Thiol/Disulfide Interconversion in Bovine Lens Aldose Reductase Induced by Intermediates of Glutathione Turnover†

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ABSTRACT: The effectiveness of cysteine and cysteinylglycine to act as protein thiolating agents was investigated using bovine lens aldose reductase (ALR2) as the protein target. Disulfides of both thiol compounds appear to be very effective as ALR2 thiolating agents. Cysteine- and CysGly-modified ALR2 forms (Cys-ALR2 and CysGly-ALR2, respectively) are characterized by the presence of a mixed disulfide bond involving Cys298, as demonstrated by a combined electrospray mass spectrometry and Edman degradation approach. Both Cys-ALR2 and CysGly-ALR2 essentially retain the ability to reduce glyceraldehyde but lose the susceptibility to inhibition by Sorbinil and other ALR2 inhibitors. Cys-ALR2 and CysGly-ALR2 are easily reduced back to the native enzyme form by dithiothreitol and GSH treatment; on the contrary, Cys and 2-mercaptoethanol appear to act as protein trans-thiolating agents, rather than reducing agents. The treatment at 37 °C of both Cys-ALR2 and CysGly-ALR2, unlikely what observed for glutathionyl-modified ALR2 (GS-ALR2), promotes the generation of an intramolecular disulfide bond between Cys298 and Cys303 residues. A rationale for the special susceptibility of Cys-ALR2 and CysGly-ALR2, as compared to GS-ALR2, to the thermally induced intramolecular rearrangement is given on the basis of a molecular dynamic and energy minimization approach. A pathway of thiol/disulfide interconversion for bovine lens ALR2 induced, in oxidative conditions, by physiological thiol compounds is proposed.

The involvement of oxidative stress in the ethiology of several diseases has been widely recognized (*1*). Cells can counteract oxidative stress both by specific enzyme systems (*2*) and through the action of a variety of oxygen radical scavengers such as amino acids (*3*, *4*), vitamins (*2*, *5*−*9*), and thiols (*4*, *10*−*13*).

Disulfides, the main products of the antioxidant action of thiols, can modify proteins at the level of cysteine residues, leading to the formation of protein−thiol mixed disulfides, which may further rearrange to form inter- or intramolecular disulfides. In most cases the formation of these species is a reversible process, and if reducing conditions are provided, modified proteins can be recovered in their native state. In this regard, S-thiolation of proteins can be seen as a defense mechanism which would avoid irreversible protein damage (*14*). In the lens, the S-thiolation process would be relevant. In fact, because of a slow lens protein turnover, irreversibly damaged proteins may accumulate with deleterious consequences for the correct functioning of the organ. The antioxidant effect induced by S-thiolation may be elicited when the target proteins are enzymes. Indeed, the reversible S-glutathionylation of a variety of enzymes suggests that S-thiolation plays an active role in enzyme regulation, as a response to oxidative conditions (*15*−*18*). In such a mechanism S-thiolation would allow the cell's reducing power to be preserved by channeling metabolic resources toward NADPH production and by reducing the NADPH flux involved in biosynthetic pathways (*15*, *19*).

GSH plays a major role in S-thiolation processes; however, products of its degradation such as Cys and CysGly[1] have the potential to actively participate to redox processes involving protein thiols.

Indeed, although it is present at rather low levels, cysteine is able to thiolate proteins. Cys−protein mixed disulfides have been found in lenses of both humans and several animal species, such as rat, rabbit, and monkey rhesus (*20*−*22*). Different ratios between glutathione−protein mixed disulfides and PS-SCys were found in lenses of different species (*23*), and differences associated with the age of the lens were observed (*20*). Thus, while in young lens mixed disulfides,

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though present, are rather low, aging induces an increase in both PS-SCys and PS-SCysGly (*21*, *22*). A different distribution of PS-SG and PS-SCys was observed within the lens, with a higher PS-SCys/PS-SG ratio in the nucleus than in the cortex (*24*). The role of CysGly in inducing the formation of protein mixed disulfides has been evaluated in human platelets exposed to oxidative stress, in which an increase of CysGly−protein mixed disulfides, as well as of PS-SG and PS-SCys, has been observed (*25*). In the lens, no specific evidence of the presence of PS-SCysGly has been provided, even because the experimental approach used to evaluate the protein mixed disulfides was not able to discriminate between PS-SCys and PS-SCysGly (*26*).

The ability of Cys and CysGly to intervene in protein S-thiolation and thiol−disulfide interconversion processes was investigated using bovine lens aldose reductase (alditol: $NADP^+$ oxidoreductase, EC 1.1.1.21). This enzyme, which was shown to be susceptible to S-thiolation by both glutathione disulfide (*27*, *28*) and HEDS (*29*), represents a rather suitable protein model, both as a structural lens protein and as an antioxidant enzyme. In fact, proteins with high homology to the ALR2 sequence were recruited as crystallin in some animal species (*30*, *31*); moreover, ALR2 itself, being a NADPH-consuming enzyme, may take part through its modulation to reduce the flux of NADPH under conditions of oxidative stress (*15*). Despite the fact that X-ray diffraction data indicate for ALR2 a structure with its Cys residues all far apart in a reduced state (*32*), the present results demonstrate that the pathway of structural modification induced by S-thiolation on the bovine lens enzyme includes the generation of an intramolecular disulfide bond. In this regard, it is clearly shown the peculiar effect of the S-thiolation process generated on the target protein when different physiological thiols other than GSH are involved.

EXPERIMENTAL PROCEDURES

Materials. Cysteinylglycine, cysteinylglycine disulfide, CysGly−glutathione mixed disulfide, NADPH, $NADP^+$, D,L-glyceraldehyde, GSH, GSSG, dithiothreitol, iodoacetamide, DTNB, TFA, and isoelectric focusing standards were purchased from Sigma Chemical Co. L-Cystine, L-cysteine, and all chromatographic solvents were from Carlo Erba. DEAE-cellulose (DE-−52) was obtained from Whatman. Orange Matrex A, Centricon 10 microconcentrators, and YM-30 and YM-10 ultrafiltration membranes were from Amicon, Inc. All electrophoresis reagents were obtained from Bio-Rad. Sephadex G-75 and ampholine PAG plates (pH 4.0−6.5) for isoelectric focusing were obtained from Amersham Pharmacia Biotech Inc. Endoprotease LysC was from Boehringer. Vydac C_4 214TP54 and C_{18} 218TP52 columns were from The Separation Group, Hesperia CA. L-[U-^{14}C]Cystine, 318.4 mCi $mmol^{-1}$ was purchased from DuPont New England Nuclear. *N*-[[5-(trifluoromethyl)-6-methoxy-1-naphthalenyl]thioxomethyl]-*N*-methylglycine (Tolrestat) was from Ayerst Research Laboratories; 2,7-difluorospiro(9*H*-fluorene-9,4′-imidazolidine)-2′,5′-dione (AL1576) was from Alcon; (*S*)-(+)-6-fluoro-2,3-dihydrospiro(4*H*-1-benzopyran-4,4′-imidazolidine)-2′,5′-dione (Sorbinil) and 7-fluoro-2-(*N*-methyl-*N*-carboxymethyl)sulfamoylxantone (BAL-ARI8) were a gift from Dr. G. Caccia, Laboratori Baldacci SpA, Pisa. All other chemicals were of reagent grade. Calf lenses for ALR2 purification were obtained from freshly slaughtered animals at a local slaughterhouse (INALCA SpA, Castelvetro, Modena).

Enzyme Activity Measurements and Purification of ALR2. The assay for enzyme activity was performed as previously described (*33*). One unit of enzyme activity is the amount of the enzyme which catalyzes the oxidation of 1 μmol of NADPH per minute. The sensitivity of the enzyme activity to inhibition by Sorbinil was tested in the above assay conditions at an inhibitor concentration of 10 μM. The sensitivity of Cys-ALR2 to aldose reductase inhibitors was tested in the above assay conditions at different inhibitor concentrations ranging from 0.025 to 10 μM. The $I_{0.5}$ (inhibitor concentration producing 50% inhibition of reaction rate) was computed by linear regression analysis of the linear portion of the dose−response curve. ALR2 was purified as previously described (*34*). The pure enzyme (1.12 units/mg) was stored at 4 °C.

Affinity Chromatography Analysis of Modified Aldose Reductase Forms. One milliliter aliquots of either Cys- or CysGly-modified aldose reductase (20 μg of protein) were applied on a column (1 × 6 cm) containing 2 mL of Matrex Orange A equilibrated in 10 mM sodium phosphate buffer, pH 7.0 (S buffer); the flow rate was 0.1 mL/min, and 0.8 mL fractions were collected. The column was washed with 4.8 mL of S buffer prior to the application of the same buffer supplemented with 0.1 mM NADPH. After measurement of the aldose reductase activity, both in the absence and in the presence of 10 μM Sorbinil, each collected fraction was supplemented with 1 mM DTT, incubated for 1 h at room temperature, and assayed again for enzyme activity and susceptibility to inhibition.

Alkylation of Aldose Reductase Samples with Iodoacetamide. Native ALR2 was denatured in reducing conditions in 0.25 M Tris-HCl and 1.25 mM EDTA, containing 6 M guanidinium chloride, pH 7.0, with a 10:1 molar excess of DTT over the SH groups for 2 h at 37 °C under nitrogen atmosphere. Similarly, native ALR2, Cys-ALR2, CysGly-ALR2, and the enzyme preparations obtained by thermal treatment (3 h at 37 °C) of Cys-ALR2 and CysGly-ALR2 were denatured in 0.25 M Tris-HCl and 1.25 mM EDTA, containing 6 M guanidinium chloride, pH 7.0, without the presence of reducing agents. All protein samples were quickly alkylated by the addition of iodoacetamide (1.1 M final concentration) at room temperature for 1 min in the dark (*35*). Protein samples were freed from salt and reagent excess by passing the reaction mixture through an analytical Vydac C_4 column using an isocratic elution of 0.1% TFA for 15 min and then a linear gradient from 0 to 70% of acetonitrile in 0.1% TFA, over a period of 20 min, at a flow

[1] Abbreviations: ALR2, aldose reductase; CAM, carboxamidomethyl; Cys-ALR2, cysteine-modified ALR2; Cys-CAM, carboxamidomethylcysteine; CysGly, cysteinylglycine; CysGly-ALR2, cysteinylglycine-modified ALR2; CyS-SG, cysteinylglutathione; DTNB, dithionitrobenzoic acid; DTT, dithiothreitol; EM, energy minimization; ESMS, electrospray mass spectrometry; HEDS, hydroxyethyl disulfide; GS-ALR2, glutathione-modified ALR2; GSSG, oxidized glutathione; LES, locally enhanced sampling; MCO, metal-catalyzed oxidation; MD, molecular dynamics; 2ME, 2-mercaptoethanol; PS-SCys, cysteine−protein mixed disulfides; PS-SCysGly, cysteinylglycine−protein mixed disulfides; PS-SG, glutathione−protein mixed disulfides; PTH, phenylthiohydantoin; RMS, root mean square; RP-HPLC, reversed-phase high-performance liquid chromatography; SD, standard deviation of the mean; TFA, trifluoroacetic acid.

rate of 1 mL/min. Protein samples were manually collected and lyophilized. The carboxamidomethyl group introduced with the quenching reaction increased the molecular mass of the different species by a fixed value (57 Da for each free SH group), blocking at the same time the reactivity of the remaining cysteine residues. This allowed the separation by mass of species containing a different number of mixed and/or intramolecular disulfide bonds and the determination of their relative concentration (*36*).

Enzymatic Hydrolysis and Peptide Purification. Endoprotease LysC digestion was performed by incubating carboxamidomethylated samples of aldose reductase (100 μg) at 37 °C overnight in 0.4% ammonium bicarbonate, pH 8.0, using an enzyme/substrate ratio of 1:50. Aliquots of the digests were directly separated on a narrow bore Vydac C_{18} column using a linear gradient from 5% to 70% of acetonitrile containing 0.1% TFA, over a period of 65 min, at a flow rate of 0.2 mL/min. Peptides were manually collected, lyophilized, and further analyzed.

Protein Sequence Analysis. Automated N-terminal degradation of the purified peptides was performed using a Procise 491 protein sequencer (Applied Biosystems) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems) for the automated identification of PTH-amino acids (*37*).

Mass Spectrometric Analysis. Electrospray mass spectra were recorded by using an API-100 single quadrupole mass spectrometer (Perkin-Elmer-SCIEX) equipped with an atmospheric pressure ionization source. A probe voltage of 5 kV and a declustering potential of 60 V were used. The analysis of the protein or peptide samples was performed by injecting a peptide solution (10 pmol/μL) directly into the ion source at a flow of 5 μL/min. Data were elaborated using the BioMultiView program version 1.3 (Perkin-Elmer-SCIEX). Mass calibration was performed by means of the multiply charged ions from a separate injection of horse heart myoglobin (molecular mass 16 951.5 Da). All masses are reported as average values.

Molecular Modeling. Energy minimizations and molecular dynamics simulations have been performed with the SANDER module of the AMBER 5.0 package (*38*). The atomic point charges of the nicotinamide moiety, missing in the AMBER residue database, have been obtained by the RESP electrostatic potential approach (*39*, *40*) from ab initio 6-31G* calculation on the 1-methyl derivative of the nicotinamide cation, performed with the GAMESS (*41*) program.

The locally enhanced sampling MD approach (*42*, *43*) has been also used to improve sampling of the orientation of glutathionyl, CysGly, and Cys moieties in the corresponding ALR2 forms.

The AMBER91 (*44*) and AMBER94 (*45*) parameter sets were used in simulations without and with explicit solvation, respectively. In the former calculations solvent effects were approximated by a distance-dependent dielectric constant $\epsilon = r$. An 8 Å cutoff for nonbonded interactions was used in all of the simulations.

The MOLMOL program (*46*) was used for structure analysis and plotting.

The structure of $NADP^+$-bound bovine ALR2 has been modeled from the crystal structure of $NADP^+$-bound human ALR2 (*47*) [entry 1ADS in the Protein Data Bank (*48*)] by replacement of the nonconserved residues and subsequent EM. Models of the glutathionyl−Cys298 disulfide bond in GS-ALR2 have been obtained by manual docking of the glutathionyl moiety structure taken from the NMR resolved structure of the covalent intermediate between GSH and the human thioltransferase C7S, C25S, C78S, C82S mutant (1B4Q entry in PDB) (*49*). Models of the Cys298 disulfide-linked CysGly-ALR2 and Cys-ALR2 have been obtained by subsequent deletion from GS-ALR2 of γ-Glu and Gly, respectively. To build starting structures for MD simulations, two different orientations of the bound thiol moiety, corresponding to N-terminal and C-terminal residues of the ligand pointing to the active site of ALR2, were used for each S-thiolated enzyme form.

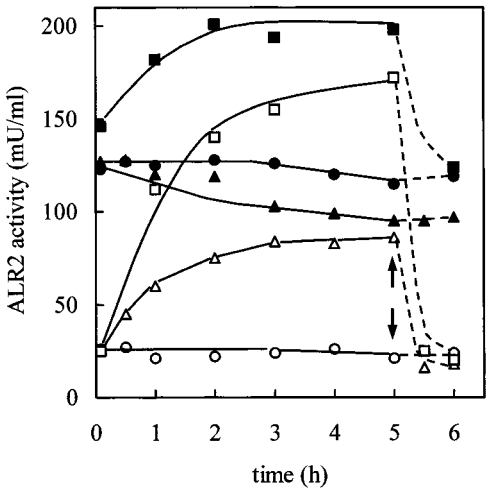

FIGURE 1: Modification of aldose reductase induced by cystine and CysGly disulfide. ALR2 (3.5 μM) was incubated at 25 °C in 10 mM sodium phosphate buffer, pH 6.8, alone (●, ○) or in the presence of 0.4 mM cystine (▲, △) or 0.4 mM CysGly disulfide (■, □). At different times the enzyme activity was measured both in the absence (closed symbols) and in the presence (open symbols) of 10 μM Sorbinil. At the time indicated by the arrows 2 mM DTT was added to the incubation mixtures.

Other Methods. Isoelectric focusing was carried out, as previously described (*27*), at 4 °C on a Biophoresis horizontal electrophoresis cell (Bio-Rad) using ampholine PAG plates (pH 4.0−6.5). Protein concentration was estimated by the Coomassie blue binding assay (*50*) with bovine serum albumin as the standard. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (*51*), and gels were stained with silver nitrate according to the method of Wray (*52*). A Beckman LS5000CE scintillation counter was used for radioactivity measurements using Opti Phase Hi Safe II scintillation fluid (Pharmacia Biotech Inc.). Measurement of radioactivity incorporation in protein bands focusing on IEF acrylamide plates was performed on gel slices (0.5 × 5 mm) smashed and dispersed in the scintillation fluid.

RESULTS

Thiol-Dependent Modification of ALR2 Induced by Cys and CysGly. Incubation of ALR2 at 25 °C with either cystine or CysGly disulfide determined changes in the kinetic properties of the enzyme. Only a modest loss of enzyme activity (approximately 20% of the initial activity) was induced by cystine (Figure 1). Despite the fact that no changes in the p*I* with respect to the native enzyme (p*I* 4.85)

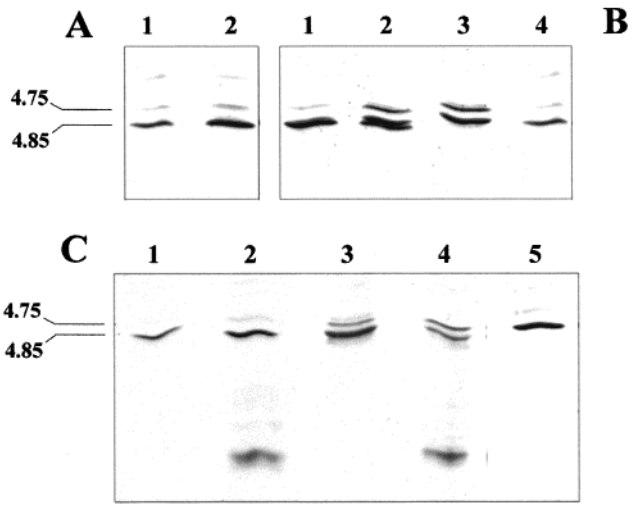

FIGURE 2: Isoelectric focusing analysis of thiol-modified ALR2 forms. Cys-ALR2, CysGly-ALR2, and GS-ALR2 were obtained by dialysis of 3.5 μM ALR2 after incubation for 5 h at 25 °C in 100 mM sodium phosphate buffer, pH 6.8, containing the following disulfides: 0.4 mM cystine, 0.4 mM CysGly disulfide, and 0.4 mM GSSG, respectively. Three micrograms of each sample was subjected to isoelectric focusing analysis as described in the Experimental Procedures section. Panel A: lane 1, native ALR2; lane 2, Cys-ALR2. Panel B: lane 1, native ALR2; lane 2, a mixture of native ALR2 and CysGly-ALR2; lane 3, CysGly-ALR2; lane 4, CysGly-ALR2 after treatment for 1 h at room temperature with 2 mM DTT. Panel C: lane 1, Cys-ALR2; lane 2, Cys-ALR2 after 3 h at 37 °C; lane 3, CysGly-ALR2; lane 4, CysGly-ALR2 after 3 h at 37 °C; lane 5, GS-ALR2 after 3 h at 37 °C. The standards used for p*I* determination were glucose oxidase (4.2), trypsin inhibitor (4.6), β-lactoglobulin A (5.1), and carbonic anhydrase (5.4).

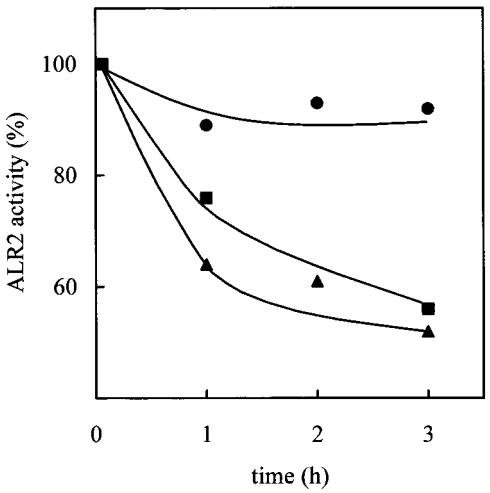

FIGURE 3: Thermally induced rearrangement of thiol-modified ALR2 forms. GS-ALR2 (●), CysGly-ALR2 (■), and CyS-ALR2 (▲) were incubated (3.5 μM) at 37 °C in 100 mM sodium phosphate buffer, pH 6.8, and the enzyme activity was measured at different times.

were observed for the cystine-treated enzyme (Figure 2A), cystine did modify ALR2, as highlighted by the progressive loss of susceptibility to the inhibitory action of Sorbinil (Figure 1). Besides Sorbinil, other ARIs were either completely (AL1576 and BAL-ARI8) or partially (Tolrestat, $I_{0.5}$ of 1.5 μM) uneffective in inhibiting Cys-ALR2. The susceptibility to inhibition was, however, recovered after further treatment (30 min at 37 °C) of the Cys-treated enzyme with 2 mM DTT (Figure 1). The modification of ALR2 induced by cystine was further investigated by the use of the ^{14}C-labeled disulfide as a modifying agent. In particular, when the Cys-ALR2 preparation, obtained by incubating at 25 °C for 5 h 5 μM ALR2 in the presence of 0.2 mM [^{14}C]-cystine, was analyzed by the affinity chromatographic approach described in the Experimental Procedures section, it was possible to elute by NADPH an enzyme population showing the incorporation of 1 equiv of cysteine (1.13 ± 0.18)/mol of enzyme. Treatment of the [^{14}C]Cys-ALR2 with DTT, together with the recovery of sensitivity to Sorbinil inhibitory action, led to the release of radioactivity (data not shown).

The incubation at 25 °C of ALR2 with CysGly disulfide led to an increase of the enzyme activity up to 1.2 times the original value (Figure 1). Also in this case the generated enzyme form was insensitive to Sorbinil. Both specific activity and susceptibility to inhibition typical of the native ALR2 form were recovered after treatment of the CysGly disulfide-treated enzyme with DTT (Figure 1). When subjected to isoelectric focusing analysis, ALR2 modified by CysGly disulfide displayed a major band focusing at pH 4.82 and one focusing at pH 4.75. Both bands disappeared, while one band with p*I* 4.85 was generated, after treatment with DTT (Figure 2B).

Thermal Rearrangement of S-Thiolated ALR2 Forms. Differently from what was observed with the glutathionyl-modified ALR2, when Cys-ALR2 and CysGly-ALR2 were incubated at 37 °C, a progressive loss of enzyme activity, up to 50% of the initial activity, was observed (Figure 3).

In both cases, a complete recovery of enzyme activity and sensitivity to inhibition by Sorbinil, compatible with the generation of the native ALR2, occurred after treatment with DTT of the thermally inactivated enzyme (data not shown). No differences with respect to Cys- and CysGly-ALR2 were observed when the products of thermal rearrangement were analyzed by SDS−PAGE in nonreducing conditions (data not shown). When the [^{14}C]Cys-ALR2 was subjected to thermal treatment and then analyzed, as above, by affinity chromatography, it appeared that the loss of the enzyme activity observed at the end of the incubation at 37 °C was paralleled by the release of approximately 50% of the bound cysteine. In these conditions, an enzyme preparation carrying an average of 0.46 ± 0.012 equiv of Cys/mol of enzyme was generated (data not shown). The isoelectric focusing analysis of the 37 °C treated Cys-ALR2 (Figure 2C) showed, in addition to the protein band at pH 4.85 associated with Cys-ALR2, another molecular species focusing at pH 5.25. A similar band distribution was observed when the modification by cystine of the native ALR2 was directly performed at 37 °C. The measurement of the radioactivity associated with protein bands isolated by isoelectric focusing from the [^{14}C]Cys-ALR2 sample (see Experimental Procedures) revealed that only the most acidic protein form (i.e., p*I* 4.85) was radioactive and no radioactivity was associated with the protein band focusing at pH 5.25.

Similarly to what was observed with Cys-ALR2, the isoelectric focusing analysis of the enzyme modified by CysGly disulfide revealed, after incubation at 37 °C, the generation of a protein form focusing at pH 5.25 (Figure 2C). A parallel decrease of the protein band focusing at pH 4.82 was observed.

The incubation at 37 °C of GS-ALR2 determined only a rather modest decrease in the enzyme activity (Figure 3);

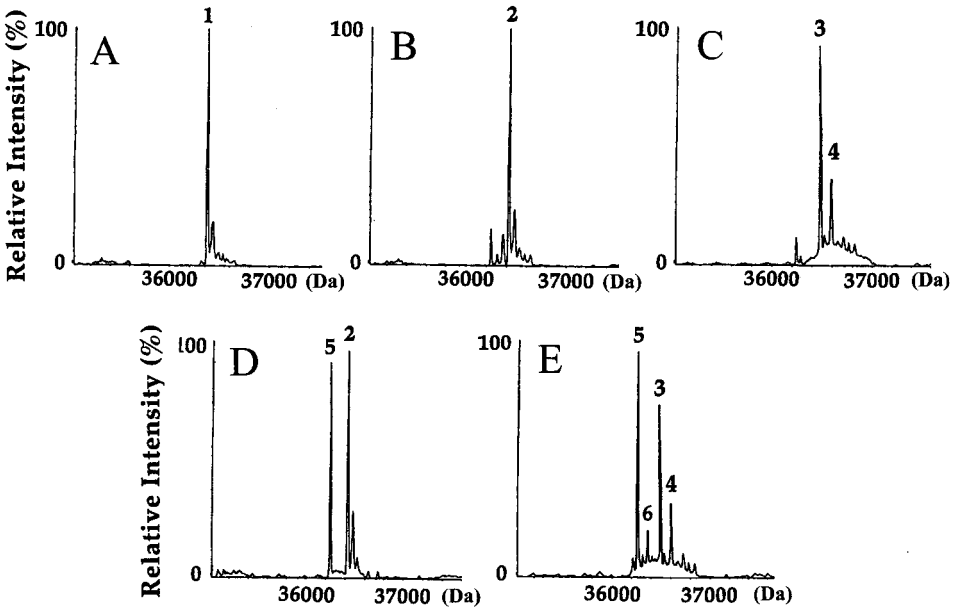

FIGURE 4: Electrospray mass spectrometric analysis of ALR2 samples. Samples were analyzed by ESMS after alkylation with 1.1 M iodoacetamide as described in the Experimental Procedures section. Panels: A, native ALR2; B, Cys-ALR2 obtained as described in the legend of Figure 2; C, CysGly-ALR2 obtained as described in the legend of Figure 2; D, sample reported in (B) after 3 h at 37 °C; E, sample reported in (C) after 3 h at 37 °C. Different molecular species are marked with different numbers (see the text for details).

however, no changes in the isoelectric focusing band distribution (Figure 2C) were observed.

Structural Analysis of ALR2, Cys-ALR2, CysGly-ALR2, and Their Products of Thermal Rearrangement. The amino acid sequence of ALR2 was verified by a combined ESMS/Edman degradation approach. The measured molecular mass for the native protein was 35 961.8 ± 3.4 Da. This value was in perfect agreement with the theoretical one (35 962.2 Da) calculated on the basis of the primary structure deposited in the Swiss-Prot database, including the presence of an acetyl group blocking the N-terminus.

Following the alkylation procedure described in the Experimental Procedures section, samples of native ALR2, Cys-ALR2, thermally treated Cys-ALR2, CysGly-ALR2, and thermally treated CysGly-ALR2 were analyzed by ESMS, and the obtained mass spectra are shown in Figure 4. In the case of native ALR2 (Figure 4A), the spectrum showed a single component (component 1: 36 360.7 ± 3.1 Da) corresponding to a protein species containing seven carboxamidomethyl groups (theoretical value 36 360.3 Da). This result was consistent with the expected fully reduced form of ALR2 and demonstrated the exhaustivity of the alkylation reaction.

The spectrum of Cys-ALR2 (Figure 4B) showed only one species (component 2: 36 422.1 ± 3.8 Da), whose molecular mass was associated to an ALR2 form containing six carboxamidomethyl groups and one mixed disulfide with exogenous Cys (theoretical value 36 422.3 Da). The mass spectrum of thermally treated Cys-ALR2 (Figure 4D) showed, besides component 2, the occurrence of a second species (component 5: 36 243.9 ± 2.9 Da) that corresponded to an enzyme form containing an intramolecular disulfide bond and five carboxamidomethyl groups (theoretical value 36 244.3 Da). The quantitative measurement of the total ion current produced by each species was in agreement with the loss of radioactivity from Cys-ALR2 generated from thermal treatment, as reported above.

Similarly, the mass spectrum of CysGly-ALR2 (Figure 4C) showed the occurrence of a main species (component 3: 36 479.8 ± 2.8 Da) corresponding to a ALR2 form presenting a mixed disulfide with exogenous CysGly and six carboxamidomethyl groups (theoretical value 36 479.3 Da). Small relative amounts of a molecular species containing two mixed disulfides with CysGly and five carboxamidomethyl groups (component 4: 36 598.6 ± 3.4 Da) (theoretical value 36 598.3 Da) were also observed. Thermally treated CysGly-ALR2 (Figure 4E) presented, besides components 3 and 4, molecular species containing one intramolecular disulfide bond. In fact, the spectrum showed the occurrence of ALR2 forms containing one intramolecular disulfide bond and five carboxamidomethyl groups (component 5: 36 244.8 ± 2.7 Da) (theoretical value 36 244.3 Da) and, in a very small amount, one intramolecular disulfide bond, one mixed disulfide with CysGly, and four carboxamidomethyl groups (component 6: 36 363.9 ± 4.8 Da) (theoretical value 36 363.3 Da). These results definitively demonstrate that thermal treatment of Cys- and CysGly-modified ALR2 promotes intramolecular rearrangement.

To identify the amino acids involved in the mixed and intramolecular disulfide bonds observed in Cys-ALR2, in CysGly-ALR2, and in their products of thermal rearrangement, all enzyme forms were digested with endoprotease LysC. The peptide mixtures obtained were resolved by reversed-phase HPLC in 30 elution peaks, and each peptide component was further characterized by ESMS and Edman degradation. In all cases peptides were identified within the protein sequence on the basis of their molecular mass and enzyme specificity. Furthermore, ESMS analysis allowed the determination of the redox state of the cysteine residues present in each peptide. In fact, the occurrence of a disulfide bridge was confirmed by reduction of the sample with DTT, followed by ESMS identification of the reduced fragments (data not shown). The peaks corresponding to Cys-containing

Table 1: Electrospray Mass Analysis of the Endoprotease LysC Peptides from ALR2 Samples[a]

time (min)	mass (Da) ALR2 reduced	ALR2 + Cys at 25 °C	Cys-ALR2, 3 h at 37 °C	ALR2 + CysGly at 25 °C	CysGly-ALR2, 3 h at 37 °C	peptide
4.2	562.3 ± 0.5	562.4 ± 0.4	562.6 ± 0.1	563.1 ± 0.3	562.9 ± 0.2	(90−94)CAM
14.5	1024.6 ± 0.9	1024.7 ± 0.8	1024.8 ± 0.4	1024.9 ± 0.4	1025.1 ± 0.6	(195−202)CAM
27.5	1121.6 ± 0.6	1121.7 ± 0.6	1122.5 ± 0.5	1121.9 ± 0.7	1122.3 ± 0.9	(78−85)CAM
31.1	1925.9 ± 0.7	1926.5 ± 0.6	1926.1 ± 0.6	1925.8 ± 0.7	1926.1 ± 0.9	(179−194)CAM
32.9	2218.8 ± 0.8	2217.9 ± 0.5	2218.2 ± 0.6	2219.1 ± 0.5	2219.2 ± 0.7	(177−194)CAM
35.3	2536.7 ± 0.9	2536.9 ± 0.6	2536.7 ± 0.8	2537.1 ± 0.4	2536.6 ± 0.5	(66−85)CAM
36.3	1675.9 ± 0.6	1676.2 ± 0.7	1675.1 ± 0.9	1676.1 ± 0.7	1676.1 ± 0.5	(86−100)CAM
37.4	3297.5 ± 0.6	3298.2 ± 0.8	3297.5 ± 1.0	3298.1 ± 0.9	3297.5 ± 1.0	(33−61)CAM
37.9	3035.3 ± 1.0	3035.8 ± 0.8	3035.1 ± 0.5	3035.9 ± 0.7	3036.1 ± 0.9	(62−85)CAM
39.5	3796.5 ± 0.8	3796.2 ± 0.7	3796.4 ± 0.9	3796.8 ± 1.0	3796.2 ± 0.9	(33−65)CAM
48.7		6520.8 ± 1.0	6520.5 ± 1.1			(263−315)CAM-Cys
				6577.3 ± 1.4	6577.6 ± 1.2	(263−315)CAM-CysGly
50.2			6342.1 ± 1.1		6342.6 ± 1.1	(263−315)S-S
51.5	6457.9 ± 0.9					(263−315)CAM_2

[a] Protein samples were carboxamidomethylated under denaturing conditions prior to enzyme digestion. The peptides obtained were separated by RP-HPLC and the individual fractions submitted to ESMS. Peptides were identified within the protein sequence on the basis of their molecular mass and enzyme specificity. For simplicityonly the peptides containing cysteine residues are indicated.

peptides, named by their retention times, are reported in Table 1.

The peptide maps obtained were almost identical, the only difference being limited to peaks eluting at 48.7, 50.2, and 51.5 min, respectively (Table 1). In the case of reduced ALR2, the peak at 51.5 min contained a component with a molecular mass of 6457.9 ± 0.9 Da that was assigned to the peptide (263−315)CAM_2. Edman degradation analysis confirmed the nature of the peptide, demonstrating the occurrence of a carboxamidomethyl group at Cys298 and Cys303. In all of the other peptides cysteine residues were carboxamidomethylated.

In the case of Cys-ALR2 (Table 1), the peptide (263−315)CAM_2 was absent. On the contrary, the peak at 48.7 min presented a clear signal at mass 6520.8 ± 1.0 Da that was associated to peptide (263−315)CAM-Cys. Peptide sequencing confirmed this hypothesis and revealed the occurrence of PTH-Cys-Cys at position 298 and of PTH-Cys-CAM at position 303 (*37*). Mass spectrometric analysis of thermally rearranged Cys-ALR2 (Table 1), in addition to the peptide (263−315)CAM-Cys (6520.5 ± 1.1 Da, peak at 48.7 min) carrying a mixed disulfide with Cys at position 298 and Cys-CAM at position 303, showed in the peak eluting at 50.2 min the presence of a clear signal at mass 6342.1 ± 1.1 Da. This value was assigned to the peptide (263−315), where both Cys298 and Cys303 were involved in an intramolecular disulfide bridge. Edman degradation analysis confirmed the nature of this peptide (*37*). The analysis of all other peptides shown in Table 1 revealed for Cys-ALR2 and for its thermally rearranged product that Cys44, Cys80, Cys92, Cys186, and Cys199 were all present in carboxamidomethylated form.

The use of the same approach allowed to demonstrate the occurrence of peptide (263−315)CAM-CysGly in CysGly-ALR2 and of peptides (263−315)CAM-CysGly plus (263−315)S-S in its product of thermal rearrangement (Table 1). Also in this case, peptide sequencing of all species confirmed the occurrence of CysGly modification at position 298 and carboxamidomethylation at position 303. ESMS analysis of these fractions clearly showed the unique occurrence of (263−315)CAM-CysGly in CysGly-ALR2 and of equimolar amounts of (263−315)CAM-CysGly and (263−315)S-S in thermally rearranged CysGly-ALR2. These results demonstrated also for CysGly-ALR2 that thermal treatment induced an intramolecular rearrangment, resulting in the Cys298-Cys303 pairing. The analysis of all other peptides shown in Table 1 revealed for CysGly-ALR2 and thermally rearranged CysGly-ALR2 that Cys44, Cys80, Cys92, Cys186, and Cys199 were present mainly in carboxamidomethylated form. Traces of CysGly-modified peptides at Cys44 or Cys186 were also detected; these products originated from the very limited amounts of the species containing two CysGly-mixed disulfides observed in the ESMS spectrum reported in Figure 4.

Table 2: Effect of Thiol Compounds on Cys-ALR2[a]

sample	activity (milliunits/mL)	activity + Sorbinil (milliunits/mL)
Cys-ALR2	78 ± 4	75 ± 7
Cys-ALR2 + DTT	107 ± 6	9 ± 2
Cys-ALR2 + GSH	93 ± 5	15 ± 3
Cys-ALR2 + Cys	83 ± 4	50 ± 2
Cys-ALR2 + 2ME	145 ± 7	110 ± 8

[a] The effectiveness of thiol compounds to modify Cys-ALR2 was tested by monitoring the enzyme activity and sensitivity to inhibition by Sorbinil after 2 h of incubation (see Experimental Procedures for details).

Effect of Monothiol Compounds on Cys-ALR2. The effectiveness of monothiol compounds in reacting with Cys-ALR2 was tested by monitoring the enzyme activity and sensitivity to inhibition by Sorbinil during an incubation at 25 °C of 3.5 μM Cys-ALR2 with either GSH, Cys, or 2-mercaptoethanol. As reported in Table 2, GSH, similarly to what was observed with DTT, is able to restore the sensitivity of the enzyme to Sorbinil with only a rather modest effect on the activity. When cysteine was used as a thiol reducing agent, only a rather low recovery of sensitivity to Sorbinil was observed. Finally, the incubation of Cys-ALR2 with 2ME induced a progressive increase in enzyme activity up to 2-fold the value expected for a complete recovery of the enzyme in its native reduced form. It is worth noting that the activity rescued upon addition of 2ME was still not susceptible to inhibition by Sorbinil.

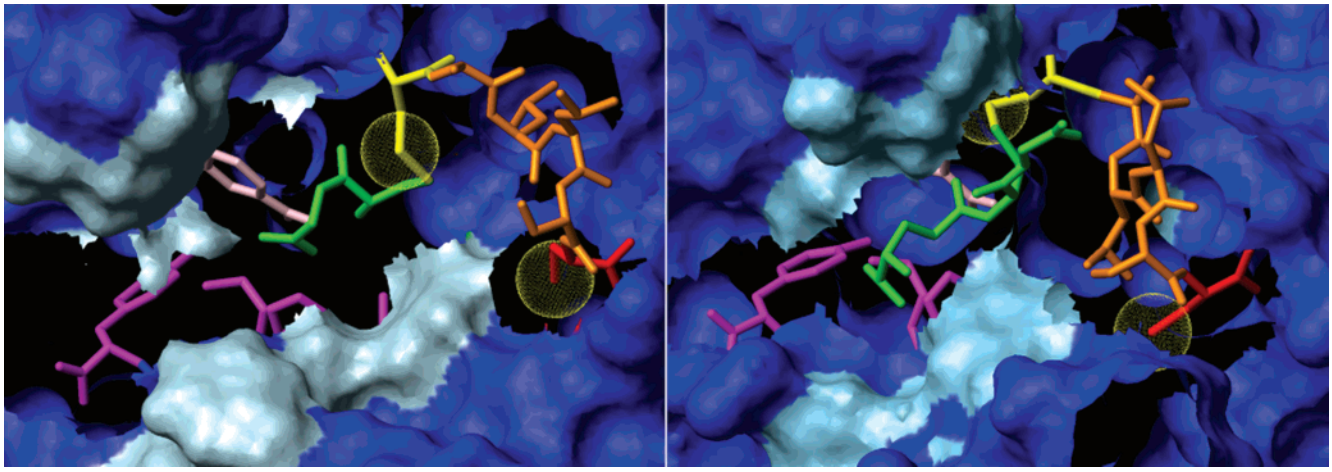

FIGURE 5: Molecular models of S-thiolated ALR2 forms. The MD frame with minimum RMS deviation from the MD average structure is shown for the active site region of CysGly-ALR2 (left panel) and GS-ALR2 (right panel). ALR2 residues are represented with a blue solvent-accessible surface, except for hydrophobic residues lining the cavity extending from the active site to Cys298 (light blue), active site residues (Tyr48, Lys77, His110, magenta sticks), the 299−302 region (orange sticks), Cys298 (yellow sticks), and Cys303 (red sticks). Pink and green sticks are used for the $NADP^+$ and GS^- moiety, respectively. Yellow dotted spheres surround the Cys298 and Cys303 sulfur atoms.

Molecular Simulations and Structural Features Associated to S-Thiolation of ALR2. A docking procedure based on a LES calculation with five copies of glutathionyl, CysGly, and Cys moieties in the corresponding ALR2 forms, and three 750 ps MD runs (250 ps of equilibration, followed by 500 ps of production run) in a water box for the most stable LES structure of each ALR2 form, allowed a characterization of both static and dynamic behavior of the different compounds at 25 and 37 °C. A detailed analysis has been performed on ALR2 regions involving the active site, the enzyme pocket extending from the active site to Cys298, and the region surrounding Cys298. The RMS fluctuations of ALR2 backbone atoms and the distances between the Cys298 S atom and all of the Cys S atoms potentially involved in intramolecular disulfide bonds have been monitored as a function of time. As a general feature, both the LES sampling procedure and MD production runs in water show a fairly different local flexibility for GS-ALR2 vs CysGly-ALR2 and Cys-ALR2 in the region surrounding Cys298. While in GS-ALR2 the calculated flexibility of the region involving residues 299−302 is lower than that observed in a reference simulation of ALR2 (the RMS fluctuations for backbone atoms at 25 °C for GS-ALR2 and ALR2 were 0.15 and 0.29 Å, respectively), it is substantially increased in CysGly-ALR2 (0.65 Å) and Cys-ALR2 (0.63 Å). In addition, the glutathionyl moiety appears to be fairly rigid inside the cavity extending from the ALR2 active site to Cys298 (RMS fluctuation for backbone atoms was 0.09 Å), while both CysGly and Cys groups in the corresponding S-thiolated enzyme forms exhibit a large mobility in water (1.11 and 0.98 Å, respectively). Also S−S distances involving Cys298 and other ALR2 Cys residues show a completely different trend on going from GS-ALR2 to either CysGly-ALR2 or Cys-ALR2. In GS-ALR2 no distance below 8 Å was detected at both 25 and 37 °C [average value: 10.2 Å at 25 °C, 9.8 Å at 37 °C, minimum distance detected at 37 °C, 8.12 Å, to be compared with 12.4 Å in the human ALR2 crystal structure (*47*)]. On the contrary, for CysGly-ALR2 and Cys-ALR2, as a consequence of the combined increased mobility of the Cys298 side chain and flexibility of the 299−302 region, both average values (CysGly-ALR2, 7.6 Å at 25 °C, 6.4 Å at 37 °C; Cys-ALR2, 7.8 Å at 25 °C, 6.5 Å at 37 °C) and minimum S−S distances (CysGly-ALR2, 5.1 Å at 25 °C, 3.9 Å at 37 °C; Cys-ALR2, 5.5 Å at 25 °C, 4.3 Å at 37 °C) are significantly shorter than those observed in the human ALR2 crystal structure. All of these features, leading to a different structural organization of GS-ALR2 and CysGly-ALR2 in the region of the active site, are depicted in Figures 5 and 6.

DISCUSSION

Cysteine and CysGly, two physiological thiols involved in the GSH turnover, were studied as S-thiolating agents, making use of bovine lens ALR2 as a model of the target protein. This enzyme has been previously shown to be especially susceptible to S-thiolation by GSSG and hydroxyethyl disulfide, which were able to generate enzyme forms, carrying a mixed disulfide at the level of Cys298, with special kinetic and structural features (*27*, *29*, *53*, *54*). In both cases the treatment of the modified enzyme with DTT led to the recovery of the native ALR2 form. Also cystine (*55*, *56*) or cysteine in oxidative conditions (*29*) was shown at 37 °C to reversibly inactivate ALR2. In this regard, the similarities in the kinetics of the reversible enzyme inactivation at 37 °C induced by GSSG and cystine would initially suggest for both cases the occurrence of a common pathway of protein modification, leading, as previously shown for GSSG and HEDS (*27*, *29*, *54*, *55*), to the formation of a mixed disulfide bond between the thiol compound and the enzyme. However, as emerging from this study, the enzyme inactivation observed when S-thiolation is induced by a cysteinyl residue occurs with a peculiar two-step mechanism which involves (i) the formation of a mixed disulfide bond and (ii) a thiol/disulfide intramolecular rearrangement. In fact, when the treatment of ALR2 with cystine was performed at 25 °C instead of at 37 °C, only a modest decrease in the enzyme activity was observed (Figure 1). Nevertheless, the progressive loss of sensitivity to Sorbinil observed during ALR2 incubation and its recovery after treatment of the enzyme with DTT (Figure 1) indicated that even at 25 °C ALR2 undergoes modification during incubation with cystine. In these conditions, when ^{14}C-labeled cystine was used as a

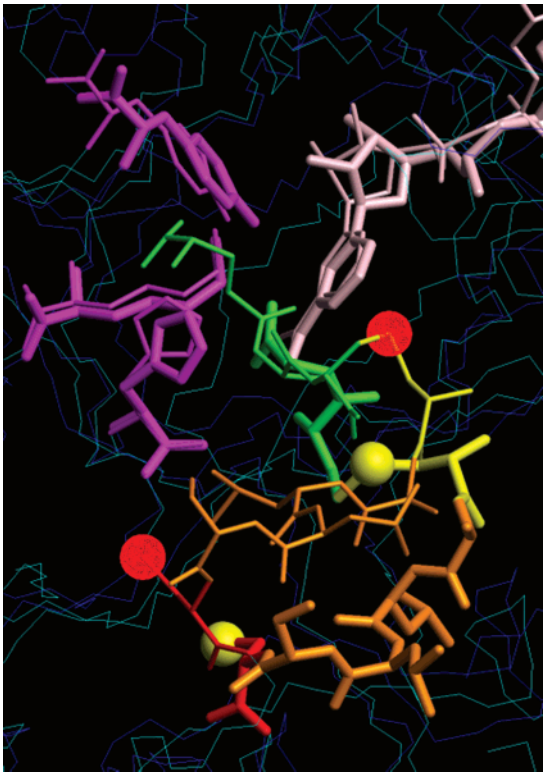

FIGURE 6: Best fit superposition of CysGly-ALR2 and GS-ALR2. ALR2 backbone atoms in the structures shown in Figure 5 have been RMS fitted. The active site region of the two enzyme forms is shown. ALR2 backbone bonds are represented with blue (CysGly-ALR2) or cyan (GS-ALR2) thin lines, except for active site residues (Tyr48, Lys77, His110, magenta sticks), the 299−302 region (orange sticks), Cys298 (yellow sticks), and Cys303 (red sticks). Pink and green sticks are used for the $NADP^+$ and ligand moieties, respectively. Thick and thin sticks are used for CysGly-ALR2 and GS-ALR2, respectively. Spheres (yellow solid for CysGly-ALR2, red wireframe for GS-ALR2) surround Cys298 and Cys303 sulfur atoms.

modifying agent, an incorporation of 1 equiv of cysteinyl residue/mol of enzyme was observed. Indeed, the ESMS analysis of the native and cystine-modified ALR2 clearly indicated that in the latter, among the seven reduced Cys residues present on the native enzyme, one was engaged in the formation of a mixed disulfide bond with a Cys residue (Figure 4B). Thus, even with cystine, the first step of enzyme modification, as previously observed for GSSG, is the generation of an enzyme form carrying a mixed disulfide, which however differently from GS-ALR2 is still able to reduce glyceraldehyde.

Concerning the reversibility of the enzyme modification, both DTT and GSH are able to reduce Cys-ALR2 back to the native enzyme form (Table 2). This is different from what was observed when 2ME and Cys were used as thiol reducing agents. While Cys is apparently ineffective on Cys-ALR2, 2ME acts by giving an enzyme form with the same kinetic properties of the *S*-mercaptoethyl-ALR2 (*29*). These results, which are indicative of the involvement of Cys298 in the formation of the mixed disulfide bond (see below), are clearly related to a specific ability of different thiols to interact with Cys-ALR2. In fact, trans-thiolation by GSH appears to occur targeting the cysteinyl moiety of the Cys-ALR2 mixed disulfide bond, generating the reduced enzyme and CyS-SG. On the contrary, 2ME and conceivably Cys apparently target the Cys residue of the enzyme, allowing the release of free cysteine.

Similarly to what was observed for the Cys-induced modification, CysGly disulfide is able to generate an active ALR2 form with a specific activity 1.2 times higher than the native enzyme, which is essentially not susceptible to inhibition by Sorbinil (Figure 1). Also in this case, the major product of S-thiolation of ALR2 is an enzyme form whose molecular mass is compatible with the insertion of one CysGly residue/mol of enzyme (Figure 4D).

The combined ESMS and Edman degradation approach to the peptide analysis of Cys-ALR2 and CysGly-ALR2 allowed to identify Cys298 as the residue involved in the formation of the mixed disulfide with the exogenous thiol compound (Table 1).

The present results support the view of Cys298 as a relevant group in substrate and cofactor positioning (*57*). In fact, taking also into account the features of the *S*-glutathionyl- and the *S*-2-mercapto-modified ALR2, both carrying the mixed disulfide at the level of Cys298 (*54*, *55*), the steric hindrance as well as the charge distribution of the different thiolating agents of Cys298 appears to differently affect the enzyme activity. Thus, D,L-glyceraldehyde, which is a poor substrate for GS-ALR2 (*27*), is reduced by Cys-ALR2 and Cys-Gly-ALR2 at a rate comparable to that of the native enzyme. Furthermore, the Cys298 free thiol group appears relevant with respect to the susceptibility of the enzyme to inhibition. In this regard, irrespective of the nature of the modifying thiol, the derivatization of Cys298 appears to interfere with the enzyme−inhibitor binding, with a consequential reduction in sensitivity to ARIs.

Unlike what was observed for GS-ALR2, Cys-ALR2 (p*I* 4.85) and CysGly-ALR2 (p*I* 4.82) are rather unstable and easily lose enzyme activity when incubated at 37 °C (Figure 3), generating an enzyme form characterized by a p*I* of 5.25 (Figure 2C). Treatment with DTT of the thermally inactivated enzyme preparations restores both the activity and the susceptibility to inhibition. When [^{14}C]Cys-ALR2 was incubated at 37 °C, a loss of [^{14}C]Cys paralleled the loss of enzyme activity. Moreover, the residual radioactivity was exclusively associated to the most acidic protein band (p*I* 4.85). These data together with the results of a PAGE−SDS analysis of the inactive enzyme forms performed in nonreducing conditions, which shows the lack of any high molecular weight aggregate (data not shown), would suggest that the loss of Cys from Cys-ALR2 and CysGly from CysGly-ALR2 is associated with the formation of an intramolecular disulfide bond.

Indeed, the ESMS analysis of the thermally treated Cys-ALR2 and CysGly-ALR2 enzyme preparations allowed to assess the presence of a component (component 5 of both panels C and E of Figure 4) carrying an intramolecular disulfide. In this regard, ESMS and amino acid sequence analysis of peptides obtained from thermally treated Cys-ALR2 and CysGly-ALR2, clearly indicated the pairing of Cys298 and Cys303 in the intramolecular disulfide bond. Thus, it is clear from the present study that even though the two Cys residues appear to be far apart at a nonbonding distance on the native ALR2 complexed with its pyridine cofactor (*58*), an intramolecular disulfide bond is formed as a result of an intramolecular trans-thiolation process, following S-thiolation of the enzyme by Cys and CysGly but

not by GSH. This fact, besides the rather low concentration of Cys and CysGly in the lens, may explain why S-thiolation of ALR2 in this organ cultured under oxidative conditions appears to be essentially due to GSH (*28*). This may be especially relevant in the nucleus where, with respect to the cortex, a higher proportion of PS-SCys was found following oxidative stress (*20*, *22*−*24*).

The reduced stability displayed by Cys-ALR2 and CysGly-ALR2 with respect to GS-ALR2 finds explanation into the special interaction generated by the glutathionyl moiety at the active site of the enzyme. A comparison among the predicted structures of GS-ALR2, CysGly-ALR2, and Cys-ALR2 forms, as derived from different conformational sampling approaches, shows some general well-defined trends and significant differences, which are fairly independent from both the computational approach and the starting orientation of the thiol residue bound to the enzyme. In particular, the glutathionyl moiety in GS-ALR2 tends to fill the ALR2 cavity extending from the loop surrounding Leu300 to the enzyme active site. Different is the case of CysGly-ALR2 and Cys-ALR2 in which the modifying thiol residues appear fairly flexible and less tightly interacting with ALR2. In both CysGly-ALR2 and Cys-ALR2 the active site of the enzyme remains fully accessible, a fact that justifies the retention in these enzyme forms of enzyme activity. Moreover, the local motion in the region surrounding Cys298 is even somewhat increased as compared with the native ALR2. Finally, the distance between sulfur atoms belonging to Cys298 and Cys303 exhibits broad fluctuations, and by increasing temperature from 25 to 37 °C, a substantial decrease in its minimum value (from 5.1 to 3.9 Å for CysGly-ALR2 and from 5.5 to 4.3 Å for Cys-ALR2) is observed.

The way a shorter length of the ligand peptide affects its flexibility inside the ALR2 binding pocket may be less obvious than it appears at a first look. In fact, in addition to the expected reduction in the number of potential interactions and the increased accessibility of these interactions to solvent (although the enhanced flexibility is also observed in simulation without explicit introduction of solvent), in both Cys- and CysGly-modified ALR2 forms either N- or C-terminal ligand charged groups would be forced in a largely hydrophobic region of the ALR2 pocket (Trp20, Trp79, Trp111, Phe122, Trp219, and Leu300) (Figure 5). On the contrary, in GS-ALR2, all N-terminal charged groups of the glutathionyl moiety pointing toward the ALR2 active site are allocated in a more hydrophilic environment. Indeed, this feature could also suggest a role of charged groups of Cys and CysGly in both Cys- and CysGly-ALR2 forms in favoring the reaction path leading to the formation of an intramolecular disulfide bridge.

On the basis of the above results concerning (i) the potential of the cysteinyl- and CysGly-residue insertion on ALR2, (ii) the tendency of CyS-ALR2 and CysGly-ALR2 to generate an inactive ALR2 form carrying an intramolecular disulfide bond, and (iii) the previously assessed susceptibility of the enzyme to be modified by GSSG and the ability of the mixed disulfides CyS-SG to thiolate ALR2 with both its thiol moieties (*27*, *28*, *55*, *56*), it was possible to draw a scheme of interconversion pathways of bovine lens ALR2 induced in oxidative conditions by GSH and by its breakdown products (Figure 7). In the scheme the different reactions of S-thiolation of ALR2, which can possibly

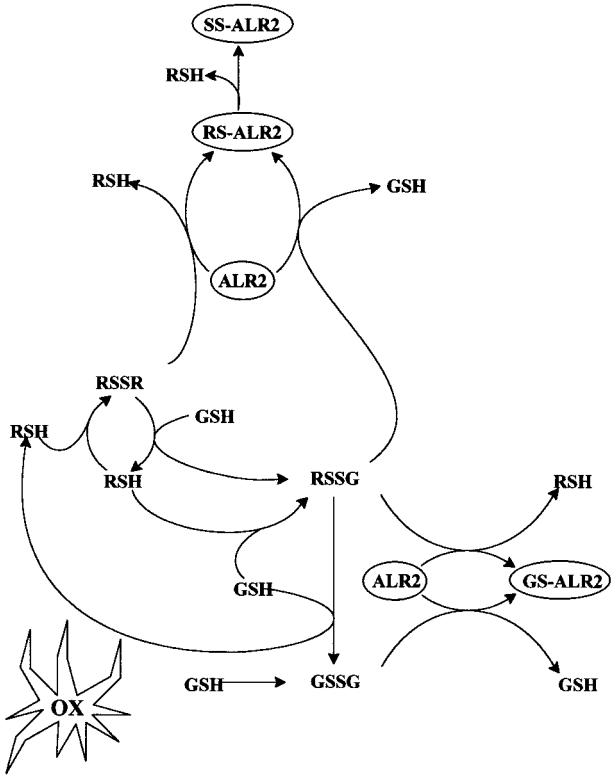

FIGURE 7: Thiol-dependent modification of ALR2 in oxidative conditions. RSH refers to both Cys and CysGly.

modulate the enzyme activity, are reported. It is difficult at this point to predict the relevance of the enzyme interconversion in oxidative conditions in vivo. So far only GS-ALR2 was specifically searched and found in the intact cultured lens undergoing oxidative stress (*28*). In this regard, the present results may represent the basis for further investigation on the involvement and relevance of S-thiolation phenomena on structural and functional modification of aldose reductase in cell systems undergoing oxidative stress.

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