# Electron Paramagnetic Resonance Study of the Active Site of Copper-Substituted Human Glyoxalase I†

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ABSTRACT: $Zn^{2+}$ in native glyoxalase I from human erythrocytes can be replaced by $Cu^{2+}$, giving an inactive enzyme. $Cu^{2+}$ was demonstrated to compete with the activating metals $Zn^{2+}$ and $Mn^{2+}$, indicating a common binding site on the enzyme for these metal ions. The electron paramagnetic resonance (EPR) spectra of $^{63}Cu(II)$ glyoxalase I at 77 K and of its complexes with glutathione and some glutathione derivatives are characteristic of $Cu^{2+}$ in an elongated octahedral coordination ($g_{\parallel} = 2.34$, $g_{\perp} = 2.09$, and $A_{\parallel} = 14.2$ mT). The low-field bands of the free enzyme are asymmetric and become symmetrical upon addition of glutathione or *S*-(*p*-bromobenzyl)glutathione but not *S*-(D-lactoyl)glutathione. The results indicate the existence of two conformations of Cu(II) glyoxalase I, in agreement with the effects caused by these compounds on the protein fluorescence. The copper hyperfine line at low field in the EPR spectrum of the *S*-(*p*-bromobenzyl)glutathione complex of $^{63}Cu(II)$ glyoxalase I shows a triplet structure, indicative of coupling to one nitrogen ligand in the equatorial plane. Similar results were obtained with the glutathione complex. By addition of the spectrum of the *S*-(*p*-bromobenzyl)glutathione complex and a spectrum corresponding to two nitrogen ligands with two different coupling constants, a good fit was obtained for the low-field region of the asymmetric spectrum of free $^{63}Cu(II)$ glyoxalase I. The first two spectra are assumed to correspond to two separate conformational states of the enzyme. The results demonstrate that at least one nitrogen ligand is involved in the binding of $Cu^{2+}$. Comparison with EPR parameters from reported model studies suggests one nitrogen plus three oxygens as the most likely in-plane ligands. A hypothetical model for the metal coordination site of glyoxalase I with different conformations of the enzyme is presented. One or two histidines are suggested to provide EPR-active nitrogen ligands to the metal ion in the two proposed conformational states. A transition between two conformations probably is linked to the dynamics of the catalytic process. Model building, using distances from earlier proton NMR studies of glutathione derivatives, showed that it is sterically possible for an imidazole group of histidine in an apical coordination position to act as the base in the catalytic mechanism.

Mammalian glyoxalase I (EC 4.4.1.5) is a dimeric metalloenzyme (Aronsson et al., 1978) with $M_r$ 23 000 per monomer (Marmstål et al., 1979). Glyoxalase I converts the hemimercaptal formed between glutathione (GSH) and methylglyoxal (or other 2-oxoaldehydes) into *S*-(D-lactoyl)glutathione, which may subsequently be hydrolyzed by glyoxalase II.


A metabolic role of the glyoxalase system is believed to be the detoxication of dicarbonyl compounds by converting them to less reactive hydroxy acids (Mannervik, 1980). Methylglyoxal, a possible natural substrate, has been shown to be highly mutagenic to a *Salmonella* tester strain (Marnett et al., 1985).

Each monomer of the native enzyme contains a $Zn^{2+}$ ion, which is essential for catalytic activity. $Mg^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Mn^{2+}$ can substitute for $Zn^{2+}$ and restore high catalytic activity (Sellin et al., 1980; Han et al., 1977), while substitution with $Cd^{2+}$ gives an inactive enzyme (Sellin et al., 1983a). Kinetic parameters, such as apparent $K_m$ and $k_{cat}$ values, are of similar magnitude for native and metal-substituted glyoxalase I (Sellin et al., 1983a). The inhibition constants for glutathione and its derivatives are more dependent on the nature of the metal in the active site (Sellin et al., 1983a).

Spectroscopically detectable metal substitutions have provided active enzyme derivatives such as Mn(II) and Co(II) glyoxalase I that have been used in studies of the essential metal binding site. Two fast-exchanging water molecules have been detected in studies of the paramagnetic effect of Mn(II) glyoxalase I on the nuclear relaxation rate of water protons (Sellin et al., 1982a). The number of rapidly exchangeable water molecules decreases to approximately one when *S*-(*p*-bromobenzyl)glutathione and *S*-(D-lactoyl)glutathione are added to the enzyme. Analyses of the paramagnetic effects of Mn(II) and Co(II) glyoxalase I on the relaxation rate of hydrogen and carbon nuclei of the product *S*-(D-lactoyl)glutathione and three additional glutathione derivatives have made possible calculations of distances between the metal ion and the responsive nuclei. Thus, an extended Y-shaped conformation of the enzyme-bound glutathione derivatives, as well as a second sphere coordination to the metal of the product and probably also of the substrate, has been defined (Sellin et al., 1982b; Rosevear et al., 1983, 1984).

Optical and EPR[1] properties of the $Co^{2+}$-substituted enzyme (Sellin et al., 1983b) and X-ray absorption studies of Zn(II)

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[1] Abbreviations: EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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glyoxalase I (Garcia-Iniguez et al., 1984) indicate an octahedral metal coordination in glyoxalase I, involving four oxygen and two nitrogen ligands. Metal dissociation constants of glyoxalase I have been determined for $Zn^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Mg^{2+}$, and the values are consistent with the proposal of nitrogen and oxygen as metal ligands in the enzyme (Sellin & Mannervik, 1984).

EPR studies of $Cu^{2+}$-substituted proteins have proven useful in the examination of the metal binding sites of proteins (Vänngård, 1972). The values of $g_\parallel$ and $A_\parallel$ often give information about the nature of the ligands. In a Zn(II) protein the ligands are usually oxygen and nitrogen and sometimes sulfur. The nitrogen ligands in some cases can be detected due to the nuclear spin of $^{14}N$ ($I = 1$), which gives rise to a characteristic superhyperfine pattern. Interaction with one or two equivalent nitrogen nuclei can usually be deduced from the low-field line in an EPR spectrum, but contributions of two or more nonequal nitrogen atoms are not always resolved and are therefore not easily detectable.

In this paper we report EPR studies of glyoxalase I using $^{63}Cu(II)$ as a spectroscopic probe. The enzyme complexes with glutathione, the product *S*-(D-lactoyl)glutathione, and the strong competitive inhibitor *S*-(*p*-bromobenzyl)glutathione are also investigated. These studies offer new insight into the nature and function of the metal binding site.

## Experimental Procedures

### *Materials*

Glyoxalase I from human glyoxalase was purified to homogeneity as previously described (Aronsson et al., 1979). Isoenzymes 1 and 3, which are homodimeric proteins (i.e., they contain identical subunits), as well as a mixture of the three isoenzymes 1–3 were used.

Isotopically pure $^{63}Cu$ (99.89 atom %) was obtained from Oak Ridge National Laboratories (Oak Ridge, TN) in the form of CuO. A stock solution of $^{63}Cu^{2+}$ was prepared by dissolving the oxide in 2 M HCl. NaOH and Tris base were used to adjust the solution to pH 7.8. Synthesis of the strong competitive inhibitor *S*-(*p*-bromobenzyl)glutathione was carried out by method A of Vince et al. (1971). *S*-D-(Lactoyl)glutathione was prepared enzymatically (Ball & Vander Jagt, 1979).

### *Methods*

*Enzyme Preparation and Assay.* Preparation of metal-free apoenzyme was performed as earlier described (Sellin et al., 1983b). $^{63}Cu(II)$ glyoxalase I was obtained by addition of excess $^{63}Cu^{2+}$ to the apoenzyme containing about 0.2 mM EDTA (which protects the apoenzyme from reactivation by spurious metal ions). The excess $^{63}Cu^{2+}$ was removed by dialysis against 10 (or 50) mM Tris-HCl buffer (pH 7.8) for 48 h. In some experiments the dialysis buffer was Hepes–NaOH (pH 7.6). Protein concentration was determined by absorbance at 280 nm ($A_{280} = 0.80$ mL·mg$^{-1}$·cm$^{-1}$). Flameless atomic absorption spectrometry was carried out by using a graphite furnace atomizer. Metallic copper dissolved in concentrated $HNO_3$ was used as a standard. The activity of glyoxalase I was measured spectrophotometrically with methylglyoxal as the 2-oxoaldehyde substrate (Marmstål et al., 1979).

*Dissociation Constant of Cu(II) Glyoxalase I.* Determination of the metal dissociation constant of Cu(II) glyoxalase I was performed in the presence of a metal buffer, essentially as described earlier (Sellin & Mannervik, 1984). Competition between $Mn^{2+}$ (which gives active glyoxalase I) and $Cu^{2+}$ (giving inactive enzyme) was applied. Samples of apoenzyme were equilibrated in 20 mM nitrilotriacetic acid (NTA) at pH 8.5 with 5 mM $Mn^{2+}$, plus 0–1 mM $Cu^{2+}$ (total concentrations). In the calculation of the free metal ion both 1:1 and 1:2 metal–NTA complexes were considered (Sellin & Mannervik, 1984).

*Electron Paramagnetic Resonance.* X-band (9.1 GHz) EPR spectra were recorded with a Varian E-9 spectrometer using 100-kHz field modulation with 0.4-mT amplitude. The samples in the rectangular cavity were kept in liquid nitrogen at 77 K by means of a Dewar insert. The incoming power was 2 mW or 10 mW in the case of amplified spectra. A 9-in. magnet was used. Calibration of the field scale and determination of *g* values were carried out by means of an AEG NMR flux meter. Spectra were collected by an on-line computer. A 1 mM CuEDTA sample was employed as a standard for the double integrals.

Computer simulations of the complete spectra were done by the program by Lefebvre and Maruani (1965). Separate simulations of the superhyperfine patterns of the low-field components of the spectra were conveniently carried out by superposition of Gaussian lines, generated by a computer. The line width at half-height ($\Delta H_{1/2}$) of the spectral components was used as a parameter. The fitting procedure allowed variation of the number of nitrogen ligands involved and their hyperfine couplings. The values of $g_\parallel$, $g_\perp$, and $A_\parallel$ for $^{63}Cu$ were measured in the experimental spectra. The relative amounts of the components in a spectrum, reflecting different metal coordination sites, were determined by computing weighted sums by best fit.

## Results

*Properties of Cu(II) Glyoxalase I.* Cu(II) glyoxalase I is catalytically inactive. The copper enzyme is almost colorless as judged from a solution of 0.8 mM concentration (enzyme binding sites). The following results demonstrate that $Cu^{2+}$ binds to the same site as the activating metal ions $Zn^{2+}$ and $Mn^{2+}$. Addition of $Cu^{2+}$ to the apoenzyme gave rise to the same 3-nm red-shift in the emission spectrum of the intrinsic tryptophan fluorescence as found by addition of $Zn^{2+}$ (Aronsson et al., 1981). The $Cu^{2+}$ dissociation constant for the active site of the enzyme was determined from the competition between $Mn^{2+}$ and $Cu^{2+}$. The following relationship [cf. Ray (1969)] was used for the determination

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{1}{V_{max}} \frac{[Cu^{2+}]K_{d,Mn}}{[Mn^{2+}]K_{d,Cu}}$$

where $v$ is the measured enzymatic activity, $V_{max}$ is the maximum activity, and $K_d$ is the dissociation constant for the metal–enzyme complex. A plot of $1/v$ against $[Cu^{2+}]$ at constant $[Mn^{2+}]$ is linear, demonstrating the competitive binding of $Cu^{2+}$ and $Mn^{2+}$ (Figure 1). An apparent $K_d$ for $Cu^{2+}$ binding to glyoxalase I was calculated from the concentration of free $Mn^{2+}$ and by the slope/intercept ratio of the plot. By use of $K_{d,Mn} = 4.9 \times 10^{-9}$ M, obtained from an earlier study (Sellin & Mannervik, 1984), $K_{d,Cu} = 0.9 \times 10^{-15}$ M was derived. The experiment shown in Figure 1 is representative of several determinations. A similar experiment in which $[Mn^{2+}]$ was varied at constant $[Cu^{2+}]$, as well as an analogous experiment involving $Zn^{2+}$ instead of $Mn^{2+}$, gave $K_{d,Cu}$ values not significantly different from the value above.

*EPR Spectra of $^{63}Cu(II)$ Glyoxalase I.* In order to improve the quality of spectra and facilitate the interpretation, we used isotopically pure $^{63}Cu$ ($I = {}^3/_2$) for the substitution. EPR spectra of $^{63}Cu(II)$ glyoxalase I and its complexes with glu-

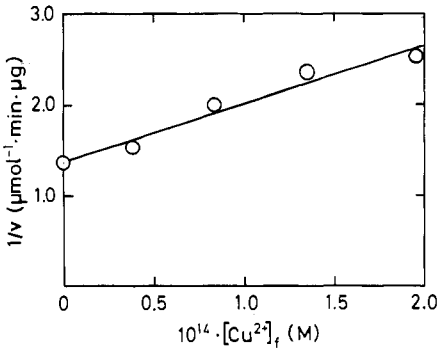


FIGURE 1: Competitive binding of $Mn^{2+}$ and $Cu^{2+}$ by glyoxalase I monitored by assay of enzyme activity. Samples of apoenzyme (5.1 μM binding sites) were equilibrated for 3 h at 20 °C with 5 mM Mn(II) plus 0–1 mM Cu(II) and 0.10 M Tris-HCl buffer, pH 8.5, containing 20 mM NTA and 0.10 M NaCl, and subsequently assayed for glyoxalase I activity.

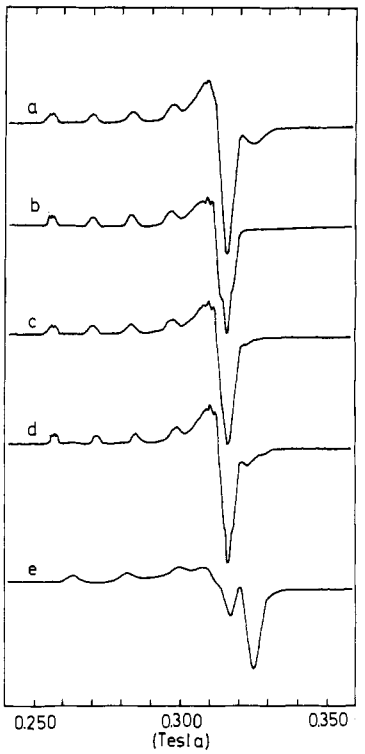


FIGURE 2: X-band EPR spectra at 77 K of $^{63}Cu(II)$ glyoxalase I and its complexes: (a) $^{63}Cu(II)$ glyoxalase I in 50 mM Tris-HCl (pH 7.8) (0.8 mM binding sites); (b) $^{63}Cu(II)$ glyoxalase I plus 5 mM glutathione in 50 mM Tris-HCl (pH 7.8); (c) $^{63}Cu(II)$ glyoxalase I plus 5 mM *S*-(D-lactoyl)glutathione in 200 mM Tris-HCl (pH 7.8); (d) $^{63}Cu(II)$ glyoxalase I plus 1 mM *S*-(*p*-bromobenzyl)glutathione in 50 mM Tris-HCl (pH 7.8). (e) Control experiment: EPR spectrum of $^{63}CuCl_2$ (0.5 mM) in 12 mM Tris-HCl (pH 7.8) and 5 mM NaCl. Spectrometer gain increased 2 times as compared to spectra a–d. Microwave frequency was 9.11 GHz.

tathione, *S*-(D-lactoyl)glutathione, and *S*-(*p*-bromobenzyl)-glutathione are shown in Figures 2a–d and 3a–d. The complexes of $^{63}Cu(II)$ glyoxalase I with *S*-(*p*-bromobenzyl)glutathione and glutathione have the following parameters: $g_{\parallel} = 2.34$, $g_{\perp} = 2.09$, and $A_{\parallel} = 14.2$ mT.

The peaks at 0.263 T in Figure 3a,c,d are due to extraneous copper, which remained even after extensive dialysis (72 h). This $^{63}Cu(II)$ signal is probably mainly due to a Tris complex, since a solution containing $^{63}Cu^{2+}$ and Tris buffer alone gave peaks (Figures 2e and 3e) with the same spectral characteristics ($g_{\parallel} = 2.25$; $A_{\parallel} = 17.9$ mT). Since this copper complex was not easily removed by extended dialysis, the enzyme may also take some part in binding of the extraneous copper.

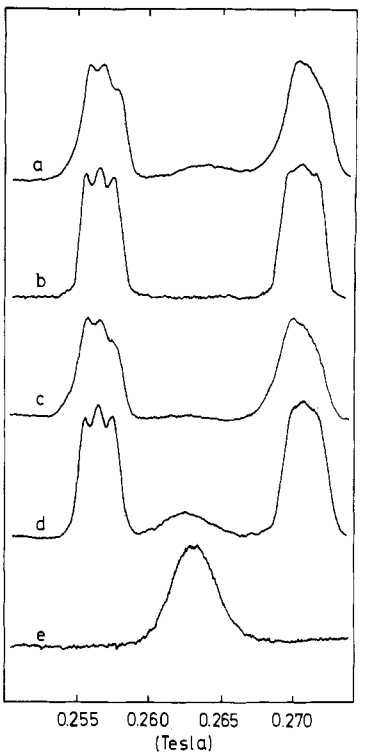


FIGURE 3: Expansion of the low-field hyperfine lines for the EPR spectra in Figure 2. Microwave power as well as gain was increased 5-fold as compared to spectra in Figure 2.

Dialysis of the Cu(II)–Tris solution in the absence of enzyme demonstrated a fast removal of $Cu^{2+}$ (half-life ca. 2 h). When the competitive inhibitor *S*-(*p*-bromobenzyl)glutathione was added, a spectrum only slightly different was obtained. The lowest hyperfine line of the $^{63}Cu(II)$–Tris spectrum is located between the two low-field hyperfine lines of $^{63}Cu(II)$ glyoxalase I and its complex with *S*-(*p*-bromobenzyl)glutathione. Consequently, the extraneous copper causes no major problem in the interpretation of the spectra of the enzyme and its complexes.

Addition of glutathione to $^{63}Cu(II)$ glyoxalase I completely extinguished the copper signal from extraneous copper (Figures 2b and 3b), probably due to chelate formation. It has been reported that glutathione forms a binuclear Cu(II) complex at a pH value below about 9 without any detectable EPR spectrum (Jezowska-Trzebiatowska et al., 1977; Kroneck, 1975).

When Hepes buffer was used for the dialysis of $^{63}Cu(II)$ glyoxalase I, extraneous copper was barely detectable. However, addition of *S*-(*p*-bromobenzyl)glutathione to the sample showed that $^{63}Cu(II)$ glyoxalase I in fact contained extraneous copper. The concentration was about the same as when Tris was used as buffer. The $^{63}Cu(II)$–Hepes complex is evidently not easily detectable with EPR.

An estimation of the amount of extraneous copper was obtained by spectral subtraction of an appropriate portion of the EPR spectrum of the $^{63}Cu(II)$–Tris complex from the spectrum of $^{63}Cu(II)$ glyoxalase I. It was estimated that approximately 15% of the total EPR-detectable signal was due to extraneous Cu. A stoichiometry of approximately 0.8 $Cu^{2+}$ bound per enzyme subunit was obtained from the measured total copper concentration after correction for the extraneous copper. Independent analysis of the total copper content by atomic absorption spectrometry gave 0.9 Cu atom per protein subunit, demonstrating that all copper was accounted for by the EPR signal. Consequently, the possibility of bias in interpretation of the EPR data caused by the presence of

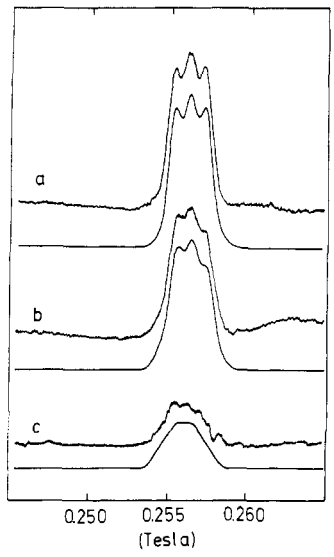


FIGURE 4: Experimental and simulated EPR spectra of the copper hyperfine lines at the lowest field for $^{63}$Cu(II) glyoxalase I isoenzyme 1 (0.75 mM binding sites): (a) $^{63}$Cu(II) glyoxalase I complexed with *S*-(*p*-bromobenzyl)glutathione in 10 mM Tris-HCl (pH 7.8); (b) pure enzyme in 10 mM Tris-HCl (pH 7.8); (c) difference between spectrum b and 60% of spectrum a. Other conditions were the same as in Figure 2. The lower curves were simulated as described in the text.

EPR-silent $Cu^{2+}$ can be ruled out.

*Analysis of the EPR Spectra.* The low-field $Cu^{2+}$ hyperfine lines in the $g_\parallel$ region of the spectra of Cu(II) glyoxalase I interacting with *S*-(*p*-bromobenzyl)glutathione, as well as with glutathione, show a distinct symmetric triplet superhyperfine splitting (Figure 3b,d). The $g_\perp$ region displays at least six unresolved components (Figure 2b,d). Also the EPR spectra of Cu(II) glyoxalase I itself, as well as the complex with *S*-(D-lactoyl)glutathione, have a triplet splitting of the lowest field hyperfine line. However, the lines are broader and asymmetric in those cases (Figure 3a,c). Moreover, there is less structure in the $g_\perp$ region (Figure 2a,c).

The results shown in Figures 2 and 3 were obtained with a mixture of the three isoenzymes of glyoxalase I, since all known data indicate that they are functionally identical (Aronsson et al., 1981). To find out whether the asymmetry of the $^{63}$Cu(II) glyoxalase I spectrum might be due to a difference in the spectral properties of the two distinct subunits of the enzyme, a study of the isolated homodimeric isoenzymes 1 and 3 was undertaken. Essentially the same results were obtained with the homodimers as with the mixture of isoenzymes.

Titration of $^{63}$Cu(II) glyoxalase I by *S*-(*p*-bromobenzyl)-glutathione gradually converted the spectrum containing the skew peaks into the simpler one of the inhibitor complex. Only a stoichiometric amount of *S*-(*p*-bromobenzyl)glutathione was needed to reach the end point. This demonstrates a strong binding of *S*-(*p*-bromobenzyl)glutathione to Cu(II) glyoxalase I, comparable to its binding to native Zn(II) glyoxalase I (Aronsson et al., 1981). Addition of an amount of *S*-(D-lactoyl)glutathione equivalent to the glutathione concentration in a $^{63}$Cu(II) glyoxalase I sample gave a mixed spectrum, indicating that the binding constants for the latter two components are similar.

*Simulation of the Low-Field Spectral Region.* Spectra for the isoenzyme 1 (Figure 4b) and for its complex with *S*-(*p*-bromobenzyl)glutathione (Figure 4a) were simulated. Special attention was paid to the low-field singularity assuming a superhyperfine coupling to one nitrogen ($I = 1$). A reasonable fit of the lowest field line of Cu(II) glyoxalase I complexed with *S*-(*p*-bromobenzyl)glutathione was obtained with $A_\parallel^N$ = 0.93 mT and $\Delta H_{1/2}$ = 0.90 mT. A good fit in this particular region was also obtained when the total Cu(II) spectrum was simulated with the same parameters. However, some center-peak intensity as well as the intensity of the broad base, as observed for the experimental spectrum (cf. Figure 4a), could not be accounted for in the above-mentioned simulation. These minor features could be modeled by a 20% contribution of an additional spectrum characterized by two equivalent nitrogen ligands with identical parameters, $A_\parallel^N$ = 0.93 mT and $\Delta H_{1/2}$ = 0.90 mT. Alternative models were also tested by using different coupling constants of the two nitrogen nuclei as well as only one nitrogen nuclei with a coupling constant smaller than 0.93 mT. However, those alternative simulated spectra tested were inferior. In the spectrum of Figure 4a, the major spectral component is considered to correspond to a single molecular structure. The experimental data do not permit a definitive conclusion about the origin of the additional minor contribution, and the following discussion is restricted to the dominating component.

The skew EPR spectrum of Cu(II) glyoxalase I, as well as that of its complex with *S*-(D-lactoyl)glutathione, can be interpreted as the sum of two different spectral components (Figure 4b). When 60% of the spectrum of the *S*-(*p*-bromobenzyl)glutathione complex (Figure 4a) is subtracted, a symmetrical difference spectrum is obtained (Figure 4c). Compared to the spectrum of $^{63}$Cu(II) glyoxalase I plus *S*-(*p*-bromobenzyl)glutathione, the difference spectrum exhibits a similar $g_\parallel$ value (2.35). However, the value $A_\parallel$ = 13.3 mT (Figure 4c) is lower than the value $A_\parallel$ = 14.2 mT for the inhibitor complex (Figure 4a). The subtraction involved the two low-field lines in the $g_\parallel$ region of the two spectra, which made a calculation of $g_\parallel$ and $A_\parallel$ of the resulting difference spectrum possible. No resolved structure could be detected in the low-field difference spectrum (Figure 4c). This spectrum was simulated by assuming various nitrogen–Cu(II) interactions in the protein. The best fit was obtained with two nitrogen ligands with different coupling constants. Figure 4c presents a simulated spectrum with the coupling constants 0.93 and 0.60 mT ($\Delta H_{1/2}$ = 0.90 mT). When simulated spectra in Figure 4a,c were added in the proportions arrived at in the above analyses, a good correspondence to the Cu(II) glyoxalase spectrum was obtained (Figure 4b).

## Discussion

$Cu^{2+}$ binds strongly to the active site of glyoxalase I. The metal dissociation constant for Cu(II) glyoxalase I is lower than that for Zn(II) glyoxalase I, in analogy with the values of dissociation constants for smaller molecules, such as complexes of Cu(II) and Zn(II) with nitrilotriacetic acid (Anderegg, 1982).

The EPR parameters for the extraneous $Cu^{2+}$ are quite similar to those for copper ions bound nonspecifically to hemoglobin and myoglobin ($g_\parallel$ = 2.22, $g_\perp$ = 2.03, and $A_\parallel$ = 16.7 mT) (Bemski et al., 1969), suggesting that the $^{63}$Cu-(II)–Tris complex is bound to glyoxalase I. Since the amount of the complex is not stoichiometric to the subunit content, the binding may be to denatured protein. An observation supporting this hypothesis is that the total concentration of copper, including extraneous copper, is equimolar to the total concentration of protein subunits.

The EPR parameters of the $^{63}$Cu(II) glyoxalase I complexes with *S*-(*p*-bromobenzyl)glutathione or glutathione are consistent with an elongated tetragonal structure with four ligands in an equatorial plane and two ligands in axial positions situated further away from $Cu^{2+}$ (Attanasio et al., 1974). Cu(II)

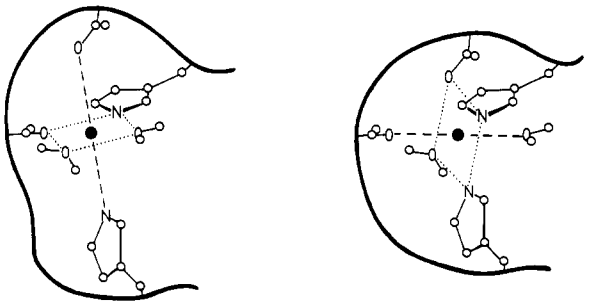


FIGURE 5: Hypothetical conformational states of glyoxalase I, with two different octahedral coordinations of the metal ion in the active site. Conformations A and B correspond to the EPR spectra of parts a and c of Figure 4, respectively. The ligands are pictured as two imidazoles, two carboxylate groups, and two water molecules.

glyoxalase I might be referred to as a so-called type 2 copper complex, designated by Vänngård (1972). These complexes are faintly colored, have $A_{\parallel}$ larger than 13 mT, and are frequent among simple copper complexes. Basically, the EPR spectrum of $^{63}$Cu(II) glyoxalase I interacting with *S*-(*p*-bromobenzyl)glutathione, as well as with glutathione, has a 1:1:1 superhyperfine splitting, indicative of a single nitrogen ligand. The values of $g_{\parallel}$ and $A_{\parallel}$ are in the same range as those of model complexes with one nitrogen and three oxygens as equatorial ligands (Peisach & Blumberg, 1974).

The interpretation of the EPR spectra of Cu(II) glyoxalase I complexed with glutathione or *S*-(*p*-bromobenzyl)glutathione is in agreement with the findings from the studies of the visible light absorption and the EPR spectrum of Co(II) glyoxalase I (Sellin et al., 1983b), as well as the X-ray absorption analysis of Zn(II) glyoxalase I (Garcia-Iniguez et al., 1984). Hexacoordination involving four oxygen and two nitrogen atoms as ligands to the metal is suggested. The axial ligands in the Cu(II) complex would then be one oxygen and one nitrogen. Only the ligands in the equatorial plane, and not the axial ligands, are expected to give rise to coupling with $Cu^{2+}$ (Vänngård, 1972). A hypothetical structure for the metal coordination site of Cu(II) glyoxalase in the presence of glutathione or *S*-(*p*-bromobenzyl)glutathione is given in Figure 5. Two of the ligands have been identified as water molecules by NMR relaxation studies with Mn(II)-substituted glyoxalase I (Sellin et al., 1982a). At least one nitrogen was revealed by the present study, in agreement with the proposal of two imidazole ligands based on the X-ray absorption studies (Garcia-Iniguez et al., 1984). The two additional oxygen ligands have been assumed to be provided by glutamate or aspartate residues (Sellin & Mannervik, 1984). The EPR spectrum of the $^{63}$Cu glyoxalase I–inhibitor complex is strongly reminiscent of that reported (Attanasio et al., 1974) for the model complex formed by one $^{63}Cu^{2+}$ and two molecules each of 3,3-dimethyl-1-nitrobutan-2-one and 4-methylpyridine as ligands ($g_{\parallel}$ = 2.335, $g_{\perp}$ = 2.095, $A_{\parallel}$ = 12.2 mT, and $A_{\parallel}^{N}$ = 0.8 mT). In both the enzyme and the model complex, coupling to a single nitrogen nucleus was evidenced by a superhyperfine triplet splitting. This particular Cu(II) complex has been suggested to be either a six-coordinate complex with one nitrogen and three oxygen ligands in a plane, and one nitrogen and one oxygen ligand out of the plane, or a five-coordinate complex (approximately square pyrimidal) with a nitrogen atom in the basal plane.

The EPR spectrum of uncomplexed $^{63}$Cu(II) glyoxalase I (Figure 4b) implies that $Cu^{2+}$ is bound at a site that can exist in two (or more) interchangeable conformations. Addition of *S*-(*p*-bromobenzyl)glutathione or glutathione, but not of *S*-(D-lactoyl)glutathione, shifts the equilibrium between these conformations toward a conformation (Figure 5A) with one EPR-active nitrogen ligand in the equatorial plane (corresponding to the spectrum in Figure 4a). The difference in EPR parameters of the spectra in Figure 4 indicates a difference in geometry of the Cu(II) binding site in the two conformations of the protein. The simulation studies suggest that the spectrum in Figure 4c corresponds to a structure in which $Cu^{2+}$ is surrounded by two nitrogen and two oxygen ligands in the equatorial plane and two oxygen ligands in the axial positions (Figure 5B). This interpretation is in agreement with the findings of X-ray absorption studies, where two imidazole ligands are suggested to change position when *S*-(*p*-bromobenzyl)glutathione is bound to the enzyme (Garcia-Iniguez et al., 1984). It is of interest to note the similarity between the EPR parameters of the difference spectrum (Figure 4c) and those reported for Cu(II) carboxypeptidase A ($g_{\parallel}$ = 2.327 and $A_{\parallel}$ = 12.4 mT) (Rosenberg et al., 1975) as well as for human $^{63}$Cu(II) carbonic anhydrase C ($g_{\parallel}$ = 2.305 and $A_{\parallel}$ = 12.8 mT) (Taylor & Coleman, 1973). In carboxypeptidase A there are two nitrogen ligands binding the metal in addition to the oxygen ligands. In carbonic anhydrase A there are three nitrogen ligands, of which only two give rise to a superhyperfine structure.

It has been demonstrated that imidazole must be present together with EDTA in the buffer for an effective removal of the metal ion from glyoxalase I (Uotila & Koivusalo, 1975). Replacement of a protein imidazole ligand by an imidazole molecule from the buffer may explain this finding. An interchange between two protein conformations, where one imidazole ligand is more distant from the metal ion in one conformation, may facilitate the metal exchange (Figure 5).

The effects of glutathione, *S*-(D-lactoyl)glutathione, and *S*-(*p*-bromobenzyl)glutathione on the EPR spectrum of $^{63}$Cu(II) glyoxalase I show similarities with those monitored by fluorescence studies of glyoxalase I. The fluorescence of tryptophan in glyoxalase I is partially quenched by addition of glutathione, *S*-(*p*-bromobenzyl)glutathione, and other glutathione derivatives, probably due to a conformational change of the enzyme upon binding of these substances (Aronsson et al., 1981). *S*-(D-Lactoyl)glutathione does not quench the fluorescence. Since the substrate for the enzyme is a derivative of glutathione, this conformational change is assumed to place the reactive groups of the active site in their correct positions for catalysis to occur. In the catalytic mechanism there is probably another conformational change that reverses the change and facilitates product release. This dynamic model is supported by the results of the EPR studies.

A catalytic mechanism has been suggested for glyoxalase I (Sellin et al., 1982a,b) involving an enediol-proton transfer (Chari & Kozarich, 1981). A base is required for the transfer of the proton. This base has not yet been identified. The position of the apical imidazole group of histidine in the active conformation of the enzyme (Figure 5A) is compatible with an involvement of this group as a base in the enzymatic process. Model building, using distances from NMR studies of glutathione derivatives (Sellin et al., 1982b; Rosevear et al., 1983, 1984), shows that this is sterically possible.

Cu(II) glyoxalase I is catalytically inactive like most Cu(II)-substituted Zn(II) enzymes (Vallee & Geldes, 1984), which raises the question of the relevance of using Cu(II) glyoxalase I to probe the structure of the native enzyme. However, the conformational transitions in the protein structure, induced by glutathione and some glutathione derivatives, seem to be independent of the particular metal

present in the active center (Aronsson et al., 1981). This finding implies essential structural similarities between the different metal derivatives of glyoxalase I. Comparisons can be made with the extensive structural similarities between the largely inactive Cd(II) and the native Zn(II) alcohol dehydrogenases demonstrated by X-ray crystallography (Schneider et al., 1985).

In conclusion, the present study has demonstrated the presence of at least two conformational forms of glyoxalase I, with at least one nitrogen as a metal ligand in the Cu(II)-substituted enzyme. NMR studies may reveal the nature of this chemical group. The proposed second nitrogen ligand (histidine) may be detectable by electron nuclear double resonance (ENDOR) spectroscopy of the Cu(II) enzyme.

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## REFERENCES

Anderegg, G. (1982) *Pure Appl. Chem. 54*, 2693–2758.

Aronsson, A.-C., Marmstål, E., & Mannervik, B. (1978) *Biochem. Biophys. Res. Commun. 81*, 1235–1240.

Aronsson, A.-C., Tibbelin, G., & Mannervik, B. (1979) *Anal. Biochem. 92*, 390–393.

Aronsson, A.-C., Sellin, S., Tibbelin, G., & Mannervik, B. (1981) *Biochem. J. 197*, 67–75.

Attanasio, D., Collamati, I., & Ercolani, C. (1974) *J. Chem. Soc., Dalton Trans.*, 1319–1324.

Ball, J. C., & Vander Jagt, D. L. (1979) *Anal. Biochem. 98*, 472–477.

Bemski, G., Arends, T., & Blanc, G. (1969) *Biochem. Biophys. Res. Commun. 35*, 599–605.

Chari, R. V. J., & Kozarich, J. W. (1981) *J. Biol. Chem. 256*, 9785–9788.

Garcia-Iniguez, L., Powers, L., Chance, B., Sellin, S., Mannervik, B., & Mildvan, A. S. (1984) *Biochemistry 23*, 685–689.

Han, L.-P. B., Schimandle, C. M., Davison, L. M., & Vander Jagt, D. L. (1977) *Biochemistry 16*, 5478–5484.

Jezowska-Trzebiatowska, B., Formicka-Kozlowska, G., & Kozlowski, H. (1977) *J. Inorg. Nucl. Chem. 39*, 1265–1268.

Kroneck, P. (1975) *J. Am. Chem. Soc. 97*, 2839–2841.

Lefebvre, R., & Maruani, J. (1965) *J. Chem. Phys. 42*, 1480–1502.

Mannervik, B. (1980) in *Enzymatic Basis of Detoxication* (Jakoby, W. B., Ed.) Vol. 2, pp 263–273, Academic, New York.

Marmstål, E., Aronsson, A.-C., & Mannervik, B. (1979) *Biochem. J. 183*, 23–30.

Marnett, L. J., Hurd, H. K., Hollstein, M. C., Levin, D. E., Esterbauer, H., & Ames, B. N. (1985) *Mutat. Res. 148*, 25–34.

Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691–708.

Ray, W. J., Jr. (1969) *J. Biol. Chem. 244*, 3740–3747.

Rosenberg, R. C., Root, C. A., Bernstein, P. K., & Gray, H. B. (1975) *J. Am. Chem. Soc. 97*, 2092–2096.

Rosevear, P. R., Chari, R. V. J., Kozarich, J. W., Sellin, S., Mannervik, B., & Mildvan, A. S. (1983) *J. Biol. Chem. 258*, 6823–6826.

Rosevear, P. R., Sellin, S., Mannervik, B., Kuntz, I. D., & Mildvan, A. S. (1984) *J. Biol. Chem. 259*, 11436–11447.

Schneider, G., Cedergren-Zeppezauer, E., Knight, S., Eklund, H., & Zeppezauer, M. (1985) *Biochemistry 24*, 7503–7510.

Sellin, S., & Mannervik, B. (1984) *J. Biol. Chem. 259*, 11426–11429.

Sellin, S., Aronsson, A.-C., & Mannervik, B. (1980) *Acta Chem. Scand., Ser. B B34*, 541–543.

Sellin, S., Eriksson, L. E. G., & Mannervik, B. (1982a) *Biochemistry 21*, 4850–4857.

Sellin, S., Rosevear, P. R., Mannervik, B., & Mildvan, A. S. (1982b) *J. Biol. Chem. 257*, 10023–10029.

Sellin, S., Aronsson, A.-C., Eriksson, L. E. G., Larsen, K., Tibbelin, G., & Mannervik, B. (1983a) in *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects* (Larsson, A., Orrenius, S., Holmgren, A., & Mannervik, B., Eds.) pp 187–197, Raven, New York.

Sellin, S., Eriksson, L. E. G., Aronsson, A.-C., & Mannervik, B. (1983b) *J. Biol. Chem. 258*, 2091–2093.

Taylor, J. S., & Coleman, J. E. (1973) *J. Biol. Chem. 248*, 749–755.

Uotila, L., & Koivusalo, M. (1975) *Eur. J. Biochem. 52*, 493–503.

Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283–430.

Vänngård, T. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R., & Borg, D. C., Eds.) pp 411–447, Wiley, New York.

Vince, R., Daluge, S., & Wadd, W. B. (1971) *J. Med. Chem. 14*, 402–404.