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REGULAR ARTICLE

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Cu, Zn Superoxide dismutase: distorted active site binds substrate without significant energetic cost

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Abstract Copper, Zinc superoxide dismutase (CuZnSOD) catalyzes the dismutation of the toxic superoxide radical into molecular oxygen and hydrogen peroxide. Dismutation is achieved by reduction and re-oxidation of the active site copper ion, where the superoxide substrate binds. This enzyme is considered to be a perfect enzyme, as the catalytic rate is very high and diffusion controlled. The redox active copper ion is coordinated by four histidine residues in a distorted square planar geometry. Much has been written about the biological significance of the geometry distortion. It is sometimes considered that it should help to tune the redox potential of the copper ion in order to efficiently reduce the first superoxide molecule and oxidize the second one. In this work we present a series of high level theoretical calculations using realistic models, which demonstrate that the distorted geometry is fundamental for the catalytic efficiency of the enzyme by allowing substrate binding without extensive geometric reorganization of the copper complex, upon changing from four to five ligands. A lower limit for the reorganization energy is calculated here in 22 kcal/mol, which would slow down the reaction kinetics by more than 13 orders of magnitude, transforming a perfect enzyme into an inefficient one.

Keywords Superoxide dismutase · Theoretical study · Active-site distortion · Superoxide binding

1 Introduction

Copper, zinc superoxide dismutase (CuZnSOD) is an important enzyme from a biochemical point of view, as well as an extremely interesting system academically speaking. It catalyses the dismutation of the superoxide radical into hydrogen peroxide and molecular oxygen [1–3]. CuZnSOD is a homodimer with one copper and one zinc ion per monomer and a molecular weight of 32 kDa[4]. The crystal structures of several copper zinc superoxide dismutases have been solved [4–12] and deposited in the Brookhaven Protein Data Bank [13] in the past. Figure 1 shows a schematic representation of the typical active centre for oxidised CuZnSOD with the two Cu and Zn ions bonded via His 61, and the copper ion further surrounded by three other histidines, 44, 46 and 118 respectively. Histidines 69 and 78, and aspartate 81 are bonded to the zinc ion.

Several mechanistic studies, relative to CuZnSOD, exist in the literature both experimentally [1, 14–17] and theoretically [18–20] derived. The main trains of thought seem to be divided between the generally accepted catalytic mechanism, which is derived from experiment [14], and another one based on theoretical studies [18–20]. The most important difference between them is that the experimental mechanism proposes that reduction of cupric ion into cuprous ion occurs spontaneously upon substrate binding, whereas the theoretical mechanism proposes the existence of a stable complex between the superoxide radical and the cupric ion, being a second superoxide molecule oxidized into molecular oxygen through an outer sphere mechanism [21]. Whichever the mechanism, the rates for the catalytic process are very high and diffusion controlled [3, 16, 17, 22, 23].

Reporting ourselves back to the geometry of the active centre, it is common knowledge by now that the four ligands around the zinc are arranged in a tetrahedral structure and that the four histidines surrounding the copper ion have a distorted square planar geometry [4, 24]. Furthermore, it seems to have been generally accepted that the Zn ion does not play a direct role in the dismutation [25], its electronic contribution being important only to assure fast protonation of the Nτ nitrogen of His61, which disconnects from the cuprous ion upon reduction [26–32]. As we became interested in CuZnSOD, we wondered on the role played by the enzyme. The biological significance of the distorted square planar geometry of the histidines arrangement surrounding the Cu ion has never really been explained.

In this work, as a first step to the establishment of the complete catalytic mechanism, we found out that the enzyme concurs to distort the square planar geometry of the Cu(II)

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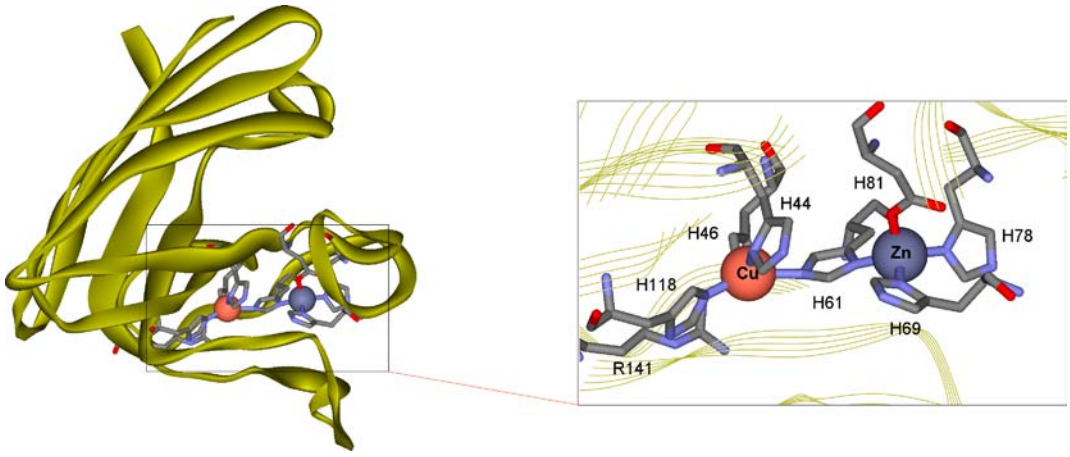

Fig. 1 The active centre of CuZnSOD, taken directly from the X-ray structure for the enzyme [4]. The copper ion is coloured red and the zinc ion coloured blue. Besides the metal ligands, the catalytically important Arg144 residue is also shown

ligands in such a way that the entrance of the superoxide radical is almost free of any geometric rearrangements upon binding the cupric ion, a fact that should greatly contribute to the very fast and diffusion controlled catalytic process.

Table 1 Energies (E) and energy differences relative to Model 1 (ΔE) obtained with Models 1, 2 and 3. All values in kcal/mol

Model	Charge	Spin	E	ΔE
I	+2	Doublet	−221168.87	0.0
II	+2	Doublet	−221146.71	22.2
III[a]	+2	Doublet	−221147.34	21.5

[a] Without superoxide

2 Computational details

Density functional theory was used in all calculations, with the Gaussian03 suite of programs [33], at the unrestricted Becke3LYP level of theory [34–36]. The quasi-relativistic Compact Effective Potentials of Stevens, Krauss, Basch and Jasien were used in all atoms as implemented in Gaussian03, using the 121 contraction scheme for the valence electrons of the light elements [37,38]. For the copper atom only the electrons from the first two levels were included in the core potential (a total of ten electrons), being the remaining 3s, 3p and 3d electrons treated explicitly.

As our system contains charged species it becomes important to evaluate the influence of the environment in the energetics. Coherently, all energies were calculated under the influence of a dielectric continuum. We have used a Polarized Continuum Model, IEFPCM, as implemented in Gaussian03 [33,39], with a dielectric constant of 4. This value has been shown to give good agreement with experimental data in the active site of proteins, and accounts for a dielectric constant of 3 for the protein and 80 for the buried water molecules [40]. It is usually assumed that geometry optimizations can be carried out in vacuum, and transferred to the continuum to calculate final energies, without introducing significant error [41].

In open-shell systems, spin contamination is a frequent problem. It is well known that DFT methods are quite robust in respect to spin contamination. In the calculations presented here, the expectation value for S^2 in the copper-superoxide complex (triplet state) was 2.011, before annihilation. After annihilation, the S^2 the expectation value was returned to the desired value of 2.000. Considering the copper complexes without the superoxide radical (doublet states) the expectation value for S^2 was always lower than 0.7530, before annihilation. After annihilation, the S^2 expectation value was returned to the desired value of 0.7500.

The histidine residues were modeled by imidazole rings. It has been shown before that these rings are the smallest histidine models which still incorporate most of the important chemical effects, like π-electron polarizability and σ-donor capability[42,43]. Another aspect of the modeling is that the zinc ion has been replaced by a proton, as usually in this kind of metalloenzymes; this approximation was demonstrated to be valid in earlier works [18,42], for the properties studied here.

3 Results and discussion

To begin with, we built a first computational model of the active site of CuZnSOD, shown in Fig. 1, including the copper ion and the four bonded histidines (modelled here by the corresponding imidazole rings). The zinc ion, which is bonded to the Nδ nitrogen of His61, was replaced by a proton, this is a valid approach already used before by other workers [18,42], and is justified by the unimportant role of the zinc ion in this first step of the mechanism. It is well known that enzymes lacking zinc have wild type activity at acidic pH and only slightly compromised activity at basic pH[44–47]. The role of the zinc ion seems to be to facilitate the protonation of the Nε nitrogen of His61, which dissociates from the complex upon copper reduction. For what we are about to

Table 2 Comparison of the Cu(II)-N distances in the models with distances taken from crystal structure and EXAFS data

Distances	EXAFS[a]	X-Ray[b]	Model I	Model II	Model III
Cu–His44	1.99	2.06	2.02	2.08	2.11
Cu–His46	1.99	2.02	2.02	2.02	2.12
Cu–His61	1.99	2.21	2.02	2.04	2.13
Cu–His118	1.99	2.07	2.02	2.06	2.11
Cu–O(proximal)	–	–	–	–	2.08
Cu–O(distal)	–	–	–	–	2.96
Mean difference from EXAFS	–	–	0.03	0.06	0.13
Mean difference from XRay	–	–	0.07	0.05	0.07

Selected distances of the optimized DFT models, EXAFS studies and crystal structures for the active site of bovine erythrocyte CuZnSOD. All distances are given in Å

[a] Values are from [27]

[b] Values are from Protein Data Bank, Brookhaven, PDB accession code 2SOD [4]

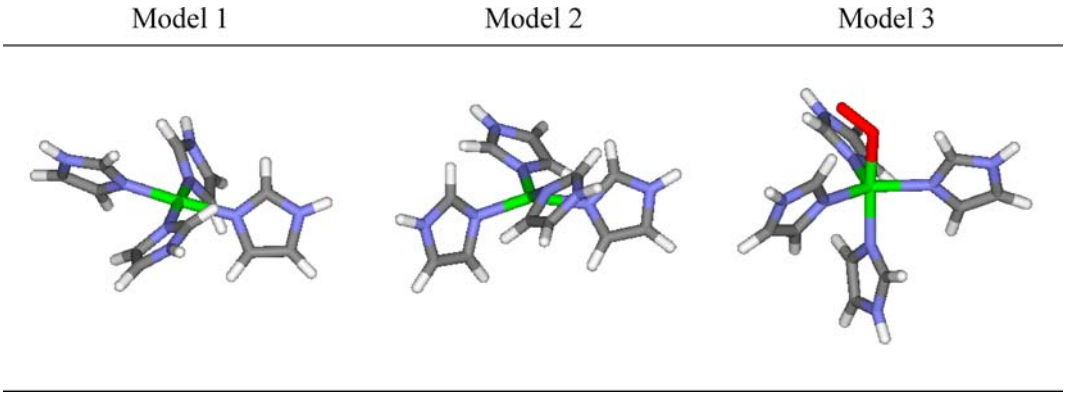

Fig. 2 Schematic representation of the three models considered in this study

discuss, the size of the model is adequate and the conclusion the same, even if we had used the whole active centre.

The coordination geometry of a metallic ion depends essentially on the nature of the ion and the ligands. Moreover there are no strong specific interactions between the enzyme backbone and the redox center. In these cases a small model, as the one used here, is very realistic and capable of reproducing the characteristics of the enzyme active site. Such modelling has been applied many times in the past with great success by other workers in the study of blue copper proteins [48,49].

We first started by adding the necessary hydrogens to the heavy atoms obtained from the X-ray coordinates and optimizing the hydrogen's positions only with CHARMm [50–52]. Subsequently, the geometry of the complex was fully optimized at the unrestricted DFT level of theory. This resulted in a perfectly regular square planar geometry. We have named this model as Model 1 and displayed it in Fig. 2, along with all the other models considered here. We have obtained an energy of −221168.87 kcal/mol for this model, as shown in Table 1.

We proceeded by building a second model starting from the X-ray structure, in which we have constrained only the necessary angular and dihedral internal coordinates in order to keep the active site distortion typical of CuZnSOD. The coordinates that were constrained are included in the Supporting Information. The resulting geometry (Model 2) is also depicted in Fig. 2. The energy of this system was determined to be −221146.71 kcal/mol, a value also shown in Table 1.

Finally, we created Model 3 by adding the superoxide radical to Model 1 and optimizing the geometry of the whole system without constraints. Interestingly enough, the geometry of Model 3 distorted in such a way that the final optimised one was very near the geometry of Model 2. Figure 3 shows Models 2 (green) and 3 (blue) superimposed [53].

In Table 2, we have collected the most relevant bond and angle values for the three models, as well as the corresponding experimental ones.

The similarity between Models 2 and 3 clearly highlight one of the biological significances of the distortion around the copper ion. The coordination geometry of the copper ion allows substrate binding without almost any geometric reorganization. This reorganization would have a very significant cost, as the histidine residues are bound to the enzyme backbone, and therefore their mobility is very limited [54]. We have used two different procedures to estimate the energetic cost associated with the distortion of the copper complex. First we compared the energy of Model 1 (with the perfect square planar geometry) with Model 2 (with the distortion imposed by the enzyme). The difference, presented in Table 1, amounts to 22.2 kcal/mol. We also compared the energy of Model 1 with Model 3, after deleting the superoxide radical. The resulting value of 21.5 kcal/mol is basically the energy cost of the distortion of the geometry that the

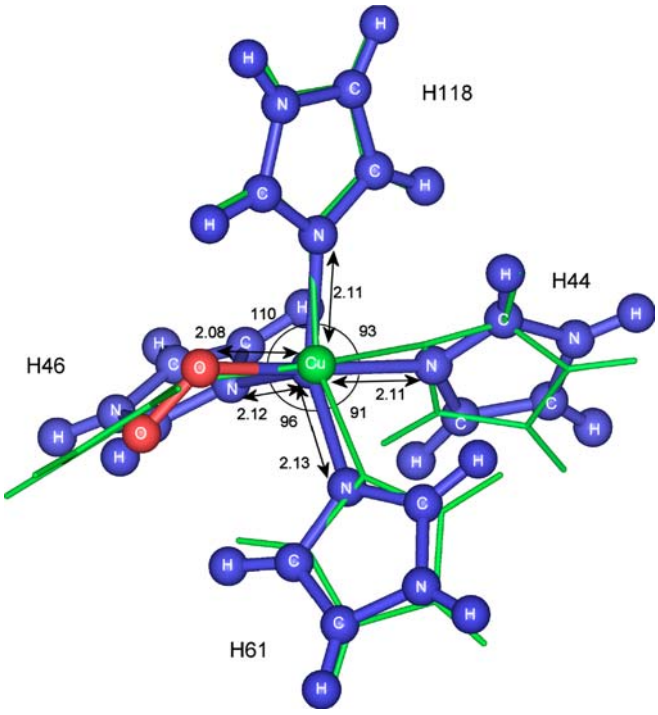

Fig. 3 Optimized geometries of models II (*green*) and model III (*blue*). Superoxide is shown in red. RMS deviation of superimposed structures is 0.40 Å. All bond distances are in Å and angles in degrees

system should suffer to accommodate the superoxide radical. This resulted in a value quite close to the earlier one, which reinforces the great similarity between the geometries of Models 2 and 3, emphasising the proposed role for the copper distorted geometry. It is interesting to note that similar theoretical studies have been performed, using a similar methodology, in an active site model of the blue copper protein family [48,49]. In that case the geometry of the theoretical model was still superimposable to the crystallographic structure even after free optimization without constrains. This led the authors to conclude that in the case of the blue copper protein family there was no strain at the coordination shell of copper. If CuZnSOD was to have a copper ion with square planar geometry, the geometric reorganization energy would be superior to 21.5 kcal/mol as the superoxide bound to the copper ion, raising the barrier for this step to a value larger than 21.5 kcal/mol. That would slow down the enzyme kinetics up to 13 orders of magnitude, basically transforming a perfect enzyme to be transformed into a very inefficient one. However, as the enzyme has the active site already distorted, the geometric reorganization energy is "paid off" during the process of protein folding, and not during catalysis, thus enabling the astonishing catalytic rates characteristic of the CuZnSOD Table 3.

4 Conclusions

CuZnSOD is an important enzyme which catalyses the dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen. By using simple models, we have shown that one of the biological significances of the distorted copper site in CuZnSOD is to keep the geometry around the Cu optimal to accommodate the superoxide radical. This should save an energy of more than 22 kcal/mol to the overall catalytic reaction, which has been calculated as being the cost of the geometry distortion. Furthermore, it greatly contributes to the very fast and diffusion controlled catalytic process that is associated to CuZnSOD, which is fundamental for its biological role.

The knowledge acquired with these calculations is only a preliminary step for a deeper investigation of this system. In fact, the findings reported here have prompted us to investigate the approach and interaction of the incoming superoxide with the whole enzyme, which we are undertaking using QM/MM hybrid calculations. We hope to be able, then, to address important aspects such as the quenching of the system's paramagnetism and corresponding electronic aspects, and the energy profile associated with the entry of the superoxide into the active site of the enzyme. The knowledge acquired by studying the whole enzyme will be invaluable for a deeper understanding of the catalytic mechanism of this fundamentally important enzyme, which still remains elusive even though much thought and effort has been put into it.

Table 3 Comparison of the angles in the optimized models with angles taken from the crystal structure

Angles[a]	X-Ray[b]	Model I	Model II	Model III
His44–Cu–His46	150	173	150	116
His44–Cu–His61	87	90	96	91
His44–Cu–His118	84	90	84	93
His46–Cu–His61	99	90	101	96
His46–Cu–His118	104	90	95	110
His61–Cu–His118	146	176	146	133
O(prox.)–O(dist.)–Cu	–	–	–	119

[a] Angle values are in degrees
[b] Values are from Protein Data Bank, Brookhaven, PDB accession code 2SOD[4]

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