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The Binding Sites of Cadmium to a Reduced Form of Glutathione

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Abstract

Glutathione is the most abundant low molecular weight thiol-containing molecule in biological cells with a strong tendency to interact with metal ions. Among the eight possible glutathione binding sites, only two are determined as groups that interact with the Cd^{2+} ion. Analysis of vibrational spectra and ^{13}C and ^{1}H NMR spectra revealed that thiol and glutamyl's carboxylic groups are groups that cooperate in interaction with Cd^{2+} ions. The coordination of Cd^{2+} with those groups was supported by the application of auxiliary molecules (D-penicillamine, glycine, cysteine and glutamic acid dipeptides, mercaptosuccinic acid and N-acetyl-L-cysteine). These molecules provide a reliable assignment of the fundamental vibrations in the glutathione vibrational spectra. Concentration-dependent measurements of Cd^{2+} ions showed that the optimal stoichiometry of coordination with the glutathione molecule is 1:1. The analysis of $^{3}J(H_{\alpha}, H_{N})$ coupling constants and conformational sensitive bands in the glutathione vibrational spectra suggest that interaction with Cd^{2+} ions significantly alters glutathione backbone conformation. The binding of ions induced the conformational change of the cysteine backbone from a predominantly β structure to P_{II}.

Keywords: Glutathione, cadmium complex, vibrational spectroscopy, NMR spectroscopy

1. Introduction

Glutathione (γ- glutamylcysteinylglycine; GSH) is an essential metabolite and the principal low-molecular weight thiol usually present at millimolar concentrations in most living cells. The fundamental and earliest recognized functions of GSH are thiol-disulphide interactions in which reduced glutathione (GSH) is continuously oxidized to a disulphide form (GSSG) that is recycled back to GSH by NADPH-dependent glutathione reductase. Because of this unique redox and nucleophilic properties, GSH serves in bio-reductive reactions as an important line of defence against reactive oxygen species, xenobiotics and heavy metals, preventing excessive oxidation of sensitive cellular components.[1,2] GSH plays an indispensable role also in many other physiological processes in plant cells such as storage and transport of nonprotein reduced sulfur, regulation of sulphur metabolism, synthesis of proteins and nucleic acids, regulation of gene expression, development of embryos and meristems, signaling in plant pathogen interactions, binding of toxic metals and others.[2–4]

Heavy metal ions are highly reactive and consequently toxic to living cells, although like all living organisms, plants have evolved a suite of mechanisms that control and respond to the uptake and accumulation of heavy metals in tissues. Biosynthesis of glutathione is markedly induced by heavy metals. Plants overexpress γ-EC synthetase, the key enzyme responsible for GSH synthesis. Other mechanisms include the chelation and sequestration of heavy metals by particular ligands[5] and compartmentation of metal ions in less metabolically active cell parts, such as vacuoles.[6,7] Cadmium for example can be transported into the vacuole as a free ion or associated with thi-

ol compounds, GSH or phytochelatins.[1] Phytochelatins $(\gamma\text{-Glu-Cys})_{2-11}$-Gly are one of the best-characterized heavy metal-binding ligands in plant cells.[8–10] They are short peptides that are synthesized from GSH by phytochelatin synthase, a specific enzyme activated by heavy metals, especially cadmium.[11,12] Although the role of phytochelatins in heavy metal detoxification is well documented,[13] studies of metal accumulation and detoxification mechanisms in metal hyperaccumulating plants have shown that increased tolerance is not explained solely by the accumulation of phytochelatin-heavy metal complexes,[14–17] but rather GSH, cysteine and other low molecular weight thiols might be involved in Cd detoxification in the cells.[17,18] Since GSH has been considered to be the first line of defence against cadmium toxicity in many cell types knowledge of the coordination chemistry of GSH is essential for understanding the molecular mechanism of the accumulation and tolerance to cadmium in plants. In principle, the glutathione molecule has eight possible binding sites. The determination of privileged binding sites of GSH with Cd^{2+} was the object of a wide range of studies. ^{1}H NMR spectroscopy indicates that metal ions bind to the potential coordination sites of GSH with a high degree of specificity, which depends on the type of metal ion and the pH value of the solution.[19] Dorcak and Krezel[20] assumed that in phytochelatins besides thiol groups, the amino groups, glutamic acid and glycine deprotonated carboxyls also participate in cadmium coordination. Li *et al*.[21] observed that in yeast, a $Cd(GSH)_2$ complex is formed, rather than the 1:1 complex, while Leverrier *et al*.[22] concluded that at the physiological pH value, Cd^{2+} ions form 1:1 complexes with GSH at micromolar concentration. The authors showed that 1:2 $Cd(GSH)_2$ complexes become dominant at higher concentrations of GSH. Delalande *et al*.[23] demonstrated that Cd^{2+} is linked by two thiol groups from distinct GSH and that Cd^{2+} ligation by nitrogen (the amino group of glutamic acid or the amide of cysteine) does not seem to occur. They also asserted that no carboxylate groups are involved in the interaction.

The present study is focused on structural studies of the GSH Cd^{2+} complex and identification of the favored coordination sites by applying vibrational (Raman and FTIR) and NMR (^{1}H and ^{13}C) spectroscopy. Mercaptosuccinic acid, *D*-penicillamine and *N*-acetyl-*L*-cysteine are thiol-containing components and were used as model compounds to assure the proper assignment of GSH vibrational spectra. Glutamic acid, glycine and cysteine dipeptides were applied for determining the effects of complex formation on the backbone conformation of the GSH molecule.

2. Materials and Methods

Glycine, glutamic acid and cysteine in the form of dipeptides, blocked by CH_3-CO- and -NH-CH_3 groups, were purchased from Bachem. The reduced form of L-glutathione, mercaptosuccinic acid and *N*-acetyl-*L*-cysteine were purchased from Sigma-Aldrich, and *D*-penicillamine from Fluka. The samples were prepared in a concentration range between 0.125 M and 1.0 M in mQ H_2O and D_2O, where the equimolar concentrations of $CdSO_4$ were added. The infrared spectra were measured using Bruker FTS-66 and Bruker Vertex 80 infrared spectrometers. Spectra were recorded in the range between 7000 and 370 cm^{-1}. Typically, 256 scans were averaged and apodized using a Gapp-Henzel function. The infrared spectra were recorded using a diamond ATR cell equipped with a heated top plate and KRS-5 lenses. The frequency differences between the bands recorded in absorption and ATR mode are large, particularly for the strong bands.[24,25] In order to eliminate anomal dispersion effects, pure absorption spectra were calculated from the ATR ones using a method proposed by Bertie and Lan[26] and Bertie and Eysel.[27] The procedure[25] is written in the Matlab environment. The regions between 1750 cm^{-1} and 1150 cm^{-1} were analyzed using the Grams band fitting procedures (GRAMS program, Thermo Electron, San Jose, CA). The shape of individual components was fitted by the sum of bands with Lorentzian and Gaussian shapes. The critical point of applying band fitting algorithm is determination of the number of model bands. We applied two mathematical tools i.e. Fourier avtodeconvolution and second derivative spectroscopy. Both methods are sensitive on the shape of the experimental bands and give the number and approximate position of overlapped component bands. These data are used as starting parameters in the band fitting procedure. The optimization procedure varies peak shape, position, intensity and band halfwidth of individual model bands to minimize the difference between the experimental spectrum and calculated one. The optimized calculated model bands are appropriate for explaining the overlapped region if the resulting difference between the experimental and calculated spectrum contains only recorded noise.

Raman spectra were recorded using the HORIBA Jobin Yvon LabRAM HR800, with a nominal resolution of 0.83 cm^{-1}/pixel, equipped with an NIR Raman source with the excitation line at 785 nm and Bruker Ram II FT Raman spectrometer with a nominal resolution of 2 cm^{-1}. The excitation laser line in the FT Raman experiments was 1064 nm. Typically, up to 5000 scans were averaged. The laser power varies from 100 mW to 300 mW.

The ^{1}H experiments were recorded at 30 °C on a Varian INOVA 600 MHz spectrometer and the ^{13}C experiments on a Varian Unity INOVA 300 MHz spectrometer equipped with automated triple broadband (ATB) probes. The concentration of GSH was 20 mM and different equivalents of concentrated $CdSO_4$ solutions were added as required. The pH values at 4.5 and 7 were adjusted by adding NaOH and HCl. All the samples were prepared in a mixed solvent of 90% H_2O and 10% D_2O. The concentration-dependent measurement of GSH was performed in the range

between 0.5 mM and 20 mM. The chemical shift data for ^{1}H were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The stoichiometry of interactions between ligand and metal was determined using the Jobs method. Series of 2D NMR experiments (COSY, gHSQC and gHMBC) were recorded on a Varian Unity INOVA 300 MHz spectrometer applying standard sequences. Peak assignments for ^{1}H resonances were carried out using COSY and gHSQC and for ^{13}C resonances using gHMBC. $^3J(H_{\alpha}, H_N)$ coupling constants were measured from H_N resonances. A band fitting procedure (GRAMS program, Thermo Electron, San Jose, CA) was used to calculate the peak frequency of the broad overlapped H_N doublet. During the fitting procedure, a Lorentzian lineshape was used. Band frequencies, bandwidths and intensities were allowed to vary simultaneously with no restrictions.

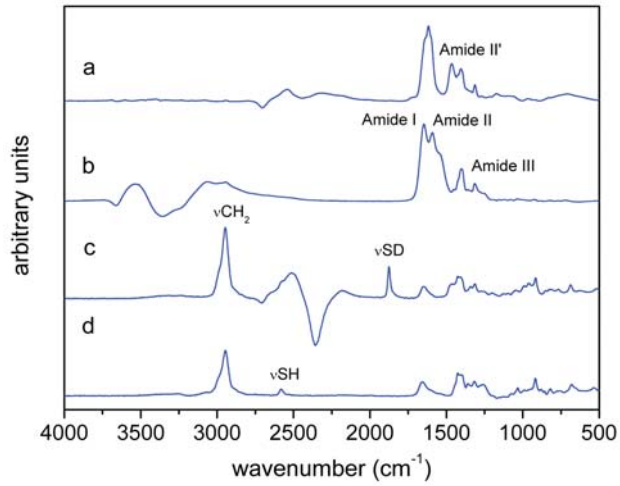

Figure 2. The infrared spectra of GSH in D_2O (a) and H_2O (b) and the Raman spectrum in D_2O (c) and H_2O (d). The spectra of bulk D_2O and H_2O are subtracted. The pH (pD) value is adjusted to 7.

3. Results and Discussion

3. 1. Assignment of Vibrational Bands

The GSH molecule consists of four groups with a structure that strongly depends on the pH value (Fig. 1). The state of protonation has severe consequences on the number and position of vibrational bands in the infrared and Raman spectra. Therefore, the prerequisite condition of successfully applying vibrational spectroscopy to study a cadmium complex with a GSH molecule is the consistent assignment of infrared and Raman spectra (Fig. 2).

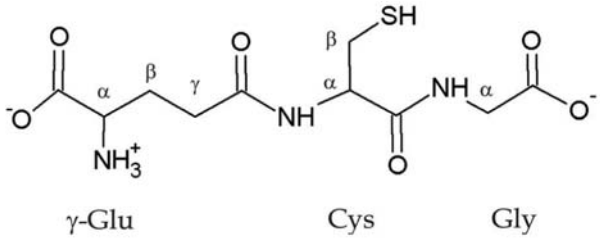

Figure 1. The structure of the GSH molecule in ionized form at pH 7.

The common characteristic of these spectra is extensive band overlapping. We separated the overlapped bands by applying the band-fitting algorithm. The basis for the assignment of the component bands presented the spectra of auxiliary molecules (glutamic acid, glycine and cysteine dipeptides, *N*-acetyl-*L*-cysteine, *D*-penicillamine and mercaptosuccinic acid), which possess only particular functional groups of interest. The detailed frequency tables and deconvolved spectra of the auxiliary molecules are added in the Supplementary file.

The infrared vibrational bands in the spectra of GSH, *N*-acetyl-*L*-cysteine and *D*-penicillamine are associated with the carboxylic acid, amide, thiol and amino functional groups that were examined in acidic, neutral and alkaline pH values. We used the pK_a values of the carboxylic acid, thiol and amino functional groups determined by the NMR study of the aqueous solution of GSH[28] and potentiometric measurement of penicillamine.[29] The results of the curve-fitting procedure of IR and Raman GSH spectra at neutral pH in H_2O and in D_2O are presented in Figs. 3 and 4 and Table 1.

The common spectral characteristic of the GSH molecule and model compounds containing the thiol gro-

Table 1. The assignment and peak frequencies in cm^{-1} of GSH dissolved in H_2O and D_2O. The assignment of the bands in D_2O is written in parentheses. † – vibration is weak or not active, * – vibration not assigned. Intensities: s-strong, m-medium and w-weak

	H_2O (D_2O)	
Assignation	**Infrared**	**Raman**
νSH (*νSD*)	†	2580s (1875s)
Amide I (*Amide I'*)	1646s (1638s)	1660s (1652s)
$\delta_{as}NH_3^+$ ($\delta_{as}ND_3^+$)	1630m (†)	1636w (†)
$\nu_{as}COO^-$	1616m	1621w
	1595s	1590w
Amide II (*Amide II'*)	1555s (1474s)	1560w (1472s)
$\delta_sNH_3^+$ ($\delta_sND_3^+{}_{sim}$)	1530m (†)	†
δCH and CH_2	1450m	1450m
	1426m	1426s
	1411m	1412s
	1361m	1360s
	1345m	1345s
	1275w	1275m
	1266w	1266m
ν_sCOO^-	1393s (Gly)	1393s (Gly)
	1316m (Glu)	1316s (Glu)
Amide III (*Amide III'*)		
P_{II}	1305 (*)	1305 (*)
α_R	1298 (*)	1298 (*)
β	1288 (*)	1288 (*)

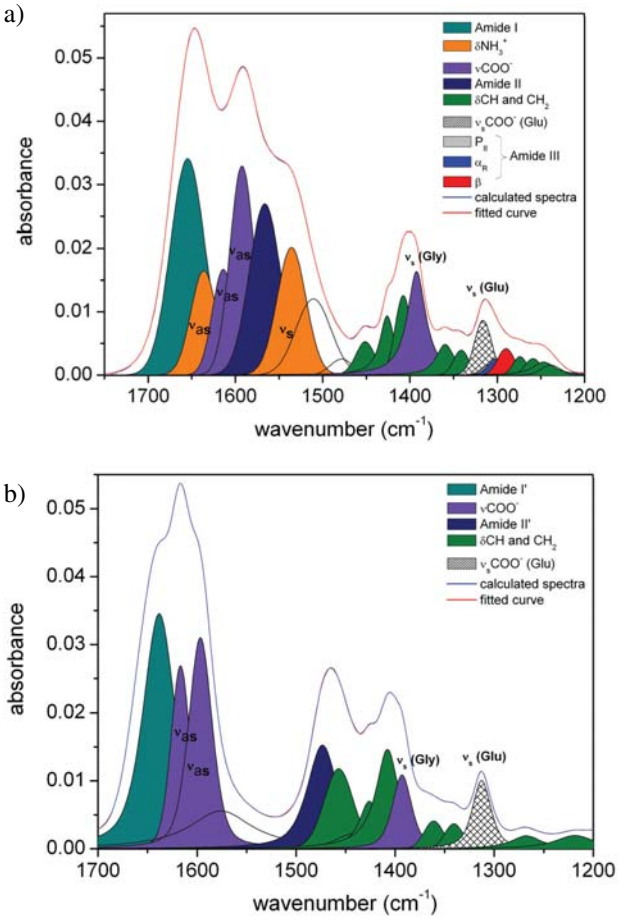

Figure 3. Model bands in the infrared fingerprint region of the GSH spectra in H_2O (a) and D_2O (b), after subtraction of the spectrum of bulk water. The pH (pD) value is adjusted to 7.

up is a band at 2580 cm^{-1} due to SH stretching vibration (Fig. 4a), which appears in acidic and neutral solutions. In the Raman spectrum, this band is always strong while the intensity in the infrared is variable and usually weak. Deuteration moves the ν SD vibration to 1875 cm^{-1}.

In acidic conditions, a carboxylic group vibration ν C=O is identified as an intense band around 1730 cm^{-1} (Fig. 3). A feature that arises together with the C=O stretching band is the C-OH deformation near 1400 cm^{-1}. The coupled C-O stretching mode absorbs predominantly in the 1250–1300 cm^{-1} region.[30] This mode is located in the spectrum of *N*-acetyl-*L*-cysteine at 1286 cm^{-1} and moves to higher wavenumbers in the spectrum of *D*-penicillamine (1293 cm^{-1}) and finally reaches 1311 cm^{-1} in the spectrum of GSH. A C=O stretching band is due to the deprotonation of a carboxylic group absent in neutral and basic solutions. The deprotonated state of the carboxylic acid groups in general gives rise to antisymmetric and symmetric stretching vibrations in the regions between 1550 cm^{-1} and 1620 cm^{-1} ($\nu_{as}COO^-$) and 1300 cm^{-1} and 1420 cm^{-1} (ν_sCOO^-), respectively. The decomposed spectra revealed that antisymmetric vibrations of both carboxylate ions in the GSH molecule are located in the infrared spectrum at 1616 cm^{-1} and 1595 cm^{-1} and in the Raman spectrum at 1621 cm^{-1} and 1590 cm^{-1}, respectively.[31,32] The assignment of both symmetric counterpart vibrations is not so evident.[33] The assignment of corresponding vibration in the spectra of glutamic acid dipeptide and mercaptosuccinic acid was used to clarify the position of both stretchings. A significant change of band intensity in the region between 1400 cm^{-1} and 1315 cm^{-1} is observed in the spectra of mercaptosuccinic acid by changing the pH value from 2 to 7. Moreover, at pH 4.9, the glutamic acid dipeptide has an indicative band at 1317 cm^{-1} in the amide III region, which significantly loses intensity by reducing the pH value to 2.9.[34] The same intensity changes were detected in GSH spectra upon changing the pH values. Therefore, we ascribe a band at 1316 cm^{-1} in the spectra of GSH with a neutral pH to symmetrical stretching of the COO^- group from the glutamic acid side chain and a band at 1393 cm^{-1} to the same type of vibration of the C-terminal end of the GSH molecule. The

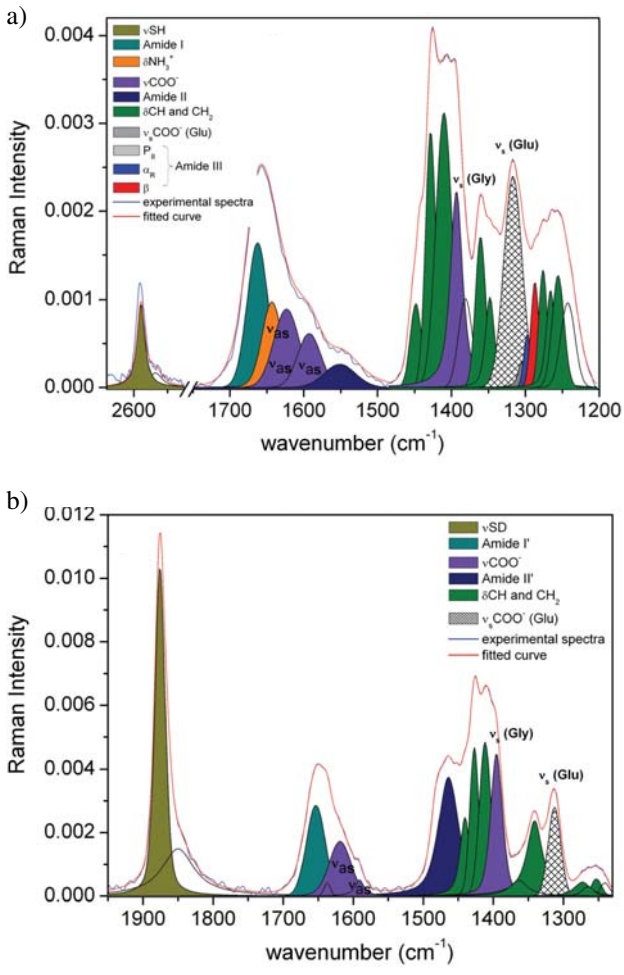

Figure 4. Model band components in the Raman fingerprint region of the GSH spectra in H_2O (a) and D_2O (b), after subtracting the spectra of bulk water. The pH (pD) value is adjusted to 7.

bands are observed in same positions in the Raman spectra of GSH as well.

A weak deformation band due to the NH_3^+ group vibration is expected at around 1600 cm^{-1} ($\delta_{as}NH_3^+$), with a more intense symmetrical counterpart in the region between 1500–1550 cm^{-1} ($\delta_sNH_3^+$).[30] The assignment of these bands is hampered by their low intensity and overlap with stronger amide I and II bands. A relatively weak band found at 1530 cm^{-1} is assigned to the symmetric NH_3^+ deformation. The antisymmetric deformation is located at 1630 cm^{-1}. This band is deprotonated in the basic solution, resulting in NH_2. The latter group exhibits a scissoring mode around 1590–1650 cm^{-1}.[35] We found a broad band at 1560 cm^{-1} in GSH, which gains in intensity upon the deprotonation of the NH_3^+ group.

Intense bands in the region between 1410 cm^{-1} and 1450 cm^{-1} and at around 1350 cm^{-1} were assigned to CH_2 scissoring modes. The latter can also be assigned to CH or CH_2 wagging modes.[36] In accordance with the literature, we assign the band at 1270 cm^{-1} to CH and CH_2 twisting.[36]

The most prominent bands in the spectra of GSH are the so-called amide I and amide II vibrations at 1646 cm^{-1} (Raman 1660 cm^{-1}) and 1555 cm^{-1} (Raman 1560 cm^{-1}), respectively. The amide III vibrations fall in region between 1308 and 1288 cm^{-1}. The shape of the amide III band suggests a complex intrinsic structure, similar to one found in dipeptides.[37] The important characteristic of these bands is connected to their sensitivity to backbone conformation. In general, the amide bonds in peptides and proteins have several vibrational modes, commonly referred as amide A, I, II and III.[38,39] All amide bands are sensitive to the conformation of the backbone. However, the amide III bands are the most applicable for structural studies of the smallest peptides.[34,37] The conformational sensitivity of the amide III band components has been proven by numerous experimental and theoretical studies.[40–44] These studies show that the frequency of the amide III band depends on both dihedral angles. The band intensities of the amide III components in infrared and Raman spectra are thus indicators of the peptide backbone conformation. It has been shown that the most populated conformations of dipeptides in water are P_{II}, β, and α_R.[34,37] Bands in similar positions to the spectra of dipeptides were also found in GSH spectra. With an analogy to dipeptides, we assign the band at 1305 cm^{-1} to the P_{II} conformation, the band at 1298 cm^{-1} to the α_R conformation and the band at 1288 cm^{-1} to the β conformation. Comparison of the area of particular band components revealed that the main population of the GSH backbone represents the β conformation. The results of conformational analysis of the GSH backbone are summarized in Fig. 5 and Table 2.

To confirm the band positions of the amide vibrations, we dissolve GSH in deuterium oxide. The shifts of the amide I, II and III bands for the solution in D_2O compared to the solution in H_2O are in the accordance with the predictions. By dissolving GSH in D_2O, an intense and sharp band appears at 1638 cm^{-1} (Raman 1652 cm^{-1}) corresponding to the amide I' band. The small frequency change of amide I is due to the weak coupling between amide C=O stretching and NH deformation modes. A more significant shift is observed for the amide II band. It redshifts to 1474 cm^{-1}. The shifted amide III' band was not identified.

3. 2. Binding Sites in the GSH Molecule

Vibrational spectroscopy provides direct monitoring of the interaction between a metal and a GSH functional group.[33] The most prominent change upon the addition of cadmium ions is the decreasing intensity of the *ν* SH band presented in Fig. 6. The decrease in intensity indicates deprotonation due to the coordination of the thiol group with cadmium ions.[35] A similar approach was used to confirm the interaction of Cu^{2+} and Co^{2+} ions with a reduced form of GSH molecules.[45,46] Additional evidence of the interaction of cadmium ions with sulfur is the appearance of a band at 389 cm^{-1} in all complexes indicating a stretching vibration of the Cd-S group (ν Cd-S).

Other spectral changes due to interaction with cadmium ions are not so evident. The decomposition of amide III regions reveals changes in the intensity of the amide III band components and band location at 1316 cm^{-1} and assigned to the ν_{sym} COO^- of the glutamic acid side chain (Fig. 5).

From band intensity changes, it is evident that only a fraction of these COO^- groups are involved in this type of interaction with cadmium ions. The level increases with the increase in the concentration of cadmium ions (Fig. 6). With a comparison of the band area of ν_{as} COO^- at the beginning and at the molar ratio metal-ligand of 1, it can be roughly estimated that approximately two thirds of the glutamyl side chains interact with cadmium ions.

The analysis of the amide III region reveals a distribution of conformations of the GSH backbone (Fig. 5). A GSH molecule has three amino acids where only glycine

Table 2. Calculated distribution of conformations (%) of the GSH (0.5 M in H_2O, pH 7) backbone with and without Cd^{2+} ions. For calculation, the amide III region from Raman spectra was applied.

	0.5M GSH	**0.0625M Cd^{2+}**	**0.125M Cd^{2+}**	**0.25M Cd^{2+}**	**0.5M Cd^{2+}**
$\mathbf{P_{II}}$	15	13	25	27	55
$\boldsymbol{\alpha_R}$	25	27	26	27	27
β	60	60	49	46	18

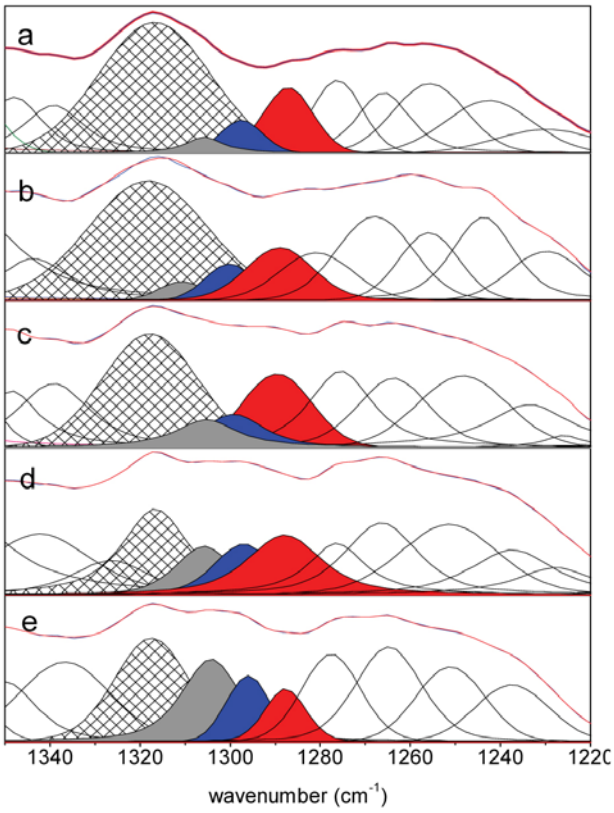

Figure 5. Raman spectra of 0.5M GSM in water with added Cd^{2+}. (a) no Cd^{2+}, (b) 0.0625 M Cd^{2+}, (c) 0.125 M Cd^{2+}, (d) 0.25 M Cd^{2+} and (e) 0.5 M Cd^{2+} added. The meshed band is assigned to the $\nu_{as}COO^-$ vibration of glutamic acid side chain and the grey, blue and red bands to amide III components that belong to the P_{II}, α_R and β conformation of the peptide backbone.

and cysteine are linked by peptide bonds. Since two peptide bonds are present in the GSH molecule, we are not able to determine which amide III band component belongs to the vibration of a particular amino acid residue. Therefore, we can only attribute the observed changes in the calculated

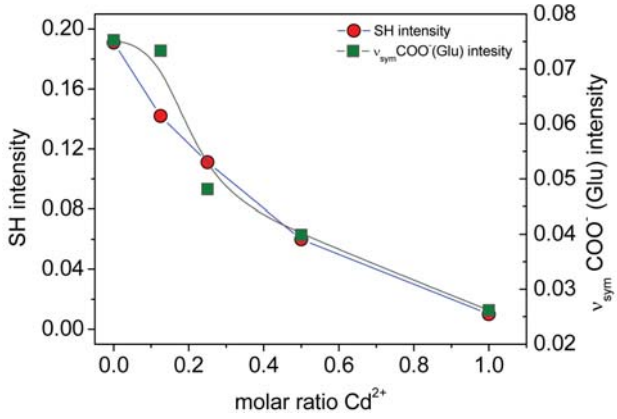

Figure 6. The intensity change of the νSH stretching and $\nu_{sym}COO^-$ (Glu) as a function of the molar ration of GSH and Cd^{2+} ions at pH 7.

distribution of the conformations presented in Table 1 to the whole GSH molecule and not to a specific ϕ, ψ map of a particular amino acid residue. However, the presented distribution of conformations for the GSH backbone reveals that interaction between the GSH molecule and Cd^{2+} ions has a severe influence on the GSH backbone conformations. The addition of metal ions substantially decreases the population of the β structure and gives rise to the P_{II} conformation. The portion of the α_R conformation remains unaffected by cadmium ions (Table 2).

3. 3. The Interaction Between Cadmium Ions and the GSH Molecule Monitored by ^1H and ^{13}C NMR Spectroscopy

The assignment of the ^1H and ^{13}C was checked by the ^1H-^{13}C correlations in the 2D gHSQC and gHMBC spectra. Spectra with corresponding assignment are presented in the supplementary file; in Fig. 7, only changes in the ^1H and ^{13}C chemical shifts induced by the presence of cadmium ions are presented. The most significant change in proton chemical shift after the addition of Cd^{2+} ions was observed for the $GluH_\alpha$, $GluH_\beta$ and $CysH_\beta$ protons (Fig. 7). The addition of Cd^{2+} ions also substantially alters the $GluCOO^-$ and $CysC_\beta$ resonance in the ^{13}C spectra. Smaller shifts were observed for the ^{13}C resonances of the $GluC_\alpha$ and $GluC_\beta$ groups.

The addition of one quarter of the equivalent of metal ions induced only minor changes in the ^1H resonance. More significant are the changes to the ^{13}C chemical shifts of the $GluCOO^-$, $GluC_\beta$ and $CysC_\beta$ carbons. Upon further increasing the Cd^{2+} ratio, considerable deshielding of $CysH_\beta$ protons was observed. The addition of cadmium ions separates the two cysteine β protons, which are strongly overlapped in the spectrum of GSH alone. Deshielding is also observed for $CysC_\beta$ carbons.

The pronounced deshielding of the $CysH_\beta$ proton upon the addition of a molar equivalent of cadmium ions indicates a strong interaction with Cd^{2+}. This interaction is also reflected in the upfield shifts of $GluH_\alpha$ protons and downfield shift of the $GluH_\beta$ protons. In the ^1H spectrum, two well-separated glutamyl β protons were observed, while the GluHγ and GlyHα protons show only negligible shifts. The ^1H chemical shift of $CysH_\beta$ and ^{13}C chemical shift of $CysC_\beta$ move linearly up to the equivalent molar concentration of cadmium ions and GSH. The large ^{13}C $GluCOO^-$ chemical shift indicates the second binding side of cadmium ions in the GSH molecules.

The variations in the chemical shifts in both the ^{13}C and ^1H spectra as a function of the molar ration between the cadmium ions and the GSH indicate that the optimal complex is formed between one cadmium ion and one GSH molecule. This prediction is confirmed by a Jobs plot[47] presented in Fig. 8. From this plot, it is evident that the metal-ligand stoichiometry is 1.

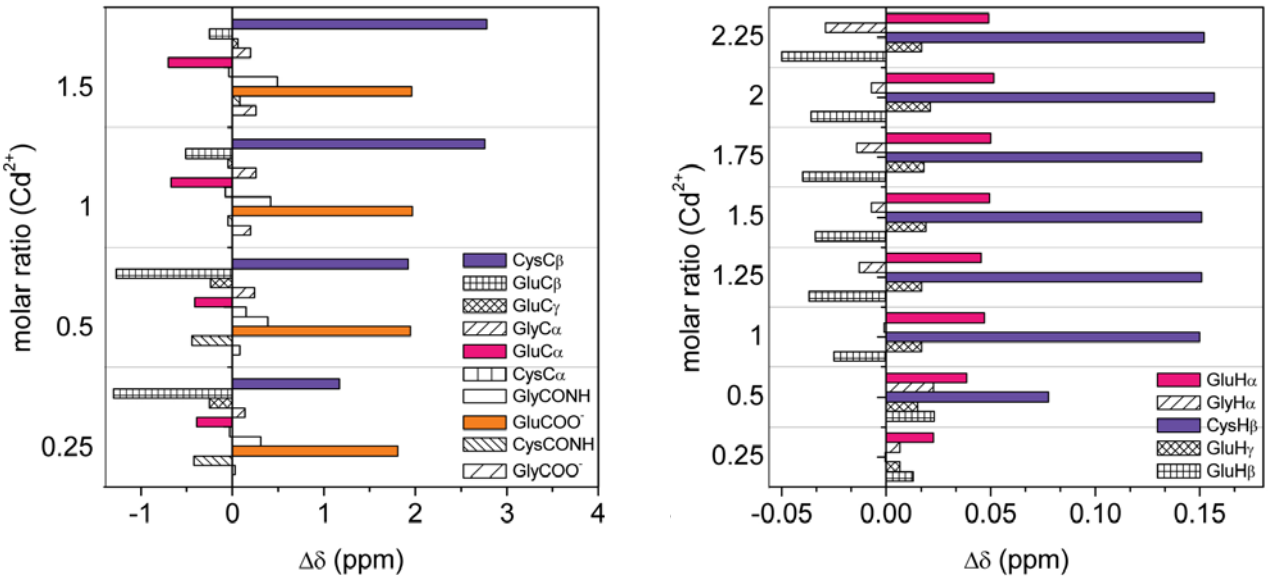

Figure 7. ^{13}C (*left*) and ^{1}H (*right*) chemical shift changes monitored from the ^{1}H and ^{13}C GSH spectra induced by the addition of different equivalents of Cd^{2+}. The concentrations of GSH were 20 mM.

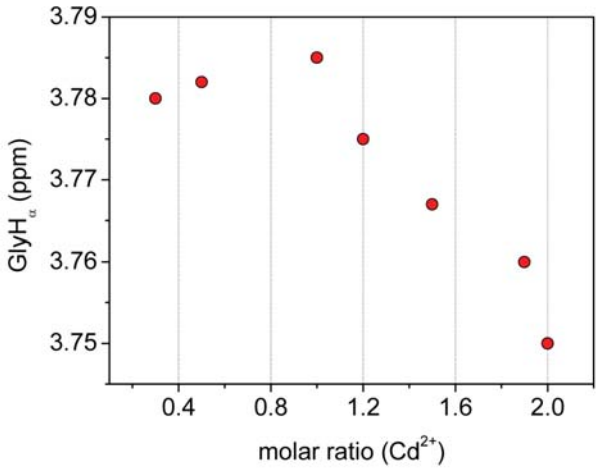

Figure 8. Jobs-plots for the interaction of $GlyH_\alpha$ with Cd^{2+}. The total molarity of the metal and ligand was 20 mM.

3. 4. Conformation of the GSH Backbone Determined by $^{3}J(H_\alpha, N_H)$ Coupling Constant

The $^{3}J(H_\alpha, N_H)$ coupling constant was efficiently used for determining the distribution of backbone conformations.[48] The vicinal coupling constants of glycine and cysteine residues are presented in Fig. 9. As expected, the interaction between GSH with cadmium ions mainly perturbed the cysteine vicinal coupling constant. The initial value of the $^{3}J(H_\alpha, N_H)$ coupling constant of the cysteine residue (7.64 Hz) monotonically decreased to 7.16 Hz after the addition of Cd^{2+} ions. The trend of the vicinal coupling constant to lower values indicates the decrease in the population of the β structure of the glutamine backbone. It has to be emphasized that the initial value cysteine coupling constant found in the GSH spectrum is larger for 0.34 Hz than the same coupling constant found in cysteine dipeptide[48] or GGCysGG blocked peptides.[49] The value of the measured coupling constant is comparable with the value of the coupling constant of the histidine dipeptide (7.76 Hz), which is known to possess a backbone conformation predominantly in the β structure.[48] The application of glycine vicinal constants for conformational studies is more complicated since the glycine ϕ, ψ map is complex. Moreover, the value of the glycine vicinal constants alternate with respect to the addition of cadmium ions.

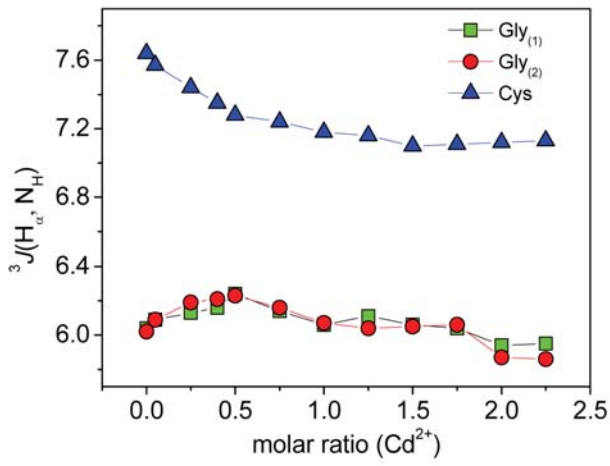

Figure 9. The $^{3}J(H_\alpha, N_H)$ coupling constant of GSH at pH 4.5. The concentrations of GSH were 20 mM. All the spectra were recorded in 90% H_2O/ 10% D_2O at 30 °C.

4. Conclusions

We have demonstrated by the application of infrared, Raman, ^{1}H and ^{13}C experiments that cadmium ions interact predominantly with the thiol group of cysteine residue and the ionized carboxylic group from the side chain of the glutamyl residue. The binding analysis between GSH and Cd^{2+} demonstrates that the chelating abilities of the GSH molecule is intimately associated with the SH group. The broadening of the cysteine β proton after cadmium addition suggests the existence of an exchange process involving the metal ion and the cysteine residue. The decreasing intensity of the SH stretching band with the addition of Cd^{2+} ions supports the thesis that this group is the primary binding site.

Another important functional group that contributes to the formation of Cd-GSH complexes, are glutamyl carboxyl group. The analyses of ^{1}H and ^{13}C resonance showed a broadening of glutamyl α and β protons and $GluCOO^{-}$ carbon after cadmium addition. The interaction of cadmium is also reflected in the changing of the chemical shifts of particular protons and carbon atoms. Additional confirmations of the presence of the second interaction site are decomposed Raman spectra. These spectra are indicative that addition of the Cd^{2+} ions substantially decreases the band area of the glutamyl COO^{-} side chain group. The functional groups in the GSH molecule involved in the coordination of metal ions have been established from changes in the chemical shifts of groups close to the binding site and changes in intensities in the vibrational spectra. Following the concentration measurements and corresponding spectral changes indicates that at physiological pH values, the 1:1 complex is the dominant form.

Our results indicate that both thiol and glutamyl carboxyl groups are essential for the formation of a tight cadmium-peptide complex. This conclusion is in agreement with the result presented by Satofuka and coworkers.[50] Negatively charged carboxyl groups in the peptide stabilize the cadmium-peptide complexes by interacting with positively charged Cd^{2+}.[50]

The formation of metal complexes has a strong influence on the GSH structure. Interaction with cadmium ions induced a conformational change in the cysteine side in the GSH molecule, where the population of the β structure is reduced upon the addition of the cadmium ions. The decrease of the β structure upon interaction was observed in the diminishing of the cysteine vicinal constant and the reduction of the area of the amide III band component assigned to this structure. Concomitant to this reduction, the area of the band characteristic for the P_{II} structure increases. Conformational changes of GSH induced by binding of cadmium ions may result in malfunction of the GSH molecule which results in impairment of its biochemical and signaling functions in the plant cell and consecutive development of toxicity symptoms. Conformational changes of GSH after the binding of cadmium may also partly explain the lack of phytochelatin synthesis in hyperaccumulating plants. When cadmium ions enter the symplast in concentrations typical for hyperaccumulating plants, the pool of functional GSH molecules decreased resulting in deficiency of precursors required for phytochelatin synthesis.

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6. References

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Povzetek

Glutation je najbolj razširjenamajhna molekula v bioloških celicah z veliko afiniteto vezave s kovinskimi ioni. Teoretično ima molekula glutationa osem funkcionalnih skupin, ki lahko sodelujejo pri vezavi s kovinskimi ioni. Z analizo infrardečih in ramanskih spektrov ter ^{13}C in ^{1}H NMR spektrov smo pokazali, da sta v kompleksacijo s Cd ioni udeleženi le dve skupini; tiolna in glutamilna karboksilna skupina. Za natančno asignacijo vibracijskih spektrov glutationa in koordinacije s kovinskimi ioni smo uporabili serijo modelnih spojin (D-penicilamin, dipeptide glicina, cisteina in glutaminske kisline ter merkaptosukcinsko kislino in N-acetil-L-cistein). Koncentracijsko odvisne meritve s Cd ioni pokažejo, da je optimalno stehiometrijsko razmerje z glutationom 1:1. Analiza $^{3}J(H_{\alpha}, H_{N})$ sklopitvenih konstant in konformacijsko odvisnih trakov v amidnem III območju v vibracijskih spektrih pokaže, da se z vezavo Cd ionov spremeni tudi konformacija glutationskega skeleta. Kompleksacija inducira prehod iz pretežno β strukture v P_{II} konformacijo.