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Copper–glutathione complexes under physiological conditions: structures in solution different from the solid state coordination

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The physiologically important copper complexes of oxidized glutathione have been examined by electron spin resonance (ESR) spectroscopy in aqueous solution at neutral pH. Low temperature measurements show that the Cu(II) binding site in oxidized glutathione has the same ligand arrangement as in the copper complexes of *S*-methylglutathione, glutamine, glutamate and glycine. The site is composed of the amino nitrogens and the carboxyl oxygens of two γ-glutamyl residues; there is no interaction with amide nitrogens, the sulphur bond or the glycyl carboxyl groups. At high metal to ligand ratios a binuclear species exists, in which each Cu(II) binds only to one γ-glutamyl residue. The previously reported forbidden transition detected at $g = 4$ is due to non-specific aggregation and not to spin coupling of intramolecular sites. Liquid solution ESR spectra show the Cu(II)–glutathione complex has a lower mobility than the corresponding Cu(II)–*S*-methylglutathione species. From the degree of spectral anisotropy the complex with glutathione is calculated to exist as a dimer. These results demonstrate that the physiologically relevant complex between copper and oxidized glutathione in solution is completely different from the known solid state structure determined by crystallography.

Keywords: copper complexes, copper transport, ESR spectroscopy, glutathione

Introduction

The tripeptide thiol glutathione (GSH) has an essential role in the cellular processes controlling the level of heavy metals. Direct interaction between GSH and metal ions can take place by two different mechanisms: the first is one-electron reduction of the metal with concomitant oxidation of GSH to GSSG; the second is chelation of metals by either GSH or GSSG, with the formation of a complex that allows transport of the metal in a controlled manner (Rabenstein 1989, Ballatori 1991). Much interest has been dedicated to studies of copper GSH complexes in biological systems; the reactivity of Cu(II) is known to be connected with GSH oxidation (Steinkühler *et al.* 1988, 1990, 1991), and an inhibitor of both *N*-methyltransferase and opiate receptor binding was identified as a Cu(II)–GSSG complex (Marzullo & Friedhoff 1977). Recently it has been shown that Cu(I)–GSH complexes serve in the intracellular transport and incorporation of copper into metallothionein (Freedman *et al.* 1989, Freedman & Peisach 1989), superoxide dismutase (Ciriolo *et al.* 1990) and hemocyanin (Brouwer & Brouwer-Hoexum 1992).

Because of this biological importance, the chemical properties of the complexes formed between copper and GSH have been studied extensively. Chelate formation involving GSH has turned out to be difficult to characterize due to the redox activity of the thiol group, but Cu(II)–GSSG complexes are stable and suitable for spectroscopic analysis. It is therefore somewhat surprising that considerable confusion exists as to the structure and stoichiometry of such complexes.

The first model of a Cu(II) GSSG structure was presented by Kroneck (1975), who proposed a binuclear complex, formed at alkaline pH, with each Cu(II) bonded to five donor atoms from one GS moiety (two deprotonated amide nitrogens, the glutamyl carboxyl oxygen and amine nitrogen, the glycyl carboxyl oxygen) and the last apical coordinate being the distant sulphur of the disulphide bond. Crystals of a similar complex, but with a completely different ESR spectrum, were isolated by Miyoshi *et al.* (1980); the

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X-ray crystallography revealed a slightly different square pyramidal Cu(II) site, with the near sulphur acting as the axial ligand and without any bonding to the glycine carboxylate. This structure was suggested to exist also at physiological pH, on the basis of spectroscopic evidence and model studies (Miyoshi *et al.* 1983a,b).

However, different findings have been reported by other groups. Formicka-Kozlowska *et al.* concluded that initially a complex between Cu^{2+} and the amine and carboxyl groups of the glutamate residue was formed, which subsequently changed into a binuclear species containing a Cu–S–S–Cu unit, at all Cu:GSH ratios tested (Formicka-Kozlowska *et al.* 1979). In contrast, Jouini and co-workers detected a variety of differently protonated complexes at low pH values (Abello *et al.* 1980, Huet *et al.* 1984), and suggested that the dominant stable form at physiological pH was a 1:1 Cu:GSSG species, with the metal bound to the amine and carboxyl groups of the two terminal glutamate residues in a symmetrical square-planar coordination structure (Huet *et al.* 1984). This model was based on comparison with the spectroscopic characteristics of similar copper complexes, in particular the resemblance with the Cu(II)–glycine chelation mode, although direct evidence was not available due to the lack of nitrogen hyperfine structure in their electron spin resonance (ESR) spectra. Indirect support for the 1:1 complex has come from potentiometric titration (Micheloni *et al.* 1978) and NMR studies of the corresponding Zn–GSSG complexes (Postal *et al.* 1985), but these two methods have also led to unexpected observations, such as the presence of an otherwise unknown Cu–$GSSG_2$ species (Micheloni *et al.* 1978, Abello *et al.* 1980, Huet *et al.* 1984) and the formation of a particular binuclear complex at physiological pH (Postal *et al.* 1985).

Normally NMR is the method of choice for characterization of small structures in solution, but with paramagnetic complexes this technique is problematic. In the present work we have taken advantage of the improvements of recent years in the sensitivity and resolution of ESR to clarify the ambiguous nature of Cu–GSSG complexes. We have determined the ligand arrangement through the nitrogen hyperfine splitting pattern and report evidence on the metal:GSH stoichiometries occurring under physiological conditions.

Materials and methods

GSH and *S*-methylglutathione (GSMe) were purchased from Sigma (St Louis, MO) and used without further purification; all other chemicals were from Merck (Darmstadt, Germany). Samples were prepared in a standard physiological buffer containing 10 mM phosphate, 127 mM NaCl and 2.7 mM KCl at pH 7.4. Control measurements were made in 10 mM HEPES to exclude specific effects of the buffer. The Cu^{2+} concentration was 0.5 mM unless otherwise stated, measurements were normally made immediately after preparation of complexes from $CuSO_4$ and Na_2GSSG stock solutions.

ESR studies were carried out using a Bruker ESP300 instrument operating at X-band frequencies, equipped with an ER4111VT variable temperature unit and a computer-based acquisition system. A standard TE_{102} cavity with a Dewar insert was used for low temperature measurements with samples in 3 mm quartz ESR tubes, whereas room temperature experiments were made with a high sensitivity TM_{110} mode cavity with samples in flat glass capillaries (Pedersen & Cox 1988). Scans of 1000 or 2000 G were recorded using 20 mW microwave power, 10 G modulation at 100 kHz, a time constant of 20 ms and a scan time of 21 s. Normally eight scans were accumulated to improve the signal to noise ratio. High resolution measurements of the hyperfine splitting in the $g_\perp$ region were done with 2 mW power and 4 G modulation, accumulating 100–200 scans; no improvement in resolution was obtained using lower power or modulation levels. Superhyperfine splitting in the $g_\parallel$ region could not be detected. A crystal of 1,1-diphenyl-2-picrylhydrazyl was used as a marker for g determinations. The interaction energy was calculated according to Peisach & Blumberg (1974).

Optical spectra were recorded with a Perkin-Elmer Lambda-9 spectrophotometer.

Results and discussion

The structure of the mononuclear complex

The ESR spectrum of the 1:1 Cu–GSSG complex at physiological pH shows a single species with spectral parameters $g_\parallel = 2.251$, $g_\perp = 2.055$ and $A_\parallel = 178$ G (Figure 1). The g determinations closely match the previously published values, apart from the usual uncertainty in the measurement of the third decimal (Table 1). In contrast, the reported hyperfine splitting constants differ widely; we ascribe this variation to the fact that it has been difficult to determine

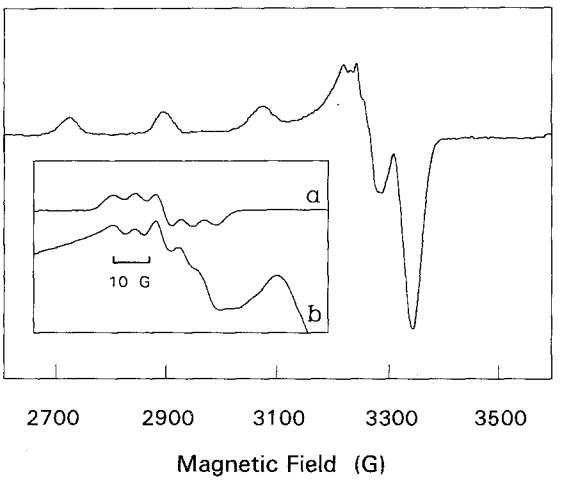


Figure 1. ESR spectrum of the equimolar Cu(II)–GSSG complex in phosphate buffer at pH 7.4, measured at 100 K. The inset shows the nitrogen hyperfine splitting region (3150–3250 G) under high resolution conditions (**b**) and the simulated hyperfine pattern (**a**).

Table 1. A comparison of reported ESR parameters for the 1:1 Cu–GSSG complex at non-extreme pH values

pH	$g_{\|}$	$g_{\perp}$	$A_{\|}$ (cm^{-1})	Reference
8.0	2.256	2.055	0.0172[a]	Miyoshi *et al.* 1983b
9.5	2.258	2.059	0.0174[a]	Miyoshi *et al.* 1983b
7.4	2.250	2.065	0.0192	Formicka-Kozlowska *et al.* 1979
8.4	2.247	2.064	0.0191	Formicka-Kozlowska *et al.* 1979
8.8	2.249	2.065	0.0191	Formicka-Kozlowska *et al.* 1979
4.6–8.0	2.248	2.059	0.0187[a]	Huet *et al.* 1984
7.0	2.250	2.055	0.0187	Postal *et al.* 1985
7.4, 7.6	2.251	2.055	0.0187	this work

[a] Interaction energy calculated from values reported in Gauss.

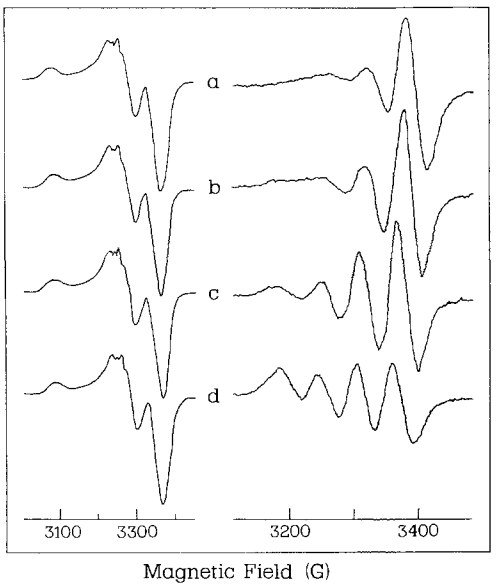


Figure 2. ESR spectra of Cu–GSSG (**a**), Cu–$(GSMe)_2$ (**b**), Cu–$(glu)_2$ (**c**) and Cu–$(gly)_2$ (**d**); measured at 100 K (left) and room temperature (right). Only the high field part of the low temperature spectra is shown. The spectrum of Cu–$(gln)_2$ is practically identical to spectrum (**c**).

this parameter exactly without the use of computerized data handling systems.

Secondary hyperfine structure in the ESR spectrum has previously been observed, but not well enough resolved to permit analysis (Postal *et al.* 1985). High resolution measurements of the $g_{\perp}$ region reveal a distinct splitting pattern (Figure 1, inset), which nevertheless does not immediately unveil the nature of the ligands. In fact, the weak secondary splittings are superimposed on the converging lines of the $g_{\perp}$ region and are further obfuscated by the different contributions of the two copper isotopes present. A very similar hyperfine pattern can be simulated by a mononuclear copper site containing two equivalent nitrogens, $A_1^N = A_2^N = 11.9$ G, with a linewidth of 13.5 G (Figure 1, inset); however, this resemblance might be fortuitous. The $g_{\|}$ absorptions do not yield any hyperfine structure even under high resolution conditions (not shown) and so cannot provide direct evidence for a binding site with two symmetrical nitrogen ligands.

An unambiguous demonstration of the ligand arrangement is instead achieved through the comparison of the ESR spectrum of Cu–CSSG with spectra of the 1:2 metal:ligand complexes formed between copper and GSMe, glutamine, glutamate and glycine (Figure 2). At room temperature the degree of spectral anisotropy varies, reflecting the different mobilities of the complexes. However, in the frozen state all of these complexes show the same spectrum with identical g and A parameters, and they all display similar nitrogen hyperfine splitting patterns in the $g_{\perp}$ region. This result shows that the coordination sphere of Cu(II) in the Cu–GSSG complex is exactly the same as in the simple copper–bisglycine complex, and eliminates the possibility of any significant apical binding to sulphur or amide groups. It can be concluded that under physiological conditions Cu^{2+} is ligated to the amino nitrogens and the carboxyl oxygens of two glutamyl residues (Zand & Palmer 1967, Crawford & Dalton 1969), in analogy with the results found for the Zn–GSSG complex (Postal *et al.* 1985) and in agreement with the model suggested by Huet *et al.* (1984). Curiously, this model was originally based on the absence of superhyperfine structure, which was believed to be consistent with glycine-like chelation.

The 2N–2O coordination system gives rise to the possibility of two different ligand configurations, *cis* (N,N,O,O) and *trans* (N,O,N,O). Many copper(II) bis(amino acid) complexes are known to exist in solution as a mixture of both isomers; they have slightly different tensors and g values, which contribute to the complexity of the nitrogen superhyperfine structure pattern (Goodman *et al.* 1981, Goodman & McPhail 1985). Isomerization is probably very rapid for small complexes in solution since the energy barrier is low (Delf *et al.* 1979), but this may not be the case for Cu–GSSG where the glutamyls are not free to move independently. Goodman and co-workers were able to detect *cis* and *trans* components of the high field peak in isotropic spectra of amino acid copper complexes, using isotopically pure Cu^{2+} in deuterated solvent (Goodman *et al.* 1981, Goodman & McPhail 1985). This procedure turned out not to be successful with Cu–GSSG (not shown), probably because the solution spectrum is very anisotropic (Figure 2).

The question of the second binding site

The binuclear model put forward by Kroneck (1975) suggested interaction between the two metal sites to account for the observed ESR spectra. The subsequent crystallization of a Cu–GSH complex at alkaline pH indeed revealed a binuclear structure, but the ESR spectrum was different from those previously reported and did not show any sign of relaxation broadening (Miyoshi *et al.* 1980).

We have examined the existence of a second binding site using low ligand concentrations. At Cu^{2+} to GSSG ratios

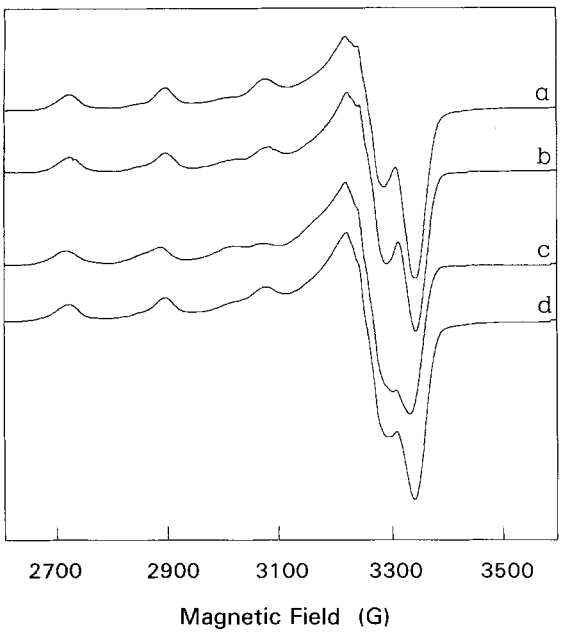


Figure 3. Low temperature spectra of complexes formed at higher metal to ligand ratios. The Cu(II) concentration was fixed at 0.5 mM, the ligand concentration was varied to obtain the following ratios: Cu–GSSG 2:1 (**a**); Cu–gly 1:1 (**b**); Cu–GSSG 4:1 (**c**) and Cu–gly 2:1 (**d**).

of 2 or higher, a second species appears in the ESR spectrum (Figure 3a). This species is characterized by a $g_{\|}=2.289$ and $A_{\|}=154$ G, corresponding to a interaction energy of 0.0165 cm^{-1}. Such g and A values are consistent with a binding site with a single nitrogen and three oxygen ligands (Peisach & Blumberg 1974). The same spectral component is found with glycine (Figure 3b) as well as with glutamate, glutamine and GSMe (spectra not shown) when copper is added in excess of the ligand concentration, indicating the presence of 1:1 complexes. The formation of the 1:1 Cu–glycine complex immediately allows identification of the ligands of the new species as one carboxyl and one amino group plus two equatorial and two axial water molecules. For GSH this means that in the Cu_2 complex each copper is bound to a single glutamate moiety and does not interact with other parts of the molecule.

The appearance of the second species in the spectrum with increasing copper levels is accompanied by a gradual loss of the nitrogen hyperfine structure in the $g_{\perp}$ region, eventually leading to the formation of a featureless strongly broadened spectrum (Figure 3c), identical to the one observed originally by Kroneck (1975). However, this is not due to intramolecular interactions in a binuclear complex, as shown by the fact that the same spectral changes are observed for the Cu–glycine complex (Figure 3d). Instead this phenomenon can be ascribed to the formation of microaggregates, giving rise to the slow formation of a precipitate. The formation of aggregates accelerates when the concentration of glutathione or amino acids is increased above 1 mM. This explains why the second spectral component has not been observed in previous studies, where the glutathione concentrations were always in the millimolar range, and even up to 50 mM for NMR measurements (Postal *et al.* 1985). The precipitation and isolation of a Cu_2–GSSG solid has been described, but the $g_{\|}=2.258$ is very different from that of the second species observed here; the $A_{\|}$ was not reported (Postal *et al.* 1985).

The appearance of an ESR signal near $g=4$ due to the forbidden $\Delta m=2$ transition in a spin coupled Cu_2 system has been mentioned by several authors, although spectra have never been shown (Kroneck 1975, Formicka-Kozlowska *et al.* 1979, Postal *et al.* 1985). We do not see this signal even at high copper to ligand ratios, as long as the samples are completely transparent (Figure 4a and b). When higher GSH concentrations are used the formation of insoluble aggregates make the samples cloudy; only in such samples can the $g=4$ transition be observed (Figure 4c). Under similar experimental conditions this signal can also be seen when copper is ligated to glycine or GSMe, indicating that it is not indicative of specific spin coupling of intramolecular sites (Figure 4d). In weakly buffered samples the addition of high concentrations of Cu^{2+} easily resulted in a lowered pH and under these conditions a much higher signal was found at $g=4$.

Aggregation is seen in both buffer systems tested in this study. At all copper to glutathione ratios tested, only the signal of the 1:1 Cu–GSSG species remains when the sample supernatants are measured after 24 h (data not shown). The aggregation process can probably also explain the time-dependent colour variations described for samples containing 15 mM Cu^{2+} (Formicka-Kozlowska *et al.* 1979). Under our experimental conditions there are no detectable

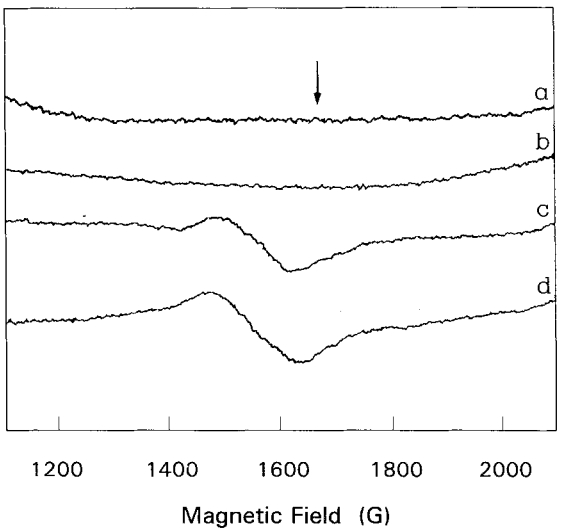


Figure 4. Low temperature ESR spectra of copper complexes measured in the region around $g=4.0$, indicated by the arrow. Samples contained 0.5 mM Cu(II) and 0.5 mM GSSG (**a**); 0.5 mM Cu(II) and 0.20 mM GSSG (**b**); 5 mM Cu(II) and 2.5 mM GSSG (**c**); and 5 mM Cu(II) and 5 mM gly (**d**).

changes in the visible range absorption spectra of dilute solutions of Cu–GSSG complexes (results not shown).

The possibility of a dimer structure

Naturally occurring Cu–GSSG has been isolated from erythrocytes by gel filtration which surprisingly gave an estimated molecular weight close to 1500, instead of approximately 700 as expected for a 1:1 complex (Marzullo & Friedhoff 1977). This suggested that Cu–GSSG could exist in solution as a dimer. ESR spectra of frozen samples cannot be used to distinguish monomers from dimers in the absence of spin coupling. However, at room temperature the degree of spectral anisotropy of small copper complexes in solution reflects the molecular size of the ligands, as seen in Figure 2. Interestingly, the Cu–GSSG spectrum appears much more anisotropic than the spectrum of Cu–$(GSMe)_2$, even though the latter compound actually has a higher molecular weight.

The effect of molecular motion on Cu(II) ESR spectra is complex. The width of each line in the quadruplet is determined by the paramagnetic relaxation times, T_1 and T_2, which depend on nuclear spin, I_Z, anisotropies in g factors and hyperfine interactions, Δg and ΔA, and the molecular correlation time, τ_c. For a small spherical copper complex the relaxation times may be calculated according to McConnell (1956):

$$T_1^{-1} \geqslant (8\pi^2/15)(\Delta g \beta H_0 + \Delta A I_Z)^2 h^{-2}(1 + 4\pi^2 \nu_0^2 \tau_c)^{-1}$$

$$T_2^{-2} \geqslant (32\pi/45)(\Delta g \beta H_0 + \Delta A I_Z)^2 h^{-2} \tan^{-1}(2\tau_c/T_2)$$

where H_0 is the strength and ν_0 the frequency of the magnetic field. The correlation time is given by

$$\tau_c = 4\pi\eta r_e^3/3kT$$

where η is the medium viscosity and r_e the hydrodynamic radius of the complex. In theory it is thus possible to calculate the size of a Cu(II) complex through spectral linewidth measurements, although a number of assumptions and approximations must be made (McConnell 1956, Stone *et al.* 1965). In practice the values of the various parameters cannot be determined with sufficient precision and the Stokes formalism does not hold for small complexes with a molecular weight below 850 (Squire & Himmel 1979). An alternative approach is to use the linewidth ratio of the two relatively well resolved high field lines as a measure of the anisotropy. The hydrodynamic radii of the copper complexes examined here are not known, but are estimated to be proportional to the molecular weight M_r according to the empirically found expression (Horiike *et al.* 1983)

$$r_e \sim M_r^{0.555}.$$

The relative linewidth ratio is obtained conveniently from the square roots of the peak heights, $(h_{-1/2}/h_{-3/2})^{1/2}$ (Stone *et al.* 1965). When this parameter is plotted against $M_r^{0.555}$ a linear relation is found (Figure 5). A presumed 1:1 Cu–GSSG complex does not lie on this line, but a 2:2 complex (Cu_2–$GSSG_2$) fits the line perfectly, confirming the probability of a dimer structure. It should be emphasized,

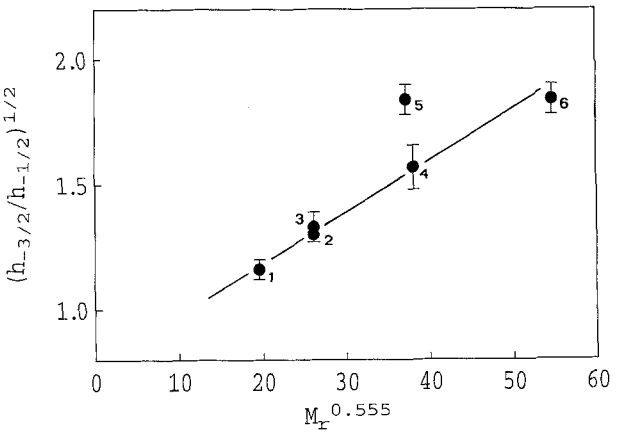
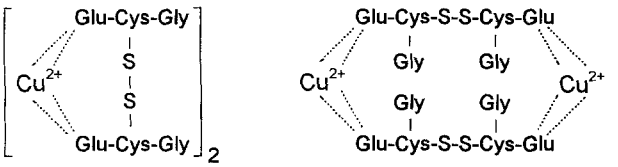


Figure 5. Effect of increasing ligand size on the spectral anisotropy of the Cu(II) complexes. The data points refers to the following complexes: Cu–$(gly)_2$ (**1**), Cu–$(glu)_2$ (**2**), Cu–$(gln)_2$ (**3**), Cu–$(GSMe)_2$ (**4**), a monomer Cu–GSSG (**5**) and a dimer $(Cu–GSSG)_2$ (**6**).

however, that this procedure can be applied here only because the ligand arrangement is the same in the different complexes and that factors other than molecular size influence spectral anisotropy.

A Cu–GSSG stoichiometry of 1:1 and a binding site composed of two identical glutamyl residues naturally leads to a structure with the metal bridging the two ends of a single folded GSSG molecule; two such complexes could make up a dimer. However, the same binding site and stoichiometry can also be found for a dimer structure where each of the two GSSG molecules provides half of the ligands for two binding sites (Scheme 1). The latter model is attractive since it explains why the binding site apparently is not affected at all by the strain imposed on the complex by the S–S bond. Steric interaction between GSSG molecules has previously been inferred from calculations based on potentiometric data (Micheloni *et al.* 1978), but so far no experimental evidence allows the distinction between the two dimer models in Scheme 1. To account for simulations of potentiometric titrations, the existence of an intermediate GSSG dimer with only one Cu^{2+} bound has been postulated (Micheloni *et al.* 1978, Abello *et al.* 1980), but this result was later ascribed to a computer program artifact (Blais & Berthon 1982).



$(CuGSSG)_2$ $Cu(GSSG)_2Cu$

Scheme 1

Conclusion

The use of analogous amino acid complexes has allowed the definite allocation of the ligand arrangement in the equimolar Cu–GSSG complex, providing experimental evidence for the model deduced by Huet *et al.* (1984). This site corresponds to the binding site of Zn^{2+} and probably also Ni^{2+} and Co^{2-}, whereas Fe^{2+}/Fe^{3+} apparently binds exclusively to carboxylate groups (Formicka-Kozlowska *et al.* 1979, Postal *et al.* 1985, Rabenstein 1989); the binding of other metals to oxidized glutathione has not yet been studied in detail.

The results also demonstrate that spectral broadening observed at copper to GSH ratios higher than 1 can occur without the presence of a second metal-binding site. The Cu–Cu interaction discussed in several reports simply reflects the non-specific formation of aggregates and cannot be taken as evidence for a binuclear complex. There is no separate second binding site; the second species detected in the spectra corresponds to a complex in which the metal is only bound to two of the ligands that make up the binding site. With the low levels of Cu^{2+} found in biological systems a binuclear complex is not likely to be physiologically relevant, in particular considering the recent finding that the GSSG to GSH ratio in some cell compartments is much higher than assumed previously (Hwang *et al.* 1992). The measurements made in solution confirm that Cu–GSSG exists as a dimer. This may also be the case for the complexes formed with several other divalent cations, and could be of importance for the metal-transporting properties of GSSG.

Taken together, the results of the present study demonstrate unequivocally that the Cu–GSH complex found in solution under physiological conditions is completely at variance with the structure known from crystal studies. This is probably due to fact that alkaline pH is required to achieve crystallization; some groups have mentioned ongoing attempts to crystallize Cu(II)–GSSG at neutral pH (Miyoshi *et al.* 1980, Postal *et al.* 1985), but no report of the successful preparation of such a crystal has appeared in the literature so far.

Acknowledgements

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