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Zofenoprilat-Glutathione Mixed Disulfide as a Specific *S*-Thiolating Agent of Bovine Lens Aldose Reductase

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ABSTRACT

The ability of Zofenoprilat, an angiotensin-converting enzyme inhibitor carrying a thiol group, to intervene in protein *S*-thiolation processes was tested on bovine lens aldose reductase (ALR2). Zofenoprilat, more susceptible to oxidation than glutathione (GSH), forms with this physiological thiol a rather stable mixed disulfide (ZSSG). ZSSG, whose generation through the transthiolation reaction between GSH and Zofenoprilat homodisulfide was shown to be enhanced by a μ-class glutathione *S*-transferase, appears to be a specific donor of the Zofenoprilat moiety in the *S*-thiolation processes. This is indicated by the apparent stability of ZSSG to reduction by GSH and by the specificity of the transfer of the group on ALR2, used as a protein model. Indeed, the *S*-thiolation of ALR2 by ZSSG occurred exclusively through the insertion of the Zofenoprilat moiety of ZSSG on the enzyme. The modified ALR2 is shown to retain the same activity of the native enzyme, but displays a reduced sensitivity to inhibition. The *S*-thiolation of specific target enzymes is proposed as an event potentially relevant for the antioxidant action of Zofenoprilat. *Antioxid. Redox Signal.* 7:841–848.

INTRODUCTION

Pharmacological interest in Zofenoprilat (ZSH) as an effective and powerful angiotensin-converting enzyme (ACE) inhibitor was recently associated with its possible capacity to intervene as an antioxidant factor in reducing oxidative damage (7, 8, 19, 20, 30, 31, 35). The complexity of the observed phenomena makes it difficult to predict for ZSH a general mechanism; its action is generally associated with its scavenging ability as a thiol-containing molecule (8, 20, 34, 35).

In fact, the presence of the thiol group was invoked to be an essential requirement in order to induce, in bovine aortic endothelial cells, a decrease in the oxidative stress that parallels an increase in the production of nitric oxide (NO) (35). Such an enhancement of NO was postulated to derive from the ability of ZSH to reduce the level of free radicals and in particular the level of superoxide ion (8), indicated as a relevant factor in decreasing the NO biological activity (37). Again, the presence of a SH group was associated with the ability of ZSH to reduce, in human umbilical vein endothelial cells, the expression of adhesion molecules, the activation of nuclear factor-κB and the reduced glutathione (GSH) decrease induced by oxidized low-density lipoprotein and tumor necrosis factor-α (8).

Because of their wide availability, physiological cell thiols can effectively compete with exogenous thiols. This should be the case for ZSH. In fact, the low level of circulating ACE inhibitor, sufficient to effectively target ACE, would hardly explain the observed antioxidant action based on a direct scavenging of reactive oxygen species (ROS). Indeed it would be conceivable that, in order to contribute to the antioxidant cell defense, the action of the thiol compound needs to be amplified. The covalent modification of proteins and enzymes, which can modify their molecular features and functions, represents one possible way to elicit the pharmacological action of drugs. In this regard, protein *S*-thiolation is a rapid, reversible modification of protein thiols that may occur as an early cellular response to oxidative stress. In oxidative conditions, protein *S*-thiolation is an important mechanism of metabolic regulation (15, 25, 48) that represents a useful

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strategy to protect enzymes and proteins from the irreversible oxidation induced by ROS (4, 6, 32).

The ability of Zofenoprilat disulfide (ZSSZ) to induce protein *S*-thiolation was previously reported for aldose reductase (ALR2) isolated from bovine lens (16). ALR2 (alditol:$NADP^+$ oxidoreductase, EC 1.1.1.21) catalyzes, with a broad catalytic specificity, the NADPH-dependent reduction of aldo-sugars and a variety of aldehydes to their corresponding alcohol. ALR2 is susceptible to thiol modification, thus giving rise to enzyme forms with altered kinetic properties and a general decrease in susceptibility to inhibition (3, 12, 13, 21, 28). The ability of ALR2 to reduce sugars in hyperglycemic conditions has been indicated as a primary factor in the etiology of diabetic complications (24, 42). More recently, the enzyme was found to be involved in the detoxification pathway of 4-hydroxy-2-nonenal (HNE), a major aldehydic product of lipid peroxidation (36, 46), whose particular reactivity toward histidine, lysine, and especially cysteine protein residues (40, 44, 45) may be a cause of cell damage (18, 27). Although ALR2 is able to reduce HNE, it has been shown to be irreversibly inactivated by its own substrate (16). In this regard, the ZSSZ-induced modification of ALR2 has been found to be able to stabilize the enzyme against the inactivation induced by HNE, without affecting its catalytic activity (16).

The present article shows that ZSH, through its oxidation to Zofenoprilat–glutathione mixed disulfide (ZSSG), has the potential to act as a specific protein *S*-thiolating agent, through a process that may be enzymatically enhanced by a μ-class glutathione *S*-transferase (GST).

MATERIALS AND METHODS

Materials

NADPH, GSH, oxidized glutathione (GSSG), dithiothreitol (DTT), and 1-chloro-2,4-dinitrobenzene were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sorbinil [(*S*)-6-fluorospiro(chroman-4,4′-imidazolidine)-2′,5′-dione] was from Pfizer Inc. (Groton, CT, U.S.A.). Zofenoprilat [[1(*S*),4(*S*)]-1-(3-mercapto-2-methyl-1-oxopropyl)-4-(phenylthio)-L-proline] was from Menarini Ricerche S.p.A. (Pomezia, Italy). Matrex Orange A resin was purchased from Amicon (Beverly, MA, U.S.A.). All other chemicals were of reagent grade from BDH (Poole, U.K.).

Synthesis of Zofenoprilat-containing disulfides

ZSSZ was prepared by incubating ZSH for 48 h at 25°C in the presence of 0.3 m*M* Fe^{2+} and 0.9 m*M* EDTA. High-performance capillary electrophoresis (HPCE) was used to test the occurrence of the complete oxidation of the thiol compound. HPCE analysis was performed as previously described (2) by a Beckman P/ACE system 2100 (Beckman, Fullerton, CA, U.S.A.) using fused silica capillaries (50 cm × 50 μm inner diameter) at a constant voltage of 30 kV, with 0.1 *M* Tris-borate buffer, pH 8.5, as an electrolyte. The migration times of ZSH, GSH, ZSSZ, ZSSG, and GSSG were 5.3, 6.2, 6.7, 6.8, and 6.9 min, respectively.

ZSSG was prepared by incubating for 48 h at 25°C 4 m*M* ZSH and 30 m*M* GSSG in 10 m*M* potassium phosphate, pH 7.0 (S-buffer). ZSSG was separated from the unreacted reagents by HPLC on a Beckman C18 reverse-phase column (1 × 25 cm) equilibrated at a flow rate of 1.8 ml/min in S-buffer. After the sample had been applied, the elution was performed by 5 min of S-buffer followed by a 15-min linear gradient from 0 to 40% acetonitrile in the same buffer and then by an isocratic step for 10 min with 40% acetonitrile in S-buffer. The peak of ZSSG monitored at 254 nm, which showed a retention time of 20.7 min, was collected, dried by a centrifugal evaporator, resuspended in Milli-Q water to a final concentration of 5 m*M*, and stored at −20°C. In the same conditions, the retention times of ZSH and GSH were 25.2 and 7.8 min, respectively. The HPLC analysis performed as above on a ZSSG sample after treatment with 2 m*M* DTT at 37°C for 1 h revealed the disappearance of the 20.7-min peak and the appearance of two peaks, consistent with the elution times of GSH and ZSH (data not shown).

Assay of enzyme activities

The assay for ALR2 activity was performed in a final assay volume of 535 μl, using 4.7 m*M* DL-glyceraldehyde as substrate in 0.25 *M* sodium phosphate buffer, pH 6.8, containing 0.38 *M* ammonium sulfate, 0.11 m*M* NADPH, and 0.5 m*M* EDTA. One unit of ALR2 activity is the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per minute. An absorption coefficient at 340 nm of 6.22 mM^{-1} cm^{-1} for NADPH was used.

GST (EC 2.5.1.18) activity was determined according to Habig and Jakoby (22). One unit of GST activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of *S*-2,4-dinitrophenylglutathione per minute. An absorption coefficient at 340 nm of 9.6 mM^{-1} cm^{-1} for *S*-2,4-dinitrophenylglutathione was used.

Enzyme purification

ALR2 was purified from bovine lens as previously described (14). The pure enzyme, which was stored at 4°C in the presence of 2 m*M* DTT, had a specific activity of 920 mU/mg. When used as a protein target of *S*-thiolation, ALR2 was dialyzed against *S*-buffer until the concentration of DTT was <1 μ*M*.

GST was purified from bovine lens as previously described (10). The pure enzyme, which was stored at −20°C, had a specific activity of 3,000 mU/mg.

Molecular modeling and energy minimization analysis

A starting structure for ZSSG was built with Ghemical v. 1.0 package (33). Atomic point charges for the ZSH moiety were derived with MOPAC7 (38), using the AM1-BCC approach (23).

Calculations were performed with the Sander module of AMBER6 package (5), using the PARM94 parametrization (9), in which the missing parameters for the Zofenoprilat group were derived by similarity from existing dataset entries.

The starting ZSSG structure underwent 200 cycles of simulated annealing (SA), using an implicit solvation model, based on a generalized Born (43) with surface area contribution (GBSA) approach (47). During each SA cycle (100,000

molecular dynamics steps), the system temperature was linearly raised from 10 K to 1,200 K (step 1 to 5,000), then kept constant at 1,200 K (step 5,001 to 45,000), and, finally, linearly decreased down to 10 K (step 50,001 to 100,000). A time step of 1 fs, with no constraints or restraints, a nonbonded cutoff of 16 Å, and a 0.05-fs time constant for heat bath coupling were used, with all the other parameters set at their default values.

The final structures were energy-minimized with 100 steps of steepest descent followed by a conjugate gradient method, with the Sander module of AMBER6, down to a gradient norm value of $<10^{-3}$ kcal mol^{-1} $Å^{-1}$. The best 100 structures in terms of total energy were clusterized and analyzed.

Analyses and figures were obtained with the MOLMOL 2K.2 program (26).

Other methods

Protein concentration was determined according to Bradford (1), using bovine serum albumin as a standard.

Isoelectric focusing was carried out at 4°C on a Biophoresis horizontal electrophoresis cell (Bio-Rad, Hercules, CA, U.S.A.) using Ampholine PAG plates (pH 4.0–6.5). Gels were prefocused for 20 min at 15 W. 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 a solution containing 25% ethanol and 8% acetic acid. Gels were stained for 15 min with 1.16 g/L Coomassie Blue R-250 in 25% ethanol, 8% acetic acid solution.

HNE was prepared by acid hydrolysis of the dimethyl acetal (11) in the presence of 0.05% trifluoroacetic acid. It was then isolated by HPLC on a C18 reverse-phase column (16).

RESULTS

Thiols oxidation

Oxidative conditions led to the oxidation of ZSH and GSH to the corresponding disulfides. Figure 1 shows the effect of 0.3 m*M* $FeSO_4$ and 0.9 m*M* EDTA on the oxidation rate of both thiols incubated at a final concentration of 3 m*M*. After 24 h at 25°C, a complete oxidation of ZSH occurred, whereas only 10% of GSH was oxidized. After oxidative treatment, a complete recovery of the reduced species was observed after 1 h of incubation of both thiol mixtures in the presence of DTT. When GSH and ZSH were present together in oxidative conditions, the formation of ZSSG was observed. In fact, ZSSG was formed (initial rate of 4 μ*M*/min) upon incubation of ZSSZ in the presence of GSH (Fig. 2). Moreover, the transthiolation process appeared to be enhanced by a μ-class GST, purified from bovine lens (10). In fact, an approximately fourfold increase in the initial rate of transthiolation was observed when 6.7 U/ml GST was added to the thiol/disulfide mixture. The ZSSG mixed disulfide appeared to be quite stable in the presence of an excess of GSH. No changes in the ZSSG level nor GSSG formation were detected by both HPLC and HPCE analyses, after 5 h of incubation at 25°C of 0.2 m*M* ZSSG with 0.5 m*M* GSH, both in the absence and in the presence of GST (data not shown).

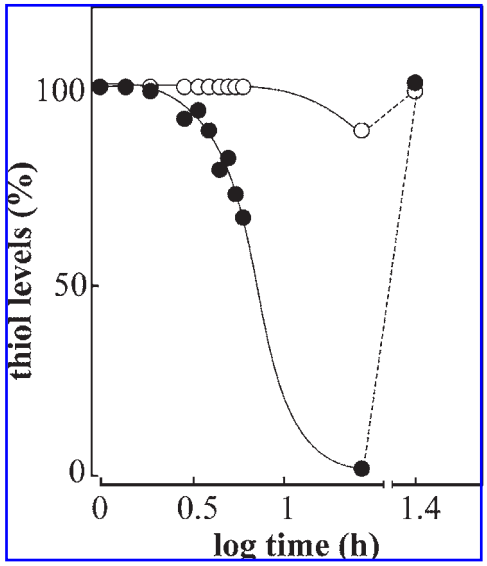

FIG. 1. Effect of the Fe^{2+}/EDTA oxidative system on ZSH and GSH. Solutions (3 m*M*) of GSH (○) and ZSH (●) were incubated in S-buffer at 25°C in the presence of 0.3 m*M* $FeSO_4$ and 0.9 m*M* EDTA. At the indicated times, aliquots of the incubating mixtures were analyzed for residual GSH and ZSH by HPCE. After 24 h of incubation, samples were supplemented with 2.5 m*M* DTT, incubated for 1 h at 25°C, and then analyzed by HPCE (dashed lines).

Conformational analysis of ZSSG

The use of an implicit solvation model substantially decreases energy differences between folded and extended structures and greatly reduces the overstabilization of intramolecularly H-bonded conformations, usually affecting *in vacuo* simulations. Thus, a rather flat potential energy distri-

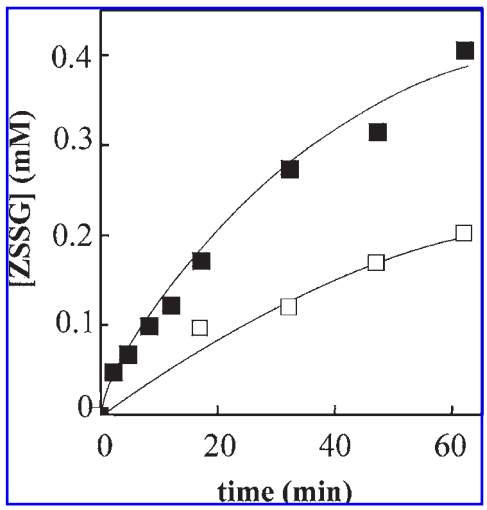

FIG. 2. Time course of ZSSG formation. Solutions (0.5 m*M*) of ZSSZ and GSH were incubated at 25°C in S-buffer, both in the absence (□) and in the presence (■) of 6.7 U/ml bovine lens μ-class GST. At the indicated times, aliquots were withdrawn and the ZSSG level was detected by HPCE.

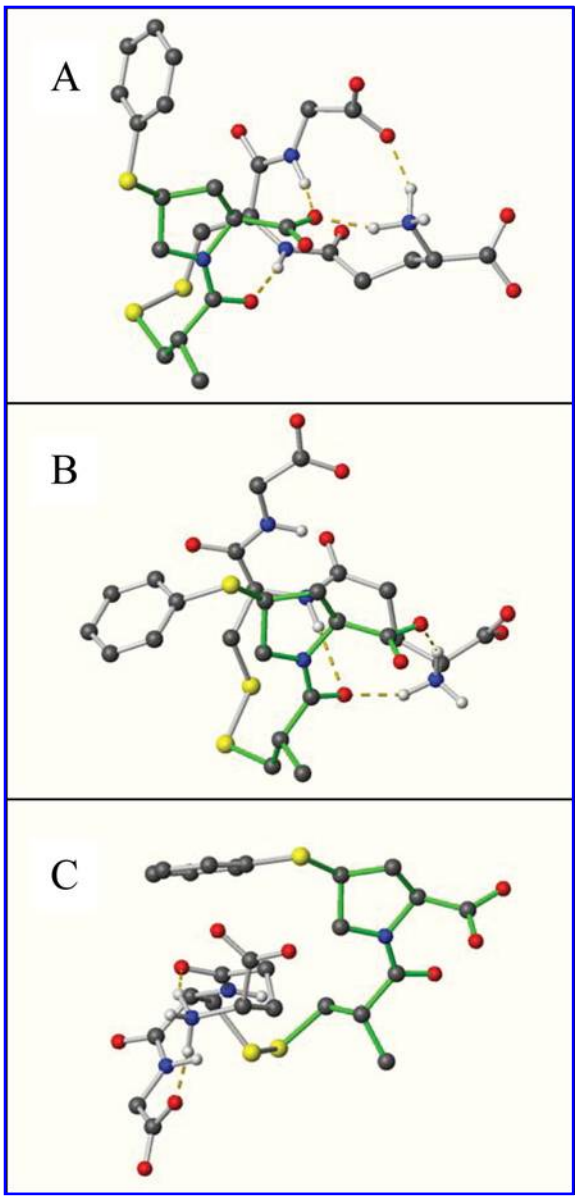

FIG. 3. Representation of ZSSG structures. Ball-and-stick representation of three ZSSG structures, corresponding to conformers 1 (**A**), 2 (**B**), and 4 (**C**) in order of increasing energy, altogether representative of the first 25 most stable ZSSG conformers. Standard color convention has been used for atoms, whereas bonds are shown in gray, except for the most structurally converged part of the Zofenoprilat moiety (root mean square deviation on heavy atoms is 0.6 Å in the best 100 conformers), which is painted green. Dotted orange sticks represent H-bonds and salt bridges. Nonpolar hydrogen atoms are omitted for clarity.

bution was obtained within the final structure set, the 100 most stable conformers spanning a range of 10.5 kcal mol^{-1}. However, the most stable conformation families all exhibited networks of salt bridges and/or H-bonds. In particular, 48% of the most stable conformers form polar interactions involving atoms in both the Zofenoprilat and the glutathione moieties, whereas in the remaining 52% of conformers the network of polar interactions is all internal to the glutathione group.

A compactness analysis of the best conformers shows that 70% of the best 100 conformers exhibit a compact structure, with either polar or hydrophobic interactions between Zofenoprilat and glutathione moieties. This percentage increases to 80% and 100% when the best 50 or 20 conformers, respectively, are considered.

The most stable conformers (Fig. 3) clearly show a typical network of H-bonds/salt bridges and hydrophobic interactions, involving either intermoiety Zofenoprilat–glutathione or intramoiety glutathione–glutathione interactions.

S-*Thiolation of ALR2 induced by Zofenoprilat-containing disulfides*

Before investigating the ability of Zofenoprilat to act as modifying agent of ALR2, a preliminary investigation on the effect of Zofenoprilat and its disulfides on the enzyme activity was performed. The results (data not shown) indicate that whereas Zofenoprilat and ZSSG were ineffective on the enzyme activity (50 μ*M*, maximal tested concentration), ZSSZ displayed an inhibitory action on the native ALR2 with an IC_{50} of 10 μ*M*.

When ALR2 was incubated in the presence of ZSSG, no changes in the enzyme activity were observed for at least 3 h; however, the incubation led to a progressive loss of sensitivity to Sorbinil (Fig. 4). A loss of sensitivity to Sorbinil was also observed when the native enzyme was incubated in the presence of ZSSZ. It is worth noting that the low activity observed at zero time when ALR2 was incubated with ZSSZ

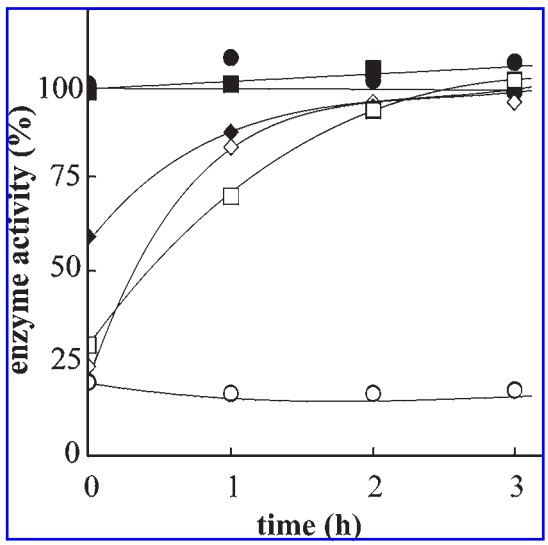

FIG. 4. *S*-Thiolation of ALR2 induced by disulfides. Purified ALR2 (3.5 μ*M*) was incubated in S-buffer at 25°C alone (circles) and in the presence of 1 m*M* ZSSZ (diamonds) and 1 m*M* ZSSG (squares). At the indicated times, aliquots of 15 μl were withdrawn and ALR2 activity was measured in standard assay conditions both in the absence (closed symbols) and in the presence (open symbols) of 10 μ*M* Sorbinil. The percentage of enzyme activity refers to the activity measured at zero time in the incubation performed in the absence of disulfides (100% equals 127 mU/ml ALR2).

was expected on the basis of the inhibitory action exerted on ALR2 by this disulfide. In such a case, the peculiar increase of ALR2 activity with the incubation time (Fig. 4) finds explanation in the failure of ZSSZ to inhibit the ALR2 activity of the enzyme subjected to incubation, as in Fig. 4, with either ZSSG or ZSSZ. In fact, an IC_{50} > 100 μ*M* was observed for ZSSZ when the enzyme coming from the incubation with either ZSSG or ZSSZ was subjected to an inhibition test after extensive dialysis against S-buffer. In this regard, Sorbinil (as also reported in Fig. 4) and ZSSG (as it occurs for the native enzyme) were ineffective as inhibitors of the disulfide-treated enzyme preparations. Finally, the enzyme activity detectable after the incubation of ALR2 with either ZSSZ or ZSSG recovered its susceptibility to inhibition by Sorbinil and ZSSZ when 2 m*M* DTT was added to the enzyme preparations (data not shown).

When subjected to isoelectric focusing analysis (data not shown), ALR2 modified by ZSSZ revealed one major band focusing at pH 4.75. An identical pI value has been previously reported (3) for the *S*-glutathionyl-modified ALR2 (GS-ALR2) and is consistent with the insertion on the enzyme of a carboxyl–containing *S*-modifying agent (the pI of the native ALR2 is 4.85).

When the enzyme modified by ZSSG was subjected to isoelectric focusing analysis, again a major band focusing at pH 4.75 was observed. To verify the degree of specific insertion on the enzyme of one of the two thiol moieties of ZSSG, ALR2 modified by ZSSZ and ZSSG as described in Fig. 4 was analyzed by affinity chromatography on Matrex Orange A. As previously reported (4, 17), whereas the native ALR2 and other-thiol modified enzyme forms bind to the chromatographic support and were eluted by 0.1 m*M* NADPH, GS-ALR2 passed through the column without interacting with the resin. The results reported in Fig. 5A show that, similar to what was observed with the native enzyme (4), the enzyme modified by ZSSZ, applied to the column after a dialysis against S-buffer, did bind Matrex Orange A and was eluted by 0.1 m*M* NADPH. However, unlike what was observed for the native enzyme, the eluted activity was not susceptible to inhibition by Sorbinil. The treatment of the eluted fractions with 2 m*M* DTT did not affect enzyme activity, although it led to a recovery of susceptibility to inhibition.

The behavior of GS-ALR2 on the affinity column is also shown (Fig. 5B). In this case, as expected (4), the enzyme did not bind the resin, and both the activity and sensitivity to Sorbinil were recovered upon treatment of the eluted fractions with DTT. The chromatographic analysis performed on the enzyme previously treated with ZSSG revealed again only one activity peak eluted by NADPH (Fig. 5C). In this case, too, the enzyme activity detectable in the eluted fractions was insensitive to Sorbinil, which recovered its inhibitory ability only after the enzyme samples had been treated with DTT.

Direct evidence of *S*-thiolation by Zofenoprilat came from the measurement of the Zofenoprilat released by DTT treatment of ZSSZ-modified ALR2. A molar ratio Zofenoprilat/ALR2 of 1:1 was evaluated when 3.2 μ*M* ZSSZ-modified ALR2 was reduced by 1 m*M* DTT, filtered through Amicon Centricon10, and both the retentate and the protein-free ultrafiltrate were analyzed for ALR2 and ZSH, respectively. An indication of the specific targeting of *S*-thiolation by ZSSZ and ZSSG comes from the failure of these disulfides to modify ALR2, whose Cys^{298} was previously specifically blocked by carboxyamidomethylation (29). In this case, no ZSH was detectable when the Cys^{298}-blocked ALR2 treated with ZSSZ was analyzed as above. In this regard, the Cys^{298}-blocked ALR2, which displays an increased specific activity with respect to the native ALR2 (28), was also insensitive to the inhibitory action of ZSSZ (data not shown).

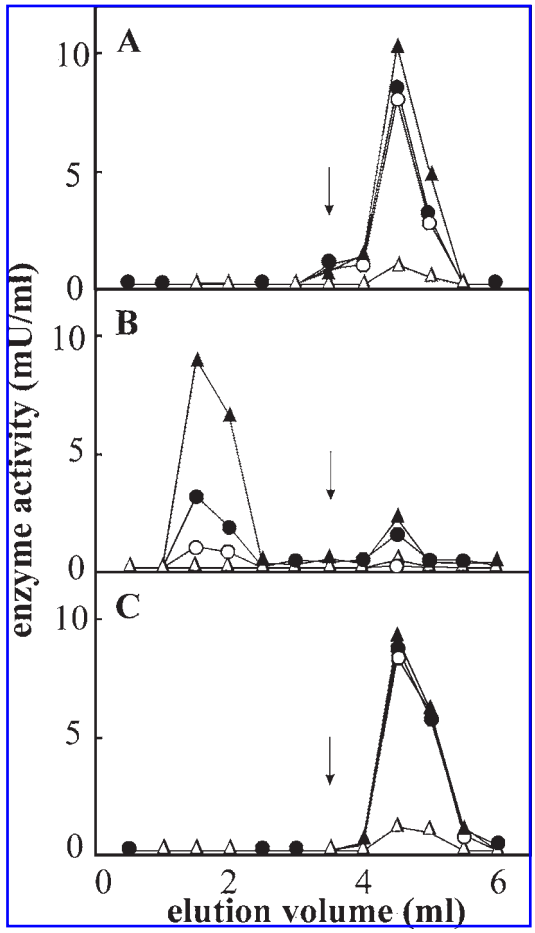

FIG. 5. Affinity chromatography of modified forms of ALR2. Native ALR2 (3.5 μ*M* final concentration) was incubated in S-buffer for 3 h at 25°C in the presence of 1 m*M* GSSG, 1 m*M* ZSSZ, or 1 m*M* ZSSG. After dialysis against S-buffer, samples (15 μg) of ZS-ALR2 (**A**), GS-ALR2 (**B**), or ALR2 modified by ZSSG (**C**) were applied to a Matrex Orange A column (1 × 3.5 cm) and elution was performed with S-buffer. Where indicated by an arrow, the elution buffer was supplemented with 0.1 m*M* NADPH. The eluted fractions were analyzed for ALR2 activity both before (circles) and after (triangles) incubation for 2 h at 25°C with 1 m*M* DTT. Closed and open symbols refer to the activity measured either in the absence or in the presence of 10 μ*M* Sorbinil, respectively.

DISCUSSION

The higher susceptibility to oxidation of ZSH as compared with GSH, and the easy chemical generation of the ZSSG by transthiolation, indicate that the mixed disulfide ZSSG is the

most favorable disulfide end product of ZSH oxidation occurring in the presence of GSH. This is strengthened by the observation that the conjugation of ZSH with GSH through the transthiolation of GSH on the ZSSZ may be enzymatically driven. In fact, a μ-class GST, an enzyme isolated from bovine lens, which has been shown to act as a thioltransferase enzyme (10), was able to enhance the formation rate of the mixed disulfide. The polar and/or the hydrophobic interactions of the most stable conformers of ZSSG and the compactness of the resulting structures (Fig. 3) may both adversely affect, in principle, the reactivity of ZSSG disulfide toward thiol-containing molecules, by decreasing the accessibility of the disulfide bridge, the flexibility of the molecule, and the availability of polar functional groups eventually involved in the reaction path.

Indeed, the apparent stability of ZSSG in thiol-reducing conditions (*i.e.,* 0.5 m*M* GSH) would support such a view. Nevertheless, even if the above stabilizing interactions were unable to prevent the reaction, the intrinsically asymmetric structure of ZSSG and of its internal interactions could strongly favor the replacement of the glutathione moiety, with formation of a new ZSSG molecule, rather than the displacement of the Zofenoprilat group. In such a view, ZSSG may represent the only potential agent that is able to induce *S*-thiolation by Zofenoprilat.

ALR2, which was shown to be modified by ZSSZ, turned out to be quite a useful model to study the effectiveness of ZSSG as a thiolating agent. Indeed, the peculiar kinetic behavior of different enzyme forms carrying a mixed disulfide with different thiol-modifying agents allowed us to follow the enzyme modification and to assess the specific transfer on the enzyme molecule of the ZSH moiety of the mixed disulfide.

The susceptibility to inhibition of the native enzyme by ZSSZ, its insensitivity to ZSSG, as well as the significant decrease in the effectiveness of ZSSZ as inhibitor of *S*-zofenoprilat-modified ALR2 (ZS-ALR2) when compared with ALR2, give an explanation of the differences in the enzyme activity during the *S*-thiolation of ALR2 by ZSSZ and ZSSG (Fig. 4). Thus, when ZSSZ is used as modifying agent, the generation of ZS-ALR2 can be monitored through the apparent recovery of the enzyme activity; on the other hand, when the enzyme is incubated with ZSSG, the ZS-ALR2 formation can be monitored by following the progressive loss of sensitivity of the enzyme to the Sorbinil inhibitory action.

These results are consistent with the occurrence of a reversible modification of ALR2 that generates an enzyme form with the same specific activity as the native enzyme, but with a reduced sensitivity to the inhibitory action of both ZSSZ and a classical ALR2 inhibitor like Sorbinil. In this regard, no changes in the enzyme activity were observed after ALR2 modification with either ZSSZ or ZSSG, when 100 μ*M* HNE rather than DL-glyceraldehyde was used as an enzyme substrate. Moreover, as expected, the enzyme *S*-thiolated by ZSSG was stabilized against HNE inactivation (data not shown), as occurred for ALR2 modified by ZSSZ (16).

The isoelectric focusing analysis confirmed the occurrence of ALR2 modification by a shift of the pI from 4.85 to 4.75. However, the identity between the pI values of ZS-ALR2 and GS-ALR2, as expected by the presence of a carboxyl group in both Zofenoprilat and glutathione, did not allow us to discriminate any preferential conjugation of the two thiol moieties of ZSSG with cysteine residues of the protein.

The retention of the enzyme activity following the incubation of ALR2 with ZSSG (Fig. 4) seems to suggest the lack of a substantial insertion of the glutathionyl moiety into the enzyme. However, a clear indication that the enzyme modification specifically occurred through the insertion of the Zofenoprilat moiety came from the results of the affinity chromatographic analysis on Matrex Orange A (Fig. 5). Through this approach, it was possible to discriminate between different modified ALR2 forms (4, 17). In this case, too, the ability of the chromatographic support to retain ZS-ALR2 allowed us to separate this enzyme from GS-ALR2 and then, eventually, quantify the relative contents of the two enzyme forms. The lack of GS-ALR2 formation upon incubation of ALR2 with ZSSG (Fig. 5C) confirmed that the mixed disulfide ZSSG is a specific Zofenoprilat donor. This is in line with the apparent stability of ZSSG in the presence of GSH, which, as mentioned above, can find explanation with the simple recycle of the glutathionyl moiety on the mixed disulfide. Concerning the potential role of ALR2 as a detoxifying enzyme against damaging aldehydic products of lipid peroxidation (*i.e.,* HNE) (36, 46), the ability of ZSSG to generate an enzyme form (ZS-ALR2) still active, and at the same time insensitive to the inactivating effect of HNE, might be relevant in preserving the enzyme function in oxidative conditions. Besides the specific effect of ZSSG on ALR2, these results revealed the potential of ZSSG as a specific protein thiol-modifying agent. This may be particularly relevant for those enzymes, such as ACE (39) or aminopeptidase W (41), that are specifically targeted by the drug. A search for different protein targets of *S*-thiolation by ZSSG may reveal further insights into the antioxidant component of the pharmacological action of Zofenoprilat.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme; ALR2, aldose reductase; DTT, dithiothreitol; GS-ALR2, *S*-glutathionyl-modified ALR2; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; HNE, 4-hydroxy-2-nonenal; HPCE, high-performance capillary electrophoresis; NO, nitric oxide; ROS, reactive oxygen species; SA, simulated annealing; ZS-ALR2, *S*-Zofenoprilat-modified ALR2; ZSH, Zofenoprilat, reduced form; ZSSG, Zofenoprilat-glutathione mixed disulfide; ZSSZ, Zofenoprilat, disulfide form.

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