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Green Tea Polyphenols: Novel Irreversible Inhibitors of Dopa Decarboxylase

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The green tea gallocatechins, (−)-epigallocatechin-3-*O*-gallate (EGCG), and (−)-epigallocatechin (EGC) were found to be inhibitors of Dopa decarboxylase (DDC). EGCG and EGC inactivate the enzyme in both a time- and concentration-dependent manner and exhibit saturation of the rate of inactivation at high concentrations, with efficiency of inactivation values (k_{inact}/K_i) of 868 and 1511 M^{-1} min^{-1}, respectively. In contrast, gallic acid behaves as a weak inhibitor of DDC. Protection against inactivation by EGCG and EGC was observed in the presence of the active site-directed inhibitor D-Dopa. Either EGCG or EGC induce changes in the absorbance and CD bands of the visible spectrum of enzyme-bound PLP. Taken together, these findings indicate the active site nature of the interaction of DDC with both polyphenols. On the basis of the properties of the EGCG-inactivated enzyme, it can be suggested that inactivation could be ascribed to a covalent modification of not yet identified residue(s) of the active site of DDC.

Key Words: **Dopa decarboxylase; pyridoxal 5′-phosphate; inactivation; green tea gallocatechins.**

The pyridoxal 5′-phosphate (PLP) dependent-enzyme Dopa decarboxylase (DDC; EC 4.1.1.28) is a key enzyme involved in the biosynthesis of biogenic amines. Its primary catalytic activity consists in the conversion of L-Dopa and L-5-hydroxytryptophan into dopamine and serotonin, respectively. The enzyme is of medical interest being the target of drugs used in the therapy of various disorders such as Parkinson's disease and hypertension. Since a potent and selective inhibition of DDC is a desirable pharmaceutical goal, the action on the enzyme of a variety of compounds has been defined by measuring their effects on the decarboxylase activity and/or on the absorbance and CD spectral properties of the PLP-bound enzyme. Schiff-base analogs (phosphopyridoxyl aromatic amino acids) and substrate analogs (catechol- or indole-related structure) have been shown to inhibit enzymic activity and/or to bind to the active site of DDC (1, 2). Their dissociation or inhibition constants range from 10^{-2} to 10^{-6} M, depending on the chemical structure of the analog. Substrate analogs endowed with a substrate hydrazine function, L-α-methyl-α-hydrazino-3,4-dihydroxyphenylpropionic acid (carbidopa or MK 485) and 2,3,4-trihydroxybenzylhydrazine (Ro-4152) were found to be powerful, but not selective inhibitors of DDC (3, 4). Comparison of the affinity constants of various substrate analogs has allowed to establish that the catechol ring gives the largest contribution to the binding, while the carboxyl, the aminic group, and the aliphatic chain are responsible for a decrease in the binding (2). These data led us to study the interaction of DDC with catechol-type polyphenols.

In this study the interaction of DDC with the green tea polyphenols, (−)-epigallocatechin-3-*O*-gallate (EGCG), and (−)-epigallocatechin (EGC) has been investigated by kinetic and spectroscopic methods. It is demonstrated that both EGCG and EGC bind to the active site of the enzyme and behave as irreversible inactivators of DDC.

MATERIALS AND METHODS

Chemicals. PLP, L-Dopa, D-Dopa, EGC, and gallic acid were purchased from Sigma. EGCG was from Alexis Biochemicals. All other chemicals were of the highest purity available.

Purification of DDC. Recombinant pig kidney DDC was purified to homogeneity as described previously (5) with slight modification (6). The enzyme concentration was determined by using an E_M of 1.3×10^5 M^{-1} cm^{-1} (7).

Enzyme and inactivation assays. DDC activity was measured as described by Sherald *et al.* (8), as modified by Charteris and John (9). The inactivation mixture contained enzyme (6 μM) and freshly diluted inactivator (EGCG, EGC, or gallic acid) at varying concentrations at 25°C in potassium phosphate buffer, $I_c = 0.1$, pH 7. At various time intervals aliquots were taken and tested for residual

Abbreviations used: DDC, Dopa decarboxylase; PLP, pyridoxal 5′-phosphate; EGCG, (−)-epigallocatechin-3-*O*-gallate; EGC, (−)-epigallocatechin.

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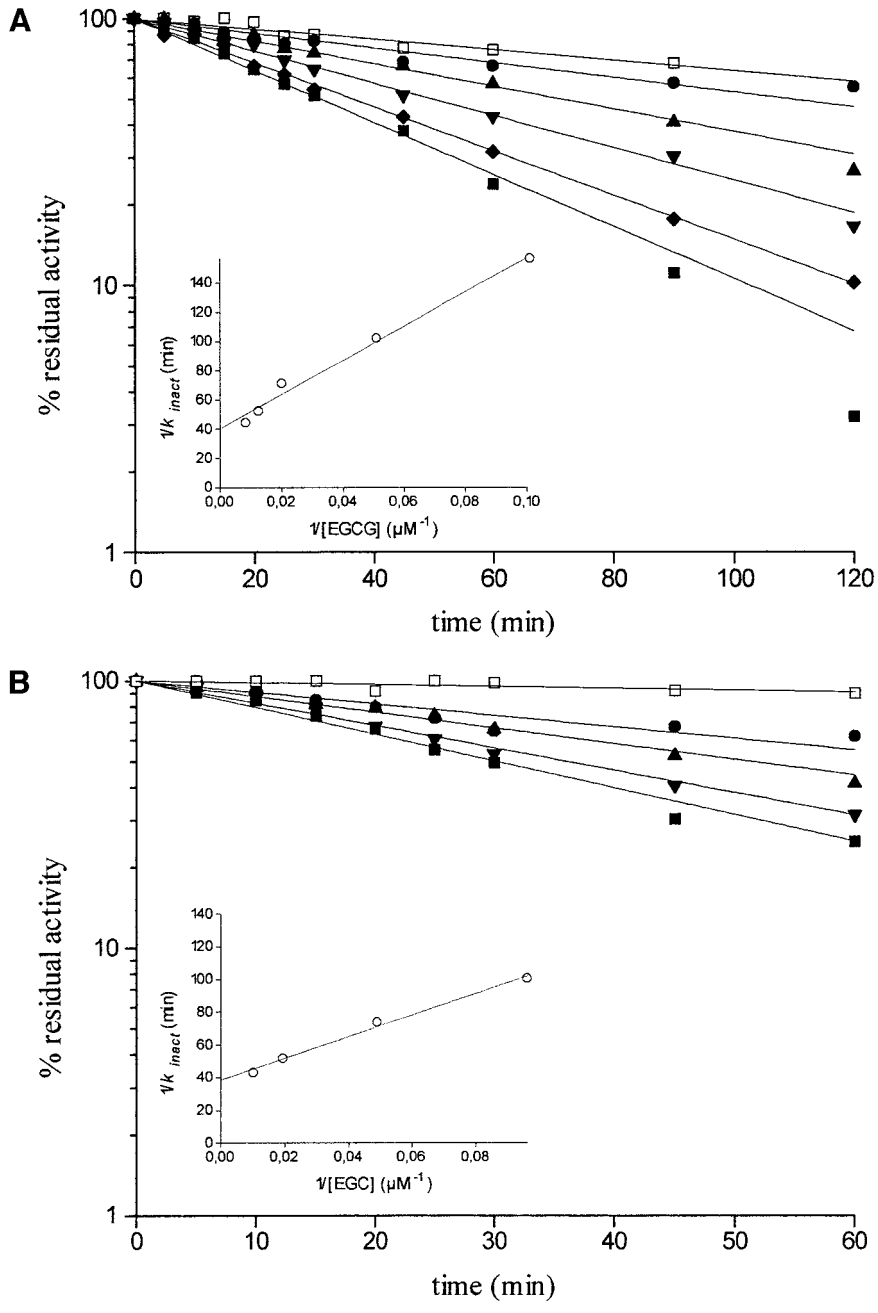

FIG. 1. Inactivation of DDC by EGCG and EGC. The enzyme (6 μM) was incubated with different concentrations of the inactivator in potassium phosphate buffer, $I_c = 0.1$, pH 7 at 25°C. At time intervals, aliquots were removed for measurements of the residual decarboxylase activity. (A) ●, 9.9 μM EGCG; ▲, 19.6 μM EGCG, ▼, 49.5 μM EGCG, ◆, 80.3 μM EGCG, ■, 122 μM EGCG, □, reaction to which 10 mM D-Dopa was added prior to the addition of 122 μM. (B) ●, 10.3 μM EGC, ▲, 20.7 μM EGC, ▼, 51.5 μM EGC, ■, 99.2 μM EGC, □ reaction to which 10 mM D-Dopa was added prior to the addition of 99.2 μM EGC. *Insets*, double reciprocal plots of the apparent rate of inactivation as a function of (A) EGCG concentration and (B) EGC concentration.

decarboxylase activity as described above. In protection experiments, D-Dopa (10 mM) was included prior to the addition of the inactivator.

HPLC analysis. HPLC analysis was carried out on a Jasco HPLC system equipped with a UV1570 detector using a Discovery (Supelco) C18 column (4.6 × 250 mm). PLP content of the native and inactivated enzymes was determined with the HPLC method described previously (10). For EGCG analysis the eluent was methanol: potassium phosphate (25 mM, pH 3.5) (25:75, v/v) at a flow rate of 0.6 ml/min. Detection was set at 280 nm. Standard curve of peak area as a function of PLP and EGCG concentration was prepared using commercially available PLP and EGCG.

Spectrophotometric measurements. Absorption spectra were carried out using a Jasco V-550 spectrophotometer at 25°C. CD spectra were obtained with a Jasco 710 spectropolarimeter with a thermostatically controlled cell at 25°C at a protein concentration of 10 μM.

Spectra were recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm.

RESULTS AND DISCUSSION

EGCG inactivates DDC in both a time- and concentration-dependent manner (Fig. 1A). Inactivation follows a pseudo-first order kinetic behavior at each fixed concentration of EGCG. A reciprocal plot of the pseudo-first-order constants at each inhibitor concentration, taken from the slopes of the lines in Fig. 1A, gives a straight line (inset of Fig. 1A) which demonstrates saturation kinetics with a K_i of 28.8 ± 3.3 μM and indicates the presence of a binding step prior to inactivation with a k_{inact} of 0.025 ± 0.002 min^{-1}. Likewise, EGC inactivates DDC in both a time- and a concentration-dependent fashion (Fig. 1B) and exhibits saturation of the inactivation at high concentration (inset of Fig. 1B). The K_i and k_{inact} of EGC are 17.2 ± 1.2 μM and 0.026 ± 0.001 min^{-1}, respectively. The addition of D-Dopa to the reaction mixture containing DDC and EGCG or EGC decreases the rate of inactivation (Figs. 1A and 1B). The k_{inact}/K_i value, which represents the efficiency of inactivation, is equal to 1511 M^{-1} min^{-1} for EGC, a value higher than that calculated for EGCG (868 M^{-1} min^{-1}). Thus, these catechol-type polyphenols without and with the esterified galloyl group show significant inhibition. On the contrary, gallic acid itself behaves as a weak inhibitor of DDC: at 500 μM the rate constant of inactivation is of 0.0036 ± 0.0003 min^{-1}. It should be noted that the presence of the galloyl moiety in EGCG appears to decrease the binding to DDC instead of providing an additive binding contribution.

As shown in Figs. 2A and 2B, upon addition of 100 μM either EGCG or EGC to DDC (6.5 μM) the absorbance and CD spectra are altered. In both cases, the absorbance and dichroic signals at 420 nm immediately decrease, more markedly in the presence of EGCG than in the presence of EGC. Concomitantly, the absorbing and dichroic peak at 335 nm shifts to 342 nm with EGCG, while remaining almost unaltered with EGC. These spectral bands do not change with time. These data suggest that the inhibition of DDC by both EGCG and EGC could be caused by specific binding of these molecules to the enzyme, presumably in close proximity to the PLP-binding site. The binding of both EGCG and EGC to the active site of the enzyme is demonstrated by (i) the protection against inactivation of DDC by each of these compounds by an active site-directed inhibitor of the enzyme, D-Dopa, and (ii) the changes in the absorbance and CD bands of the visible spectrum of enzyme-bound PLP induced by the addition of EGCG and EGC. Catechol-type polyphenols with and without galloyl moiety have been reported to be selective inhibitors of many enzymes, mainly oxidases and reductases for which it has been proposed

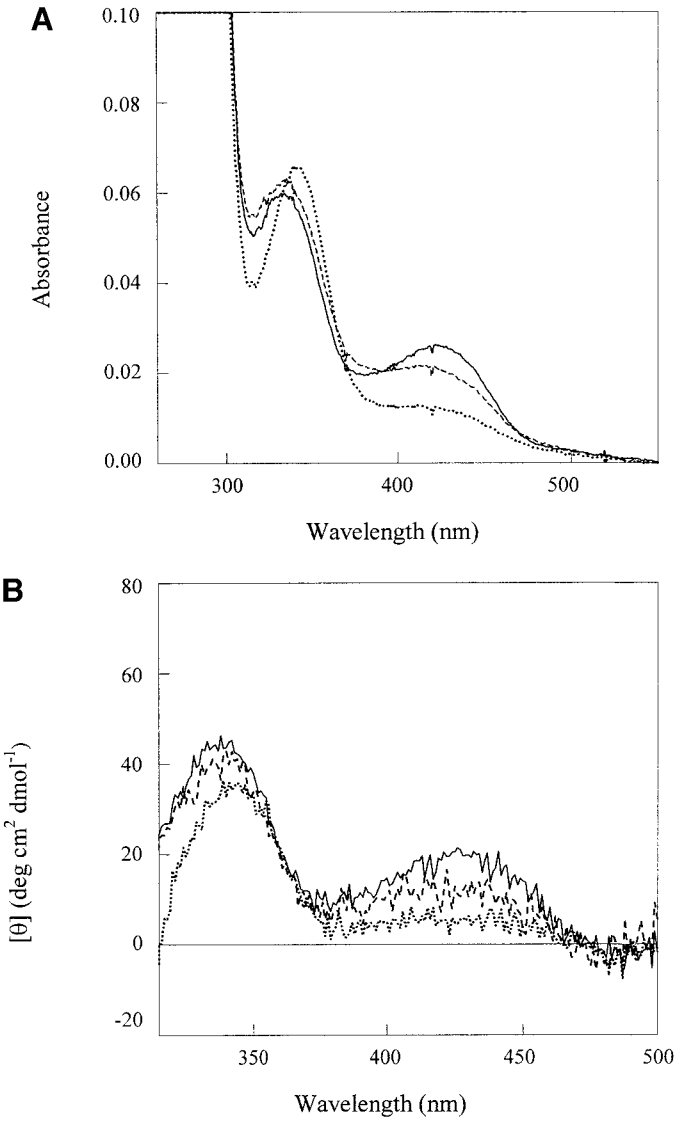

FIG. 2. Absorbance and CD spectra of DDC in the presence of EGCG and EGC. (A) Absorption spectra of DDC (—) and immediately after the addition of 100 μM EGCG (· · ·) or 100 μM EGC (– – –). The enzyme concentration was in each case 6.5 μM. (B) CD spectra as absorption.

that inhibition would be caused by scavenging reactive oxygen species required for enzyme reaction (11–14). In addition, molecular modeling has predicted the manner by which EGCG binds to urokinase (15) and *p*-hydroxybenzoate hydroxylase (14). No other information are presently available on the inhibition mechanism exerted by these compounds. In order to determine if the inactivation of DDC involves a covalent attachment to protein and/or coenzyme, the properties of the EGCG-inactivated enzyme have been studied. Following inactivation of DDC by 100 μM EGCG as shown in Fig. 1A and removal of unreacted EGCG by PD-10 column gel filtration, the enzyme exhibits absorption and CD spectral features identical to those of

Figs. 2A and 2B, and does not show recovery of activity even after addition of exogenous PLP. Treatment of the inactivated enzyme with 6 M guanidine hydrochloride at pH 7 or with trichloroacetic acid causes the release of PLP but not that of EGCG, as judged by HPLC analysis. The peak corresponding to PLP is about 80% with respect to the original content of the unmodified enzyme. Various attempts have been made to release EGCG from the protein. However, treatment of the inactivated-DDC with NaOH, hydroxylamine or by heating for 1 min at 100°C causes the appearance in HPLC of a number of peaks absorbing at 280 nm that possibly are degradation species of EGCG. This interpretation is supported by the fact that a sample of free EGCG maintained under identical experimental conditions displays the same chromatographic pattern. These data could suggest, although not definitely proving, that in the inactivated enzyme EGCG is covalently bound to the protein and not to the coenzyme. This view is consistent with the observation that the immediate modification of the visible absorbance and CD spectra induced by EGCG was not followed by further changes with time. Furthermore, addition of L-Dopa to the inactivated-DDC causes the immediate increase of the 420 nm absorbance band (data not shown), thus indicating the ability of the inactive enzyme species to form a Schiff-base.

Taken together, these results indicate that the inactivation (i) does not involve release of the bound PLP, (ii) does not produce nonspecific steric or conformational changes which may prevent substrate binding, and (iii) could be possibly due to a covalent bond of EGCG to amino acidic residue(s) of the protein.

In conclusion, this study reports for the first time that the naturally occurring EGCG and EGC bind to the active site of DDC, behave as effective inhibitors of the enzyme, and might be potential useful therapeutic agents. The unavailability of radioactive labelled EGCG makes the identification of the residue(s) involved in polyphenol binding to DDC difficult. Further studies are now in progress to overcome this trouble.

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