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Electron Spin Resonance Investigation of the Copper(II)–β-Glucosidase Interaction in Aqueous Solution

Franco Laschi* and Claudio Rossi

Department of Chemistry, University of Siena, Pian dei Mantellini 44, Siena, Italy

Electron spin resonance spectroscopy has been used to investigate the coordination behaviour of β-glucosidase aqueous solutions in the presence of divalent metal ions such as copper(II). The strong affinity of the paramagnetic metal ions towards the enzyme was verified; two metal–enzyme interaction sites were characterized as a function of pH and temperature and a N_3/O_1 copper(II) atom donor set proposed. In the presence of cellobiose solutions the Cu^{II}–β-glucosidase electron spin resonance parameters were unaffected in the range of pH and molar ratios investigated.

The enzymatic hydrolysis of the β-glucoside linkage is due to the presence of β-glucosidase.[1] A full understanding of the mechanism of β-glucoside bond cleavage by cellulolytic enzymes is crucial in determining the complete pattern of the hydrolysis cellulose to glucose. An analysis of the metal-ion–β-glucosidase interaction and its possible role in enzymatic activity provides a key to understanding the role of metal ions as enzymatic modulators. For the investigation of biomolecular metal complexes, magnetic resonance is the most suitable technique because of its non-invasive nature.[2] In particular, electron spin resonance (e.s.r.) is a very specific and widely applied technique for studying the interaction of paramagnetic metal ions such as Cu^{II} with macromolecules.[3,4] The aim of this paper is to contribute to a deeper understanding of the nature and the mechanisms of the Cu^{II}–β-glucosidase interaction, as well as the extent and the limits of the copper–enzyme chemical equilibrium, in an attempt to clarify the hydrolytic process of cellulose.

Experimental

β-Glucosidase (β-D-glucoside glucohydrolase; FC 3.2.1.21) enzyme and cellobiose [D(+)cellobiose, 4-*O*-β-D-glucopyranosyl-D-glucopyranose] were obtained from Sigma; both were used without further purification. $Cu(ClO_4)_2 \cdot 6H_2O$ (Aldrich) was used as a source of Cu^{II} ions in order to minimize anionic complexation. The pH was varied by adding KOH to the copper(II) aqueous solutions, and the pH values were measured with a Metrohm model E-388 potentiometer.

E.s.r. spectra were recorded with a Bruker ER 200D-SRC spectrometer operating in the X-band ($\omega_0 = 9,78$ GHz). The temperature was controlled with a Bruker ER 4111 VT variable-temperature assembly (accuracy ± 1 K). The magnetic parameters were measured by direct field calibration with the diphenylpicrylhydrazyl (DPPH) free radical ($g = 2.0036$) as field marker. In order to have quantitative reproducibility, the solutions were introduced into a calibrated quartz capillary permanently positioned in the resonance cavity.

Results and Discussion

General Remarks on the E.S.R. Copper(II) Spectra

In this paper the Cu^{II}–β-glucosidase interaction is analysed in terms of the X-band e.s.r. hyperfine structure of the aqueous-solution copper(II) spectra. The $3d^9$ copper(II) ion can be suitably treated as a 'd^1' unpaired system and its e.s.r. spectrum interpreted in terms of the corresponding general Hamiltonian:[5]

$$\mathcal{H} = \beta \boldsymbol{H}\mathbf{g}\boldsymbol{S} + \boldsymbol{I}\mathbf{a}\boldsymbol{S}. \quad (1)$$

In eqn (1) the first term refers to the Zeeman interaction between the applied external magnetic field (H) and the unpaired electron spin (S). The second term refers to the nuclear hyperfine energy, taking into account the magnetic interaction between the copper(II) ion nucleus ($I_{Cu} = \frac{3}{2}$) and the unpaired electron ($S = \frac{1}{2}$). In eqn (1) $\boldsymbol{H}$, $\boldsymbol{I}$ and $\boldsymbol{S}$ are expressed in vector notation and **g** and **a** in tensor notation.[6,7]

E.s.r. theory forecasts for the Cu^{II} hyperfine interaction a pattern of four lines of equal intensity (N, the number of hyperfine lines, is given by $2I+1 = 4$) equally spaced by a, the so-called hyperfine coupling constant, which is assumed to be a measure of the entity of the isotropic interaction between $\boldsymbol{I}$ and $\boldsymbol{S}$.

The rapid random tumbling of the copper species in solution rules out the original anisotropic nature of **g** and **a** in eqn (1), so that room-temperature copper(II) solutions show characteristic isotropic e.s.r. spectra with single values of both the **g** and **a** terms. On the other hand, liquid-nitrogen-temperature (100 K) spectra reveal the anisotropic nature of the **g** and **a** tensors as a consequence of the inhibited motion of the paramagnetic metal–ligand complex.

In the case of axially symmetric structures the explicit form of the Hamiltonian can be written as[6]

$$\mathcal{H} = \beta[g_{\parallel} H_z S_z + g_{\perp}(H_x S_x + H_y S_y)] + a_{\parallel} I_z S_z + a_{\perp}(I_x S_x + I_y S_y). \quad (2)$$

The first term in square brackets is the Zeeman interaction with anisotropy in **g** referred to the parallel component ($\parallel = z$ axis) and the perpendicular component ($\perp = xy$ plane). The second and third terms represent the nuclear hyperfine interaction with anisotropy in **a** referred to the z axis and the xy plane, respectively.

E.s.r. theory states that there are changes in the magnitude of the **a** hyperfine splitting as well as in the value of the g-factor, depending on the nature and the number of the ligand atoms in the first coordination sphere of the copper(II) ion;[7,8] the analysis of such variations can be suitably carried out both at room temperature and in frozen solutions in order to obtain information about the structure and the stability of the copper(II) complexes. This approach is particularly useful in tentatively proposing the nature and number of binding atoms lying in the first coordination sphere of the copper(II) ion.[7,9]

The Cu^{II}–β-Glucosidase System

Fig. 1 shows the observed changes in the X-band e.s.r. lineshape of the Cu^{II}–β-glucosidase binary system in aqueous solutions at room temperature as a function of the pH ($[Cu^{2+}] = 8 \times 10^{-3}$ mol dm^{-3}; [β-glucosidase] = 20 g dm^{-3}). A lineshape analysis suggests the presence of two different Cu^{II}–β-glucosidase interaction sites in the pH range 4.0–6.8 [fig. 1(*b*)–(*d*)].

The corresponding isotropic e.s.r. parameters are reported in table 1; they support the existence of two different enzyme-atom donor sets directly interacting with the metal ion. Here the A and B notation refers to the two copper(II) binding pathways.

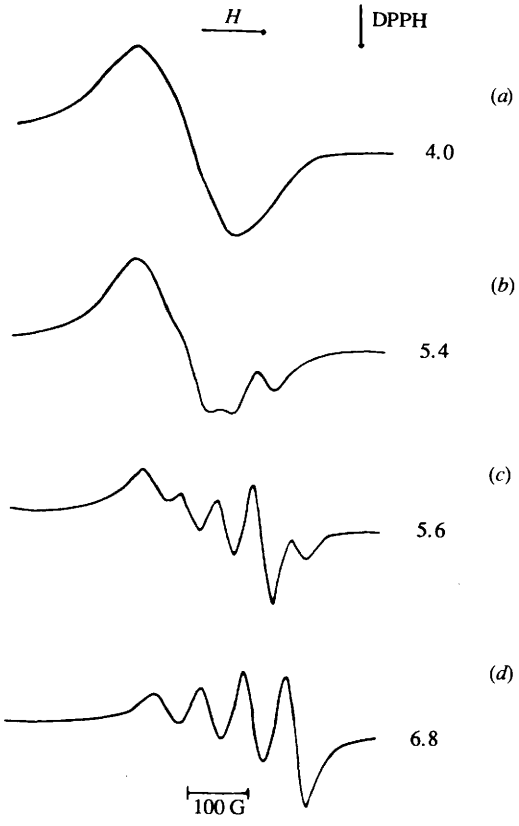

Fig. 1. X-Band e.s.r. spectra of Cu^{II}–β-glucosidase solution for various pH values. $T = 300$ K; $[Cu^{2+}] = 8 \times 10^{-3}$ mol dm^{-3}; [β-glucosidase] = 20 g dm^{-3}.

Table 1. X-Band e.s.r. isotropic a and g parameters of the Cu^{II}–β-glucosidase A- and B-type interactions in a Cu^{II}–β-glucosidase solution ($T = 300$ K)

complex	a_{iso}/G	g_{iso}	pH
$[Cu^{II}$–β-Glu$]_A$	61.3	2.150	5.5
$[Cu^{II}$–β-Glu$]_B$	73.3	2.121	6.8

$[Cu^{2+}] = 8 \times 10^{-3}$ mol dm^{-3}; [β-glucosidase] = 20 g dm^{-3}; $T = 300$ K.

The presence of two Cu^{II}–β-glucosidase coordination sites can be explained by taking into account the following chemical equilibrium:

$$Cu^{II}\text{–}H_2O + \beta\text{-Glu} \underset{K_A}{\rightleftharpoons} [Cu^{II}\text{–}\beta\text{-Glu}]_A + H_2O \overset{+OH^-}{\underset{K_B}{\rightleftharpoons}} [Cu^{II}\text{–}\beta\text{-Glu}]_B. \quad (3)$$

An analysis of the spectra in fig. 1(*b*) and (*c*) shows that the A-type complex is difficult to characterize as a pure form owing to the presence of large amounts of the Cu^{II}–H_2O

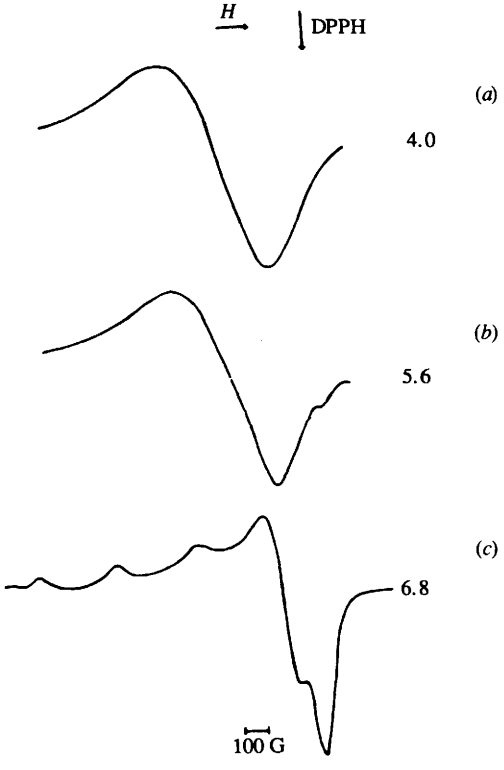

Fig. 2. X-Band e.s.r. spectra of a Cu^{II}–*β*-glucosidase frozen solution for various pH values. $T = 100$ K; $[Cu^{2+}] = 8 \times 10^{-3}$ mol dm^{-3}; [*β*-glucosidase] = 20 g dm^{-3}.

complex which produce a signal that overlaps that of the A-type complex; in contrast, on increasing the pH the $[Cu^{II}$–*β*-glucosidase$]_B$ complex becomes the major species, and is largely predominant in the pH range 6.0–7.2. High *β*-glucosidase concentrations cause pH changes: for [*β*-glucosidase] = 70 g dm^{-3} the corresponding pH is 7.2. Under these experimental conditions and in the presence of copper(II) ions there is no evidence of the Cu^{II}–$(OH)_2$ light-blue precipitate: this means that the copper(II) is totally involved in the direct interaction with the enzyme at acid as well as at neutral pH values. Moreover, on decreasing the pH the lineshape varies from that shown in fig. 1(*c*) to that in fig. 1(*a*): this demonstrates that the equilibrium (3) is reversible with pH.

These findings can be explained assuming that the *β*-glucosidase enzyme acts as an effective weak base, making previously protonated coordination sites available to the copper ions; the competing precipitation of copper(II) hydroxide is therefore ineffective.

The lineshape analysis of fig. 1(*b*)–(*d*) does not show superhyperfine Cu^{II}–N splittings at room temperature: it is reasonable to suppose that the relatively large linewidth cancels possible superhyperfine contributions.

Further information about the extent of equilibrium (3) and some structural evidence of the different characteristics of the two previously identified metal–enzyme interaction sites can be obtained by recording e.s.r. spectra at liquid-nitrogen temperatures (100 K). Assuming axial symmetry for both the copper(II)–*β*-glucosidase A and B sites as the lineshape analysis suggests, several hypotheses concerning the composition of the 'atom donor set' in the *xy* equatorial plane can be verified by taking account of the so-called Peisach–Blumberg approach.[8,9] In this approach, depending on the nature of the four

Table 2. X-Band e.s.r. anisotropic a and g parameters of the Cu^{II}–β-glucosidase B-type interaction in a Cu^{II}–β-glucosidase frozen solution ($T = 100$ K)

complex	a/G	g
$[Cu^{II}$–β-Glu$]_B$(exptl)	$a_\parallel = 198.2$	$g_\parallel = 2.236$
$[Cu^{II}$–β-Glu$]_B$(calcd)	$a_\perp = 13.0$	$g_\perp = 2.063$
$[Cu^{II}$–β-Glu$]_B$	$a_{iso} = 73.8$	$g_{iso} = 2.121$

$[Cu^{2+}] = 8 \times 10^{-3}$ mol dm^{-3}; [β-glucosidase] = 50 g dm^{-3}; pH 6.8; $T = 100$ and 300 K. The $a_\perp$ and $g_\perp$ parameters are obtained by the following formulae:

$$a_{iso} = \tfrac{1}{3}(a_\parallel + 2a_\perp);\ g_{iso} = \tfrac{1}{3}(g_\parallel + 2g_\perp).$$

coordinating atoms in the xy equatorial plane around the metal ion, a close relationship between the experimental g and a spectral parameters can be inferred, particularly in the case of N and O coordinating atoms. Our experimental data in frozen solutions permit an evaluation of the g and a parameters for the [Cu^{II}–β-glucosidase] B-type interaction site [fig. 2(c)], while the A-type site has not been characterized as a pure form [fig. 2(a)]. Table 2 reports the B-type frozen solution parameters.

The well resolved spectrum of fig. 2(c) can be interpreted, in agreement with the previous discussion, on the basis of a total shift to the right equilibrium (3). A lineshape analysis of the B-type spectrum does not show the parallel low-field component ($M_I = -\frac{3}{2}$) resolution of the single absorption lines of the copper isotopes:[10,11] this means that the width of both the $M_I = -\frac{3}{2}$ parallel absorption lines is larger than the main separation between the two corresponding Cu^{63} and Cu^{65} absorption lines ($Cu^{63} = 69.1\%$, $Cu^{65} = 30.9\%$ in natural abundance). In this situation the two lines overlap, giving rise to a single absorption. It is known that the largest copper isotopic separation is 6–10 G at room temperature and 11–15 G at liquid-nitrogen temperature;[12] in our discussion this value can be assumed as the lower limit for the linewidth of the parallel components:

$$\delta_\parallel^{Cu^{63}\text{–}Cu^{65}}([Cu^{II}\text{–}\beta\text{-glucosidase}]_B) \leqslant \Delta H_\parallel([Cu^{II}\text{–}\beta\text{-glucosidase}]_B) \qquad (4)$$

where $\delta_\parallel^{Cu^{63}\text{–}Cu^{65}}$ is the separation between the $M_I = -\frac{3}{2}Cu^{63}$ and $M_I = -\frac{3}{2}Cu^{65}$ parallel absorption lines.

On the other hand, from table 2 it is evident that in the perpendicular region of the e.s.r. spectrum the value of the calculated hyperfine constant ($a_\perp = 13.0$ G) is less than the true value of the corresponding [Cu^{II}–β-Glu]$_B$ linewidth:

$$a_\perp([Cu^{II}\text{–}\beta\text{-glucosidase}]_B) < \Delta H_\perp([Cu^{II}\text{–}\beta\text{-glucosidase}]_B). \qquad (5)$$

In such a situation, neither a hyperfine or superhyperfine splitting is evident in the perpendicular region of the spectrum.

Recently a large number of copper(II) biomolecular species have been analysed on the basis of the Blumberg–Peisach approach[7] in order to verify the actual atom-donor pattern. Taking into account the hypothesis for O and N donor atoms, the Cu^{II} ion could experience the interaction of four binding atoms from the N_0/O_4 to N_4/O_0 coordinating set in the equatorial plane. Assuming that for the [Cu^{II}–β-glucosidase]$_B$ form the coordination site is characterized by a neutral or single negative total charge (pH 6.5), the $a_\parallel/g_\parallel$ ratio (88.7 G) can be explained by a N_3/O_1 equatorial atom-donor set.

Table 3. X-Band e.s.r. a and g parameters of Cu^{II}–β-glucosidase–cellobiose systems at room and liquid-nitrogen temperatures

complex	g_{iso}	a_{iso}/G	pH
	$T = 298$ K		
Cu^{II}–β-Glu + Cell	2.121	73.3	6.8
Cu^{II}–Cell + β-Glu	2.120	73.8	6.5
Cu^{II} + Cell–β-Glu	2.120	73.2	6.8
complex	$g_{\parallel}$	$a_{\parallel}$/G	pH
	$T = 100$ K		
Cu^{II}–β-Glu + Cell	2.235	198.2	6.8
Cu^{II}–Cell + β-Glu	2.238	195.0	6.5
Cu^{II} + Cell–β-Glu	2.236	195.0	6.5

$[Cu^{2+}] = 8 \times 10^{-3}$ mol dm^{-3}; [β-glucosidase] = 50 g dm^{-3}.

Cu^{II}–Cellobiose

Addition of cellobiose to copper(II) aqueous solutions at room temperature in the pH range 4.0–6.8 does not alter the e.s.r. parameters characteristic of the Cu^{II}–H_2O system. On the other hand, pH values > 6.2 cause loss of the absorption signal due to the massive precipitation of Cu^{II}–$(OH)_2$.

The analysis of a number of e.s.r. measurements performed in the pH range 4.0–6.8 at various ligand metal molar ratios and at liquid-nitrogen temperature does not show resolution of the broad copper(II) glassy spectrum. This fact excludes any direct binding interaction between the metal ion and the sugar.

Cu^{II}–β-Glucosidase–Cellobiose System

In order to characterize the influence of the cellobiose molecule on the Cu^{II}–β-glucosidase binary system, e.s.r. measurements have been carried out by adding increasing amounts of cellobiose to the Cu^{II}–β-glucosidase system (i) and by adding β-glucosidase to the Cu^{II}–cellobiose system (ii). The aim of this double treatment was to reveal a possible competing interaction between the metal ion and the sugar molecule towards the enzyme. The two series of experiments (i) and (ii) were performed at room and liquid-nitrogen temperatures and at three different pH values (4.5, 5.5 and 6.8).

Under the same experimental conditions (T, pH and $[Cu^{2+}]$) the e.s.r. analysis of the corresponding solutions (i) and (ii) showed that (*a*) the e.s.r. features of solutions (i) and (ii) are almost identical both at room and liquid-nitrogen temperatures and (*b*) pH variations in the range 4.0–6.8 do not induce changes in the e.s.r. parameters of (i) and (ii) with respect to those of the Cu^{II}–β-glucosidase binary system at the same metal–enzyme molar ratios. Further e.s.r. experiments were carried out by adding Cu^{II} solutions to the β-glucosidase–cellobiose system at pH 4.5, 5.5 and 6.8 (iii). The experimental data of system (iii) strongly agree with those of solutions (i) and (ii); table 3 summarizes the e.s.r. parameters of the Cu^{II}–β-glucosidase + cellobiose system (i), the Cu^{II}–cellobiose + β-glucosidase system (ii) and the Cu^{II} + cellobiose–β-glucosidase system (iii), both at room and liquid-nitrogen temperatures and at pH 6.8.

Conclusions

The e.s.r. analysis of the experimental results carried out on the Cu^{II}–β-glucosidase binary system and on the two Cu^{II}–β-glucosidase–cellobiose ternary systems (i) and (ii) underlines the strong affinity between the enzyme and the copper(II) ion and allows one to identify structurally different copper(II) coordination sites on the enzyme molecule. The marked pH dependence of the binding activities in the pH range 4.0–6.8 is characterized in terms of e.s.r. lineshape analysis and both isotropic and anisotropic g and a spectral parameters.

The e.s.r. technique provides experimental verification of the ineffectiveness of the cellobiose interaction on the $[Cu^{II}$–β-glucosidase$]_A$ and $[Cu^{II}$–β-glucosidase$]_B$ systems, even at high sugar concentrations; moreover, our e.s.r. analysis excludes any direct interaction between the copper(II) and the cellobiose molecule in the pH range considered.

On the basis of these findings we conclude that in the pH range 4.0–6.8 two different copper(II)–β-glucosidase interaction sites are active, each characterized by significantly different e.s.r. parameters, *i.e.* by different complexation pathways; in particular, for the $[Cu^{II}$–β-glucosidase$]_B$ form a N_3O atom-donor set can be proposed for the equatorial coordination. It is clear that these two metal-ion binding sites are not related to the active site between the substrate and the enzyme. Our results also suggest that metal complexation in the limits of our experimental conditions does not affect the hydrolytic activity of the enzyme.

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