Trapping tyrosinase key active intermediate under turnover†‡

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This paper shows for the first time that the spectral features of the ternary complex of tyrosinase/O_2/phenol, trapped at low temperature using the very slow substrate 3,5-difluorophenol, are those of a μ–η²:η²-peroxidodicopper(II) species, and that this remains the only enzyme species under turnover and substrate saturation conditions.

Tyrosinase (EC 1.14.18.1, Ty) is a dinuclear copper enzyme catalyzing the hydroxylation of monophenols and the oxidation of *ortho*-diphenols to *ortho*-quinones;[1] it is the only enzyme known to be able to catalyze both of these reactions. Tyrosinase is found in bacteria, plants and animals, where it is involved in the formation of melanin pigments and other polyphenolic compounds.[1b,c] A recent X-ray structure determination of various forms of the enzyme from *Streptomyces castaneoglobisporus* showed that each copper ion is bound to three highly conserved histidines residues.[2] The region around these Cu-binding ligands is also well conserved in Tys from different species, as well as in hemocyanins and catechol oxidases.[3] Native tyrosinase is predominantly in the met form, with Cu^{II} ions bridged by a small ligand, likely OH^-.[2] Upon reduction, metTy is converted to the deoxy-form, which can readily bind dioxygen in a μ–η²:η² coordination mode producing oxyTy, the only form supporting the monophenolase reaction. This species exhibits characteristic LMCT features at 345 and 590 nm, and a very low vibrational frequency at 750 cm^{-1} in the Raman spectrum, indicative of a peroxido ligand.[1a] The most challenging step of the monophenolase cycle is the insertion of an O atom into the C–H bond *ortho* to the phenol C-OH group by oxyTy. Cleavage of the peroxidic O–O bond could take place *before* the attack on the aromatic ring (a, Fig. 1), *after* that (b), or *concerted with* it (c).[4]

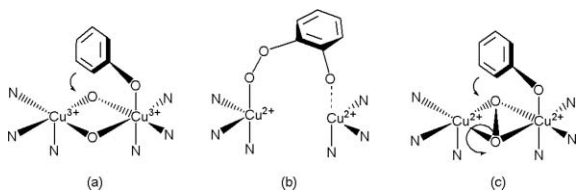

Fig. 1 Phenol hydroxylation mechanism.

In general, one of the last two possibilities is assumed,[5] but a recent report by Mirica *et al.*[6] showed that in the model μ-peroxido complex $[Cu_2(\mu\text{–}\eta^2\text{:}\eta^2\text{-}O_2)(DBED)]^{2+}$ (DBED = *N*,*N*′-di-*tert*-butyl-ethylenediamine) binding of a phenolate ligand at very low temperature induced an isomerisation to the corresponding bis(μ-oxido)dicopper(III) species $[Cu_2(\mu\text{-}O)_2(DBED)]^{2+}$ *prior* to the O-atom transfer reaction. Another possibility exists, in which binding of the phenol to oxyTy may occur with proton transfer to the electron-rich peroxido moiety, which would be converted into a hydroperoxido group (bound as a bridging or terminal ligand to the coppers).[7] It is generally accepted in biomimetic copper chemistry, that both the μ–η²:η²-peroxidodicopper(II)[8a–d] and bis(μ-oxido)dicopper(III) complexes[6,9] are capable of performing the hydroxylation of a phenolate ligand. In this report, we prove that the ternary complex of Ty/O_2/phenol, trapped at low temperature and followed during initial turnover, maintains all the spectroscopic characteristics of oxyTy, and hence that the peroxidodicopper(II) species is the enzymatically competent active species in the monophenolase reaction.

To succeed in the attempt of kinetically trapping the elusive ternary complex of the enzyme, two requirements need to be fulfilled: (i) to reach a sufficiently low temperature and (ii) to find a slowly reacting substrate, such that even using high enzyme concentration, the detection of the active enzyme intermediate is possible. In fact, it is well known that tyrosinase undergoes extremely rapid inactivation by the quinone species generated upon oxidation of phenolic substrates.[10] In this investigation, we used tyrosinase from *Streptomyces antibioticus*, which bears 82% identity with the structurally characterized enzyme from *S. castaneoglobisporus* and can be obtained in excellent purity,[11] and studied its activity in the mixed solvent of 34.4% methanol-glycerol (7/1 v/v) and 65.6% (v/v) aqueous 50 mM Hepes buffer at pH 6.8, the cryosolvent previously used for variable temperature studies of the fungal enzyme.[5b] Preliminarily, we studied some representative natural substrates to show that the enzyme works normally in the mixed aqueous-organic solvent at low temperature. The activation parameters and substrate binding data, deduced from k_{cat} and K_M, respectively, are comparable to those obtained previously for the fungal enzyme[5b] (Table 1), showing the substantial analogy in the behavior of the enzymes from bacterial and fungal sources, in

Table 1 Activation parameters and substrate binding data for *Streptomyces antibioticus* tyrosinase in the mixed solvent of 34.4% methanol-glycerol (7:1 v/v) and 65.6% (v/v) aqueous 50 mM Hepes buffer, pH 6.8; L-TyrOMe is L-tyrosine methyl ester

Substrate	$\Delta H^{\neq}$/kJ mol^{-1}	$\Delta S^{\neq}$/J K^{-1} mol^{-1}	ΔH°/kJ mol^{-1}	ΔS°/J K^{-1} mol^{-1}
Dopamine	+64.8 ± 2.6	−3 ± 9	−75.1 ± 2.7	−198 ± 9
L-Dopa	+59.5 ± 3.4	+8 ± 11	−37.9 ± 2.3	−96 ± 8
L-TyrOMe	+59.3 ± 2.9	+8 ± 10	−45.6 ± 1.6	−106 ± 5
Tyramine	+67.6 ± 3.2	−13 ± 11	−42.0 ± 1.7	−77 ± 6

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spite of the large differences in size and structural organization.[1c,2] The activation free energy ($\Delta G^{\neq}$ = 62.5 ± 5.5 kJ mol^{-1} for *S. antibioticus* Ty, 64.4 ± 3.7 kJ mol^{-1} for fungal Ty,[5b] at 25 °C) is always dominated by the enthalpic term, with $\Delta H^{\neq}$ values in the range of 63 ± 8 kJ mol^{-1} for *S. antibioticus* Ty, irrespective of substrate and reaction type (monophenolase or catecholase), while the activation entropies are small (still smaller for the bacterial Ty), indicative of a very high degree of preorganization of the enzyme active site.[5b]

As phenol hydroxylation by tyrosinase occurs through an electrophilic aromatic substitution,[12a] and we previously showed that a fluorine atom on the phenol ring significantly depresses the rate of enzymatic oxidation,[12b] we thought that a difluorophenol could be a suitably "slow" substrate to enable the observation of the enzyme species during turnover, even in conditions of large enzyme concentration and substrate saturation. The reactivity of both *S. antibioticus* Ty and mushroom Ty with 3,5-difluorophenol is indeed extremely weak, so that to show that this phenol is a substrate of the enzyme rather high enzyme concentration had to be used. On the other hand, as with monofluorophenols,[12b] the primary 3,5-difluoroquinone product is an extremely reactive electrophile towards excess phenol present in solution, which rapidly gives rise to a mixture of complex oligomeric products, preventing its isolation. The quinone intermediate was therefore trapped using glutathione (GSH), a tripeptide containing a cysteine thiol group very reactive with the quinone. Two quinone addition compounds were isolated from the enzymatic reaction, and their characterization (by MS, ^{19}F-NMR, ^{1}H -NMR and ^{1}H-DQF-COSY spectra, electronic supplementary information, ESI†) showed they consist of the isomeric adducts **3** and **4**, containing two GSH residues bound to the quinone moiety (Fig. 2).

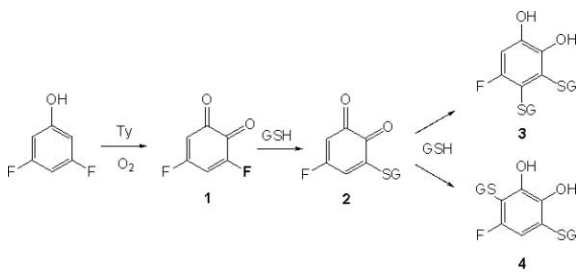

Fig. 2 Trapping the reactive 3,5-difluoroquinone primary product by glutathione.

These products can be accounted for by the extreme susceptibility of 3,5-difluoroquinone **1** to nucleophilic fluorine substitution by GSH; this reaction is regiospecific and yields the monofluoro-quinone adduct **2**, which undergoes a Michael addition reaction producing the two isomeric bis-GSH adducts **3** and **4** in comparable amounts. Release of inorganic fluoride upon GSH addition to compound **1** was confirmed by means of ^{19}F-NMR.

The extremely low reactivity of 3,5-difluorophenol makes it nearly impossible to carry out substrate-dependent kinetic studies, for the large quantity of enzyme that would be required. Therefore, to demonstrate that 3,5-difluorophenol is a "normal", albeit slowly reacting, substrate for tyrosinase, its competition in the enzymatic oxidation of L-Dopa was investigated. The presence of 3,5-difluorophenol bears a reduction in k_{cat} and an increase in K_M for L-Dopa oxidation in a concentration dependent fashion, indicating an inhibition of mixed type (ESI†). This behavior can be accounted for by considering that 3,5-difluorophenol can bind to the active site, competing with L-Dopa, but at the same time, it can react with oxyTy, partly consuming this species, and may also interact with the enzyme at non-specific sites, probably on the protein surface. The competitive inhibition constant (K_I) deduced from the analysis of the kinetic data of L-Dopa oxidation is 32 mM. This constant is important because it enables an estimation of the 3,5-difluorophenol concentration required to saturate oxyTy.

The direct observation of the oxyTy active form during turnover at low temperature with conventional spectrophotometry is difficult. The experiment requires enzyme at least in the 0.03 mM concentration range, for appropriate monitoring the evolution of the oxyTy optical band, but the organic component of the cryosolvent limits the solubility of the protein. Therefore, a minimum amount of this component allowing the mixed solvent to reach temperatures where the enzymatic reaction is not too fast had to be found, otherwise the absorption of the oxidation products would rapidly prevent monitoring of the enzyme. We found that a cryosolvent with a 55% (v/v) content of organic component (7:4 ratio, v/v, of methanol:glycerol) and Hepes buffer provides sufficient solubility for the enzyme and allowed us to reach temperatures slightly below −30 °C, maintaining the solution sufficiently fluid to enable rapid mixing of reagents. In these conditions, the characteristic near-UV band of oxyTy at 345 nm could be easily generated by anaerobic reduction of metTy (35 μM) with a small excess of hydroxylamine,[13] followed by exposure to air oxygen. When a conventional substrate, such as L-tyrosine or tyramine, is added to this enzyme solution at −30 °C, an extremely fast reaction takes place, producing immediate darkening of the solution during the mixing time. Even upon addition of an enzyme-saturating amount of 3,5-difluorophenol (70 mM), the reaction is rapid, but in this case, the enzyme species could be observed during several turnovers until, after about 60 s, the oxyTy band was depleted and became progressively covered by the broad optical absorption of the reaction products (Fig. 3). It is clear from this experiment that the oxyTy species observed immediately after the addition of saturating substrate is still the μ–η^2:η^2-peroxidodicopper(II) form, and therefore phenol binding does not induce an isomerisation to the bis(μ-oxido)dicopper(III) form, as observed in the model system described by Mirica and coworkers.[6] A change to the latter species would be signalled by a marked shift of the LMCT band above 410 nm.[14] In addition, phenol binding to oxyTy is accompanied by the appearance of an absorption feature at 460 nm ($\Delta\varepsilon \sim 1000$ M^{-1} cm^{-1}), which is consistent with the expected LMCT band typical of the copper(II)-phenolate chromophore.[15] By contrast, we have recently shown that binding of phenolate to the bis(μ-oxido)dicopper(III) core of the biomimetic complex $[Cu_2(\mu\text{-}O)_2(m\text{-}XYL^{MeAN})]^{2+}$ is accompanied by development of an LMCT band at 560 nm.[9] The oxyTy band remains well defined and basically unshifted for about 20 s reaction time, the progressive reduction in intensity being due to consumption of dioxygen in solution and, possibly, by some enzyme inactivation by the reactive quinone compounds produced in the reaction. In parallel, also the

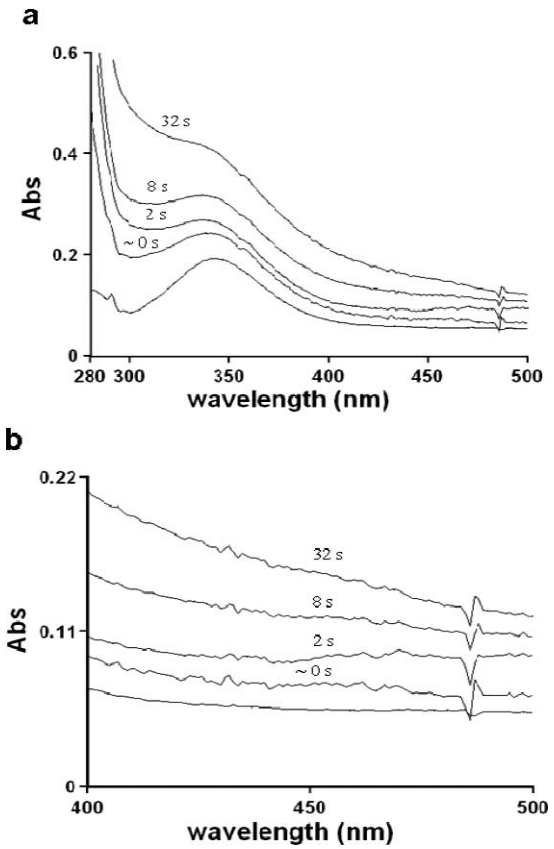

Fig. 3 Monitoring tyrosinase Cu_2O_2 intermediate during turnover at low temperature. (a) UV-Vis spectra recorded at –30 °C in the initial phase of the reaction of *S. antibioticus* oxytyrosinase (35 μM) and 3,5-difluorophenol (70 mM) in the mixed solvent of 35% methanol, 20% glycerol and 45% 50 mM Hepes buffer pH 6.8 (v/v/v). OxyTy was generated at –20 °C from the met form by reaction with 10 equiv. NH_2OH under anaerobic conditions, followed by exposure to dioxygen. The spectra are difference spectra with respect to deoxyTy (with oxyTy shown as lower trace), and were taken at different times after the addition of 3,5-difluorophenol (the spectrum at ~0 s time being the first one obtained after mixing). (b) The panel shows an expansion of the low-energy spectral region. Absolute spectra of deoxyTy, oxyTy, and enzyme under turnover are given in the ESI.†

LMCT band loses definition and the absorption throughout the near-UV range increases due to the formation of the oligomeric addition products of the initially formed 3,5-difluoroquinone with excess difluorophenol.

Unfortunately attempts to detect the signature of oxyTy in Raman experiments, around 700–800 cm^{-1}, failed due to excessive fluorescence of the sample,[16a] which is likely stronger here than in the previously investigated enzyme from *Neurospora crassa*.[16b] The experiments were carried out both on frozen and fluid solutions (see ESI†), with excitation wavelengths of 370, 380, 390, and 410 nm in a triple subtractive mode. Fluorescence by the sample was already high at 410 nm excitation and increased exponentially at the lower wavelengths.

The observation of the ternary complex of oxyTy with a bound phenolate during enzyme turnover, and the kinetic data at variable temperature, confirm our previous hypothesis,[5b] which appears to be supported by theoretical calculations,[17] that peroxide O–O bond cleavage represents the rate determining step of the enzymatic reaction. The O–O bond cleavage occurs in the transition state, not in the ground state ternary complex of Cu_2/O_2/phenol, as it occurs in the Cu-DBED model system.[6] This is confirmed by the striking difference in the activation parameters for the enzymatic and model reactions,[8d,9] which may reflect the (evolutionary optimised) stabilization of the transition state by the enzymatic system. Even though the intrinsic barrier for the interconversion between the μ-peroxido and bis(μ-oxido) Cu_2O_2 cores appears to be small on theoretical grounds, and is therefore observed in a number of low-molecular weight biomimetic copper systems,[14,18] such a change may involve considerable conformational change in the active site of the protein, to meet the necessary requirement of a strong tetragonal field by the copper(III) species.

Our results can neither be reconciled with the recent quantum chemical study of the reaction mechanism of *S. castaneoglobisporus* tyrosinase,[17b] according to which binding of the phenolic substrate to oxyTy occurs with initial isomerization of the μ–η^2:η^2-peroxidodicopper(II) species to a μ–η^1:η^2-peroxidodicopper(II) form, followed by proton transfer from the phenol to the peroxo group and phenolate binding to Cu_B. There is only one precedent of μ–η^1:η^2-peroxidodicopper(II) complex in the literature, reported by Tachi *et al.*,[19] and this bears spectroscopic signatures quite different from that of the μ–η^2:η^2 peroxido isomer, with an LMCT band strongly red shifted to 483 nm. Such a spectral change is clearly not detected in our experiment, and therefore we believe that no isomerization of oxyTy occurs upon formation of the enzyme ternary complex. The evolution of the theoretical μ–η^1:η^2-hydroperoxidodicopper(II) species along the reaction coordinate involves one-electron oxidation of the bound phenolate with production of a phenoxyl radical.[17b] This feature is common to other previous theoretical studies[17a] and deserves more general consideration on alternative formulations of the tyrosinase mechanism functioning through intermediates different from μ–η^2:η^2-peroxidodicopper(II). Also the bis(μ-oxido)dicopper(III) species, in fact, exhibits strong tendency to induce radical reactions,[14,18a,20] and indeed the formation of a 1:1 mixture of catechol/quinone, from the phenolate, in the Cu-DBED system reported by Mirica and co-workers[6] likely reflects the initial formation of a semiquinone radical, followed by disproportionation. In contrast, we proved some time ago that no radical species is formed in tyrosinase reactions,[21] and found no trace of the characteristic sharp optical feature of the phenoxyl radical[18b,22] in our present low temperature experiments. As it is well known by the work of Itoh's group, the enzymatic phenol hydroxylation proceeds through an electrophilic aromatic substitution,[12a] and the reactivity of the μ–η^2:η^2-peroxidodicopper(II) intermediate fully conforms to this mechanism.

In conclusion, we have shown by a cryoenzymatic study that tyrosinase functions through a μ–η^2:η^2-peroxidodicopper(II) active species, that remains the only detectable species during turnover under substrate saturation conditions. The bis(μ-oxido)dimetal core, with its powerful oxidizing properties, is relevant for dinuclear iron enzymes, like methane monooxygenase,[20] where radical chemistry is necessary, but is not observed with tyrosinase. Though, it bears strong interest for copper mediated oxidation processes[20,23] and remains a potential candidate for other Cu-dependent biochemical systems promoting oxidative reactions.

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