-buffer alone (\circ) or in the presence of either 1.5 mM GSSG (\bullet) or 0.4 mM cystine (\blacksquare), and the enzyme activity was measured at different times. *Inset*, recovery of enzyme activity when the above-mentioned 90-min GSSG-treated (\square) and cystine-treated (\triangle) enzyme samples were dialyzed against S-buffer on YM-3 membranes, followed by incubation (0.1 mg/ml) at 25 $^{\circ}\text{C}$ in the same buffer supplemented with 5 mM DTT. Samples were in the process of modification for 150 min before treatment with DTT. Also shown in the *inset* is a control incubation of the inactivated enzyme forms in the absence of DTT (\blacksquare and \blacktriangle). B, purified mutants C80S (*triangles*), C303S (*squares*), and C298S (*circles*) (60 $\mu\text{g/ml}$, corresponding to 0.245, 0.258, and 0.620 units/ml for C80S, C303S, and C298S, respectively) were incubated at 25 $^{\circ}\text{C}$ in S-buffer alone (*empty symbols*) or in the presence of 1.5 mM GSSG (*filled symbols*), and the enzyme activity was measured at different times. ALR2 activity is reported as percent of the initial value.

the wild-type binary enzyme-NADPH complex as evidenced by a r.m.s. deviation of only 0.52 \AA .

When incubated with GSSG, the C80S and C303S mutants were readily inactivated, whereas the activity of the C298S mutant was unaffected (Fig. 1B). Incubation of mutants with 0.4 mM cystine gave similar results (data not shown). Isoelectric focusing analysis for all the mutants prior to GSSG treatment revealed a pI of 6.1 ± 0.05 . While a decrease of 0.15–0.2 pH units was observed in the pI of the wild-type enzyme (Fig. 2, lanes c and d) and the C80S and C303S mutants (data not shown) after GSSG treatment, no apparent change in pI was observed after GSSG treatment of the C298S mutant (Fig. 2, lanes a and b).

Wild-type ALR2 as well as the C80S and C303S mutants, unlike the C298S mutant, were effectively labeled by [^3H]GSSG. Incorporation of 0.74 ± 0.04 , 0.62 ± 0.05 , and 0.81 ± 0.05 molar eq of glutathione/mol of enzyme was measured for C80S, C303S, and wild-type ALR2, respectively. This result is consistent with the incorporation of 1 mol of glutathione residue/mol of enzyme. The relatively lower stoichiometry

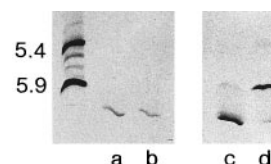


FIG. 2. Isoelectric focusing analysis of glutathione-modified recombinant human ALR2. Wild-type ALR2 and the C298S mutant were incubated for 90 min at 25 $^{\circ}\text{C}$ with 1.5 mM GSSG in S-buffer and then subjected to isoelectric focusing analysis as described under "Experimental Procedures." Lanes a and b, C298S mutant before and after incubation in the presence of GSSG, respectively; lanes c and d, wild-type ALR2 before and after incubation in the presence of GSSG, respectively. The pI values of the isoelectric focusing standards are shown on the left.

of thiolation observed for the C303S mutant may be ascribed to incomplete thiolation of the mutant under the indicated conditions (100-min incubation with [^3H]GSSG as described in the legend to Fig. 1B). Indeed, affinity chromatographic analysis of C303S after modification (as in Fig. 3) showed that $\sim 20\%$ of the total enzyme eluted with the unmodified fraction. In contrast, treatment of the C298S mutant resulted in incorporation of only background levels of radioactivity ($\sim 0.03 \pm 0.008$ mol of glutathione/mol of enzyme). Matrex Orange A chromatography of [^3H]GSSG-treated C298S revealed that all enzyme activity was recovered in column fractions corresponding to the unmodified enzyme (Fig. 3B). Moreover, no changes in the activity profile were observed after treatment of the eluted fractions with DTT (data not shown). In addition, virtually no radioactivity was detected in column fractions corresponding to the elution position of the GSSG-modified enzyme (Fig. 3B). These results, together with our failure to observe a GSSG-induced anionic shift in the pI of the C298S mutant, suggest that Cys-298 is the predominant target residue for GSSG-induced thiolation of human ALR2.

The capability of ALR2 to tightly bind the cofactor (25), well depicted by x-ray diffraction analysis (35, 36), indicates that the binary complex would exist even in purified enzyme preparations. The availability of both NADP $^{+}$ -bound and NADP $^{+}$ -depleted ALR2 allowed us to test the interaction between the pyridine cofactor and the target site of the enzyme modification. ALR2 depleted of NADP $^{+}$ was prepared by incubating the enzyme at high ionic strength (0.5 M NaCl) under reducing conditions (2 mM DTT). Dialysis of this enzyme sample resulted in complete removal of the pyridine cofactor as judged by CD analysis in the near-UV spectral region (Fig. 4, curve 2). Indeed, the spectrum is essentially superimposable to that of S-2-hydroxymethyl-mercaptyl-modified bovine lens ALR2, an enzyme form shown to be lacking in the bound pyridine cofactor (25). When NADP $^{+}$ -depleted ALR2 was dialyzed first against 10 mM sodium phosphate buffer (pH 7) supplemented with 0.1 mM NADP $^{+}$ and 2 mM DTT and then against the same buffer supplemented only with 2 mM DTT, replenishment of the enzyme with the cofactor was observed (Fig. 4). Comparison of the time course of inactivation between NADP $^{+}$ -depleted ALR2 and the NADP $^{+}$ -bound enzyme (Fig. 4, *inset*) shows that the rate of inactivation of the NADP $^{+}$ -depleted enzyme was ~ 4 -fold higher. A complete protection against GSSG-induced inactivation was observed only when NADP $^{+}$ was added to the incubating mixture at a cofactor/enzyme ratio ≥ 3 (data not shown). The protective effect provided by the cofactor was observed for the wild-type enzyme as well as the C80S and C303S mutants. In contrast, the C298S mutant was insensitive to inactivation by GSSG irrespective of the presence of NADP $^{+}$. These results are concordant with the previously reported interaction of the pyridine cofactor with Cys-298 apparently affecting the susceptibility of the enzyme to inhibition and the

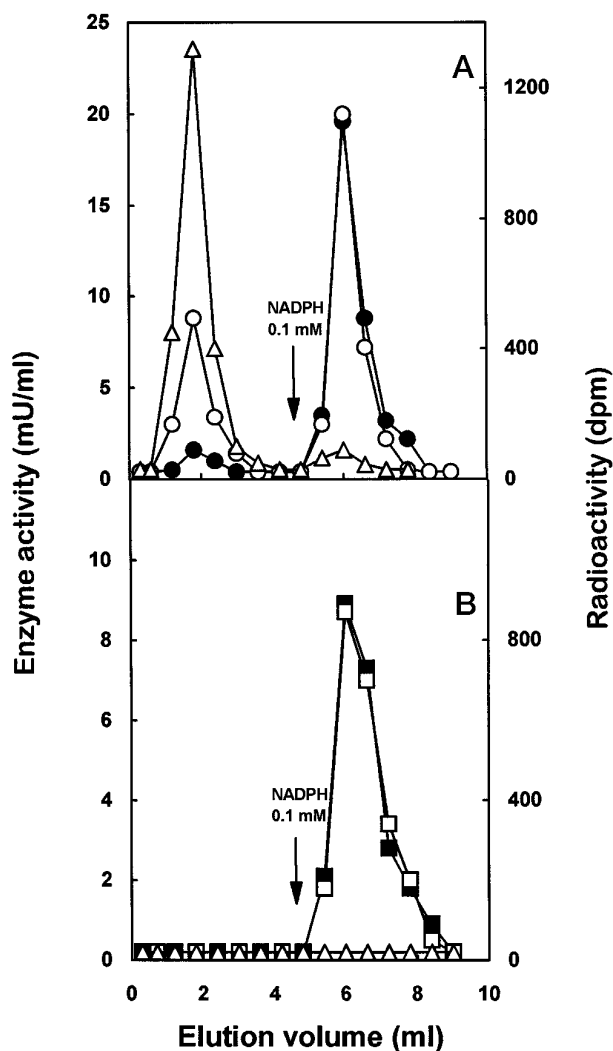


FIG. 3. Affinity chromatographic analysis of native and glutathione-modified ALR2. A, purified ALR2 (0.2 mg/ml) was incubated at 25 °C in S-buffer supplemented with 0.15 mM [³H]GSSG. After 100 min of incubation, the protein was dialyzed in a Centricon 10 microconcentrator against S-buffer supplemented with 0.1 M NaCl until no radioactivity was detectable in the ultrafiltrate; 0.2 ml of the dialyzed sample (20 μg of protein) was then applied to a Matrex Orange A column (1 × 2.5 cm), and elution was performed with S-buffer. After the elution volume indicated by the arrow, the elution buffer was supplemented with 0.1 mM NADPH. After measurement of ALR2 activity (●), each eluted fraction was supplemented with 1 mM DTT, incubated for 2 h at room temperature, and assayed again for enzyme activity (○). B, purified mutant C298S (0.2 mg/ml) was incubated at 25 °C for 100 min in S-buffer supplemented with 0.15 mM [³H]GSSG and subjected to affinity chromatography as described in A for the wild-type enzyme. The enzyme activity was measured in each eluted fraction (□). The elution profile of an untreated C298S mutant is also reported (■). In both A and B, the radioactivity measured in each eluted fraction is shown (△).

accessibility of the cysteine residue to thiol modification reagents (21).

Binding of NADPH induces a conformational isomerization involving hinge movement of a loop, resulting in the formation of a bridge over the pyrophosphate portion of NADPH. A bidentate salt link from Asp-216 to Lys-21 and Lys-262 on the other side of the cleft locks NADPH into position. In addition, a portion of the loop composed of residues 213–226 contributes a number of side chains that form a part of the pocket. The three-dimensional structure of ALR2 complexed to NADP(H) shows that the side chain of Cys-298 is directed into the substrate-binding pocket (35). In the absence of cofactor, this loop

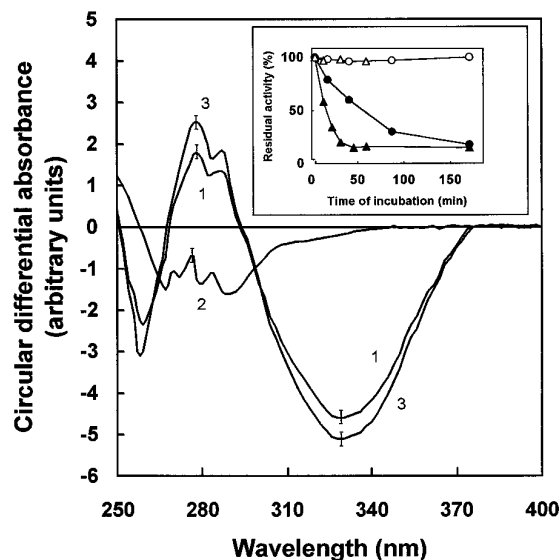


FIG. 4. Effect of cofactor-ALR2 binding on GSSG-dependent inactivation of ALR2. Circular dichroic spectra, obtained as described under “Experimental Procedures,” for NADP⁺-bound (curve 1) and NADP⁺-depleted (curve 2) ALR2 (0.5 mg/ml final protein concentration) are shown. Curve 3 represents the CD spectrum of the NADP⁺-depleted enzyme supplemented with 20 μM NADP⁺. No changes were observed in the CD spectrum after further addition of NADP⁺. A spectrum superimposable to curve 1 was obtained when the NADP⁺-bound enzyme was supplemented with 20 μM of NADP⁺ (data not shown). Δε at 330 nm for NADP⁺-bound ALR2 ($M_r = 35,800$) was 12 M⁻¹ cm⁻¹. Vertical bars on the spectra indicate the instrumental noise level. Inset, GSSG-dependent inactivation of NADP⁺-bound (●) and NADP⁺-depleted (▲) enzymes. Incubation conditions were the same as described in the legend to Fig. 1. Also shown is a control incubation of either NADP⁺-bound ALR2 (○) or NADP⁺-depleted ALR2 (△) performed in the absence of GSSG.

of residues would be expected to have considerable mobility, which would result in greater access of GSSG to the S-γ of Cys-298. Movement of this loop on NADPH binding would be expected to reduce the accessibility of GSSG to the S-γ of Cys-298. The distance from its S-γ to the C-α of Trp-219, a prominent active-site residue contained in this loop, is only 10.18 Å. In contrast, the distances from the C-α of Trp-219 to the S-γ atoms of Cys-80 and Cys-303 are 17.84 and 14.05 Å, respectively, indicating that the NADPH-induced movement of the loop would have much less effect on the modification of these residues. Furthermore, neither side chain of Cys-80 or Cys-303 is directed into the substrate- or NADPH-binding sites.

Structural Modeling of Glutathione-modified Active-site Cysteines—To interpret the pattern of GSSG-induced modification of ALR2 in a structural context, we constructed structural models with glutathione adducts at Cys-80, Cys-298, and Cys-303. Examination of the crystal structure of human ALR2 complexed to NADPH and Zopolrestat, a high affinity inhibitor, shows that Cys-80 is far removed from the active site of the enzyme. The distance from its S-γ to the C-4 atom of the NADPH cofactor is 10.90 Å. Furthermore, it is almost completely sequestered from solvent, with only 0.4-Å² solvent accessibility. Energy minimization of an enzyme form hypothetically modified by glutathione at Cys-80 showed that the protein would have to undergo a relatively large and thermodynamically unfavorable shift to accommodate the extra steric volume of the adduct. The r.m.s. deviation between the starting and minimized coordinates of all atoms included in a sphere of 10-Å radius centered upon the S-γ of Cys-80 was 1.12 Å. From a structural perspective, this cysteine is probably not reactive since it is almost completely sequestered from the solvent and

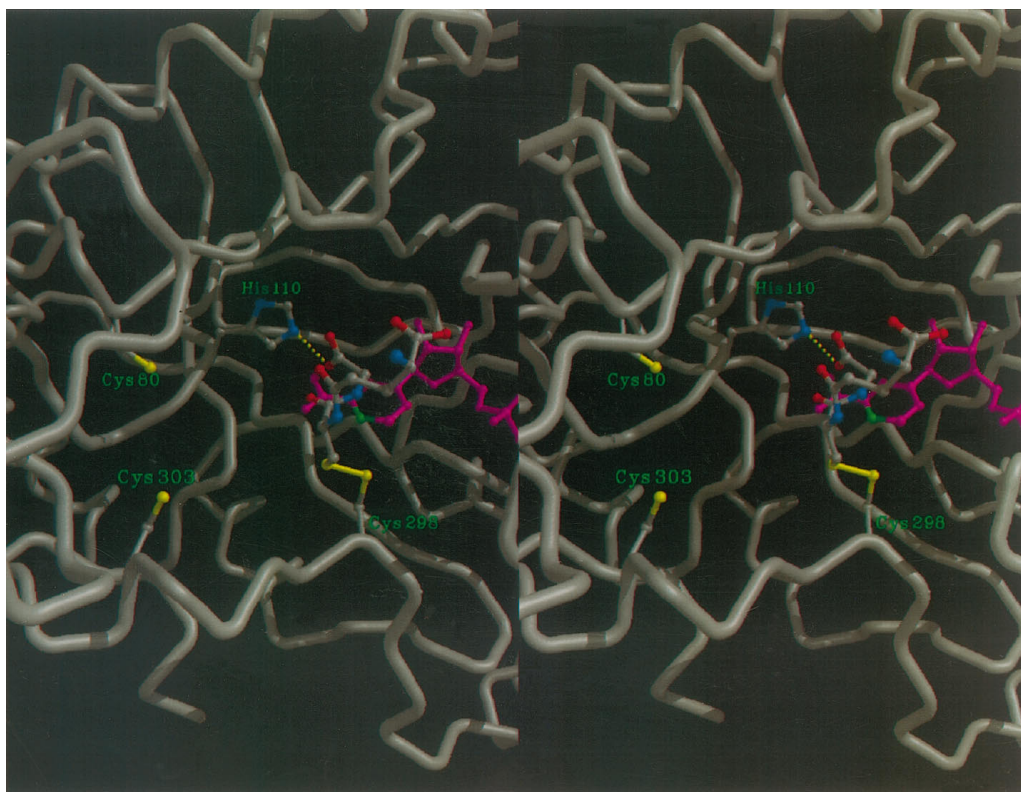


FIG. 5. **Stereo view of energy-minimized ALR2 active site with Cys-298 modified by glutathione.** The α -carbon backbone is shown in gray; the NADPH cofactor is in pink, with its C-4 carbon, which provides the hydride, in green. All other atoms are colored using standard colors for atom type. The salt link between the glutathione glycyl carboxylate and His-110 is shown by a dashed yellow line. This figure was produced using MIDAS (37).

the enzyme. However, if modified at this position, the energy minimization suggests that the structure would be markedly disrupted.

In contrast to Cys-80, the S- γ of Cys-298 is predicted to be susceptible to oxidation by GSSG because it has 6.1 Å² of solvent accessibility. Modification of Cys-298 would likely have a dramatic effect on enzyme activity as the S- γ of Cys-298 is only 3.91 Å from the reactive C-4 of the nicotinamide. Energy minimization of the ALR2 thiolated at Cys-298 suggests a small 0.49-Å r.m.s. shift from the original structure. Examination of this structure reveals that the carboxylate from the glycyl moiety of the glutathione is able to be positioned in a manner strikingly similar to the carboxylate in the potent inhibitor Zopolrestat (Fig. 5) (30). Since binding of this carboxylate appears to closely resemble the binding arrangement for a substrate (aldehyde) carbonyl group, it is clear that the catalytic site would be completely blocked by this glutathione adduct. An alternative model containing the glutamyl carboxylate proximal to the active site was found to be much less favorable. The predicted charged interaction between the glycyl carboxylate of glutathione and His-110 is reminiscent of a similar interaction demonstrated by x-ray crystallography to occur between His-110 and the carboxylate moiety of Zopolrestat (30). This charged interaction is believed to be a defining structural feature of the large class of carboxylate-containing ALR2 inhibitors (38).

When compared with Cys-298, the Cys-303 S- γ exhibits a similar solvent accessibility (6.6 Å²) and a slightly larger r.m.s. shift upon energy minimization (0.72 Å). Although these calculations indicate that Cys-303 is potentially oxidizable by GSSG and that the adduct may be modestly disruptive to the structure of the enzyme, the 12.83-Å distance from the S- γ to the C-4 of the active site leaves the binding site free for substrate binding if this modification were to occur. On the other hand,

based on both the stoichiometry of the glutathionyl incorporation on ALR2 evaluated by radioactivity measurements and the extremely limited modification of the C298S mutant by GSSG (Fig. 3B), it seems unlikely that Cys-303 is a significant target for adduct formation.

In the direction of aldehyde reduction, the catalytic mechanism of aldose reductase involves transfer of a hydride from NADPH to the substrate carbonyl carbon and abstraction of a proton by the substrate carbonyl oxygen from a general acid at the enzyme's active site. X-ray crystallography studies of human aldose reductase complexed to NADP(H) revealed that Tyr-48 and His-110 could potentially serve as the proton donor. Tyr-48 appeared to be the more likely candidate on account of its involvement in a hydrogen-bonding interaction with Lys-77, which, together with the arrangement of neighboring residues (Ala-45, Trp-79, and Trp-111), would serve to depress the pK_a of the phenolic hydroxyl of Tyr-48 (35). Subsequent mutagenesis studies confirmed the role of Tyr-48 as the proton donor and suggested a role for His-110 in orientation of substrates in the active site (39, 40). A role for Tyr-48 and His-110 in the binding of carboxylic acid-containing inhibitors was clearly demonstrated by x-ray crystallography of a ternary complex of human ALR2 bound to NADP(H) and Zopolrestat, which showed one of the inhibitor's carboxylate oxygen atoms to be located within 2.65 Å of the phenolic oxygen of Tyr-48 and 2.89 Å from the N- ϵ 2 of His-110 (30).

We propose that inactivation of human ALR2 by GSSG-dependent thiolation of Cys-298 occurs as a result of the interaction of the glycyl carboxylate of glutathione and His-110 in a manner similar to the interaction between His-110 and the carboxylate oxygen of Zopolrestat described previously (30). While the ternary complex involving Zopolrestat appears to be stabilized by extensive hydrophobic contacts between the inhibitor and the enzyme (30), we propose that the ternary com-

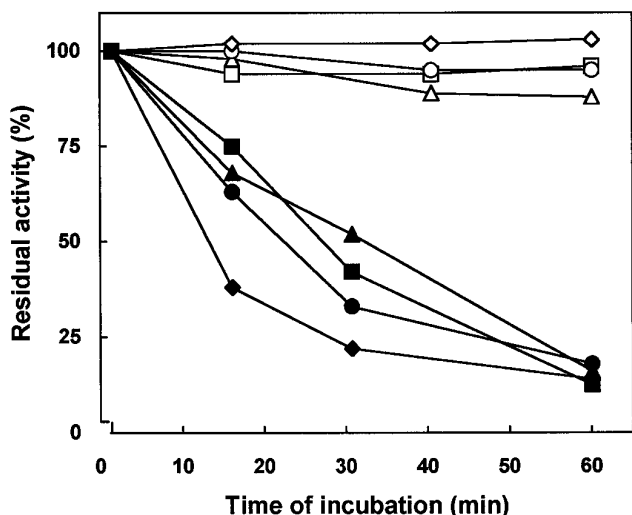


FIG. 6. Effect of cystamine on ALR2 activity. Purified wild-type ALR2 (circles) and mutants C80S (diamonds), C303S (triangles), and C298S (squares) (60 μ g/ml, corresponding to 0.240, 0.245, 0.258, and 0.620 units/ml for wild-type, C80S, C303S, and C298S ALR2, respectively) were incubated at 25 °C in S-buffer supplemented with 1.5 mM cystamine. Filled symbols refer to NADP⁺-depleted enzymes, while empty symbols refer to NADP⁺-bound enzymes (see "Results and Discussion"). Control incubations for the wild-type and all mutant enzymes, performed in the absence of the disulfide, are reported in Fig. 1 (A and B, empty symbols). ALR2 activity is reported as percent of the initial value.

plex involving glutathione is stabilized by covalent "tethering" of the adduct resulting from the mixed disulfide bond involving glutathione and Cys-298. In the GSSG-modified enzyme, we predict that the interaction between His-110 and the glycol carboxylate of glutathione prevents access of aldehyde substrate to the catalytic center defined by the side chains of His-110, Tyr-48, and the C-4 of the coenzyme nicotinamide ring. As predicted from this model, DTT treatment of GSSG-inactivated aldose reductase resulted in essentially complete recovery of catalytic activity (see Fig. 1, inset) presumably as a result of reduction of the mixed disulfide at Cys-298 and the consequent release of GSH from the active site.

Enzyme Inactivation by Cystamine—As observed in GSSG-induced modification experiments, NADP⁺ afforded protection against cystamine-induced inactivation of ALR2 (Fig. 6). However, when tested with enzyme forms depleted of NADP⁺, cystamine caused inactivation of both the wild-type enzyme and all three cysteine mutants. This suggests that the mechanism of cystamine modification of ALR2 is distinctly different from that of GSSG and involves modification of one or more residues near the cofactor-binding site.

Glutathione-mediated thiolation of ALR2 has been previously observed in organ-cultured bovine lenses subjected to oxidative stress (28). Given the similarities between bovine and human ALR2 in their susceptibility to thiol-dependent modification, it seems likely that similar modifications of human ALR2 may occur in tissues subjected to oxidative insult. Whether such post-translational modifications result in a substantially altered flux of glucose through the polyol pathway in oxidatively stressed tissues such as in diabetes mellitus is a subject for future study.

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