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COVALENT BINDING OF CATECHOLS TO PROTEINS THROUGH THE SULPHYDRYL GROUP

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Abstract—The cytotoxicity of catechols has been ascribed to covalent binding of the *o*-quinone oxidation products to proteins through the sulphydryl group. We have previously shown that dopaquinone can bind covalently to proteins through cysteine residues to form protein-bound cysteinyldopas. In this study, we have compared the reactivities of *o*-quinones, derived from tyrosinase oxidation of various catechols, with the cysteine residue in bovine serum albumin. *o*-Quinone forms of dopamine, norepinephrine, *N*-acetyldopa, *N*-acetyldopamine, 3,4-dihydroxyphenylacetic acid, pyrocatechol and 4-methylcatechols were much more reactive than dopaquinone, while *o*-quinone forms of 5-*S*-cysteinyldopa and epinephrine were much less reactive. The yield of protein-bound cysteinylcatechols appears to depend on a competition between the intermolecular nucleophilic reaction of sulphydryl groups in protein and the intramolecular nucleophilic reaction of an amino group in the side chain.

Cytotoxicity of catechols has been a subject of extensive studies because of their physiological roles or therapeutic use. Such catechols as 3,4-dihydroxyphenylalanine (dopa†) and α-methyldopa have been used to treat Parkinson's disease, hypertension, and other diseases. Dopa, dopamine and their derivatives have been evaluated as possible antitumour agents, especially against melanoma and neuroblastoma [1–6]. On the other hand, Graham *et al.* [7] have postulated that Parkinson's disease may be a consequence of life-long exposure of dopaminergic neurons to cytotoxic dopamine.

Two mechanisms have been proposed for the cytotoxicity of catechols: (a) covalent binding of *o*-quinone oxidation products to enzymes through highly nucleophilic sulphydryl groups, thus inhibiting enzymes such as DNA polymerase [7–11]; (b) production of active oxygens such as hydrogen peroxide, superoxide radical and hydroxyl radical, by autoxidation of catechols [7, 12, 13]. It is conceivable that the *o*-quinone mechanism may predominate in melanocytes where a specific enzyme tyrosinase catalyses the oxidation of catechols to *o*-quinone forms [1, 2, 11].

We have recently reported that dopaquinone produced by tyrosinase oxidation of dopa can bind covalently to proteins through cysteine residue to form cysteinyldopa residue (protein-bound cysteinyldopas), the yield of which depends on the reactivity of cysteine residue [14]. The binding reaction is in a competition with an intramolecular cyclisation yielding dopachrome [14]. In the present study, we have examined the covalent binding of *o*-quinones, derived from tyrosinase oxidation of various catechols, to bovine serum albumin that contains one cysteine residue per molecule. Catechols examined include dopa, dopa derivatives, catecholamines, 3,4-dihydroxyphenylacetic acid (dopac), pyrocatechol and 4-methylcatechol. The products protein-bound cysteinylcatechols were quantified, after HCl hydrolysis, by HPLC with electrochemical detection of the free cysteinylcatechol derivatives (Fig. 1).

MATERIALS AND METHODS

Materials. Mushroom tyrosinase (2430 units/mg), bovine serum albumin, L-dopa, L-dopa methyl ester, dopamine, *N*-acetyldopamine, norepinephrine, epinephrine, dopac, DL-α-methyldopa and L-cysteine were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-acetyldopa was prepared as previously described [15]. All other chemicals were of analytical grade and from Wako Pure Chemical Industries. HCl was purified by distillation at 110° (concentration: 6 M HCl). Ultrapure water prepared by use of the Milli-Q-system (Millipore Corp.) was used throughout this study.

Preparation of cysteinylcatechols. The preparation of most of cysteinylcatechol derivatives has been reported by us [4, 16], except for the following new compounds.

Cysteinylnorepinephrines were prepared by tyrosinase oxidation of norepinephrine in the presence of cysteine. The reaction mixture contained 0.05 mmol norepinephrine, 0.10 mmol L-cysteine and 5 mg tyrosinase in 5.0 ml of 0.05 M sodium phosphate buffer (pH 6.8). The oxidation was carried out at room temperature and after 60 min it was stopped by acidification. The products 5-*S*- and 2-*S*-cysteinylnorepinephrine were partially separated by chromatography twice on Dowex 50W-X2

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† Abbreviations: dopa, 3,4-dihydroxyphenylalanine; dopac, 3,4-dihydroxyphenylacetic acid; HPLC, high-performance liquid chromatography.

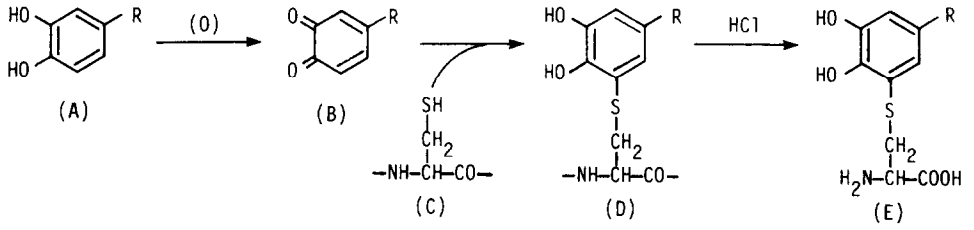

Fig. 1. Formation of protein-bound cysteinylcatechols by tyrosinase oxidation of catechol in the presence of protein and their acid hydrolysis. (A) Catechol; (B) *o*-quinone; (C) cysteine residue in protein; (D) cysteinylcatechol residues in protein (protein-bound cysteinylcatechols); (E) free cysteinylcatechol derivatives.

(2.0 × 5.5 cm; equilibrated and eluted with 2 M HCl). The yields, determined by assuming the molar extinction coefficients to be identical to those of the corresponding cysteinyldopas [4], were 39% (97% purity by HPLC) and 13% (79% purity) for 5-*S*- and 2-*S*-cysteinylnorepinephrine, respectively.

5-*S*-Cysteinylepinephrine was prepared as described above for cysteinylnorepinephrines, in a yield of 37% (97% purity). The 2-*S* isomer could not be separated well from the 5-*S* isomer.

5-*S*-Cysteinyldopac was prepared as follows. The reaction mixture contained 0.05 mmol dopac, 0.10 mmol L-cysteine, 5 mg tyrosinase and 0.05 mmol Na_2HPO_4 in 5.0 ml of water. The oxidation was carried out at room temperature for 30 min. The product 5-*S*-cysteinyldopac was isolated by chromatography twice on Dowex 50W-X2 (2.0 × 6.0 cm; equilibrated and eluted with 1 M HCl): 91% yield (94% purity by HPLC), based on the molar extinction coefficient of 2800 $(M.cm)^{-1}$ [17].

Formation and acid hydrolysis of protein-bound cysteinylcatechols. A reaction mixture contained 100 μM catechol and 200 μM bovine serum albumin in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4). After adding 10 μg (or an appropriate amount) of mushroom tyrosinase, the mixture was incubated at 37°, and the reaction was stopped after 5 min by adding 1 ml of 10% trichloroacetic acid. In some experiments, tyrosinase was omitted or replaced with tyrosinase that was previously inactivated by heating in the buffer at 100° for 5 min. The precipitate was kept at 4° for 1 hr and collected by centrifugation. The supernatant was subjected to the determination of catechol remaining unoxidised that was determined by comparing with the blank experiment in which the reaction was stopped without incubation. The precipitate was washed twice with 5% trichloroacetic acid and hydrolysed with 1.0 ml of 6 M HCl containing 5% thioglycolic acid at 110° under argon atmosphere. After 16 hr, 10 μl of 100 μg/ml α-methyldopa in 0.1 M HCl was added as an internal standard.

Catechols in the hydrolysate were then isolated by alumina extraction as follows. In a conical plastic tube (1.5-ml volume) were placed 50 mg acid-washed alumina, 50 μl of 4% $Na_2S_2O_5$, and 100 μl of either the hydrolysate or a standard solution which contained appropriate amounts of cysteinylcatechols to be analysed, and 1 μg α-methyldopa per ml of 6 M HCl/5% thioglycolic acid. Catechols were adsorbed onto alumina at pH 8.6 by adding 500 μl of 2.7 M Tris/2% Na_2EDTA and immediately shaking for 5 min on a JASCO multi-tube mixer (Japan Spectroscopic Co.). The alumina was washed three times with about 1 ml of water, and then catechols were eluted with 150 μl of 0.4 M $HClO_4$. The extract was analysed by HPLC with electrochemical detection.

Chromatographic conditions. A Yanaco Model L-2000 liquid chromatograph (Yanagimoto) was used with a Yanaco Model VMD-101 electrochemical detector. The detector was set at +750 mV vs an Ag/AgCl reference electrode. Separation was achieved on a C_{18} reversed-phase column (Yanaco ODS-A, particle size 7 μm, 250 × 4.6 mm). The mobile phase contained 10 g phosphoric acid, 7 g methanesulphonic acid and 0.1 mmol Na_2EDTA per liter of water, pH being adjusted to 2.35 with 5 M NaOH [17]. Column temperature was 40 or 50° and flow rate was 0.7 or 1.0 ml/min.

RESULTS

Various catechols were incubated with bovine serum albumin and mushroom tyrosinase, and protein-bound cysteinylcatechols formed were analysed after acid hydrolysis and alumina extraction. The amount of tyrosinase was 10 μg per ml of the reaction mixture for preliminary experiments and was changed, when necessary, to achieve the oxidation of catechol by 30–80%. The results shown in Table 1 indicate that tyrosinase mediated the binding of catechols to bovine serum albumin to form protein-bound cysteinylcatechol derivatives. In the blank experiments in which tyrosinase was omitted or was added after heat inactivation, the formation of protein-bound cysteinylcatechols was minimal except for 4-methylcatechol. The data for the extents of catechol oxidised indicate that *N*-acetyldopamine, pyrocatechol and 4-methylcatechol were good substrates for mushroom tyrosinase, while 5-*S*-cysteinyldopa, norepinephrine and epinephrine were poor substrates.

The catechols examined in this study can be classified into three groups: (a) a highly reactive group, *N*-acetyldopa, dopamine, *N*-acetyldopamine, norepinephrine, dopac, pyrocatechol and 4-methylcatechol; (b) the least reactive group, 5-*S*-cysteinyldopa and epinephrine; and (c) an intermediate group, dopa and dopa methyl ester. When compared among dopa and catecholamines, the reactivities were in the order dopamine, norepinephrine > dopa > epinephrine. When compared among dopa and its derivatives, the reactivities were in the order

Table 1. Formation of protein-bound cysteinylcatechols by tyrosinase oxidation of catechols in the presence of bovine serum albumin*

Catechol	Tyrosinase (μg/ml)	Catechol oxidised (A)† (μM)	Protein-bound cysteinylcatechols (B)‡ (μM)		B/A (%)
Dopa	2.0	33	3.4		10
	10	81	6.5	5-S-Cys-dopa§	8.1
Dopa methyl ester	2.0	71	9.8	+	14
	10	99	13	2-S-Cys-dopa	13
N-Acetyldopa	2.0	48	33	+	69
	10	97	65	6-S-Cys-dopa	67
5-*S*-Cysteinyldopa	200	29	0.06	Dicys-dopa	0.2
Dopamine	2.0	55	37	5-S-Cys-DA	67
	10	86	70	+	82
N-Acetyldopamine	0.5	51	41	2-S-Cys-DA	80
	2.0	97	76	+	79
				6-S-Cys-DA	
Norepinephrine	50	46	35	5-S-Cys-NE	77
	200	84	60	+	72
				2-S-Cys-NE	
Epinephrine	50	63	0.6	5-S-Cys-E‖	1.0
	200	97	0.6		0.6
Dopac	10	72	34		47
	50	98	43	5-S-Cys-dopac‖	44
	0.0¶	13	3.2		25
Pyrocatechol	0.5	61	57	3-S-Cys-cat	93
	2.0	98	97	+	99
	0.0¶	11	2.3	4-S-Cys-cat	21
4-Methylcatechol	0.5	80	52		64
	2.0	100	66	3-S-Cys-Me-cat‖	68
	0.0¶	45	22		48

* The reaction mixture contained 100 μM catechol and 200 μM bovine serum albumin in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4). After adding the indicated amount of tyrosinase, the mixture was incubated at 37° for 5 min. Results are averages for two separate experiments.

† In experiments without tyrosinase or with heat-denatured enzyme, the amounts of catechol oxidized were less than 10% except for dopac, pyrocatechol and 4-methylcatechol.

‡ Products and their total yield after acid hydrolysis. The yields were corrected for the recoveries (89–100%) of the major isomers that were subjected to the acid hydrolysis. Cysteinylnorepinephrine and cysteinylepinephrine were unstable under the hydrolysis conditions, being converted to other derivatives, possibly by substitution of the hydroxyl group with chlorine atom.

§ Cys-dopa, cysteinyldopa; dicys-dopa, 2,5-*S,S'*-dicysteinyldopa; cys-DA, cysteinyldopamine; cys-NE, cysteinylnorepinephrine; cys-E, cysteinylepinephrine; cys-dopac, cysteinyldopac; cys-cat, cysteinylcatechol; 3-*S*-cys-Me-cat, 3-*S*-cysteinyl-5-methylcatechol.

‖ Yields of minor isomers were not added to these values.

¶ Experiments with heat-denatured enzyme gave similar results.

N-acetyldopa > dopa methyl ester, dopa. Autoxidation of catechols, dopac, pyrocatechol and 4-methylcatechol, resulted in lower yields of protein-bound cysteinylcatechols. From dopac and 4-methylcatechol, the percent yields were a half to two-thirds those by tyrosinase-mediated binding, while from pyrocatechol, the yield was one-fifth.

The ratio of 3-*S*- and 4-*S*-cysteinylcatechol formed in the reaction of pyrocatechol with bovine serum albumin was found to be 2:1. When 100 μM pyrocatechol was incubated with 200 μM L-cysteine and 2 μg/ml tyrosinase at 37° for 5 min, the ratio of free 3-*S*- and 4-*S*-cysteinylcatechol was 50:1. We have found similar, but not so striking, differences in the previous study [14]; in the reaction of dopaquinone with proteins the relative yields of minor isomers of cysteinyldopas were much higher than with free cysteine.

DISCUSSION

As a possible mechanism of cytotoxicity of catechols, it has been proposed that *o*-quinone oxidation products bind covalently to sulphydryl enzymes through the cysteine residues [7–11]. The present study demonstrates that *o*-quinones produced by tyrosinase oxidation of catechols can bind to bovine serum albumin through the cysteine residue to form the protein-bound cysteinylcatechol residue (Fig. 1). The degree of the covalent binding varied greatly with the catechol oxidised (Table 1).

It is well known that dopaquinone undergoes an intramolecular cyclisation (followed by oxidation) to yield a red pigment dopachrome [19]. The same type of cyclisation can take place in catecholamines to form aminochromes [20]. Although the reaction proceeds very rapidly [21, 22], significant differences

were observed in the rates of cyclisation of dopamine, norepinephrine and epinephrine, the order being the reverse [20, 21]. *o*-Quinone forms of *N*-acetyldopa and *N*-acetyldopamine also undergo cyclisation, although the rates are one magnitude lower than that of dopaquinone cyclisation [22]. On the other hand, the nucleophilic addition reaction of sulphydryl compounds, such as cysteine and glutathione, is more rapid than the cyclisation by a factor of 10^3 [22, 23].

On the basis of these previous data, it appears that the yield of protein-bound cysteinylcatechol per catechol oxidised depends on a competition between the intermolecular nucleophilic reaction (sulphydryl group of cysteine residue in protein) and the intramolecular nucleophilic reaction (side chain amino group) with the *o*-quinone form. Specifically, (a) dopac, pyrocatechol and 4-methylcatechol gave high yields of protein-bound cysteinylcatechols on account of the lack of the side chain amino group; (b) *N*-acetyl derivatives of dopa and dopamine also gave high yields on account of the slow rates of intramolecular cyclisation [22]; (c) 5-*S*-cysteinyldopa and epinephrine gave only trace amounts of protein-bound cysteinylcatechols on account of the extremely rapid intramolecular cyclisation [21, 22]; and (d) dopamine and norepinephrine gave high yields, suggesting that the cyclisation proceeds much slower than in dopa, although no data have been reported for the difference in the cyclisation of dopa and dopamine. Graham *et al.* [7] have shown that dopamine is more toxic to neuroblastoma cells than dopa and that epinephrine is not toxic at all. We have also shown that toxicity of 5-*S*-cysteinyldopa to neuroblastoma cells is mediated by hydrogen peroxide [13]. The present findings fit well with these previous results.

Tyrosinase converts catechols to *o*-quinones by two-electron oxidation [24], while autoxidation of catechols gives rise to semiquinone radicals, by one-electron oxidation, disproportionation of which yields *o*-quinones [25]. The difference in the mechanism may account for the difference in the rates of covalent binding obtained with tyrosinase oxidation and autoxidation.

Finally, the present results may provide a chemical basis for developing better chemotherapeutic agents against melanoma and for controlling adverse, cytotoxic effects of catechols now being used as drugs.

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