THIOL OXIDASE ABILITY OF COPPER ION IS SPECIFICALLY RETAINED UPON CHELATION BY ALDOSE REDUCTASE

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Abstract

Bovine lens aldose reductase is susceptible to a copper mediated oxidation, leading to the generation of a disulfide bridge with the concomitant incorporation of two equivalents of the metal and inactivation of the enzyme. The metal complexed by the protein remains redox-active, being able to catalyze the oxidation of different physiological thiol compounds. The thiol oxidase activity displayed by the enzymatic form carrying one equivalent of copper ion (Cu_1-AR) has been characterized. The efficacy of Cu_1-AR in catalyzing thiol oxidation is essentially comparable to the free copper in terms of both thiol concentration and pH effect. On the contrary, the two catalysts are differently affected by temperature. The specificity of the AR-bound copper towards thiols is highlighted being Cu_1-AR completely ineffective in promoting the oxidation of both low density lipoprotein and ascorbic acid.

Introduction

The role of transition metals in inducing cell damage has been widely recognized [1, 2]. They promote the generation of reactive oxygen species (ROS), thus leading to oxidative stress which represents a co-causative factor in several pathological states linked to proteins, lipids and nucleic acid damage [3-8].

Copper ion is one of the most active transition metals in eliciting ROS generation. Given that it is the cofactor of several enzyme systems [9, 10], copper plays an important role as a trace element, thus making its deficiency detrimental for living cells [11, 12]. On the other hand, the fact that copper promotes ROS production through the Fenton reaction, means that it is important to control cellular levels of copper to prevent metal toxicity.

Several protein systems (i.e. transporter, storage proteins and Cu-chaperones) have thus evolved to regulate the uptake, distribution and delivery of copper [13-17]. Despite this strict control, diseases characterized by abnormally either elevated or low copper levels have been described, such as Wilson or Menkes disease, respectively [18]. Recently it has been postulated that elevated free copper levels may predict the risk for the onset of diabetes and Alzheimer disease [19].

The binding of copper to proteins normally warrants the maintenance of the metal in a redox inactive form, in order to avoid metal-catalyzed oxidation of cell components. However, pro-oxidant features of copper-binding proteins, such as superoxide dismutase, caeruloplasmin and β-amyloid, have been documented [20-23].

Despite not being a metal binding protein, aldose reductase (alditol: $NADP^+$oxidoreductase, EC 1.1.1.21) (AR) is extremely sensitive to Cu(II), which overall seems to act as a tightly binding modifying agent. In fact, AR from bovine lens has been shown to be readily inactivated in the presence of stoichiometric amounts of the metal through an oxygen independent modification [24]. The loss of activity relies on the formation of a disulphide bridge between Cys298 and Cys303, and is accompanied by the incorporation of two equivalents of copper per enzyme mol [24, 25]. Molecular modelling studies [25] indicated that, of these two copper ions, one was completely embedded in the protein, while the other resulted free to interact with water molecules. Moreover, these studies suggested an active role of Thr113, Trp111 and Leu 300 in the interaction between AR and the copper ion embedded in the protein structure. These predictions were consistent with the observation that only one of the two copper ions can be detected by direct bathocuproine titration, while the second copper ion resulted accessible to bathocuproine only after prolonged thermal

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treatment of the copper-modified AR in the presence of either reducing or chelating agents [24].

In this paper, we report that the copper bound to AR remains redox active toward thiol compounds, but not toward other copper sensitive targets (i.e. ascorbic acid and low density lipoprotein), thus making the copper-modified AR able to act as a thiol oxidase.

Materials and Methods

Materials

Bathocuproinedisulfonic acid (BCDS), cysteine, CysGly, dithiothreitol (DTT), reduced glutathione (GSH), glutathione disulfide (GSSG), homocysteine, human low density lipoprotein (LDL), NADPH, penicillamine, monothioglycerol, γ-glutamylcysteine, cysteamine, thiobarbituric acid (TBA), D,L-glyceraldehyde (GAL), ascorbic acid (AA), ascorbate oxidase from *Cucurbita sp* (E.C. 1.10.3.3) were from Sigma-Aldrich. All other chemicals were of reagent grade from BDH Chemicals.

Enzymatic assay and purification of aldose reductase. Aldose reductase activity was measured at 37°C as previously described [26] using GAL as a substrate.

The enzyme was purified from bovine lens as previously described [27]. The pure enzyme (1.2 U/mg) was stored at 4°C in 10 mM sodium phosphate buffer pH 7.0 (S-buffer) containing 2 mM DTT. The molar concentration of AR was calculated on the basis of a molecular mass of 34 kDa, and of the protein concentration determined by the Coomassie Blue binding assay [28] performed using bovine serum albumin as a standard.

Preparation of copper-modified AR. The copper-modified AR was prepared as described [24], by incubating at 25°C in the S-buffer the purified bovine lens enzyme (3.5 μM final concentration), after an extensive dialysis against the S-buffer, in the presence of a 2.5 fold molar excess of $CuCl_2$. After 90 min of incubation, the enzyme (which displayed a residual activity of about 15% compared to the native enzyme) was again dialyzed against S-buffer and stored at 4°C for no more than two days. As expected [24], this enzyme form (Cu_2-AR) contained two equivalents of copper as determined by complexometric titration (see below).

Preparation of glutathione-modified aldose reductase. AR carrying a mixed disulphide with glutathione at the level of Cys298 (GS-AR) was prepared as previously described [29] upon incubation of 3.5 μM native AR for 3 h at 25°C with 1.5 mM GSSG, followed by extensive dialysis against the S-buffer.

Evaluation of LDL oxidation. LDL oxidation was detected essentially according to Yagi [30]. LDL was suspended (2 mg/mL) in S-buffer and then dialyzed against the same buffer

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in order to remove the ethylenediamine tetraacetic acid (EDTA) present in the commercial preparation. Dialyzed LDL was incubated (70 μg/mL) in S-buffer at 37°C alone or in the presence of 1μM of either $CuCl_2$ or Cu_1-AR. At appropriate times (from 0 to 5 h), aliquots of 400 μL were withdrawn and 10 μL of 10 mM EDTA and 1 mL of 10% trichloroacetic acid were added. After the addition of 1 mL of 0.67 % TBA, samples were incubated at 95°C for 2 h and, after cooling, the fluorescence at 554 nm (excitation 525 nm) was measured on a Jasco FP6500 spectrofluorimeter.

Other methods. The concentration of thiol compounds was determined by Ellman titration [31].

Copper concentration was determined by BCDS titration as previously described [24] using an extinction coefficient for the $(BCDS)_2Cu(I)$ complex of of 12,250 $M^{-1}cm^{-1}$, which was evaluated by calibration curves obtained using standard $CuCl_2$ solutions. When the metal was measured on Cu-treated AR, 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 the same concentration of native AR.

The concentration of ascorbic acid was determined by evaluating the decrease in the absorbance at 265 nm following the oxidation of AA upon the addition of 250 mU of ascorbate oxidase. The extinction coefficient at 265 nm, obtained using known concentration of ascorbic acid, was 12.6 ± 0.9 $mM^{-1}cm^{-1}$.

Unless otherwise stated, all the data are expressed as the mean ± standard deviation of at least four independent measurements. Statistical analysis was performed using GraphPad 6.0 Software.

RESULTS AND DISCUSSION

Thiol oxidase ability of copper-modified AR
An important aspect of the oxidative modification of AR induced by Cu(II) is the retention by the oxidized enzyme of two equivalents of copper, which are bound to the protein (Cu_2-AR) with a different level of efficiency [24, 25]. In our study, while one copper ion was released in solution after the incubation of 3.5 μM Cu_2-AR at 25 °C in S-buffer for 2 h, the second metal ion was removed from the protein only by a dialysis against the S-buffer performed after a prolonged incubation (at least 3 h) at 37°C in the presence of 5 mM DTT. Both modifications occurring on AR upon Cu(II) treatment (i.e. the binding of the metal ion and the oxidation of protein thiols) were thus reversed.

When 1 μM Cu_2-AR was incubated at 25° C in S- buffer with 1 mM cysteine, there was a decrease in the reduced thiol, evaluated by DTNB titration (Fig. 1). There was no significant oxidation of cysteine with either 1 μM native AR or with a solution obtained after protein removal by ultrafiltration through an Amicon YM10 membrane of a freshly prepared 1 μM Cu_2-AR. The oxidation rate (29 ± 4 μM/min) was approximately twofold the value measured, under the same conditions, with 1 μM $CuCl_2$ (14 ± 1 μM/min). These results suggest that both copper ions of Cu_2-AR remained active as catalysts of thiol oxidation. The removal of one copper ion upon incubation of Cu_2-AR at 25°C for 2 h followed by dialysis against S-buffer led to the generation of an enzyme form carrying only one (1.0 ± 0.1) copper ion per enzyme mol (Cu_1-AR), as evaluated by complexometric analysis, which was still able to induce cysteine oxidation (Fig. 1). A rate of cysteine oxidation of 13 ± 2 μM/min was measured with 1 μM Cu_1-AR, which was comparable with that measured the presence of 1 μM $CuCl_2$.

In order to evaluate the contribution of protein moiety on the thiol oxidase ability of AR, the second copper ion was removed in not reducing conditions by dialysis against S-buffer after a prolonged incubation (3 h at 37°C) in S-buffer containing 1 mM EDTA (data not shown). In this case, the removal of the metal left AR in an inactive status, both as aldose reductase and as thiol oxidase (Fig. 1). When this copper-depleted enzyme was incubated (3.5 μM) with $CuCl_2$ (8.75 μM) at a ratio of approximately 1 to 3, it was able to bind approximately two (1.8 ± 0.2) equivalents of the metal per enzyme mol, thus regaining its ability to catalyze cysteine oxidation (data not shown). These results highlight the importance of copper as the triggering species for thiol oxidation, as the oxidized AR is simply a protein scaffold that reversibly binds the metal ion.

The significance of the disulphide between Cys298 and Cys303 occurring in Cu_1-AR [25] for copper binding was tested using the glutathionylated AR. This enzyme form (GS-AR), characterized by the S-glutathionylation at Cys298 [32], the most accessible Cys residue of AR, generated by GSSG transthiolation on AR (see Methods) and found in the bovine lens undergoing oxidative stress [33], was used as a protein target for Cu(II) oxidation. The incubation for 2 h of 3.5 μM GS-AR at 25°C in the presence of a 2.5 molar excess of $CuCl_2$ led to a progressive inactivation of the enzyme, as occurred for the native AR (data not shown).

Copper evaluation on the inactive enzyme revealed the presence of approximately one (1.1 ± 0.2) equivalent of the metal ion per enzyme mol. When copper-modified GS-AR was used as a catalyst of CysGly oxidation, oxidation rates of 11 ± 1 μM/min were measured, which

were essentially the same values observed for equivalent concentrations of free copper in solution. This suggests that the structural restraints linked to the intramolecular disulphide on Cu_1-AR are not a key factor in copper binding.

Kinetic features of thiol oxidation catalyzed by Cu$_1$-AR

Due to the relatively easy release of the first copper ion from Cu_2-AR, it is conceivable that this copper ion may leave the enzyme, either wholly or partially, while acting as thiol oxidase and may exert its oxidant ability in solution. Thus, in order to evaluate the oxidant ability of the AR bound metal, Cu_1-AR was used in further experiments. It is conceivable that the copper remaining in Cu_1-AR was the ion that molecular modelling studies predicted as completely embedded in the protein structure [25]. The possible release of copper from Cu_1-AR during incubation with different thiol compounds was tested and ruled out. In fact, the same rate of oxidation of cysteine was observed using both freshly prepared Cu_1-AR and Cu_1-AR recovered after the enzyme had been used as a catalyst of the complete oxidation of 1 mM cysteine and then dialyzed against S-buffer.

Different thiols showed a different susceptibility to oxidation by Cu_1-AR, as occurs when using the free copper in solution. Also the pH, as expected, affects the oxidation, being pH 8 the optimal condition for oxidation for most of the tested thiols, except for cysteine and Cys-Gly, whose maximal oxidation rates were observed at pH 7.4 and 6.5, respectively. Table 1 reports the oxidation rates measured for different thiol compounds when incubated at 25°C at their optimal pH with 1 µM of either Cu_1-AR or $CuCl_2$. For both catalysts, the values ranged within more than one order of magnitude between the oxidation rate of glutathione and homocysteine and the oxidation rate of cysteamine. The results underline the similarity of the thiol oxidative process in the presence of the two oxidation inducers (i.e. Cu_1-AR and free copper ion). The only exception concerns Cys-Gly, which appeared to be slightly more susceptible to the oxidation induced by Cu_1-AR compared to $CuCl_2$ (29 ± 5 µM/min versus 18 ± 2 µM/min). This difference was constantly observed at different pH values and at different concentrations (ranging from 0.25 to 2 µM) of both free and AR-bound copper (data not shown).

The substrate dependence of thiol oxidation catalyzed by Cu_1-AR and $CuCl_2$ was evaluated using cysteine as a target. The oxidation rate of cysteine increased with increasing thiol concentration, reaching an apparent saturation, as reported in Fig. 2. An apparent K_M of 1.3 ± 0.1 (SE) mM and a V_{max} of 25 ± 3 (SE) µM/min were measured with 0.8 µM Cu_1-AR. A similar result was obtained when 0.8 µM $CuCl_2$ was used as catalyst. In this case, a

concentration of cysteine leading to a half saturation of the oxidative process of 1.0 ± 0.1 (SE) mM and a maximal rate of oxidation of 28 ± 1 (SE) μM/min were measured. Temperature appeared to be the most effective parameter in discriminating the free copper from the AR-bound copper dependent thiol oxidation. The oxidation rate of cysteine was evaluated at temperatures in the range 25-40°C, using both Cu_1-AR and $CuCl_2$ as catalysts (Fig. 3). In the range 20-33°C, no differences were observed between the two catalysts. When the temperature was increased above 33°C, the oxidation rate in the presence of Cu_1-AR diverges, at 40°C reaching a value (44 ± 1 μM/min) that accounts for an overall four-fold increase compared to the value measured at 25°C. In the same conditions, the increase in temperature from 25 to 40°C in the presence of $CuCl_2$ led to an increase in the rate of cysteine oxidation of only 1.3 fold.

Thiol oxidation induced by copper proceeds through the preliminary formation of complexes with different low molecular weight ligands, including the same thiol undergoing oxidation [34-37]. It is conceivable that the intramolecular copper complex characterizing Cu_1-AR is more stable than the intermolecular complexes that copper may form with cysteine undergoing oxidation, which may explain the observed difference.

Target specificity of the oxidase activity of copper-bound AR

The ability of copper bound to AR to act as a catalyst of the oxidation of other molecules, different from thiols, was investigated. The susceptibility of LDL to oxidative modification by Cu(II), a key step in the aetiology of atherosclerosis, is a well-known event, although the conditions governing the oxidative process are still under investigation [38-41]. Ascorbic acid is another target of copper dependent oxidation [42, 43]. In fact, ascorbic acid and Cu(II) represent a pro-oxidant cell-damaging system, in which the reducing features of ascorbic acid enhance the redox potential of the metal ion, thus favouring, in the presence of oxygen, ROS generation [44, 45]. Both LDL and ascorbate were used as targets to compare the oxidative trigger effect exerted by the free copper ion and the AR-bound metal. LDL and ascorbic acid were incubated with either $CuCl_2$ or Cu_1-AR, at a final concentration of 1 μM. The results reported in Figs. 4 a and b clearly show the complete inability of Cu_1-AR to catalyze the oxidation of both LDL and ascorbic acid. Besides the oxidation potential of the metal ion bound to AR, as judged by its ability to oxidize thiols, it is evident that its action may be restricted. In particular, it is relevant that, when reduced thiols are added to copper modified AR, the generation of a mixed disulphide between the thiol and Cys298 has been described [25]. Thus, it is conceivable that this may be the first step in the interaction

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between Cu_1-AR and the thiol compound and may represent the only way for a molecule to interact with a copper ion which is completely embedded in the protein scaffold. The copper ion trapped on the protein at an interactive, even though not bonding, distance from Cys 303 (the counterpart of Cys298 in the disulfide bridge) may act as an electron sink when the trans-thiolation between the exogenous thiol compound and the intramolecular disulfide takes place. This site must then be accessible to oxygen; even though no predictions can be made at this point for the mechanism of the electron transfer, oxygen reduction will ultimately be the driving force of the oxidative process. The above considerations may be the rationale of the insensitivity of ascorbic acid and LDL to the oxidant activity of the copper bound AR.

The ability of AR to efficiently interact with copper ion leads to a stable 1:1 copper:AR complex in which the redox potential of the bound metal ion is preserved. Thus, following Cu(II) treatment, the activity of AR is switched from the aldehyde reductase activity of the enzyme to a thiol oxidase activity. Indeed, except for the slight, but consistent, increase in susceptibility of Cys-Gly to oxidation and for the significant temperature effect observed with Cu_1-AR with respect to free copper, the pro-oxidant activity of the AR-bound metal ion towards thiols does not appear to be especially enhanced with respect to the free metal ion. On the contrary, the metal ion retained by the oxidized enzyme appears to be less active in triggering oxidation of non-thiol targets. In fact, ascorbate and LDL, which are very susceptible to the oxidation promoted by free copper ion, are essentially preserved when copper is bound on AR. Thus, the AR protein appears adequate in controlling the pro-oxidant action of the metal ion in eliciting the oxidative stress phenomena of lipid peroxidation or ascorbate-dependent ROS generation. On the other hand, AR is apparently unable to buffer the redox ability of copper towards thiols. Thus, the generation of copper modified AR, which could occur in situations of abnormally elevated cellular copper levels, as those reported in Wilson disease [46] or in diabetes [47], might, through the reported prooxidant action of Cys and CysGly [48], ultimately promote glutathione oxidation.

Conflict of interest: The authors declare that they have no conflict of interest.

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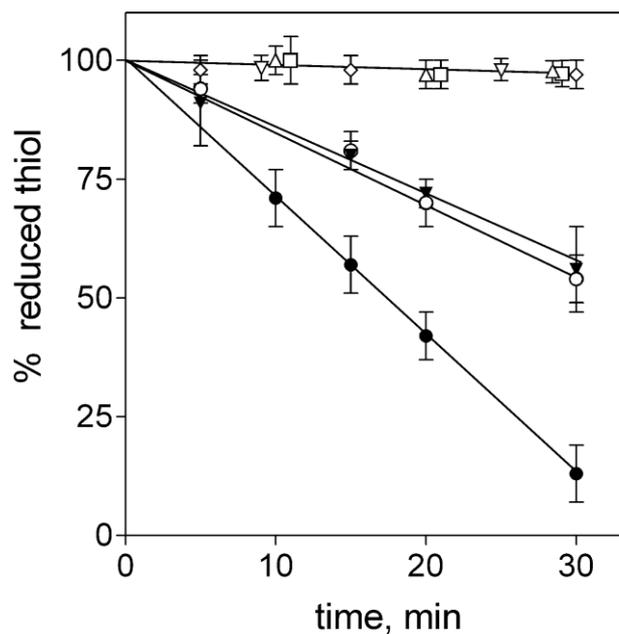
Figure 1 Cysteine oxidation induced by copper-modified aldose reductase. Cysteine (1 mM) was incubated at 25°C in S-buffer in the presence of the following: none, (∇); 1 μM Cu_2-AR, ($\bullet$); 1 μM Cu_1-AR, ($\blacktriangledown$); 1 μM $CuCl_2$, ($\circ$); 1 μM native AR, ($\triangle$); solution obtained by ultrafiltration through an Amicon YM10 membrane of a freshly prepared 1 μM Cu_2-AR, ($\square$); 1 μM Cu_1-AR after EDTA treatment, ($\diamondsuit$). At different times, residual reduced thiol was evaluated through Ellman titration and reported as percent of the initial value. Error bars represent the standard deviation from three independent measurements
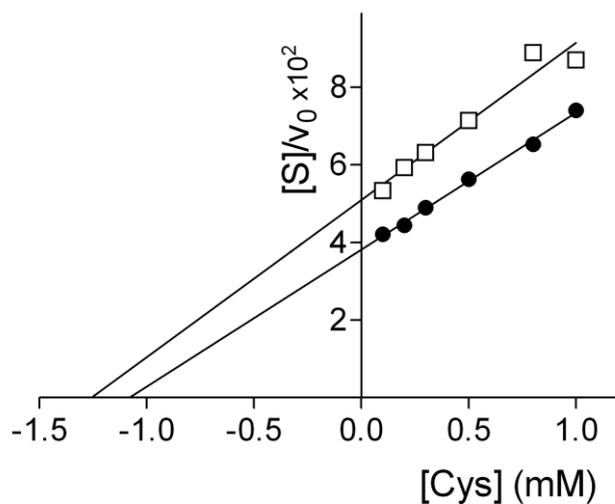
Figure 2 Effect of cysteine concentration on the thiol oxidation rate in the presence of AR-bound copper or free copper ion. Different concentrations of cysteine were incubated at 25°C in 50 mM sodium phosphate buffer pH 7.4 in the presence of 0.8 μM of either Cu_1-AR (●) or $CuCl_2$ (□). At different times aliquots were withdrawn and the residual reduced thiol was evaluated. On the basis of measurements performed at 4 different times the oxidation rates were calculated and reported as a function of cysteine concentration. Data are reported as Hanes-Woolf plot; linear regression analysis was performed in order to estimate both the maximal rate of oxidation and the cysteine concentration leading to half saturation
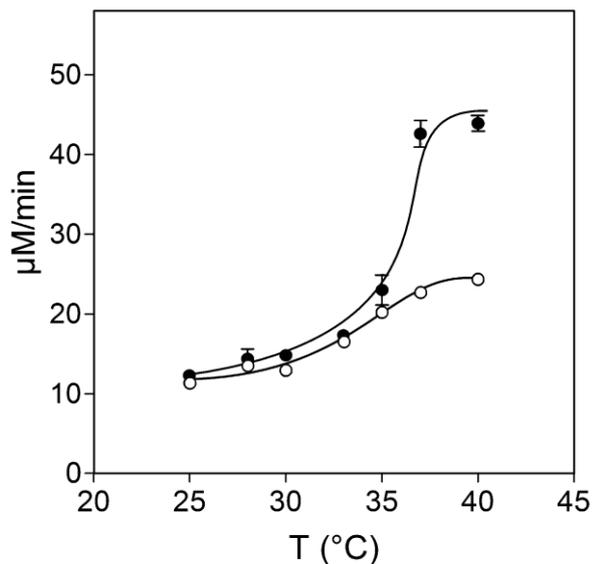
Figure 3 Effect of temperature on cysteine oxidation induce by AR-bound copper or free copper ion. Cysteine (1 mM) was incubated in 50 mM sodium phosphate buffer pH 7.4 at the indicated temperatures in the presence of 1 µM of either Cu_1-AR (●) or $CuCl_2$ (○). At different times aliquots were withdrawn and the residual reduced thiol was evaluated. On the basis of measurements performed at 4 different times the oxidation rates were calculated and reported as a function of cysteine concentration. Error bars (when not visible are within the symbol size) represent the standard deviation from three independent measurements
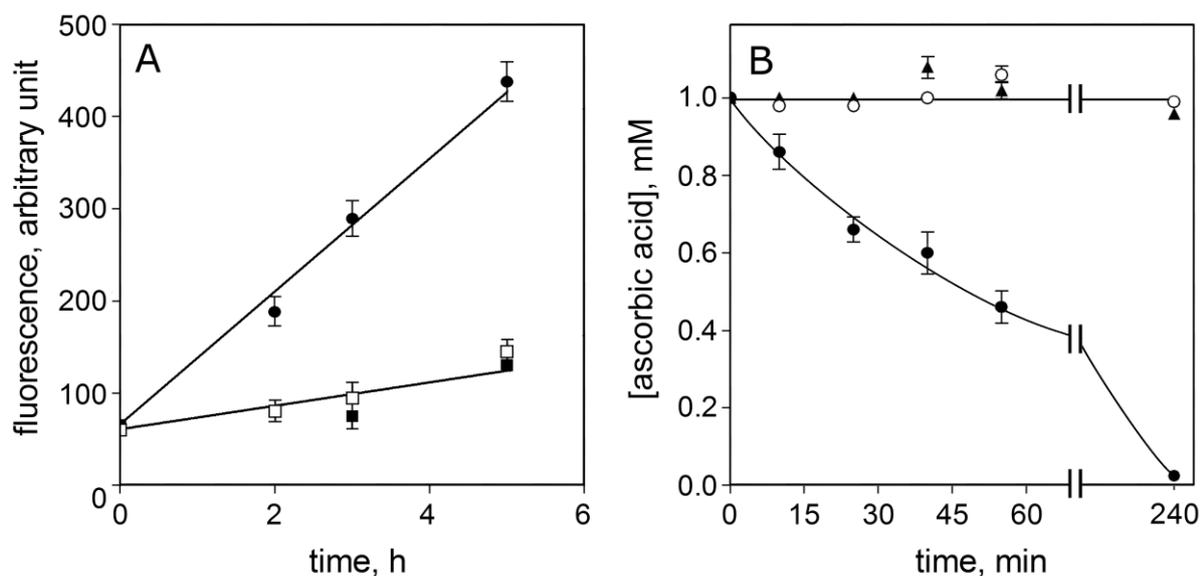
Figure 4 LDL and ascorbic acid are not oxidized by AR-bound copper. *Panel a*: LDL were incubated (70 µg/mL) in S-buffer at 37°C alone (■) or in the presence of 1µM of either $CuCl_2$ (●) or Cu_1-AR (□). At the indicated times aliquots were withdrawn for the evaluation of TBA reactive compounds (see Methods). *Panel b*: ascorbic acid (1 mM) was incubated in S-buffer at 37°C alone (▲) or in the presence of 1µM of either $CuCl_2$ (●) or Cu_1-AR (○). At the indicated times aliquots were withdrawn for the evaluation of the residual ascorbic acid (see Methods). Error bars represent the standard deviation from three independent measurements

Table 1

Oxidation rates of different thiols at optimal pH in the presence of Cu_1-AR or $CuCl_2$.

Thiol compound	pH	Cu_1-AR	$CuCl_2$
Homocysteine	8.0	0.6 ± 0.1	0.7 ± 0.1
Glutathione	8.0	0.8 ± 0.1	0.6 ± 0.2
Monothioglycerol	8.0	0.8 ± 0.2	0.8 ± 0.1
γ-Glu-Cys	8.0	1.2 ± 0.2	1.8 ± 0.1
β-mercaptoethanol	8.0	1.2 ± 0.1	1.0 ± 0.1
Penicillamine	8.0/6.5	$1.5 \pm 0.2/1.2 \pm 0.1$	$1.3 \pm 0.2/1.6 \pm 0.2$
Cysteine	7.4	23 ± 2	22 ± 2
Cys-Gly	6.5	29 ± 5	18 ± 2
Cysteamine	8.0	37 ± 2	30 ± 6

Different thiols were incubated (1 mM final concentration) at 25°C in 50 mM sodium phosphate buffer at the indicated pH in the presence of 1 µM of either Cu_1-AR or $CuCl_2$. Oxidation rates (mean ± standard deviation from at least three independent measurements) are expressed as µM/min.