Oxidative Modification of Aldose Reductase Induced by Copper Ion. Factors and Conditions Affecting the Process†

Ilaria Cecconi, Maria Moroni, Pier Giuseppe Vilardo, Massimo Dal Monte, Paola Borella,‡ Giulio Rastelli,§ Luca Costantino,§ Donita Garland,|| Deborah Carper,|| J. Mark Petrash,⊥ Antonella Del Corso, and Umberto Mura*

Dipartimento di Fisiologia e Biochimica, Università di Pisa, via S. Maria, 55-56100 Pisa, Italy, Dipartimento di Scienze Biomediche, Università di Modena, via Campi, 287, 41100 Modena, Italy, Dipartimento di Scienze Farmaceutiche, Università di Modena, via Campi, 183, 41100 Modena, Italy, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892, and Departments of Ophthalmology and Visual Sciences and of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

Received May 18, 1998; Revised Manuscript Received August 13, 1998

ABSTRACT: Bovine lens aldose reductase (ALR2) is inactivated by copper ion [Cu(II)] through an oxygen-independent oxidative modification process. A stoichiometry of 2 equiv of Cu(II)/enzyme mol is required to induce inactivation. While metal chelators such as EDTA or *o*-phenantroline prevent but do not reverse the ALR2 inactivation, DTT allows the enzyme activity to be rescued by inducing the recovery of the native enzyme form. The inactive enzyme form is characterized by the presence of 2 equiv of bound copper, at least one of which present as Cu(I), and by the presence of two lesser equivalents, with respect to the native enzyme, of reduced thiol residues. Data are presented which indicate that the Cu-induced protein modification responsible for the inactivation of ALR2 is the generation on the enzyme of an intramolecular disulfide bond. GSH significantly interferes with the Cu-dependent inactivation of ALR2 and induces, through its oxidation to GSSG, the generation of an enzyme form linked to a glutathionyl residue by a disulfide bond.

Copper is a trace element involved in the mechanism of action of several enzymes and other functional proteins (*1, 2*). In vivo, the level of the free metal is strictly controlled by chelating proteins, such as ceruloplasmin, transcuprein, and albumin, which are all devoted to storage and transport (*1*). There are, however, pathological conditions (*3*−*7*) in which the concentration of the metal ion increases and causes damage. Similar to iron, copper promotes the Fenton reaction and therefore may contribute to oxidative stress in biological systems (*8*−*13*). Moreover, due to its effective binding capability to polypeptides (*14, 15*) and nucleic acids (*16*−*18*), copper ion has the potential for site-directed action on these molecular targets, effectively interfering with normal cell function and proliferation (*19*). Different metal-catalyzed oxidation (MCO)[1] systems have been used in order to study the ability of copper to induce oxidative damage (*20*−*22*). In this study, aldose reductase (alditol:$NADP^+$ oxidoreductase, EC 1.1.1.21), purified from bovine lens was chosen as the protein target.

Aldose reductase, which catalyzes the NADPH-dependent reduction of aldoses, as well as a variety of aliphatic and aromatic aldehydes to the corresponding alcohols (*23*), was shown to be especially susceptible to thiol-mediated oxidative modification induced by the oxygen radical generating system Fe^{2+}/EDTA (*24*). Thus, different thiol compounds under oxidative conditions, lead in vitro to enzyme forms displaying altered kinetic and structural properties (*25*−*28*). Among these enzyme forms, the S-glutathionyl-modified ALR2, can be generated in vitro by treatment of both bovine lens and human ALR2 (*27, 29*) with GSH in the presence of Fe^{2+}/EDTA or with GSSG. This enzyme form was detected in intact bovine lens subjected to hyperbaric oxygen treatment (*30*). In vitro studies demonstrate that the modification of ALR2 appears to proceed by a preliminary oxidation of thiols to disulfides which would be the true modifying agents of the protein. The MCO system would act by generating or propagating oxygen radicals in the bulk solution without a significant direct interaction with the enzyme molecule, and the subsequent enzyme modification would be the result of the increasing concentration of GSSG formed as a consequence of the GSH scavenging action. When oxidative stress is induced by copper ion, GSH counteracts the stress by its well-known oxidant scavenging action as well as by chelating the copper, which would then be more easily transferred from the bulk solution to metal-binding proteins (*31, 32*).

We describe here the special effectiveness of copper ion in inducing bovine lens ALR2 inactivation, either in the absence or in the presence of GSH, the most highly represented lens thiol.

† This work was supported in part by the "Target Project on Cellular Oxidative Stress" of the Italian National Research Council and in part by Italian Board of Education (MURST).

* To whom correspondence should be addressed at the Università di Pisa. Tel: 39-50-500292. Fax: 39-50-502583. E-mail: cmario@dfb.unipi.it.

‡ Dipartimento di Scienze Biomediche, Università di Modena.

§ Dipartimento di Scienze Farmaceutiche, Università di Modena.

|| National Institutes of Health.

⊥ Washington University School of Medicine.

[1] Abbreviations: MCO, metal catalyzed oxidation; ALR2, aldose reductase; BCDS, bathocuproinedisulphonic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; GS-ALR2, glutathione-modified ALR2; OP, *o*-phenantroline; SD, standard deviation of the mean; SOD, superoxide dismutase.

S0006-2960(98)01159-3 CCC: $15.00
Published on Web 09/17/1998

EXPERIMENTAL PROCEDURES

Materials. NADPH, D,L-glyceraldehyde, dithiothreitol, GSH, GSSG, catalase (EC 1.11.1.6; 17 600 units/mg), and superoxide dismutase (EC 1.15.1.1; 13 300 units/mg) were purchased from Sigma Chemical Co. Bathocuproinedisulfonic acid was from Jannsen Pharmaceutical. All electrophoresis reagents and isoelectric focusing standards were from Bio-Rad. Ampholine PAG plate, pH 4.0−6.5, for isoelectric focusing was from Pharmacia. Copper(II) chloride and all inorganic chemicals were of reagent grade from BDH. The ALR2 inhibitor (*S*)(+)-6-fluoro-2,3-dihydrospiro-(4H-1-benzopyran-4,4′-imidazolidine)-2′,5′-dione (Sorbinil) (*33*) was a gift from Dr. G. Caccia, Laboratori Baldacci S.p.A., Pisa, Italy. The complex $(BCDS)_2Cu(I)$ was a gift from Dr. R. L. Levine, Laboratory of Biochemistry, NHLBI, NIH, Bethesda, Maryland.

Enzyme Purification. The purification of bovine lens ALR2 was performed as previously described (*34*). The pure native enzyme (specific activity, 1.12 units/mg) was stored at 4 °C in 10 mM sodium phosphate buffer, pH 7.0, supplemented with 2 mM DTT. Just before use, the enzyme was extensively dialyzed against 100 mM sodium phosphate buffer, pH 6.8 (S-buffer).

Measurement of Enzyme Activity. The ALR2 activity was measured at 37 °C, using 4.7 mM D,L-glyceraldehyde as substrate, in 0.25 M sodium phosphate buffer containing 0.38 M ammonium sulfate, 0.11 mM NADPH, and 0.46 mM EDTA. One unit of enzyme activity is the amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH/min. The sensitivity of ALR2 to inhibition by Sorbinil was tested in the above assay conditions in the presence of 10 μM of the inhibitor. Superoxide dismutase and catalase activities were measured as previously described (*35*, *36*).

Electrophoretic and Isoelectric Focusing Analyses. Gel electrophoresis in the presence of SDS, both in reducing and nonreducing conditions, was performed according to the method of Laemmli (*37*) using 0.75 mm thick slab gels and 12% acrylamide. Gels were stained with silver nitrate according to the method of Wray et al. (*38*). The following standards were used for calibration: ovalbumin (45 000 Da), glyceraldehyde-3-phosphate dehydrogenase (36 000 Da), carbonic anhydrase (29 000 Da), and trypsinogen (24 000 Da). Isoelectric focusing was run at 4 °C on a Biophoresis horizontal electrophoresis cell (Bio-Rad) using Ampholine PAG plate, pH 4.0−6.5. Gels were prefocused for 20 min at 15 W using an LKB 2103 power supply. Samples were applied approximately 2 cm from the cathode and focusing was allowed to proceed for 90 min. After the gel was focused, it was immediately fixed in TCA 10%, 0.135 M sulfosalicylic acid for 30 min, then rinsed for 5 min with 25% ethanol−8% acetic acid. The gel was stained for 15 min with 1.16 g/L Coomassie blue R250 in 25% ethanol−8% acetic acid and then destained with 25% ethanol−8% acetic acid. The following standards were used for pI determination: phycocyanin (pI 4.65), β-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), and human carbonic anhydrase (pI 6.5).

Measurement of Copper. *Complexometric Determination*. The concentration of Cu(I) was determined by measuring the formation of the complex between the metal ion and BCDS (*39*).

Aliquots (380 μL) containing reduced copper were supplemented with 25 μL of 10 mM EDTA, and then a 5-fold excess of BCDS was added. The absorbance at 483 nm was immediately read against a blank solution containing no copper. When the metal was measured on Cu-treated ALR2, the increase in absorbance at 483 nm was followed at 25 °C until no more increase was observed (approximately 120 min). Calculation was done after subtraction of absorbance values observed for control samples containing native ALR2. Total concentration of copper [Cu(II) plus Cu(I)] could be determined by supplementing the samples of 3 mM DTT 15 min before addition of BCDS. The copper concentration was determined by using an extinction coefficient for the $(BCDS)_2Cu(I)$ complex of 12 250 M^{-1} cm^{-1}, which was evaluated by calibration curves (ranging 1.5−30 μM) obtained by standard solutions of the complex as well as by standard $CuCl_2$ solutions.

Atomic Absorption. Copper was measured by an atomic absorption spectrophotometer (Perkin-Elmer model 5000) equipped with a graphite furnace system (HGA model 400) after proper sample dilution by MilliQ-grade purified water (Millipore, Medford, MA). The stock calibrator solution, containing 15 mM Cu(II) as copper cloride, and working solutions were prepared immediately before analysis.

Thiol and Disulfide Determination. Reduced thiol residues on both native and Cu-treated ALR2 were measured as follows in denaturing conditions by using DTNB as titrating agent (*40*). Enzyme samples after extensive dialysis against standard buffer were added (2 μM final concentration) to mixtures containing, in 0.1 M potassium phosphate buffer, pH 7.5, 6 M urea, 0.33% SDS, and 0.4 mM DTNB. The absorbance at 412 nm was followed by a Beckman DU-6 spectrophotometer at 37 °C until the rate of absorbance increase was the same as observed in control assays (approximatively 4 min). Control assays were performed on the ultrafiltrate of both enzyme solutions obtained through YM5 membrane. The −SH concentration was evaluated by a calibration curve obtained, in the above experimental conditions, by using GSH as standard thiol.

Reduced and oxidized glutathione were measured by a free zone capillary electrophoresis method (*41*) after acidification of samples to pH 2 by 1 M hydrochloric acid.

Oxygen Removal. The anaerobic conditions adopted to study the effect of oxygen on the Cu-induced inactivation of ALR2 were as follows. The enzyme solution (183 μL of 28.5 μM ALR2) and the Cu(II) solution (17 μL of 500 μM $CuCl_2$), while kept separated into the two arms of a sidearmed tube, were subjected to three cycles of vacuum-nitrogen treatment and then kept under nitrogen flow for at least 30 min. The ALR2 inactivation was started by mixing the two solutions and stopped at the proper time by addition under nitrogen flow of 20 μL of 10 mM EDTA. Traces of oxygen possibly present in the pure grade of nitrogen used were removed by allowing the gas to pass through a solution of 0.1% of pyrogallol in a gas scrubber bottle before reaching the Cu-enzyme incubating mixture.

Other Methods. Protein concentration was determined according to the method of Bradford (*42*) using bovine serum albumin as standard.

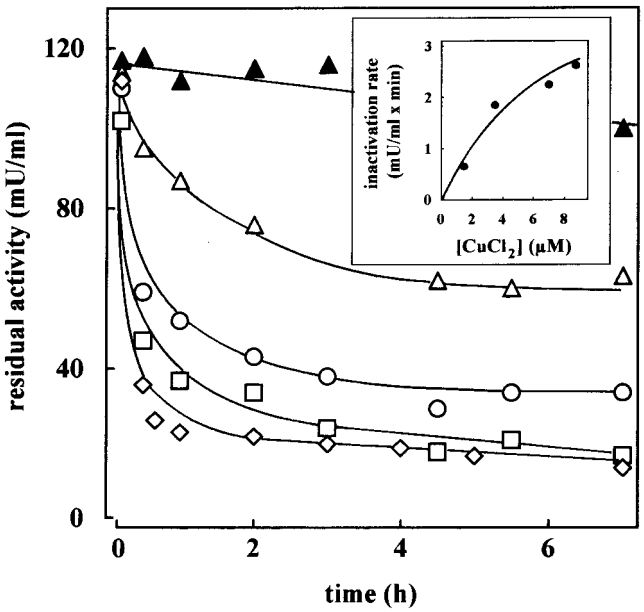

FIGURE 1: Effect of $CuCl_2$ on ALR2 inactivation. Aldose reductase at the final concentration of 3.5 μM was incubated at 25 °C in S-buffer both alone and in the presence of the following $CuCl_2$ (μM) concentrations: none (▲), 1.5 (△), 3.5 (○), 5 (□), 7 (◇), and the enzyme activity (residual activity) was measured. In the inset, the initial rate of enzyme inactivation (milliunits/mL × min) is reported.

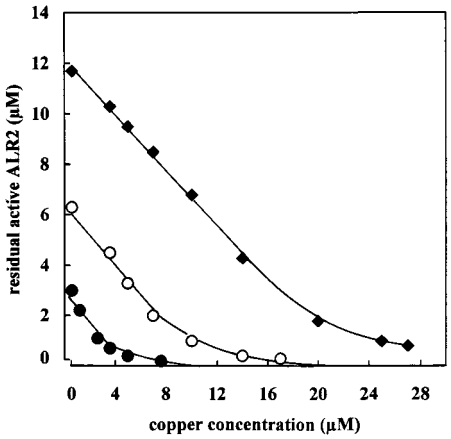

FIGURE 2: Stoichiometry of the ALR2 inactivation process induced by $CuCl_2$. ALR2 was incubated at 25 °C in S-buffer in the presence of different concentration of $CuCl_2$ and the inactivation monitored until equilibrium was reached. The residual activity was used to evaluate the concentration of the residual active enzyme taking into account that the Cu-modified ALR2 displays a specific activity of 20% with respect to the native enzyme. The concentration of residual active enzyme, measured in incubation performed at 3.5 (●), 7 (○) and 14 (◆) μM of ALR2, is plotted against copper ion concentration.

RESULTS

Copper-Dependent Inactivation of Bovine Lens ALR2. The incubation of ALR2 in the presence of low levels of Cu(II) caused a progressive inactivation of the enzyme. Both the rate and maximal extent of inactivation were dependent on the metal ion concentration (Figure 1). The time course of inactivation of ALR2, at different enzyme concentrations (3.5, 7, and 14 μM of ALR2), allowed the evaluation of the requirement of copper needed to induce the loss of enzyme activity (Figure 2). The equilibrium concentration of inactive ALR2 as a function of the copper ion concentration at subsaturating levels of the metal ion indicated a stoichiometry of [Cu(II)]/[inactive ALR2] close to 2:1. The SDS−PAGE analysis performed under reducing and nonreducing conditions revealed that most of the Cu-inactivated ALR2 migrated as a monomer of 34 kDa (Figure 3B). Protein bands appearing in nonreducing conditions in the proximity of the 34 kDa band of both the untreated and Cu-treated enzyme samples (Figure 3B, lanes 4 and 6, respectively) may be ascribed to partial refolding of the protein molecule due to the nonsufficient protection of SH groups. These protein bands, as well as those with higher molecular weight present in nonreducing conditions, were absent in the electrophoretic run performed in the presence of 2ME. The isoelectric focusing analysis of the Cu-inactivated ALR2 (Figure 3A) indicated a major protein band focusing at pH 5.25, 0.4 units higher than the native enzyme. The most acidic band (pI 4.6) (Figure 3A, lane 1) can be ascribed to the complex NADP−ALR2; such a complex appeared to be stabilized by DTT, which is actually present as preservative in the native enzyme preparations (Cappiello et al., unpublished results). When the incubation of ALR2 and copper was performed at relatively higher concentrations of the metal ion, the enzyme inactivation was followed by protein precipitation. Incubations performed at different ALR2 levels (3.5, 7, and 21 μM) clearly indicate that the molecular ratio [Cu(II)]/[ALR2] rather than the absolute concentration of the metal ion is the parameter which is related to the occurrence of the aggregation phenomena (data not shown). Thus, only when the [Cu(II)]/[ALR2] ratio was higher than 3, protein precipitation was observed. Even though DTT was able to reverse Cu-induced enzyme inactivation (see below), this thiol agent was ineffective in dissolving the protein aggregate which could be solubilized, irrespective of the presence of the thiol agent, only when a detergent (0.4% SDS) was added.

Metal Specificity and Oxygen Dependence of ALR2 Inactivation. As shown in Figure 4, in which ALR2 treatment performed under different conditions is reported, oxygen did not appear to be a relevant factor in ALR2 inactivation. A rapid decline in the enzyme activity was observed in the presence of Cu(II) irrespective of the presence of oxygen. Moreover, no effect on the enzyme inactivation process was detectable when mannitol as well as SOD and catalase were present in the incubation mixture (data not shown).

Zinc ion is a divalent cation that, like copper, is known to strongly bind to proteins, but does not act as a redox catalyst. Zn(II) did not interfere in the Cu-induced inactivation process, up to [Zn(II)]/[ALR2] ratios of 10. In addition, no interference in the rate of copper-dependent inactivation of ALR2 and in the residual activity at equilibrium was observed when Zn(II), at Zn(II)/Cu(II) ratios as high as 10^3, was added to the enzyme either before or after the copper. Similarly, no effect on the ALR2 activity was exerted by Cd(II) and Ni(II) up to Me(II)/[ALR2] ratios of 200 (data not shown).

Reversibility of the Cu(II)-Induced Inactivation of ALR2. When the ratio Cu(II)/ALR2 was kept lower than 3, so that no aggregation phenomena occurred, the loss of enzyme activity induced by copper ion was reversed by reducing conditions. In fact, the addition of DTT to the enzyme after incubation with copper allowed the recovery of most of the

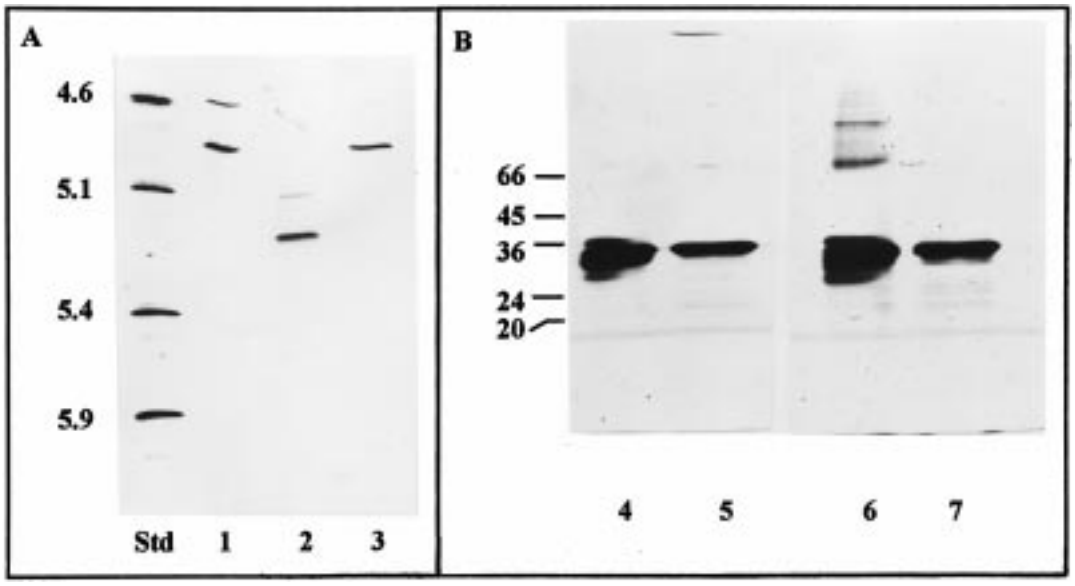

FIGURE 3: IEF and SDS−PAGE analysis of native and copper-modified ALR2. ALR2 and Cu-modified ALR2, obtained after incubation for 90 min at 25 °C of 7 μM ALR2 with 14 μM $CuCl_2$, were dialyzed against S-buffer containing 0.5 mM EDTA and then analyzed both by isoelectric focusing (panel A) and SDS−PAGE (panel B) as described in the Experimental Procedures. (Panel A) Std, standards; lanes 1, 2, and 3: 4.7 μg of standards, ALR2, Cu-modified ALR2, and Cu-modified ALR2 treated with DTT, respectively. The numbers alongside the gel represent the isoelectric point of proteins used as standards. See Experimental Procedures for details. (Panel B) Lanes 4 and 6, 2.4 μg of ALR2 and Cu-modified ALR2 in nonreducing conditions, respectively. Lanes 5 and 7, 2.4 μg of ALR2 and Cu-modified ALR2 in reducing conditions, respectively. The numbers alongside the gel represent apparent molecular weights, divided by 1000, of the polypeptide species used as standards. See Experimental Procedures for details.

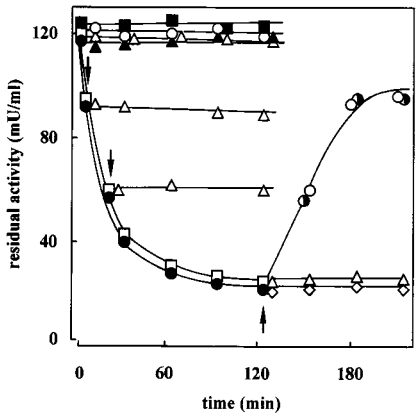

FIGURE 4: Inactivation of bovine lens ALR2 induced by the Cu(II) ion. Aldose reductase at the final concentration of 3.5 μM was incubated at 25 °C in S-buffer both alone (■) and in the presence of 7 μM $CuCl_2$ (□) and assayed at different times for enzyme activity. At the time indicated by arrows, as well as at zero time, aliquots of the copper containing mixture were withdrawn, supplemented as follows and again incubated at 25 °C: (△), 1 mM EDTA; (▲), 1 mM OP; (○), 0.2 and 3 mM DTT at zero time and 120 min, respectively. (●) The mixture of ALR2 and copper ion incubated under nitrogen atmosphere after oxygen depletion (see Experimental Procedures). (◑) Incubation after addition of 3 mM DTT to the enzyme in nitrogen atmosphere.

enzyme activity (Figure 4). EDTA, as well as *o*-phenanthroline, were ineffective in enzyme reactivation (see below). Moreover, incubation of the copper-inactivated enzyme at 37 °C in the presence of 1 mM EDTA, followed by extensive dialysis against the same metal chelator in S-buffer, also did not allow reactivation. Again DTT, added to the dialyzed sample, causes a progressive recovery of enzyme activity culminating in an enzyme form with specific activity and susceptibility to Sorbinil inhibition comparable to the native ALR2 (data not shown).

While ineffective in restoring enzyme activity after treatment of ALR2 with Cu(II), EDTA, as well as OP, was able, when present during the incubation, to prevent enzyme modification. EDTA, added at different times during the inactivation, abruptly stopped the loss of enzyme activity induced by copper (Figure 4). Indeed, this fact, together with the lack of any interference in the enzyme assay by EDTA up to 0.5 mM, is the basis for the procedure adopted to stop the inactivation process and then follow the kinetics of enzyme modification.

Effect of Thiol Compounds on the Copper-Induced Modification of Bovine Lens ALR2. Thiol compounds protected the ALR2 from inactivation induced by copper. DTT (0.2 mM final concentration) present in the inactivating mixture containing ALR2 and copper ion completely prevented enzyme inactivation (Figure 4). Under these conditions, analysis of the copper redox state by the BCDS-complexometric method revealed that the metal ion was in a reduced state and that both the rate and extent of metal reduction were dependent on the concentration of DTT (data not shown). The capacity of GSH to protect ALR2 against the copper-induced inactivation is shown in Figure 5. A significant decrease in the rate of enzyme inactivation was observed when 3 mM GSH was present in an incubation mixture containing 3.5 μM ALR2 and 15 μM $CuCl_2$. Moreover, a reduced maximal extent of ALR2 inactivation was observed. Isoelectric focusing analysis of the Cu(II)/GSH-treated enzyme (sample obtained as in Figure 5 after 4 h of incubation, closed triangles) revealed only protein bands focusing at pH 4.85 and at pH 4.75 (data not shown) consistent with the presence of the native enzyme and GS-ALR2 (*27*), respectively. Moreover, analysis of the enzyme preparation by affinity chromatography on Matrex orange A, a method optimized for detection of GS-ALR2 in bovine lens (30), revealed the presence of this enzyme form in the

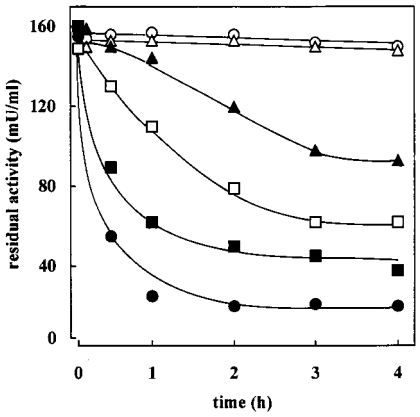

FIGURE 5: Effect of GSH and GSSG on the copper-induced modification of bovine lens ALR2. Aldose reductase at the final concentration of 3.5 μM, was incubated at 37 °C in S-buffer supplemented with 15 μM $CuCl_2$ in the absence (●) or in the presence of both 1.5 mM GSSG (■) and 3 mM GSH (▲) and assayed for enzyme activity. Open symbols refer to incubations performed as control in which the enzyme was incubated in the absence of metal ion alone (○) or in the presence of 3 mM GSH (△) or 1.5 mM GSSG (□).

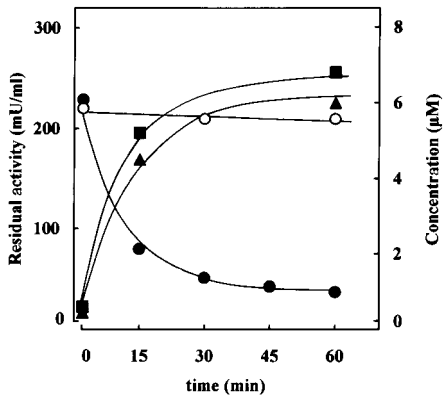

FIGURE 6: Redox state of copper ion during ALR2 inactivation. Aldose reductase at the final concentration of 7 μM, was incubated in S-buffer at 25 °C in the presence of 14 μM $CuCl_2$. At indicated times aliquots were withdrawn, supplemented with 0.5 mM EDTA and analyzed for residual activity (●) and Cu(I) content (■). Closed triangles refer to the μM concentration of the inactive enzyme calculated from the residual catalytic activity values as described in Figure 2. Open circles refer to a control incubation of ALR2 performed in the absence of copper.

treated enzyme preparation. On the other hand, protein bands focusing at pH 4.75 and pH 5.25, corresponding to GS-ALR2 and Cu-inactivated ALR2, respectively, were observed when GSSG instead of GSH was present with copper during ALR2 incubation. Under these conditions, the inactivation rate of ALR2 was very close to that observed when only Cu(II) was present during the incubation (Figure 5).

ALR2−Cu(II) Interaction. The interaction between copper and ALR2 was assessed by measuring the metal ion bound to the protein. Taking advantage of the specificity of bathocuproin for Cu(I), the complexometric analysis described in the Experimental Procedures allowed us to follow the redox state of copper during ALR2 inactivation. A direct comparison between the formation of Cu(I) and the loss of enzyme activity is shown in Figure 6. By evaluating the concentration of the inactivated ALR2 from the measurement of the activity, a ratio of [Cu(I)]/[inactive ALR2] of approximately 1:1 was calculated. The retention of copper

Table 1: Evaluation of Copper Content in Cu-Modified ALR2 Samples[a]

samples	[Cu]/[enzyme] 1	[Cu]/[enzyme] 2	% activity −DTT	% activity +DTT
(1) Cu-ALR2	1.1 ± 0.1	ND	20	80
(2) 1 + S-buffer + EDTA	0.9 ± 0.1	0.7	21	83
(3) 2 + 3 h at 37 °C	2.1 ± 0.5	ND	13	52
(4) 2 + 3 h at 37 °C in DTT	2.0 ± 0.2	ND	95	82
(5) 2 + 3 h at 37 °C in EDTA	2.2 ± 0.3	ND	22	69
(6) 3 + S-buffer	1.7 ± 0.2	1.8	6	70
(7) 3 + S-buffer + EDTA	0.9 ± 0.1	1.1	8	57
(8) 4 + S-buffer	0.6 ± 0.2	0.3	92	86
(9) 4 + S-buffer + EDTA	0.2 ± 0.1	0.2	100	98

[a] Cu-modified ALR2 (sample 1) was obtained by incubating native ALR2 at the final concentration of 3.5 μM with 7.0 μM $CuCl_2$ at 25 °C for 90 min, followed by dialysis at 4 °C against 10 vol of S-buffer. Sample 1 was then subjected to the following treatments: (a) dialysis against 10 vol of S-buffer containing 0.5 mM EDTA (sample 2); (b) treatment described in a followed by incubation at 37 °C for 3 h in S-buffer both alone (sample 3) and in the presence of 5 mM DTT (sample 4) or of 0.5 mM EDTA (sample 5). Samples 3 and 4 were dialyzed against S-buffer either alone (samples 6 and 8, respectively) or in the presence of 0.5 mM EDTA (samples 7 and 9, respectively). Bound copper was measured both as Cu(I) by the BCDS-complexometric method directly on the enzyme preparation (samples 1 and 2) and as total copper, by using both complexometric (column 1) and atomic absorption (column 2) analyses (see Materials and Methods). The enzyme activity measured on different samples, either in the absence or in the presence of 5 mM DTT, is reported as a percentage of the initial activity of native ALR2. Activity values in the presence of DTT are corrected for the 15% inhibition exerted by DTT on the enzyme in the adopted assay conditions. All measurements are within a SD of 10% of the mean value. Standard deviation is the result of at least three determinations. ND = not detected.

by ALR2 after inactivation is evident from the results reported in Table 1, which shows the content of copper ion in the Cu(II)-inactivated ALR2 and in enzyme subjected to further manipulation. The inactive enzyme, generated by 1 h of incubation as described in Figure 6 and subjected to extensive dialysis against EDTA, still showed a [Cu(I)]/[ALR2] ratio of 1:1 (Table 1, samples 1 and 2). However, when the dialyzed enzyme was incubated for 3 h either alone or in the presence of EDTA or DTT, approximately 2 equiv of copper/enzyme mol were detected (Table 1, samples 3−5). In all cases, the complexometric detection of the second equivalent of copper required the presence of DTT in the assay mixture (see Experimental Procedures). While making detectable the second equivalent of copper, the incubation of the Cu-inactivated enzyme for 3 h in S-buffer did not cause the release of the metal ion. In fact, after the dialysis of sample 3 against S-buffer, 2 equiv of copper/enzyme mol were still detectable in the enzyme preparation (Table 1, sample 6). On the other hand, only one metal ion was retained by the enzyme when the dialysis buffer was supplemented with 0.5 mM EDTA (Table 1, sample 7). Finally, a complete removal of copper (Table 1, samples 8 and 9) was achieved by the dialysis of the Cu-inactivated ALR2 subjected to a 3 h incubation at 37 °C with DTT (Table 1, sample 4). As shown in the left-hand column of the right-hand section of Table 1, the treatment with DTT was the only one that allowed the recovery of the enzyme activity (Table 1, samples 4, 8, and 9). For all the samples, however, it was possible to rescue the activity by supplementing the enzyme assay mixture with DTT.

Protein Thiols Determination. The thiol assay performed in denaturing conditions on the native and Cu-modified ALR2 allowed the determination of 6.8 ± 0.1 and 4.9 ± 0.1 thiol equiv/enzyme mol, respectively.

DISCUSSION

The incubation of ALR2 in the presence of low levels of copper ion leads to a progressive inactivation of the enzyme (Figure 1) and to the generation of an enzyme form characterized by a pI of 5.25, 0.4 pH units higher with respect to the native enzyme (Figure 3). Oxygen radical scavenging systems [i.e., mannitol (*43*), SOD, and catalase] and the removal of oxygen from the incubating mixture and its substitution with nitrogen do not affect the inactivation process (Figure 5). Thus, the well-known capacity of copper to promote the formation of oxygen-activated species does not seem to be involved in the observed inactivation of ALR2.

Metal chelators such as EDTA or OP, prevent the copper-induced inactivation of ALR2. Because of the special properties of EDTA and OP either to impair or activate, respectively, the capacity of Cu(II) as a catalyst of thiol oxidation (*11, 44, 45*), the protective action exerted by both metal chelators on the enzyme activity can simply be ascribed to their ability to compete with the enzyme for copper, thus interfering with effective metal-enzyme binding. On the other hand, the failure of metal chelators to allow the recovery of the enzyme activity and the fact that the Cu-inactivated ALR2 can be activated by the addition of DTT (Figure 4) would suggest the occurrence of an oxidative process, which probably involves protein cysteine residues.

The enzyme inactivation appears to occur through a specific interaction with the copper ion since both the rate and the extent of inactivation are proportional to the Cu(II) concentration (Figure 1). Indeed, although it is not possible to speculate in terms of the mechanism of the inactivation process, the plot of the maximal extent of inactivation as a function of copper ion concentration (Figure 2) indicates, for the overall process, a stoichiometry [Cu(II)]/[ALR2] of 2:1.

The relatively low levels of Cu(II) required to inactivate ALR2, as well as the high rate of enzyme inactivation, are indicative of the extraordinary effectiveness of the metal−ALR2 interaction. Zn(II), a metal ion which, like Cu(II), readily binds proteins but does not have the potential to catalyze redox processes, is unable to affect the ALR2 activity and does not interfere with copper-dependent inactivation. These results indicate either a significant specificity of ALR2 for copper [Cd(II) and Ni(II) do not affect ALR2 activity] and/or that the metal binding, as such, is not sufficient for enzyme inactivation. In fact, a complexometric analysis for Cu(I) performed during ALR2 inactivation revealed the formation of reduced copper to give a ratio [Cu(I)]/[inactive ALR2] of approximately 1:1 (Figure 6). We therefore hypothesize either the formation of a specific inactive adduct ALR2−Cu(I)/Cu(II) or an oxidation of ALR2 induced by the metal ion. However, if the latter is the case, the defined stoichiometry of the inactivation process implies that the metal ion must, in any event, remain bound to the enzyme so that its oxidation and recycling by oxygen is impaired.

Indeed, 2 equiv of copper/mol of enzyme were detectable on Cu-inactivated ALR2 (Table 1). The complexometric analysis for Cu(I) performed on the Cu-inactivated ALR2 revealed that at least one of the two protein-bound copper ions was present as Cu(I) (Table 1). The second equivalent of bound copper could not be directly detected by the complexometric assay method because either it was Cu(II) or it was Cu(I) buried in a BCDS-inaccessible site. It became detectable only after the prolonged incubation of the inactive enzyme at 37 °C. The requirement of DTT for the complexometric detection of the second equivalent of copper following incubation at 37 °C would suggest its disclosure as Cu(II). However, although thermal treatment was unable to determine the release of the metal ion in the solution, it may well allow its oxidation; thus, it was not possible to ascertain the redox state of the second copper equivalent detectable on the enzyme.

The presence on the enzyme of two bound copper ions, one of which like Cu(I), confirms the hypothesis that a redox process occurred on ALR2 and that the metal ion is firmly bound to the enzyme. The presence of copper is sufficient, in principle, to explain the loss of enzyme activity but, obviously, the possibility of a specific oxidative modification of the protein molecule cannot be excluded. In this regard, the effectiveness of DTT in rescuing enzyme activity (Figure 5) while making all bound copper accessible to BCDS titration (Table 1) might be interpreted as a reduction of protein disulfides. However, the unique capability of DTT to interact with ALR2 (*34*), together with its potential for chelating and reducing copper (*46*), may restore ALR2 activity simply by removing the metal ion from sites inaccessible to other metal chelators. Nevertheless, the complete removal of copper by EDTA at 37 °C, which occurs without the recovery of enzyme activity (Table 1) and without changes in the pI (data not shown), together with the susceptibility of such a Cu-depleted inactive enzyme form to be reduced by DTT to an active native ALR2 (Table 1), is a strong indication that the inactivation of ALR2 induced by copper ion is associated with an enzyme form containing both bound copper and disulfide bonds. The occurrence of the latter modification is sufficient to keep ALR2 in an inactive state. Protein bands with apparent molecular weights of approximately 70 and 115 kDa were detected after SDS−PAGE under nonreducing conditions (Figure 3), suggesting the formation of dimeric and trimeric structures. However, most of the enzyme is present as a monomer. Therefore, the majority of any disulfide bonds formed must be intramolecular. Indeed, titration by DTNB indicated that the Cu-inactivated ALR2 contained two lesser equivalents of reduced thiols as compared to native enzyme, which would be consistent with the formation of one disulfide bond per enzyme molecule. Such event is not peculiar for ALR2. Indeed other proteins and enzymes have been shown to be oxidized by copper ion by electron-transfer mechanism at level of Cys residues (*47*−*49*).

The buffering action exerted by GSH on the Cu-induced ALR2 inactivation is reminiscent of its antioxidant, scavenging function. In fact when ALR2 was treated with copper in the presence of GSH (Figure 5), the thiol was readily oxidized and the rate and maximal extent of ALR2 inactivation was significantly reduced. The decrease in enzyme activity observed under these conditions can be linked to a

GSSG-dependent formation of GS-ALR2 rather than to a direct action of the metal ion on the enzyme. This is supported by the isolectric focusing analysis of Cu(II)/GSH-treated ALR2, which revealed protein bands focusing as the native (pI 4.85) and the glutathionyl-modified (pI 4.75) ALR2, but not as the Cu-inactivated ALR2 (pI 5.25). Direct interaction between metal ion and ALR2 was more readily observed when GSSG rather than GSH was present with Cu(II) during ALR2 incubation (Figure 5). Under these conditions, while the inactivation rate of ALR2 observed in the presence of Cu(II) /GSSG is significantly higher than that observed in the presence of either Cu(II) /GSH or GSSG alone, the isoelectrofocusing analysis revealed not only GS-ALR2 but also the Cu-inactivated ALR2 form.

These results indicate that an increase of GSSG concentration as such is not sufficient for the change in the oxidative modification pathway of ALR2 observed when GSH was supplemented with Cu(II). On the contrary, the sequestration of Cu(II) engaged in redox cycling in the bulk solution by GSH (*46*, *50*) may significantly contribute to the protective effect exerted by the thiol compound against Cu-induced inactivation of the enzyme. From these results, it is evident that the stability of the GSSG/Cu(II) complex (*50*) is such that GSSG does not compete effectively with ALR2 for metal binding, as occurs for metal chelators such as EDTA or OP (Figure 4). In fact, when the thiol/disulfide redox step is circumvented, as in the case of the incubation in the presence of GSSG, the fast ALR2 inactivation process induced by Cu(II) occurs despite the favorable conditions (i.e., high GSSG concentration) for S-glutathionylation of the enzyme. Thus, it appears conceivable that alteration of the normal thiol redox status may enhance the modifying capability of copper ion toward target proteins. Maintenance of the GSH pool in a reduced state with high [GSH]/[GSSG] ratios may be important in counteracting pathological conditions resulting in elevated cellular copper levels.

ACKNOWLEDGMENT

We are indebted to Dr. Nando Benimeo (IN.AL.CA., Castelvetro, Modena) for the kind supply of bovine lenses, and to Dr. Giovanni Sorlini and the veterinary staff of IN.AL.CA. for their valuable cooperation in bovine lens collection.

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BI981159F