Mechanistic studies of tyrosinase suicide inactivation

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Abstract

Tyrosinase is a copper containing enzyme that oxidises both phenols and catechols to *ortho*-quinones. The oxidation of catechols (oxidase activity) is associated with a gradual and irreversible inactivation of the enzyme. This suicide inactivation has been interpreted in terms of the enzyme occasionally oxidising catechols as phenols (oxygenase activity) leading to reductive elimination of copper. In this account experimental evidence supporting the authors' mechanism is reviewed and discussed.

**Keywords:** Tyrosinase, suicide inactivation, catechols, oxidase, oxygenase, reductive elimination

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1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper metalloenzyme that is widely distributed in nature and is involved in many biologically significant transformations.[1] The chemistry of tyrosinase includes a number of interesting features, including two discrete mechanisms of oxidation, a lag period during *in vitro* oxidation of tyrosine, and suicide inactivation of the active site during catechol oxidation. In this account we review experimental evidence for the mechanism of suicide inactivation of tyrosinase by catechols.

    The active site of tyrosinase contains two copper atoms and the enzyme can occur in three forms (Scheme 1). *Deoxy*-tyrosinase **2** binds dioxygen to form *oxy*-tyrosinase **1**. The third form, *met*-tyrosinase **3**, cannot bind oxygen but is reduced to *deoxy*-tyrosinase **2** by catechols.
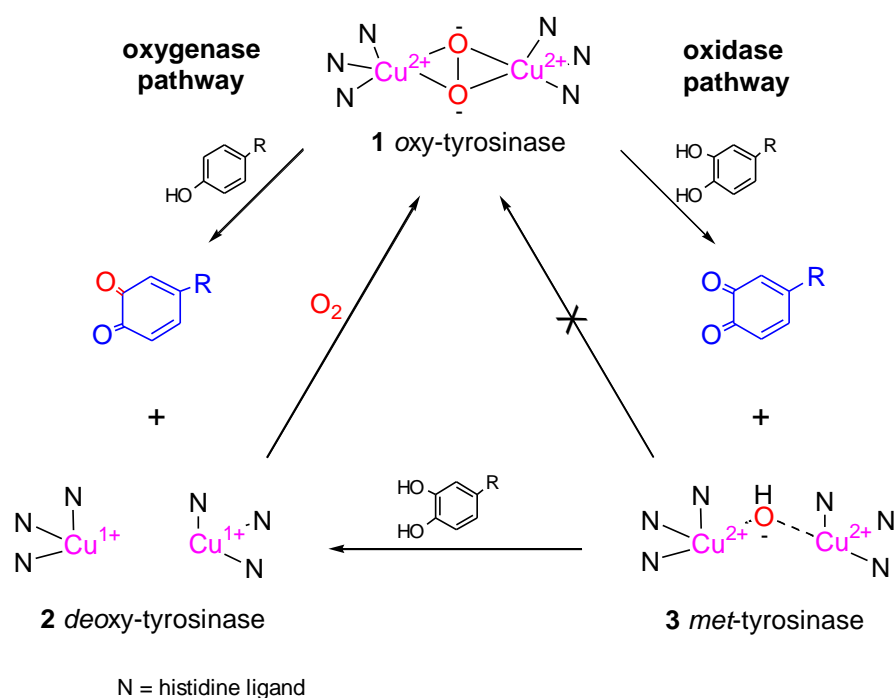
Scheme 1

    In mammals tyrosinase is the principal enzyme of the pigmentation pathway giving rise to the melanin pigments (Scheme 2).[2] The natural substrate tyrosine **4** is oxidised by *oxy*-tyrosinase **1** to give dopaquinone **6** which either cyclises to cyclodopa **7** leading to eumelanin (black/brown pigment) or reacts with cysteine to give cysteinyldopa **5** leading to pheomelanin (red/yellow pigment).
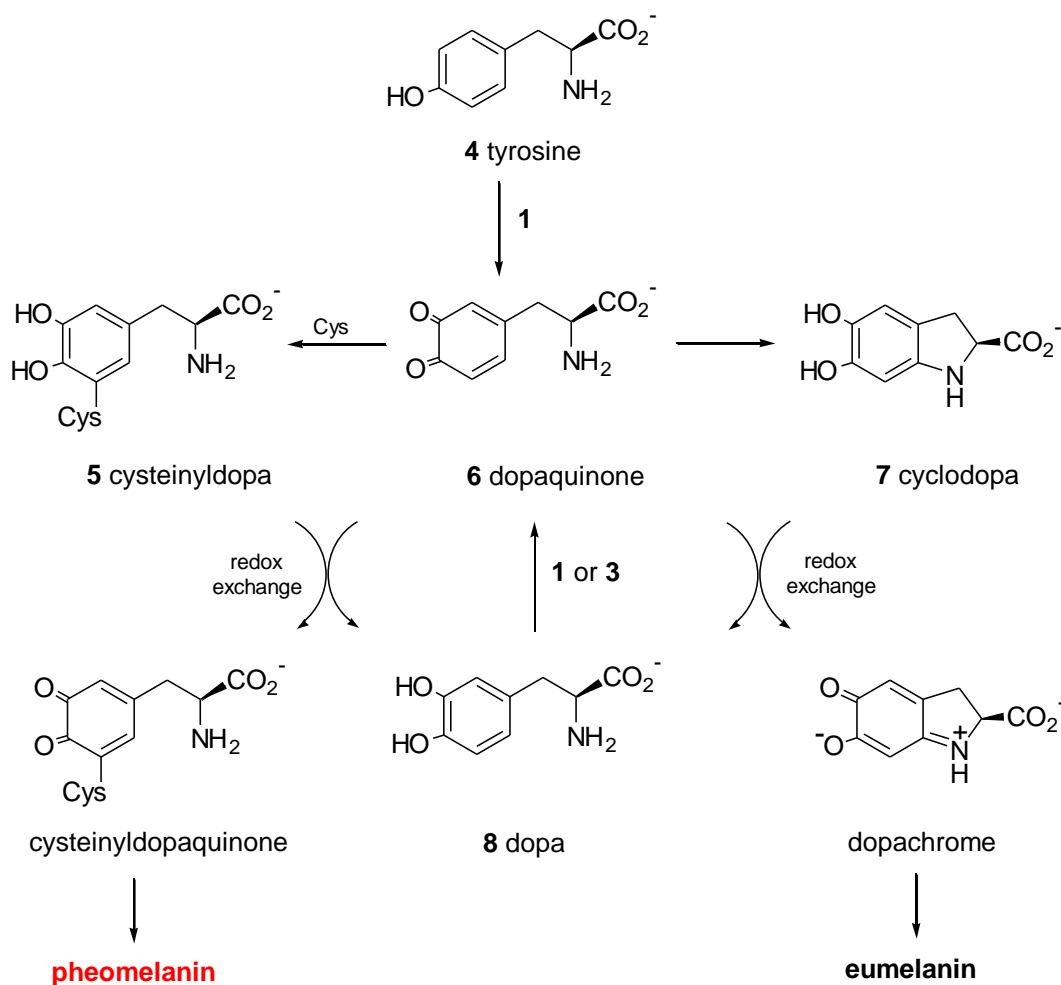
Scheme 2

Tyrosinase is unusual in that it exhibits two catalytic functions: (a) oxygenase activity ('cresolase' action) in which monohydric phenols have oxygen substituted at the 2-position giving an *ortho*-quinone (Scheme 1);[3,4] and (b) oxidase activity ('catecholase' action)[5] in which a catechol is oxidised to an *ortho*-quinone (Scheme 1). One of the characteristics of *in vitro* oxidation of tyrosine **4** is a 'lag period'.[6] Native tyrosinase occurs mainly as *met*-tyrosinase **3**, which cannot oxidise phenols, e.g. tyrosine **4**, and needs to be reduced to *deoxy*-tyrosinase **2** by a catechol before phenol oxidation can begin (Scheme 1).[7] *In vitro* this activating catecholic substrate is generated indirectly by fast redox exchange of dopaquinone **6** formed slowly by the small amount of *oxy*-tyrosine **1** present in native tyrosinase. The lag period ends when all the enzyme has been activated by this indirect and relatively slow non-enzymatic formation of dopa **8** (Scheme 2).[8,9]

2. Suicide inactivation by catechol substrates

Another unusual feature of tyrosinase is that the oxidase pathway (Scheme 1) exhibits inactivation kinetics.[10-16] It was shown in 1982 by Dietler and Lerch[17] that this inactivation is linearly correlated with the loss of 50% of the copper atoms from the active site (Figure 1) but the mechanism to account for this loss of copper remained elusive. Dietler and Lerch suggested that it might be the result of oxidative modification of the histidine residues that coordinate the copper atoms in the active site of the enzyme,[17] but radical scavengers were without effect on the inactivation process,[14,17] and it is probable that the histidine oxidation observed was secondary to the inactivation process.
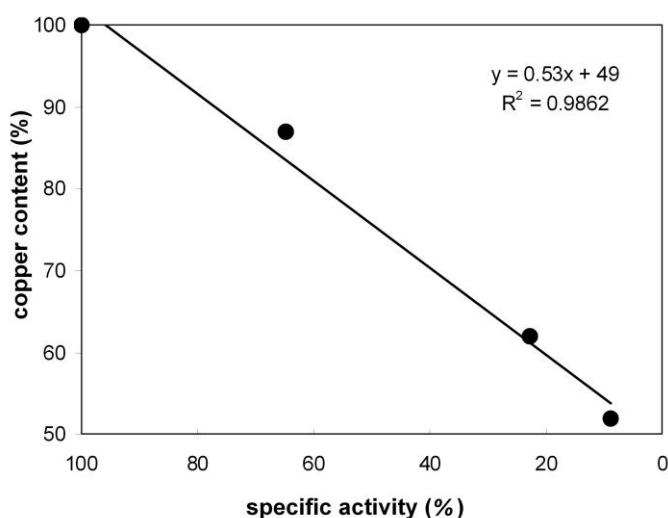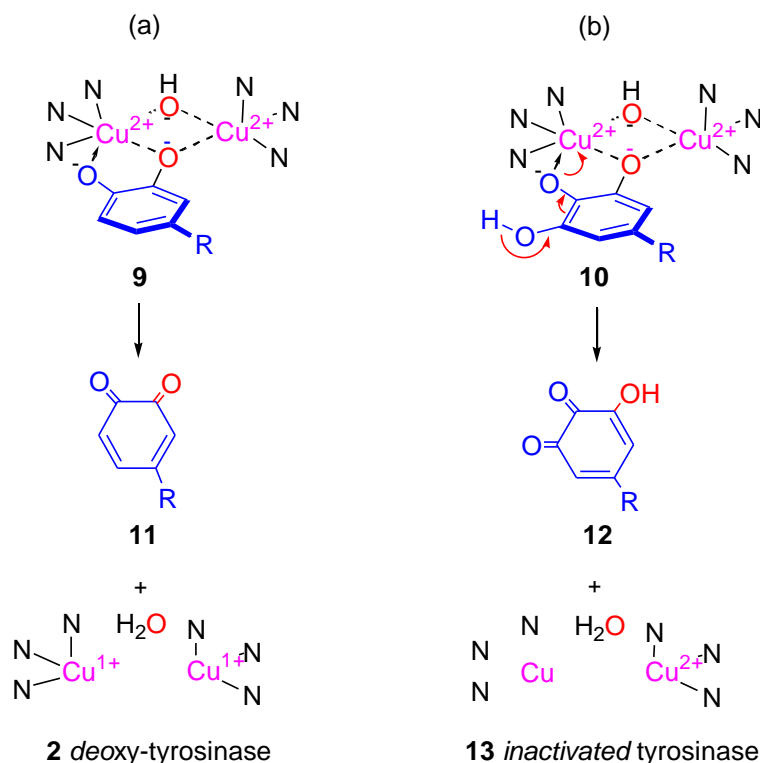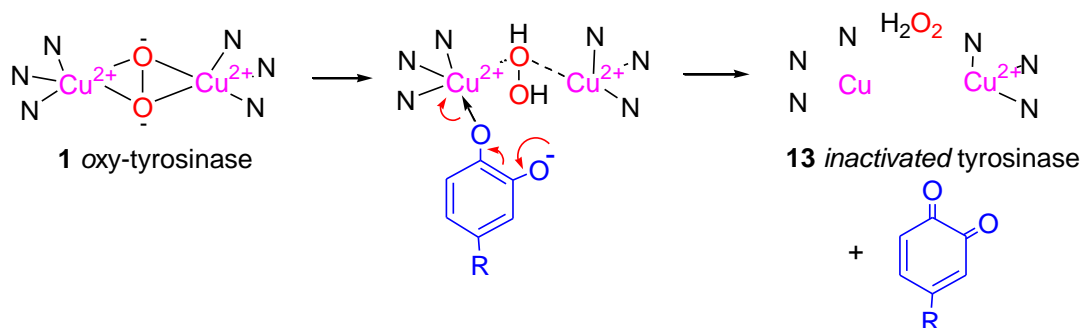

Figure 1. Data of Dietler and Lerch on the correlation of suicide inactivation and 50% copper loss from *Neurospora* tyrosinase.[17]

Recently we proposed a mechanism that accounts for the copper loss during inactivation.[18] Briefly stated, this mechanism postulates that *oxy*-tyrosinase **1** sometimes binds catechol substrates in the oxygenase mode, i.e. as if they were phenols. Scheme 3(a) shows the intermediate **9** formed by binding of a phenol to *oxy*-tyrosinase **1** followed by elimination of an *ortho*-quinone **11** and generation of *deoxy*-tyrosinase **2**. If a catechol occasionally binds in a similar way (**10**, Scheme 3(b)), the extra hydroxy substituent can deprotonate leading to reductive elimination of Cu(0), a 3-hydroxy-*ortho*-quinone **12** and an irreversibly inactivated form of tyrosinase **13**. This processing of catechols as phenols in the oxygenase cycle nicely accounts for the observed suicide-inactivation mechanism and we have described further experimental studies that support this mechanism.[19,20] Recognising the novelty and significance of our studies and proposals, another group has subsequently proposed a variation on the reductive-elimination theme to account for copper loss.[21] This alternative suggestion is summarised in Scheme 4. In this account we review the experimental evidence regarding the reductive-elimination mechanism of suicide inactivation.

(a)   (b)

9   **10**

11   **12**

+   +

2 *deo*xy-tyrosinase   **13** *inactivated* tyrosinase

Scheme 3

1 *o*xy-tyrosinase   **13** *inactivated* tyrosinase

+

Scheme 4

At the outset we wish to emphasize that, to our knowledge, there are no studies unequivocally demonstrating the formation of zero-valent copper during the suicide inactivation of tyrosinase. However, this seems to be the most plausible explanation for the loss of enzymatic activity (Figure 1), and while attempts have been made to re-activate tyrosinase by addition of copper salts, the interpretation of the results of these experiments has been hampered by significant non-enzymatic oxidation.

Another caveat is that the majority of the recent experimental data bearing on the question of suicide inactivation are derived from work with commercially available tyrosinase from

mushrooms (*Agaricus bisporus*). This limits the extent to which the conclusions drawn from them can be generalized, but it is our view that the strongly conserved active centre of tyrosinases from different sources[22] makes it probable that very similar, if not identical, mechanisms apply to tyrosinases in general.

The current state of the evidence can be conveniently discussed under the following subheadings:

2.1 The requirement for oxygenase activity

Catechol oxidase (EC 1.10.3.1) is an enzyme closely related to tyrosinase but which lacks oxygenase activity due to subtle differences in the active site.[23-25] A strong prediction arising from the mechanistic hypothesis proposed by the Quintox group[18] is that catechol oxidases, which do not possess oxygenase activity, will fail to exhibit suicide-inactivation kinetics since in this mechanism the copper reduction requires oxygenase generation of a hydroxylated intermediate (Scheme 3b). This is not predicted by the alternative mechanism advanced by Muñoz-Muñoz and co-workers[21] since their proposal depends on a protonation of the peroxy oxygen in a bidentate catechol complex (Scheme 4).

The behaviour of a catechol oxidase extracted from bananas (*Musa cavendishii*) indicates that, in contrast to tyrosinase, it oxidises a number of catecholic substrates without displaying suicide-inactivation kinetics.[19] Earlier results published by Padrón and co-workers[26] show first-order kinetics exhibited by the banana enzyme which are claimed as evidence of enzyme inactivation. However, in this study[26] the exponential decrease in oxidation rate probably reflects the fall in oxygen concentration as shown by the reported polarimetric results. In contrast we have shown that the banana enzyme exhibits no activity towards four monohydric phenols tested (4-methylphenol, 4-methoxyphenol, tyrosine and tyramine) under the same experimental conditions used for catechol oxidation. The absence of oxygenase activity was shown not to be due to lack of activation of the enzyme (Figure 2) or the presence of an inhibitor of oxygenase activity.[19] All these data are consistent with the requirement for oxygenase activity for suicide inactivation of tyrosinase and provide very strong support for the Quintox mechanism (Scheme 3).
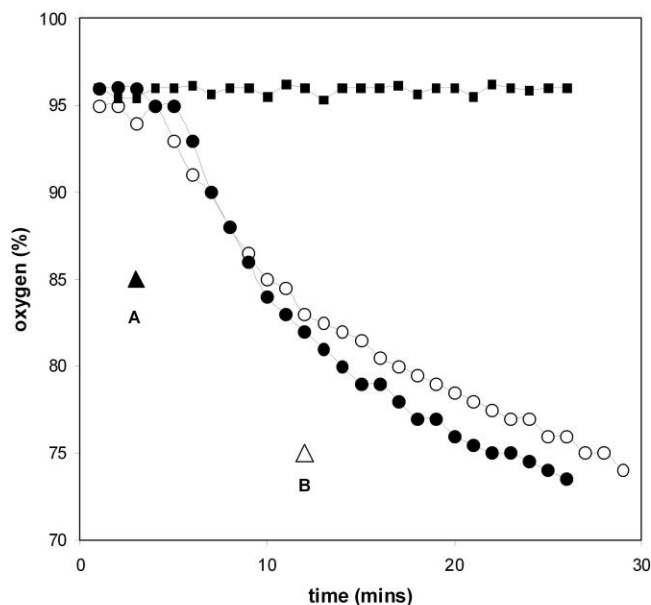
Figure 2. Lack of oxygenase activity of catechol oxidase from *Musa cavendishii*. The chart shows the polarimetric results of three experiments conducted in 0.1 M phosphate buffer (pH 6.3). In all cases the enzyme was added at the time point indicated by **A**. In two cases (○) and (●) 4-methylcatechol (140 µM) was present from t = 0 and in one of these (○) 4-methylphenol (280 µM) was added at **B**. The control enzyme solution (■) also had 4-methylphenol added at the point indicated by **B**. There is no evidence of 4-methylphenol oxidation in the control (■) and no activation of oxygenase following pre-exposure of the enzyme to the catecholic substrate (○) in comparison with the pre-exposed control (●).

Further indirect evidence of the involvement of an oxygenase presentation in the inactivation mechanism (Scheme 3) is the inhibitory effect of addition of a monohydric phenol to the catechol oxidation mixture. We have shown that the relative inactivation rate (the ratio of the inactivation rate to the initial rate of oxidase activity) of 4-methylcatechol oxidation by tyrosinase is diminished with increasing concentration of 4-methylphenol.[18] While this is consistent with the Quintox mechanism (Scheme 3), this result would not be anticipated on the basis of the alternative proposal (Scheme 4)[21] since both the catechol oxidation rate and the inactivation rates would be similarly affected by a competing substrate.

2.2 Alternative substrate-binding orientations

The kinetic data of Muñoz-Muñoz and co-workers[21] indicate a correlation between the catalytic rate and the maximum apparent inactivation rate for a range of substituted diphenols tested (Figure 3), which is consistent with their mechanism which does not involve a difference in substrate binding.
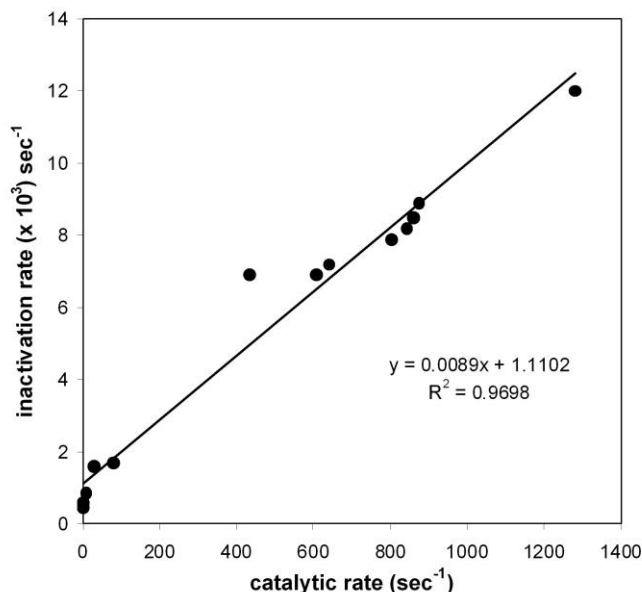
Figure 3. Relationship between the inactivation rate and catalytic rate (data of Muñoz-Muñoz et l.[21])

However, excluding the case of pyrogallol (discussed separately in Section 2.5), comparison of the data on four catecholic substrates tested by both Muñoz-Muñoz and co-workers[21] and the Quintox group[18] shows significant discrepancies in the ratio of the initial oxidation rate to the inactivation rate (k_1/k_2) (Table 1).

Table 1. Comparison of kinetic data for four catecholic substrates.

Substrate	k_1/k_2	
	Muñoz-Muñoz et al.[21]	Quintox group[18]
catechol	97.9	68.4
4-chlorocatechol	100.5	29.6
4-methylcatechol	100.5	24.1
4-ethylcatechol	100.9	9.6

The Quintox group ratios were obtained directly from plots of the total oxygen utilization versus the initial amount of enzyme, the slope of the line being defined as k_1/k_2, whereas the Muñoz-Muñoz *et al.* results were derived from kinetic parameters. It is possible that the estimates of k_1 differ for technical reasons, since the assay procedure used by Muñoz-Muñoz and co-workers is designed to maintain a steady-state concentration of the substrate and depends on the calculation of the catalytic rate from the oxidation of a redox indicator. Nevertheless, the substantial discrepancy in the apparent inactivation rates is difficult to explain. Substituting the (essentially constant) k_1 values obtained by Muñoz-Muñoz *et al*. in the Quintox Group data

yields a ten-fold variation in k_2 whereas the corresponding Muñoz-Muñoz et al. data for k_2[21] differ by only 10%.

According to the Quintox mechanism[18] the inactivation comes about by a proportion of the substrate binding to the active site in an alternative orientation. The catechol may bind either in the usual bidentate oxidase mode to the active site copper atoms **14**, or like a phenol in the oxygenase mode **15**. The orientation of these binding modes to *oxy*-tyrosinase differs in respect of the plane defined by the copper atoms and the coordinated molecular oxygen.[27-30] Both modes (**14** and **15**) have well-defined and inflexible locations of the catechol unit and its associated substituents. It might be anticipated, therefore, that structural constraints associated with different binding modes would be reflected by a differential influence of substituents on the rate of oxidation (k_1) and the rate of inactivation (k_2).

14   **15**
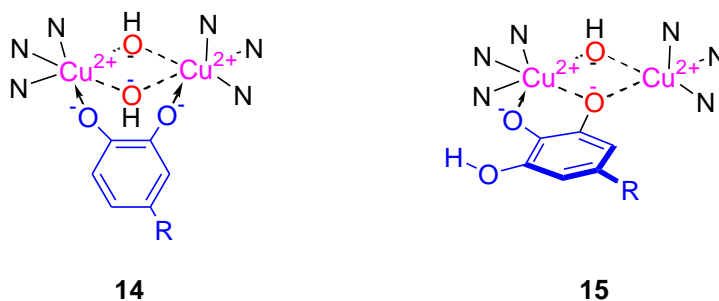
Such differences have been observed by quantitative analysis of a series 4-substituted catechols with ring substituents chosen to give a wide variation and minimal correlation of substituent properties.[31] In this study the statistically-significant relationships shown in Equations 1 and 2 were found between the rate constants (k_1 and k_2) and the length (L) and hydrophobicity (π) of the catechol substituents. The substituent effects for oxidation (k_1) and inactivation (k_2) are clearly different.

$$\log k_1 = 0.364 \, (\pm 0.097) \, \pi - 0.297 \, (\pm 0.077) \, L + 2.369 \tag{1}$$

$$\log k_2 = 1.143 \, (\pm 0.229) \, \pi - 0.819 \, (\pm 0.243) \, \pi^2 + 0.061 \tag{2}$$

This result would appear to be at variance with the mechanism proposed of Muñoz-Muñoz and co-workers[21] since the binding orientation is not critical in their mechanism shown in Scheme 4.

2.3 Hydroxylation products

There is evidence in the literature of the 5-hydroxylation of L-3,4-dihydroxyphenylalanine (dopa **8**) which confirms the ability of tyrosinase from different sources to process catechols by the oxygenase pathway,[32-35] and a strong prediction of the Quintox mechanism is the generation of a hydroxylation product **12** of the catecholic substrate during the inactivation reaction (Scheme 3b). Because the inactivation reaction is relatively rare and involves small quantities of enzyme, in the experimental system used the *ortho*-hydroxylated quinone product **12** is a minor component of the reaction mixture. Nevertheless, the product **12** has been identified by hplc-

mass spectrometry in the case of 4-methylcatechol oxidation by tyrosinase and shown to be correlated with the loss of enzyme activity.[19]

2.4 Blocking the hydroxylation site

Since the Quintox suicide-inactivation mechanism requires *ortho*-hydroxylation to take place, the inactivation kinetics of tyrosinase during catechol oxidation should be prevented if an unsubstituted ring carbon adjacent to the catecholic function is not available. Since this is not a requirement of the mechanism suggested by Muñoz-Muñoz and co-workers[21] this provides a further opportunity for a critical experimental test of the mechanisms. As predicted by the Quintox mechanism, 3,6-difluorocatechol **16**, which cannot act as an oxygenase substrate, acts as an oxidase substrate for tyrosinase but does not exhibit suicide-inactivation kinetics.[20]
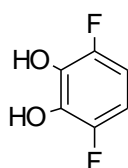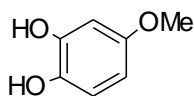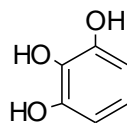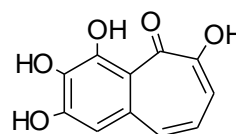
16   **17**   **18** pyrogallol   **19** purpurogallin

The oxygen utilization during the oxidation of 3,6-difluorocatechol **16** by tyrosinase shows first-order kinetics and contrasts with the kinetics of an inactivating substrate **17** in an incubation mixture exhibiting a similar initial oxidation rate (Figure 4). This result, together with the results described in Section 2.1, very strongly supports the Quintox mechanism.

2.5 Interference with oxygenase binding

An alternative aspect of the prevention of cresolase binding is offered by the trihydric phenol pyrogallol **18** which, because of the restrictions imposed by hydroxyl groups in positions 4 and 5,[18] is only capable of binding to the active site of mushroom tyrosinase as a catechol. The Quintox mechanism predicts that this substrate will not be subject to suicide inactivation and this has been observed in relation to the kinetics of oxygen utilization.[18] However, the data have been challenged on the grounds that the observed oxygen uptake is due to auto-oxidation and that pyrogallol is a strongly inactivating substrate.[21] There is no doubt that there are some complications with this substrate arising from rapid secondary reactions, which may involve auto-oxidative steps, during the formation of the major oxidation product purpurogallin **19**;[36] so it is not possible to make strong inferences from the data presently available. Since the *ortho*-quinone formed from pyrogallol **18** rapidly forms purpurogallin **19**, any assay system related to *ortho*-quinone formation may give the impression that the enzyme is inhibited and *ortho*-quinone formation has ceased. Based on oxygen uptake, we have found that pre-exposure to pyrogallol did not inactivate tyrosinase as shown by the subsequent oxidation of 4-methylcatechol (Figure 5).[18] The claim that pyrogallol is an inactivating substrate is clearly incorrect.
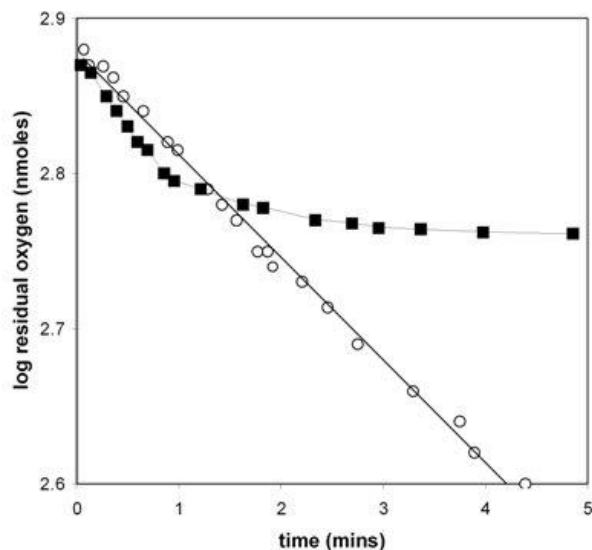
Figure 4. Comparison of pseudo-first-order oxidation kinetics of 3,6-difluorocatechol **16** (○) (incubated with 30 units tyrosinase) and the suicide inactivation kinetics of 4-methoxycatechol **17** (■) (incubated with 9 units tyrosinase).
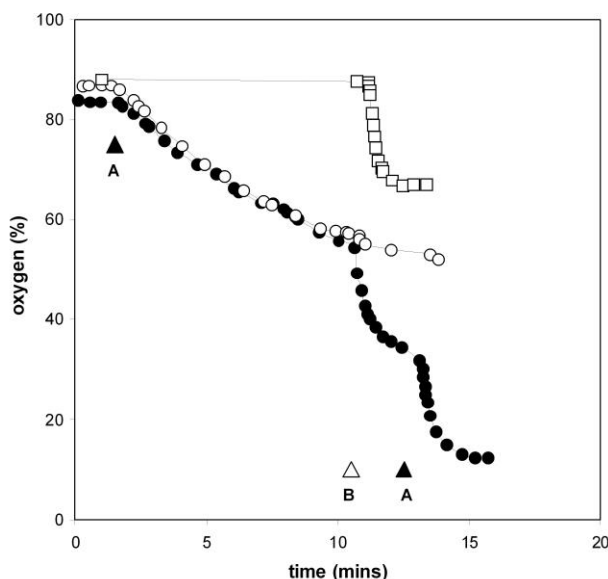

Figure 5. Failure of tyrosinase inactivation by pre-exposure to pyrogallol **18**. The chart shows the polarimetric results of three experiments in 0.1 M phosphate buffer (pH 6.3). Tyrosinase additions (15 units) were made at points marked **A**. In two experiments, indicated by (○) and (●), pyrogallol (180 μM) was present from t = 0. At the point indicated by **B** 4-methylcatechol (500 μM) was added to the test incubation mixture (●). This exhibited the characteristic catecholic oxidation pattern in comparison with the control (○). The oxidation was equivalent to that shown by the unexposed control (□) and to that following additional enzyme addition at **A**.

2.6 Sensitivity to pH

We have shown that the relative inactivation rate of 4-methylcatechol oxidation by tyrosinase is diminished at lower pH of the incubation mixture. Over a limited range the reduction in relative inactivation was approximately log-linear with a negative slope of 6.7 x 10^{-2} per pH unit.[18] Although this trend is predicted by the Quintox mechanism, since the alternative model[21] also involves a deprotonation step this characteristic does not distinguish between them.

3. Conclusions

It is well known that resorcinols also irreversibly inactivate tyrosinase.[37] Although the details of the tyrosinase inactivation by resorcinols are not fully resolved, the Quintox mechanism is able to rationalize the process by a reductive elimination of zero-valent copper **20** (Scheme 5)[18] whereas this is not possible by the alternative proposal **21**.
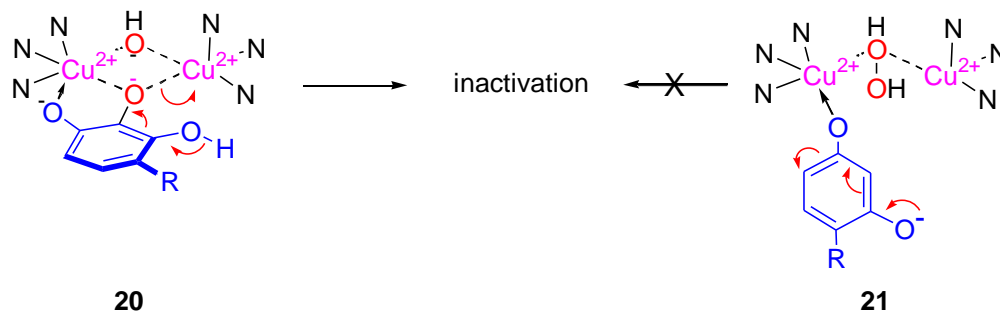
Scheme 5

Furthermore, a major difficulty posed by the alternative mechanism (Scheme 4) is that it implies that hydroquinone, as well as catechol, should be capable of acting as a suicide-inactivator with formation of 1,4-benzoquinone, which is contrary to observation.[38]

All the experimental tests so far carried out have been consistent with the predicted outcome of the Quintox inactivation mechanism of tyrosinase (Scheme 3). In contrast, the alternative mechanism (Scheme 4) does not explain all of the experimental observations, as discussed above.

Acknowledgements

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Authors' Biography
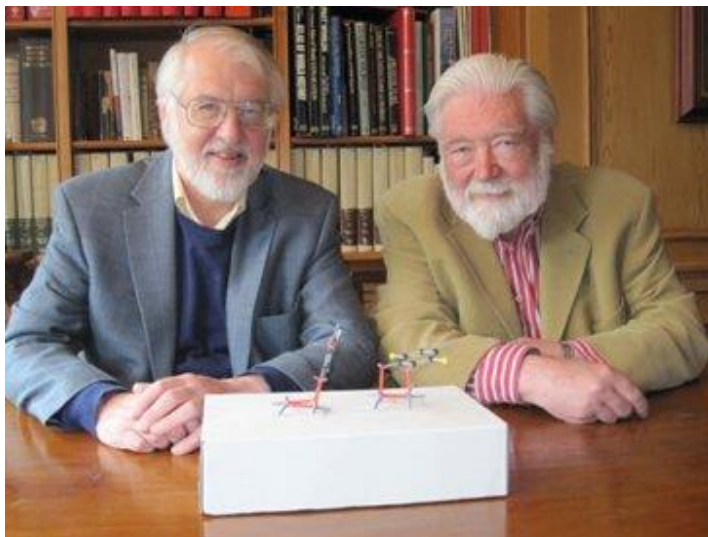
Chris Ramsden (left) and Patrick Riley (right) are members of the Quintox Group which is a multi-disciplinary group that has a long-standing interest in the properties of tyrosinase and the chemistry of related *ortho*-quinones.

Chris Ramsden was born in Manchester, U.K. He is a graduate of Sheffield University and received his Ph.D. (W. D. Ollis) in 1970 and D.Sc. in 1990. After post-doctoral work at the University of Texas with M. J. S. Dewar (1971-3) and the University of East Anglia with A. R. Katritzky (1973-6), he worked as a medicinal chemist in the pharmaceutical industry. He moved to Keele as Professor of Organic Chemistry in 1992. His research interests are in heterocycles,

ortho-quinones and three-centre bonds and the application of their chemistry to biological problems. He has been a Scientific Editor for Arkivoc since 2000 and is a member of the Arkivoc Steering Committee. He has been a member of the Quintox Group since 1993.

Patrick A Riley was born in Neuilly-sur-Seine, France. He qualified in medicine (M.B., B.S.) from University College Hospital Medical School, London, in 1960. Work for his Ph.D. (1965) was carried out in the Department of Dermatological Histopathology under Arthur Jarrett. Subsequently he joined the Chemical Pathology research laboratories under the direction of Claude Rimington. He was successively appointed Lecturer, Senior Lecturer, Reader and Professor of Cell Pathology at University College London. His D.Sc. was awarded by the University of London in 1990. He is currently Emeritus Professor of Cell Pathology at University College London and Director of the Totteridge Institute for Advanced Studies. He has published over 250 scientific papers. His main current research interests are in the field of melanogenesis.