Oxidative Modification of Aldose Reductase Induced by Copper Ion

DEFINITION OF THE METAL-PROTEIN INTERACTION MECHANISM*

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Aldose reductase (ALR2) is susceptible to oxidative inactivation by copper ion. The mechanism underlying the reversible modification of ALR2 was studied by mass spectrometry, circular dichroism, and molecular modeling approaches on the enzyme purified from bovine lens and on wild type and mutant recombinant forms of the human placental and rat lens ALR2. Two equivalents of copper ion were required to inactivate ALR2: one remained weakly bound to the oxidized protein whereas the other was strongly retained by the inactive enzyme. Cys303 appeared to be the essential residue for enzyme inactivation, because the human C303S mutant was the only enzyme form tested that was not inactivated by copper treatment. The final products of human and bovine ALR2 oxidation contained the intramolecular disulfide bond Cys298-Cys303. However, a Cys80-Cys303 disulfide could also be formed. Evidence for an intramolecular rearrangement of the Cys80-Cys303 disulfide to the more stable product Cys298-Cys303 is provided. Molecular modeling of the holoenzyme supports the observed copper sequestration as well as the generation of the Cys80-Cys303 disulfide. However, no evidence of conditions favoring the formation of the Cys298-Cys303 disulfide was observed. Our proposal is that the generation of the Cys298-Cys303 disulfide, either directly or by rearrangement of the Cys80-Cys303 disulfide, may be induced by the release of the cofactor from ALR2 undergoing oxidation. The occurrence of a less interactive site for the cofactor would also provide the rationale for the lack of activity of the disulfide enzyme forms.

Transition metals have a relevant role among systems that can induce or modulate oxidative stress. They are both able to promote the formation of reactive oxygen species (1) and to act as cofactors in enzymatic systems devoted to counteract oxidative stress. The important role of the copper ion as an effective prosthetic group for special protein functions and its role as a potential toxic agent in cell function are handled by the cell through a fine control of the free copper level by highly efficient metal chelating proteins (2, 3). In this regard, it is worth noting the extensive cell damage associated with pathologies resulting from both excess and a deficit of copper, such as Wilson’s and Menkes’s disease, respectively (4, 5).

When the concentration of free copper increases, either by environmental or pathological causes, cell damage likely occurs (6–10). The effectiveness of copper ion in inducing protein as well as nucleic acid oxidation, by eliciting the generation of reactive oxygen species through a Fenton-type reaction, is well documented (11–16). Moreover, because of its ability to bind proteins and nucleic acids, copper has the potential to specifically promote in situ oxidative modification reactions. Thus, it is important to define the mechanisms underlying processes induced by copper-protein interaction.

The effect of the copper ion on aldose reductase (alditol: NADP\textsuperscript{+} oxidoreductase, EC 1.1.1.21) (ALR2),\textsuperscript{1} isolated from bovine lens was previously described (17). It appears that the enzyme, which was previously shown to be especially susceptible to thiol-mediated oxidation (18–22), is highly sensitive to Cu(II). The enzyme is readily inactivated by the metal ion through an oxygen independent modification process. The modified enzyme, fully reactivated in the presence of dithiothreitol, was postulated to contain an intramolecular disulfide bond and to carry two equivalents of bound copper ion. Based on the characterization of the inactivation process and on the measurement of the redox state of the bound copper on the enzyme, it was concluded that the metal ion responsible for ALR2 inactivation was directly involved in a site specific oxidation mechanism of the enzyme.

In this paper, the rationale for the definition of the copper binding site(s) and the formation of the disulfide bond in ALR2 is put forward through mass spectrometry, circular dichroism, and molecular modeling approaches on the enzyme and its mutants from different species.

** Experimental Procedures

Materials

NADPH, d,L-glyceraldehyde, dithiothreitol, GSH, EDTA, endoprotease Lys-C, iodoacetamide, DTT, and myoglobin were purchased from Sigma. Bathocuproinedisulfonic acid was from Janssen Pharma.

The abbreviations used are: ALR2, aldose reductase; b-ALR2, bovine lens ALR2; b-ALR2, human placental recombinant ALR2; h-C298S, h-C80S, h-C303S, cysteine to serine mutated h-ALR2; r-ALR2, rat lens recombinant ALR2; r-C298S, cysteine to serine mutated r-ALR2; CAM, carboxamidomethyl; DTT, dithiothreitol; GS-2-ME, 2-mercaptoethanol; MD, molecular dynamics; MM, molecular mechanics.

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ceutical. All electrophoretic reagents and isoelectric focusing standards were from Bio-Rad. Ampholine PAG plates, pH 4.0–6.5, for isoelectric focusing were from Amersham Biosciences. Copper(II) chloride and all inorganic chemicals were of reagent grade and were from BDH. The ALR2 inhibitor (S)-(+)-6-fluoro-2,3-dihydrospinripro(4H-1-benzopyran-4,4'-imidazolidine)-2',5'-dione (Sorbinil) (23) was a gift from Dr. G. Caccia, Laboratori Baldacci S.p.A., Fisa, Italy. The complex (bathocuproinedisulfonic acid,Cu(I)) was a gift from Dr. R. L. Levine, Laboratory of Biochemistry, NHLBI, National Institutes of Health, Bethesda, MD. γ-Glutamyl-cysteinyl-2'-[7H]glycine ([7H]GSH), 1 Ci/mol was purchased from PerkinElmer Life Sciences.

Generation of Mutated Enzymes and Enzyme Purification

The purification of b-ALR2 was performed as previously described (24). The pure native enzyme (specific activity 1.12 units/mg) was stored at 4 °C in 10 mM sodium phosphate buffer, pH 7.0 (S-buffer), supplemented with 2 mM DTT. Expression of h-ALR2 in Escherichia coli was done as previously described (25). Recombinant aldose reductase was extracted from host cells by osmotic shock and stored at −70 °C until used. Wild type and mutated forms of h-ALR2 were purified to electrophoretic homogeneity by the same chromatographic steps used for the bovine lens enzyme (24); the pure enzymes were stored at 4 °C in S-buffer supplemented with 2 mM DTT. The specific activities of h-ALR2 and its C298S, C80S, and C303S mutants were 3.9, 9.8, 3.1, and 6.2 units/mg, respectively. E. coli expressing r-ALR2 and its mutants were grown as previously described (26). The cells were washed twice in 20 mM imidazole buffer, pH 7.2, and centrifuged. The cell pellet was resuspended in 10 ml of the same buffer, sonicated, and stored at −70 °C until used. r-ALR2 and its mutants were purified to electrophoretic homogeneity by the same chromatographic steps used for the bovine lens enzyme (24); the pure enzymes were stored at 4 °C in S-buffer supplemented with 2 mM DTT. The specific activities of r-ALR2 and its C298S, C80S, and C303S mutants were 4.5, 5.2, 0.6, 4.0, 4.0, and 5.2 units/mg, respectively.

measurement of Enzyme Activity

The ALR2 activity and sensitivity to inhibition by Sorbinil was measured as previously described by using d,L-glyceraldehyde as substrate (17).

Enzyme Inactivation

Before use, the enzyme forms were extensively dialyzed against S-buffer. If not otherwise specified, copper treatment of h-ALR2 forms was performed by supplementing the enzyme after dialysis with a stoichiometric amount of NADP⁺. When r-ALR2 was used, 30 μM DTT was present in the dialysis buffer and 4 μM DTT was constantly present in all further incubations. All tested enzyme forms, from 3 to 8 μM final concentrations, were incubated at the proper time at 25 °C in S-buffer supplemented with CuCl₂ to give final ratios of [Cu(II)/enzyme] from 0.5 to 5, as specifically indicated. At the end of the incubation, 0.5 mM EDTA was added and the enzyme activity was measured. To detect copper bound to ALR2, the samples were extensively dialyzed at 4 °C against S-buffer containing 0.5 mM EDTA.

Measurement of Copper

The concentration of Cu(I) was determined by a complexometric method as previously described (17) by measuring the formation of the complex between the metal ion and bathocuproinedisulfonic acid.

Circular Dichroism Analysis

Circular dichroism spectra were obtained on a Jasco J400AS spectropolarimeter with a cylindrical 10-mm path length cuvette kept at 10 °C. A spectral bandwidth of 2 nm was used.

Alkylation of Aldose Reductase Samples with Iodoacetamide

To block reduced cysteines, ALR2 samples were alkylated with 1.1 mM iodoacetamide in 0.25 mM Tris-HCl, 1.25 mM EDTA, containing 6 M guanidinium chloride, pH 7.0, at room temperature for 1 min in the dark. Proteins were freed from salt and reagent excess by passing the reaction mixture through an analytical Vydac C₂ column as previously reported (22). Protein samples were manually collected and lyophilized.

ESIMS Analysis

Electrospray mass spectra of intact protein species were recorded by using an API-100 single quadrupole mass spectrometer (Applied Biosystems) equipped with an atmospheric pressure ionization source as previously reported (22). 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.

Enzymatic Hydrolysis

Samples of carboxamidomethylated aldose reductase (150 μg) were digested with endoproteinase Lys-C in 0.4% ammonium bicarbonate, pH 8.0, at 37 °C overnight, using an enzyme/substrate ratio of 1:100 (w/w).

LC-ESIMS Analysis

ALR2 digests were analyzed using a LCQ Deca mass spectrometer (ThermoFinnigan) equipped with an electrospray source connected to a HP1100 chromatographic system (Agilent, Palo Alto, CA). Peptide mixtures were separated on a narrow bore Vydac C₂ column (The Separation Group) using a linear gradient from 5 to 70% acetonitrile containing 0.1% trifluoroacetic acid, over a period of 65 min, at a flow rate of 0.2 ml/min. The column effluent was split 1:1 into the mass spectrometer connected on-line. The remaining part was spectrophotometrically detected at 220 nm. In the last case, peptides were manually collected and lyophilized for further characterization. Spectra were acquired in the range m/z 250–2000. Data were elaborated using the Excalibur software provided by the manufacturer. The instrument was calibrated using a mixture of caffeine, MRFA peptide, and Ultramark 1621.

A determination of the relative abundance of the peptides containing Cys⁶⁰, Cys⁹², and Cys⁴⁰ was performed as previously reported by Vinci et al. (27). Briefly, because different peptides containing a specific Cys residue could exist, the ion current for peptides containing a specific Cys residue (in reduced or oxidized form) was obtained by summing the ion current relative to all peptides containing that cysteine (in reduced or oxidized form). To obtain the relative abundance of a specific cysteine in reduced or oxidized form, this value was divided by the ion current produced by all peptides containing Cys⁶⁰, Cys⁹², and Cys⁴⁰. Because different peptides may ionize with different efficiencies, it was not possible to evaluate the absolute abundance of each reduced or oxidized cysteine. It was, nevertheless, possible to compare the trends in the oxidation of the different cysteine residues.

Protein Sequence Analysis

Automated N-terminal degradation of the purified peptides was performed by using Procise 491 protein sequencer (Applied Biosystems) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems) for the automated identification of phenylthiohydantoin-derivative.

Other Methods

Protein concentration was determined according to Bradford (28) using bovine serum albumin as the standard. Electrophoretic and isoelectrofocusing analyses were performed as previously described (17). Radioactivity measurements were done using a Beckman LS5000CE scintillation counter and Optiphase HiSafe scintillation fluid with a counting efficiency of 50% as determined by the tritium standard quench curve of the instrument.

Molecular Modeling

Molecular mechanics and molecular dynamics simulations were performed with the sander classic module of AMBER6 (29), using the Cornell et al. (30) force field. Calculations were performed on a IBM-SP3 computer. Graphical display and manipulation programs were performed on Silicon Graphics O2 workstations using MIDAS (31).

Endogenous metal content and copper-induced inactivation of aldose reductase

The coordination geometry of copper extracted from the crystal structure of plastocyanin (34) was used for charge calculations of copper; to this aim, the Cu carbons of the four amino acids coordinating copper (His⁶⁷, His⁹⁷, Cys⁴⁴, and Met⁴⁵) were truncated with methyl groups, and cysteine was modeled as anionic (35, 36). The atomic charge of Cu(II) was obtained from an electrostatic potential fit to STO-3G and 6–31G*
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RESULTS

Copper-dependent Inactivation of Human and Rat Recombinant ALR2—The recombinant h-ALR2 and r-ALR2 were readily inactivated by low concentrations of Cu(II) (Fig. 1). The rate and extent of inactivation were dependent on copper ion concentration. Moreover, in both cases enzyme activity was recovered upon addition of DTT. Following incubation with

FIG. 1. Inactivation of human and rat aldose reductase induced by copper ion. ALR2 at the final concentration of 3.5 µM was incubated at 25 °C both in the absence (●) and presence of the following CuCl2 µM concentrations: 1.75 ( ○ ), 3.5 ( △ , ● ), 7.0 ( ■ , ○ ). The incubations were performed either in S-buffer for h-ALR2 (panel A) or in S-buffer containing 4 µM DTT for r-ALR2 (panel B). At the times indicated by the arrows, 3 mM DTT was added and the mixtures were again incubated at 25 °C (dashed lines). Closed and open symbols refer to the enzyme activity measured in the absence and presence of 10 µM Sorbinil, respectively.

two copper ions close to Cys290 and Cys303.

Disulfide Bond Formation—Because the sulfur atoms of Cys290 and Cys303 after the 300 ps MD with copper, turned out to be much closer than the corresponding atoms in the crystal structure of the holoenzyme, the last minimized structure obtained from the noncovalent simulation described in the force field parameter section was used as the starting point for building a covalent disulfide bond between these cysteines. Five thousand steps of minimization and 800 ps of MD at 27 °C were performed on the enzyme carrying the Cys290-Cys303 disulfide with coordinates collected for the subsequent analysis.

Because, after 800 ps of MD, Cys290 and Cys303 were not sufficiently close to form a disulfide in the noncovalent complexes, a different strategy was used to build a model structure carrying this disulfide. Using the homology modeling software Modeler6 (45), the sequence of ALR2 was artificially aligned with itself, with the only difference being that while the template did not contain any disulfide (the crystal structure of the holoenzyme), a disulfide was explicitly requested in the noncovalent simulation described in the force field parameter section was used as the starting point for building a covalent disulfide bond between these cysteines. Five thousand steps of minimization and 800 ps of MD at 27 °C were performed on the enzyme carrying the Cys290-Cys303 disulfide with coordinates collected for the subsequent analysis.

Two copper ions were docked into the structure of the human ALR2 holoenzyme (44). Because Cys290, Cys299, and Cys303 are the three cysteines involved in the formation of a disulfide bridge, copper ions were initially positioned to interact with these residues. One copper ion was positioned close to Cys290 and one close to Cys303 to investigate the formation of the Cys290-Cys303 disulfide, and one copper ion was positioned close to Cys299 and one close to Cys303 for the Cys299-Cys303 disulfide. When coordinating copper, cysteines were assigned a deprotonated form (55, 36). The ALR2 structures have been prepared using a procedure similar to that described for plastocyanin, with hydrogens added and counterions placed. The parameters for the cofactor were taken from previous work. Structures were solvated with spherical caps of more than 2000 TIP3P (42) water molecules centered on the center of mass of ALR2. The following protocol was adopted for minimization and dynamics. A few steps of minimization with MM were performed on the two copper ions keeping the protein fixed at its original position to adjust their initial position with respect to the two cysteines. Prior to energy minimization of ALR2, only the water molecules were energy minimized and then subjected to 50 ps of MD at 27 °C to let the solvent equilibrate around the solute. Then, 5000 steps of minimization were performed on the whole system. 300 ps of MD at 27 °C was then performed starting from the minimized structure, using the same conditions described for plastocyanin. The whole structure was allowed to move during MD. MD was continued for over 800 ps in the case of the enzyme loaded with the

ab initio wave functions, using GAUSSIAN98 (37), followed by standard RESP fit (38, 39). In the first case, a STO-3G basis set for copper and the whole coordination sphere was used, whereas a mixed 6–31G* basis set for the amino acids and a STO-3G basis set for copper was used in a second case. Atomic charges of copper of +0.71 and +1.13 were obtained, respectively. All calculations limited to position close to Cys80 and one close to Cys303 to investigate the initial position for building a model structure carrying this disulfide. This approach produced an initial structure of ALR2 that while the template did not contain any disulfide (the crystal structure of the holoenzyme), a disulfide was explicitly requested in the noncovalent simulation described in the force field parameter section was used as the starting point for building a covalent disulfide bond between these cysteines. Five thousand steps of minimization and 800 ps of MD at 27 °C were performed on the enzyme carrying the Cys290-Cys303 disulfide with coordinates collected for the subsequent analysis.

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RESULTS

Copper-induced Inactivation of Aldose Reductase

Aldose Reductase (ALR2) is a two-step process in which copper initially forms a noncovalent complex with ALR2 and, subsequently, induces the disulfide bond formation. When coordinating copper, cysteines were assigned a deprotonated form (55, 36). The ALR2 structures have been prepared using a procedure similar to that described for plastocyanin, with hydrogens added and counterions placed. The parameters for the cofactor were taken from previous work. Structures were solvated with spherical caps of more than 2000 TIP3P water molecules centered on the center of mass of ALR2. The following protocol was adopted for minimization and dynamics. A few steps of minimization with MM were performed on the two copper ions keeping the protein fixed at its original position to adjust their initial position with respect to the two cysteines. Prior to energy minimization of ALR2, only the water molecules were energy minimized and then subjected to 50 ps of MD at 27 °C to let the solvent equilibrate around the solute. Then, 5000 steps of minimization were performed on the whole system. 300 ps of MD at 27 °C was then performed starting from the minimized structure, using the same conditions described for plastocyanin. The whole structure was allowed to move during MD. MD was continued for over 800 ps in the case of the enzyme loaded with the

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CuCl₂ and extensive dialysis at 4 °C against EDTA, r-ALR2 and h-ALR2 contained 1.8 ± 0.1 and 2.1 ± 0.1 equivalents of total metal ion per enzyme mole, respectively. This is consistent with that reported for the b-ALR2 (17). Moreover, EDTA or o-phenanthroline (1 mM), when initially present or when added at different times in the Cu(II)/ALR2 incubations mixtures, was able to prevent or block the enzyme modification (data not shown).

Some distinct differences were observed among the bovine, rat, and human enzymes. The rat lens enzyme, possibly because of the presence of an extra cysteine residue with respect to the human and bovine enzymes (47), is not stable unless low DTT concentrations (in the micromolar range) are present. On the other hand, h-ALR2, because of its reduced ability to retain the bound cofactor with respect to b-ALR2, requires supplementation with NADP⁺, which is always present during enzyme manipulation and incubation at a concentration ratio of [NADP⁺]/[enzyme] of 1:1. Despite species-specific differences in the amino acid sequence among these enzymes, the high sequence homology can be ascribed as the reason for the substantially identical behavior of these enzymes with respect to copper-induced inactivation. Rat and human enzymes, mutated at different Cys residues (Fig. 2), as well as the rat enzyme mutated at several His residues (data not shown), are also inactivated by copper and their activities could be rescued by DTT treatment. The h-C298S and r-C298S enzymes were as sensitive to inactivation by copper as their respective wild type enzymes (Fig. 2). The h-C80S enzyme was slightly less susceptible to copper-induced inactivation than the native enzyme, but was still reactivated by DTT.

The h-C303S enzyme was insensitive to copper treatment; this enzyme retained 90% of the initial activity after 90 min of incubation in the presence of copper ion at a ratio of [Cu(II)]/[ALR2] of 2 (Fig. 2). A significant loss of enzyme activity was observed only when the concentration of copper was raised to a [Cu(II)]/[ALR2] ratio higher than 3. However, under these conditions enzyme inactivation paralleled protein aggregation and enzyme activity was not recovered with DTT treatment (data not shown). The h-C303S enzyme also differed from wild type and other human mutated forms in the content of bound copper after treatment with the metal ion. Only 1.0 ± 0.1 eq of total copper ion per enzyme mole was detected on Cu(II)-treated, but still active, h-C303S. The h-C303S enzyme became sensitive to copper only when the pyridine cofactor, normally supplemented in the incubating mixtures of human enzymes, was omitted. However, under these conditions the activity of the h-C303S enzyme was recovered by incubation for 3 h at 37 °C with 0.5 mM EDTA. This was the only case among all of the ALR2 enzyme forms tested in which the inactivation could be reversed without addition of DTT. Thus, it appeared that Cys³⁰³ was the most relevant residue for the copper-induced inactivation of ALR2. The susceptibility to inactivation by copper was tested for a series of His mutants of r-ALR2 (i.e., H200Q, H110Q, H41Q, H187Q); in all cases at ratios of [Cu(II)]/[enzyme] of 3, the mutated enzymes were inactivated and the activity was recovered upon treatment with DTT (data not shown).

Effect of Thiol Compounds on the Copper-modified ALR2—DTT was able to rescue aldose reductase activity from Cu(II)-inactivated r-ALR2 and h-ALR2 (Fig. 1) as previously shown for the bovine lens enzyme (17). In fact, treatment with this reducing agent generated enzyme forms with a specific activity and sensitivity to inhibition by Sorbinil comparable with those of the respective native enzymes. GSH and 2-ME allowed a recovery of the enzyme activity, which was consistent with the generation of enzyme forms carrying the specific thiol reagent linked to the protein by a mixed disulfide bond. This conclusion was drawn from the results obtained with both b-ALR2 and h-ALR2 which, after inactivation by copper ion and removal of excess metal ion by extensive dialysis against EDTA, were treated at 37 °C with different reducing thiol compounds. In particular, for b-ALR2 (Fig. 3) 2-ME caused an increase of the activity ~2-fold that expected for the native ALR2, and the enzyme was scarcely affected by Sorbinil. These properties are...
all comparable with those of a previously characterized 2-ME-modified ALR2 form (18, 48). GSH treatment of the copper-modified b-ALR2 induced a progressive recovery of enzyme activity and susceptibility to Sorbinil inhibition that was compatible with the formation of native ALR2. However, in this case, during the GSH-dependent reactivation process, the generation of GS-ALR2, an intermediate enzyme form, was observed. In fact, an enzyme form that did not bind the Matrix Orange A resin, and was not sensitive to Sorbinil (49), was detectable (data not shown). Finally, cysteine appeared to be a poor reducing agent of the oxidized enzyme, inducing a very modest recovery of ALR2 activity without a parallel recovery of the susceptibility to Sorbinil inhibition.

Concerning the human enzyme (data not shown), the activity of the Cu(II)-inactivated ALR2 was rescued by thiols compounds in a slightly different fashion than observed for the bovine enzyme. In this case, GSH was unable to induce a full reduction of the inactivated enzyme. Instead, GS-ALR2, in which Cys\textsuperscript{306} was previously shown to be involved in the formation of the mixed disulfide bond (19), is the only product of the reaction. In fact, after GSH treatment of the Cu(II)-inactivated h-ALR2, only one protein band in isoelectric focusing analysis (pI 5.9) was detectable; this enzyme form was insensitive to Sorbinil and did not bind the Matrix Orange A. When \textsuperscript{3}H\textsubscript{GSH} was used to recover enzyme activity from the Cu(II)-modified h-C80S enzyme, an incorporation of radioactivity consistent with the insertion of 1 (0.82 ± 0.02) glutathionyl residue per enzyme mole was observed. A further support of the involvement of Cys\textsuperscript{306} in the modification of the cysteine redox state of the enzyme subjected to copper treatment comes from the effect of the metal ion on the h-C298S enzyme. As shown above (Fig. 2) this form was readily inactivated by Cu(II), and the enzyme activity was fully recovered upon treatment with DTT. In the case of h-C80S and h-C298S enzymes, GSH and 2-ME were largely ineffective, allowing recoveries of only 30–40% of the expected enzyme activity value (data not shown).

**Structural Analysis of Bovine and Human ALR2 and Their Products following Cu(II) Treatment**—The amino acid sequences of b-ALR2, h-ALR2, and the h-C298S mutant were verified by ESIMS; the measured masses were 35,961.8 ± 3.4 Da, 35,721.3 ± 3.1 Da, and 35,704.9 ± 3.9 Da, respectively, in perfect agreement with the theoretical values (35,961.2, 35,722.5, and 35,706.2 Da, respectively).

b-ALR2 and h-ALR2 (3.5 \textmu{}M) were incubated in S-buffer either in the absence or presence of 7 \textmu{}M CuCl\textsubscript{2} for 180 min, quickly alkylated, and then analyzed by ESIMS as described under “Experimental Procedures.” In the case of untreated b-ALR2 and h-ALR2, the spectra showed a single component at 36,360.9 ± 2.9 Da and 36,120.8 ± 3.2 Da, respectively, corresponding to a protein species containing seven carboxamidomethyl groups (theoretical values 36,360.5 Da and 36,121.5 Da). These results were consistent with the expected fully reduced form for both ALR2 species and demonstrated that the alkylation reaction went to completion. Similarly, the spectra of Cu(II)-treated b-ALR2 and h-ALR2 showed, in both cases, a main component at 36,244.9 ± 2.5 and 36,005.8 ± 2.7 Da, respectively, corresponding to an ALR2 form containing an intramolecular disulfide bond and five carboxamidomethyl groups (theoretical values 36,244.4 and 36,005.5 Da, respectively), with traces of fully reduced species.

To identify the amino acids involved in the intramolecular disulfide bond observed following Cu(II) treatment, all enzyme species were digested with endoproteinase Lys-C. The peptide mixtures obtained gave similar peptide maps when analyzed by LC-ESIMS. The fractions obtained were eventually characterized by Edman degradation for their peptide components.

**Table I**

| Product | Time (min) | Mass (Da) | Peptide |
|---------|-----------|-----------|----------|
| b-ALR2 | 10.2 | 1018.3 ± 0.6 | 960.1 ± 0.8 | (86–94)-CAM\textsubscript{2} |
| h-ALR2 | 4.5 | 562.3 ± 0.5 | 563.3 ± 0.7 | (90–94)-CAM\textsubscript{2} |
| Cu(II)-treated b-ALR2 | 37.4 | 3297.5 ± 0.9 | 3237.2 ± 0.8 | (33–61)-CAM\textsubscript{1/2} |
| Cu(II)-treated h-ALR2 | 14.7 | 1025.6 ± 0.9 | 1040.1 ± 0.7 | (195–202)-CAM\textsubscript{1/2} |
| h-C298S | 35.0 | 2218.8 ± 0.8 | 2218.5 ± 0.5 | (38–51)-CAM\textsubscript{1/2} |
| h-C80S | 26.9 | 1122.6 ± 0.6 | 1136.6 ± 0.9 | 35,721.3 and 35,704.9 Da, respectively |
| h-C298S | 37.4 | 3297.5 ± 0.9 | 3237.2 ± 0.8 | (33–61)-CAM\textsubscript{1/2} |
| h-C80S | 37.4 | 3297.5 ± 0.9 | 3237.2 ± 0.8 | (33–61)-CAM\textsubscript{1/2} |
| h-C80S | 37.4 | 3297.5 ± 0.9 | 3237.2 ± 0.8 | (33–61)-CAM\textsubscript{1/2} |

**Experimental Procedures.** Table I indicates mono- and di-carboxamidomethylated species for h-ALR2 C298S mutant and b- or h-ALR2, respectively.
Cys298 and Cys303 were involved in an intramolecular disulfide bridge pairing between Cys 298 and Cys303. The same molecular rearrangement resulting in the specific S-S form. Therefore, these results clearly demonstrate that Cu(II) treatment induces in both bovine and human enzymes the oxidized enzyme showed the occurrence of a clear signal at 7,365.2 ± 1.5 Da that was associated with peptides-(78-85) and -(263-315), respectively, where Cys298 and Cys303 were involved in an intramolecular disulfide bridge. Peptide sequencing of both fractions confirmed this hypothesis as demonstrated from the presence of the corresponding phenylthiohydantoin-derivative at the relative cycles. Also, in this case minor components originating from hydrolysis at Lys282 (bovine) and Lys 274 and Lys 307 (human) were observed.

The analysis of all the other fractions shown in Table I revealed, in all the enzyme species, that Cys34, Cys186, Cys199, Cys298, and Cys303 were in a fully carboxamidomethylated form. Therefore, these results clearly demonstrate that Cu(II) treatment induces in both bovine and human enzymes the same molecular rearrangement resulting in the specific S-S bridge pairing between Cys298 and Cys303.

Following the alkylation reaction, ESIMS analysis of the h-C298S enzyme showed a single component at 36,049.4 ± 3.4 Da corresponding to a protein species containing six carboxamidomethyl groups (theoretical value 36,048.5 Da). Furthermore, the spectrum of the Cu(II)-treated h-C298S enzyme showed a main molecular species, whose molecular mass (35,932.9 ± 3.2 Da) was consistent with an ALR2 form containing an intramolecular disulfide bond and four carboxamidomethyl groups (theoretical value 35,932.5 Da), and traces of the fully reduced species. Peptide mapping experiments on the oxidized enzyme showed the occurrence of a clear signal at 7,365.2 ± 1.5 Da that was associated with peptides-(78-85) and -(263-315), containing Cys80 and Cys303 joined by a disulfide bridge (Table I). The nature of this species was confirmed by Edman degradation. A minor component originating from hydrolysis at Lys274 was also observed. Traces of peptides-(78-85)-CAM and -(263-315)-CAM were also present. These data demonstrated that in the case of h-C298S mutant, ALR2 oxidation can proceed through the alternative formation of a specific S-S bridge between Cys80 and Cys303.

To definitively clarify the mechanism of Cu(II)-induced oxidation of h-ALR2, protein aliquots were taken at different times during copper treatment and quickly alkylated with iodoacetamide. Samples were digested as previously reported and a determination of the relative abundance of the peptides containing Cys80, Cys298, and Cys303 was calculated from the LC-ESIMS analysis by using the approach of Vinci et al. (27). The results reported in Fig. 4 clearly show that the ALR2 inactivation parallels Cys oxidation. The oxidation initially proceeds with the simultaneous formation of the disulfide Cys80-Cys303 and Cys298-Cys303. However, the relative amount of the disulfide Cys80-Cys303 decreases as reaction proceeds, whereas a concomitant increase in the disulfide Cys303-Cys303 concentration can be observed. These results suggest that the disappearance of the disulfide Cys80-Cys303 at long times of incubation can be tentatively associated with an intramolecular disulfide rearrangement that results only in the Cys298-Cys303 species as already reported in Table I. As expected, a parallel decrease in the relative abundance of peptides containing these cysteines in the reduced form was observed.

Noncovalent Interactions between ALR2 and Copper—After two copper ions were positioned on the human holoenzym as described under “Experimental Procedures,” MM and MD in water were performed on the noncovalent complexes. MD simulations performed on the Cu(II)-Cys80-Cys303 complex resulted in a substantial conformational rearrangement of the ALR2 C-terminal end, as graphically reported in Fig. 5A. Compared with the crystal structure, the C terminus carrying Cys303 moves significantly toward Cys80 thereby reducing the distance between the two S atoms from 6.9 Å to an average value of 3.7 Å. It is interesting to observe that the conformation of the segment carrying Cys80 is almost unaltered during MD, so this conformational change may be ascribed only to the C terminus. This finding is in agreement with the relative B-factor of the two regions, and finds precedents in the substantial conformational reorganization of segment 298–303 observed in the crystal structures of ALR2 complexed with the inhibitors Zopolrestat (50) and Tolrestat (51).

In the structures of this noncovalent complex, a stable complex between Cys80, Cys303, and the two copper ions was detected (Fig. 5A, inset). Each copper ion coordinates both cys-

![Image](http://example.com/image.png)
MD are graphically reported in Fig. 5B. Compared with the noncovalent structures described above, the C-terminal end moves even closer to the segment carrying Cys80 in the disulfide-containing structure. Cys298 and Cys303 are still far away from Cys80 and Cys303. The loops L4 and L7, adjacent to the C-terminal end, exhibited the highest conformational flexibility during MD. Whereas one of the two copper ions that coordinate Cys80 and Cys303 in the noncovalent complex is now significantly distant from both cysteines and free to interact with water molecules and loop L4 (Cu2 in Fig. 5B), the other copper ion (Cu1) remains completely embedded into the protein, interacting with Thr113, Trp111, Leu112, and only one water molecule.

As the 300 ps MD simulation with the copper ions close to Cys298 and Cys303 failed to reach a noncovalent complex in which these cysteines were sufficiently close to conceive the formation of a disulfide, a starting structure with the Cys298-Cys303 disulfide bond was modeled using Model er6, and then this structure was refined with 900 ps MD. Fig. 6 reports the averaged structures collected during MD. As expected, formation of the disulfide bond results in a significant refolding of the C-terminal segment. Three folded conformations of the C-terminal segment were sampled during MD, as it can be inferred from the clustering of conformations reported in the figure. In all cases, these conformations were significantly different from those observed in the crystal structure of ALR2, in which this segment is in an extended conformation. One of the two copper ions (Cu2) is, again, significantly distant from Cys298 and Cys303 and free to interact with water molecules and residues at the enzyme surface. The other (Cu1) remains embedded into the protein in a position similar to that already described for the Cys80-Cys303 disulfide, even though solvent-exposed positions of this copper were also sampled during MD.

ALR2 Oxidation and Active Site—Tyr48 and His110 are two residues playing a fundamental role in the catalytic reduction of aldehydes by ALR2 (52, 53). A close inspection of the active site architecture reveals that Tyr48 and His110 are still properly located with respect to the C4-carbon of the nicotinamide of the cofactor after formation of the Cys80-Cys303 and Cys298-Cys303 disulfides. Fig. 7 reports a superimposition between these and a few nearby residues in the crystal structure of the holoenzyme and those in the MD disulfide-containing ALR2 structures. Whereas the phenol ring of Tyr48 superimposes very well, slight differences in the position of His110 have been observed. However, despite these differences, the average distance between the reactive C-4 of nicotinamide and the C2 nitrogen of His110 was 5.6 ± 0.4 Å in the Cys80-Cys303 disulfide structure and 5.4 ± 0.6 Å in the Cys298-Cys303 disulfide structure. These compare well with the value of 5.1 Å in the ALR2 crystal structure. Similarly, the distances between C-4 of nicotinamide and the phenol oxygen of Tyr48 were 4.6 ± 0.4 and 4.4 ± 0.4 Å in the Cys80-Cys303 and Cys298-Cys303 MD structures, respectively. These also compare well with the distance of 4.5 Å in the crystal structure. In contrast, major differences were observed in the conformation of the side chain of Trp111, which is more affected by the nearby disulfides Cys80-Cys303 and Cys298-Cys303 (Fig. 7, A and B, respectively). Conformational changes at Trp111 clearly depend on the C terminus rearrangement caused by the formation of the disulfides. Based on these results, the possibility that slight differences at His110, coupled with marked differences at Trp111, might result in an impaired kinetics cannot be ruled out. On the other hand, a small aldehyde like D-glyceraldehyde could still be manually docked into the structures of the modified active sites with the carbonyl of the aldehyde hydrogen bonded to Tyr48 and His110, and without steric conflicts with the Trp111 side chain.
Enzyme Cofactor Binding following Copper-induced Inactivation—The possibility of a loss of the pyridine cofactor, following treatment of b-ALR2 with copper ion, was considered by evaluating the relative amount of NADP$^+$ bound to the copper-modified enzyme by CD spectroscopy. This evaluation was possible because the addition of DTT to the ALR2-cofactor complex generates a dichroic signal that peaks at 335 nm with an intensity proportional to the complex concentration (24). Fig. 8 shows the appearance of such a dichroic signal after addition of 5 mM DTT to copper-modified b-ALR2 previously dialyzed against S-buffer. A further increase of the signal intensity was observed when 10 mM NADP$^+$ was added to the enzyme sample. No further changes in the 280–370 nm spectral region were observed by further increases of the cofactor concentration to 20 mM (data not shown). The relative intensity of the CD signal at 335 nm of the DTT-treated, copper-modified ALR2 before and after addition of saturating NADP$^+$ accounted for a loss of ~60% of the pyridine cofactor from the Cu(II)-inactivated enzyme. This was evaluated by taking into account the dilution factor of 1.12 on the CD signal after addition of NADP$^+$. The same dilution factor was adopted to correct the protein contribution to the overall observed elongation.

**DISCUSSION**

The human and rat recombinant forms of aldose reductase exhibited the same response to copper ion as the bovine lens enzyme. Both were inactivated by Cu(II) and ALR2 activity could be recovered by treating the inactive enzyme with DTT but not with EDTA or $o$-phenanthroline. Copper remained bound on the protein after inactivation. Whereas 1 eq of copper per enzyme mol is directly detectable as Cu(1), a total of 2 eq of copper per enzyme mol can be detected only after a 3-h treat-
Copper-induced Inactivation of Aldose Reductase

Fig. 9. Model of copper induced oxidation of ALR2.

ment at 37 °C of the inactive enzymes with either 3 mM DTT or 0.5 mM EDTA.

Cys™ is the only residue whose presence is essential for ALR2 inactivation. In fact, the h-C303S mutant reacts with copper quite differently from all other enzyme forms tested. When this mutant was incubated with copper in the presence of a stoichiometric amount of NADP⁺, the enzyme activity remained essentially stable until the copper concentration was raised to values causing protein aggregation (17). Furthermore, when NADP⁺ was not supplemented to the enzyme preparation, the copper treatment caused C303S mutant inactivation, which did not require DTT to be reversed, at variance with all other enzymes. In fact, in this case treatment with EDTA was sufficient to rescue the enzyme activity.

In this study, mass spectrometric analysis of Cu(II)-inactivated ALR2 of bovine and human, as well as, the mapping analysis of their peptides (Table I), revealed that the final product generated by copper treatment is an enzyme form carrying an intramolecular disulfide bond Cys298-Cys303. The involvement of Cys298 in the disulfide bridge explains the special reactivity of the oxidized ALR2 form with respect to monothiol compounds (Fig. 3). In fact, it appears clear that both GSH and 2-ME can disrupt the disulfide bond and generate mixed disulfide enzyme forms either as intermediate species (i.e. reduction of oxidized b-ALR2 by GSH) and/or as end products (i.e. reduction of oxidized h-ALR2 and b-ALR2 by GSH and 2-ME). In this regard, the apparent lack of effectiveness of Cys in rescuing the enzyme activity, both in terms of recovery of the native enzyme form and of generation of the Cys-ALR2 mixed disulfide form, is in complete agreement with the rather low, unusual stability of Cys-ALR2 that was shown to rearrange at 37 °C back to the Cys298-Cys303 disulfide-containing enzyme (22). The behavior of the h-C80S enzyme is in line with these results. However, the inactivation observed for the h-C298S enzyme following Cu(II) treatment (Fig. 2) and the presence of the intramolecular disulfide Cys80-Cys303, verified for the oxidized human mutant (Table I), raise the question on the mechanism underlying the modification processes. Indeed, whereas the kinetics of copper-induced inactivation of b-ALR2 is paralleled by the accumulation of the Cys298-Cys303 disulfide, a transient formation of the Cys80-Cys303 disulfide bond was also observed (Fig. 4). Thus the disulfide Cys80-Cys303 can be generated not only in the Cys298 mutants, but also in the native enzyme. The transient appearance of the Cys80-Cys303 disulfide enzyme and the progressive accumulation of the Cys298-Cys303 disulfide enzyme (Fig. 4) necessarily imply the occurrence of an intramolecular rearrangement of the former enzyme form to the latter. Even though the results related to the h-C80S enzyme indicates the possible direct formation of the Cys298-Cys303 disulfide, the exact oxidation pathway occurring on the native enzyme that gives rise to the end product of the copper-induced ALR2 oxidation remains an open question.

By inspection of the distances between the sulfur atoms of the cysteines in the crystal structure of the h-ALR2/NADP⁺ holoenzyme (44) it turns out that while Cys298 and Cys303 are too far apart (12.3 Å) to be likely candidates for formation of a disulfide bond, Cys80 and Cys303 are relatively closer (6.9 Å). However, given that these cysteines are not close to a bonding distance, the formation of disulfide bridges must be accompanied by remarkable conformational changes in ALR2.

The structural changes allowing the generation of intramolecular disulfide bonds were investigated by a molecular modeling approach. Indeed, simulations were performed with the aim to investigate both steps of the ALR2 modification by copper (i.e. the early formation of a noncovalent complex between copper and ALR2 and the subsequent formation of the disulfide bond). Following the definition of the force field parameters for copper, a four-centered complex between Cys80, Cys303, and 2 copper ions could be envisaged for ALR2. During a molecular dynamics simulation of the copper-ALR2 noncovalent complex, the distance between the sulfur atoms of Cys80 and Cys303 dropped significantly (Fig. 5A). In this configuration each copper ion coordinates both cysteines. The closer distances between the two cysteines and between these residues and the copper ions were compatible with an electron transfer process that can proceed through the formation of the disulfide Cys80-Cys303. Molecular dynamics simulations of the ALR2 structure containing the disulfide were then performed to allow the conformational changes arising from the disulfide modification (Fig. 5B). One interesting observation was that, following the disulfide formation, the two copper ions appeared to interact to a lesser extent with the cysteine residues. In fact, whereas Cu2 significantly drifted apart from both residues (>10 Å) interacting with water molecules, Cu1, yet at non-coordinating distance from both the cysteines, appeared to remain completely embedded in the protein (Fig. 5B). This finding is in agreement with a previous experimental observation (17) that only one of the two copper ions bound to ALR2 can be detected by direct bathocuproinedisulfonic acid titration (Cu2 in our case). Moreover, the second copper ion (Cu1), which would be trapped by the enzyme upon formation of the disulfide, became detectable.
Cys<sup>298</sup> remained too far apart from other cysteines to participate in any disulfide bond. Any attempt to shorten its distance from Cys<sup>303</sup> through the interaction with copper ions by MM and MD approaches using the crystal structure of the NADP<sup>+</sup>-ALR2 complex failed. A substantial reduction of the distance between these residues could occur only after a refolding of the C-terminal segment. Conformational changes at the C terminus were previously observed in the crystal structures of ALR2 complexed with the inhibitors Zopolrestat (51) and Tolrestat (51), but in none of these structures were Cys<sup>298</sup> and Cys<sup>303</sup> closer than 11 Å. However, different experiments involving formation of mixed disulfides at 37 °C showed that thermal rearrangements of Cys- and Cys-Gly mixed disulfide containing ALR2 likely occurred by closer Cys<sup>298</sup>-Cys<sup>303</sup> interactions (22). Nevertheless, when the disulfide Cys<sup>298</sup>-Cys<sup>298</sup> was imposed, the two copper ions, initially positioned at a coordinating distance between Cys<sup>298</sup> and Cys<sup>298</sup>, drifted apart and became situated in a way similar to that described in the case of the Cys<sup>90</sup>-Cys<sup>303</sup> disulfide (Fig. 6).

One possible structural change that could allow Cys<sup>298</sup> to move closer and interact either directly with Cys<sup>298</sup> (as may be the case of the C80S enzyme) or with the disulfide bond Cys<sup>298</sup>-Cys<sup>303</sup> may derive from the loss of the pyridine cofactor from the enzyme undergoing oxidation. A schematic representation of ALR2 modification induced by the copper ion is depicted in Fig. 9. The loss of the cofactor should induce significant changes in the ALR2 conformation. In fact, the release of the cofactor, which is the rate-limiting step in the direction of aldehyde reduction, is known to be associated with remarkable conformational changes of loop 7 (54–57). Indeed, loop 7, which is adjacent to the Cys<sup>298</sup>-Cys<sup>303</sup> disulfide (Fig. 6), showed the highest conformational variability during MD, supporting the hypothesis that formation of the disulfide might affect the NADP<sup>+</sup> binding site and NADP<sup>+</sup> release. Such an event may also give the rationale for the lack of activity of the disulfide bond Cys<sup>298</sup>-Cys<sup>303</sup> disulfide, as seen in the release of the cofactor, leading Cys 303 to reach at bonding distance Cys 298 is suppose to result in a conformational rearrangement leading to ALR2 at 37 °C with EDTA.

Only after the reduction of Cys<sup>298</sup> and Cys<sup>303</sup> through the interaction with copper ions by MM and MD approaches using the crystal structure of the NADP<sup>+</sup>-ALR2 complex failed. A substantial reduction of the distance between these residues could occur only after a refolding of the C-terminal segment. Conformational changes at the C terminus were previously observed in the crystal structures of ALR2 complexed with the inhibitors Zopolrestat (51) and Tolrestat (51), but in none of these structures were Cys<sup>298</sup> and Cys<sup>303</sup> closer than 11 Å. However, different experiments involving formation of mixed disulfides at 37 °C showed that thermal rearrangements of Cys- and Cys-Gly mixed disulfide containing ALR2 likely occurred by closer Cys<sup>298</sup>-Cys<sup>303</sup> interactions (22). Nevertheless, when the disulfide Cys<sup>298</sup>-Cys<sup>298</sup> was imposed, the two copper ions, initially positioned at a coordinating distance between Cys<sup>298</sup> and Cys<sup>298</sup>, drifted apart and became situated in a way similar to that described in the case of the Cys<sup>90</sup>-Cys<sup>303</sup> disulfide (Fig. 6).

One possible structural change that could allow Cys<sup>298</sup> to move closer and interact either directly with Cys<sup>298</sup> (as may be the case of the C80S enzyme) or with the disulfide bond Cys<sup>298</sup>-Cys<sup>303</sup> may derive from the loss of the pyridine cofactor from the enzyme undergoing oxidation. A schematic representation of ALR2 modification induced by the copper ion is depicted in Fig. 9. The loss of the cofactor should induce significant changes in the ALR2 conformation. In fact, the release of the cofactor, which is the rate-limiting step in the direction of aldehyde reduction, is known to be associated with remarkable conformational changes of loop 7 (54–57). Indeed, loop 7, which is adjacent to the Cys<sup>298</sup>-Cys<sup>303</sup> disulfide (Fig. 6), showed the highest conformational variability during MD, supporting the hypothesis that formation of the disulfide might affect the NADP<sup>+</sup> binding site and NADP<sup>+</sup> release. Such an event may also give the rationale for the lack of activity of the disulfide enzyme forms. In fact, from a closer view of the active site of the disulfide-carrying enzymes (Fig. 7), besides the conformational change observed at Thr<sup>111</sup>, which may possibly affect enzyme activity, there are no apparent reasons for the oxidized holoenzyme to be inactive. Unfortunately, the high values of the kinetic constants of the reversible movement of loop 7 in the mechanism of action (i.e. 0.5 s<sup>−1</sup>) (55), as well as the lack of useful crystal structure data on the ALR2 apoenzyme, made it very difficult to handle this problem by a molecular modeling approach. However, the involvement of the release of NADP<sup>+</sup> in the conformational rearrangements leading Cys<sup>293</sup> to reach at bonding distance Cys<sup>298</sup> is supported by the CD data. In fact, the analysis of CD spectra of the Cu(II)-modified b-ALR2 reveals that 60% of the NADP<sup>+</sup>, usually firmly bound to native ALR2, was lost after copper treatment (Fig. 8).

In conclusion, the site-specific oxidative action of copper ion on ALR2 ends with one of the most easy to predict modifications (i.e. thiol oxidation to disulfides) that this metal ion is able to induce on target proteins. In this case, the evolution of the oxidative insult on the protein structure leads to an enzyme form that is inactive but still convertible to the active enzyme form and to an efficient copper sequestration. Whether such an apparent scavenging action exerted by ALR2 contributes to the control of oxidative insult is still a matter of investigation.

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