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Thermal stability and redox properties of *M. tuberculosis* CuSOD

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ABSTRACT

The superoxide dismutase from *Mycobacterium tuberculosis* is the only Cu-containing superoxide dismutase that lacks zinc in the active site. To explore the structural properties of this unusual enzyme, we have investigated its stability by differential scanning calorimetry. We have found that the holo-enzyme is significantly more stable than the apo-protein or the partially metallated enzyme, but that its melting temperature is markedly lower than that of all the other characterized eukaryotic and prokaryotic Cu,Zn superoxide dismutases. We have also observed that, unlike the zinc-free eukaryotic or bacterial enzymes, the active site copper of the mycobacterial enzyme is not reduced by ascorbate, confirming that its redox properties are comparable to those typical of the enzymes containing zinc in the active site. Our findings highlight the role of zinc in conferring stability to Cu,Zn superoxide dismutases and indicate that the structural rearrangements observed in *M. tuberculosis* Cu,SOD compensate for the absence of zinc in achieving a fully active enzyme.

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Introduction

Cu,Zn superoxide dismutases (Cu,ZnSODs)[1] are antioxidant enzymes that protect cells from the toxic effects of reactive oxygen intermediates by converting superoxide radicals into hydrogen peroxide and oxygen [1]. Enzymes of this class are widespread in nature and can be found in the cytoplasm and/or in the mitochondria of all the eukaryotic cells [2] and in the extracytoplasmic compartments of several prokaryotes [3].

Despite showing significant differences in subunit assembly and active site channel organization, prokaryotic and eukaryotic Cu,ZnSODs share a similar structure, based on a flattened antiparallel β-barrel fold and a common arrangement of the dinuclear metal center [4]. Here, in the oxidized form, the Cu^{2+}ion is coordinated to four histidine residues and a water molecule with a distorted square pyramidal geometry, while Zn^{2+} is coordinated by three histidine and one aspartate residues in a distorted tetrahedral geometry. The two metal ions are bridged by the imidazole ring of a histidine residue, which acts as a ligand to both metals, and loses coordination with Cu upon reduction of Cu^{2+} to Cu^{+} [5]. This structural arrangement of the active site has been selected very early during evolution, as it can be observed also in the Cu,ZnSODs isolated from the very ancient eubacterium *Aquifex aeolicus* [6, D'Orazio and Battistoni, unpublished observations]. While it is clear that the copper ion plays a central role in the catalytic mechanism, the functions of the zinc ion in Cu,ZnSODs have not been yet completely understood. Zinc contributes to the exceptional thermal stability of the holo-enzyme [7–9] and to its remarkable resistance to denaturing agents and proteolytic enzymes [7]. Besides this unequivocal structural function other roles have been attributed to zinc. For example, the zinc ion contributes to the formation of the positively charged electric field that efficiently drives the superoxide ion to the catalytic site [10]. In addition, zinc favours formation of the copper site and modulates the affinity for this metal [11–13]. Intriguingly, evidences have been provided for the involvement of zinc in modulating the reactivity of the Cu ion. Unlike the holo-enzyme, which shows constant activity over a wide pH range, the activity of the zinc-free enzyme is normal at physiological pH, but falls off rapidly above neutral pH [13]. Moreover, Zn-deficient Cu,ZnSODs react more readily with peroxynitrite to nitrate tyrosine residues [14] and are rapidly reduced by ascorbate and other reductants [15,16]. This is likely due to disorder in the protein loops shaping the active site channel of the zinc-free

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[1] *Abbreviations used:* Cu,ZnSODs, Cu,Zn superoxide dismutases; T_m, temperature of maximum heat capacity; ΔH_c, calorimetric enthalpy of denaturation; ΔH_{vH}, vant'Hoff enthalpy of denaturation.

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enzyme, favouring access to copper of low molecular weight intracellular reductants [17]. It has been hypothesized, but never demonstrated, that the abnormal copper reactivity in the zinc-free enzyme could make possible the transfer of electrons from cellular antioxidants to molecular oxygen and the generation of superoxide by the enzyme, thus contributing to Cu,ZnSOD-related pathological conditions [16].

On the whole, the above mentioned studies indicate that zinc greatly contributes to Cu,ZnSOD stability, catalytic efficiency and specificity of activity. However, the idea that zinc is an indispensable requirement for the construction of a fully active Cu-containing superoxide dismutase has been challenged by the identification in *Mycobacterium tuberculosis* of a novel superoxide dismutase (MtSOD) lacking zinc in the active site [18]. Interestingly, the catalytic rate of MtSOD at neutral pH is similar to that of other Cu,ZnSODs and shows a pH-dependence comparable to that of the zinc-containing bacterial Cu,ZnSODs. In addition, unlike zinc-devoid Cu,ZnSODs, MtSOD spectral properties are pH-independent, a behaviour typical of the holo-enzymes of this class [18]. This atypical enzyme, which is lipid-modified and associated to membranes [19], is structurally homologous to the other Cu,ZnSODs, but shows peculiar structural rearrangements. The absence of zinc, in fact, is due to a considerable reorganization of the zinc-subloop, which is much shorter than in the other Cu,ZnSODs, and to deletion or mutation of some zinc ligands [18]. MtSOD also shows a unique kind of dimer architecture, characterized by a very long and rigid loop that protrudes from the enzyme core in a direction roughly perpendicular to the β-barrel axis. This loop (denominated dimerization loop) significantly increases the interface area of the MtSOD dimer compared to either prokaryotic or eukaryotic SODs and, therefore, it was proposed that the introduction of this novel structural element might compensate the loss of stability associated to the lack of the zinc ion [18]. In consideration of the peculiar properties of MtSOD, we decided to further characterize its molecular properties. The here reported results highlight the prominent role of zinc in conferring structural stability to Cu,ZnSODs, and confirm that MtSOD copper reactivity is similar to that typically observed in other enzymes of this class.

Materials and methods

MTB expression and purification

Recombinant MtSOD was purified in a soluble form from *E. coli* 71/18 cells harbouring plasmid pΔCysMycSOD, essentially as described previously [19]. To better control expression of the *lac* promoter, bacteria were cultivated in LB broth supplemented with 0.2% glucose. $CuSO_4$ (125 μM) was added to the medium to ensure proper metallation of the enzyme. The quality and homogeneity (at least 98%) of the enzyme was checked by SDS–PAGE analysis. Protein concentration was evaluated by the method of Lowry [20], using bovine serum albumin as standard. Metal content of the recombinant enzyme was measured by atomic absorption spectroscopy using a PerkinElmer Life Sciences AAnalyst 300 spectrometer equipped with a HGA-800 graphite furnace. The copper content was about 0.7 eq/enzyme subunit, whereas only trace amounts of zinc were found.

Differential scanning calorimetry

Heat capacity versus temperature profiles were obtained with a VP-DSC differential scanning calorimeter (MicroCal.Inc., Northampton, MA). Protein samples were dissolved at 0.2–0.4 mg/ml concentration, dialyzed against 0.1 M potassium phosphate buffer at the appropriate pH and degassed before the calorimetric experiment. The reference cell was filled with degassed dialysis buffer. Both cells were kept under an excess pressure of ~30 psi (~200 kPa) to avoid bubbling during the scan. A scan rate of 60 °C/h was used in all the experiments. At the end of each run the solutions were cooled and subjected to a second heating cycle under the same conditions to determine the reversibility of the transitions. After each scan, samples were analyzed for integrity by SDS–PAGE and for residual activity using the pyrogallol method [21]. The presence of EDTA in some of the samples assayed for SOD activity had negligible effects on the rate of pyrogallol autoxidation. Thermograms were corrected by subtracting the instrumental base line, obtained with both cells filled with the same solvent, and normalized for protein concentration. Data analysis was performed with the ORIGIN software provided by MicroCal, after subtraction of a cubic base line connecting the pre- and post-transition traces. For each peak T_m (temperature of maximum heat capacity), ΔH_c (calorimetric enthalpy of denaturation), and ΔH_{vH} (vant'Hoff enthalpy of denaturation, equal to ΔH_c for a two-state transition) were obtained by deconvolution. Thermodynamic data at pH 6.0 were fitted assuming the calorimetric transitions of the deconvoluted thermogram to be two-state. The validity of this assumption was confirmed by the reversibility and the good agreement between the experimental and the calculated curves for the native holo-enzyme in the absence of EDTA, supporting the assumption that metals remain bound upon unfolding [22] and for the apo-enzyme at all pHs. Native Cu,ZnSOD was deconvoluted according to a two-state model after subtracting the appropriate baseline. At pH 7.8 the thermogram shows again complete reversibility in the absence of EDTA, but the deconvolution of the calorimetric profile yields three non two-state transitions. In the presence of EDTA, the denaturation is irreversible at all pHs. Errors are estimated to be ±0.1 °C for T_m and ±10% for ΔH.

Attempts to improve the copper content by incubation with copper or to obtain homogeneous preparations of the Cu-free enzyme by standard demetallation procedures [9] were unsuccessful. At the same time, the apo-enzyme obtained by heating MtSOD in presence of EDTA did not efficiently regained superoxide dismutase activity upon incubation with copper. These difficulties prevented the possibility to carry out DSC experiments on samples prepared by titrating the apo-protein with known equivalents of copper.

Assays

Bovine Cu,ZnSOD was obtained by Sigma and further purified by ion-exchange chromatography on a HiLoad 16/10 Q-Sepharose FPLC column (GE-Healthcare) equilibrated with 20 mM Tris–HCl, pH 7.0. Zn-free bovine superoxide dismutase was obtained by extensive dialysis against 50 mM sodium acetate, pH 3.6 of a 40 mg/ml solution of the enzyme. The loss of zinc was confirmed by analysis of the enzyme absorption spectrum. Holo- and Zn-free *E. coli* Cu,ZnSOD were prepared as previously described [9].

Holo- and Zn-free bovine and *E. coli* SODs and MtSOD were dissolved in 0.1 M potassium phosphate pH 7.0, at a 10^{-4} M concentration. Reduction of active site $Cu^{2+}$to Cu^{+}in solution was monitored following the addition of an equimolar amount of sodium ascorbate. The electronic spectra of the enzyme were recorded with a DU 800 UV/Vis Beckman Coulter spectrophotometer.

The effect of EDTA on MtSOD activity was analyzed as described [23], by incubating protein samples at a concentration of 0.04 mg/ml at 37 °C in 100 mM phosphate buffer, 0.1 mM EDTA, pH 7.8. Aliquots were withdrawn at different times and immediately assayed for residual activity by the pyrogallol method.

The apparent molecular weight of MtSOD before and after heating to 80 °C in the presence of EDTA was analyzed by gel filtration chromatography carried out on a HiLoad™ 16/60 Superdex™ 75 gel filtration FPLC column (GE-Healthcare), calibrated with bovine

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