Tyrosinase Autoactivation and the Chemistry of *ortho*-Quinone Amines†

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ABSTRACT

Tyrosinase oxidizes tyrosine to dopaquinone, which undergoes nonenzymatic reactions leading to precursors of melanin pigments. Cyclization of dopaquinone gives cyclodopa, which participates in redox exchange with dopaquinone to give the eumelanin precursor dopachrome plus dopa. The indirect formation of the catechol (dopa) from the phenol (tyrosine) leads to unusual enzyme kinetics. Using a combination of enzyme oximetry, pulse radiolysis, and chemical oxidation, the study of structurally modified dopaquinones provides firm evidence of nonenzymatic catechol formation during tyrosinase oxidation of phenols and reveals significant differences in their modes of reaction.

1. Introduction

The evolutionary importance to living organisms of being able to generate *ortho*-quinones is attested to by the widespread retention of their ability to oxidize phenols and catechols to *ortho*-quinones. The main set of enzymes with this property comprises a highly conserved group of copper-containing proteins that include the tyrosinases. Specifically, tyrosinase refers to those copper-containing enzymes that are able to oxidize both phenols and catechols to *ortho*-quinones. Tyrosinases exhibit unusual kinetic properties that are explained by the nonenzymatic reactivity of the *ortho*-quinone oxidation products, and these nonenzymatic reactions also have wider biological impact.

Ted Land was born in 1937 in Birmingham, UK. A graduate of Sheffield University, he received his PhD (G. Porter) in 1962. At the Paterson Institute for Cancer Research, Manchester (1963−1997), he continued to study fast reactions of free radicals and excited states relevant to biology and medicine. In 1998 he became a Research Fellow in Chemistry at Keele University, where his current interests include the action of anti-oxidants and the chemistry of early steps of melanogenesis.

Chris Ramsden was born in Manchester, UK, in 1946. He is a graduate of Sheffield University and received his PhD (W. D. Ollis) in 1970 and DSc in 1990. After postdoctoral work at the University of Texas (M. J. S. Dewar) (1971−3) and University of East Anglia (A. R. Katritzky) (1973−6), he worked in the pharmaceutical industry. He moved to Keele University as Professor of Organic Chemistry in 1992. His research interests are heterocycles and three-center bonds, and applications of their chemistry to biological problems.

Patrick Riley was born in Paris, France in 1935. He is a graduate of University College London (UCL), qualifying in medicine in 1960. He received his PhD (A. Jarrett) in 1965 and DSc in 1990. He joined the laboratory of Claude Rimington in 1966 and worked on photosensitization and pigmentation, finally specialising in cellular pathology. He was Professor of Cell Pathology at UCL (1984−2000) and is currently Emeritus Professor in the University of London. His research interests include quinone chemistry and melanogenesis.

The intramolecular cyclization of *ortho*-quinones with aminoalkyl side chains provides the biosynthetic pathway to the indole moieties that form the structural basis of the melanin pigments. The structure and chemistry of melanins are not as well understood as those of other natural polymers (e.g., DNA, proteins, polysaccharides) that are ubiquitous in the animal kingdom. One feature that distinguishes melanin from the other polymers is its generation by C−C bond formation. An early stage in melanin biosynthesis is tyrosinase-catalyzed oxidation of tyrosine leading to reactive melanin precursors. Tyrosinase is therefore essential for the formation of melanin (melanogenesis).[1] In vertebrates the tyrosinase gene is selectively activated in specialized dendritic cells embryologically derived from the neural crest and in the retinal pigment epithelium of the eye. The neural crest cells migrate to various tissues, including the skin, where they produce melanin. In mammalian systems the starting material for the melanogenic pathway is tyrosine **1**, although the initial demonstration of the enzyme was with 3,4-dihydroxyphenylalanine **3** (dopa) as substrate (Scheme 1). In both cases the substrate is oxidized by tyrosinase to dopaquinone **2**, which either cyclizes to give a dihydroxyindole precursor of eumelanin (black or brown pigment) or reacts with cysteine giving a thioether precursor of pheomelanin (reddish brown pigment). The early steps in melanin biosynthesis were established by Raper[2] and Mason[3] but uncertainty still remains regarding the later reactions leading to various forms of the pigment. Other biological transformations that have been related to *ortho*-quinone formation by tyrosinase include antibiosis, sclerotization of insect cuticles, balanid adhesion, and strengthening of plant walls.

Textbooks often show the formation of dopaquinone **2** from tyrosine **1** as involving initial formation of dopa **3** that undergoes further oxidation. Although dopa is formed during tyrosinase oxidation of tyrosine, it is largely formed by an indirect nonenzymatic mechanism and tyrosinase oxidizes tyrosine **1** to dopaquinone **2** in one step without requiring significant intermediate release of dopa **3**. The study of this mechanism and its biological significance has revealed a variety of *ortho*-quinone amine chemistry.

2. Type-3 Copper Proteins and the Oxidation States of Tyrosinase

Tyrosinase belongs to a larger class of copper-containing proteins that are able to bind dioxygen and which are widely distributed in living organisms.[4] These class-3 copper proteins include the hemocyanins, which act as oxygen carriers in the hemolymph of arthropods and

† Dedicated to the memory of George Porter (1920−2002), Nobel Laureate (1967).

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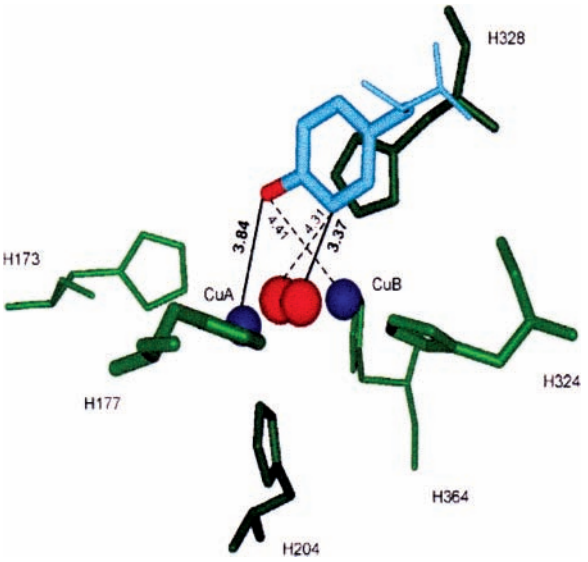
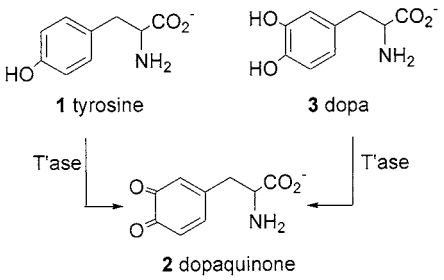


FIGURE 1. View of the potential substrate in the oxygen binding site of *Limulus polyphemus* hemocyanin based on the X-ray structure. (Reprinted with permission of Elsevier Science from Decker, H.; Tuczek, F. Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. *TIBS 25,* 392−397, copyright 2000.)

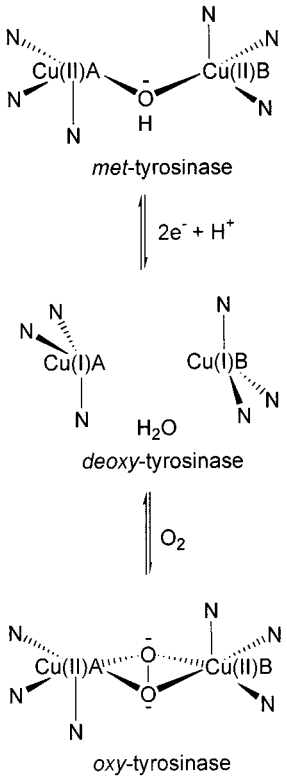
crustacea, and a variety of polyphenoloxidases that can oxidize either catechols (catecholoxidases) or phenols and catechols (tyrosinases).

X-ray structures of several hemocyanins and a catecholoxidase are now available and this has enabled important structural comparisons to be made.[5−7] Despite large differences in primary, tertiary and quaternary structure, the oxygen binding site is highly conserved suggesting that these proteins have all evolved from the same origin. An important common feature is a binuclear copper site coordinated by six histidines. This arrangement is shown in Figure 1, which is based on the X-ray structure of hemocyanin from the arthropod *Limulus polyphemus*. A potential tyrosine substrate is also shown in the active site. Although X-ray diffraction data are not available for tyrosinase, there is strong evidence to suggest that the active site resembles that shown in Figure 1. The differences in function of type-3 copper proteins are ascribed to the extent to which access of potential substrates to the oxygen-binding site is permitted. In *Eurypelma californicum* hemocyanin access of substrate is prevented by the presence of a phenylalanine residue (Phe-49) that occupies the active site in the native protein. However, it acquires phenoloxidase activity on limited proteolysis with trypsin, chymotrypsin,[8] or the nonnatural activator SDS,[9] and it is postulated that this dual role may have been the origin of polyphenoloxidases generated by gene duplication.[5] Differences in substrate selectivity, as in the case of enzymes showing preferential catecholoxidase activity (e.g., from *Ipomoea batatas*), are proposed to result from partial shielding of one of the copper sites (Figure 1, CuA) by a Phe-371 residue so that substrate binding to CuB is enforced, which is unfavorable to *ortho*-hydroxylation required for monophenoloxidase activity.[7]

Scheme 1

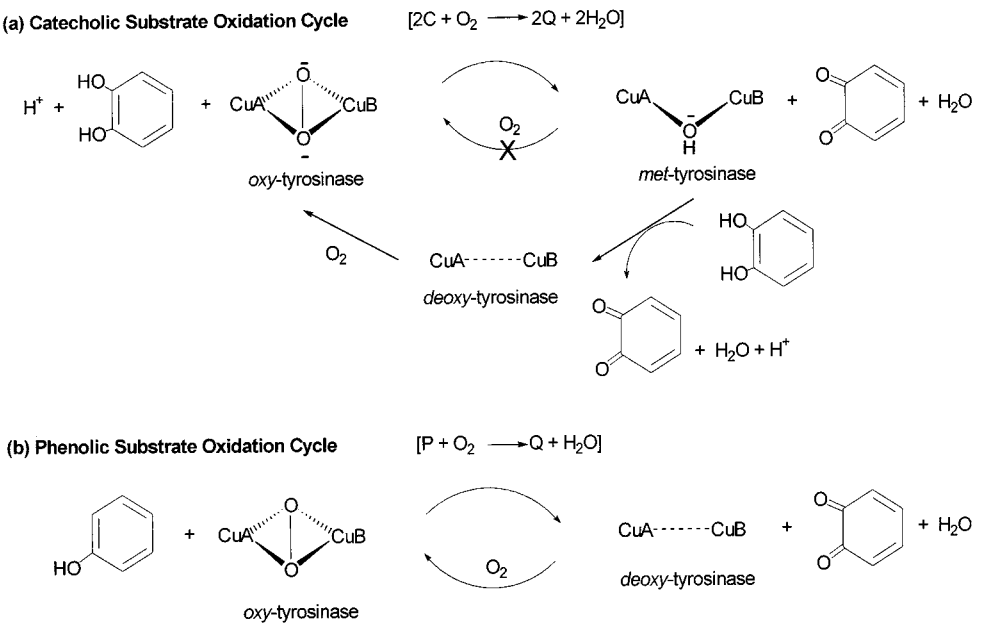


Scheme 2



Native tyrosinase occurs in the inactive *met*-form in which the binuclear copper site is in the wrong oxidation state [Cu(II)] to bind dioxygen.[10] Two-electron reduction by a catechol converts *met*-tyrosinase to *deoxy*-tyrosinase, which readily binds dioxygen giving *oxy*-tyrosinase (Scheme 2). Both phenols and catechols are oxidized to *ortho*-quinones by *oxy*-tyrosinase but the mechanisms of these oxidations are different. Oxidation of a catechol leads to *met*-tyrosinase, which cannot bind oxygen to regenerate *oxy*-tyrosinase. Only in the presence of a second catechol molecule is the *met*-tyrosinase reduced to *deoxy*-tyrosinase which then regenerates the *oxy* form (Scheme 3a). This activation of *met*-tyrosinase by a catechol has important mechanistic implications and provides evidence of the indirect formation of catecholamines in the oxidation of phenolamines by tyrosinase (Section 4). In contrast to catechols, oxidation of monophenols by *oxy*-tyrosinase gives an *ortho*-quinone plus *deoxy*-tyrosinase, which binds further oxygen, and the oxidation cycle continues to completion (Scheme 3b). Thus, it can be appreciated that

Scheme 3



while all forms of the enzyme oxidize catechols to *ortho*-quinones, *met*-tyrosinase is inert toward phenols, although they bind reversibly to the active site. Reaction of phenols with native tyrosinase therefore does not occur unless a catechol (added or formed by an indirect mechanism) leads to *deoxy*-tyrosinase formation. This aspect of phenol oxidation is primarily responsible for the "lag period" that is characteristic of in vitro tyrosinase oxidations.

3. Tyrosinase Oximetry and the Lag Period

There are, in essence, three methods to monitor the progress of tyrosinase-catalyzed oxidation: (i) chromatography of aliquots of reaction mixture; (ii) spectrophotometry of the reaction mixture; and (iii) oximetry. Oxygen levels can be measured by a variety of methods, including fluorometric measurements, electron paramagnetic resonance, and polarimetry. We have studied the tyrosinase system by a technique employing a combination of spectrophotometry and polarimetry. This utilizes a quartz cuvette modified to accept the tip of a Clark electrode positioned orthogonally to the light path of the spectrophotometer. The reaction mixture is stirred by a magnet mounted on the side of the cuvette opposite to the electrode tip. The reaction chamber is filled with buffered enzyme solution and closed with a capillary stopper that permits additions using a fine-tipped pipet. Employing this system the progress of the reactions can be monitored and the oxygen stoichiometry of the system under differing experimental conditions investigated. Also, because of the secondary reactions that may occur, the most reliable index of the reaction velocity is the oxygen utilization rate.

Figure 2 illustrates the difference in the in vitro kinetics of oxidation of phenolic and catecholic substrates. When 3,4-dihydroxyphenylethylamine (dopamine) is used as substrate, the reaction follows Michaelis−Menten kinetics

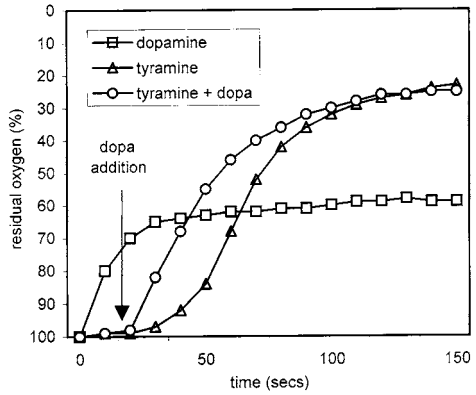


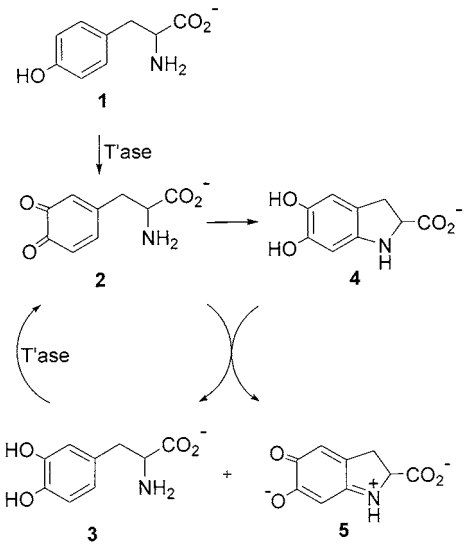
FIGURE 2. Kinetics of phenol and catechol oxidation by tyrosinase.

and the initial velocity of the reaction corresponds to the maximum rate. Data obtained in the case of the corresponding monohydric phenol, 4-hydroxyphenylethylamine (tyramine), show that there is an initial delay in oxygen utilization. This so-called "lag period" has been observed for tyrosinases derived from many sources and appears to be a characteristic feature of the enzyme. The lag period is the period during which the rate of oxidation accelerates to reach the maximum velocity equivalent to the maximal rate of catechol oxidation. This lag period is abolished by the addition of a small amount of catechol (Figure 2), which rapidly generates *deoxy*-tyrosinase from *met*-tyrosinase (Scheme 3a). It was shown by Raper[2] that dopa **3** accumulates in the reaction mixture during tyrosine oxidation, and it is recognized that in vitro dopa is a cofactor in facilitating tyrosine oxidation.[11]

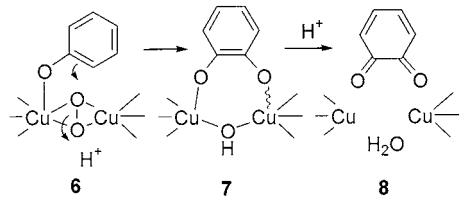
4. The Mechanism of Tyrosinase-Mediated Phenolamine Oxidation

It was long thought that tyrosinase oxidizes tyrosine **1** to dopa **3**, which is then further oxidized by the enzyme to dopaquinone **2**. This incorrectly implies that the *ortho*-

Scheme 4



Scheme 5



hydroxylation of tyrosine, shown by the oxygen isotope incorporation studies of Mason and co-workers,[3] takes place separately from the dehydrogenation of dopa. However, it is clear that since the rate-limiting step is the conversion to dopa, this does not account for the observation of dopa accumulation in the reaction system. Although dopa **3** is formed, the sequence of events leading to both catechol and *ortho*-quinone products is more subtle than direct dopa formation (**1** → **3**), and most of the catechol (i.e., **3**) is formed by an indirect nonenzymatic mechanism. Initial oxidation of phenolamines by native tyrosinase is slow because there is little *oxy*-tyrosinase present. A critical concentration of dopaquinone **2** is eventually produced, and this partially cyclizes to give cyclodopa **4** (Scheme 4). A fast nonenzymatic redox reaction then occurs between the bicyclic catechol and its precursor giving dopa **3** and the eumelanin precursor dopachrome **5**. The dopa **3** formed by this indirect mechanism can now activate more *met*-tyrosinase (Scheme 3a) leading to acceleration of the oxidation rate and the formation of a second equivalent of dopaquinone **2**. A mechanism involving direct enzyme oxidation of phenol to catechol (e.g., **1** → **3**) without redox exchange (**2** + **4** → **3** + **5**) does not rationalize the enzyme kinetics including the lag period.

The mechanism of the enzymatic phenol to *ortho*-quinone transformation is believed to involve initial bonding of the phenolic oxygen to CuA of *oxy*-tyrosinase (**6**) (Scheme 5) followed by electrophilic mono-oxygenation of the ring giving a catecholic ligand, probably bonded to both Cu atoms of *met*-tyrosinase **7**.[12] This bidentate complex **7** may then dissociate to *ortho*-quinone and *deoxy*-tyrosinase **8**, leading to the overall transformation shown in Scheme 3b. It has been suggested that before *ortho*-quinone formation can occur a configurational change of the complex **7** must take place (possibly $CuA_{axial}-CuB_{equatorial} \rightarrow CuA_{axial}-CuB_{axial}$) and that the necessary cleavage of the equatorial CuB−O bond with consequent weakening of binding can result in some catechol ligand dissociating from the enzyme.[13] Alternatively, it may be that monodentate binding to CuA is required for phenolase activity whereas monodentate binding to CuB is necessary for catecholase activity and in skipping between copper atoms some catechol ligand dissociates. Whatever the mechanism, recent studies suggest that some catechol is produced directly from simple phenolic substrates. Experiments using 4-*tert*-butylphenol have shown that 4-*tert*-butylcatechol is released into the medium.[13,14] However, studies using site specific mutagenesis of mouse tyrosinase have provided evidence that for L-dopa there is additional binding between the amino acid carboxyl group and histidine-389 located near the active site.[15] This additional interaction can be expected to contribute to holding the partially oxidized endogenous substrate in the active site. Whether any L-dopa dissociates from the enzyme−substrate complex during oxidation of L-tyrosine to L-dopaquinone therefore remains unclear. Regardless of the extent of direct catechol formation, this monophenol oxidase activity results in formation of *met*-tyrosinase that can only be reactivated by reaction with the equivalent of catechol that was generated during its formation. Thus, catechol release is a deactivating process, which may allow access of phenolic substrate to the inactive *met*-form and prolong the lag period.

To provide evidence to support the indirect mechanism of catechol formation summarized in Scheme 4, we have investigated a wide range of phenol and catechol amines as substrates for both tyrosinase and chemical oxidation. Using pulse radiolysis we have also investigated the modes and rates of reaction of the reactive, short-lived *ortho*-quinones (e.g., **2**) that are formed in these oxidations. These studies have revealed interesting variations in *ortho*-quinone amine chemistry.

5. Pulse Radiolysis and the Study of *ortho*-Quinones

The method of choice for studying the kinetics of unstable *ortho*-quinones is pulse radiolysis.[16] In this technique[17] the absorbance change induced in a sample solution by a pulse of ionising radiation is monitored by an analyzing light beam passing through the sample reaching a detector via a monochromator. Basically, the equipment is a very fast spectrophotometer connected to a linear accelerator or other source of ionising radiation.

Within 1 ns of radiation deposition in water, the following primary radicals predominate: $H_2O \rightsquigarrow e^-_{aq} + HO^\bullet + H^+$. The hydrated electron (e^-_{aq}) and hydroxyl radical ($HO^\bullet$) are vigorous one-electron reducing and

oxidizing species, respectively. In the presence of oxygen, e^-_{aq} reacts to form the superoxide radical anion, but if the solution is deoxygenated by saturating with nitrous oxide, the reducing hydrated electrons are converted quantitatively to further oxidizing HO• radicals:

$$e^-_{aq} + N_2O + H_2O \rightarrow N_2 + HO^- + HO^\bullet$$

The HO• radical is so vigorous an oxidizing agent that it tends to add to solutes also present as well as abstracting electrons or hydrogen atoms. Milder one-electron oxidizing agents are the radicals $Br_2^{\bullet-}$ and $N_3^\bullet$, which can be produced from HO• by adding high concentrations of the corresponding halide or pseudohalide:

$$HO^\bullet + Br^- \rightarrow HO^- + Br^\bullet$$

$$Br^\bullet + Br^- \rightarrow Br_2^{\bullet-}$$

$$HO^\bullet + N_3^- \rightarrow HO^- + N_3^\bullet$$

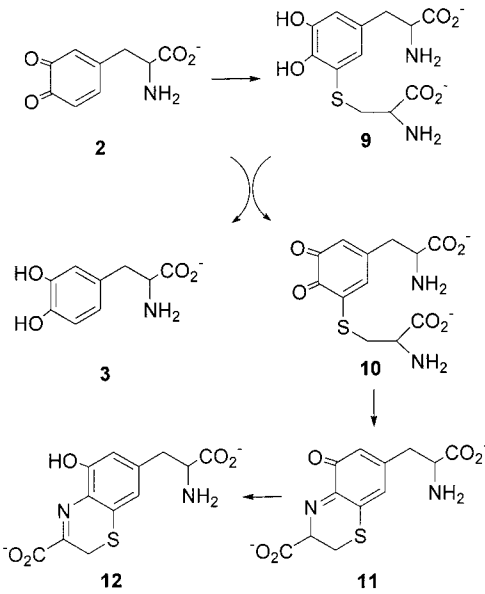
Addition of sufficient catechol (e.g., $\sim 10^{-3}$ M), such as dopa **3**, to N_2O-saturated 10^{-1} M KBr results in the exclusive formation of dopasemiquinone radicals which under typical pulse radiolysis conditions disproportionate within around a millisecond to dopaquinone **2** ($\lambda_{max} \sim 400$ nm, $\epsilon \sim 2000$ M^{-1} cm^{-1}). By following the subsequent changes in absorption at 400 nm, or by looking at products appearing at other wavelengths, one can measure quantitatively the rate constants for various reactions of dopaquinone. Similarly, the reactions of *ortho*-quinones derived from other catechols can be monitored.

In the absence of further reactants, dopaquinone **2** cyclizes ($k \sim 7$ s^{-1}) to cyclodopa (leucodopachrome) **4**. The latter, being readily oxidized, undergoes rapid redox exchange with remaining dopaquinone to form dopachrome **5** ($\lambda_{max} = 480$ nm) and regenerate dopa **3** (Scheme 4). Over much longer periods of time dopachrome **5** tautomerises and partially decarboxylates to dihydroxyindole-2-carboxylic acid and dihydroxyindole, which ultimately go on to form eumelanin. This generation of dopa by disproportionation of dopaquinone and cyclodopa is consistent with the mechanism proposed for tyrosinase autoactivation.

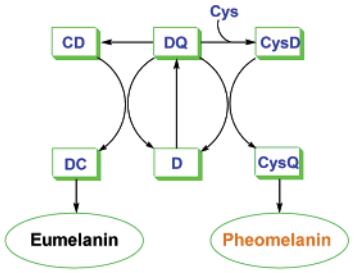
When dopaquinone **2** is generated in the presence of cysteine, it rapidly decays via a nucleophilic addition reaction ($k = 3 \times 10^7$ M^{-1} s^{-1})[18] to form predominantly 5-*S*-cysteinyldopa **9** (Scheme 6), together with smaller amounts of the 2-isomer. Although cysteinyldopa **9**, unlike cyclodopa **4**, is stable, it can also undergo an efficient redox exchange[19] with dopaquinone **2** ($k = 8.8 \times 10^5$ M^{-1} s^{-1}) to form cysteinyldopaquinone **10** and dopa **3**. Cysteinyldopaquinone **10** spontaneously cyclizes to the unstable bicyclic quinonimine **11**[20] which subsequently decays ($k = 6.0$ s^{-1}) to form the aromatic 1,4-benzothiazine **12**. This series of reactions (Scheme 6) represents the early steps of pheomelanogenesis.

The balance between eumelanogenesis and pheomelanogenesis (Scheme 7) is determined by competition between (a) dopaquinone cyclization followed by redox exchange to form dopachrome and (b) reductive addition of cysteine to dopaquinone forming cysteinyldopa followed by redox exchange generating cysteinyldopaquinone. Our current best estimates for all the rate constants involved in this competition (Scheme 7) suggest that the point where both rates are equally favored occurs when the concentration of cysteine is around 10^{-7} M. Below this cysteine concentration, eumelanogenesis predominates, and above this concentration, pheomelanin is the main product.

Scheme 6



Scheme 7

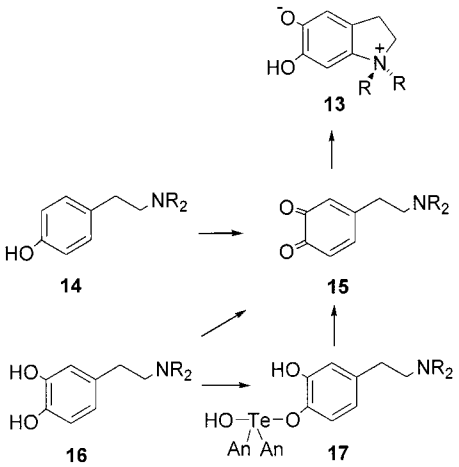


CD = cyclodopa **4**, DQ = dopaquinone **2**, CysD =cysteinyldopa **9**, DC = dopachrome **5**, D = dopa **3**, CysQ = cysteinyldopaquinone **10**.

6. 2,3-Dihydro-1*H*-indolium-5-olate Formation and Tyrosinase Activation

To provide enzymatic evidence that auto-activation of *met*-tyrosinase requires indirect catechol formation via cyclization and redox exchange (Scheme 4), we investigated oxidation of the phenolic and catecholic tertiary amine substrates **14** and **16** (R = alkyl)(Scheme 8).[21,22] In contrast to tyramine **14** (R = H), which is oxidized with an oxygen uptake stoichiometry of 1.0 O_2 per mol of substrate after an initial lag period, the *N*,*N*-dialkyltyramines **14** remained unoxidized upon prolonged exposure to native tyrosinase. However, addition of a catalytic amount of dopa or use of preactivated tyrosinase

Scheme 8



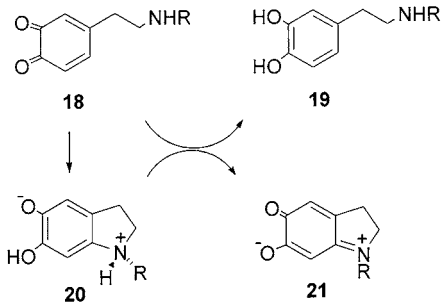
led to rapid formation of a stable product with an oxygen uptake stoichiometry of 0.5. Using *N*,*N*-di-*n*-propyltyramine **14** (R = nPr) as substrate, the final product was extracted and the crude material identified by ^{1}H NMR spectroscopy as the 2,3-dihydro-1*H*-indolium-5-olate **13** (R = nPr), formed by intramolecular cyclization of the *ortho*-quinone amine **15** (R = nPr)(Scheme 8). Since the betaines **13** are not catechols and cannot tautomerise to catechols, redox exchange with their *ortho*-quinone precursors **15** is precluded, and indirect formation of catechol amines (i.e., **16**) from the phenol amines **14** cannot occur. This result demonstrates the essential role of redox exchange for autoactivation of tyrosinase and, by inference, the general participation of this mode of reaction in eumelanin formation.

As expected, the catechol tertiary amines **16** were oxidized to the indolium-5-olates **13** by tyrosinase without the need for preactivation or catalysis. In a separate experiment, we demonstrated that the betaines **13** could not activate tyrosinase in the way that catechols do. In a tyrosinase oximetry assay using *N*,*N*-dimethyltyramine **14** (R = Me) as substrate neither preincubation with nor addition to the incubated mixture of synthetic betaine **13** modified the rate of oxygen uptake.

Authentic samples of the betaines **13** were prepared by chemical oxidation of the catechol amines **16**.[22] Although there are many oxidizing agents available for oxidation of catechols to *ortho*-quinones, in these studies we have found dianisyltellurium oxide ($An_2Te{=}O$)(DAT) to be particularly convenient due to its selectivity for the catechol function and its ease of handling.[23] We believe that the selectivity of this reagent is associated with the acidity of the catecholic OH groups. DAT is a hindered base that removes a proton from the catechol and the conjugate acid (An_2TeOH^+) then reacts with the catechol anion to form a hypervalent tellurium intermediate **17**. This product then fragments, giving water, dianisyltellurium, and the desired *ortho*-quinone **15**. Using this approach the betaines **13** were isolated in good yield as stable solids and full characterized.

A priori it was not clear how rapidly the tertiary amine quinones **15** would cyclize. In practice cyclization was too

Scheme 9



rapid for the *ortho*-quinones to be detected by ^{1}H NMR spectroscopy, but they could be detected by pulse radiolysis. The *ortho*-quinone **15** (R = Me) was generated from the catechol **16** (R = Me) and found to be very short-lived with a lifetime of a few milliseconds at pH 7.0.[22] *ortho*-Quinones derived from catecholamines can become longer-lived on changing from neutral to acidic conditions and this was found to be the case for the *ortho*-quinone **15** (R = Me). At pH 6.2 this quinone (λ_{max} = 390 nm) was observed to decay unimolecularly with a rate constant of 300 s^{-1} to a stable species identified as the betaine **13** (R = Me) (λ_{max} = 290 nm).

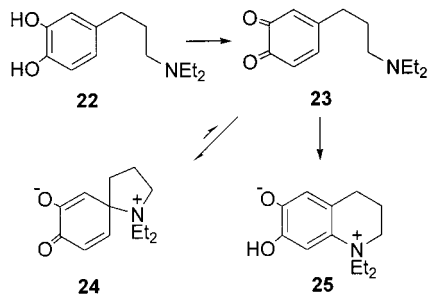
In contrast to the tertiary amines, secondary amine analogues of dopaquinone behaved like the endogenous primary amines.[24] The *ortho*-quinone **18** (R = nPr)(λ_{max} = 400 nm) was detected after 2.1 ms by pulse radiolysis and this was replaced by the aminochrome **21** (R = nPr)-(λ_{max} = 480 nm) after 18 ms at pH 8.0 (Scheme 9). Neither the betaine **20** (R = nPr) nor its catechol tautomers was detected in this system, and in contrast to the tertiary amines, the redox exchange (Scheme 9) can clearly proceed with catechol **19** formation due to the labile proton on the secondary amine function.

7. Spiro Cyclization of *ortho*-Quinone Amines

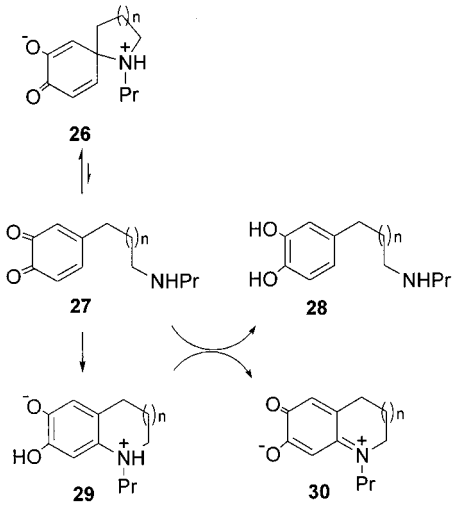
Investigation of the tertiary amine **22**, in which the amine chain has been extended by one carbon atom relative to the dopamine derivatives **16**, gave enzymatic and chemical oxidation results analogous to those obtained for the lower homologues **16**. Thus, treatment with one equivalent of DAT gave a good isolated yield of the quinolinium-6-olate **25** formed by cyclization of the transient *ortho*-quinone **23**.[22] Similarly, tyrosinase catalyzed oxidation of this tertiary amine **22** at physiological pH (7.4) gave a final product with the same UV spectrum as the isolated betaine **25** (λ_{max} = 286 nm) and an oxygen uptake stoichiometry of 0.5. However, when the cyclization of the *ortho*-quinone **23** was studied by pulse radiolysis, a subtle difference in mechanism compared to that of the *N*,*N*-dialkylated dopamines **16** was observed.

Pulsed irradiation of the catechol **22** (pH 6.2) gave the corresponding semiquinone which decayed bimolecularly with evidence of formation of the *ortho*-quinone **23** (λ_{max} = 400 nm), with a half-life of a few milliseconds, as the semiquinone decayed. However, the decay of this *ortho*-quinone **23** did not lead directly to the stable betaine **25**,

Scheme 10



Scheme 11



as previously observed for the dopamine derivatives **16**. In this case a new transient absorption ($\lambda_{max} = 250$ nm) increased as the *ortho*-quinone absorption ($\lambda_{max} = 400$ nm) decayed ($k = 230$ s^{-1}). This new intermediate is the spiro-betaine **24**, which in turn unimolecularly decays over several hundred milliseconds ($k = 7.1$ s^{-1}) to give the quinolinium-6-olate **25** as the final product (Scheme 10).

This spiro cyclization was not unexpected. The 5-*exo-trig* cyclization of the quinone amine **23** to the spiro-cyclic betaine **24** is clearly favored kinetically over the alternative 6-*exo-trig* cyclization leading directly to the bicyclic betaine **25**. However, the latter is the thermodynamically more stable and rapidly accumulates as the final product. Originally we suggested that the spiro-betaine **24** rearranges directly to the final product. However, further studies on secondary amines and modeling of the kinetics suggests that the spiro-betaine equilibrates with the *ortho*-quinone with the equilibrium strongly favoring the spiro isomer (Scheme 10). Transient spiro-betaine formation does not occur in the case of dopaquinone cyclization (Scheme 4) since 4-*exo-trig* cyclization to give a four-membered ring is highly unfavorable.

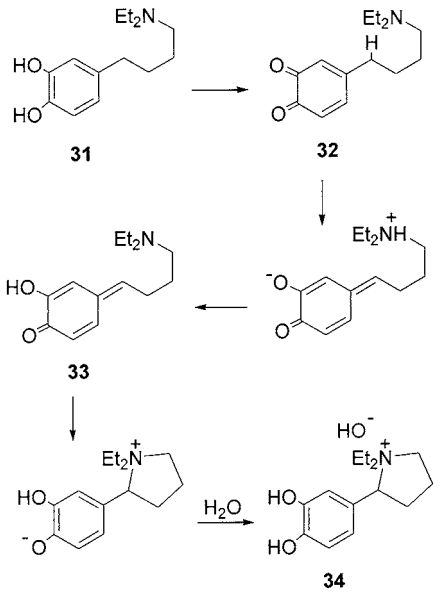
A study of the related secondary amine **27** ($n = 1$) (Scheme 11) also showed evidence of spiro-betaine formation but a significant difference in the end products of reaction was observed between the tyrosinase oximetry and pulse radiolysis studies.[24] When the *ortho*-quinone **27** ($n = 1$) was generated at pH 8.0 from the corresponding catechol by pulsed irradiation, formation of the spiro-betaine **26** ($n = 1$)($\lambda_{max} = 250$ nm) was too fast ($k > 10^3$ s^{-1}) for the *ortho*-quinone **27** ($n = 1$) to be detected. After 166 ms all of this intermediate **26** ($n = 1$) had been converted ($k = 39$ s^{-1}) to the quinolinium-6-olate **29** ($n = 1$)($\lambda_{max} = 310$ nm), which was the final product. However, in the enzyme system the oxygen uptake stoichiometry was 0.96, and the final product was the aminochrome **30** ($n = 1$), formed by further oxidation of the betaine **29** ($n = 1$). This observation is entirely consistent with the enzymatically formed betaine being further oxidized by a redox exchange reaction with the *ortho*-quinone precursor **27** ($n = 1$) (Scheme 11). This cannot happen in the pulse radiolysis system in which all the *ortho*-quinone is produced in a single pulse and is rapidly removed by conversion to the spiro-betaine **26** ($n = 1$). The concentration of *ortho*-quinone in this system is too low for redox exchange to occur. However, in the enzyme system the *ortho*-quinone **27** ($n = 1$) is produced continuously over a long period from both the original substrate and the redox product **28** ($n = 1$), and this ensures sufficient material for the redox exchange to occur.

When the next higher homologue **27** ($n = 2$) was studied, the formation of the aminochrome product **30** ($n = 2$) was observed in both the enzymatic and the pulse radiolysis experiments. In this case, 6-*exo-trig* formation of the six-membered spiro-betaine **26** ($n = 2$), which was detected, is not as favorable as formation of the five-membered spiro-betaine **26** ($n = 1$), and sufficient *ortho*-quinone **27** ($n = 2$) remains in equilibrium to ensure that even under pulse radiolysis conditions the betaine **29** ($n = 2$), or its catechol tautomer, is oxidized to the aminochrome **30** ($n = 2$) by redox exchange (Scheme 11). In fact, the betaine **29** ($n = 2$) could not be detected in this system, presumably because the redox exchange reaction is very fast.

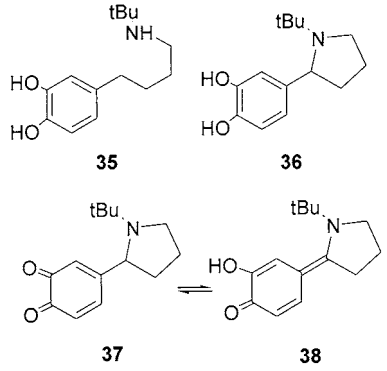
8. Quinone−Quinomethane Tautomerism

Although we anticipated the possibility of spiro-betaine formation by the higher homologues **27**, we did not anticipate the mode of reaction of the higher tertiary amine homologue **32**. Oxidation of the catechol **31** with 1 equiv of DAT gave a single product, isolated in good yield, and this was identified as the quaternary salt **34** (Scheme 12).[25] Tyrosinase oxidation similarly terminated with formation of product **34** ($\lambda_{max} = 282$ nm) via the *ortho*-quinone **32** ($\lambda_{max} = 400$ nm), whose intensity peaked after about 30 s. When the *ortho*-quinone **32** was generated by pulse radiolysis, it was found to be stable over a period of at least several seconds. This result is in complete contrast to the behavior of the corresponding secondary amine **27** ($n = 2$) which gave an aminochrome **30** ($n = 2$) via intermediate betaines **26** and **29** ($n = 2$)-(Scheme 11). Presumably steric hindrance prevents the tertiary amine **32** reacting in a manner similar to the secondary amine **27** ($n = 2$). The hindered amine **32** therefore reacts as a base rather than as a nucleophile giving the *para*-quinomethane **33** via the pathway shown in Scheme 12. This reaction is too slow to be observed by

Scheme 12



Scheme 13

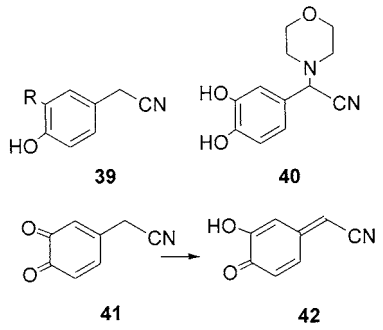


pulse radiolysis. The *para*-quinomethane **33** then undergoes favorable 5-*exo*-*trig* cyclization to give the isolated product **34**.

The role of steric hindrance in bringing about this change in mode of reaction is supported by similar behavior of a sterically hindered secondary amine.[24] Thus, when the *tert*-butyl amine **35** (Scheme 13) was investigated, the corresponding *ortho*-quinone, like *ortho*-quinone **32**, was also found to be stable by pulse radiolysis, in contrast to the *N*-propyl derivative **27** ($n = 2$). Tyrosinase oxidation of the amine **35** initially gave the *ortho*-quinone with an oxygen stoichiometry of 0.46. This was followed by slower oxidation giving a final product with absorbance peaks 274 and 476 nm, which is not an aminochrome (e.g., **30**). The initial *ortho*-quinone gives the catechol **36** by a mechanism analogous to that for the tertiary amine **31** (Scheme 12). This then undergoes further enzymic oxidation to the quinone **37** which tautomerises to the quinomethane **38**, accounting for the UV spectrum of the final product.

The apparent role of intramolecular deprotonation in facilitating *ortho*-quinone to *para*-quinomethane tautomerism led us to consider *ortho*-quinone derivatives that may have an α-proton sufficiently acidic to isomerize

Scheme 14



spontaneously. Our attention was directed to 4-hydroxybenzylcyanide **39** (R = H) (Scheme 14), which, unexpectedly, failed to achieve auto-activation of tyrosinase after the usual lag period and was only oxidized by preactivated enzyme. Using 3,4-dihydroxybenzylcyanide **39** (R = OH), the *ortho*-quinone **41** ($\lambda_{max} = 380$ nm) was generated by pulse radiolysis at neutral pH and found to decay ($k = 7.5\ s^{-1}$) to a new species that was identified as the *para*-quinomethane **42** ($\lambda_{max} = 480$ nm).[26] Rapid isomerism is clearly favored by the acidity of the α-proton. Further evidence of this rapid tautomerism was provided by chemical oxidation. Treatment of the catechol **39** (R = OH) with one equivalent of DAT in the presence of morpholine gave a quantitative yield (monitored by NMR) of the amino-nitrile **40**, which was isolated in good yield and fully characterized.[27]

The rapid conversion of the *ortho*-quinone **41** to the *para*-quinomethane **42** is particularly significant in the context of tyrosinase catalyzed oxidation of 4-hydroxybenzylcyanide **39** (R = H). Using this substrate, autoactivation of the enzyme never occurs because the *ortho*-quinone is rapidly removed by tautomerisation and therefore cannot be reduced to the activating catechol **39** (R = OH) by redox exchange. In contrast, using the catechol **39** (R = OH) as substrate, oxidation was rapid with O_2 uptake stoichiometry of 0.5 and formation of a product with the spectral characteristics of a *para*-quinomethane ($\lambda_{max} = 480$ nm).[26] In the phenolic incubation mixtures, any *para*-quinomethane generated can form catechols by reaction with thiol and amino groups on the enzyme, or other proteins. However, these catechols are enzyme bound and can neither reach the active site nor reduce *ortho*-quinone **41**, which has been removed by tautomerism. Like *N*,*N*-disubstituted betaine formation (Scheme 8), rapid quinomethane formation provides a further example of the prevention of indirect catechol formation by redox exchange and the resulting lack of tyrosinase autoactivation and acceleration of reaction rate using phenolic substrates. These experimental observations are entirely consistent with the mechanism summarized in Scheme 4 and provide further support for this route to melanin formation.

9. Conclusions

From the biological perspective the nonenzymatic reactions of *ortho*-quinones have wide impact. First, their

reactivity with nucleophiles renders them capable of covalent bonding to a wide range of structural and functional molecules such as proteins and nucleic acids and also small molecules such as glutathione. This type of "tanning" reaction is the basis of a number of toxic effects of significance in antibiosis. The possibility of using the melanogenic pathway for endogenous generation of potentially cytotoxic quinones has been proposed as a strategy for targeted chemotherapy for malignant melanoma.[28] Progress in the elucidation of the chemistry of substituted *ortho*-quinones may lead to useful therapeutic applications.[29,30]

In addition, the facile redox exchange reactions between *ortho*-quinones and related catechols, including those derived from the quinones by Michael addition reactions, permit successive oxidations of the same molecule to occur, thus enabling these agents to take part in cross-linking reactions which have important structural roles in biological systems. The improved understanding of the reaction mechanisms involved may permit constructive interference with some of these biologically significant processes.

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