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Chromate resistance in *Cupriavidus metallidurans* CH34: molecular modeling from ChrC superoxide dismutase

Resistencia a cromato en *Cupriavidus metallidurans* CH34: modelamiento tridimensional de la superóxido dismutasa ChrC

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Abstract

Chromate has become an environmental pollutant present in different ecosystems due to its use in industry. Bacteria have evolved to resist stress produced by chromate. Among chromate-resistance mechanisms we can list Reactive Oxygen Species detoxification systems. Cme-SOD (ChrC) protein from *Cupriavidus metallidurans* CH34 is a superoxide dismutase that mitigate oxidative stress caused by chromate. Cme-SOD protein belongs to Fe and Mn-dependent SOD family (pfam02777). The objective of this study was to analyze the three-dimensional structure of the Cme-SOD protein, for which monomer and tetramer models of the enzyme were built. In the monomer model it was observed that Cme-SOD has a characteristic two-domain structure from iron-dependent SOD, additionally, Cme-SOD has an iron-binding site formed by conserved residues H26 and H75 in the N-terminal domain, and D157 and H161 in the domain C-terminal domain. It was show that chromate stress response SODs have a non-conserved residues in the active site (R37, N59, S71, D143 and Y164). These findings suggest the presence of a novel active site in this family of enzymes.

***Cupriavidus metallidurans* CH34, Fe-superoxide Dismutase, Enzyme**

Resumen

El cromato se ha convertido en un contaminante ambiental presenten en distintos ecosistemas. Las bacterias han evolucionado para resistir el estrés producido por este contaminante. Entre estos mecanismos de resistencia se encuentran los sistemas de detoxificación contra las especies reactivas de oxígeno. La proteína Cme-SOD (ChrC) de *Cupriavidus metallidurans* CH34 es una superóxido dismutasa que ayuda a mitigar el estrés oxidativo causado por el cromato. La proteína Cme-SOD pertenece a la familia de SOD dependientes de Fe y Mn (pfam02777). El objetivo de este estudio fue analizar la estructura tridimensional de la proteína Cme-SOD, para lo cual se construyeron modelos del monómero y del tetrámero de la enzima. El modelo del monómero reveló que Cme-SOD presenta la estructura de dos dominios característica de las SOD dependientes de hierro, cuenta con un sitio de unión a hierro formado por los residuos conservados H26 y H75 en el dominio N-terminal, y D157 e H161 en el dominio C-terminal. Se observó que las SOD que responden al estrés por cromato tienen residuos no conservados en el sitio activo (R37, N59, S71, D143 y Y164). Estos hallazgos sugieren la presencia de un sitio activo novedoso en esta familia de enzimas.

***Cupriavidus metallidurans* CH34, Fe-superóxido-Dismutasa, Enzima**

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Introduction

Heavy metals (HMs) are those metals with a density greater than 5 g/cm3 (Nies, 1999), among which arsenic, lead, mercury, chromium, cadmium, nickel, selenium and zinc can be named (Duffus, 2002). Some PMs, such as zinc, nickel and copper, are important as trace elements at low concentrations in organisms, however, at high concentrations PMs are toxic, as they produce reactive oxygen species (ROS), alter DNA, disrupt cellular functions and form toxic organic compounds (Nanda *et al*., 2019; Nies, 1999; Ramírez-Díaz *et al*., 2008).

Chromium is a PM that is widely used in industry for electroplating, tanning, in metallurgy, in welding, in the production of pigments and agricultural fertilisers, and in the manufacture of ammunition, so it has now become an environmental pollutant (*Alvarez et al*., 2021). Bacteria have developed several mechanisms to cope with the stress produced by toxic forms of chromium, such as chromate; among these strategies are expulsion by ChrA chromate transport (Aguilar *et al*., 2008; Nies, 2003; Ramírez-Díaz *et al*., 2008), specific and non-specific reduction (Baldiris *et al*., 2018; Mala *et al*., 2020) and the expression of protective systems against ROS (Branco & Morais, 2016; Miranda *et al*., 2005).

Cupriavidus metallidurans CH34, originally named Alcaligenes eutrophus and later *Ralstonia metallidurans* CH34, is a Gram-negative, bacillary bacterium isolated in 1976 (Houba, 1976). Plasmids pMOL28 and pMOL30 were isolated from *C. metallidurans* CH34, which contain genes conferring resistance to zinc, cadmium, cobalt, mercury, arsenic, lead, silver, copper and chromate (Mergeay & Van Houdt, 2021). Chromate resistance was found to be determined by the chrIBACEF genes found in the pMOL28 plasmid, which encode for the proteins ChrI, ChrA, ChrB, ChrC, ChrE and ChrF (Table 1). Of these proteins, the chromate transporter ChrA is the best studied and is indispensable for chromate resistance (Monsieurs *et al*., 2015).

Among the protection systems against reactive oxygen species are the superoxide dismutase (SOD) proteins. SODs are metalloenzymes that catalyse the dismutation of the superoxide anion O^{2-} into oxygen and hydrogen peroxide and are the first line of defence against ROS (Zhao *et al*., 2021). In the chromate protection system of C. metallidurans CH34 are the SODs ChrC and ChrF. Although ChrF has not been functionally characterised, it has 76% sequence identity to the Mn-SOD, ChrF, from *Ochrobactrum tritici* 5bvl1 (Branco & Morais, 2016). SOD ChrC (Cme-SOD) has been biochemically characterised, it is a 197-residue, Fe-SOD-functional protein with a molecular mass of 24 KDa as a monomer; by analytical ultracentrifugation its active form was determined to have a molecular mass of 98 KDa, which means that the functional protein is a tetramer (Juhnke *et al*., 2002). None of these proteins have been structurally characterised by crystallography, nuclear magnetic resonance or electron cryomicroscopy.

Protein	Funtion
ChrI	Transcriptional activator-like protein
ChrA	Transmembrane chromate transporter protein
ChrB	Transcriptional regulator-like protein
ChrC	iron-dependent superoxide dismutase (Fe-SOD)
ChrE	Rhodase-like protein
ChrF	Manganese-dependent SOD-like protein (Mn-SOD)

Table 1 Chromate resistance-related proteins encoded by plasmid pMOL28

Although the SOD ChrC of C. metallidurans CH34 has been experimentally characterised, its three-dimensional structure has not yet been investigated due to the technical difficulties associated with this methodology. One possible solution to obtain its three-dimensional structure is bioinformatics analysis using molecular modelling (Kuhlman & Bradley, 2019). Such a technique has proven to be applicable in numerous cases of SOD (Sánchez-Calderón *et al*., 2019), whose structures have been previously solved and are indispensable to carry out this type of approach accurately. Therefore, the aim of this study is to examine the three-dimensional structure *of C. metallidurans* CH34 Cme-SOD (ChrC) using molecular modelling of the protein, with the purpose of gaining knowledge about its structure and deepening the understanding of the mechanism of action of this enzyme.

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Method

Both the monomeric and tetrameric structures of Cme-SOD were modelled. First, a preliminary monomer model was generated using the SWISS-MODEL platform (Waterhouse *et al*., 2018), taking the structure of Clostridium difficile SOD (PDB: 3TJT) as a template. For the construction of the final monomer model, moulds were searched by BlastP (Altschul *et al*., 1997), using the Cme-SOD sequence (Accession number: CAC42412), in the PDB database, with default parameters. Several structures were used as templates; the Fe-SOS from Aquifex pyrophilus (PDB: 1COJ), the Cme-SOD from the AlphaFold database (AlphaFold id: AF-P17550-F1) and the preliminary model generated with SWISS-MODEL. Sequence alignments were carried out in the T-Coffee program (Di Tommaso *et al*., 2011). The final monomer model was generated using Modeller 9v10 (Webb & Sali, 2016). The tetramer model was generated in a similar manner using a primary tetrameric model of the Fe-SOS from *Aquifex pyrophilus* (PDB: 1COJ), a tetrameric overlay of the Cme-SOD from the AlphaFold database (AlphaFold id: AF-P17550-F1) and the previously obtained monomer model as templates. All models were validated using the PROCHECK 3.5 program (Laskowski *et al*., 1993).

The molecular models were visualised and the figures were generated with the PyMOL Molecular Graphics System, Version 2.1.0 (Open-Source), Schrödinger, LLC.

Results and discussion

Obtaining the Cme-SOD model of C. metallidurans CH34

Chromate resistance in the bacterium *C. metallidurans* CH34 is due to a set of proteins that have several functions (Table 1). Among these proteins, the Cme-SOD (ChrC) protein has been characterised, whose three-dimensional structure is not known. To gain a better understanding of the active site of this enzyme, three-dimensional models of the monomer and tetramer of this enzyme were generated using homology modelling.

Although a three-dimensional model of this protein is currently available in the AlphaFold database (Jumper *et al*., 2021), this model does not have its cofactor, an iron ion per subunit, which is vital for the activity of the enzyme, so it was decided to model the protein using a solved structure containing the cofactor. In order to have a better quality model, it was decided to make an initial approximation by generating a model using the SWISS-MODEL system. In this system, the Clostridium difficile Fe-SOD protein (PDB: 3TJT), whose structure was solved with the Fe ion, was used as a template. The generated model contains residues in the areas not physically allowed, so it was decided to generate a better quality model using the Modeller program.

In the search for templates, it was found that the protein of known structure with the highest sequence similarity to Cme-SOD is the Fe-SOS from Aquifex pyrophilus (Apy-SOD, PDB:1COJ). These proteins have 34% sequence identity. This percentage is very low, and is at the limit of what is required to generate a molecular model suitable for structural studies (Khor *et al*., 2015). To generate a high quality model, it was decided to use several structures or models as templates. The casts selected were the monomeric SOD structure of A. pyrophilus, the Cme-SOD model obtained from the AlphaFold database and the model previously generated using SWISS-MODEL. The use of the Apy-SOD and the SWISS-MODEL model allowed us to model the Cme-SOD with the iron ion, and using the Cme-SOD model from AlphaFold does not ensure a high quality mould with 100% coverage of the protein residues. Ten models of the SOD monomer were generated, from which the one with the best structure quality parameters was selected according to the PROCHECK programme that checks the quality of the combinations of the angles φ and ψ on a Ramachandran plot.

The molecular model of the Cme-SOD monomer consists of 197 amino acids, in which a conserved domain characteristic of Mn-, Fe-, Zn- and Cu-dependent SODs (pfam02777) was located. The model presented more than 97% of the residues in the zone of suitable angles and 100% within the zone of allowed angles, and more importantly, none of the angles of the main chain fall within the non-allowed zones (Table 2), which indicates that we have a high quality model (Dalton & Jackson, 2010).

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The obtained model was compared by structural alignment with the Apy-SOD structure used as a template, obtaining an RMSD of 0.887 Å. A model with an RMSD deviation of less than 1 Å is indicative of a high-quality model (Dalton & Jackson, 2010). The Cme-SOD monomer shares the characteristics of other Fe-SODs by presenting an architecture with two subdomains, N-terminal and C-terminal. The N-terminal domain consists of α-helices, where part of the active site is located in the first and last helices. The C-terminal domain is formed by three β-strands, surrounded by α-helices, between these helices is the other part of the active site (Figure 1A).

Fe-SODs are conserved proteins found in all three domains of life. It has been reported that in bacteria most Fe-SODs are dimeric, tetrameric Fe-SODs can be found, as is the case for Cme-SOD (Sheng *et al*., 2014). A similar strategy was followed to model the tetramer. The tetramer structure of Apy-SOD, and a tetramer reconstruction of Cme-SOD obtained from the AlphaFold database and the high-quality monomeric model were used as templates. Ten molecular models were reconstructed and verified with PROCHECK, where 100% of the main chain angles were within the allowed angle zone (Table 2). The alignment of the tetrameric model structures yielded an RMSD value of 0.891 Å. These results together do not allow us to conclude that this is a valid model, and of high quality to carry out structural studies. At the interface of the monomers, it can be seen that a loop characteristic of dimeric Fe-SODs is absent, which allows for the correct formation of the tetramer (Sheng *et al*., 2014).

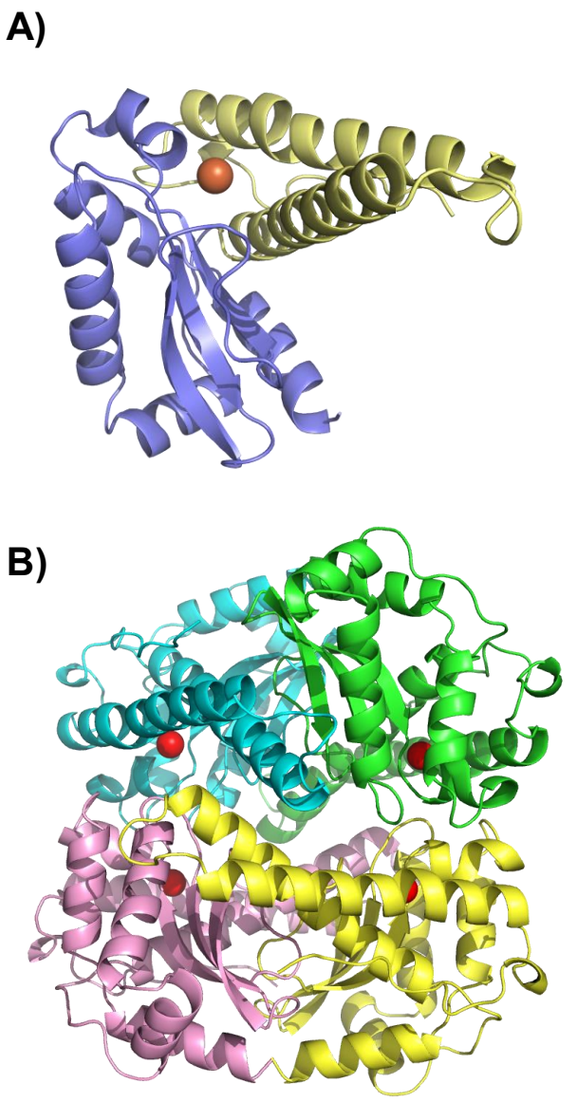

Figure 1 Three-dimensional structure of the Cme-SOD of *C. metallidurans* CH34. A) The structure of the Cme-SOD monomer model is shown. The C-terminal domain is shown in yellow and the C-terminal domain in purple. The iron ion is shown in red. B) The structure of the Cme-SOD tetramer is shown. Each subunit is shown in a different colour. The iron ion of each subunit is shown in red

	Peptide bond angles by region (%)			
Structure	A	P	G	N
1COJ	92.5	7.0	0.5	0.0
Cme-SOD monomer	97.7	2.3	0.0	0.0
Cme-SOD tetramer	97.2	2.8	0.0	0.0

Table 2 Model quality data of the SOD ChrC model of *C. metallidurans* CH34. Ramachandran plot values obtained from the PROCHECK 3.5 program of the molecular model and the Fe-SOD templated protein (1COJ) of A. pyrophilus are shown. A-Adequate. P- Allowed. G-General. N- Not allowed.

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Cme-SOD active site analysis of C. metallidurans CH34

Evolutionarily speaking, Fe-SODs were the SODs to appear due to the large amount of iron present and the low oxygen concentration during the emergence of life on earth (Sheng *et al*., 2014). Under modern earth conditions, the retention of iron ion as a cofactor can be explained by the high affinity of the enzyme for iron. The high affinity of Fe-SOD for its cofactor can be explained by a chelating effect of residues in the enzyme active site, in the case of Cme-SOD, the chelating effect is due to four residues with donor groups, in the N-terminal domain H26 and H75, and in the C-terminal domain D157 and H161 (Figure 2). One of the possible advantages of Cme-SOD binding its cofactor by interaction with four residues is its stability despite the entropy generated during the dismutation reaction. The active site is complemented by a water molecule, which connects the four residues that coordinate with the cofactor and gives this region its characteristic trigonal bi-pyramidal geometry (Figure 2).

In the *Escherichia coli* Fe-SOD (PDB:1ISA) it has been observed that residues Y34 and Q69 are part of the active site, being part of the residues that interact with the reaction intermediates (Lah et al., 1995). Comparing the sequence of Cme-SOD with the sequence of 1ISA, it is observed that Q69 of 1ISA has been replaced by S71 in Cme-SOD (Figure 3). However, it is observed that the Y34 position of 1ISA is replaced by A34 in Cme-SOD (Figure 3), which is unable to carry out the interactions necessary for enzyme function, which would undermine the functionality of the enzyme. When analysing the residues in the active site region of Cme-SOD, it is observed that the function of Y34 can be replaced by R37. In Apy-SOD, residues R65, D146 and Y180 have been reported as an important part of the function of this enzyme (Lim et al., 1997), corresponding to residues N59, D143 and Y164 in Cme-SOD, respectively (Figure 3). Both R37, N59 and S71 are conserved in the SOD ChrC protein of O. tritici 5bvl1 and C. metallidurans CH34 and differ in the other Fe-SODs, suggesting that a novel active site has evolved in the chromate stress-responsive Fe-SODs, different from the other Fe-SODs.

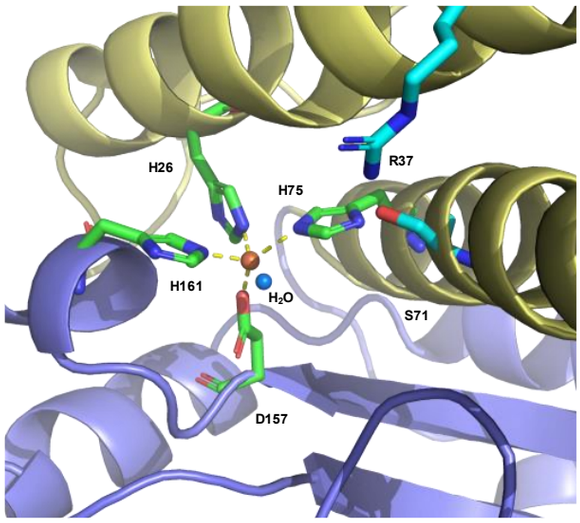

Figure 2 Active site Cme-SOD of *C. metallidurans* CH34. The four conserved residues of the protein that coordinate with cofactor Fe (red) are shown in green. Probable residues complementing the active site are shown in blue. The yellow lines represent the binding between the protein residues and the Fe atom. The complementing water molecule necessary for Cme-SOD function is shown as a blue sphere. Yellow and purple show the amino-terminal and carboxyl-terminal domains respectively

Conclusions

Chromate, a highly toxic form of chromium, induces oxidative stress, damage to organelles, DNA and proteins once it enters cells. There are bacteria that live in environments contaminated with this metal, and these bacteria have developed protective systems against this PM. The ChrC enzyme is one of the enzymes that has been biochemically characterised as an SOD and is present in several chromate-resistant bacteria, such as *P. aeruginosa*, *O. tritici* 5bvl1 and C. metallidurans CH34. The results of this study allow us to conclude that the Cme-SOD protein belongs to a conserved superfamily (pfam02777) with a structure characteristic of iron-dependent SODs and has a conserved iron binding site among the members of this superfamily. In addition, the active site was identified, which is not completely evolutionarily conserved, suggesting that chromate-responsive SOD enzymes possess a novel active site. Knowledge of the structure of Cme-SOD will help us to deepen the understanding of this enzyme family, to better understand the chromate detoxification process and to develop biotechnological tools for bioremediation.

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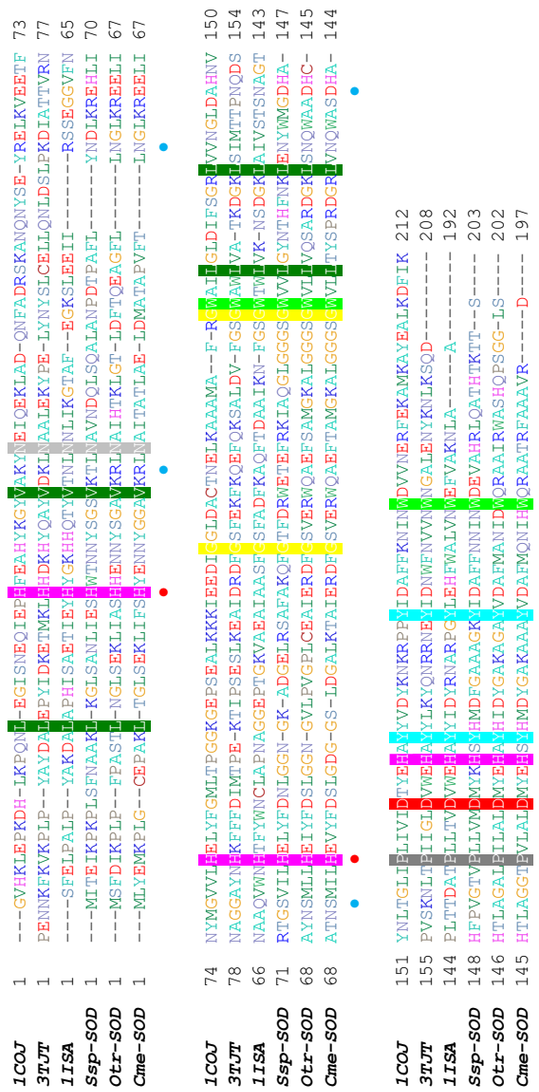

Figure 3 Sequence alignment of SOD proteins. Sequences of two SODs related to chromate resistance (ChrC) and three SODs that have been crystallised are compared. The sequences used were: Cme-SOD, SOD-ChrC from *Cupriavidus metallidurans* CH34 (accession number: CAC42412). Otr-ChrC, SOD-ChrC from *Ochrobactrum tritici* (accession number: ABO70324). Ssp-SOD, SOD-ChrC from *Shewanella* sp. ANA-3 (Accession No: WP_041413376). 1COJ, SOD from *Aquifex pyrophilus*. 3TJT, SOD of *Clostridium difficile* 630. 1ISA, SOD of *Escherichia coli*. Red ● marks the conserved residues forming the Fe-binding site. Blue ● indicates other residues forming the active site

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