

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/5816728>

# Insight into stability of CotA laccase from the spore coat of *Bacillus subtilis*

Article in *Biochemical Society Transactions* · January 2008

DOI: 10.1042/BST0351579 · Source: PubMed

CITATIONS

18

READS

39

4 authors, including:



**Eduardo Pinho Melo**

Universidade do Algarve

82 PUBLICATIONS 1,502 CITATIONS

[SEE PROFILE](#)



**Paulo Duraó**

Instituto Gulbenkian de Ciência (IGC)

35 PUBLICATIONS 519 CITATIONS

[SEE PROFILE](#)



**Ligia O Martins**

New University of Lisbon

102 PUBLICATIONS 3,034 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Biocatalysis for Tackling Lignin Recalcitrance [View project](#)



COST Action FP1306 - Valorisation of lignocellulosic biomass side streams for sustainable production of chemicals, materials & fuels using low environmental impact technologies [View project](#)

# Insight into stability of CotA laccase from the spore coat of *Bacillus subtilis*

E.P. Melo<sup>\*†‡</sup>, A.T. Fernandes<sup>‡</sup>, P. Durão<sup>‡</sup> and L.O. Martins<sup>‡</sup>

<sup>\*</sup>Instituto de Biotecnologia e Bioengenharia, Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal, <sup>†</sup>Instituto de Biotecnologia e Bioengenharia, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal, and <sup>‡</sup>Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2781-901 Oeiras, Portugal

## Abstract

The axial ligand of the catalytic mononuclear T1 copper site (Met<sup>502</sup>) of the CotA laccase was replaced by a leucine or phenylalanine residue to increase the redox potential of the enzyme. These mutations led to an increase in the redox potential by approx. 100 mV relative to the wild-type enzyme but the catalytic-centre activity  $k_{\text{cat}}$  in the mutant enzymes was severely compromised. This decrease in the catalytic efficiency was unexpected as the X-ray analysis of mutants has shown that replacement of methionine ligand did not lead to major structural changes in the geometry of the T1 centre or in the overall fold of the enzyme. However, the mutations have a profound impact on the thermodynamic stability of the enzyme. The fold of the enzyme has become unstable especially with the introduction of the larger phenylalanine residue and this instability should be related to the decrease in the catalytic efficiency. The instability of the fold for the mutant proteins resulted in the accumulation of an intermediate state, partly unfolded, in-between native and unfolded states. Quenching of tryptophan fluorescence by acrylamide has further revealed that the intermediate state is partly unfolded.

## Introduction

Laccases are members of the multicopper oxidase family of enzymes that includes ascorbate oxidase and ceruloplasmin [1,2]. These are potential biocatalysts for biotechnological applications [3,4], mainly owing to their high relative non-specific oxidation capacity, lack of a requirement for cofactors and the use of readily available oxygen as an electron acceptor. The initial substrate reaction products are dioxygen-centred radicals or cation radicals, which usually react further through non-enzymatic routes for the oxidative coupling of monomers or the degradation of polymers. Recently, we undertook a multidisciplinary study of the CotA laccase from *Bacillus subtilis* as a model bacterial laccase system. CotA laccase is a thermoactive and intrinsically thermostable enzyme [5]. The X-ray structure have shown that the enzyme has a typical three-domain laccase fold [6] with a T1 mononuclear copper centre in domain 3 and a trinuclear copper cluster (two T3 and one T2 copper ions) located between domains 1 and 3. T1 mononuclear copper site is the primary acceptor site for electrons derived from the reducing substrate. The trinuclear cluster acts in dioxygen binding and reduces the molecular oxygen to water upon receipt of four electrons forwarded from the mononuclear centre T1 [2,7,8]. The conserved co-ordinating amino acids

for the T1 copper site are two histidine residues and a cysteine residue, and the natural variations occur in the so-called axial position, most commonly a methionine. Fungal laccases have a non-co-ordinating phenylalanine or leucine residue at the axial position and these may contribute, at least in part, to the high redox potential observed in these enzymes. To test this hypothesis, we have performed site-directed mutagenesis of the methionine residue in the axial position of the T1 copper site of CotA (position 502) to leucine and phenylalanine residues and then analysed the effect of these mutations on the kinetics, redox potential and stability of the enzyme [9].

## Catalytic and redox properties of wild-type and mutant proteins

The site-directed replacement of Met<sup>502</sup>, an axial ligand of the T1 copper site, by the non-co-ordinating residue leucine and phenylalanine resulted in enzymes with the characteristic fold of the wild-type protein [9]. Overall, the geometry of the T1 copper centre is maintained in the mutant proteins displaying only small changes. The C<sup>β</sup> Leu<sup>502</sup> atom is located further way from the T1 copper and no longer co-ordinates with it, as observed in other laccases that have a leucine residue at that position. In the M502F mutant the phenylalanine residue is positioned further away from the copper atom and does not co-ordinate with it. The slight movement of the mutated residue towards the protein surface, and away from the T1 copper atom, leads to a concerted movement of this region, pushing it away towards the solvent and slightly increasing the exposure

**Key words:** *Bacillus subtilis* laccase, CotA laccase, fluorescence, multicopper oxidases, protein stability, redox potential.

**Abbreviations used:** GdmCl, guanidinium chloride; I, intermediate; N, native; NATA, *N*-acetyltryptophanamide; SGZ, syringaldazine; U, unfolded.

<sup>†</sup>To whom correspondence should be addressed, at Instituto de Biotecnologia e Bioengenharia, Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal (email emelo@ualg.pt).

of the copper centre. Copper content was determined by the trichloroacetic acid/bicinchoninic acid method [10] and confirmed by atomic absorption and revealed a stoichiometry close to 4 Cu per protein for both mutants. The wild-type enzyme previously produced with a stoichiometry of 2 Cu per protein [9] was now produced full loaded using a new production strategy (unpublished work) and therefore all the results presented in this paper were obtained with full loaded enzymes, with the obvious exception of apo-CotA.

Both mutations were successful regarding the increase in redox potential as described by Durão et al. [9] (Table 1). The replacement of the axial methionine residue by a leucine and a phenylalanine in the CotA laccase leads to an increase in the redox potential by approx. 100 and 60 mV respectively. It was not expected that the mutations would have major effects on the substrate-binding site, and indeed no major alterations regarding the  $K_m$  value for SGZ (syngaldazine; Table 1) and other substrates were observed [9]. Significant changes were found, however, for both mutants, in the values of  $k_{cat}$ . The M502L exhibits a 2.5-fold decrease and M502F a 1840-fold decrease in  $k_{cat}$ . Clearly, no direct correlation was found between the redox potentials and the oxidation rates of the substrate. Both mutants exhibited higher redox potentials than the wild-type and lower turnover rates. The parameter  $k_{cat}$  sets the lower limit to the first-order rate constant in the enzymatic reaction (reflects the rate-limiting step). For an electron-transfer reaction, its rate is mainly determined by the donor-acceptor electronic coupling, where the exact geometry of the protein matrix has an important role, the redox potential and the reorganization energy, which also depends on the structure and dynamics of the protein [11]. In the present case, we can hypothesize that mutations could have affected the electronic tunnelling and/or the reorganization energy of the electron-transfer processes to find possible reasons for discrepancies between  $k_{cat}$  values and redox potentials.

## Thermodynamic stability of wild-type and mutant proteins

The thermodynamic stability of CotA was assessed by fluorescence to evaluate the effect of copper and mutations on the overall stability of the tertiary structure (Figure 1). CotA has nine tryptophan residues (excited selectively at 296 nm) with percentages of exposure to water between 0 and 8% compared with a tryptophan residue in a standard Gly-Trp-Gly extended peptide [12]. This buried location is in accordance with the emission maximum of 331–333 nm observed for the native state of the three proteins presented in Figure 1. A maximum of 330–332 nm was assigned to tryptophan residues buried in non-polar regions of proteins [13]. Upon unfolding, the wavelengths at the emission maxima shifted to the red, reflecting clearly the exposure of tryptophan residues to the high polarity of water at the surface of the protein. The CotA structure is very stable, displaying a GdmCl (guanidinium chloride) concentration of 4.6 M at the midpoint (where 50% of the molecules

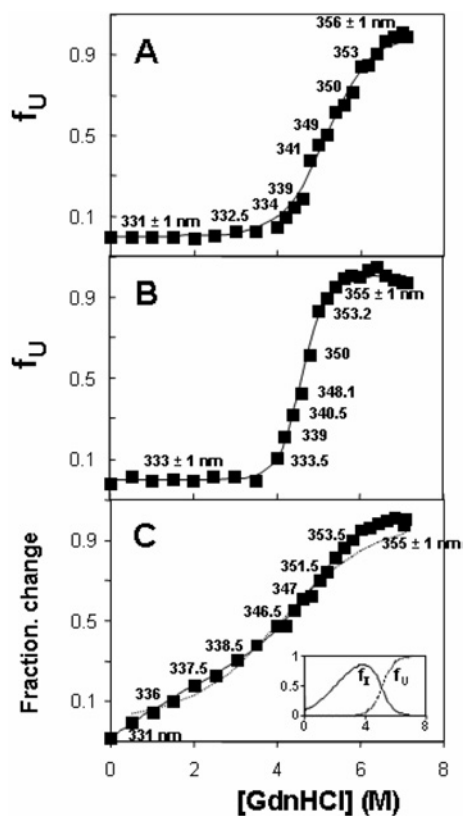
**Table 1 | Redox potential, kinetic and stability parameters of the wild-type and apo-CotA laccase and mutant enzymes**

1st, First transition, meaning from native to unfolded for the wild-type and apoprotein and from native to an intermediate '1' for mutants; 2nd, second transition, meaning from 1 to the unfolded state, which exists only for mutants. NA, no activity for the apo-CotA.

	$E^0$ (mV)	Kinetics with SGZ		Stability parameters					
		$K_m$ ( $\mu$ M)	$k_{cat}$ ( $s^{-1}$ )	$\Delta G_{1st}^{water}$ (kcal/mol)	$m_{1st}$ (kcal $\cdot$ mol $^{-1}$ $\cdot$ M $^{-1}$ )	Midpoint $_{1st}$ (M)	$\Delta G_{2nd}^{water}$ (kcal/mol)	$m_{2nd}$ (kcal $\cdot$ mol $^{-1}$ $\cdot$ M $^{-1}$ )	Midpoint $_{2nd}$ (M)
Wild-type	455	10 $\pm$ 1	18.4 $\pm$ 0.4	10.0 $\pm$ 0.1	2.2 $\pm$ 0.0	4.6 $\pm$ 0.1			
Apo-CotA	NA	NA	NA	6.5 $\pm$ 0.4	1.2 $\pm$ 0.1	5.1 $\pm$ 0.2			
M502L	548	9 $\pm$ 1	7.4 $\pm$ 2.2	1.4 $\pm$ 0.0	0.7 $\pm$ 0.1	1.9 $\pm$ 0.2	7.1 $\pm$ 0.6	1.4 $\pm$ 0.1	5.0 $\pm$ 0.0
M502F	515	8 $\pm$ 1	0.01 $\pm$ 0.003	1.4 $\pm$ 0.1	0.9 $\pm$ 0.1	1.6 $\pm$ 0.0	6.2 $\pm$ 0.5	1.3 $\pm$ 0.2	4.8 $\pm$ 0.2

**Figure 1 |** Unfolding of tertiary structure of apo- (A), wild-type (B) and mutant M502L CotA (C) induced by GdmCl as measured by fluorescence emission

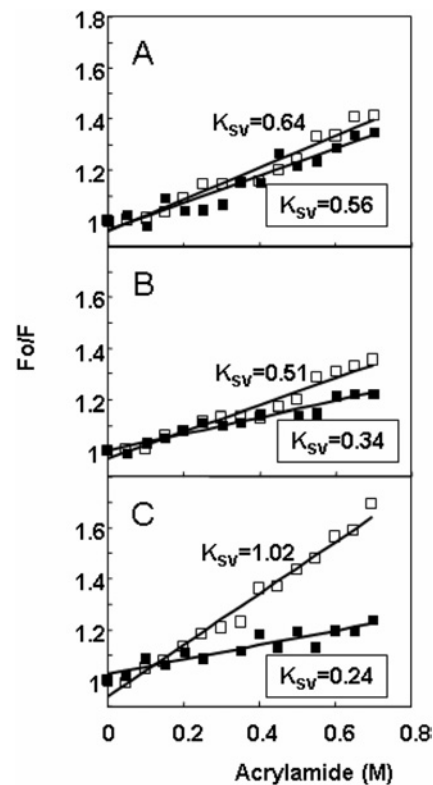
Numbers are the wavelengths at the fluorescence emission maximum. The continuous line in (A, B) is the fit according to the equation  $f_U = \exp(-\Delta G/RT) / (1 + \exp(-\Delta G/RT))$ , which assumes the equilibrium  $N \leftrightarrow U$ . The broken line in (C) is also the fit according to a two-state process and cannot describe the unfolding of the mutant protein. The solid line is the fit according to a three-state model ( $N \leftrightarrow I \leftrightarrow U$ ) using an equation described elsewhere [9]. The inset in (C) shows the accumulation of I and its disappearance to give the unfolded state and was calculated as described previously [9].



are unfolded), and the native state is more stable than the unfolded state by 10 kcal/mol ( $1 \text{ cal} \approx 4.184 \text{ J}$ ; Table 1). The apo-CotA is less stable at room temperature but as the transition is less co-operative ( $m$  value is lower) leading to a midpoint slightly larger. The T1 copper, at least, is bleached at lower GdmCl concentrations compared with the unfolding of tertiary structure [9] and therefore no strong stabilization by copper was expected for CotA wild-type. The unfolding process for the wild-type and apoprotein (Figures 1A and 1B) was accurately described according to a two-state process with native (N) and unfolded (U) states being the only ones that accumulate significantly ( $N \leftrightarrow U$ ). The mutations M502L and M502F have a profound impact on the stability of CotA. Only the stability of M502L is shown in Figure 1(C) but the mutant M502F shows basically the same pattern (Table 1). First, the unfolding of the tertiary structure is clearly a non-

**Figure 2 |** Acrylamide quenching of tryptophan fluorescence of apo- (A), wild-type (B) and mutant M502L CotA (C) in the absence (closed symbols) and presence of 2 M GdmCl (open symbols)

Quenching of fluorescence by acrylamide was fitted (solid line) by the Stern-Volmer equation ( $F_0/F = 1 + K_{SV}[Q]$ , where  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of acrylamide respectively,  $K_{SV}$  is the Stern-Volmer quenching constant and  $[Q]$  is the quencher concentration).  $F$  values were corrected to account for acrylamide absorbance [14].  $K_{SV}$  values are shown in the plots with the ones framed reporting the quenching in the absence of GdmCl.



two-state process. The accumulation of an intermediate (I) in-between N and U that occurs at low GdmCl concentration (at 1.9 M GdmCl, 50% of the molecules are in the I state) allows the accurate fit of the unfolding process. The native state is only 1.4 kcal/mol more stable than the I state, explaining its accumulation at low GdmCl concentration. Tryptophan residues are partially exposed to water in the I state as shown by the wavelength of the emission maximum, indicating that the I state is a partially unfolded state. The conversion from I into the unfolded state occurs only at high GdmCl concentrations and is thermodynamically more similar to the conversion of N into U in the wild-type and apoprotein.

Quenching resulting from collisional encounters between the fluorophore and quencher, which is called collisional or dynamic quenching, probes the accessibility of fluorophores and can give information on protein conformational dynamics. Quenching of tryptophan fluorescence by acrylamide was carried out for the wild-type, apo- and mutant M502L CotA and is shown in Figure 2. Assuming that acrylamide

quenching is dynamic, as it seems to be indicated by the linear Stern–Volmer plot, the  $K_{SV}$  constant reflects the accessibility of tryptophan residues to collisions with acrylamide [15]. A linear Stern–Volmer plot is generally also indicative of a single class of tryptophan residues, all equally accessible to acrylamide. Indeed all tryptophan residues in CotA are similarly buried as stated above. The  $K_{SV}$  constants of the analogue of a tryptophan residue totally exposed to water [NATA (*N*-acetyltryptophanamide)] is much larger (approx.  $25 \text{ M}^{-1}$  [16]) than the values reported for CotA (Figure 2). To our knowledge,  $K_{SV}$  values for proteins, even denatured proteins, never reach the values observed for NATA, which indicates that the polypeptide chain always impose some stereochemical limitations. A fully exposed residue in the polypeptide corticotropin has a  $K_{SV}$  value of  $13.5 \text{ M}^{-1}$  [17]. The  $K_{SV}$  values reported for the wild-type and mutant M502L are small, reflecting the buried position of tryptophan residues. The value for apo-CotA increases slightly relatively to the holoprotein pointing to a small degree of structure that might be inducible by copper binding. The most distinctive feature of acrylamide quenching is clearly the identification of the intermediate 'I' revealed during guanidinium denaturation experiments. In the presence of 2 M of GdmCl where the intermediate I accumulates significantly for the mutant protein only, the  $K_{SV}$  value increases 4.3-fold compared with 1.1- and 1.5-fold increases for the apoprotein and wild-type protein. Quenching by acrylamide indicates that the intermediate state I is partially unfolded, exposing tryptophan residues to collisions with acrylamide in accordance with the red-shift observed for the wavelengths at the emission maximum in Figure 1.

## Concluding remarks

The replacement of the weak so-called axial ligand of the T1 site of bacterial CotA laccase leads to an increase in the redox potential relative to that of the wild-type enzyme as expected based on fungal laccases. The role of the T1 site within the multicopper oxidases is related to the long-range intramolecular electron transfer, shuttling the electrons from the reduced substrate to the trinuclear centre, where  $\text{O}_2$  is reduced to water. X-ray structural comparison of M502L and M502F mutants with the wild-type CotA shows that the geometry of the T1 copper site is maintained as well as the overall fold of the proteins. However, the M502L mutant exhibits a 2.5-fold decrease in  $k_{\text{cat}}$  value for the substrate SGZ and the catalytic-centre activity in M502F is even more severely compromised with a 1840-fold decrease. Therefore no direct correlation was found between the redox potential and the oxidation rates. The thermodynamic stability of the mutant proteins and wild-type CotA has shown why such a correlation was not found. The mutations in the axial ligand

have a profound impact on the thermodynamic stability of the enzyme. While the unfolding of the tertiary structure in the wild-type enzyme is a two-state process displaying a midpoint at a GdmCl concentration of 4.6 M and a free energy exchange in water of 10 kcal/mol, the unfolding for both mutant enzymes is clearly not a two-state process. At 1.6 or 1.9 M GdmCl, depending on the mutant, half of the molecules are in an intermediate conformation, only 1.4 kcal/mol less stable than the native state. This intermediate state is a partly unfolded state with tryptophan residues partly exposed at the surface of the protein as revealed by collisional quenching of fluorescence by acrylamide. The instability introduced by leucine and phenylalanine in the fold of the CotA enzyme that led to the accumulation of the intermediate state at very low guanidinium concentrations has probably slightly changed the exact geometry of the protein matrix and the dynamics of the protein, affecting thus the rate of the electron-transfer reaction.

This work was supported by project grant POCl/BIO/57083/2004. We thank Dr Cláudio Soares from the Instituto de Tecnologia Química e Biológica for calculating the percentages of exposure to water of tryptophan residues.

## References

- 1 Messerschmidt, A. (1997) *Multi-copper Oxidases*, World Science Press, Singapore
- 2 Lindley, P.F. (2001) in *Handbook on Metalloproteins* (Bertini, I., Sigel, A. and Sigel, H., eds), pp. 763–811, Marcel Dekker, New York
- 3 Xu, F. (1999) in *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation* (Flickinger, M.C. and Drewin, S.W., eds), pp. 1545–1554, John Wiley and Sons, New York
- 4 Gianfreda, L., Xu, F. and Bollag, J.-M. (1999) *Bioremed. J.* **3**, 1–25
- 5 Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H. and Henriques, A.O. (2002) *J. Biol. Chem.* **277**, 18849–18859
- 6 Enguita, F.J., Martins, L.O., Henriques, A.O. and Carrondo, M.A. (2003) *J. Biol. Chem.* **278**, 19416–19425
- 7 Solomon, E.I., Sundaram, U.M. and Machonkin, T.E. (1996) *Chem. Rev.* **96**, 2563–2605
- 8 Stoj, C.S. and Kosman, D.J. (2005) in *Encyclopedia of Inorganic Chemistry*, Volume II (King, R.B., ed.), 2nd edn, pp. 1134–1159, John Wiley and Sons, New York
- 9 Durão, P., Bento, I., Fernandes, A.T., Melo, E.P., Lindley, P.F. and Martins, L.O. (2006) *J. Biol. Inorg. Chem.* **11**, 514–526
- 10 Brenner, A.J. and Harris, E.D. (1995) *Anal. Biochem.* **226**, 80–84
- 11 Bourbonnais, R., Leech, D. and Paice, M.G. (1998) *Biochim. Biophys. Acta* **1379**, 381–390
- 12 Chothia, C. (1976) *J. Mol. Biol.* **105**, 1–14
- 13 Burstein, E.A., Vedenkina, N.S. and Ivkova, M.N. (1973) *Photochem. Photobiol.* **18**, 263–279
- 14 Andrade, S.M. and Costa, S.M.B. (2002) *Biophys. J.* **82**, 1607–1619
- 15 Lakowicz, J.R. (1999) *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York
- 16 Merrill, A.R., Palmer, L.R. and Szabo, A.G. (1993) *Biochemistry* **32**, 6974–6981
- 17 Eftink, M.R. and Ghiron, C.A. (1977) *Biochemistry* **25**, 5546–5551

Received 2 July 2007