The role of Glu^{498} in the dioxygen reactivity of CotA-laccase from *Bacillus subtilis*†

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The multicopper oxidases couple the one-electron oxidation of four substrate molecules to the four electron reductive cleavage of the O–O bond of dioxygen. This reduction takes place at the trinuclear copper centre of the enzyme and the dioxygen approaches this centre through an entrance channel. In this channel, an acidic residue plays a key role in steering the dioxygen to the trinuclear copper site, providing protons for the catalytic reaction and giving overall stability to this site. In this study, the role of the Glu^{498} residue, located within the entrance channel to the trinuclear copper centre, has been investigated in the binding and reduction of dioxygen by the CotA-laccase from *Bacillus subtilis*. The absence of an acidic group at the 498 residue, as in the E498T and E498L mutants, results in a severe catalytic impairment, higher than 99%, for the phenolic and non-phenolic substrates tested. The replacement of this glutamate by aspartate leads to an activity that is around 10% relative to that of the wild-type. Furthermore, while this latter mutant shows a similar K_m value for dioxygen, the E498T and E498L mutants show a decreased affinity, when compared to the wild-type. X-ray structural and spectroscopic analysis (UV-visible, electron paramagnetic resonance and resonance Raman) reveal perturbations of the structural properties of the catalytic centres in the Glu^{498} mutants when compared to the wild-type protein. Overall, the results strongly suggest that Glu^{498} plays a key role in the protonation events that occur at the trinuclear centre and in its stabilization, controlling therefore the binding of dioxygen and its further reduction.

Introduction

CotA-laccase is a structural component of the outer layer of the spore coat from *Bacillus subtilis*,[1] and is thought to be involved in the synthesis of the brown pigment that protects the spore against UV light. Similarly to all the members of the multi-copper oxidase (MCO) family, it is able to couple one-electron oxidation of four substrate equivalents with the four-electron reduction of dioxygen to water.[2-5] The catalytic motif in these enzymes includes three distinct copper centres.[6] Type 1 (T1) Cu is characterized by an intense $S(\pi)\rightarrow Cu(d_{x^2-y^2})$ charge transfer (CT) absorption band at around 600 nm, responsible for the intense blue colour of these enzymes, and a narrow parallel hyperfine splitting [$A_{\parallel} = (43–90) \times 10^{-4}$ cm^{-1}] in the electron paramagnetic resonance (EPR) spectra. This is the site of substrate oxidation and in this respect the MCO family can be separated into two classes; enzymes that oxidize small organic substrates with high efficiency and those that oxidize metal ion substrates.[5] The trinuclear centre, where dioxygen is reduced to water, comprises two type 3 (T3) and one type 2 (T2) copper ions. The two T3 Cu ions, which are usually antiferromagnetically coupled through a bridging ligand and therefore EPR silent, show a characteristic absorption band at 330 nm. The T2 Cu site lacks strong absorption bands and exhibits a large parallel hyperfine splitting in the EPR spectra [$A_{\parallel} = (150–201) \times 10^{-4}$ cm^{-1}].

CotA-laccase has the dual advantage that it is both thermostable and thermoactive and is able to oxidise a variety of substrates including non-phenolic and phenolic compounds, and also a range of synthetic azo and anthraquinonic dyes, thus having a potential use for diverse biotechnological applications.[7-9] Several structure–function relationship studies have been performed with this enzyme.[10-13] These revealed redox properties of the T1 copper site and gave structural insights into the principal stages of the mechanism of dioxygen reduction at the trinuclear centre. Studies on the reduced and oxidized forms of the enzyme and in the presence of peroxide and the inhibitor, azide, gave significant structural insights into the principal stages of the mechanism of dioxygen reduction.[10] Thus, after diffusing through the solvent channel leading to the trinuclear centre, the dioxygen binds to the centre so that it is sited approximately symmetrically between the two type 3 copper ions with one oxygen atom close to the type 2 copper ion. Further stages involve the formation of a peroxide intermediate in which the O–O axis is inclined by some 51° to the vector linking the two type 3 copper ions and following the splitting of this intermediate, the migration of the hydroxide moieties towards the solvent exit channel. The migration steps are likely to involve a significant movement of the type 2 copper

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ion and its environment. In order to convert dioxygen to two molecules of water four protons are required in addition to the 4 electrons obtained from four individual substrate molecules. Careful comparison of crystal structures shows that MCOs have, with very rare exceptions, conserved negatively charged residues close to the trinuclear site, a glutamate or an aspartate within the entry channel for dioxygen, and an aspartate in the exit channels of the trinuclear centre.[10, 14-23] These residues may facilitate binding of dioxygen and supply of protons to the reaction intermediate as suggested by mutagenesis studies performed with CueO from *Escherichia coli* and yeast Fet3p metallo-oxidases.[24-28] It has already been suggested that Glu^{498} (Fig. 1) plays a crucial role in providing protons for the reduction of dioxygen in the bacterial laccase CotA[10] and further studies involving both structural and modelling data[29] have added strength to this proposition. In order to explore these putative roles in CotA-laccase, site directed mutagenesis has been employed to produce the mutants E498D, E498T and E498L. This is the first study that combines structural, spectroscopic and biochemical data in a (bacterial) laccase, and is complementary to what has been previously undertaken with the metallo-oxidases CueO and Fet3p.

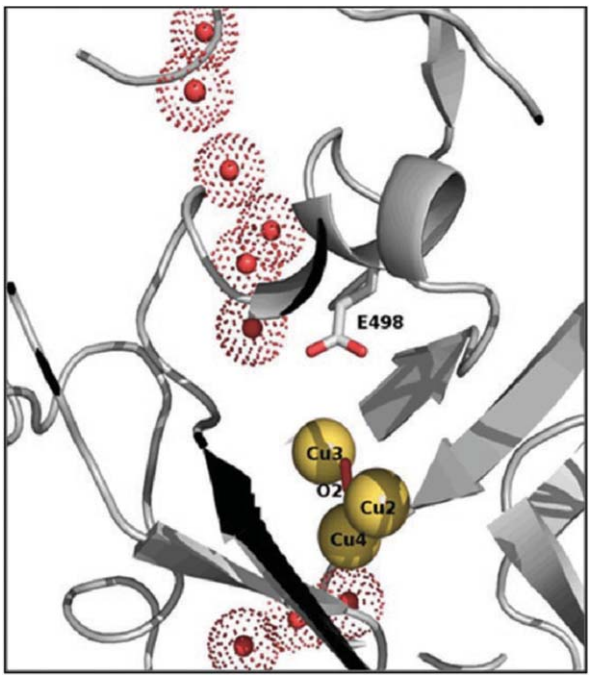

Fig. 1 Structural detail of the trinuclear Cu site in the native CotA laccase showing the Glu^{498} residue that forms a hydrogen bond with a water molecule in the access solvent channel to the trinuclear centre that in turn is hydrogen bonded to dioxygen.

Materials and methods

Construction of CotA mutants

Single amino acid substitutions in the trinuclear centre were created using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid pLOM10 (containing the wild-type cotA sequence) was used as a template[1] and the primers forward 5′-TGC CAT ATT CTA GAG CAT TTA GAC TAT GAC ATG-3′ and reverse 5′-CAT GTC ATA GTC TAA ATG CTC TAG AAT ATG GCA-3′ were used to generate the E498L mutant. The primers forward 5′-TGC CAT ATT CTA GAG CAT ACA GAC TAT G-3′ and reverse 5′-CAT AGT CTG TAT GCT CTA GAA TAT GGC A-3′ were used to generate the E498T mutation. Finally, the primers forward 5′-TGC CAT ATT CTA GAG CAT GAT GAC TAT G-3′ and reverse 5′-CAT AGT CAT CAT GCT CTA GAA TAT GGC A-3′ were used to generate the E498D mutant. The presence of the desired mutations in the resulting plasmids (pLOM53 (carrying the E498L point mutation), pLOM54 (bearing the E498T point mutation), and pLOM56 (with the E498D mutation) and the absence of unwanted mutations in other regions of the insert were confirmed by DNA sequence analysis. Plasmids pLOM53, pLOM54 and pLOM56 were transformed into *E. coli* Tuner (DE3) strains (Novagen) to obtain strains LOM408, LOM413 and LOM417, respectively.

Overproduction and purification

Strains AH3517 (containing pLOM10), LOM408, LOM413 and LOM417 were grown in Luria–Bertani medium supplemented with ampicillin (100 μg mL^{-1}) at 30 °C. Growth was performed as described by Durão *et al.*[30] according to the optimized protocol to yield a protein population fully-loaded with copper. Cell harvesting and disruption and subsequent purification of proteins by using a two-step protocol procedure were performed as previously described.[1,10] Purified enzymes were stored at –20 °C until use.

UV-visible, EPR and RR spectroscopy

UV-visible spectra were acquired using a Nicolet Evolution 300 spectrophotometer from Thermo Industries. EPR spectra (100 to 160 μM protein contents) were measured with a Bruker EMX spectrometer equipped with an Oxford Instruments ESR-900 continuous-flow helium cryostat, with a microwave frequency of 9.39 GHz, power of 2.0 mW and modulation amplitude of 0.9 mT. The spectra obtained under non-saturating conditions were theoretically simulated using the Aasa and Väangard approach.[31] RR spectra of 1 mM CotA wild-type, 2 mM E498T, 1.5 mM E498D and 0.8 mM E498L mutants (in 20 mM Tris buffer, pH 7.6) were measured as described before,[12] with 568 nm excitation, at –190 °C.

Redox titrations and enzyme assays

Redox titrations were performed at 25 °C in 20 mM Tris-HCl buffer pH 7.6 containing 0.2 mM NaCl under an argon atmosphere, and were monitored by visible spectroscopy (300–900 nm), using a Shimadzu Multispec-1501 spectrophotometer, as previously described by Durão *et al.*[11] The laccase-catalysed oxidation of ABTS (pH 4) and 2,6-dimethoxyphenol (2,6-DMP, pH 7) were photometrically monitored at 37 °C, in air saturated conditions by using the methods described by Durão *et al.*[11] The optimum pH value for the oxidation of both substrates is in the case of E498L and E498D similar to the wild-type, while the E498T mutant displays a 1.6 unit decrease in optimal pH for the oxidation of 2,6-DMP. Activities were also performed following the rate of oxygen consumption varying the concentrations of dioxygen at a fixed concentration of the reducing substrates (ABTS or 2,6-DMP)

Table 1 X-ray data collection[a]

	E498D	E498T	E498L
Beam line at ESRF	ID23-2	ID14-1	ID14-1
Wavelength/Å	0.8726	0.934	0.934
Detector distance/mm	223.2	193.3	162.0
Resolution/Å	2.1	2.0	1.7
Space group	$P3_12_1$	$P3_12_1$	$P3_12_1$
Cell parameters/Å, *a*	101.83	101.90	101.57
c	136.57	136.93	136.29
Mosaicity (°)	0.85	0.66	0.50
Oscillation angle (°)	1.0	0.5	1.0
Oscillation range (°)	65	60	60
No. of unique *hkl*	48362 (7000)	55879 (8055)	89089 (12978)
Completeness (%)	99.9 (100.0)	99.6 (99.7)	99.5 (100.0)
$I/\sigma(I)$	6.6 (2.0)	9.2 (2.2)	10.4 (2.4)
R_{symm}	0.083 (0.37)	0.069 (0.348)	0.053 (0.321)
Multiplicity	4.0 (4.0)	5.1 (3.6)	5.5 (4.6)

[a] Values in parentheses refer to the highest resolution shells as follows; E498D (2.21–2.10 Å); E498T (2.11–2.00 Å); E498L (1.79–1.70 Å).

by using an oxygen electrode (Oxygraph; Hansatech, Cambridge UK). Stock solutions of 10 mM of ABTS (pH 4) and 50 mM 2,6-DMP (pH 7 or pH 5.6) were prepared in Britton Robinson buffer. The oxygen electrode was calibrated at 37 °C using buffer saturated with air and buffer deoxygenated with sodium dithionite. The initial concentration of dioxygen in solution was controlled by flushing the reaction vessel with a mixture of nitrogen and oxygen with the aid of two flow meters. All the reactions were corrected for background autoxidation. The kinetic constants K_m and k_{cat} were determined using the Michaelis–Menten model (Origin-Lab, Northampton, MA, USA). All enzymatic assays were performed at least in triplicate. The protein concentration was measured by using the absorption band at 280 nm (ε_{280} = 84,739 M^{-1} cm^{-1}) or the Bradford assay using bovine serum albumin as a standard.[32]

Azide inhibition

Enzymes were incubated with sodium azide (0 to 100 mM, final concentration) for different periods of time (0, 30, 60, 120 min) followed by activity measurements towards ABTS oxidation (1mM) with wild-type (0.0001 mg ml^{-1}) and mutant enzymes (0.02 to 2 mg ml^{-1}) in Britton–Robinson buffer at the optimum pH value.

Crystallization

Crystals of the CotA mutant proteins were obtained by the vapour diffusion method, at room temperature, from crystallisation media containing 8–10% of PEG 4 K, 26–30% of isopropanol and 0.1 M of sodium citrate pH 5.5. The crystals were harvested and cryo-frozen with a solution that contained the crystallisation medium plus 22% of ethylene glycol.

X-ray data collection and refinement

X-ray diffraction data were collected at 110 K using the macromolecular crystallography beam lines as indicated in Table 1, at the European Synchrotron Radiation Facility at Grenoble, France. Data sets were processed and scaled using MOSFLM[33] and SCALA from the CCP4 program suite.[34] The structure solution for each CotA mutant was found by the molecular replacement method, using the MOLREP program.[35] The structure of CotA (PDB code: 1W6L)[10] from which all the copper atoms and solvent molecules were removed served as a search model. For each mutant a unique solution was determined; typical *R*-factors and scores were 0.40 and 0.74 respectively. Refinement was performed using the maximum likelihood functions in the REFMAC program,[36] while model building and improvement were achieved with COOT.[37] Solvent molecules were positioned after a few cycles of refinement as well as several molecules of ethylene glycol. Isotropic refinement of the atomic displacement parameter was performed for all atoms. The occupancies of the copper ions were adjusted so that their isotropic thermal vibration parameters refined approximately to those observed for the neighbouring atoms. The careful use of omit and standard difference Fourier syntheses, as well as monitoring of thermal vibration coefficients during refinement, enabled the identification of a diatomic species placed in between the type 3 copper sites in all three mutants. These species were assumed to be a dioxygen type species and refinement proceeded constraining the O–O distances to target values of 1.207 Å. Similarly to what has been observed for the native fully loaded CotA, additional electron density was observed close to the Cys 35 and this was modelled as an oxidised cysteine residue in two of the three mutants. In the E498T mutant two alternate conformations were modelled for the side chain of the OXY-Cys 35, whereas in the E498L mutant a mixture of Oxy-Cys and Cys was modelled according to the electron density map. In the E498D mutant no alteration was observed and a cysteine side-chain was modelled in two alternate positions. As observed in the other crystal structures of CotA, the loop comprising residues 89 to 97 was poorly defined in the electron density maps in all three mutants and was not modelled. The final statistics of the refinement procedure are listed in Table S1 in the ESI.†

Results

Kinetic characterization of mutant enzymes

Site-directed replacement of Glu^{498} by leucine, threonine or aspartate resulted in enzymes that show the same chromatographic pattern during purification when compared to the wild-type CotA-laccase. Metal analysis indicated that all "as isolated" proteins

Table 2 Apparent steady-state kinetic constants for ABTS and 2,6-DMP by the CotA proteins measured at saturating concentrations of O_2

	ABTS			2,6-DMP		
CotA	K_m/μM	k_{cat}/s^{-1}	k_{cat} /K_m/s^{-1}μM^{-1}	K_m/μM	k_{cat}/s^{-1}	k_{cat} /K_m/s^{-1}μM^{-1}
Wild type	110 ± 14	251 ± 30	2.3	227 ± 41	36 ± 5	0.16
E498D	61 ± 28	16 ± 2	2.6×10^{-1}	208 ± 46	1.3 ± 0.6	0.6×10^{-2}
E498T	120 ± 12	1.7 ± 0.6	1.4×10^{-2}	137 ± 28	0.05 ± 0.03	0.4×10^{-3}
E498L	106 ± 10	0.09 ± 0.03	0.6×10^{-3}	180 ± 4	0.006 ± 0.004	0.3×10^{-4}

Table 3 $K_{m\ app}(O_2)$ of wild-type and mutants measured with 1 mM of ABTS and I_{50} after a 30 min incubation period with sodium azide (similar values were obtained after 60 and 120 min of incubation)

CotA	$K_{m\ app}(O_2)$/μM	Azide I_{50}/mM
Wild type	25 ± 13	2.3 ± 0.0
E498D	19 ± 13	2.3 ± 0.0
E498T	58 ± 15	4.7 ± 0.2
E498L	92 ± 10	9.8 ± 0.1

contained around 4 moles of copper ions per mol of protein (with an associated 15% error, Table 2S, ESI†). Two reducing substrates, one non-phenolic (ABTS) and one phenolic (2,6-DMP), were used to identify specific changes in the catalytic properties of the studied mutant proteins. A comparison of the catalytic activities at the optimum pH shows that mutations in the Glu^{498} residue result in a lower catalytic efficiency when compared with the wild-type CotA (Table 2). Mutant E498L is the most affected in this respect showing virtually no enzymatic activity, with 3 to 4-orders of magnitude decreased efficiency (k_{cat}/K_m). The E498T mutant shows 2 to 3-orders of magnitude lower efficiency in relation to the wild-type. On the other hand, the E498D mutant shows catalytic efficiency closest to that of the wild-type strain, with a decrease between 10 and 45 times, depending on the substrate. Considering that only half of the E498D protein population has a fully loaded T1 site (see below), efficiency could be calculated as only 5 to 23 times lower than that of the wild-type. Neither of the mutations resulted in major and consistent alterations of K_m for the tested substrates and thus lower efficiency values are mainly due to a decrease of k_{cat} values.

The Michaelis–Menten constants for dioxygen reactivity (K_m (O_2)) of wild-type and mutants at the optimal pH are shown in Table 3. The K_m (O_2) value measured for the wild-type and the E498D mutant was similar and, at the same time close to the values reported for other laccases.[38] The mutants E498T and E498L exhibited around 2 to 4-fold lower values, respectively, suggesting a different binding interaction with O_2 in the steady state. The use of ABTS or 2,6-DMP as the reducing substrate did not significantly affect the K_m (O_2) (data not shown). The k_{cat} (O_2) values are similar to the k_{cat} (ABTS) or k_{cat} (2,6-DMP) (Table 2), as anticipated for a steady state with efficient coupling of the two redox halves of the catalysis. The ability of the trinuclear cluster to bind molecular oxygen, and to retain its partially reduced forms during the catalytic cycle is reflected in the relatively high affinity of this site for a number of anions.[4] Azide is one of the most studied laccase inhibitors and in the CotA-laccase crystal structure soaked with azide, this molecule bridges the type 3 copper ions.[10] The inhibition of CotA and Glu^{498} mutants was followed in the presence of increasing concentrations of sodium azide. Total inhibition was observed at a concentration of 80 mM sodium azide, after a preincubation period of 30 min. For the wild-type enzyme and E498D mutant similar I_{50} values (concentration of inhibitor required for achieving 50% reduction of the activity) were measured, while the E498T and E498L mutants show 2 and 4-fold higher I_{50} values as compared with CotA-laccase (Table 3).

Spectroscopic characterization of Glu^{498} mutants

The UV-visible absorption spectrum of wild-type CotA is dominated by two charge transfer (CT) bands; a intense band centred at 609 nm originating from the T1 site and a shoulder at 330 nm associated with the T3 site, both with ε values around 4000 M^{-1} cm^{-1} (Fig. 2 and Table S2, in the ESI†). All mutants in their as-isolated state appear to be partially reduced, as after treatment with potassium iridate an increase of intensity of both absorption bands was observed. Nevertheless, after the oxidation step, the maximal absorption intensity at 609 nm was lower than in the wild-type, by 55% for E498D, 27% for E498T and 10% for E498L, suggesting some T1 copper depletion in all mutant proteins. Mutants E498T and E498D seem to be fully reactive with dioxygen, since transition bands at 609 and 330 nm, rapidly recover after exposure to air, from dithionite-reduced proteins, in a similar manner as observed for the wild-type enzyme. However, the mutant E498L remained in the reduced state even after being exposed to the air for 24 h, revealing highly impaired reactivity towards dioxygen.

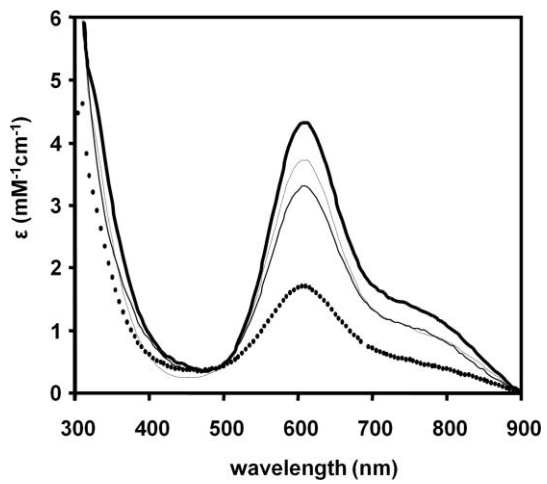

Fig. 2 UV-visible absorbance spectra of wild-type (thick line), and CotA mutants: E498L (semi-dotted line), E498T (thin line) and E498D (dotted line). Proteins were treated with the oxidizing agent potassium iridate.

The S(Cys)-Cu vibrational modes of the T1 site are selectively enhanced in RR spectra obtained by excitation into

Table 4 EPR parameters used in the simulation of wild-type and CotA mutants spectra

CotA	Cu centres	Contribution	g_{min}	g_{med}	g_{max}	A_{max} (x 10^{-4} cm^{-1})
Wild type	T1	1	2.042	2.046	2.228	78
	T2	1	2.035	2.094	2.250	195
E498D	T1	0.5	2.042	2.047	2.230	78
	T2	1	2.035	2.094	2.245	210
	T2′	1	2.035	2.094	2.330	110
E498T	T1	1	2.042	2.046	2.235	75
	T2′	1	2.035	2.094	2.325	124
E498L	T1	1	2.042	2.046	2.225	78
	T2	1	2.035	2.090	2.250	190

$S(\pi) \rightarrow Cu(d_{x^2-y^2})$ CT[4]. The spectra of the wild type, E498T and E498D mutants show several vibrational modes with stretching character centred at 400 cm^{-1}, with some subtle differences (Fig. 3). The most intense band, which appears to have the greatest Cu–S(Cys) stretching character, is downshifted from 425 cm^{-1} to 417 cm^{-1} in the E498L mutant. However, the intensity weighted frequency of all stretching coordinates for all mutant proteins (409 cm^{-1}) is very similar to that of the wild type (410 cm^{-1}), revealing an absence of substantial differences on the level of electronic configuration of the T1 site.[12] Accordingly, all mutants present similar redox potentials of the T1 Cu site of the wild-type enzyme (around 525 mV, Table S2 in the ESI†). Moreover, the signal to noise ratio is much lower in the spectrum of E498D, under the same experimental conditions and comparable protein concentration, indicating that this mutation decreased the stability of the T1 site.

The EPR spectra show strong similarities between wild-type CotA and E498L mutant, while the E498T and E498D mutants spectra show remarkable differences (Fig. 4). Spectral deconvolution (data not shown) revealed that the copper T1 site in the latter mutants has the same conformation as in the wild-type protein, since it was simulated using the same spin Hamiltonian parameters (Table 4). However, for the E498D mutant, depletion in the T1 site has been observed as revealed by spectral integration, indicating that only half of the E498D population contains copper at this site. Nonetheless, the EPR spectra reveal the most striking differences between the mutants at the T2 copper site. In the case of E498T, the g_{max} for this centre is higher than in the wild-type, while the hyperfine constant is lower, indicating that the copper T2 site in this mutant has a different conformation or experiences a different electronic vicinity (named the T2′ form). Interestingly, both forms of the type 2 Cu, the one observed for the wild-type and E498L (T2) and that of E498T (T2′), are present in the E498D mutant. This protein spectrum can be simulated using the parameters of the T2 and T2′ forms, integrated in a 1 : 1 ratio. A careful inspection of the EPR spectrum of the wild-type CotA reveals the presence of a very small signal identical to that assigned to the T2′ form (Fig. 4).

Structural details of the mutant enzymes

The overall structure of the Glu^{498} mutant CotA proteins is essentially the same as the one obtained for the wild-type enzyme. Superposition of the backbones of each of the mutants onto the fully-copper loaded CotA structure[34] gives root mean square deviations of only 0.131 Å, 0.124 Å and 0.131 Å for E498D, E498L and E498T mutants, respectively. The observed differences are located at the copper centres and in their neighbourhood. In the E498D mutant the orientation of the side chain of the mutated aspartate is different from the one observed for the native glutamate residue. In the native CotA-laccase the glutamate is pointing towards the solvent channel, forming a H-bond with a water molecule that interacts with the dioxygen moiety found in between the two T3 copper ions (Fig. 5A). In the E498D mutant, the aspartate points towards the interior of the protein having its $O^{\delta 1}$ atom at an H-bond distance from the $N^{\delta 1}$ atom of His^{491} (Fig. 5B). In this mutant, the water molecule interacting with the dioxygen moiety in the native structure no longer exists. Moreover, all copper atoms in the trinuclear centre are fully occupied, as observed in the wild-type enzyme (Table S3 in the ESI†). However, the occupancy of the T1 copper site in E498D is significantly less than one (0.7), corroborating the UV-Vis, RR and EPR data, although at first sight there appears to be no obvious structural reason for this finding. In the E498T mutant containing a polar residue with a shorter side-chain than the native glutamate, no difference was found in copper occupancy in the type 1 copper centre, but a decrease of occupancy to 0.8 was observed for all the copper ions in the tri-nuclear centre (Fig. 5C). The same was observed for the E498L mutant (Fig. 5D). This effect was even more pronounced for the type 2 copper ion, which refined to an occupancy of only 0.3. In all three mutants a dioxygen moiety was modelled in between the two type 3 copper ions and refined with the same occupancy that was observed for the type 3 copper ions (Fig. 5).

Discussion

This study aimed to address the role of residue Glu^{498} (Fig. 1) present in the second coordination sphere of the trinuclear centre of CotA-laccase. Three mutant enzymes were designed, expressed and purified. Their catalytic properties were determined, and furthermore their structural differences were addressed by spectroscopic techniques and X-ray crystallography. All MCO appear to have at least one acidic residue (Glu^{498} in CotA) accessible to the entrance channel by which dioxygen reaches the trinuclear centre. Site-directed mutagenesis studies performed with CueO from *E. coli* and yeast Fet3p indicated that Glu506 and Glu487 (equivalent to Glu^{498} in CotA) participate in the proton assisted reductive cleavage of the O–O bond at the trinuclear site, respectively.[24,26] Other mutagenesis studies have indicated that an aspartate (corresponding to Asp^{116} in CotA-laccase) located at the "outersphere" of the trinuclear centre, also plays a role in channelling protons in the O–O bond cleavage process.[24-28] The

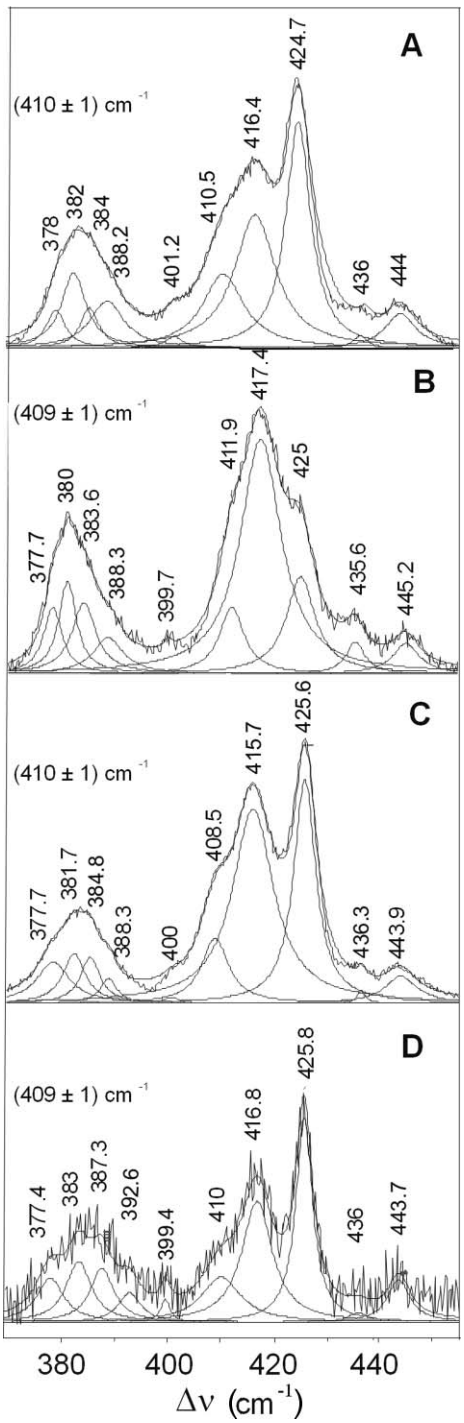

Fig. 3 Experimental and deconvoluted RR spectra of wild-type CotA (A), E498L (B), E498T (C) and E498D (D) mutants obtained with 568 nm excitation and 5 mW laser power at 77 K, accumulation time 40 s. The calculated intensity weighted frequencies are reported by the respective spectra.

carboxylic moiety of this aspartate residue, is hydrogen bonded to a water molecule which, in turn, is hydrogen bonded to the water molecule that interacts with T2 copper. In the CotA laccase, Glu^{498} is hydrogen bonded to a water molecule that is located within the hydrogen bonded distance of the dioxygen moiety observed almost symmetrically positioned in between the type 3 copper

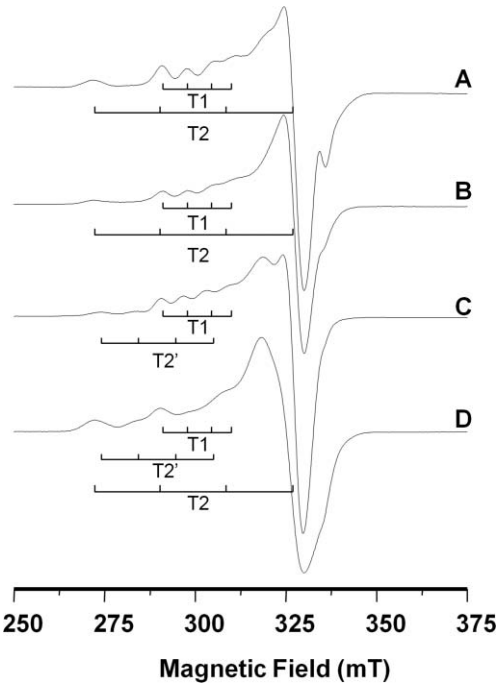

Fig. 4 EPR spectra of the wild-type (A), E498L (B), E498T (C) and E498D (D), obtained at 10 K and microwave frequency, 9.39 GHz; power, 2.0 mW; modulation amplitude: 0.9 mT. The spectra were simulated using the spin Hamiltonian parameters presented in Table 4.

ions.[10] Simulations of equilibrium protonation using structures of CotA indicated that Glu^{498} is involved in channelling protons for the reaction of reduction of dioxygen to water.[29] Therefore, mutation of this residue by aspartate, threonine and leucine has been undertaken to access its role in channelling protons to the trinuclear centre, in the stabilization and binding of dioxygen to this centre.

The E498D mutant is the only mutant that retains enzymatic activity, showing a similar dioxygen-binding affinity and azide inhibition when compared with the wild type enzyme. The orientation of the side chain of the mutated aspartate is different from that observed for the native glutamate residue and E498T and E498L mutants. However, kinetic data show that the shorter side chain carboxylate is still able, even with a lower efficiency, to assist the reductive cleavage of the O–O bond. On the other hand, when the glutamate is mutated to threonine or to leucine, residues without proton donating ability, a severe catalytic impairment, higher than 99%, is observed. These results indicate that protons from the bulk solvent, supplied through channels leading to the trinuclear centre,[10] without the final assistance of Glu^{498}, are insufficient to provide the needed protons for the catalytic process. Moreover, they also suggest the absence of alternative pathways participating in the transfer of protons to the trinuclear centre of CotA-laccase.

A perturbation of the T1 copper centre in the E498D mutant was observed by spectroscopic and crystallographic data. The different orientation of the aspartate residue, with its $O^{\delta 1}$ atom at a H-bond distance from the $N^{\delta 1}$ atom of His^{491}, is likely to cause destabilisation of the type 1 copper site, by opening this centre and promoting its depletion, as observed experimentally. In fact, His^{491} is coordinated to one of the two T3 copper ions in the tri-

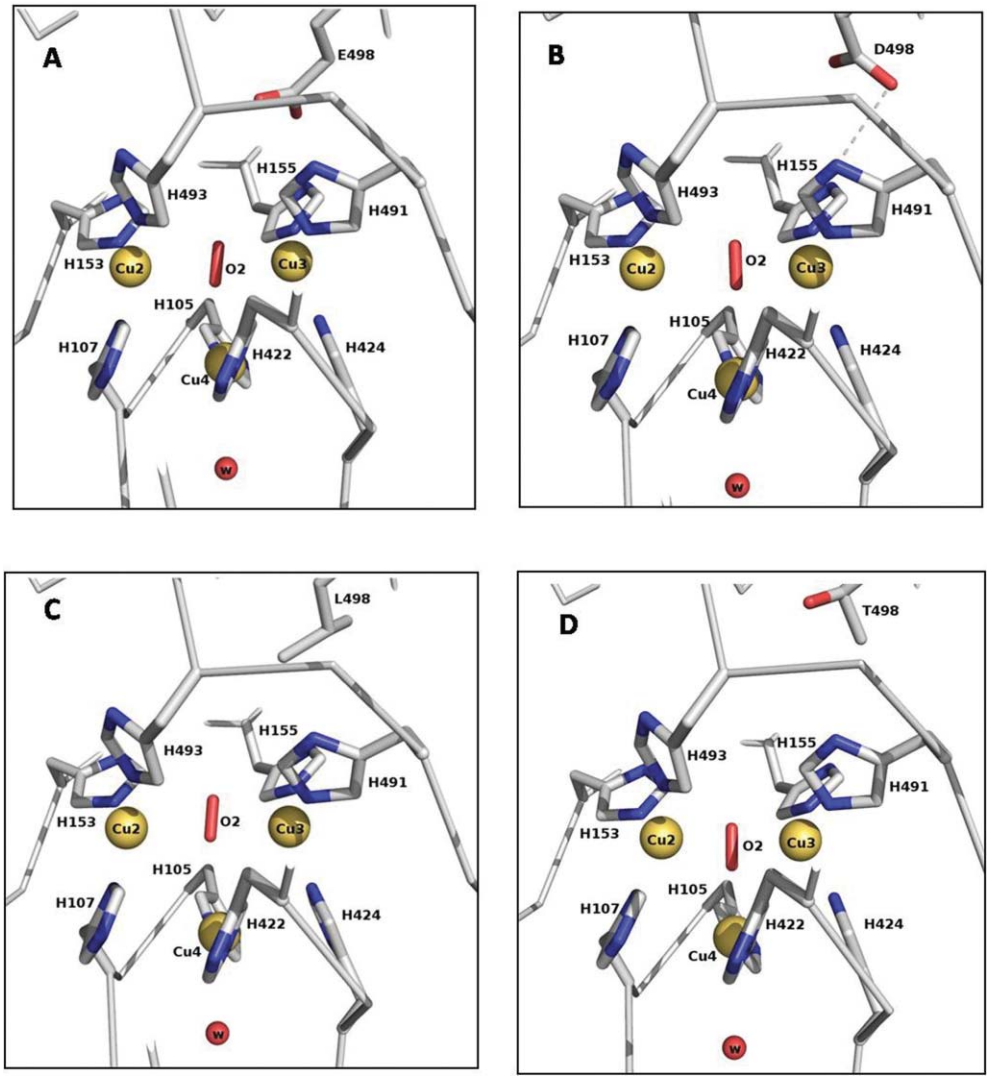

Fig. 5 Structural detail of the trinuclear centre and its neighbourhood in CotA (A), in E498D (B), in E498T (C) and in E498L (D) mutants.

nuclear centre (Cu3) while the targeted residue 498 is adjacent to His[497] which coordinates directly to the type 1 copper atom. In the crystal structure of the E498D mutant no significant difference in the bond length between the His[497] residue and the copper atom in the type 1 centre was observed. However, a 2 fold-lower K_m was measured in this mutant for the reducing substrate ABTS (Table 2). This finding could be indicative of subtle alterations near His[497], a residue suggested to be involved in both ABTS binding and electron transfer to the T1 Cu site of CotA-laccase.[13]

The geometry of the trinuclear centre of Glu[498] mutants is essentially the same as that found previously in the native structure (see for example ref. 10) except for the lower occupancy at T3 and T2 centres in the E498T and E498L mutants (Table S3 ESI†). However, the EPR spectra of the E498L mutant are similar to those of the wild-type in apparent contradiction with the X-ray results. It is possible that this discrepancy results from a destabilising effect of the mutation at the trinuclear centre making it more prone to losing copper during crystallization. Some heterogeneity of the T2 species in E498D and E498T mutants are revealed in the EPR spectra. The nature of the "new" T2′ form is not clear at this point. It can be due to direct structural (or conformational) alterations caused by the mutations or alternatively, it could result from an intermediate state stabilized by the mutations, scarcely detected in the wild-type protein. The existence of different forms of type 2 copper was also observed in the case of bilirubin oxidase where an aspartate residue close to that site has been mutated.[25] The partial reduction of the dioxygen moiety and/or the simultaneous presence of a hydroxyl and a water molecule bound to the T2 centre, could give rise to two types of centre. The resolution of the X-ray crystal data does not, however, permit any significant structural insights in the geometry of the T2 copper centre that would confirm the spectroscopic data. Nevertheless, there has to be some flexibility at this centre to allow the water molecules, formed by the reduction of dioxygen, to access the exit channel. The concomitant movement of the T2 copper may be reflected in the EPR data.

The present study unequivocally indicates that the electrostatic environment of the trinuclear centre of CotA-laccase is influenced by the presence of Glu[498]. The lack of reactivity of the E498L and E498T mutants towards dioxygen confirms that Glu[498] plays

an important role in channelling the protons to the mechanism of dioxygen reduction and that no other alternative pathway is observed, as suggested previously.[29] Furthermore, the replacement of this residue by an aspartate, allowing the enzyme to retain part of the catalytic activity, also corroborates these findings. The kinetic data additionally suggest that Glu^{498} is involved not only in the dioxygen reduction catalytic step(s) but also in the binding of dioxygen and presumably, of peroxide. Further research is in progress to provide more insight into the dioxygen reduction mechanism in CotA-laccase.

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