3-Hydroxykynurenine as a substrate/activator for mushroom tyrosinase

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Received 16 December 2002, and in revised form 30 January 2003

Abstract

3-Hydroxykynurenine is a tryptophan metabolite with an o-aminophenol structure. It is both a tyrosinase activator and a substrate, reducing the lag phase, stimulating the monophenolase activity, and being oxidized to xanthommatin. In the early stage of monophenol hydroxylation, catechol accumulation takes place, whereas 3-hydroxykynurenine is substantially unchanged and no significant amounts of the o-quinone are produced. These results suggest an activating action of 3-hydroxykynurenine toward o-hydroxylation of monophenols. 3-Hydroxykynurenine could therefore well act as a physiological device to control phenolics metabolism to catechols and quinonoids.

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Keywords: 3-Hydroxykynurenine; Tyrosinase; Polyphenoloxidase; Monophenolase; Activation; Lag time

Tyrosinases, better termed polyphenoloxidases (monophenol, o-diphenol:oxygen oxidoreductase; EC 1.14.18.1), are copper-containing monoxygenases catalyzing the o-hydroxylation of monophenols to the corresponding catechols (monophenolase activity) and the oxidation of catechols to the corresponding o-quinones (diphenolase activity). “Catecholases” lacking monophenolase activity have been considered polyphenoloxidases, whose full activity was not seen as a consequence of inappropriate assays, and therefore tyrosinases and catecholases could well be treated as a unicum [1]. These enzymes are widespread in the living world and are known also as phenolases and phenol oxidases. These terms are often used without regard to any rule, even if tyrosinase is the term usually adopted for animal including human enzymes, and refers to the “typical” substrate, tyrosine. Tyrosine is also a good substrate for the enzyme from the champignon mushroom Agaricus bisporus, and this enzyme is therefore usually called “mushroom tyrosinase.” This has been and still is the enzyme of choice as a tool to study the general behavior of polyphenoloxidases.

The polyphenoloxidase monophenolase activity is characterized by a typical lag time, which has been explained by means of kinetic [2] and allosteric models [3]. From a kinetic point of view, the lag time is the time required for the met form, unable to react with the monophenol substrate, to be drawn into the active deoxy form by the corresponding catechol, arising by the action of the small amounts of the oxy form usually accompanying the met form. Accordingly, this lag time is shortened or abolished when a reaction mixture containing polyphenoloxidase and a monophenol substrate is fed with a catechol; moreover, many reducing agents, capable of reducing the met form, shorten or abolish the lag time [4]. Polyphenoloxidases have an active site containing a dicopper center [5], formed by two cupric ions, each coordinated by three histidine residues. The resting state of the enzyme (met) can be reduced to the dicuprous form (deoxy, the only one capable of reacting with molecular oxygen), which can bind dioxgen as a peroxy bridge \((\gamma^2: \gamma^2)\) between the cupric ions (oxy).

Abundant data on the fine structure of the active site has accumulated in the recent years, but the wide variety of
enzymes studied and the different analytical methods used have given rise to highly contradictory information. For example, widely varying Cu–Cu distances have been calculated for the metpolyphenoloxidase, ranging from 2.8 to 3.6 A [6–8]: obviously, this interionic distance is of the highest importance in the binding mode of substrates, inhibitors, and modulators. The two cupric ions show some differences with regard to their coordination geometry [5,9] and have been therefore identified as CuA and CuB; no agreement exists with regard to whether CuA [7] or CuB [6] is the site for substrate binding.

Some o-aminophenols have been shown to be relatively poor substrates for polyphenoloxidase, behaving as catechols and leading to imino-o-quinones and their degradation products [10]. In particular, 3-hydroxyanthranilic acid has drawn our attention [11], as it is a tryptophan metabolite for several kinds of organisms. 3-Hydroxyanthranilic acid, in addition to being slowly oxidized to the quinoneimine and further to the phenoxazinone derivative, cinnabaric acid, acts as a catechol analog with respect to its capability of stimulating the monophenolase activity of mushroom tyrosinase and shortening the lag time. In mammals, and most probably in several other organisms, 3-hydroxyanthranilic acid comes from 3-hydroxykynurenine upon hydrolytic cleavage catalyzed by a kynureninase [12]. Therefore, 3-hydroxykynurenine can be well regarded as a key intermediate in tryptophan metabolism and has been studied with respect to both its chemical and its biochemical properties [13–15]. Its (auto)oxidation product is xanthommatin, a polycyclic quinonoid widely spread in the living world. Xanthommatin is typical for insect eyes, but is also widespread in other animals; its formation and deposition has been related to the degenerative process leading to cataractous eye lenses in humans [16,17]. 3-Hydroxykynurenine is an effective antioxidant in eye lenses [18] where it s gradually converted to xanthommatin with aging. It has been recognized as a carcinogen [19] acting with a metal-mediated oxidative mechanism. 3-Hydroxykynurenine is also a neurotoxin [20–23]. Kynurenine-3-hydroxylase, the key enzyme for 3-hydroxykynurenine production, has a very low activity within the brain [24], but 3-hydroxykynurenine formed elsewhere easily crosses the blood–brain barrier [25], with the aid of the large neutral amino acid carrier. The prominent importance of 3-hydroxykynurenine in tryptophan metabolism and its strict biochemical relationship with 3-hydroxyanthranilic acid, which shares with 3-hydroxykynurenine many physiopathological properties, have therefore prompted us to study its interactions with polyphenoloxidase, with regard to contributing to the understanding of its general metabolic behavior. In fact, the presence of tyrosinase in human tissues and body fluids has been well ascertained [26,27], so physiological interactions between 3-hydroxykynurenine and tyrosinase seem highly probable.

In the present work, we examine 3-hydroxykynurenine as a mushroom tyrosinase substrate and/or activator in the light of its peculiar o-aminophenol structure.

Materials and methods

Chemicals

3-Hydroxykynurenine was purchased from Sigma–Aldrich (Milan, Italy). As 3-hydroxykynurenine is only slightly water soluble at neutral pH, stock solutions of 3-hydroxykynurenine in 10% dimethyl sulfoxide were freshly prepared just prior to use. 4-Tert-butyl-phenol and 4-tert-butyl-catechol were supplied from Fluka (Milan, Italy). Since 4-tert-butyl-phenol also is practically insoluble in water, a 5 mM stock solution in 10% iso-propyl alcohol was prepared. 4-Tert-butyl-1,2-benzoxoquinone used for HPLC analysis was prepared as described in [28]. All other chemicals were of analytical grade, purchased from Fluka, and used without further purification. Xanthommatin, the (auto)oxidation product of 3-hydroxykynurenine, was prepared by incubating known amounts of 3-hydroxykynurenine with excess Pleurotus sajor-caju laccase until reaction went to completion. An e value of 10,000 M⁻¹ cm⁻¹ was found and used for kinetic measurements.

Enzymes

Mushroom tyrosinase (43,000 enzyme units (E.U.)/mg protein) was prepared as described in [29]. Two consecutive chromatographic steps with Bio-Gel Hydroyxylapatite HTP (Bio-Rad, Milan, Italy) and anion exchangeMacro-Prep DEAE (Bio-Rad) were carried out. One E.U. caused an increase in A₃₂₀ of 0.001/min at pH 6.5 at 25°C in a 3-mL reaction mix where the final concentrations were 18 mM potassium phosphate buffer, 0.3 mM L-tyrosine, and 50–100 units of tyrosinase. The activity of mushroom tyrosinase on 3-hydroxykynurenine was monitored by measuring the formation of xanthommatin. Steady state rate was defined as the value of 10,000 M⁻¹ cm⁻¹ was found and used for kinetic measurements.

Laccase was partially purified from a culture medium of the fungus P. sajor-caju, grown as reported in [31]. The culture medium containing the extracellular laccase was submitted to two consecutive chromatographic steps with Macro-Prep DEAE and Sephacryl HR-200 (Pharmacia, Sweden). Laccase activity was measured
photometrically by checking absorbance increase at 525 nm when 50 μM syringaldazine was present in a final volume of 1 mL of a buffered (50 mM K phosphate, pH 6) solution of suitably diluted medium. One laccase unit was defined as in [32].

Spectrophotometric measurements were performed with an Ultrospec 4000 UV/Vis Spectrophotometer (Pharmacia).

HPLC analysis

Before performing HPLC analysis, protein-containing samples were deproteinized by adding 70% perchloric acid (about 0.5 M in final concentration) and centrifuging at 10,000 g for 10 min. The resulting supernatant was then filtered through a 0.45-μm pore membrane filter (Millipore).

Identification of the products of enzymatic activity was carried out with a Beckman System Gold apparatus equipped with an UV-Vis detector module. The column used for chromatographic separations was a Chromolith RP-18e ODS-Hypersil (100 x 4.6 mm i.d.) purchased from Merck (Darmstadt, Germany). Separations of the compounds were achieved with 0.085% phosphoric acid in water, v/v, (solvent A) and acetonitrile (solvent B) as mobile phases.

Separations were achieved at room temperature by initial gradient elution, 20–50% B in 1 min, followed by a second gradient elution, 50–90% B, in 3.5 min. The detector was set at 280 nm. More details are reported in the corresponding figure legends.

Electrophoresis

Polyacrylamide gel electrophoresis runs were carried out by using a MiniProtean II (Bio-Rad) apparatus and following its instruction manual for the preparation of both running buffers and slab gels. Electrophoretograms were developed with a solution of syringaldazine (1 mM in ethanol). The pink spots on a pale yellow background corresponded to enzyme activity. Alternatively, laccase activity was revealed as very sharp and intensely blue spots when a combination of 4-tert-butyl-catechol and 4-amino-N,N-diethylaniline sulfate was used [33].

Results

To unambiguously assess the substrate nature of 3-hydroxykynurenine, the need for a purified tyrosinase, reasonably void of extraneous enzymatic activities and particularly of laccase, arose. It is common knowledge that o-aminophenols are good substrates for fungal laccases; most commercial tyrosinase preparations contain significant laccase amounts [34]. This was shown to be the case in the commercial mushroom tyrosinase preparation routinely used in most laboratories: after a native PAGE run, a significant laccase activity was readily seen [33]. When the same gels were soaked in a solution of 3-hydroxykynurenine, an intensely yellow spot corresponding to the laccase activity quickly appeared, whereas a feeble spot corresponding to tyrosinase became visible only after a few minutes. This preliminary observation indicated that 3-hydroxykynurenine is a much better substrate for laccase than for tyrosinase. That 3-hydroxykynurenine is a good laccase substrate has been confirmed with a partially purified preparation of laccase from P. sajor-caju (Fig. 1); in this way xanthommatin, the main “final” (auto)oxidation product of 3-hydroxykynurenine, was prepared to obtain an ε value used for kinetic calculations. From the above considerations, the need to work with a purified polyphenoloxidase preparation arose. For this reason, we have used a purified mushroom tyrosinase, obtained as previously described [11,29], which does not contain any laccase trace.

It has to be pointed out that no accidental autooxidation of 3-hydroxykynurenine took place under the experimental conditions adopted for this study. Xanthommatin was obtained also from tyrosinase-catalyzed 3-hydroxykynurenine oxidation, as shown in Fig. 2; 3-hydroxykynurenine is a substrate with a K_m of 127 μM and a V_max of 367 nmol/min/mg protein. The substance shows a relatively high affinity for the enzyme, whereas it is only slowly oxidized in comparison with catechols. As expected, no lag time was observed, so 3-hydroxykynurenine could be ascribed to the family of o-aminophenol-type substrates of tyrosinase that behave as catechol analogs rather than o-aminosubstituted monophenols. This observation is not so obvious as it may appear: 3-aminotyrosine, which is an o-aminophenol...
and, moreover, strictly resembles the structure of a “typical” substrate, tyrosine, has been described as one of the most potent and specific competitive inhibitors for mushroom tyrosinase [35]. Once that 3-hydroxykynurenine belongs to the \( \alpha \)-aminophenol-type tyrosinase substrates was ascertained, its capability of acting as an enzyme activator, as is well known for catechols [1–4] and as was more recently found for 3-hydroxyanthranilic acid, was tested. 4-\( \text{tert} \)-butyl-phenol was chosen as the monophenol substrate because it shows a high affinity for the enzyme \( (K_m = 16 \mu M) \) but a relatively low \( V_{\text{max}} \) (4.5 \( \mu \text{mol/min/mg protein} \)) for a monophenol [11]. Moreover, its quinonization product 4-\( \text{tert} \)-butyl-1,2-benzoquinone is remarkably stable as an \( \alpha \)-quinone and cannot cyclize; it simply accumulates in the reaction mixture and can be easily detected. On the contrary, when tyrosine is used as the tyrosinase substrate, the produced dopaquinone quickly cyclizes. The arising leukodopachrome is the starting point of a complex chemical and enzymatic pathway leading to melanin. Cross-reactions between dopaquinone and 3-hydroxykynurenine also take place and make data interpretation problematic. Fig. 3 shows the dramatic decrease of the lag time in the oxidation of 4-\( \text{tert} \)-butyl-phenol in the presence of 3-hydroxykynurenine. Fig. 4 shows a plot of \( (T - t) \) vs 3-hydroxykynurenine concentrations, where \( T \) is the lag time for 4-\( \text{tert} \)-butyl-phenol alone and \( t \) is the lag time in the presence of 3-hydroxykynurenine. As \( T \) is not easily determined due to the very slow oxidation rate of 4-\( \text{tert} \)-butyl-phenol by mushroom tyrosinase, it has been calculated with the Pomerantz equation [30] and found to be 16.6 min. Consequently, the \( K_m \) value for 3-hydroxykynurenine as an activator is 21 \( \mu M \). The activating effect of 3-hydroxykynurenine toward 4-\( \text{tert} \)-butyl-phenol oxidation by tyrosinase has been studied also at different 3-hydroxykynurenine concentrations: the obtained pattern strictly resembles that already found for 3-hydroxyanthranilic acid [11] and is shown in
The concentration decreases of both 4-tert-butyl-phenol and 3-hydroxykynurenine were monitored by means of HPLC analysis (after 1 and 6 min, respectively), in addition to the formation of 4-tert-butyl-catechol and, in a reaction mixture containing 4-tert-butyl-phenol, 3-hydroxykynurenine and tyrosinase (Fig. 6). The presence of some secondary peaks most probably indicates the rise of oxidation products of 3-hydroxykynurenine and/or by-products arising from cross-reactions between remainder reagents and reaction products. Fig. 7 shows the results from an additional experiment where the same reaction mixture was incubated for 20 min; samples were drawn at established times prior to HPLC analysis. This experiment taken together with other separate tests confirmed that in the absence of 3-hydroxykynurenine only a marginal amount of 4-tert-butyl-phenol is converted by mushroom tyrosinase.

**Discussion**

The study of the system 4-tert-butyl-phenol/4-tert-butyl-catechol/4-tert-butyl-1,2-benzoquinone in the presence of mushroom tyrosinase is particularly intriguing and represents the starting point for some considerations. 4-tert-butyl-phenol shows a high affinity for the enzyme but is only slowly oxidized. 4-tert-butyl-catechol is a good substrate, being very quickly oxidized to 4-tert-butyl-1,2-benzoquinone, but its affinity for the tyrosinase active site is comparatively low ($K_m = 990$ µM). Therefore, if the assumption of a mutual exclusion (i.e., competition) between monophenol and catechol substrates from the polyphenoloxidase active site is made, relatively high concentrations of 4-tert-butyl-catechol are required to displace 4-tert-butyl-phenol from the bicupric center of metpolyphenoloxidase, thus starting the catalytic cycle. This consideration may be reasonably extended to the case of the couple 4-tert-butyl-phenol/3-hydroxykynurenine.

3-Hydroxykynurenine is not among the best substrates for mushroom tyrosinase but shows a relatively high affinity. The chosen 3-hydroxykynurenine concentration of 80 µM allows for a substantial binding of 4-tert-butyl-phenol to the enzyme, while efficiently elicit the oxidation of that monophenol and dramatically shortening the lag time. This latter observation fully meets a $K_m$ value for 3-hydroxykynurenine as a tyrosinase activator of 21 µM.

A closer inspection of Fig. 7 also shows that a substantial amount of 4-tert-butyl-catechol (up to a concentration of about 70 µM) is formed within the very early stage of the reaction, whereas the 3-hydroxykynurenine concentration is still almost unchanged. By then, almost no 4-tert-butyl-1,2-benzoquinone was detectable, according to the poor affinity of 4-tert-butyl-catechol for mushroom tyrosinase. By comparing the $K_m$ values for 4-tert-butyl-catechol and 3-hydroxykynurenine (as a substrate), the obvious consideration that 4-tert-butyl-catechol must be substantially excluded...
from the active site of the enzyme arises, and therefore only 3-hydroxykynurenine can behave as an activator. In other words, 3-hydroxykynurenine efficiently stimulates \( o \)-hydroxylation of phenolic substrates, but their further quinonization by polyphenoloxidase is not allowed due to the persistence of 3-hydroxykynurenine within the enzyme active site, until a comparatively high catechol concentration is reached.

As expected, 3-hydroxykynurenine remains almost unchanged within the first reaction stage, due to its low \( V_{\text{max}} \). It is worth noting that completion of the 4-\textit{tert}-butyl-phenol oxidation becomes comparatively slow when a sufficient amount of 4-\textit{tert}-butyl-1,2-benzoquinone—a well-known polyphenoloxidase inhibitor—is produced.

In conclusion, 3-hydroxykynurenine seems to be a reasonably good candidate for a monophenolase positive effector. In mammals, 3-hydroxykynurenine represents by far the most abundant tryptophan metabolite, and an important fraction of the compound is in turn converted to 3-hydroxyanthranilic acid [12]; the participation of tryptophan metabolites and in particular of 3-hydroxykynurenine in disorders of melanogenesis has been ascertained [36]. Generally speaking, tryptophan is therefore deeply involved in modulation of monophenol (mainly tyrosine) metabolism, taking into due account that the immediate metabolite of 3-hydroxykynurenine in mammals, 3-hydroxyanthranilic acid, also shows a quite similar positive modulating effect toward monophenolase activity.

References


