Biochemical and spectroscopic characterization of catechol oxidase from sweet potatoes (*Ipomoea batatas*) containing a type-3 dicopper center

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Abstract Two catechol oxidases have been isolated from sweet potatoes (*Ipomoea batatas*) and purified to homogeneity. The two isozymes have been characterized by EXAFS, EPR-, UV/Vis-spectroscopy, isoelectric focusing, and MALDI-MS and have been shown to contain a dinuclear copper center. Both are monomers with a molecular mass of 39 kDa and 40 kDa, respectively. Substrate specificity and NH_2-terminal sequences have been determined. EXAFS data for the 39 kDa enzyme reveal a coordination number of four for each Cu in the resting form and suggest a Cu(II)-Cu(II) distance of 2.9 Å for the native met form and 3.8 Å for the oxy form.

Key words: Catechol oxidase; Dicopper enzyme; EXAFS; N-terminal sequence; *Ipomoea batatas*

1. Introduction

Hemocyanin and tyrosinase are the two prominent members among the dicopper proteins containing a single type-3 center in their active site [1–7]. In the met form they are characterized by an antiferromagnetically coupled EPR-silent Cu(II) pair and weak d-d transitions at 600 to 700 nm. A typical Cu(II) EPR signal appears only after denaturation of the protein. After binding of dioxygen two intensive absorption maxima at $\lambda \approx 345$ nm ($\varepsilon \approx 19\,000$ cm^{-1} M^{-1}) and $\lambda \approx 600$ nm ($\varepsilon \approx 1000$ cm^{-1} M^{-1}) are observed, due to $O_2^{2-} \rightarrow$ Cu(II) charge transfer transitions [8–11].

While hemocyanins are oligomeric oxygen transport proteins in molluscs and arthropods, tyrosinases [12,13] from fungi and vertebrates catalyze the initial step in the formation of the pigment melanin from tyrosine. Thus tyrosinases reveal two catalytic activities: (a) the hydroxylation of monophenols like tyrosine to *o*-diphenols (cresolase activity) and (b) the oxidation of *o*-diphenols (catechols) like DOPA to *o*-quinones (catecholase activity). Spectroscopic studies suggest that the catalytic dicopper site is similar to that of hemocyanin which has been structurally characterized by X-ray crystallography [14,15]. EXAFS studies of *Neurospora crassa* tyrosinase revealed a Cu(II)-Cu(II) distance of 3.6 Å for the oxy species and of 3.4 Å for the met form [16], respectively. Whereas hemocyanin has identical sets of ligands for both Cu ions evidence for an asymmetric ligand environment for the metal ions in tyrosinase has been obtained from kinetic data of the reconstitution reaction of the apoenzyme with Cu(II) [17].

Catechol oxidases, in contrast to tyrosinases, catalyze exclusively the oxidation of catechols to the corresponding *o*-quinone, without acting on monophenols. The resulting highly reactive quinones auto-polymerize to form brown polyphenolic catechol melanins, a process thought to protect the damaged plant from pathogens or insects [18–20]. Although the high specificity of this reaction is of great importance in the medical diagnosis for the determination of hormonal catecholamines (adrenalin, noradrenaline, and dopamine) only little is known about catechol oxidases. In this paper we describe the isolation, purification and spectroscopic characteristics of two monomeric catechol oxidase isozymes from *Ipomoea batatas* (ibCO).

2. Materials and methods

2.1. Chemicals and plant material

All chemicals used were of analytical grade and purchased from Sigma or Merck. Chromatographic media used were Sephacryl S-200 HR, Sephadex G-25, Sepharose DEAE-FF, Blue Sepharose CL-6B, Superdex 75 (Pharmacia Biotech, Sweden), and Macro-Prep Ceramic Hydroxyapatite (40 μm) (Bio-Rad Laboratories, USA). All buffers used in this study were degassed and adjusted to their respective pH values at 25°C. All column chromatography procedures employed an FPLC system (Pharmacia) at 20°C. Mature tubers of sweet potatoes, *Ipomoea batatas* (Bushbuck, South Africa) were purchased at a local market.

2.2. Enzyme purification

Diced potato tubers (5 kg) were homogenized in 2 l isolation buffer (50 mM NaAc, 0.1 M NaCl, 0.5% sodium ascorbate, pH = 6.0) using a blender. In order to precipitate low molecular mass phenolic compounds 200 g polyvinylpolypyrrolidone (PVP, Sigma, P 6755) were added. The homogenate was squeezed through cheese cloth. All following precipitation steps were performed at 4°C.

Solid $(NH_4)_2SO_4$ (25 g/100 ml solution) was added to the extract to 35% saturation. After 30 min the precipitate was removed by centrifugation (10 000 × *g*, 4°C, 90 min) and $(NH_4)_2SO_4$ was added to the supernatant to 85% saturation. The precipitate was collected by centrifugation (10 000 × *g*, 4°C, 90 min) after 60 min and redissolved in 2 l of isolation buffer. $(NH_4)_2SO_4$ was added to the solution up to 35% saturation prior to lowering the pH to 4.0 by adding 2 N HCl. After stirring for 10 min, the precipitate was removed by centrifugation (14 000 × *g*, 4°C, 20 min). The resulting supernatant was adjusted to pH 6.0 by 0.2 M NaOH and brought to 85% saturation by the stepwise addition of $(NH_4)_2SO_4$. After 60 min a brown-colored pellet was collected by centrifugation and redissolved in 50 mM NaAc buffer, pH 6.0, containing 0.5 M NaCl.

This solution was loaded on a Sephacryl S-200 HR column (5 × 100 cm, flow rate 5 ml/min) equilibrated with the same buffer. The fractions showing catecholase activity were pooled and concentrated with an ultrafiltration cell. After buffer exchange by gel filtration on a Sephadex G-25 column (50 mM sodium phosphate, pH 7.5), fractions from 20 kg potato tubers were pooled and the resulting solution was

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Abbreviations: CT, charge transfer; DOPA, 3,4-dihydroxyphenylalanine; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; ibCO, catechol oxidase from *Ipomoea batatas*; MALDI-MS, matrix assisted laser desorption/ionization mass spectrometry; XANES, X-ray absorption near edge structure

In memoriam to Prof. Dr. Dr. H. Witzel.

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applied to a pre-equilibrated DEAE-FF Sepharose column. Both isozymes were eluted separately with a gradient of increasing NaCl concentration up to 1 M. After this step, the isozymes were purified separately using the following procedure.

After concentration by ultrafiltration the pooled fractions were separately applied to a hydroxyapatite (40 μm) column (1.3×12 cm) equilibrated with 60 mM sodium phosphate, pH 7.0. The bound proteins were eluted with a linear gradient of 60–600 mM sodium phosphate, pH 7.0 (total buffer volume: 200 ml).

The concentrated hydroxyapatite fractions were diluted with 50 mM NaCl, 0.01 M NaAc buffer (pH 6.0) to reduce the ionic strength of the solution. The proteins were injected on a pre-equilibrated Blue Sepharose column (1×10 cm) and eluted using a linear gradient up to 2 M NaCl in the same buffer, after washing with 50 ml of the same buffer.

Final buffer exchange was performed with a Superdex 75 HR(16/60), using 0.5 M NaCl, 50 mM sodium phosphate buffer at pH 6.7.

2.3. Enzyme assay and determination of concentration

Catechol oxidase activity was measured at a substrate concentration of 3 mM catechol in 0.1 M sodium phosphate pH = 6.5 at 20°C by monitoring the increase of absorbance at 420 nm. One unit (U) of enzyme activity was defined as the amount which increased the absorbance by 1 per min [21]. The kinetic parameters were determined at a protein concentration of 2 nM using Lineweaver-Burk plots [22] for determination of K_M and V_{max}.

For the determination of the pH optimum the following buffers were used: 0.1 M sodium phosphate (pH = 3.0–10.3), 0.1 M NaAc (pH = 3.4–5.8), 0.1 M glycine/NaOH (pH = 8.9–10.3).

In order to determine the pH stability 5 μl (13.3 U) catechol oxidases were incubated in 495 μl sodium phosphate (pH = 1.5–13.8) for 1 h, 2.5 h and 20 h. After incubation 20 μl were taken from the solution to measure the enzymatic activity.

The concentration and the extinction coefficients at 280 nm of the pure proteins were determined using the Bradford method [23]. The Warburg and Christian method [24] was used for the estimation of protein concentrations in partially purified protein solutions.

2.4. Spectroscopic and analytical methods

UV-visible absorbance spectra of CO samples were monitored at room temperature in 0.5 M NaCl, 50 mM sodium phosphate pH 6.7 using a Shimadzu UV-1601PC spectrophotometer interfaced to a personal computer. For the studies on the peroxo complex the concentration of ibCO was 0.01 mM and amounts of 1 eq., 2 eq., 3 eq., 4 eq. and 6 eq. of 10 mM H_2O_2 were added to the sample.

EPR spectra were recorded with a Bruker ESP 300 spectrometer at 77 K with a microwave frequency of 9.37 GHz and a modulation frequency of 100 kHz. The enzyme concentration was 10^{-4} M in 200 μl of 0.5 M NaCl, 50 mM sodium phosphate buffer at pH 6.7. Denaturation was achieved by addition of 100 μl 6 M HCl.

SDS-PAGE (12%, polyacrylamide) was performed according to a modification of the Laemmli method [25]. Molecular mass markers ranging from 14 400 to 94 000 Da (Pharmacia Biotech, Sweden) and Coomassie staining were used.

The isoelectric point was determined by analytical IEF Phast System electrophoresis (Pharmacia Biotech, Sweden) using the p*I* calibration kit from Pharmacia as marker.

MALDI-MS was performed on a reflectron-type time-of-flight mass spectrometer (Lamma 1000, Leybold Heraeus) according to Hillenkamp et al. [26,27] by S. Hahner, Institut für Medizinische Physik, Universität Münster. The concentrations of the proteins were 1–10 μM in double-distilled water. 0.01 M 2,5-dihydroxybenzoic acid was used as ultraviolet-absorbing matrix. The laser emission wavelength was 337 nm.

The NH_2-terminal sequences of desalted native protein samples were determined using automated Edman degradation (B. Schedding, Inst. für Physiologische Chemie, Universität Münster).

2.5. EXAFS measurements

A highly purified sample of 39 kDa catechol oxidase in 0.5 M NaCl, 50 mM sodium phosphate at pH 6.7 was concentrated by ultrafiltration with membrane cones (Amicon, 25 kDa cut off) until a concentration of $c_{Cu} = 3\times10^{-3}$ mol/l was reached. 10% glycerol was added to prevent aggregation and water crystallite formation. The sample was enclosed in appropriate cells (15×10×1 mm) with capton foil windows.

The X-ray absorption spectra at the Cu-K edge were recorded at the Synchrotron Radiation Source (Daresbury, UK) at beamline 8.1. Data collection was performed at 2 GeV and a maximum current of 325 mA using a Si(220) double crystal monochromator. Harmonic rejection was accomplished by detuning the first monochromator crystal by 80%. The spectra were recorded at 80 K in fluorescence mode over the energy range from 8870 to 9530 eV using a 13-element Ge solid state detector [28]. Approximately 20 spectra were recorded, checked individually and finally averaged. Sample integrity before and after the experiment was proved by testing the enzyme activity which was not affected by irradiation. No shift in the edge position was observed between the first and the last scan.

The background subtraction, the normalization of the spectra, the determination of the edge position, and the extraction of the fine structure have been performed as described earlier [29]. An E_0 value of 8982.0 eV has been used for the data reduction. The $\chi(k)$ values were multiplied by k^3 and Fourier transformed. Prior to curve fitting analyses, the individual peaks in these functions were back-transformed into single-shell EXAFS spectra according to the Fourier filtering approach (transformation range first shell: native 0.8–2.0 Å; oxy 1.0–1.9 Å; higher shells: transformation range: native I: 2.2–2.8 Å; native II: 3.2–4.0 Å; oxy: 2.9–3.9 Å).

In order to include the multiple scattering effects of the histidine ligands, we also used wide shell filtered spectra [30] (transformation range: native: 0.6–4.0 Å; oxy: 0.9–3.9 Å). The final data analysis was carried out by least-squares curve fitting using a full curved wave approach from the program EXCURV92 [31]. For the single-shell filtered data the number of scatterers (cn), the absorber-scatterer distance (R), the Debye-Waller factors (σ^2), and the value E_0 were refined. The fitting of the wide shell filtered data was done by constrained refinement of the histidine residues, including their multiple scattering contributions as described in [32]. Debye-Waller factors of similar atoms in a similar distance were refined together. For a detailed description of this procedure see [33]. The number of parameters allowed to vary simultaneously in the curve fitting analysis was always equal to or lower than the maximum number of free parameters as defined in [34,35].

3. Results and discussion

3.1. Purification of the ibCO isozymes

No general purification scheme is shown for two reasons: no enzyme activity could be measured during the first purification steps due to the presence of sodium ascorbate. Secondly, very different ratios of the isozymes and strongly varying total amounts of ibCO were found depending on the season the potato tubers were harvested. Fig. 1 shows an example for the effectiveness of each purification step.

As shown in Fig. 2 the isozymes could be easily separated

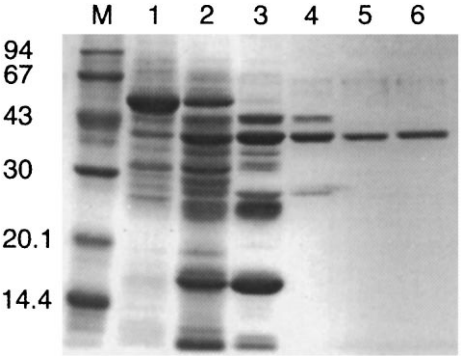

Fig. 1. SDS-PAGE of the 39 kDa and 40 kDa ibCO. Lane M: protein standards; lane 1: crude extract; lane 2: after S-200 HR; lane 3: 39 kDa ibCO after DEAE-FF; lane 4: 39 kDa ibCO after hydroxyapatite; lane 5: 39 kDa ibCO after Blue Sepharose; lane 6: 40 kDa ibCO after Blue Sepharose.

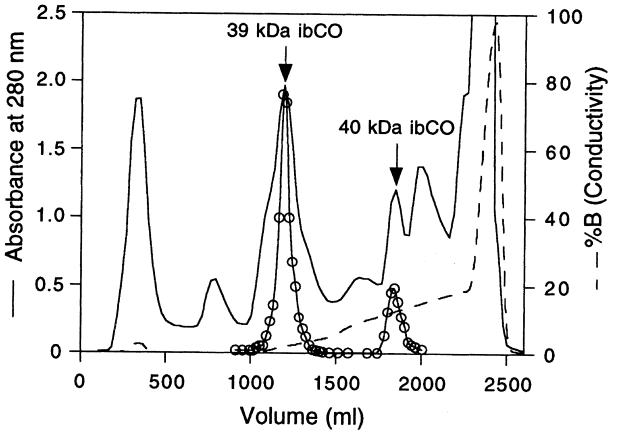

Fig. 2. Separation of both COs from *Ipomoea batatas* by DEAE-FF chromatography. The dotted line shows the catechol oxidase activity, the dashed line is representing the salt gradient.

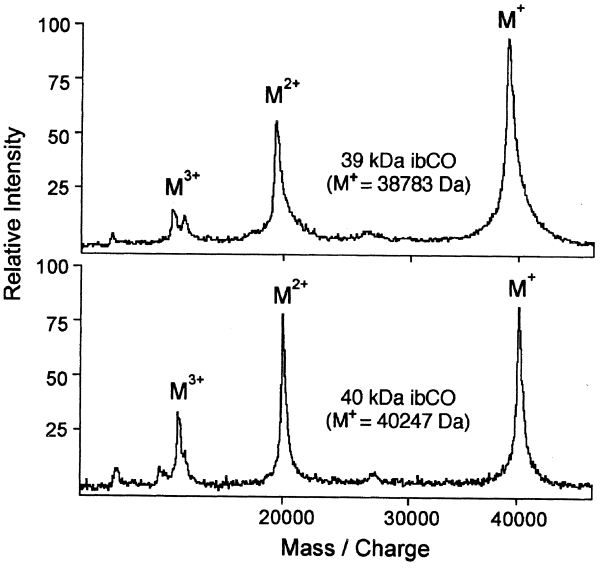

Fig. 3. MALDI-MS of both ibCO isozymes in 2,5-dihydroxybenzoic acid as matrix.

by anion exchange chromatography. The purification procedure led to electrophoretically homogeneous preparations of both isozymes, as stated by SDS-PAGE (Fig. 1) and MALDI-MS (Fig. 3). Furthermore, elution profiles from a Superdex 75 HR column showed a single symmetrical peak. On the average more than 15 mg of pure ibCO were obtained in total from 20 kg mature tubers.

3.2. Molecular mass, subunit composition, and isoelectric focusing

Denaturing SDS-PAGE (12%) of reduced and heated (100°C, 5 min) samples showed a single band for both isozymes at around 40 kDa. MALDI-MS analysis however reveals a difference in molecular mass. Singly charged molecules with M_r of 38 783 Da (39 kDa ibCO) and M_r of 40247 Da (40 kDa ibCO) are detected, respectively (Fig. 3). No evidence is found for the covalent attachment of caffeic acid quinones generated during the isolation procedure. The elution profiles of ibCO samples running on a Superdex 75 (16/60, 0.5 M NaCl, 50 mM sodium phosphate buffer, pH = 6.7, calibrated with a protein standard) indicate a monomeric quaternary structure of both ibCOs under chosen conditions.

The isoelectric point is 3.6 for the 39 kDa ibCO and 8.6 for the 40 kDa ibCO, indicating a significant difference between the amino acid sequences of the isozymes.

3.3. Spectroscopic properties

The UV/Vis spectrum of the native 39 kDa catechol oxidase of *Ipomoea batatas* in 0.5 M NaCl, 50 mM sodium phosphate buffer at pH = 6.7 is presented in Fig. 4. Both isozymes show similar spectra. A significant shoulder at 292 nm indicates the presence of several tryptophan residues. As seen for all type-3 copper enzymes a weak absorption in the range of 310 to 330 nm is observed, probably due to a μ-hydroxo→Cu(II) CT transition [36]. Concentrated solutions of ibCO show a further absorption maximum in the range of 700 to 800 nm, which can be assigned to d-d transitions (Fig. 4, inset), similar to those observed in the met hemocyanin UV/Vis spectrum [11].

The extinction coefficients at 280 nm for the native isozymes were calculated to $\varepsilon_{280} = 0.957$ cm^{-1} mg^{-1} ml (39 kDa ibCO) and $\varepsilon_{280} = 1.261$ cm^{-1} mg^{-1} ml (40 kDa ibCO) using the method by Bradford [23].

Addition of H_2O_2 causes an additional absorption band at 343 nm. Upon addition of two equivalents of H_2O_2 saturation of the 40 kDa catechol oxidase is reached, while six equivalents are required for the 39 kDa enzyme (Fig. 5, inset). Apart from this, both peroxo complexes show similar characteristics. Based on UV/Vis data on hemocyanin [9], showing an absorption maximum at 345 nm, the absorption band at 343 nm observed for the oxy form of the catechol oxidase can be assigned to an O_2^{2-} (π^*_σ)→Cu(II) ($\varepsilon \approx 6500$ cm^{-1} M^{-1}) CT transition. Such a CT band has been also reported for the oxy form of hemocyanin and tyrosinase [8,10]. The absence

Table 1
Substrate specificity of 39 kDa ibCO

Substrate	K_M (mM)	k_{cat} (s^{-1})
Tyrosine	no reaction	–
Hydrochinon	no reaction	–
Catechol	2.5	2293
4-Methylcatechol	3.8	3756
Caffeic acid	3.0	7852
L-DOPA	234	4533
Pyrogallol	no reaction	–

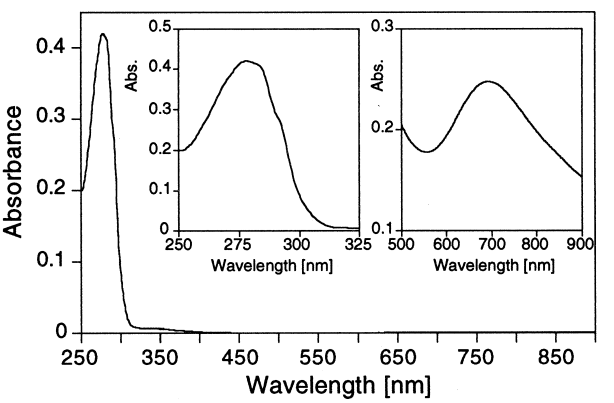

Fig. 4. UV-Vis absorption spectrum of the native 39 kDa ibCO in 0.5 M NaCl, 50 mM sodium phosphate pH = 6.7.

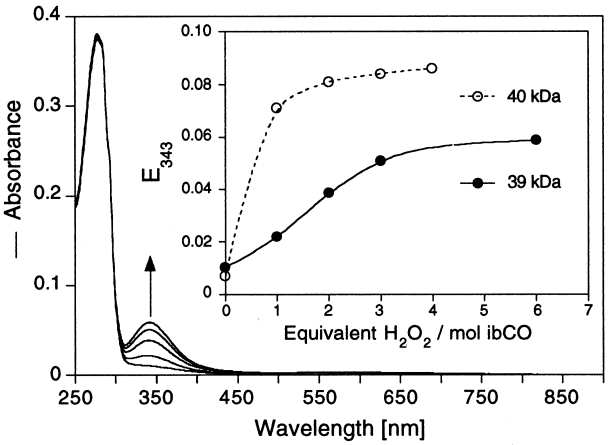

Fig. 5. Titration of the 39 kDa ibCO in 0.5 M NaCl, 50 mM sodium phosphate pH = 6.7 with H_2O_2. Inset: Absorption at 343 nm without and after addition of one, two, four, and six equivalents of H_2O_2.

of any absorbance in the range of 343 nm for both isozymes before addition of H_2O_2 reveals that ibCO is isolated in the met form. After addition of H_2O_2 both enzymes show a weak band at 580 nm ($\varepsilon \approx 450$ cm^{-1} M^{-1}) which corresponds to the second peroxo (π_v^*) → Cu(II) CT transition as also found in oxy hemocyanin and oxy tyrosinase [8–11]. The disappearance of CT transitions after a gel filtration step indicates that the peroxide complex of ibCO is not as stable as that formed by tyrosinase [8].

Native samples of ibCO isozymes (0.5 M NaCl, 50 mM sodium phosphate, pH = 6.7) are EPR silent. After addition of HCl however a signal typical for Cu(II) appears (data not shown). The EPR data thus reveal the presence of an antiferromagnetically coupled type-3 dicopper center in native ibCO.

3.4. Substrate specificity, inhibition, pH dependence of enzymatic activity

Table 1 lists the kinetic parameters (K_M, k_{cat}) of the purified 39 kDa ibCO as estimated from Lineweaver-Burk plots [22] revealing that the enzyme specifically catalyzes the oxidation of *o*-diphenolic substrates with K_M values in the mM range. The turnover number k_{cat} for caffeic acid, which is very abundant in the plant cell and has been proposed to be the substrate in vivo, is higher than for all other substrates. Only weak interaction with the enzyme is observed for L-DOPA which might result from steric hindrance.

As observed for other proteins with a copper center of type-3, competitive inhibition is observed with *N*-phenylthiourea ($IC_{50} = 4.3 \times 10^{-5}$ M) which causes an additional absorbance maximum in the range of 600 to 650 nm. This band can be assigned to a S(−II) → Cu(II) CT transition. Interestingly, also phenol shows inhibition with an estimated IC_{50} of

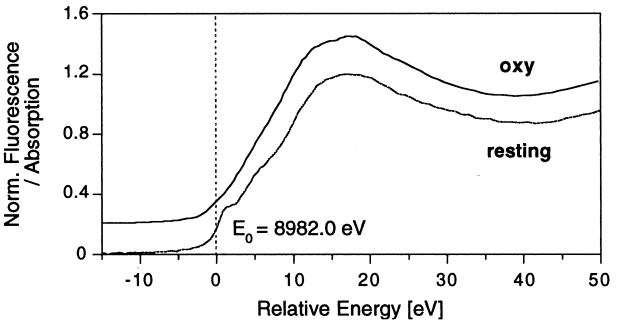

Fig. 6. X-ray absorption spectra of the copper-K edge spectra of the 39 kDa ibCO, $E_0 = 8982.0$ eV.

1.5×10^{-5} M, which is in the range of the K_M for catechol indicating a similar binding mode.

The purified 39 and 40 kDa ibCOs show a broad pH optimum around pH = 7.8 and a rapid decrease of activity below pH = 5 and above pH = 9. Both ibCOs are stable in 0.5 M NaCl, 0.05 M sodium phosphate buffer at pH = 6.7 at 4°C for months without loss of activity. Loss of activity has been also observed in buffers with low ionic strengths.

3.5. Amino acid NH_2-terminal sequence

The NH_2-terminal sequence of both isozymes is reported in Table 2 revealing 85% identity for the first 20 residues. *Vitis vinifera* [37] shows the highest homology (45% identity) to the 39 kDa ibCO isozyme among all known CO sequences.

3.6. EXAFS analysis of the 39 kDa ibCO isozyme

The 39 kDa ibCO isozyme was investigated by EXAFS in the resting met form and in its oxy form after addition of six equivalents of H_2O_2. The edge positions (resting form: 8992.0 eV; oxy form: 8991.2 eV) and shapes of the XANES spectra (Fig. 6) are consistent with Cu(II) coordinated by N/O-donor ligands [33,38,39]. The k^3-weighted fine structures (Fig. 7) have been Fourier transformed and the first shell peaks have been filtered and back-transformed. By using the program EXCURV92 these data could be best fitted with O at 1.92 Å or N at 1.94 Å. The coordination number was found to be four for each copper atom in the native and oxy form by applying the bond valence sum approach [38,39]. Free refinement of the coordination numbers gave low values, which are not very reliable because of high correlations with Debye-Waller factors. However a small increase of the coordination number for the oxy form is detectable. Therefore a fifth weakly coordinated ligand for the copper atoms in the oxy form can not be excluded.

The determination of the metal-metal distance is rendered more difficult by other scatterers, mostly carbon, which have similar distances to the absorber. Because of this and due to the bad signal-to-noise ratio (Fig. 7), a wide shell filter was used. Fig. 8 shows the obtained k^3-weighted fine structures of

Table 2
NH_2-terminal sequence alignment of catechol oxidases from *Ipomoea batatas* and *Vitis vinfera*

Source	1	10	20	
Ipomoea batatas (39 kDa)	A **P I** Q **A P** E **I**	**S K** X V V P P A D L	P P	This work
Ipomoea batatas (40 kDa)	E **P I** H **A P** E **I**	**S K** X V V P P K D L	P P	This work
Vitis vinfera	A **P I** Q **A P** D **I**	**S K** C G T A T V P D	G V	[37]

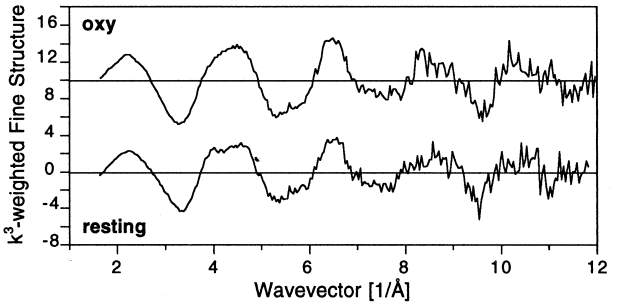

Fig. 7. k^3-weighted fine structure of unfiltered EXAFS data of the 39 kDa ibCO for the resting met form and the oxy form.

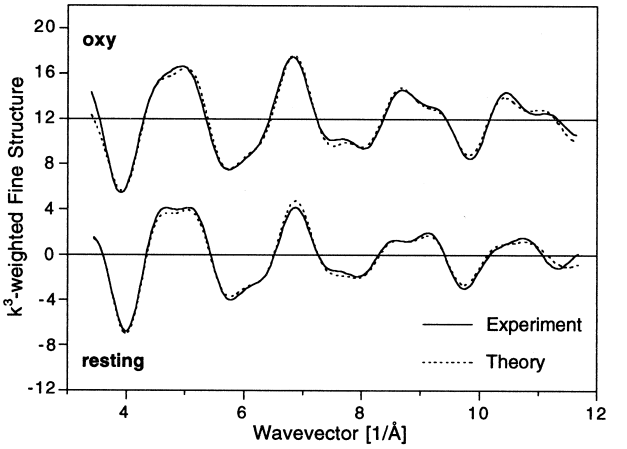

Fig. 8. Comparison of the k^3-weighted fine structures of the filtered data (solid lines) and the fits (dashed lines) of the resting enzyme (Cu-Cu distance = 2.9 Å, 3 His, 1 oxygen) and the oxy form (Cu-Cu distance = 3.8 Å, 3 His, 2 oxygens) of the 39 kDa enzyme.

both data sets. The Fourier transform of the resting met enzyme (Fig. 9) reveals two strong peaks in the higher shell region, one at ~2.9 Å and a second one at ~4.0 Å, which could be the metal-metal contribution. For the oxy form only one strong peak at 3.8 Å is observed (Fig. 9). The other peak at 2.9 Å is too weak and could not been fitted as a metal-metal contribution. Data were fitted by multiple scattering calculations using constrained refinement of the imidazole groups. Parameters from Engh and Huber [40] were used for the histidine rings. The number of imidazoles was varied during the refinement and the coordination sphere was completed with oxygen. Inclusion of an additional N-scatterer at a longer distance did not influence the fits significantly. Initial fits calculated without any metal-metal contribution resulted in significantly higher R factors confirming the presence of a dinuclear copper center.

For the native met form models for both possible Cu-Cu distances were tested to fit the experimental data. Because of a better fit (based on R values) we favor the shorter Cu-Cu distance of 2.89 Å against a longer Cu-Cu distance of 3.95 Å. This model is also in agreement with the presence of a coupled dinuclear copper center as revealed by EPR, with the EXAFS data on CO from *Lycopus europaeus* [41] and dinuclear model compounds [33].

In the oxy form of 39 kDa ibCO the Cu-Cu distance was refined to 3.82 Å. Single-shell fits for the higher shells result in almost identical Cu-Cu distances for both forms.

Due to very similar values for the R factor the number of histidine ligands could not be determined unambiguously for both enzyme forms. This problem is known from analogous experiments on model complexes [33]. Table 3 summarizes the fit parameters for both investigated samples, using the corresponding most likely coordination spheres. For the oxy form we considered the trend for an additional ligand as found by free refinement of the coordination numbers. Therefore five coordinated copper atoms are assumed in agreement with experiments on tyrosinase [42]. The resulting fits are in good agreement with the experimental data and are compared to these in Figs. 8 and 9.

The short Cu-Cu distance of 2.89 Å and the antiferromagnetic coupling in 39 kDa ibCO indicates that a monoatomic ligand is bridging the two Cu(II) ions. The absorption spectra show no indication for a phenoxo ligand which would cause CT bands in the range of 400 to 500 nm. Therefore it seems reasonable to assume one or two μ-hydroxo bridges for the Cu ions. For the oxy form the refined distance is 0.2 Å longer than that observed in oxy hemocyanin [43] and model complexes with a μ-η^2:η^2 binding mode for the peroxide [44,45]. However based on the overall similarity of the UV/Vis data we assume a similar μ-η^2:η^2 binding mode for peroxide in ibCO. A trans-μ-1,2 binding mode of the peroxide that yields

Table 3
Results of the wide shell fit using multiple scattering calculations and constrained refinement of the histidine groups for the 39 kDa enzyme, assuming a Cu-Cu scattering contribution (native: Cu-Cu distance 2.9 Å; oxy: Cu-Cu distance 3.8 Å)

Sample	cn[a] and scatterer	R (Å)	σ^2 (10^{-3} Å^2)	E_0	ϕ (deg)	R-factor
Resting form	1 O	*1.89 (1)*	*17.6 (4)*	*19.4 (2)*	*149 (0.4)*°	12.7
	3 N (His)	*1.930 (2)*	*3.8 (1.5)*			
	3 C (His)	2.594 (2)	*52.1 (9.4)*[b]			
	3 C (His)	3.135 (2)	52.1 (9.4)[b]			
	3 N (His)	4.095 (2)	*6.9 (4)*[c]			
	3 C (His)	3.858 (2)	6.9 (4)[c]			
	1 Cu	*2.883 (4)*	*12.1 (5)*			
Oxy form	2 O	*1.971 (7)*	*5.7 (4)*	*16.4 (4)*	*131.8 (0.7)*°	14.2
	3 N (His)	*1.933 (2)*	*3.3 (4)*			
	3 C (His)	2.865 (2)	*8.5 (3.1)*[b]			
	3 C (His)	2.979 (2)	8.5 (3.1)[b]			
	3 N (His)	4.078 (2)	*28.8 (5.6)*[c]			
	3 C (His)	4.033 (2)	28.8 (5.6)[c]			
	1 Cu	*3.822 (2)*	*5.8 (6)*			

[a]cn = coordination number, not refined; [b]refined together; [c]refined together; all refined parameters in italics.

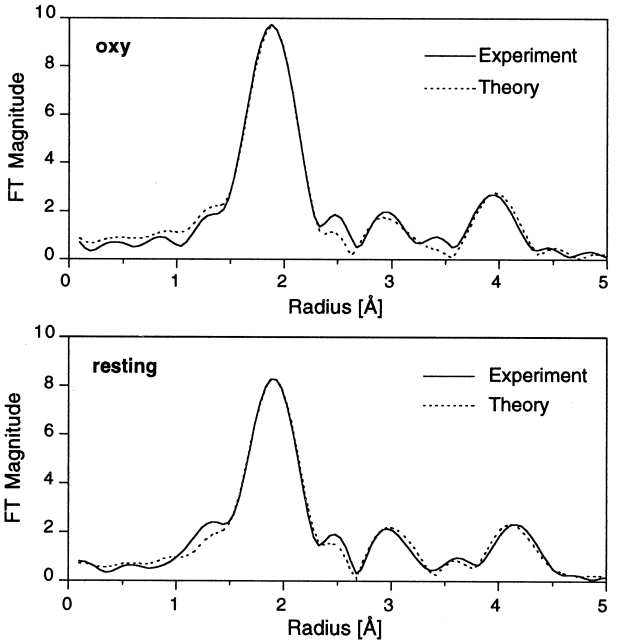

Fig. 9. Comparison of the Fourier transforms of the filtered data (solid lines) and the fits (dashed lines) of the 39 kDa enzyme in the resting met form (Cu-Cu distance = 2.9 Å, 3 His, 1 oxygen, bottom) and the oxy enzyme (Cu-Cu distance = 3.8 Å, 3 His, 2 oxygens, top).

a significant longer distance (4.36 Å) as observed in a model complex [46], can be excluded.

4. Conclusions

The spectroscopic data, including EPR, UV/Vis, and EXAFS reveal that both COs contain a catalytically active type-3 copper center. The monomeric isozymes differ by 1464 in their molecular mass, show considerably different isoelectric points (39 kDa ibCO: p*I* 3.6; 40 kDa ibCO: p*I* 8.6) and a sequence identity of ~85% (in their N-terminal region). The UV/Vis and EPR features of both catechol oxidase isozymes resemble those of tyrosinase and hemocyanin, indicating structurally related dimetal centers. Differences in the active site region, however, must exist to hold for the different activity and function of these copper proteins. This is also reflected by the shorter Cu-Cu separation (2.9 Å for the 39 kDa ibCO) seen in our EXAFS studies.

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