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Superoxide dismutase activity of a Cu–Zn complex—bare and immobilised

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Received (in Durham, UK) 13th January 2005, Accepted 18th March 2005
First published as an Advance Article on the web 6th April 2005

A binuclear, imidazolato-bridged complex (Cu(II)-diethylenetriamino-μ-imidazolato-Zn(II)-tris(aminoethyl)amine perchlorate) was prepared and immobilised on silica gel or among the layers of montmorillonite. Immobilisation occurred *via* hydrogen bonding for the silica gel and through electrostatic forces for the montmorillonite. The obtained substances were characterised by EPR and FT-IR spectroscopies and their thermal behaviour was studied by thermogravimetry. The superoxide dismutase (SOD) activity of the complex before and after immobilisation was studied by a SOD assay. It was found that the SOD activity of the host-free complex increased by more than an order of magnitude and approached the efficiency of the real enzyme when silica gel was used as host. The enhanced activity could be assigned to the formation of magnetically isolated centers in the silica pores. On the other hand, the immobilisation with montmorillonite slightly reduced the SOD activity.

DOI: 10.1039/b500579e

Introduction

A number of biological reactions in aerobic organisms have been proposed to involve the generation of superoxide anion. The superoxide radical ion is hazardous to living matter. There are indications that it reacts with thiol and other groups of proteins.[1] However, living systems have a defence, in that they are able to eliminate the superoxide radical ion or at least decrease its concentration level through a dismutation reaction catalysed by enzymes called superoxide dismutases (SODs). In fact, these enzymes are of two main types, the manganese and iron SODs are found in prokaryotes (Mn, Fe), mitochondria (Mn) and plants (Fe), and the copper–zinc SODs are most frequent in eukaryotic cells and in this version a Cu(II)–Cu(I) cycle does the catalysis. The reactions are as follows:

$$Cu^{2+} + O_2^- \rightarrow O_2 + Cu^+$$

$$O_2^- + Cu^+ + 2H^+ \rightarrow H_2O_2 + Cu^{2+}$$

The active centre of superoxide dismutase (SOD) enzyme is known: it consists of copper(II) and zinc(II) ions bridged by a histidyl imidazolate anion.[2] One water and three L-histidine molecules further coordinate the copper(II) ion. Beside the imidazolato bridge the zinc(II) ion is bonded to an L-aspartic acid and two L-histidine molecules. This arrangement is depicted in Fig. 1.

It has been shown that the copper ion plays a direct role in the catalytic electron transfer (it can change its coordination number, geometric arrangement of the ligands and its valence state), while the role of the zinc ion is to organise the peptide chains around the active centre.

Generally speaking, enzymes are very active and selective, but quite sensitive, catalysts. Changes in temperature, solvating properties, *etc.* may easily lead to denaturation (frequently irreversibly) that means the end of their catalytic activities. It would certainly be nice to have in our hands materials as active and selective as enzymes but less sensitive to the environment than they are. In order to achieve these goals complexes keeping most of the structural features of the active prosthetic groups can be and actually have been prepared; for recent reviews, see refs. 3–6. Fitting into this line of research, obviously, several superoxide dismutase-mimicking complexes have been prepared and investigated by various methods previously.[7] They were either mononuclear copper(II)- or binuclear copper(II)- and zinc(II)-containing models.[6–14] These models had SOD activity, however, they were by far less efficient, but nevertheless, less sensitive to reaction variables than the real enzyme. There is a way of further decreasing sensitivity. It is the immobilisation of complexes in/on solid or semisolid matrices.[15–19] When it is done enzyme mimics may be obtained, which might be efficient and selective catalysts in a large variety of reactions. The methods of introducing complexes to solids, mostly to zeolites, have been reviewed.[17] Four

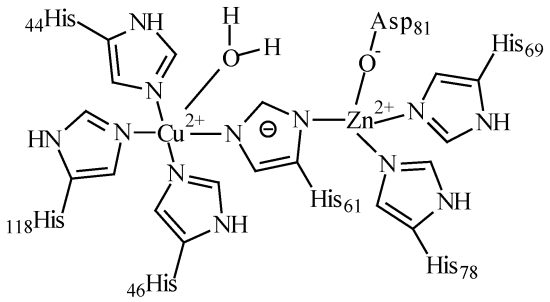

Fig. 1 The structure of the active centre of the Cu–Zn SOD enzyme.

procedures may be distinguished, (i) preparation of the complex in the intrazeolite space, (ii) introducing the preformed complex into the voids of the zeolite (*ex situ* method), (iii) introducing the ligands into the zeolite and letting the complex form with the ion-exchanged transition metal ion (*in situ* method), (iv) using the preformed complex as a template for zeolite synthesis. The first three methods require intrazeolite spaces large enough to accommodate the complexes and/or windows large enough to allow ligand precursors or ligands or the preformed complexes to enter into the channels or cavities of the molecular sieve. The fourth procedure may be the most flexible by excluding or minimizing space constraints.

Inorganic compounds such as silica gel, clays and zeolites have been extensively investigated as matrices for the immobilisation of metalloporphyrins, enzymes and enzyme mimicking metal complexes. Immobilisation of some native enzymes was also studied earlier.[20–22]

During the work leading to this contribution we have prepared a Cu–Zn binuclear complex hoping that it would effectively mimic the active centre of Cu–Zn SOD. In order to increase stability the complex has been immobilised either on silica gel or in montmorillonite. The resulting materials were characterised structurally and also their SOD activities were tested. Results of these experiments are communicated in the following.

Experimental

Materials

For the preparation of Cu(II)-diethylenetriamino-μ-imidazolato-Zn(II)-tris(aminoethyl)amine perchlorate (denoted as C in the following) $Cu(ClO_4)_2 \cdot 6H_2O$ and $Zn(ClO_4)_2 \cdot 6H_2O$ (products of Fluka), imidazole (Reanal), diethylenetriamine (Fluka), tris(aminoethyl)amine (Aldrich) were applied.

As hosts, montmorillonite (Bentolite-H, Laporte, ion-exchange capacity: 1.05 mmol g^{-1}, BET surface area: 90 m^2 g^{-1}) or silica gel (Aldrich, TLC high-purity grade, average particle size: 5–25 μm, BET surface area: ~500 m^2 g^{-1}, Fe^{3+} ≤0.001%, Cl^- ≤0.003%, pH [10% aqueous suspension]: ~6.8, average pore diameter: 6 nm) were used.

For probing the SOD activity an assay was applied consisting of nitroblue tetrazolium (NBT), riboflavin (products of Sigma), L-methionine (Reanal) and ethylenediamine tetraacetate (EDTA—Reanal).

The preparation of the complex and its immobilisation in montmorillonite or on silica gel

The C complex was prepared on the basis of the general recipe published in ref. 10. In this procedure the zinc(II) or copper(II) perchlorate was dissolved in ethanol, then the ligands (imidazole and diethylenetriamine for the copper(II) salt solution and tris(aminoethyl)amine for the zinc(II) salt solution) were added under continuous stirring. For preparing the imidazolato-bridged binuclear complex the two complex solutions were added in 1 : 1 molar ratio under stirring. The obtained blue material was filtered and dried *in vacuo*.

The immobilised complex catalysts were prepared using isopropanol as solvent. First, 1 g of complex **C** (1.13 mmol) was dissolved in 100 ml of isopropanol and from this solution the complex was introduced into/on montmorillonite or silica gel (0.5 g each) suspended in isopropanol. The mixture was stirred for 24 hours at room temperature, then filtered. After that, the solid material was suspended again in isopropanol, and was stirred for 24 hours. The obtained light blue solid material was filtered and dried. Immobilisation occurred with cation exchange in montmorillonite and hydrogen bonding on the silica gel.

The reaction for probing the SOD activity

The SOD activity was investigated by the method of Beauchamp and Fridovich.[23] Its description is as follows. On illumination under aerobic conditions riboflavin is reduced by L-methionine, and the reduced form reacts with oxygen forming a peroxide derivative, which after decomposition provides the superoxide radical anion. The ions are captured by the nitroblue tetrazolium (NBT). This compound changes colour upon the reaction (reduction occurs). The original yellow colour turns blue. The SOD probe reaction was carried out at room temperature in an aqueous solution (host-free complex) or suspension (immobilised complex) at pH = 7 ensured with a phosphate buffer. The reaction mixture contained 0.1 cm^3 of 0.2 mmol dm^{-3} riboflavin, 0.1 cm^3 of 5 mmol dm^{-3} NBT, 2.8 cm^3 of 50 mmol dm^{-3} phosphate buffer (Na_2HPO_4 and KH_2PO_4) containing EDTA (0.1 mmol dm^{-3}) and L-methionine (13 mmol dm^{-3}) and the catalyst (varying the quantity of the catalyst varies the quantity of the complex—its amount is known from atomic absorption measurements—and thus, its concentration in the slurry). Riboflavin was added last and the reaction was initiated by placing the tubes under two 15 W fluorescent lamps. It was allowed to run for 10 min to reach equilibrium. The role of EDTA is to remove the disturbing trace metal ions, since the metal ion–EDTA complexes have no SOD activity. There was no reaction without illumination and neither the pure silica gel nor the pure montmorillonite displayed SOD activity even on illumination.

Methods of characterisation and analytical

The host-free complex and the catalysts were studied by FT-IR spectroscopy by the KBr/Nujol technique in the reflectance mode. The FT-IR spectra of the host and guest materials and the host–guest complexes were taken and compared. The 400–4000 cm^{-1} range was investigated. Spectra were recorded with a BIO-RAD Digilab Division FTS-65 A/896 FT-IR spectrophotometer with 2 cm^{-1} resolution. For a spectrum 126 scans were collected. Spectra were evaluated by the Win-IR package.

The structure of the host-free and the immobilised complex **C** was investigated by electron paramagnetic resonance (EPR) spectroscopy. The EPR spectra were recorded at 298 K on a Bruker Elexsys 500 X-band spectrometer equipped with NMR Gauss-meter and frequency counter with 100 kHz field modulation. The EPR parameters were calculated by a computer program.[24]

The host-free and the immobilised complexes were also studied by thermal (TG, DTA) methods. The thermal behaviour of the substances was investigated by a Derivatograph Q instrument in air (mass of the sample 100 mg, heating rate 10 °C min^{-1}, temperature range 30–1000 °C).

Mass spectrometric measurements were performed by a Finnigan TSQ-7000 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with a Finnigan ESI source. The instrument was scanned in positive ion mode in a

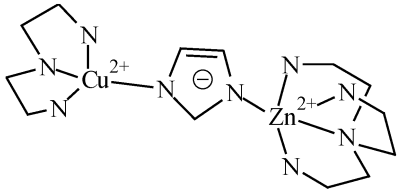

Fig. 2 Structure of the Cu(II)-diethylenetriamino-μ-imidazolato-Zn(II)-tris(aminoethyl)amine cation.

mass range of 10–1500 with scanning time of 1.5 s. Spectra were produced *via* infusion of the sample with a Harvard Apparatus 22 syringe pump (South Natick, MA) driving a 250 μl glass syringe with a stainless steel needle attached to 50 mm id fused silica capillary tubing *via* Teflon coupling at the syringe needle. The electrospray needle is adjusted to 4.5 kV and N_2 was used as a nebuliser gas. The computer program used for simulating the theoretical isotope distributions is included in the ICIS 8.3 package.

The amounts of zinc(II) and copper(II) on/in the solid hosts were measured by atomic absorption spectrometry (AAS – Perkin Elmer 3110 instrument). Before measurements the solid materials were dissolved in *aqua regia*.

The probe reaction was followed by UV–Vis spectrophotometry. The absorbance was measured at 560 nm by a Hewlett Packard 8452A Diode Array spectrophotometer. Quartz cuvettes were used. In the range of measurements the signal–concentration relationship was linear.

Results and discussion

General considerations

The structure of the binuclear host-free complex cation (Cu(II)-diethylenetriamino-μ-imidazolato-zinc(II)-tris(aminoethyl)amine cation) is shown in Fig. 2.

The complex itself has shown considerable activity and fair stability in the decomposition of H_2O_2 (*catalase* activity).[15,18] Upon immobilisation on montmorillonite the *catalase* activity was retained, while it disappeared when MCM-41 was the host material. Thus, in this study the latter was dropped as possible guest and silica gel was chosen instead.

Characterisation of the host-free complex and its immobilised versions

On the basis of pH metric,[25] magnetic susceptibility,[8,9,12] pH-dependent EPR spectroscopy[9,11,12] and X-ray diffractometry[8,9] measurements on very closely related Cu–Zn complexes, one may rightly suspect that the Cu–Zn complex of this study remains heterobinuclear under the reaction conditions applied for testing the SOD activity as well as during immobilisation (see later). Further support is the experimental fact that the prosthetic group in the Cu–Zn SOD enzyme decomposes only at low pH; then decomposition occurs at the Zn-side.[25–27]

In addition to these strong but indirect literature indications we do have our own experimental evidence as further support:

(1) Mass spectrometric detection of the parent ion of complex **C** verified that the complex remained in "one piece" in aqueous solution (Fig. 3). The measurement was performed at pH = 7, exactly at the pH of SOD activity testing. Note the perfect simulation of the isotope distribution.

(2) The room-temperature EPR spectrum of the crystalline host-free complex was also registered (Fig. 4, spectrum 1).

The $g_x = g_y$ and the g_z values are shown in Table 1 together with these parameters of other imidazolate-bridged copper–zinc systems and also of the Cu–Zn SOD enzyme. The measured values of our host-free complex closely resemble those of the other imidazolate-bridged complexes verifying again that our complex remains binuclear. Moreover, the EPR parameters of complex **C** are close to those found in the enzyme indicating close structural resemblance to the real prosthetic group.

(3) Complex **C** and its substructural relatives displayed different SOD activities (see later).

Immobilisation on silica gel was performed by adsorbing complex **C** on the surface of the solid material taking advantage of OH groups abundant on the silica gel surface. After washing off the portion that was only weakly held, the remaining complex was attached *via* hydrogen bonding. The oxygen of the OH groups could play the role of a hydrogen acceptor, while there were both hydrogen donors (carbon) as well as acceptor (nitrogen) atoms in the complex.

The complex could be adsorbed on the silica gel surface successfully and more or less unchanged, as the comparison of the FT-IR spectra of the pure silica gel, the host-free complex and the immobilised complex revealed. The latter spectrum is the superposition of the previous two. All features of the complex remained visible in the IR spectrum of the immobilised material (Fig. 5).

Even though after dissolving the solid material a 1 : 1 Cu(II) : Zn(II) ratio was found on the basis of atomic absorption spectroscopy measurement (second row in Table 2), it may have happened that part of the complex fell apart or it did not but its structure was distorted somewhat.

The powder EPR spectra of the host-free and silica gel immobilised complexes differ (compare spectra 1 and 3 in Fig. 4). The latter is a composition of two component spectra (Fig. 6); for the extracted EPR parameters see rows 7 and 8 (a) and (b) in Table 1.

It is important to note that species (a) does not give hyperfine splitting, while species (b) does: A_z = 189 G and A_x and A_y are smaller than 10 G. The relative quantity of species (a) is 77%, while that of species (b) is 23%. The decrease in g_z value for species (b) compared to species (a) indicates stronger ligand field. Hyperfine splitting only found

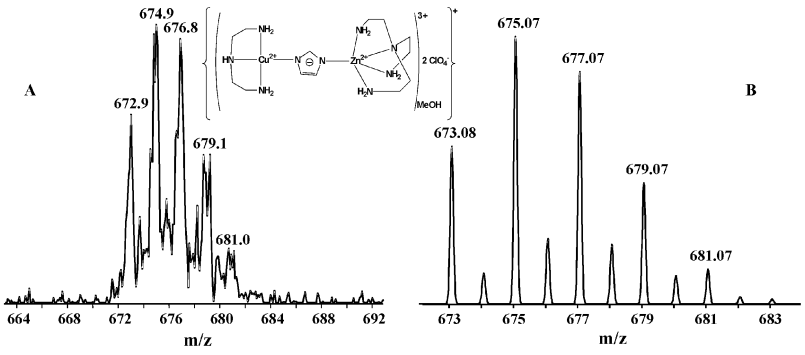

Fig. 3 Measured (A) and simulated (B) positive ion ESI mass spectrum of complex **C**.

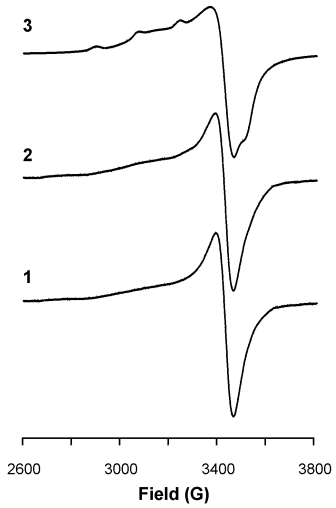

Fig. 4 Powder EPR spectra of the host-free complex **C** (**1**), complex **C** immobilised in montmorillonite (**2**) and complex **C** immobilised on silica gel (**3**).

Table 1 EPR parameters of various Cu–Zn binuclear complexes free of hosts, immobilised and in the superoxide dismutase enzyme

Materials	$g_x = g_y$	g_z	Reference
Cu–Zn SOD	2.07	2.26	9
Cu(II)–Im–Zn(II)–L	2.07	2.21	8
Na[(glygly)Cu(II)–Im–Zn(II)(glygly)]	2.04	2.21	11
[(dien)Cu(II)–Im–Zn(II)(dien)]	2.05	2.20	12
[(PDMT)Cu(II)–Im–Zn(II)(PDMT)]	2.03	2.22	12
Complex **C**[a]	2.05	2.28	This work
Complex **C** immobilised	(a) 2.05	2.26	This work
on silica gel [(a) 77%, (b)[b] 23%]	(b) 2.05	2.22	
Complex **C** immobilised in montmorillonite[c]	2.05	2.28	This work

Im: imidazole, glygly: glycylglycine, L: macrobicyclic ligand (1,4,12,15,18,26,31,39-octaazapentacyclo[13,13,13,1]-tetratetracontane -6,8,10,20,22,24,33,35,37-nonaene), dien: diethylenetriamine, PDMT: pentamethyldiethylene triamine. Line shapes are described by anisotropic signal width tensors.[a] $-W_x = W_y = 42$ G, $W_z = 238$ G. [b] Resolved parallel hyperfine splitting was observed: $A_z = 189$ G. [c] $-W_x = W_y = 51$ G, $W_z = 225$ G.

for species (b) reveals that spin–spin interaction is weak, *i.e.* the support enlarges the distance between the paramagnetic centres. Definitely, species (b) differs from species (a) whose EPR spectrum and the relevant parameters are nearly identical to complex **C** immobilised on montmorillonite.

In further experiments the silica gel support was replaced with montmorillonite. Montmorillonite is a layered material with cation exchange ability. It is capable of swelling, thus, the interlayer volume increases tremendously in a large variety of solvents, such as water and different alcohols. The complex was immobilised in this support exploiting its cation exchange and swelling capabilities. Again the excess complex was washed off during the aftertreatments. AAS measurements revealed that the amount of the complex fixed in the host corresponded to the ion-exchange capacity of montmorillonite and the Cu to Zn ratio was found to be one. Relevant data are to be seen in the third row of Table 2. The quantity of the immobilised complex was high enough to be detectable by IR spectroscopy. Spectra are displayed in Fig. 7.

Inspection of the figure reveals that in spectrum (1) the spectrum of complex **C** (2) is superimposed onto the spectrum of montmorillonite (3), consequently, complex **C** was anchored to montmorillonite indeed.

The powder EPR spectra of the montmorillonite immobilised material (Fig. 4, spectrum 2) and the EPR parameters (Table 1, last row) also reveal that the complex did not fall apart upon immobilisation. The spectra of the host-free and the montmorillonite immobilised complex are identical indicat-

Table 2 The amounts of the metal ions of the silica-, and montmorillonite-immobilised complex **C** measured by atomic absorption spectrometry

Materials	Amount of copper(II)/ mmol g^{-1}	Amount of zinc(II)/ mmol g^{-1}	Cu : Zn ratio
Complex **C** immobilised on silica gel	0.0159	0.0160	0.99 : 1
Complex **C** immobilised in montmorillonite	1.0210	1.0232	1 : 1

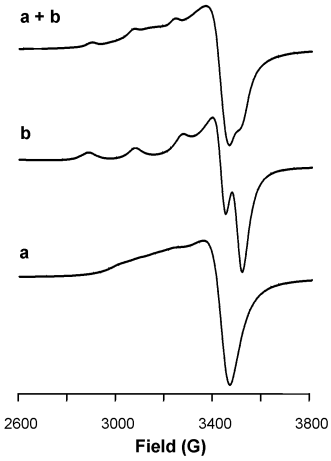

Fig. 6 The component spectra (a and b) of that of complex **C** immobilised on silica gel (a + b).

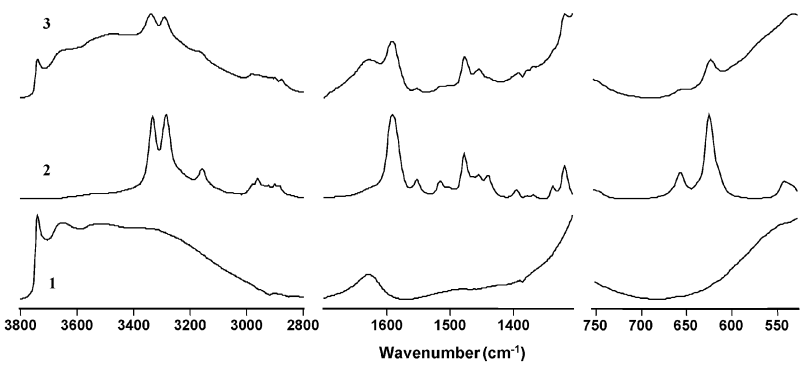

Fig. 5 FT-IR spectra of the air-dried silica gel (**1**), the host-free complex (**2**) and complex **C** immobilised on silica gel (**3**).

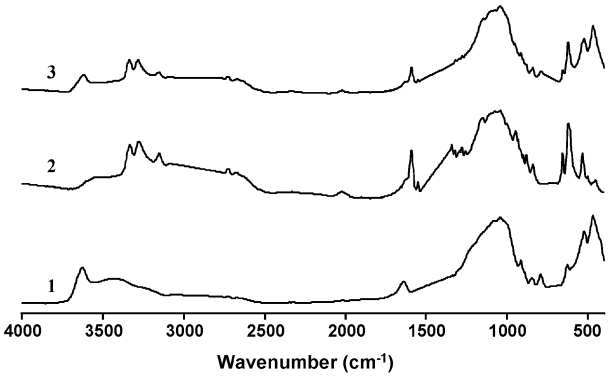

Fig. 7 FT-IR spectra of air-dried montmorillonite (**1**), host-free complex (**2**) and complex **C** in montmorillonite (**3**).

ing that there was no change in the coordination geometry upon immobilisation.

The thermal behaviour of the materials was investigated by thermogravimetry (TG) and differential thermal analysis (DTA). The obtained curves for the silica gel anchored complex are displayed in Fig. 8, while those corresponding to complex **C** and its montmorillonite-immobilised derivative have been published earlier.[15]

Between 120 °C and 220 °C dehydration could be observed for both host materials. From around 440 °C the organic ligands started to depart from the silica gel immobilised sample. This temperature was 610 °C for the montmorillonite immobilised substance due to the strong (electrostatic) interaction between the complex and the host. Both immobilised complexes are thermally more stable than the host-free complex, which fully decomposes by 300 °C.

The superoxide dismutase activity of the host-free complex

The superoxide dismutase activity of the materials was measured by the method of Beauchamp and Fridovich (the riboflavin/NBT)[23] described in the Experimental section. In order to determine the concentration of complex **C** required to achieve 50% inhibition (IC_{50}) of the reaction (a generally used indicator for comparing the efficiencies of enzymes and enzyme mimics), the percentage of inhibition against copper(II) concentration was plotted in Fig. 9. The value corresponding to IC_{50} was 69.1 μmol dm^{-3}.

For comparison we have measured the IC_{50} values of some related copper complexes as well. These data are collected in Table 3. (The IC_{50} value of the native enzyme is also included.) As has already been indicated in a previous paragraph the differing IC_{50} values of complex **C** and its substructural complexes reveal that complex **C** is indeed different structurally, *i.e.* it is heterobinuclear at the pH (=7) of SOD activity testing.

It can be seen that complex **C** has higher activity than the other copper(II) complexes, however, it is significantly less

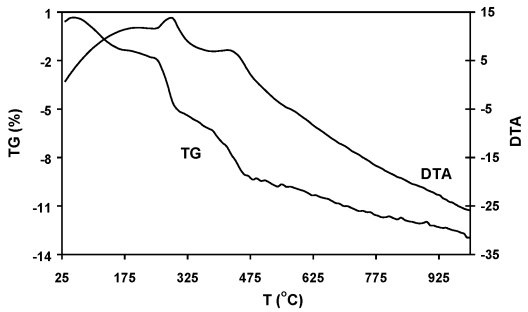

Fig. 8 TG and DTA curves of complex **C** immobilised on silica gel.

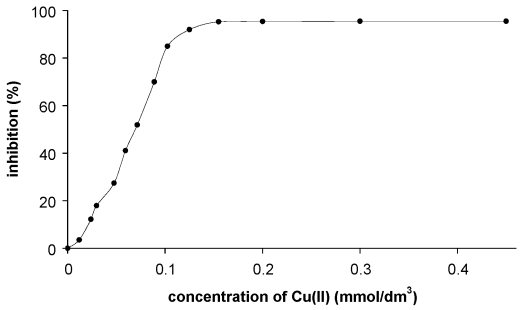

Fig. 9 Inhibition of NBT reduction by superoxide anion radical *vs.* Cu(II) concentration of the host-free complex **C**.

active than the native enzyme. Nevertheless, complex **C** is a potent SOD mimic considering its very low molecular weight compared to that of the native enzyme.

The SOD activity of the immobilised complexes

The SOD enzyme is a complex entity. It contains the appropriately coordinated metal ions and the protein matrix, which is also a crucial part of the enzyme. It not only holds the metal ions, but among many things, it is involved in the transportation of the reactants and the product molecules and due to the flexibility it contributes to the formation of the geometrically most favourable environment for the optimal performance of the enzyme. The application of rigid supports like silica gel and montmorillonite is just a crude approximation of the proteomic skeleton. The flexibility is significantly decreased or even completely lost, however, the complexes immobilised on the more durable solid supports are expected to provide catalysts usable under more rigorous conditions than the enzyme. This expectation is indeed fulfilled, since investigations with thermal methods revealed increased heat stability for the immobilised materials compared with the host-free complex.

For immobilisation we used supports of different structural features. Silica gel is a system with three-dimensional pores, while montmorillonite by virtue of the layered structure may be taken as a material with a two-dimensional pore system. Without the immobilised complex the supports were inactive in the SOD test reaction. After immobilising complex **C** on silica gel the material displayed SOD activity. The SOD activity did not merely appear but it increased with an order of magnitude compared to that of the host-free complex. The IC_{50} value was found to be 6.0 μmol dm^{-3}. The immobilised complex could be reused several times without losing activity, proving that it is a real catalyst and leaching does not occur during the reaction. To double-check this statement the used catalyst was filtered from the mixture and the solid-free system did not show activity of any kind. The immobilised substructures also displayed SOD activity, however, they were significantly less active than complex **C** after immobilisation. They were even less active than the host-free complex **C**.

After immobilisation in montmorillonite complex **C** also displayed SOD activity, however, its IC_{50} value was 91 μmol dm^{-3}, higher but comparable to that of the host-free complex.

Table 3 Data (IC_{50} values) measuring SOD activity of the Cu–Zn SOD enzyme, complex **C** and some related copper complexes

Complex	IC_{50}/μmol dm^{-3}	Reference
Complex **C**	69.1	This work
Cu(II)-dien	162.0	This work
Cu(II)-dien-Im	100.5	This work
Cu–Zn SOD	0.4	This work

dien: diethylenetriamine, Im: imidazole.

Table 4 Data (IC_{50} values) measuring SOD activity of the Cu–Zn SOD enzyme, the host-free complex and the immobilised complexes

Materials	IC_{50}/μmol dm^{-3}	Reference
Complex **C**	69.1	This work
Complex **C** immobilised on silica gel	6.0	This work
Complex **C** immobilised in montmorillonite	91.0	This work
Silica gel immobilised Cu(II)-dien	102.9	This work
Silica gel immobilised Cu(II)-dien-Im	81.6	This work
Cu–Zn SOD	0.4	This work

Dien: diethylenetriamine, Im: imidazole.

Data corresponding to SOD activities (IC_{50} values) of the immobilised materials are shown in Table 4. The IC_{50} values of the host-free complex and the native enzyme are included again to facilitate easier comparison.

One would expect decrease in activity upon immobilisation, since the accessibility of the complex is decreased by the solid host. This was indeed found for the montmorillonite immobilised complex. However, very significant increase in activity was observed for the silica gel immobilised complex. This must have been due to those molecules that displayed weak spin–spin interactions revealed by the appearance of hyperfine splitting in the powder EPR spectra. Here, paramagnetic centres are farther away from each other than for those molecules where hyperfine splitting is lacking (species (a) immobilised on silica gel or complex **C** immobilised in montmorillonite). These molecules are probably adsorbed in the pores of silica gel, while those with strong spin–spin interaction might have attached to the outer surface of this support.

Conclusions

Results described above make it clear that the imidazolato-bridged Cu–Zn complex (complex **C**) studied is a good superoxide dismutase mimic. Upon immobilisation its SOD activity was preserved and even if it was not increased (immobilisation in montmorillonite) the obtained heterogeneous catalyst allows easier workup and recycling than the host-free complex. The major result is, however, the finding that when the complex was anchored *via* hydrogen bonds to silica gel, part of the molecules with weak spin–spin interaction formed an immobilised substance having superior activity to that of the host-free complex and at the same time having the advantages of a heterogeneous system over the homogeneous one (easy workup and recyclability). It was also proven, by the combination of instrumental methods and the SOD activity testing of the host-free and the silica gel immobilised complex and the host-free and the silica gel immobilised substructural complexes, that the complex **C** remained heterobinuclear in solution as well as upon immobilisation.

Acknowledgements

This work was supported by the National Science Fund of Hungary through grant OTKA T049417. The financial support is highly appreciated.

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